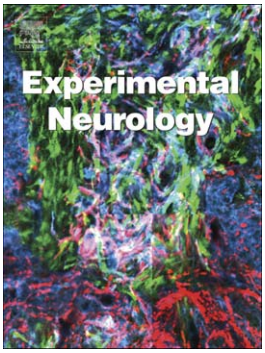
Accepted Manuscript



MHC-I promotes apoptosis of GABAergic interneurons in the spinal dorsal horn and contributes to cancer induced bone pain

Qiaochu Fu, Dai Shi, Yaqun Zhou, Hua Zheng, Hongbin Xiang, Xuebi Tian, Feng Gao, Anne Manyande, Fei Cao, Yuke Tian, Dawei Ye

PII: S0014-4886(16)30270-9
DOI: doi: 10.1016/j.expneurol.2016.09.002
Reference: YEXNR 12396

To appear in: *Experimental Neurology*

Received date: 28 March 2016
Revised date: 20 August 2016
Accepted date: 7 September 2016

Please cite this article as: Fu, Qiaochu, Shi, Dai, Zhou, Yaqun, Zheng, Hua, Xiang, Hongbin, Tian, Xuebi, Gao, Feng, Manyande, Anne, Cao, Fei, Tian, Yuke, Ye, Dawei, MHC-I promotes apoptosis of GABAergic interneurons in the spinal dorsal horn and contributes to cancer induced bone pain, *Experimental Neurology* (2016), doi: 10.1016/j.expneurol.2016.09.002

MHC-I promotes apoptosis of GABAergic interneurons in the spinal dorsal horn and contributes to cancer induced bone pain

Qiaochu Fu[a], Dai Shi[a], Yaqun Zhou[a], Hua Zheng[a], Hongbin Xiang[a], Xuebi Tian[a], Feng Gao[a], Anne Manyande[b], Fei Cao[c], Yuke Tian[a]*, Dawei Ye[d]*

[a] Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030,China.

[b] School of Psychology, Social Work and Human Sciences, University of West London, London, UK.

[c] Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030, USA

[d] Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030,China.

*__Corresponding author:__ Yuke Tian, Department of Anesthesiology, Tongji Hospital, Tongji MedicalCollege, Huazhong University of Science and Technology, 1095 Jiefang Avenue, 430030 Wuhan, People's Republic of China. E-mail: yktian@tjh.tjmu.edu.cn, Tel: 00862783663173 Fax: 00862783662853 or Dawei Ye, Department of Oncology, Tongji Hospital, Tongji MedicalCollege, Huazhong University of Science and Technology, 1095 Jiefang Avenue, 430030 Wuhan, People's Republic of China. E-mail: dy0711@gmail.com, Tel: 00862783663409 Fax: 00862783662853

Abstract:

Cancer induced bone pain (CIBP) remains one of the most intractable clinical problems due to poor understanding of its underlying mechanisms. Recent studies demonstrate the decline of inhibitory interneurons, especially GABAergic interneurons in the spinal cord, can evoke generation of chronic pain. It has also been reported that neuronal MHC-I expression renders neurons vulnerable to cytotoxic $CD8^+$ T cells and finally lead to neurons apoptosis in a variety neurological disorders. However, whether MHC-I could induce the apoptosis of GABAergic interneurons in spinal cord and contribute to the development of CIBP remains unknown. In this study, we investigated roles of MHC-I and underlying mechanisms in CIBP on a rat model. Our results showed that increased MHC-I expression on GABAergic interneurons could deplete GABAergic interneurons by inducing their apoptosis in the spinal dorsal horn of tumor-bearing rats. Pretreatment of MHC-I RNAi-Lentivirus could prevent the apoptosis of GABAergic interneurons and therefore alleviated mechanical allodynia induced by tumor cells intratibial injection. Additionally, we also found that $CD8^+$ T cells were colocalized with MHC-I and GABAergic neurons and presented a significant and persistent increase in the spinal cord of tumor-bearing rats. Taken together, these findings indicated that MHC-I could evoke CIBP by promoting apoptosis of GABAergic interneurons in the dorsal horn, and this apoptosis was closely related to local $CD8^+$ T cells.

Keywords: MHC-I Cancer induced bone pain GABAergic interneurons disinhibition $CD8^+$ T cells

Introduction

Cancer induced bone pain (CIBP) is a serious complication of bone sarcomas or other malignant tumors that metabolize to bone. It is reported that skeletal metastases affect more than 400,000 persons in the United States every year and among them about 75%–95% of patients experience CIBP (Jimenez-Andrade et al., 2010; Sabino and Mantyh, 2005). Patients with CIBP often experience moderate to severe pain and suffer from a poor quality of life (Jimenez-Andrade et al., 2010). Unfortunately, persistent pain is often difficult to treat effectively due to complicated underlying mechanisms (Payne et al., 1998). In recent years, significant progresses have been made in exploring the pathophysiology of CIBP via a variety of bone cancer animal models. Nonetheless, the detailed molecular mechanism of CIBP is still relatively unknown.

Chronic pain, such as neuropathic pain and CIBP, is characterized by mechanical allodynia and hyperalgesia. The transmission of nociceptive information from primary afferent neurons to higher cerebral centers is regulated through a balance between excitatory and inhibitory signals (Kuner, 2010). Recent studies have demonstrated that the decline of tonic and phasic inhibitory control or “disinhibition” in the dorsal horn accounts for the expansion of pain information which then induce hyperalgesia and allodynia (Sivilotti and Woolf, 1994; Yaksh, 1989). Since γ-aminobutyric acid (GABA) is a well-known major inhibitory neurotransmitter in the central nervous system (CNS), the downregulation of the GABA-releasing interneurons in the spinal cord has been thought to be responsible for chronic pain (Kingery et al., 1988; Xu et al., 1992; Yaksh, 1989). This was also evidenced by the reduction of the GABAergic interneurons after nerve and spinal injury (Thams et al.,

2008; Zhang et al., 1994) and the fact that intrathecal application of GABA analog gabapentin or GABA could alleviate chronic pain (Eaton et al., 1999; Matthews and Dickenson, 2002). We hypothesized that the loss of GABAergic interneurons leading to disinhibition in spinal dorsal horn could contribute to CIBP, however, the underlying mechanism was still unclear.

Accumulating evidence indicated that neuronal MHC-I molecule had great effects on neuronal signaling in the CNS (Corriveau et al., 1998; Glynn et al., 2011; Huh et al., 2000). Neurons could express high levels of MHC-I mRNA and/or protein in response to axotomy (Linda et al., 1998; Maehlen et al., 1988; Oliveira et al., 2004), cytokines treatments (Neumann et al., 1995; Victorio et al., 2012; Wong et al., 1984), and changes in electrical activities (Corriveau et al., 1998; Neumann et al., 1995). Recent studies have also indicated that MHC-I expression renders neurons vulnerable to $CD8^+$ T cells and finally result in neuron apoptosis in vivo (Medana et al., 2000) and cause Parkinson's disease (Cebrian et al., 2014). We hypothesized that MHC-I expressed on GABAergic interneurons could induce the apoptosis of GABAergic interneurons and involve in the development of CIBP.

To test this notion, short hairpin RNA lentivirus was constructed to knockdown the expression of MHC-I in spinal dorsal horn. A rat model of tumor cells intratibial injection was established to evaluate pain related behaviors. The apoptosis of GABAergic interneurons was detected by TUNEL staining. Cleaved (active) caspase-3 (Casp-3a) was examined to illustrate the apoptosis mechanisms. Flow cytometry and immunoblot were used to test the expression of $CD8^+$ T cells. Our result indicated that tumor cells implantation induced the upregulation of MHC-I on GABAergic interneurons and pain related behaviors which were reversed by MHC-I RNAi lentivirus. The apoptosis of GABAergic interneurons induced by

MHC-I in the spinal cord were also inhibited by MHC-I RNAi lentivirus. Additionally, we also found that $CD8^+$ T cells were colocalized with MHC-I and GABAergic neurons and presented a significant and persistent increase in the spinal cord of tumor-bearing rats. These findings indicated that MHC-I could evoke CIBP by promoting apoptosis of GABAergic interneurons in the dorsal horn, and this apoptosis was closely related to local $CD8^+$ T cells.

Materials and methods

Animals

According to the literatures(Helferich et al., 2008) (Bu et al., 2014; Guan et al., 2015; Ke et al., 2013; Liu et al., 2013; Xu et al., 2013; Yang et al., 2015), all experiments were performed on female adult Sprague-Dawley rats (180–230 g), provided by Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, PR China. r . All rats were habituated in a 22°C ± 0.5°C, relative humidity 40%–60%, a standard 12-h light/12-h dark cycle temperature- and humidity-controlled environment with abundant food and drinking. Rats were then randomly divided into four experimental groups (Sham, Tumor, Tumor+NC-LV, Tumor+RNAi-LV). Nontargeting control lentivirus (NC-LV) or β2-microglobulin RNAi-Lentivirus (RNAi-LV) were injected into the lumbar spinal cord of Tumor+NC-LV or Tumor+RNAi-LV rats respectively three days before the establishment of bone cancer rat model. The experimental procedures were implemented under the supervision of Experimental Animal Care and Use Committee of HUST and were in accordance with the guidelines published in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Construction of β2-microglobulin RNAi -Lentivirus

In accordance with previous reports, we selected β2-microglobulin as the siRNA target to knockdown the expression of MHC-I (Cebrian et al., 2014; Yang et al., 2007). Rat β2-microglobulin siRNA was generated by Shanghai GeneChem Co. according to these sequences: sense 5'-GTATATGCTTGCAGAGTTA -3' antisense 5'-TAACTCTGCAAGCATATAC-3'. And the nontargeting control lentivirus (NC-LV) was similar to β2-microglobulin RNAi –Lentivirus (RNAi –LV) except for expressing shRNAs.

Stereotaxic injection of virus in dorsal horn

NC-LV or RNAi-LV were injected into the lumbar spinal cord of Tumor+NC-LV or Tumor+RNAi-LV rats respectively three days before the establishment of bone cancer rat model. Injection of virus in the dorsal horn was prepared following the previous studies (Inquimbert et al., 2013; Meunier et al., 2007). Briefly, after deep anesthesia, cutaneous incisions were performed to expose the backbone under semi-sterile conditions. Then we found T13 and L1 vertebrae and performed L1 vertebra laminectomy to expose L3-L5 lumbar spinal cord. Two holes of 1 mm diameter (separated 2 mm) were cautiously drilled on the right 0.5mm besides the spinal cord midline. Then the piamater was opened, a glass capillary (35 ± 10mm diameter) filled with lentivirus solution was directed stereotaxically to the superficial laminae of the spinal dorsal horn (2μL/site; 0.5 μL/min). The needle was then left for 2 minutes in place before gentle withdrawal. After surgery, rats were kept in an insulation can for 30 min to recover from anesthesia before being returned to their separated cages.

Preparation of tumor cells

Walker 256 rat mammary gland carcinoma cells suspension (1ml, 1×10^7 cells/ml) was injected into the abdominal cavity of female rats. A week later, tumor cells were extracted and washed with D-Hanks solution. Cell suspension was adjusted to a concentration of 10^6 cells/ml. Tumor cells of identical final concentration that had been boiled for half an hour were used in sham groups.

Bone cancer model

Since CIBP is usually caused by various metastatic bone disease such as metastatic breast and prostate carcinomas. Thus, in this study, Walker 256 rat mammary gland carcinoma cells was used to build bone cancer model to mimic the clinic pathological process of CIBP. Three days after lentivirus injection, the bone cancer model was constructed using the above mentioned ascites tumor cells in accordance with previous studies (Huang et al., 2014; Shen et al., 2014). First, under deep anesthesia, superficial incisions were made to expose the tibia. A hole was cautiously drilled at the lower one third of the right tibia. Then a volume of 10 μL either containing Walker 256 cells (10^4 cells), or boiled cells was injected into the bone cavity with a 50 μL micro-syringe. The micro-syringe was then left for 1 minute in place to allow cells to fill the bone cavity. After that the hole was immediately closed with bone wax.

Behavioral studies

Pain related behaviors were measured on day 0 (before surgery), 3d, 6d, 9d, 12d, 15d,

18d, and 21d after intratibial injection of either tumor cells or boiled cells. Mechanical allodynia was evaluated using an Analgesy-Meter (Ugo Basile 37400, Italy), a programmed version of the von Frey hair as described before (Clark et al., 2007; Zhou et al., 2008a, b). Animals were kept in individual Plexiglas boxes with a wire mesh floor and accustomed for half an hour. A computer driven filament was located under the floor and focused on the hind paw. Continuous and incremental pressure was applied onto the plantar surface of the hind paw until the paw was withdrawn or the preset cut-off point was reached. A cut-off of 50g was set to prevent any tissue damage (Marchand et al., 2009; Thacker et al., 2009; Wodarski et al., 2009). The force in grams needed to induce paw withdrawal was named paw withdrawal threshold (PWT). Every rat model accepts the four values following the 5 minute interstimulus interval.

Isolation of mononuclear cells and flow cytometry

Rats were deeply anesthetized and perfused with ice-cold 1× Hanks' Balanced Salt Solution (HBSS), the spinal cords and lumbar local lymph nodes (LN) of sham and tumor-bearing rats were collected on day 7, 14d, and 21d post tumor cell inoculation. Then the tissues were homogenized and filtered through a 200-mesh sieve to obtain total cells suspensions. Mononuclear cells were isolated from the above mentioned cell suspensions by discontinuous Percoll gradients according to previously described procedures (Pino and Cardona, 2011). In brief, the cell pellet was resuspended in 7 mL 30% Percoll [one part of 10 × HBSS mixed with nine parts of Percoll solution (GE healthcare, USA)]. A disjointed Percoll gradient was achieved by adding 3 ml of 70% Percoll below and 8 ml of 30% Percoll

solution above. Samples were then centrifuged for 30 min at 500G 18°C without breaking. The resulting mononuclear cell part (30/70％ Percoll interface) was carefully collected and rinsed twice with 1× HBSS. The number of total mononuclear cells was counted using a hemacytometer with trypan blue before using a fluorescence activating cell sorter (FACS).

Monoclonal antibodies were purchased from Biolgend (CA, USA) and determined the optimal concentrations in preliminary trials. Approximately 100 μL of the cell suspensions derived from spinal cords and lumbar local LN, respectively, were double-labeled with Alexa Fluor 647-conjugated anti-rat CD3 and phycoerythrin (PE)-conjugated anti-rat CD8 for the detection of $CD8^+$ T cell. After incubating on ice for 30 min, the cell suspensions were washed twice with 0.1 M phosphate-buffered saline (PBS). All stained cells were finally resuspended in 2% paraformaldehyde /PBS and kept at 4 °C until analysis. About 10,000 events (for spinal cord mononuclear cells and cells from lumbar LN) were analyzed using an ACEA NovoCyte™ flow cytometer. Non-stained cells from identical tissue with identical procedures were used as negative control for data analysis.

Immunohistochemistry

After being deeply anesthetized, the animals' lumbar spinal cord on day 7, 14d, and 21d post tumor cells inoculation was rapidly removed, snap-frozen in 2-methylbutane (Sigma, USA) and stored at −80°C. For immunohistochemistry, cryostat sections of 15um were fixed in ice-cold acetone for 10 min. After increasing membrane permeability and blocking nonspecific binding, the slides were incubated for 24h at 4°C with primary antibodies: anti-GAD65 + GAD67 (1:100, ab11070; Abcam, Cambridge, UK), anti-MHC class I (1:100,

ab22367, OX-18; Abcam, Cambridge, UK), anti- Cleaved Caspase-3 (1:200, 9664s; Cell signaling Technology, CST), anti-CD8 (1:200, sc-7188; Santa Cruz),In Situ Apoptosis Fluorescein Detection Kit (**Cat.** S7111, Millipore). Except for cleaved caspase-3- slides which were treated by Alexa Fluor® 488-conjugated affiniPure fab fragment immunoglobulin G (Jackson ImmunoResearch Laboratories), all the other sections were treated with either Dylight-488 or Dylight-594-coupled secondary antibodies diluted in PBS for 2h at room temperature(RT).

Image acquisition and analysis were conducted using the Image-pro plus 6.0 software. About 9-16 images from 4 rats were collected and analyzed for each group within an experiment. For cell quantifications, positive cells were counted manually in 10000μm^2 of each section and 24 sections per sample from 4 rats were used.

Real-time PCR

Rats lumbar spinal cord were obtained on day 7, 14d, and 21d post tumor cells inoculation. Total RNA from the above tissues were extracted using Trizol Reagent (Invitrogen, USA). Samples were then synthesized to cDNA by reverse transcription. The following forward (F) and reverse (R) primers were used: β2-microglobulin: (F):5'ACCCTCATGGCTACTTCTGCTTT 3', (R): 5'CCTTTCTTAGTCCCTTTCCTCTG 3'; β–actin: sense primers (F):5' CACGATGGAGGGGCCGGACTCATC3', (R): 5'TAAAGACCTCTATGCCAACACAGT3'; the constitutively expressed *β*–actin gene was used as an internal control. Thermal cycling was completed on real-time PCR system (StepOne Plus, Applied Biosystems) at 95°C for 30 seconds followed by 45 cycles at 95°C

for 8 seconds and 60°C for 32 seconds.

Western blot analysis

Tissues of spinal lumbar enlargements (L4–L5) on day 7, 14d, and 21d post tumor cells inoculation were quickly removed and homogenized in ice-cold RIPA lysis buffer. Tissue lysates were collected and equal amounts of protein (30-60 mg) from each sample were separated in SDS/PAGE gel, and then transferred to polyvinylidene difluoride membranes (Roche, USA). After blocking in 5% Bovine Serum Albumin in Tris buffered saline with Tween 20 (TBST) buffer for 1.5h at RT, the membranes were incubated overnight at 4°C with primary antibodies: anti- GAD65 + GAD67 (1:1000, ab11070; Abcam, Cambridge, UK), anti-MHC class I (1:500, ab22367, OX-18; Abcam, Cambridge, UK), anti- Cleaved Caspase-3 (1:1000, 9664s; Cell signaling Technology, CST), anti-CD8 (1:500, sc-7188; Santa Cruz). Subsequently, blots were incubated with horseradish peroxidase-linked secondary antibodies for 40 min at RT. After extensive washing, protein bands were finally detected by electrochemiluminescence (ECL) solution (Pierce, USA) exposed to films. For densitometry analysis, blots were scanned and quantified with Scion Image free software (Meyer Instruments).

Statistical analyses

All data were analysed using SPSS (V16.0) and expressed as mean ± SEM. To analyze the differences among groups, one-way repeated measures analysis of variance or two-way analysis of variance were used followed by the Bonferroni post-hoc test. Significance was

indicated by $P < 0.05$.

Results

Elevation of MHC-I in the spinal dorsal horn initiated by tumor cells implantation

To investigate whether MHC-I was involved in the development of CIBP, we tested the location and expression of MHC-I in the lumbar dorsal horn by double immunofluorescence with MHC-I and GABAergic interneurons marker GAD65+67. Immunostaining results showed that MHC-I predominantly expressed on GABAergic interneurons in the dorsal horn of tumor-bearing rats (Fig.1a). Immunostaining analysis showed that GABAergic interneurons were significantly decreased in a time-dependent manner while MHC-I was increased significantly, reaching a peak at day 7 post tumor cells inoculation (Fig.1b). However, the upregulation of MHC-I on GABAergic interneurons in the dorsal horn of tumor-bearing rats was reversed by pretreatment with reconstructed β2-microglobulinRNAi-lentivirus (RNAi- LV), but not NC-LV. (Fig. 1a-b). Lentivirus transfection was confirmed by the expression of enhanced green fluorescent protein (EGFP) in spinal dorsal horn (Fig. 1c). Quantification analysis of β2-microglobulin mRNA (represent for MHC-I mRNA) and MHC-I protein agreed with the result of immunofluorescence. Pretreatment with RNAi-LV prevented the increase of MHC-I mRNA and protein in the spinal cord (Fig. 1d-e). Together, these results suggested change of MHC-I expression associated with CIBP and that specific RNAi-LV could efficiently decline MHC-I expression.

MHC-I was essential for the development of CIBP

To mimic clinical CIBP, a rat model of CIBP was established by walker256 mammary gland carcinoma cells intratibial injection. The behavior tests were performed every three days after surgery. PWT was tested as a measurement of mechanical allodynia. , Tumor-bearing rats showed a persistent decline of PWT during the development of CIBP (n=6, $*P$ <0.05). To examine whether MHC-I was involved in CIBP, RNAi–LV was injected into the lumbar spinal cord three days before the establishment of bone cancer rat model. Our data indicated RNAi-LV, instead of NC-LV, attenuated mechanical allodynia initiated by tumor cells injection ((n=6, $^{\#}P < 0.05$). Therefore, behavioral test data suggested that MHC-I contributed to CIBP.

MHC-I induced apoptosis of GABAergic interneurons in spinal dorsal horn

To determine whether the apoptosis of GABAergic interneurons was involved in the development of CIBP, we first examined the presence of apoptosis cells by terminal deoxynucleotidyl transferase (TdT)-mediated DNA nick-end labeling (TUNEL) staining in dorsal horn of tumor-bearing rats. TUNEL labeling revealed a large number of TUNEL-positive cells in the spinal dorsal horn of tumor-bearing rats which was prevented by pretreatment of RNAi-LV (Fig. 3a, 3c). To investigate whether the apoptosis cells were GABAergic interneurons, we performed fluorescent double-labeling with GAD and TUNEL. The results showed thatTUNEL staining was localized in the GABAergic interneurons. The merged picture indicated the apoptosis of GABAergic interneurons in the dorsal horn of tumor-bearing rats (Fig.3b). Quantitative analysis of GAD protein indicated that GABAergic interneurons decreased in a time dependent manner in tumor-bearing rats which was

prevented by RNAi-LV (Fig. 3d). These results suggested that MHC-I induced apoptosis of GABAergic interneurons in the spinal cord of tumor-bearing rats.

Apoptosis of GABAergic interneurons via caspase-3 activation

To demonstrate whether the initiation of apoptosis was associated with the executioner caspase-3 in the dorsal horn, we assayed the co-localization of cleaved (active) caspase-3 (Casp-3a) and GAD in the spinal dorsal horn. The Casp-3a immunoreactivity was localized in GABAergic interneurons and markedly elevated in the spinal cord of tumor-bearing rats which was reversed by RNAi-LV, but not NC-LV (Fig. 4a, 4c). We then performed fluorescent double-labeling with Casp-3a and TUNEL in the dorsal horn. The merged picture showed that TUNEL staining was localized in the Casp-3a positive cells (Fig.4b). Combined with the previous double staining of GAD and TUNEL, our results indicated that the apoptosis of GABAergic interneurons was mediated by caspase-3 activation. Quantitative analysis of Casp-3a protein indicated that Casp-3a increased in a time dependent manner in tumor-bearing rats which was prevented by RNAi-LV (Fig. 4d). These findings suggested that the apoptosis of GABAergic interneurons induced by MHC-I was dependent on active caspase-3.

Apoptosis of GABAergic interneurons induced by MHC-I was closely related to $CD8^+$ T cells.

It has been reported that MHC-I induced neuronal apoptosis is closely associated with $CD8^+$ T cells in vivo (Cebrian et al., 2014; Sanchez-Ruiz et al., 2008). To investigate whether

$CD8^+$ T cells were increased in the development of CIBP, we used flow cytometry to check the expression of $CD8^+$ T lymphocytes by double labeling CD3 and CD8 with mononuclear cells extracted from the tumor-bearing rats lumbar spinal cord. The number of $CD8^+$ T cells ($CD3^+$ $CD8^+$) showed a persistent increase in the spinal cord of tumor-bearing rats (Fig. 1a-b), which was in parallel with the continuous decrease of GABAergic interneurons. Phenotypic characterization of tumor-bearing rats' spinal cord lymphocytes showed that $CD8^+$ T cells ($CD3^+CD8^+$) composed of 74% ± 2.8% among total $CD3^+$ T cells, while $CD4^+$ T cells ($CD3^+CD4^+$) constituted 24% ± 2.3% of all $CD3^+$ T cells (Fig. 5a). The 2.8 to 1 ratio of $CD8^+$ T cells to $CD4^+$ T cells in the spinal cord contrasted with the constant 1 to 1.89 ratio detected in the lumber lymph nodes in the same tumor-bearing rats, indicating that a larger percentage of $CD8^+$ T cells infiltrated into the spinal cord during the pathological process of CIBP (Fig. 5c). The western blot confirmed this result (Fig. 5d). Immunofluorescent staining of CD8 showed that $CD8^+$ T cells expressed in the spinal dorsal horn of tumor-bearing rats (Fig. 5d). To investigate whether MHC-I inducing apoptosis of GABAergic interneurons was related to $CD8^+$ T cells, we performed double immunostaining with CD8 and MHC-I as well as double immunostaining with CD8 and GABAergic neurons. The results indicated $CD8^+$ T cells were colocalized with MHC-I and GABAergic neurons.. Taken together, these findings suggested apoptosis of GABAergic interneurons induced by MHC-I was closely related to $CD8^+$ T cells..

Discussion

In the present study, we showed that MHC-I expression increased on GABAergic

interneurons in the spinal dorsal horn of tumor-bearing rats. Increased expression of MHC-I could deplete GABAergic interneurons in the spinal cord by promoting its apoptosis via caspase-3 signaling pathway. Pretreatment of RNAi-LV could prevent the apoptosis of GABAergic interneurons in the spinal cord and therefore reverse mechanical allodynia of tumor-bearing rats. Since previous studies revealed that MHC-I induced neuronal apoptosis was closely associated with $CD8^+$ T cells in vivo (Cebrian et al., 2014; Medana et al., 2000), we next showed that $CD8^+$ T cells were markedly elevated in the spinal cord of tumor-bearing rats and were colocalized with MHC-I and GABAergic interneurons. The persistent increase of $CD8^+$ T cells was in parallel with the continuous decrease of GABAergic interneurons. Taken together, these results demonstrated that MHC-I could evoke CIBP by promoting apoptosis of GABAergic interneurons in the spinal cord and this apoptosis is related to $CD8^+$ T cells in the CNS.

The MHC genes can approximately be separated into three major groups (class I –III), among which MHC class I exhibits an amazing polymorphism (Thams et al., 2008). MHC-I molecules are trimeric proteins which consist of a transmembrane heavy chain, a soluble β2-microglobulin light chain, and a peptide bound to the heavy chain. Since β2m is required for stable cell-surface expression of most MHC-I proteins, the lack of β2m could make MHC-I detach from the cell surface (Chacon and Boulanger, 2013). Thus in our study, β2-microglobulin RNAi–LV was constructed to effectively deplete the expression of MHC-I. Despite the CNS “immune privilege”, there is now clear evidence for a high-level expression of MHC-I genes in the CNS, and a significant role of certain class I molecules in neuronal differentiation, brain development, selective maintenance of synapses, as well as influence of

MHC-I antigens on behavior (Huh et al., 2000; Lidman et al., 1999). Furthermore, neuronal MHC-I has also participated in several pathological conditions such as injury (Maehlen et al., 1988), infection and treatment with cytokines (Victorio et al., 2012). This evidence suggests that MHC-I plays a vital role in both physiological and pathological processes in the CNS. In this study, we found that MHC-I was markedly elevated in the spinal cord in tumor-bearing rats. Knockdown expression of MHC-I attenuated mechanical allodynia initiated by tumor cell implantation. These findings implied that MHC-I had a significant effect on mediating CIBP.

Expression of MHC-I molecules on the plasma membrane is the key prerequisite for antigen presentation and for activation of inhibitory or stimulatory natural killer (NK) cell receptors. It has been shown that MHC-I expression in dissociated hippocampal neurons can be targeted by cytotoxic T cells (CTLs) and subsequently induce neuronal apoptosis (Medana et al., 2000). This is further demonstrated in various classic neurodegenerative disorders such as Amyotrophic lateral sclerosis (Holmoy, 2008), Alzheimer's disease (Itagaki et al., 1988; Town et al., 2005) and Parkinson's disease that the expression of MHC-I can lead to a substantial loss of neurons. In the present study, we showed upregulation of MHC-I expressed on GABAergic interneurons in the dorsal horn on day 7 post operation. With the increase of MHC-I, we found a significant depletion of GABAergic interneurons. Then, due to the loss of GABAergic interneurons, MHC-I expression showed a slow decrease on day 14 post operation. However, the decrease of GABAergic interneurons can be alleviated by specific knockdown of MHC-I in tumor-bearing rats. These results suggest that MHC-I expression on GABAergic interneurons contributes to the loss of GABAergic interneurons in

tumor-bearing rats.

Millan (Millan, 2002) postulated that GABAergic interneurons are a critical part of the descending inhibitory system in the superficial laminae. The loss of inhibitory tone, or loss of modulation by the GABA-releasing interneurons could result in different types of chronic pain (Kingery et al., 1988; Xu et al., 1992; Yaksh, 1989). In this study, we showed that TUNEL staining was localized in GABAergic interneurons. The result indicated that the loss of GABAergic interneurons was caused by neurons apoptosis. We next investigated the mechanism of GABAergic interneurons apoptosis induced by MHC-I. Since caspase-3 activation is an important mediator in the process of apoptosis, we detected the change of cleaved caspase-3 during the development of CIBP. Our results revealed that casp-3a immunoreactivity was localized in GABAergic interneurons and TUNEL positive cells in the spinal cord. Knockdown of MHC-I inhibited the activation of cleaved caspase-3 and prevented the apoptosis of GABAergic interneurons. These combined evidences, indicated that MHC-I contributed to CIBP by promoting apoptosis of GABAergic interneurons via active caspase-3.

Emerging evidence has highlighted that MHC-I induced neuronal apoptosis is closely associated with $CD8^+$ T cells in vivo (Cebrian et al., 2014; Sanchez-Ruiz et al., 2008). In fact, recently, a body of reports has uncovered the role of CTLs in various autoimmune and infectious inflammatory CNS disorders (Friese and Fugger, 2005; Sauer et al., 2013). In these disorders, $CD8^+$ T cells are considered significant effector cells resulting in neuronal damage. Previous studies demonstrated that peripheral immune cells, including T-cells and macrophages, are likely to be recruited by chemoattraction, with the leukocytes traveling

across concentration gradients produced by chemokines and cytokines in various pathological processes (Cao et al., 2014; Jiang et al., 2016; Sapienza et al., 2014; Wu et al., 2015). Interestingly, our previous study verified that the chemokines CXCL10 and its receptor CXCR3 were markedly elevated in CIBP (Guan et al., 2015; Ye et al., 2014; Zhou et al., 2015). Moreover, recent studies also demonstrated that CXCR3 could express on the surface of $CD8^+$ T cells (Laragione et al., 2011; Tokuriki et al., 2002). The increase of CXCL10 and CXCR3 in CIBP may provide a prerequisite for the existence of $CD8^+$ T lymphocytes in the CNS. Thus, in this study, we clearly detected $CD8^+$ T cells in thelumbar spinal cord of tumor-bearing rats.. Interestingly, the sustained increase of $CD8^+$ T cells in the spinal dorsal horn was in parallel with the decrease of GABAergic interneurons. Moreover, $CD8^+$ T cells were colocalized with MHC-I and GABAergic neurons. Together with our current findings, these results indicated that MHC-I inducing apoptosis of GABAergic interneurons was closely related to $CD8^+$ T cells.

In conclusion, this study has proposed an immunologically-based mechanism involved in CIBP. MHC-I is a critical molecule for the process of antigen presentation. Expression of MHC-I molecules on the neuron membrane is the key prerequisite for $CD8^+$ T cells recognizing and attacking neurons. $CD8^+$ T cells could identify neuronal MHC-I and finally lead to neuron death through Fas/Fas ligand and/or perforin/granzyme pathways (Cebrian et al., 2014; Medana et al., 2000; Sauer et al., 2013). And it is also known that no neuronal death was triggered in MHC-I knockout mice even with the existence of $CD8^+$ T cells (Cebrian et al., 2014). In the present study, we provided evidence that MHC-I inhibited GABAergic interneurons by promoting its apoptosis and contributed to the development of

CIBP.This effect was closely related to local $CD8^+$ T cells. However, further studies of detailed functions of $CD8^+$ T cells in CIBP are necessary before definite conclusions can be drawn.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (NO. 81571053, 81400917, 81371250). The authors gratefully acknowledge Zhijun Zhang, PHD for his technical assistance in this study.

References

Bu, H., Shu, B., Gao, F., Liu, C., Guan, X., Ke, C., Cao, F., Hinton, A.O., Jr., Xiang, H., Yang, H., Tian, X., Tian, Y., 2014. Spinal IFN-gamma-induced protein-10 (CXCL10) mediates metastatic breast cancer-induced bone pain by activation of microglia in rat models. Breast cancer research and treatment 143, 255-263.

Cao, D.L., Zhang, Z.J., Xie, R.G., Jiang, B.C., Ji, R.R., Gao, Y.J., 2014. Chemokine CXCL1 enhances inflammatory pain and increases NMDA receptor activity and COX-2 expression in spinal cord neurons via activation of CXCR2. Experimental neurology 261, 328-336.

Cebrian, C., Zucca, F.A., Mauri, P., Steinbeck, J.A., Studer, L., Scherzer, C.R., Kanter, E., Budhu, S., Mandelbaum, J., Vonsattel, J.P., Zecca, L., Loike, J.D., Sulzer, D., 2014. MHC-I expression renders catecholaminergic neurons susceptible to T-cell-mediated degeneration. Nature communications 5, 3633.

Chacon, M.A., Boulanger, L.M., 2013. MHC class I protein is expressed by neurons and neural progenitors in mid-gestation mouse brain. Molecular and cellular neurosciences 52, 117-127.

Clark, A.K., Yip, P.K., Grist, J., Gentry, C., Staniland, A.A., Marchand, F., Dehvari, M., Wotherspoon, G., Winter, J., Ullah, J., Bevan, S., Malcangio, M., 2007. Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proceedings of the National Academy of Sciences of the United States of America 104, 10655-10660.

Corriveau, R.A., Huh, G.S., Shatz, C.J., 1998. Regulation of class I MHC gene expression in the developing and mature CNS by neural activity. Neuron 21, 505-520.

Eaton, M.J., Martinez, M.A., Karmally, S., 1999. A single intrathecal injection of GABA permanently reverses neuropathic pain after nerve injury. Brain research 835, 334-339.

Friese, M.A., Fugger, L., 2005. Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? Brain : a journal of neurology 128, 1747-1763.

Glynn, M.W., Elmer, B.M., Garay, P.A., Liu, X.B., Needleman, L.A., El-Sabeawy, F., McAllister, A.K., 2011. MHCI negatively regulates synapse density during the establishment of cortical connections. Nature neuroscience 14, 442-451.

Guan, X.H., Fu, Q.C., Shi, D., Bu, H.L., Song, Z.P., Xiong, B.R., Shu, B., Xiang, H.B., Xu, B., Manyande, A., Cao, F., Tian, Y.K., 2015. Activation of spinal chemokine receptor CXCR3

mediates bone cancer pain through an Akt-ERK crosstalk pathway in rats. Experimental neurology 263, 39-49.

Helferich, W.G., Andrade, J.E., Hoagland, M.S., 2008. Phytoestrogens and breast cancer: a complex story. Inflammopharmacology 16, 219-226.

Holmoy, T., 2008. T cells in amyotrophic lateral sclerosis. European journal of neurology : the official journal of the European Federation of Neurological Societies 15, 360-366.

Huang, Z.X., Lu, Z.J., Ma, W.Q., Wu, F.X., Zhang, Y.Q., Yu, W.F., Zhao, Z.Q., 2014. Involvement of RVM-expressed P2X7 receptor in bone cancer pain: mechanism of descending facilitation. Pain 155, 783-791.

Huh, G.S., Boulanger, L.M., Du, H., Riquelme, P.A., Brotz, T.M., Shatz, C.J., 2000. Functional requirement for class I MHC in CNS development and plasticity. Science 290, 2155-2159.

Inquimbert, P., Moll, M., Kohno, T., Scholz, J., 2013. Stereotaxic injection of a viral vector for conditional gene manipulation in the mouse spinal cord. Journal of visualized experiments : JoVE, e50313.

Itagaki, S., McGeer, P.L., Akiyama, H., 1988. Presence of T-cytotoxic suppressor and leucocyte common antigen positive cells in Alzheimer's disease brain tissue. Neuroscience letters 91, 259-264.

Jiang, B.C., Cao, D.L., Zhang, X., Zhang, Z.J., He, L.N., Li, C.H., Zhang, W.W., Wu, X.B., Berta, T., Ji, R.R., Gao, Y.J., 2016. CXCL13 drives spinal astrocyte activation and neuropathic pain via CXCR5. The Journal of clinical investigation 126, 745-761.

Jimenez-Andrade, J.M., Mantyh, W.G., Bloom, A.P., Ferng, A.S., Geffre, C.P., Mantyh, P.W.,

2010. Bone cancer pain. Annals of the New York Academy of Sciences 1198, 173-181.

Ke, C., Li, C., Huang, X., Cao, F., Shi, D., He, W., Bu, H., Gao, F., Cai, T., Hinton, A.O., Jr., Tian, Y., 2013. Protocadherin20 promotes excitatory synaptogenesis in dorsal horn and contributes to bone cancer pain. Neuropharmacology 75, 181-190.

Kingery, W.S., Fields, R.D., Kocsis, J.D., 1988. Diminished dorsal root GABA sensitivity following chronic peripheral nerve injury. Experimental neurology 100, 478-490.

Kuner, R., 2010. Central mechanisms of pathological pain. Nature medicine 16, 1258-1266.

Laragione, T., Brenner, M., Sherry, B., Gulko, P.S., 2011. CXCL10 and its receptor CXCR3 regulate synovial fibroblast invasion in rheumatoid arthritis. Arthritis Rheum 63, 3274-3283.

Lidman, O., Olsson, T., Piehl, F., 1999. Expression of nonclassical MHC class I (RT1-U) in certain neuronal populations of the central nervous system. The European journal of neuroscience 11, 4468-4472.

Linda, H., Hammarberg, H., Cullheim, S., Levinovitz, A., Khademi, M., Olsson, T., 1998. Expression of MHC class I and beta2-microglobulin in rat spinal motoneurons: regulatory influences by IFN-gamma and axotomy. Experimental neurology 150, 282-295.

Liu, S., Liu, Y.P., Song, W.B., Song, X.J., 2013. EphrinB-EphB receptor signaling contributes to bone cancer pain via Toll-like receptor and proinflammatory cytokines in rat spinal cord. Pain 154, 2823-2835.

Maehlen, J., Schroder, H.D., Klareskog, L., Olsson, T., Kristensson, K., 1988. Axotomy induces MHC class I antigen expression on rat nerve cells. Neuroscience letters 92, 8-13.

Marchand, F., Tsantoulas, C., Singh, D., Grist, J., Clark, A.K., Bradbury, E.J., McMahon, S.B., 2009. Effects of Etanercept and Minocycline in a rat model of spinal cord injury.

European journal of pain 13, 673-681.

Matthews, E.A., Dickenson, A.H., 2002. A combination of gabapentin and morphine mediates enhanced inhibitory effects on dorsal horn neuronal responses in a rat model of neuropathy. Anesthesiology 96, 633-640.

Medana, I.M., Gallimore, A., Oxenius, A., Martinic, M.M., Wekerle, H., Neumann, H., 2000. MHC class I-restricted killing of neurons by virus-specific CD8+ T lymphocytes is effected through the Fas/FasL, but not the perforin pathway. European journal of immunology 30, 3623-3633.

Meunier, A., Latremoliere, A., Dominguez, E., Mauborgne, A., Philippe, S., Hamon, M., Mallet, J., Benoliel, J.J., Pohl, M., 2007. Lentiviral-mediated targeted NF-kappaB blockade in dorsal spinal cord glia attenuates sciatic nerve injury-induced neuropathic pain in the rat. Molecular therapy : the journal of the American Society of Gene Therapy 15, 687-697.

Millan, M.J., 2002. Descending control of pain. Progress in neurobiology 66, 355-474.

Neumann, H., Cavalie, A., Jenne, D.E., Wekerle, H., 1995. Induction of MHC class I genes in neurons. Science 269, 549-552.

Oliveira, A.L., Thams, S., Lidman, O., Piehl, F., Hokfelt, T., Karre, K., Linda, H., Cullheim, S., 2004. A role for MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy. Proceedings of the National Academy of Sciences of the United States of America 101, 17843-17848.

Payne, R., Mathias, S.D., Pasta, D.J., Wanke, L.A., Williams, R., Mahmoud, R., 1998. Quality of life and cancer pain: satisfaction and side effects with transdermal fentanyl versus oral morphine. Journal of clinical oncology : official journal of the American Society of

Clinical Oncology 16, 1588-1593.

Pino, P.A., Cardona, A.E., 2011. Isolation of brain and spinal cord mononuclear cells using percoll gradients. Journal of visualized experiments : JoVE.

Sabino, M.A., Mantyh, P.W., 2005. Pathophysiology of bone cancer pain. The journal of supportive oncology 3, 15-24.

Sanchez-Ruiz, M., Wilden, L., Muller, W., Stenzel, W., Brunn, A., Miletic, H., Schluter, D., Deckert, M., 2008. Molecular mimicry between neurons and an intracerebral pathogen induces a CD8 T cell-mediated autoimmune disease. J Immunol 180, 8421-8433.

Sapienza, A., Reaux-Le Goazigo, A., Rostene, W., Melik-Parsadaniantz, S., 2014. [Chemokines and attraction of myeloid cells in peripheral neuropathic pains]. Biologie aujourd'hui 208, 31-44.

Sauer, B.M., Schmalstieg, W.F., Howe, C.L., 2013. Axons are injured by antigen-specific CD8(+) T cells through a MHC class I- and granzyme B-dependent mechanism. Neurobiology of disease 59, 194-205.

Shen, W., Hu, X.M., Liu, Y.N., Han, Y., Chen, L.P., Wang, C.C., Song, C., 2014. CXCL12 in astrocytes contributes to bone cancer pain through CXCR4-mediated neuronal sensitization and glial activation in rat spinal cord. Journal of neuroinflammation 11, 75.

Sivilotti, L., Woolf, C.J., 1994. The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. Journal of neurophysiology 72, 169-179.

Thacker, M.A., Clark, A.K., Bishop, T., Grist, J., Yip, P.K., Moon, L.D., Thompson, S.W., Marchand, F., McMahon, S.B., 2009. CCL2 is a key mediator of microglia activation in

neuropathic pain states. European journal of pain 13, 263-272.

Thams, S., Oliveira, A., Cullheim, S., 2008. MHC class I expression and synaptic plasticity after nerve lesion. Brain research reviews 57, 265-269.

Tokuriki, A., Seo, N., Ito, T., Kumakiri, M., Takigawa, M., Tokura, Y., 2002. Dominant expression of CXCR3 is associated with induced expression of IP-10 at hapten-challenged sites of murine contact hypersensitivity: a possible role for interferon-gamma-producing CD8(+) T cells in IP-10 expression. J Dermatol Sci 28, 234-241.

Town, T., Tan, J., Flavell, R.A., Mullan, M., 2005. T-cells in Alzheimer's disease. Neuromolecular medicine 7, 255-264.

Victorio, S.C., Cartarozzi, L.P., Hell, R.C., Oliveira, A.L., 2012. Decreased MHC I expression in IFN gamma mutant mice alters synaptic elimination in the spinal cord after peripheral injury. Journal of neuroinflammation 9, 88.

Wodarski, R., Clark, A.K., Grist, J., Marchand, F., Malcangio, M., 2009. Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats. European journal of pain 13, 807-811.

Wong, G.H., Bartlett, P.F., Clark-Lewis, I., Battye, F., Schrader, J.W., 1984. Inducible expression of H-2 and Ia antigens on brain cells. Nature 310, 688-691.

Wu, C.H., Yuan, X.C., Gao, F., Li, H.P., Cao, J., Liu, Y.S., Yu, W., Tian, B., Meng, X.F., Shi, J., Pan, H.L., Li, M., 2015. Netrin-1 Contributes to Myelinated Afferent Fiber Sprouting and Neuropathic Pain. Molecular neurobiology.

Xu, Q., Zhang, X.M., Duan, K.Z., Gu, X.Y., Han, M., Liu, B.L., Zhao, Z.Q., Zhang, Y.Q., 2013. Peripheral TGF-beta1 signaling is a critical event in bone cancer-induced hyperalgesia

in rodents. The Journal of neuroscience : the official journal of the Society for Neuroscience 33, 19099-19111.

Xu, X.J., Hao, J.X., Aldskogius, H., Seiger, A., Wiesenfeld-Hallin, Z., 1992. Chronic pain-related syndrome in rats after ischemic spinal cord lesion: a possible animal model for pain in patients with spinal cord injury. Pain 48, 279-290.

Yaksh, T.L., 1989. Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: effects of modulatory receptor systems and excitatory amino acid antagonists. Pain 37, 111-123.

Yang, J., Zhang, X., Wang, J., Qian, J., Zhang, L., Wang, M., Kwak, L.W., Yi, Q., 2007. Anti beta2-microglobulin monoclonal antibodies induce apoptosis in myeloma cells by recruiting MHC class I to and excluding growth and survival cytokine receptors from lipid rafts. Blood 110, 3028-3035.

Yang, Y., Li, H., Li, T.T., Luo, H., Gu, X.Y., Lu, N., Ji, R.R., Zhang, Y.Q., 2015. Delayed activation of spinal microglia contributes to the maintenance of bone cancer pain in female Wistar rats via P2X7 receptor and IL-18. The Journal of neuroscience : the official journal of the Society for Neuroscience 35, 7950-7963.

Ye, D., Bu, H., Guo, G., Shu, B., Wang, W., Guan, X., Yang, H., Tian, X., Xiang, H., Gao, F., 2014. Activation of CXCL10/CXCR3 signaling attenuates morphine analgesia: involvement of Gi protein. Journal of molecular neuroscience : MN 53, 571-579.

Zhang, A.L., Hao, J.X., Seiger, A., Xu, X.J., Wiesenfeld-Hallin, Z., Grant, G., Aldskogius, H., 1994. Decreased GABA immunoreactivity in spinal cord dorsal horn neurons after transient spinal cord ischemia in the rat. Brain research 656, 187-190.

Zhou, Q., Price, D.D., Caudle, R.M., Verne, G.N., 2008a. Visceral and somatic hypersensitivity in a subset of rats following TNBS-induced colitis. Pain 134, 9-15.

Zhou, Q., Price, D.D., Caudle, R.M., Verne, G.N., 2008b. Visceral and somatic hypersensitivity in TNBS-induced colitis in rats. Dig Dis Sci 53, 429-435.

Zhou, Y.Q., Gao, H.Y., Guan, X.H., Yuan, X., Fang, G.G., Chen, Y., Ye, D.W., 2015. Chemokines and Their Receptors: Potential Therapeutic Targets for Bone Cancer Pain. Curr Pharm Des 21, 5029-5033.

Figure Captions

Fig.1. Upregulation of MHC-I in spinal cord after cancer development. (a). Double immunostain with MHC-I (green) and GABAergic interneurons marker GAD (red) in the dorsal horn of sham, tumor, tumor+NC-LV, tumor+RNAi-LV rats on day 14 post tumor cells implantation. Amplified pictures with white squares (upper right) showed the co-localization of MHC-I (green) and GAD (red). Scale bar: 100μm.(b) Quantification of MHC-I positive cells, GABAergic interneurons on day 7, 14d and 21d after tumor cells implatation. Data are expressed as mean ± s.e.m, n=4, $*P < 0.05$, versus sham group; $^{\#}P < 0.05$, versus Tumor+NC-LV rats.Scale bar: 200μm. (d) Quantified data of β2-microglobulin mRNA by Real-time PCR in the dorsal horn of different groups. Data are expressed as mean ± s.e.m, n=5, $*P < 0.05$, versus sham group; $^{\#}P < 0.05$, versus Tumor+NC-LV rats. (e) Immunoblot analyses show time course of MHC-I protein in the dorsal horn of different groups post-sarcoma implantation. Data are expressed as mean ± s.e.m, n = 4, $*P < 0.05$, versus sham group; $^{\#}P < 0.05$, versus Tumor+NC-LV rats. GAD: Glutamate decarboxylase 65+67; EGFP: enhanced green fluorescent protein. (TIFF format, 500 dpi, 1.5-column fitting image)

Fig.2. Inhibition of MHC-I by specific β2-microglobulin shRNA attenuated mechanical allodynia initiated by sarcoma implantation. Mechanical allodynia were measured by paw withdrawal threshold (PWT) before and every 3 days after surgery. Application of β2-microglobulin RNAi–Lentivirus showed the reverse of the decline in paw withdrawal threshold induced by CIBP.. Data are expressed as mean ± s.e.m, n = 6, $*P < 0.05$, versus sham; $^{\#}P < 0.05$, versus Tumor+NC-LV rats. (TIFF format, 1000 dpi, single column fitting

image)

Fig.3. MHC-I induced GABAergic neuronal apoptosis and reduced GAD levels in cancer bearing rats. (a) Immunofluorescence showed the expression of TUNEL positive cells (green) in the spinal dorsal horn in sham, tumor, tumor+NC-LV and tumor+RNAi-LV rats on day 14 post tumor cells implantation. Scale bar: 100μm. (b) TUNEL (green), combined with immunostaining for GABAergic neuronal marker GAD (red), revealed the apoptosis of GABAergic interneurons (white arrow) in spinal dorsal horn at day 14 after cancer implantation. Scale bar: 25μm. (c) Quantification of TUNEL positive cells at days 7, 14 and 21 post-sarcoma inoculation. Data are expressed as mean ± s.e.m, n=6, $*P < 0.05$, versus sham; $^{\#}P < 0.05$, versus Tumor+NC-LV. (d) Immunoblot analyses show the time course of GAD protein in the dorsal horn of different groups after tumor cells injection. Data are expressed as mean ± s.e.m, n=4, $*P < 0.05$, versus sham; $^{\#}P < 0.05$, versus Tumor+NC-LV.. TUNEL:terminal deoxynucleotidyl transferase (TdT)-mediated DNA nick-end labeling; GAD:Glutamate decarboxylase 65+67. (TIFF format, 500 dpi, 1.5-column fitting image)

Fig.4. Apoptosis of GABAergic interneurons via caspase-3 activation. (a) Double immunostain with active GABAergic interneurons marker GAD (red) and Casp-3a (green) in the dorsal horn of sham, tumor, tumor+NC-LV, tumor+RNAi-LV rats on day 14 post tumor cells implantation. Amplified pictures with white squares (upper right) showed the co-localization of GAD (red) and Casp-3a (green). Scale bar: 100μm. (b) Casp-3a (green), combined with Tunel positive cells (red), revealed Caspase-3 activation in TUNEL positive

cells (white arrow). Scale bar: 25μm. (c) Quantification of Casp-3a positive cells at days 7, 14 and 21 in the dorsal horn of tumor-bearing rats. Data are expressed as mean ± s.e.m, n=4, $*P < 0.05$, versus sham; $^{\#}P < 0.05$, versus Tumor+NC-LV. (d) Immunoblot analyses show the time course of casp-3a protein in the dorsal horn of different groups post-sarcoma implantation. Data are expressed as mean ± s.e.m, n=4, $*P < 0.05$, versus sham; $^{\#}P < 0.05$, versus Tumor+NC-LV. Casp-3a: cleaved (active) caspase-3; GAD: Glutamate decarboxylase 65+67. (TIFF format, 500 dpi, 1.5-column fitting image)

Fig.5. Apoptosis of GABAergic interneurons induced by MHC-I was closely related to $CD8^+$ T cells. (a) FACS show representative dot plot examples of CD3 and CD8 expression in the monocytes extracted from the spinal cord of the sham group and tumor group as well as the lymph nodes in tumor-bearing rats. (b) Quantification of $CD8^+$ T cells at days 7, 14d and 21d in tumor- bearing rats by FACS. Data are expressed as mean ± s.e.m, n=4, $*P < 0.05$, versus sham. (c) Percentages of $CD8^+$ versus $CD4^+$ among $CD3^+$ T cells purified from the spinal cord or the lumbar lymph nodes of tumor-bearing rats on day 14 post tumor cells implantation. Data are expressed as mean ± s.e.m, n=4, $^{\#}P < 0.05$, versus lumbar lymph nodes. (d) Western blot analyses and immunofluorescence results showed up-regulation of $CD8^+$ T cells in spinal dorsal horn in tumor-bearing rats. Scale bar: 100μm. Data are expressed as mean ± s.e.m, n=4, $*P < 0.05$, versus sham. (e) Double-labeling experiments revealed that $CD8^+$ T cells were colocalized with MHC-I and GABAergic neurons in the spinal dorsal horn of tumor-bearing rats on day 14 post tumor cells inoculation. Scale bar: 50μm. FACS: fluorescence activated cell sorter. (TIFF format, 500 dpi, 1.5-column fitting

image)

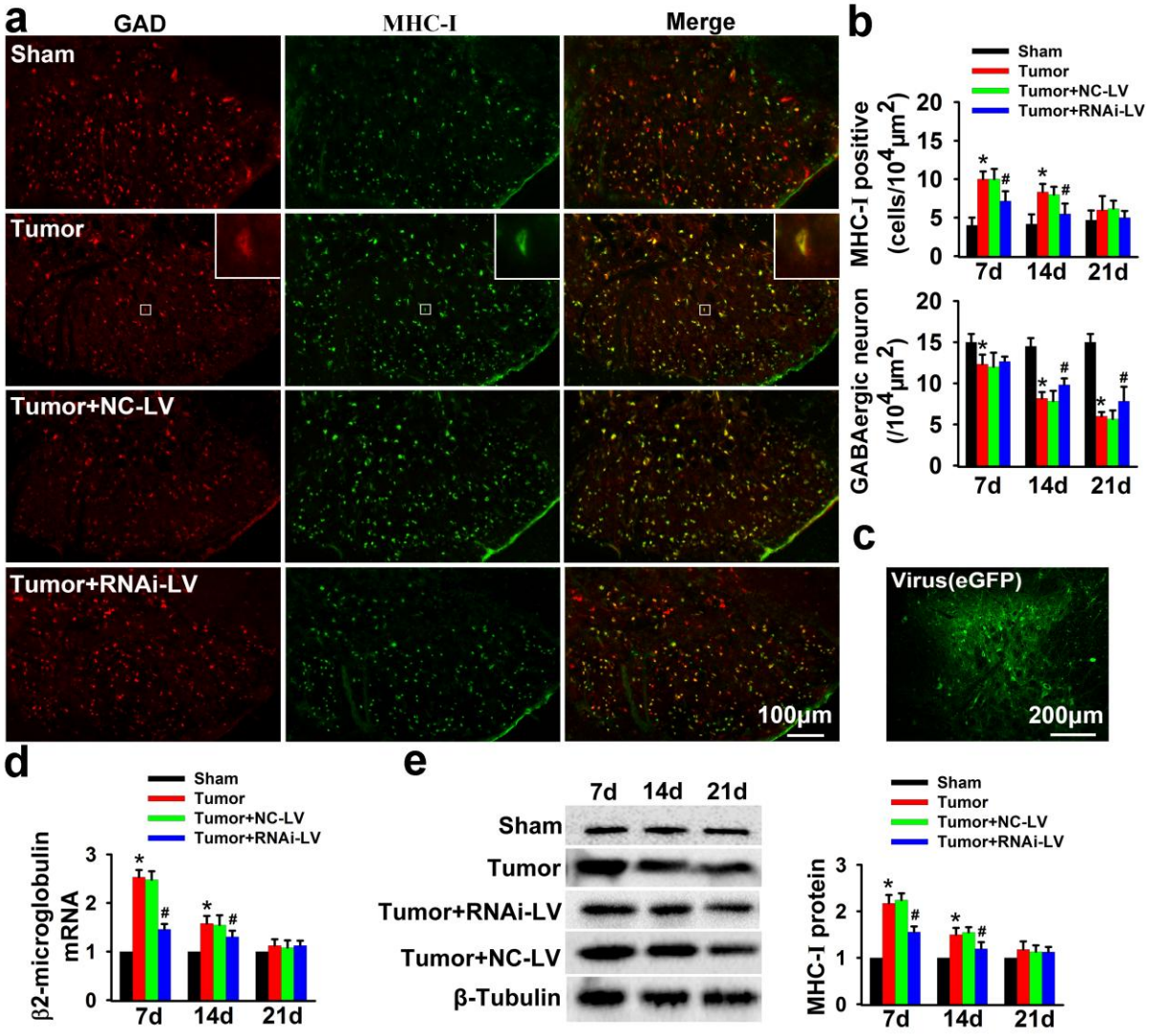


Fig. 1

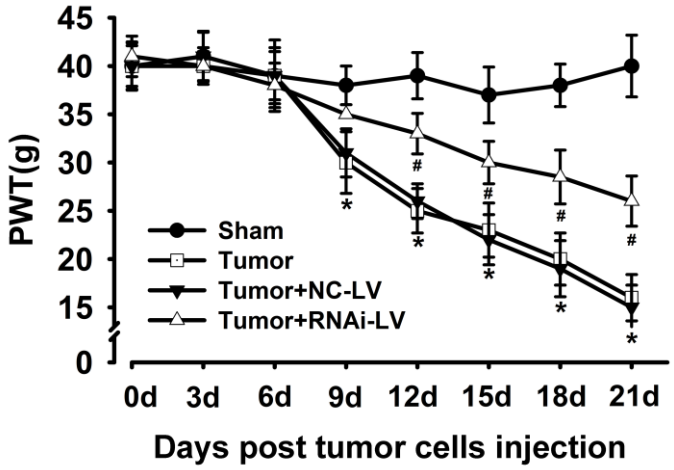


Fig. 2

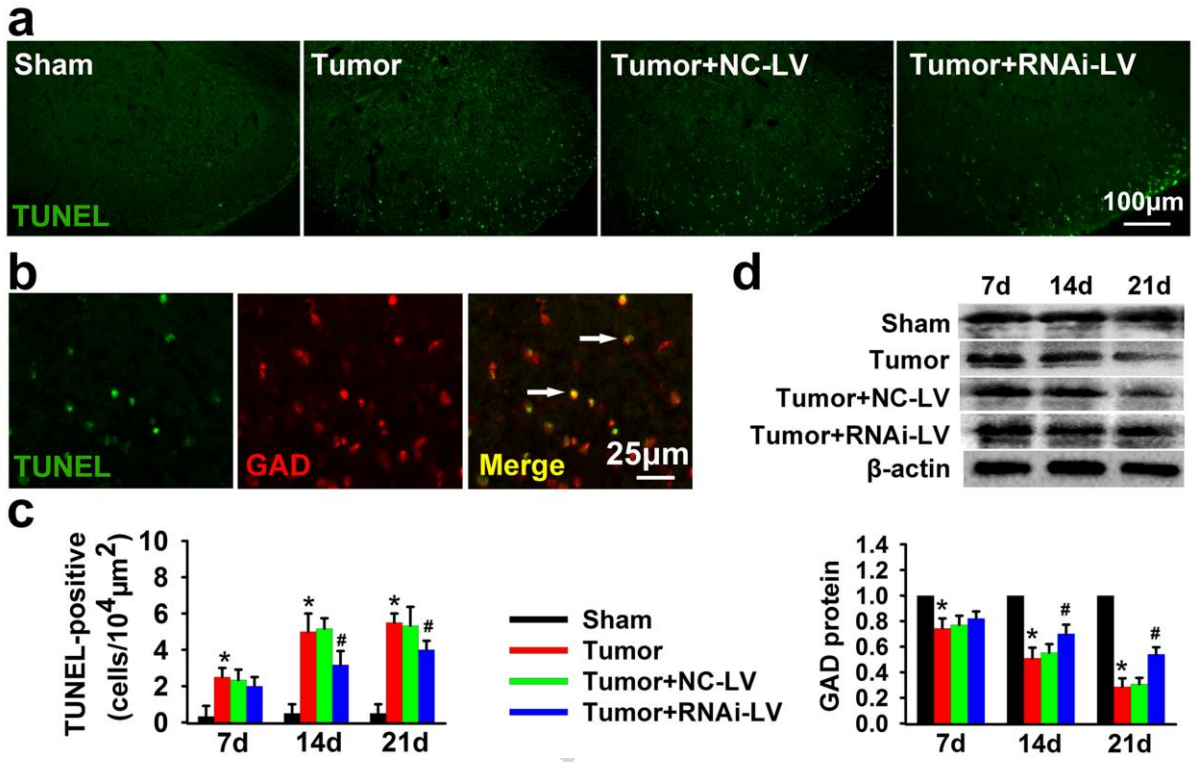


Fig. 3

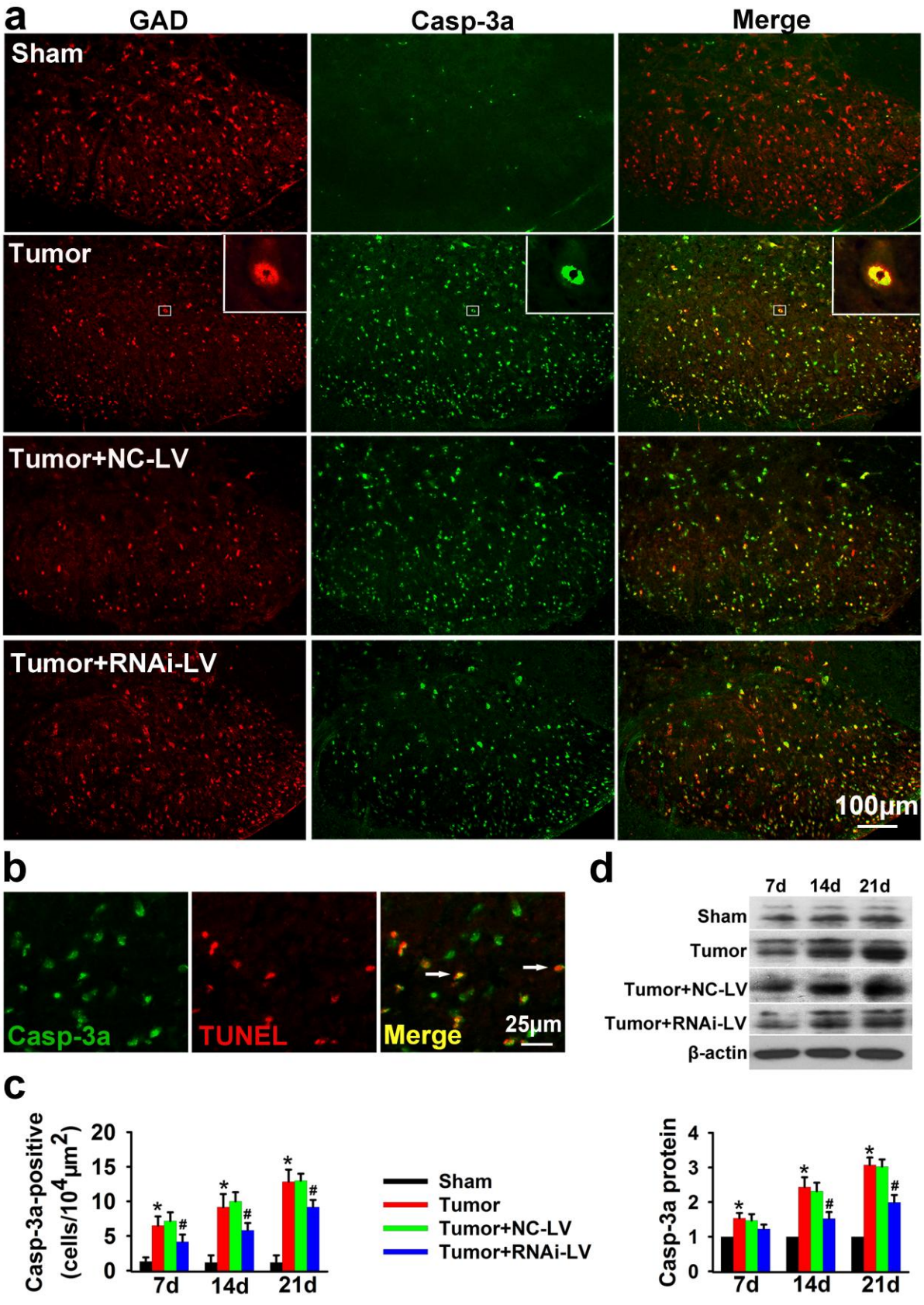

a
GAD
Casp-3a
Merge
Sham
Tumor
Tumor+NC-LV
Tumor+RNAi-LV
100μm
b
Casp-3a
TUNEL
Merge
25μm
d
7d
14d
21d
Sham
Tumor
Tumor+NC-LV
Tumor+RNAi-LV
β-actin
c
Casp-3a-positive (cells/10^4μm^2)
20
15
10
5
0
7d
14d
21d
Sham
Tumor
Tumor+NC-LV
Tumor+RNAi-LV
Casp-3a protein
4
3
2
1
0
7d
14d
21d

Fig. 4

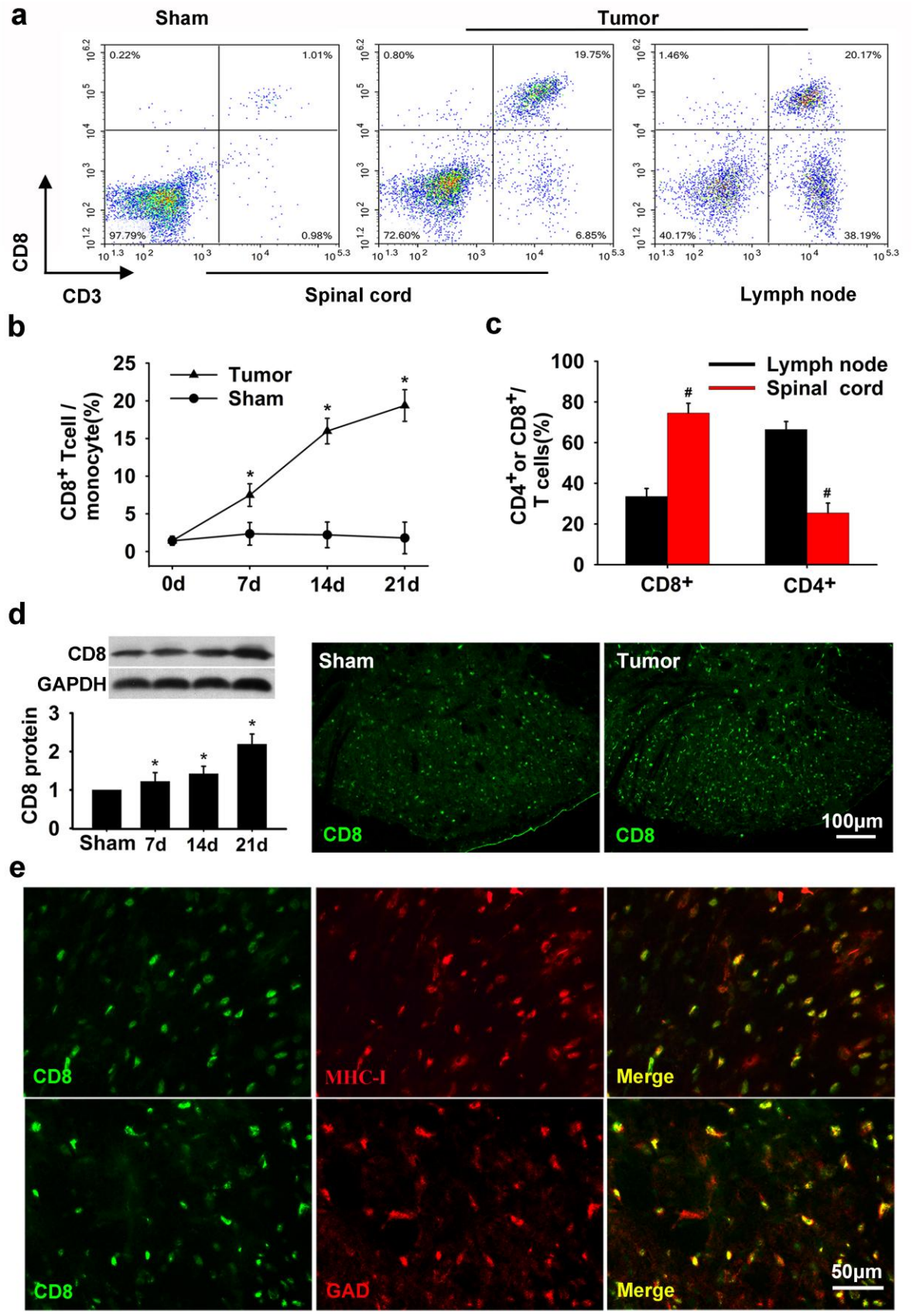

a
Sham
Tumor
0.22%
1.01%
97.79%
0.98%
0.80%
19.75%
72.60%
6.85%
1.46%
20.17%
40.17%
38.19%
CD8
CD3
Spinal cord
Lymph node
b
Tumor
Sham
CD8+ Tcell / monocyte(%)
0d
7d
14d
21d
c
Lymph node
Spinal cord
CD4+or CD8+/ T cells(%)
CD8+
CD4+
d
CD8
GAPDH
CD8 protein
Sham
7d
14d
21d
Sham
Tumor
CD8
100μm
e
CD8
MHC-I
Merge
GAD
50μm

Fig. 5

Highlights:

- MHC-I expression increased on GABAergic interneurons in the spinal dorsal horn of bone cancer rats.
- Increased expression of MHC-I can remove GABAergic interneurons in the spinal cord by promoting its apoptosis via caspase-3 signaling pathway.
- Knockdown of MHC-I with RNAi lentivirus can alleviate the apoptosis of GABAergic interneurons in the spinal cord and reversed mechanical allodynia of bone cancer rats.
- MHC-I induced the apoptosis of GABAergic interneurons is related to $CD8^+$ T cells in the CNS.