Inhibition of tumor-derived CCL2 expression attenuates tactile allodynia in NCTC 2472 fibrosarcoma-inoculated mice

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CCL2 is therapeutic target of tumor-induced allodynia

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Abbreviations:
CCL2 (C-C motif chemokine ligands 2)
PWT (paw withdrawal threshold)
PLGA (poly lactic-co-glycolic acid)
ABSTRACT

Neuropathic pain associated with cancers is caused by tumor growth compressing and damaging nerves, which would also be enhanced by inflammatory factors through sensitizing nociceptor neurons. A troublesome hallmark symptom of neuropathic pain is hypersensitivity to innocuous stimuli, a condition known as “tactile allodynia”, which is often refractory to NSAIDs and opioids. The involvement of chemokine CCL2 (monocyte chemoattractant protein-1) in cancer-evoked neuropathic pain is well established, but opinions remain divided as to whether CCL2 is involved in the production of tactile allodynia with tumor growth. In this study, we constructed Ccl2 knockout NCTC2472 (Ccl2-KO NCTC) fibrosarcoma cells and conducted pain behavioral test using Ccl2-KO NCTC-implanted mice. Implantation of naïve NCTC cells around the sciatic nerves of mice produced tactile allodynia in the inoculated paw. Although the growth of Ccl2 KO NCTC-formed tumors was comparable to that of naïve NCTC-formed tumors, Ccl2-KO NCTC-bearing mice failed to show tactile pain hypersensitivity, suggesting the involvement of CCL2 in cancer-induced allodynia. Subcutaneous administration of controlled-release nanoparticles containing the CCL2 expression inhibitor NS-3-008 significantly attenuated tactile allodynia in naïve NCTC-bearing mice accompanied by a reduction of CCL2 content in tumor masses. Our present findings suggest that inhibition of CCL2 expression in cancer cells is useful strategy to attenuate tactile allodynia induced by tumor growth. Development of a controlled release system of CCL2 expression inhibitor may be a preventive option for the treatment of cancer-evoked neuropathic pain.

SIGNIFICANCE STATEMENT

The blockade of chemokine/receptor signaling, particularly for C-C motif chemokine ligands 2 (CCL2) and its high-affinity receptor CCR2, has been implicated to attenuate cancer-induced inflammatory and nociceptive pain. This study demonstrated that continuous inhibition of CCL2 production from cancer cells also prevents the development of tactile allodynia associated with tumor growth. Development of controlled release system of CCL2 expression inhibitor may be a preventative option for management of cancer-evoked tactile allodynia.
INTRODUCTION

‘Pain’ is one of the most serious symptoms of cancer patients. Chronic pain is experienced by 90% of patients with advanced cancer (Meuser et al., 2001), which is sometimes refractory to opioids, NSAIDs, and other adjuvant therapies (Fallon et al., 2018). Cancer-evoked pain is caused by the tumor progression into bone, nerves, or other organs, and it is characterized by multiple attributions including nociceptive, neuropathic, and inflammatory pain (Edwards et al., 2019). This complicated pathological condition makes it difficult to understand the mechanism of cancer-evoked pain and also hinders the identification of effective targets for pharmacological treatment.

Recently, the role of the chemokine-chemokine receptor axis has attracted interest as a potential mechanism for cancer-associated pain (Aloyouny et al., 2020; Shen et al., 2014). The C-C motif chemokine ligands 2, 3 and 5 (CCL2, CCL3 and CCL5), C-X-C motif chemokine ligand 12 (CXCL12) or C-X3-C motif chemokine ligand 3 (CX3CL1) and their specific receptors are detected in both peripheral and central sites of the nervous system relevant to the transmission of nociceptive signals (Bhangoo et al., 2007; Old and Malcangio, 2012; Kiguchi et al., 2012; Réaux-Le Goazigo et al., 2013; Pevida et al., 2014). There is also ample evidence that CCL2, also known as monocyte chemoattractant protein-1 (Yoshimura, 2018), plays a critical role in the cancer-evoked neuropathic pain by acting through its high-affinity receptor CCR2 (Dansereau et al., 2008; Gao et al., 2009; Thacker et al., 2009). Tactile allodynia, hypersensitivity to normally innocuous stimuli, is also one hallmark symptom of cancer-evoked neuropathic pain (Menéndez et al., 2005; Yoon and Oh, 2018; Fallon, 2013). An animal model of bone cancer pain induced by the intrafemoral implantation of NCTC2472 fibrosarcoma cells produces tactile allodynia (Mao et al., 2019), but administration of CCR2 antagonist to bone cancer animal model fails to attenuate tactile pain hypersensitivity, although suppresses mechanical hyperalgesia: enhanced pain perception of noxious stimuli (Pevida et al., 2012). In contrast to this observation, mice overexpressing CCL2 show hypersensitivity to innocuous stimuli (Jung et al., 2009). Furthermore, CCR2 knockout mice are also resistant to the
development of tactile allodynia following nerve ligation (Abbadie et al., 2003). Therefore, despite a considerable number of studies on the role of CCL2/CCR2 signaling in the development of neuropathic tactile allodynia, there is still no clear understanding of its contribution to cancer-evoked tactile pain hypersensitivity.

Chemical screening is a powerful tool for investigating diverse biological pathways (Zhu et al., 2016). Several recent studies have used cell-based chemical screening to identify bioactive small molecules (Jiang et al., 2022). In the previous study, we performed a high-throughput chemical screening of 9600 synthetic small molecules from a chemical library (Open Innovation Center for Drug Discovery, The University of Tokyo) and found a small compound, NS-3-008 (N-benzyl-N'-hexylguanidine), which inhibits the transcriptional activity of G0/G1 switch 2 (G0S2). This small compound significantly suppressed G0S2-mediated transactivation of Ccl2 (Matsunaga et al., 2016), suggesting the utility of a chemical biology approach to investigate the role of CCL2 in the development of neuropathic pain induced by tumor growth.

To elucidate the role of CCL2 in the production of cancer-induced tactile allodynia, we constructed Ccl2 knockout NCTC 2472 (Ccl2-KO NCTC) cells and performed pain behavioral test using Ccl2-KO NCTC-implanted mice. After demonstrating the failure of development of tactile allodynia in mice inoculated with Ccl2-KO NCTC cells, we applied a novel CCL2 expression inhibitor NS-3-008 to elucidate its analgesic effects on cancer-induced tactile hypersensitivity.
MATERIALS AND METHODS

Cell culture and treatment

NCTC2472 fibrosarcoma cell line (NCTC) was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in dulbecco’s modified eagle medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 5% fatal bovine serum (FBS; Moregate Biotech, Bulimba, Australia) and 0.5% penicillin-streptomycin (Invitrogen, Grand Island, NY). We used the third passage cells (10-15 cell generation) for inoculation into mice. We also confirmed no microbial and chemical contamination in cells.

Construction of CCL2-KO NCTC cells

To construct CCL2 KO cells, NCTC cells were reverse transfected with MCP-1 CRISPR/Cas9 KO Plasmid (m) (Cat# sc-422838; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and MCP-1 HDR Plasmid (m) (Cat# sc-422838-HDR; Santa Cruz Biotechnology) using Lipofectamine LTX Reagent (Invitrogen). Control cells were transfected with a plasmid encoding Cas9 and a non-targeting sgRNA. 48 h after transfection, cultured cells were suspended in DMEM and sorted by a BD FACSAria III Cell Sorter (Becton Dickinson Biosciences, San Diego, CA). GFP-positive cells were collected and then incubated in media containing 6 µg/mL puromycin. To verify cleavage of the mouse CCL2 genomic DNA, genomic DNA was extracted from naive, GFP-positive, and puromycin-resisted GFP positive NCTC cells with GenElute™ Mammalian Genomic DNA Miniprep Kit (Cat# G1N70, Sigma-Aldrich Co., St. Louis, MO). Genomic DNA was then PCR amplified with primers flanking the terminal region of gene encoding CCL2. The sequences of the forward/reverse primers were as follows: 5’-ACTCTCACTGAAGCCAG-3’/ 5’-CTCAAACACAAAGTTTACCC-3’. PCR products were detected by agarose gel electrophoresis and ethidium bromide staining.

Animals and treatments

Male C3H/HeJ mice (CLEA Japan, Inc.) were housed in groups from 6 to 10 per cage in a light-controlled room (7:00AM, lights on; 7:00PM, lights off) at 24 ± 1°C and humidity of 60 ±
10% with food and water ad libitum. This strain was chosen for their histocompatibility with the NCTC 2472 cells as previously reported (Clohisy et al., 1995), and implantation of NCTC 2472 cells to C3H/HeJ mice also produces tactile pain hypersensitivity (Gu et al., 2010). Implantation of cancer cells to mice was conducted under isoflurane anaesthesia. The right thigh was shaved and the sciatic nerve was exposed through an incision. NCTC2472 (2.5 × 10^5 cells) suspended in 10 µL of 50% Matrigel (Cat# 354234, Corning, Corning, NY)/DMEM (1:1) was implanted around the sciatic nerve of right hindlimb. The wound was closed in layers. To prepare control mice, sciatic nerve of right hindlimb was also exposed and the same volume of 50% Matrigel/DMEM was injected instead of cell suspension. All experimental procedures were performed under the approval and guidance stipulated by animal care and use committee of Kyushu University.

**Assessment of cancer-induced tactile allodynia**

Cancer-induced tactile allodynia was assessed using von Frey filaments (0.02-2.0 g; Muromachi Kikai Co., Ltd., Tokyo, Japan). Prior to paw withdrawal threshold (PWT) assessment, mice were placed in plastic cages with a wire mesh floor for 0.5 h to allow for acclimation to the new environment. Acclimation was repeated for at least 3 days before pain behavioral experiments. Calibrated von Frey filaments were then applied to the plantar surfaces of the hind paws of the tumor-implanted side. The 50% PWT was determined using the up-down method (Dixon, 1980; Chaplan et al., 1994). The PWT to tactile stimuli was assessed at a fixed time between 1:00 and 15:00 pm, because breakthrough pain in cancer patients shows diurnal variation (Saini et al., 2013; Campagna et al., 2018). If the PWT was less than 1.0 g before the implantation of cancer cells, the animals were excluded from the study. Observers were blinded to genetic background and drug treatment.

**Determination of CCL2 protein levels**

After changing culture media, naïve and Ccl2 KO NCTC cells were incubated for 4h. Naïve NCTC cells were also treated with vehicle (0.1% DMSO in saline) or NS-3-008 for 24 h. On day
21 after implantation of cancer cells, tumor masses and healthy thigh muscle were removed and homogenized in CellLytic MT Cell Lysis Reagent (Cat# C3228, Sigma-Aldrich)-supplemented protease inhibitor cocktail, which contained 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μM Phenylmethylsulfonyl fluoride, and 200 μM sodium vanadate. Then, homogenates were centrifuged at 12,000 × g for 10 min at 4°C, and protein concentration of supernatants were assessed using the BCA Protein Assay kit (Cat# 71285, Merck Millipore, Bedford, MA). Blood samples were drawn by cardiac puncture on day 21 after implantation of cancer cells. Plasma was separated by centrifugation (1200 × g). The CCL2 concentration in cell culture media, tumor masses, and plasma were assessed by using the mouse CCL2 ELISA Duo-Set (Cat# MJE00B, R&D Systems Inc, Minneapolis, MN) according to the manufacture’s protocol.

**Immunofluorescence staining**

Animals were anesthetized and sacrificed on day 21 after implantation of cancer cells. The sciatic nerve eroded by tumor mass and spinal cord were removed and postfixed at 4°C for 5 h with 4% paraformaldehyde/PBS (pH7.4). The postfixed sciatic nerve and spinal cord were transferred to 15% sucrose/PBS for 24 h and then 30% sucrose/PBS for 24 h. The samples were frozen at -80°C, serially cut with a cryostat (15 μm) and mounted on silane-coated slides. After washing with ice cold PBS, sciatic nerve and spinal cord were blocked in solution containing 10% normal goat serum and 0.1% Triton X-100 for 2 h at 4°C. The sections were then incubated at 4°C with primary antibodies against CD11b (1:1000; Cat# MCA711G, AbD Serotec, Oxford, UK), Iba1 (1:1,000; Cat# 01919741, FUJIFILM Wako Pure Chemical Tokyo, Japan), CD68 (1:500, Cat# 137001, BioLegend, San Diego, CA), or myelin-basic protein (1:500, Cat# 836504, BioLegend) for 48 h. After washing, the sections were incubated with a fluorescent-conjugated secondary antibody (Alexa 488, Alexa 546, Alexa 555 or Alexa 647, 1:1,000; Abcam, Cambridge, UK) at 4°C for 2 h. The slides were covered with one drop of Vectashield (Vector Laboratories, Burlingame, CA), and then cover-slipped. Fluorescent images were obtained with confocal fluorescence microscopy.
Drug administration

Poly lactic-co-glycolic acid (PLGA)-based nanoparticles have the potential to be used as drug delivery units for sustained release. To investigate the effect of the CCL2 expression inhibitor NS-3-008 (Matsunaga et al., 2016) on tactile allodynia, we encapsulated the compound in PLGA as previously described (Liu et al., 2007), with slight modifications. Briefly, a 0.5 mL internal aqueous phase containing 15 mg NS-3-008 was emulsified in a mixed with organic solution (dichloromethane and acetone) containing PLGA (250 mg). Thereafter, the emulsion was poured into 50 mL of 1% polyvinyl alcohol (PVA) aqueous solution. The resulting suspension was stirred for 3 h to evaporate the organic phase. After centrifugation at $190 \times g$ for 5 min, the precipitates were added to 10 mL of deionized water, then vortexed for 5 min and sonicated for 3 min. The nanoparticles were purified by repeating the procedure of centrifugation and resuspension in distilled water 3 times. Finally, the product was dried by lyophilization and stored at 4°C. Tumor-bearing mice were randomized into three groups: vehicle or two doses of NS-3-008 (50 mg/kg and 100 mg/kg). On day 14 after tumor cell implantation, mice were subcutaneously administered a single dose of PLGA nanoparticles containing NS-3-008.

Statistical analysis

Values presented are expressed as the mean with S.D. Statistical significance of differences among groups was analyzed by one-way or two-way ANOVA followed by Tukey-Kramer post hoc tests. Sample size was determined by power analysis using G*Power 3.1 software (Heinrich-Heine-University, Düsseldorf, Germany). Statistical power was set at 0.8 for all experiments. Equal variances were not formally tested. $P < 0.05$ was considered significant.
RESULTS

Construction of Ccl2-KO NCTC cells

To construct Ccl2 KO cancer cells, we applied the CRISPR/Cas9 system to NCTC2472 fibrosarcoma (Fig.1A). Since the Ccl2-targeted CRISPR/Cas9 plasmid contains GFP reporter and puromycin resistance cassettes, the plasmid-transfected cells were sorted by FACS, and then GFP-positive cells were incubated in the media containing 5 µg/mL puromycin to select Ccl2 KO cells. The results of PCR analysis revealed the cleavage of Ccl2 gene in GFP-positive CRISPR/Cas9-transfected NCTC cells (Fig.1B). Indeed, a significant reduction of CCL2 levels was observed in the culture media of Ccl2 KO cells (P<0.01, Fig. 1C). Since transfection with control CRISPR/Cas9 plasmid had a negligible effect on CCL2 production, we used naïve NCTC2472 cells as a comparative control and performed further experiments.

Failure to develop tactile allodynia in mice inoculated with Ccl2-KO NCTC cells

To investigate the role of cancer cell-produced CCL2 in the development of tactile allodynia, Ccl2 KO NCTC cells were implanted around the sciatic nerve of male C3H/HeJ mice and the severity of cancer-induced tactile allodynia was assessed. The paw withdrawal threshold (PWT) to tactile stimuli was gradually decreased in mice inoculated with naïve NCTC cells (Fig. 2A). Compared to control mice, a significant reduction in PWT was observed on day 10 after implantation of naïve NCTC cells. Ccl2 KO NCTC-inoculated mice also showed a reduction in PWT on the next day after implantation, but did not show a further reduction similar to naïve NCTC-inoculated mice (Fig. 2A), or rather showed a rapid recovery from tactile allodynia. On day 21 after cancer cell implantation, the weight of Ccl2 KO NCTC-formed tumors was comparable to that of naïve NCTC-formed tumors (Fig. 2B), although the CCL2 content in Ccl2 KO NCTC-formed tumors was significantly lower than that observed in naïve NCTC-formed tumors (P<0.01, Fig. 2C). Similarly, plasma CCL2 levels were also decreased in Ccl2 KO NCTC-inoculated mice (P<0.05, Fig. 2D). These results suggest that CCL2 released from cancer cells contributes significantly to the production of tactile allodynia.
Suppression of macrophage infiltration into sciatic nerves of mice inoculated with Ccl2-KO NCTC cells

CCL2 acts as a potent inducer of monocyte and macrophage recruitment (Gschwandtner et al., 2019; Baggiolini, 1998). Infiltration of macrophages into primary sensory nerves and enhancement of microglial phagocytosis in the spinal cord have been implicated in the development and maintenance of pain hypersensitivity after peripheral nerve injury or inflammation (De Logu et al., 2017). Results of immunofluorescence labeling for CD11b, a marker of macrophage, showed that the number of CD11b-positive cells was increased in the sciatic nerve of mice inoculated with naïve NCTC cells (Fig. 3A). In contrast, the number of CD11b-positive cells in the sciatic nerve of mice inoculated with Ccl2 KO NCTC cells was significantly lower than that observed in mice implanted with naïve NCTC cells.

After peripheral nerve injury, molecules released from damaged primary afferents contribute to microglial activation in the spinal cord, resulting in increased phagocytic activity (Austin and Moalem-Taylor, 2010). Iba1-positive microglia had myelin-basic protein (MBP), which was also increased in the spinal cord in naïve NCTC-bearing mice (Fig. 3B). These results suggest that macrophage infiltration into the primary sensory nerve and enhanced microglial phagocytosis in the spinal cord are involved in the production of tactile allodynia induced by CCL2 released from NCTC cells.

CCL2 expression inhibitor NS-3-008 attenuates tactile allodynia in naïve NCTC cell-implanted mice

We have previously demonstrated that the small molecule NS-3-008 has the ability to inhibit LPS-induced Ccl2 expression in mouse hepatoma cells (Matsunaga et al., 2016). This small molecule also suppressed the CCL2 release from naïve NCTC cells in a concentration-dependent manner (Fig. 4A). Significant suppression of CCL2 release was observed when cells were treated with NS-3-008 over 5 µM (P<0.01). Therefore, we also investigated whether NS-3-008 is able to alleviate cancer-induced tactile allodynia.
CCL2 exerts its physiological actions through its high-affinity receptor CCR2 (Gschwandtner et al., 2019; Mellado et al., 1998). However, it has been reported that a single administration of the CCR2 antagonist RS504393 does not attenuate tactile allodynia in mice intratibially inoculated with NCTC2472 cells (Pevida et al., 2012; Baamonde et al., 2007). The previous fact, together with the present findings that implantation of Ccl2 KO NCTC cells in mice failed to produce tactile alldynia, raised the possibility that continuous inhibition of CCL2 activity is required for attenuation of cancer-evoked alldynia. Since PLGA nanoparticles are capable of controlled delivery of bioactive agents suitable for continuous administration of drug (Dinarvand et al., 2011; Su et al., 2021; Mahapatro and Singh, 2011), we prepared PLGA nanoparticles containing NS-3-008 and administrated them subcutaneously to mice on day 14 after implantation of naïve NCTC cells. A single subcutaneous administration of this PLGA nanoparticle (100 mg/kg NS-3-008) to tumor-bearing mice significantly decreased the intratumoral content of CCL2 for at least for 7 days (Fig.4B). Similarly, plasma CCL2 levels were significantly decreased in mice on day 7 after administration of the nanoparticle containing 100 mg/kg NS-3-008 (P<0.01, Fig. 4C). The PWT of mice inoculated with naïve NCTC cells was also gradually increased after administration of PLGA nanoparticles containing NS-3-008 (Fig.4D). Significant alleviation of cancer-induced tactile alldynia was observed when the compound was administered to mice at a dose of 100 mg/kg body weight (P<0.01). Administration of PLGA nanoparticle-encapsulated NS-3-008 for 7 days had a negligible effect on the tumor growth (Fig. 4E). These results demonstrated that sustained inhibition of intratumoral expression of CCL2 by NS-3-008 resulted in amelioration of cancer-induced tactile alldynia.
DISCUSSION

As observed in a previous study using mice with intratibial inoculation of NCTC2472 cells (Pevida et al., 2012; Baamonde et al., 2007), implantation of the fibrosarcoma cells around the sciatic nerve also induced tactile allodynia. Since CCL2 KO NCTC-implanted mice failed to show the production of tactile allodynia, our present study indicates that CCL2 produced by NCTC2472 fibrosarcoma is involved in the developmental process of cancer-induced tactile allodynia. Although several mechanisms have been proposed to explain about the CCL2-induced sensitization of nociceptors (Piotrowska et al., 2016), a recent study shows that tactile allodynia induced by intrasciatic nerve injection of CCL2 is associated with macrophage infiltration and oxidative stress in the nerve (De Logu et al., 2017). This damage to primary sensory neurons would induce the activation of spinal microglia, as evidenced by an increase in their phagocytic activity (Guan et al., 2016; Calvo and Bennet, 2012). In Ccl2 KO NCTC-implanted mice, we observed the suppression of macrophage infiltration in the sciatic nerve and decreased phagocytosis of spinal microglia. Therefore, previous and our present findings suggest that CCL2-induced sciatic nerve macrophage infiltration and spinal microglia activation are involved in the underlying mechanism of cancer-evoked tactile allodynia.

Inflammatory monocytes are recruited to sites of injury and inflammation in a CCL2/CCR2-regulated manner and release a variety of factors, many of which exert pronociceptive functions (Ingersoll et al., 2011). In the sciatic nerve, the CCR2 is also activated by CCL2, which is produced by endothelial cells, monocytes/macrophages, and sensory neurons (Langhert et al., 2013; Sierra-Filardi et al., 2014; Zhang et al., 2013). The contribution of CCL2/CCR2 signaling to the production of neuropathic pain hypersensitivity is well established, but a previous study has shown that subcutaneous administration of the CCR2 antagonist RS504393 does not attenuate tactile allodynia in mice with intratibial inoculation of NCTC2472 cells (Pevida et al., 2012). There are several reasons to explain the inability of the CCR2 antagonist to attenuate neuropathic allodynia. Primary sensory neurons expressing transient receptor potential ankyrin-1 (TRPA1) channels have been implicated in the
development of CCL2-induced allodynia (De Logu et al., 2017). TRPA1-expressing neurons may be more sensitive to CCL2 stimuli. Therefore, selective blockade of CCR2 may be insufficient to suppress CCL2-induced tactile allodynia. On the other hand, cancer-evoked neuropathic pain is likely caused by tumor growth that compresses or damages peripheral nerves. After nerve injury, spinal microglial cells are activated and express a specific type of purinergic receptor on the membrane (Trang et al., 2009; Inoue and Tsuda, 2018). Extracellular ATP stimulates purinergic receptors on activated microglia, resulting in the release of BDNF, and the neurotrophic factor acts on TrkB in lamina I neurons, causing an altered transmembrane anion gradient by downregulating K⁺/Cl⁻ cotransporter 2 (KCC2). Downregulation of KCC2 switches GABA- and glycine-evoked responses from inhibitory to excitatory (Coull et al., 2005), resulting in pain hypersensitivity in response to normally innocuous stimuli. Our present results have shown that CCL2 produced by cancer cells contributes to the recruitment of macrophages into the sciatic nerves and the increase in the phagocytic activity of spinal microglia, suggesting the involvement of this chemokine in the early developmental stage of neuropathic allodynia. Therefore, it may be possible that a single administration of CCR2 antagonist is insufficient to suppress the spinal microglia activation as well as its downstream events to induce tactile allodynia.

C3H/HeJ mice have a missense mutation in the third exon of the toll-like receptor 4 (TLR4) gene, which interferes with signaling and results in a defective response to lipopolysaccharide (LPS) (Poltorak et al., 1998; Qureshi et al., 1999). Stimulation of TLR4 by LPS induces the expression of CCL2 in macrophages (Qian et al., 2021). Although attenuation of mechanical pain hypersensitivity is observed in peripheral nerve-injured C3H/HeJ mice (Tanga et al., 2005), implantation of NCTC 2472 cells in the same strain mice significantly induces tactile allodynia (Gu et al., 2010), suggesting that TLR4 is unlikely to be the major contributor to the development of cancer-induced tactile allodynia in C3H/HeJ mice.

A single subcutaneous administration of PLGA nanoparticles containing NS-3-008 to tumor-bearing mice decreased intratumoral levels of CCL2 for 7 days, demonstrating the utility
of this nanoparticle to continuously inhibit CCL2 expression. Administration of NS-3-008-encapsulated nanoparticles also attenuated cancer-induced tactile allodynia. These results suggest that sustained inhibition of CCL2/CCR2 signaling may be required for the attenuation of cancer-evoked neuropathic allodynia. We previously identified NS-3-008 by conducting a high-throughput screen of 9600 synthetic small molecules from a chemical library (Open Innovation Centre for Drug Discovery, The University of Tokyo) (Matsunaga et al., 2016). NS-3-008 inhibits CCL2 expression by suppressing the transcriptional activity of the G0/G1switch 2 (G0S2) protein. This small compound has also been shown to ameliorate renal inflammation in an animal model of chronic kidney disease (Matsunaga et al., 2016). Consistent with the observation in Ccl2 KO NCTC-implanted mice, continuous inhibition of tumoral expression of CCL2 had a little effect on the tumor growth. Therefore, delivery of NS-3-008 via PLGA nanoparticles appeared to attenuate cancer-induced neuropathic allodynia without interfering with cancer progression.

This study demonstrated a significant role of CCL2 produced by NCTC2472 fibrosarcoma in the development of tactile allodynia associated with tumor growth. Although the blockade of CCL2/CCR2 signaling may be useful strategy for attenuating cancer-induced inflammatory and nociceptive pain, continuous inhibition of the chemokine signal seemed to be required for exerting antiallodynic effect. Therefore, development of controlled release system of CCL2 expression inhibitor may be a preventative option for management of cancer-evoked tactile allodynia.
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DATA AVAILABILITY STATEMENT
The authors declare that all the data supporting the findings of this study are contained within the paper.

AUTHOR CONTRIBUTIONS
Participated in research design: Taniguchi, Matsunaga, Koyanagi, Ohdo.
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REFERENCES


Langhert KA, Von Zee CL, Stubbs Jr EB (2013) Tumour necrosis factor α enhances CCL2 and ICAM-1 expression in peripheral nerve microvascular endoneurial endothelial cells. ASN Neuro 5:e00104.


Footnotes

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FIGURE LEGENDS

Figure 1. Construction of Ccl2-KO NCTC cells. (A) Schematic procedure for construction of Ccl2-KO NCTC cells using the CRISPR/Cas9 system. Cells were co-transfected with CRISPR/Cas9 Ccl2-KO plasmids and HDR plasmids. Fluorescence-activated cell sorting was conducted to isolate GFP-positive NCTC cells, and the isolated cells were incubated in media containing 6 µg/mL puromycin. (B) Verifying cleavage of the mouse Ccl2 genomic DNA by CRISPR/Cas9. The upper scheme shows the strategy for the detection of the Ccl2 gene. The lower photograph shows PCR products in DNA extracted from naïve, GFP-positive, or puromycin-resisted GFP-positive NCTC cells. Arrow heads indicate expected fragment sizes for each locus. (C) CCL2 protein levels in the culture medium of native or Ccl2-KO NCTC cells. Values are shown as means with S.D. (n = 3). **P < 0.01 compared between the two groups (F2, 6 = 138.667, P < 0.001, one-way ANOVA, Tukey-Kramer post-hoc test).

Figure 2. Cancer-induced tactile allodynia is prevented by genetic ablation of CCL2. (A) Time course of PWT in mice inoculated with naïve and Ccl2-KO NCTC cells. Values are shown as means with S.D. (n = 8). **P < 0.01 compared with control mice (F15, 112 = 14.226, P < 0.001, two-way ANOVA, Tukey-Kramer post-hoc test). ##P < 0.01 compared with naïve NCTC-implanted mice (F15, 112 = 8.725, P < 0.001, two-way ANOVA, Tukey-Kramer post-hoc test). (B) Tumor weight on day 21 after implantation of naïve and Ccl2-KO NCTC cells. (C) CCL2 content in tumor masses of mice on day 21 after implantation of naïve and Ccl2-KO NCTC cells. Values are shown as means with S.D. (n = 5–8). **P < 0.01 compared between the two groups (F2, 14 = 39.276, P < 0.001, one-way ANOVA, Tukey-Kramer post-hoc test). (D) Plasma CCL2 levels in mice on day 21 after implantation of naïve and Ccl2-KO NCTC cells. Values are shown as means with S.D. (n = 6). **P < 0.01, *P < 0.05 compared between the two groups (F2, 14 = 7.201, P = 0.006, one-way ANOVA, Tukey-Kramer post-hoc test).

Figure 3. Cancer cell-produced CCL2 induces macrophage infiltration into the sciatic nerve and microglial phagocytosis in the spinal cord. (A) Immunostaining microphotographs of a semithin cross-section of sciatic nerves in mice inoculated with naïve or Ccl2-KO NCTC cells. The left panels show CD11b staining with arrows indicating infiltration of macrophage into the sciatic nerve. The sciatic nerve areas are encircled by dashed lines. Scale bars represent 100 µm. The right panel shows the quantification of the number of
CD11b-positive cells. Values are shown as means with S.D. (n = 6). **P < 0.01 compared between the two groups (F_{2, 15} = 38.255, P < 0.001, one-way ANOVA, Tukey-Kramer post-hoc test). (B) Immunostaining microphotographs of Iba1-positive microglia containing MBP within CD68+ lysosomes in the spinal cord of mice inoculated with naïve or CCL2-KO NCTC cells. In the left panel, Iba1 (green)-positive microglial cells merged with CD68 (red) and MBP (magenta) in the tumor implanted side of the spinal cord, indicated by yellow allows. Scale bars represent 100 µm. The right panel shows the quantification of the number of Iba1-positive microglia containing MBP within CD68+ lysosomes. Values are shown as means with S.D. (n = 4–7). *P < 0.05 compared between the two groups (F_{2, 13} = 5.861, P = 0.015, one-way ANOVA, Tukey-Kramer post-hoc test).

Figure 4. Alleviation of cancer-induced tactile allodynia by CCL2 expression inhibitor NS-3-008. (A) Inhibition of CCL2 release from naïve NCTC cells by NS-3-008. Cells were treated with vehicle (0.1% DMSO in saline) or NS-3-008 at the indicated concentrations. Values are shown as means with S.D. (n = 4–6). **P < 0.01, a significant difference between the two groups (F_{3,18} = 15.230, P < 0.001, one-way ANOVA, Dunnett’s post-hoc test). (B) Time course of intratumoral content of CCL2 in naïve NCTC-implanted mice after subcutaneous administration of PLGA nanoparticles containing NS-3-008 or vehicle (0.1% DMSO in saline). Drugs were administrated on day 14 after tumor implantation. Arrow indicates the subcutaneous administration of drugs. Values are shown as means with S.D. (n = 6). *P < 0.05 compared with vehicle-treated group (F_{5, 30} = 6.028, P < 0.001, two-way ANOVA, Tukey-Kramer post-hoc test). (C) Plasma CCL2 levels in naïve NCTC-implanted mice on 7 after administration of PLGA nanoparticle containing NS-3-008. Values are shown as means with S.D. (n = 6). **P < 0.01, compared between the two groups (F_{2, 14} = 6.754, P = 0.006, one-way ANOVA, Dunnett’s post-hoc test). (D) Time course of PWT in naïve NCTC-implanted mice after subcutaneous administration of PLGA nanoparticles containing NS-3-008 or vehicle (0.1% DMSO in saline). Drugs were administrated on day 14 after tumor implantation. Arrow indicates the subcutaneous administration of drugs. Values are shown as means with S.D. (n = 6). **P < 0.01, *P < 0.05; compared with vehicle-treated group (F_{15, 80} =17.425, P < 0.001, two-way ANOVA, Tukey-Kramer post-hoc test). (E) Tumor weight on day 7 after subcutaneous administration of PLGA nanoparticles containing NS-3-008 or vehicle (0.1% DMSO in saline). Drugs were administrated on day 14 after tumor implantation.
Figure 1

A

Contorl or Ccl2-target CRISPR/Cas9 plasmids

GFP

HDR plasmids

Transfection

(1) Naive NCTC cells

Sorting of GFP positive cells

(2) GFP positive cells

Selection of puromycin-resistant cells

(3) Ccl2 KO NCTC cells

- Verify the cleavage of Ccl2 genomic DNA
- Determination of CCL2 concentration in the media

B

PCR product of the mouse Ccl2 gene (1,628 bp)

1500bp

500bp

Marker

Naive NCTC

Control NCTC

Ccl2 KO NCTC

C

CCL2 concentration (pg/mL)

Naive NCTC

Control NCTC

Ccl2 KO NCTC

**

Figure 1
Figure 2
Figure 3
Figure 4