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# Spinal microglial activation in a murine surgical model of knee osteoarthritis

P.B. Tran †, R.E. Miller † ‡, S. Ishihara †, R.J. Miller §, A.M. Malfait † ‡ *

† Department of Internal Medicine, Division of Rheumatology, Rush University Medical Center, 1611 W. Harrison St, Suite 510, Chicago, IL, USA
‡ Department of Biochemistry, Rush University Medical Center, 1611 W. Harrison St, Suite 510, Chicago, IL, USA
§ Department of Pharmacology, Northwestern University, Chicago, IL, USA

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SUMMARY

*Objective:* Microgliosis, the activation of microglial cells, is thought to contribute to synaptic transmission in the dorsal horn and thereby promote chronic pain. The primary aim of this study was to document the temporal profile of dorsal horn microgliosis after destabilization of the medial meniscus (DMM) in wild type (WT) and *Adamts5* null mice. Since neuronal fractalkine (CX3CL1) contributes to microgliosis, we assessed its release from dorsal root ganglia (DRG) cultures after DMM.
*Design:* DMM or sham surgery was performed in the right knee of 10-week old male WT, CX3CR1-green fluorescent protein (GFP), or *Adamts5* null C57BL/6 mice. Hind paw mechanical allodynia was monitored using von Frey fibers. L4 dorsal horn microgliosis was assessed 4, 8 and 16 weeks after surgery, based on the morphology of Iba1-immunoreactive microglia. DRG cells (L3–L5) were cultured and supernatants collected for fractalkine (FKN) ELISA.
*Results:* In WT mice, numbers of activated microglia were increased 8 and 16 weeks, but not 4 weeks, after DMM but not sham surgery. DRG cultures showed increased basal FKN release at 8 and 16 weeks. *Adamts5* null mice did not develop mechanical allodynia up to 16 weeks after DMM. Accordingly, DRG cultures from these mice did not exhibit increased FKN release and dorsal horn microgliosis did not occur.
*Conclusion:* DMM surgery leads to late stage dorsal horn microgliosis. The temporal correlation with DRG FKN release suggests it may contribute to microgliosis. Reduced microgliosis in *Adamts5* null mice, which are protected from joint damage and associated mechanical allodynia after DMM, suggests that microgliosis is associated with joint damage and accompanying persistent pain.
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## Introduction

Chronic pain is the major symptom associated with osteoarthritis (OA), and its clinical presentation is complex. Osteoarthritic joint pain has a strong mechanical component, and can be triggered by specific activities such as climbing stairs. As the disease progresses, pain can become more persistent and manifest at rest[1]. Ongoing peripheral input from the affected joint appears to drive the pain, as evidenced by the observation that local joint pain can be abolished by intra-articular anesthetics[2] and – in most cases – by total joint replacement (reviewed in Ref. 3). In the course of OA, nociceptors (specialized sensory neurons that detect potentially harmful stimuli) innervating the affected joint can become sensitized by locally generated products, such as nerve growth factor, inflammatory cytokines and chemokines, prostaglandins[4,5], and disease-associated molecular patterns (DAMPs)[6] ("peripheral sensitization"). In addition, increased nociceptive input from the periphery heightens activity and excitability in the spinal cord ("central sensitization")[7]. Thus, OA pain may be driven by abnormal excitability in pain pathways of both the peripheral and central nervous systems (CNSs). Indeed, clinical researchers are increasingly reporting signs of sensitization, both peripheral and central, in OA patients. This includes decreased pressure withdrawal thresholds and temporal summation at sites remote from the affected joint[8–11].

Small animal models of OA in rats and mice increasingly incorporate pain-related behaviors as outcome measures

* Address correspondence and reprint requests to: A.-M. Malfait, Rush University Medical Center, 1611 W. Harrison St, Suite 510, 60612, Chicago, IL, USA.
*E-mail address:* anne-marie_malfait@rush.edu (A.M. Malfait).

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(reviewed in Ref. 12). The best-characterized model in terms of pain behaviors uses intra-articular injection of the glycolysis inhibitor, mono-iodoacetate (MIA), into the rat or mouse knee in order to kill articular chondrocytes. This causes severe cartilage and subchondral bone damage, and pronounced inflammation[13,14]. The aggressive joint pathology is accompanied by pain behaviors (reviewed in Ref. 15), including mechanical hypersensitivity, weightbearing deficits[13], and ongoing pain[16], all driven both by peripheral and central mechanisms[16–18].

Surgically induced knee OA is also associated with mechanical allodynia, as has been described in the rat medial meniscal tear model[19], after partial meniscectomy in the mouse knee[20], and after destabilization of the medial meniscus (DMM) in the mouse[21–23]. In the latter, mechanical allodynia in the ipsilateral hind paw develops early after surgery and is maintained for the 16-week follow-up period[21,22]. Since mechanical allodynia is a behavioral indicator of sensitization, it is likely that central mechanisms contribute to its development and/or maintenance. It has, however, never been demonstrated whether changes in the spinal cord can be detected in surgical models of OA.

In the current study, we focused on microglial cells in the dorsal horn of the spinal cord, because mounting evidence shows that activation of these cells contributes to the plasticity of synaptic transmission that is associated with chronic pain states[24]. Therefore, the primary aim of this study was to document the temporal profile of microglial activation in the dorsal horn after DMM surgery. Additionally, we assessed fractalkine (FKN, CX3CL1) release by dorsal root ganglia (DRG), because in neuropathic pain models it has been demonstrated that FKN derived from the central terminals of DRG neurons contributes to the activation of dorsal horn microglia, expressing the FKN receptor, CX3CR1[25–28]. Finally, we explored the role of joint damage in establishing microgliosis, by assessing microglial activation in *Adamts5* null mice. Since *Adamts5* null mice do not develop joint damage or associated mechanical allodynia after DMM surgery[21,29], we hypothesized that these mice would not develop microgliosis or FKN release purely from the DMM surgery itself.

## Materials and methods

### *Animals*

All animal experiments were approved by the Institutional Animal Care and Use Committee at Rush University Medical Center. Animals were housed with food and water *ad libitum* and kept on 12-h light cycles. We used a total of 107 C57BL/6 mice, including in house wild types (WT), *Adamts5* null mice[21], and CX3CR1-green fluorescent protein (GFP) reporter mice[30]. We used CX3CR1-GFP mice that were heterozygous for the GFP reporter gene.

### *Surgery*

DMM or sham surgery was performed in the right knee of 10-week old male mice, as previously described[31]. Briefly, after medial parapatellar arthrotomy, the anterior fat pad was dissected to expose the anterior medial meniscotibial ligament, which was severed. The knee was flushed with saline and the incision closed. Sham surgery was identical to DMM except that the medial meniscotibial ligament remained intact.

### *Mechanical allodynia*

Mice were tested for sensitivity to von Frey monofilaments, using the up–down staircase method of Dixon[32,33]. The threshold force required to elicit withdrawal of the paw (median 50% withdrawal) was determined twice on each hind paw (and averaged) on each testing day, with sequential measurements separated by at least 5 min. Baseline thresholds were assessed prior to surgery, and thresholds were assessed 4, 8, 12, and 16 weeks after DMM.

### *DRG cell culture*

Four, 8, or 16 weeks after DMM, ipsilateral and contralateral innervating DRG, L3–L5, were collected and pooled from four mice. L3–L5 DRG collected from surgically naïve mice older than 10 weeks or from sham mice 4 or 8 weeks after surgery were used as controls. Cells were acutely isolated from DRG via collagenase 4 (1 mg/mL) and papain (30 U/mL, Worthington Biochemical Corp, Lakewood, NJ) digestion. Cells were plated on poly-L-lysine and laminin (20 μg/mL) coated glass coverslips within 6-well plate wells, and cultured at 37°C with 5% $CO_2$ for 4 days in adult neurogenic medium: F12 with L-glutamine, 0.5% FBS, 1× N2 (Life Technologies), penicillin and streptomycin (100 μg/mL and 100 U/mL). Medium was changed on day 2, and supernatants collected on day 4 for protein analysis. For cell culture experiments, a total of 48 C57BL/6 male mice were used (4 mice pooled per DRG culture in two independent experiments). Additionally, 6 *Adamts5* null mice (three DMM and three surgically naïve mice in one experiment). Each plot shows the result from one independent experiment where each dot represents an individual culture well.

### *Analysis of basal FKN levels in DRG culture supernatants under naïve, sham, and DMM conditions*

Cell culture supernatants were concentrated via 3-kDa molecular weight cut-off Millipore Centrifugal Filters (Billerica, MA). Total protein levels were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Inc., Rockford, IL). Levels of soluble FKN were measured via ELISA (R&D Systems Inc, Minneapolis, MN), following manufacturer recommendations.

### *Immunofluorescence*

Mice were anesthetized by ketamine and xylazine and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS[34]. The spinal column was dissected and post fixed overnight in 4% paraformaldehyde followed by cryopreservation in 30% sucrose in PBS. The post fixed spinal column was decalcified in 6% Trichloroacetic acid overnight, followed by cryopreservation in 30% sucrose in PBS[35]. The spinal column was sectioned into corresponding L3–L5 lumbar segments and each segment was embedded in optimal cutting temperature compound, frozen on dry ice and cryosectioned into 20 μm sections[18]. Spinal cord sections were then immunostained with an antibody against ionized calcium-binding adaptor molecule-1 (Iba1) (Wako Chemicals USA, Richmond, VA), followed by a secondary antibody, anti-rabbit conjugated AlexaFluor 488. For CX3CR1-GFP mice, spinal cord sections were immunostained with anti-GFP antibody (Abcam, Cambridge, MA) and anti-Iba1 antibody, followed by the secondary antibodies, anti-chicken conjugated AlexaFluor 488 and anti-rabbit conjugated AlexaFluor 633. As a negative control, spinal cord sections were immunostained with only the secondary antibodies. All images were captured using a laser-scanning confocal microscope and exported to Adobe Photoshop CS5.1 (Adobe, San Jose, CA). Adjustments were made to brightness and contrast to reflect true colors[34]. All images were treated exactly the same in terms of adjustments to brightness and contrast to minimize bias. For immunofluorescence experiments, a total of 38 male WT mice were used for Iba1 staining. Most of the mice within the group

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