CCR2 chemokine receptor signaling mediates pain in experimental osteoarthritis

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Osteoarthritis is one of the leading causes of chronic pain, but almost nothing is known about the mechanisms and molecules that mediate osteoarthritis-associated joint pain. Consequently, treatment options remain inadequate and joint replacement is often inevitable. Here, we use a surgical mouse model that captures the long-term progression of knee osteoarthritis to longitudinally assess pain-related behaviors and concomitant changes in the innervating dorsal root ganglia (DRG). We demonstrate that monocyte chemotactant protein (MCP)-1 (CCL2) and its high-affinity receptor, chemokine (C-C motif) receptor 2 (CCR2), are central to the development of pain associated with osteoarthritis, clinical presentation of movement-provoked pain behaviors, and concomitant changes in the innervating dorsal root ganglia at 8 wk after surgery. This result correlated with the presentation of movement-provoked pain behaviors, which were maintained up to 16 wk. Mice that lack Ccr2 also developed mechanical allodynia, but this started to resolve from 8 wk onwards. Despite severe allodynia and structural knee joint damage equal to wild-type mice, Ccr2-null mice did not develop movement-provoked pain behaviors at 8 wk. In wild-type mice, macrophages infiltrated the DRG by 8 wk and this was maintained through 16 wk after surgery. In contrast, macrophage infiltration was not observed in Ccr2-null mice. These observations suggest a key role for the MCP-1/CCR2 pathway in establishing osteoarthritis pain.

The results show that MCP-1/CCR2 signaling is up-regulated in DMM DRG compared with age-matched naïve DRG. Eight weeks postsurgery, both MCP-1 and CCR2 mRNA were greatly upregulated in DMM DRG compared with age-matched naïve and with sham DRG. Finally, at 16 wk after DMM, MCP-1 and CCR2 expression had returned back to base levels, and they were even somewhat down-regulated compared with age-matched naïve DRG.

In situ hybridization at 8 wk postsurgery on L3–L5 DRG sections showed expression of MCP-1 and CCR2 in DRG neuronal cells of different sizes, and this was strongly up-regulated after DMM (Fig. 1B; higher magnification images in Fig. S1). To assess protein levels of MCP-1, DRG were harvested at 4 and 8 wk postsurgery, and neurons were acutely isolated and cultured for 4 d. Four weeks postsurgery, supernatants from DMM cultures contained similar MCP-1 protein levels compared with sham and age-matched naïve cultures (Fig. 1C). By 8 wk postsurgery, DMM cultures produced increased amounts of MCP-1 compared with both naïve and sham mice (Fig. 1C). Together with the in situ hybridization results, these data suggest that neurons are a source of the increased levels of MCP-1 observed in the DRG in progressive DMM.
One way that chemokines can produce pain is by directly exciting DRG nociceptive neurons, which is associated with increases in intraneuronal calcium concentrations, [Ca\[^{2+}\]]\(_i\) (18). We therefore assessed the sensitivity of DRG neurons to stimulation by MCP-1 by measuring the effects of MCP-1 on neuronal [Ca\[^{2+}\]]\(_i\). Mobilization can be measured on an individual cell basis by using a fluorescent calcium indicator combined with digital video microscopy (18) (Fig. 1D). At 4 wk postsurgery, a similar percentage of neurons in cultures from DMM, sham, and age-matched naïve mice responded to MCP-1 (Table 1). At 8 wk postsurgery, an increased percentage of DMM neurons displayed enhanced [Ca\[^{2+}\]]\(_i\) in response to MCP-1 stimulation compared with neurons from age-matched naïve and sham mice (Table 1), implying that an increased number of DMM neurons expressed functional CCR2 receptors. The increased effects of MCP-1 were specific, as responses to capsaicin or potassium did not change over the same time period.

**Macrophage Infiltration of Innervating DRG.** In addition to the cell bodies of sensory neurons, the DRG contain glial cells and may be infiltrated by immune cells, particularly macrophages. All these cell types may contribute to pain signaling (5, 19, 20). MCP-1 can act on all of these cell types (12) and has been shown to promote infiltration of the spinal cord and DRG following nerve injury (21–23). Therefore, we examined changes in the DRG macrophage population following DMM surgery to assess whether these cells were infiltrating the innervating DRG as a result of the up-regulation of MCP-1. Four weeks postsurgery, few macrophages appeared in DMM and age-matched naïve DRG (Fig. S2). By 8 wk postsurgery, macrophages infiltrated the DRG in wild-type DMM only (Fig. 2 and Fig. S2). This increase in macrophages in DMM DRG was maintained through 16 wk postsurgery (Fig. 2). Macrophage infiltration was not noted in Ccr2-null DRG at 8 wk after DMM nor in age-matched naïve Ccr2-null mice (Fig. 2).

**Wild-Type Mice Develop Mechanical Alloodynia and Decreases in Locomotion at Different Stages After DMM.** Our findings suggest a temporary role for MCP-1/CCR2 signaling in the DRG after DMM surgery, with 8 wk postsurgery apparently being a crucial time point. To understand the biological role of MCP-1/CCR2 signaling in mediating pain-related behaviors, we compared mechanical allodynia and locomotive behaviors for 16 wk after DMM in wild-type vs. Ccr2-null mice. In wild-type mice, mechanical allodynia occurred in the operated hindpaw after DMM but not sham surgery, as we previously reported (17). This allodynia progressed up to 4 wk and was maintained at a constant level up to 16 wk after surgery; age-matched naïve and sham-operated mice did not develop mechanical allodynia (Fig. 3A). We previously reported that morphine and, to a lesser extent, acetaminophen reversed this mechanical allodynia (17). During the second stage, beginning at 8 wk after DMM surgery, behaviors indicative of movement-provoked pain were observed. When monitored overnight on a LABORAŠ (Laboratory Animal Behavior Observation Registration Analysis System) platform (24), DMM mice 4-wk postsurgery and age-matched naïve mice traveled, climbed, and reared as much as age-matched naïve Ccr2-null mice. In wild-type mice, mechanical allodynia occurred in the operated hindpaw after DMM but not sham surgery, as we previously reported (17). This allodynia progressed up to 4 wk and was maintained at a constant level up to 16 wk after surgery; age-matched naïve and sham-operated mice did not develop mechanical allodynia (Fig. 3A). We previously reported that morphine and, to a lesser extent, acetaminophen reversed this mechanical allodynia (17). During the second stage, beginning at 8 wk after DMM surgery, behaviors indicative of movement-provoked pain were observed. When monitored overnight on a LABORAŠ (Laboratory Animal Behavior Observation Registration Analysis System) platform (24), DMM mice 4-wk postsurgery and age-matched naïve mice traveled similar distances (Fig. 3B). The mice also climbed (hanging upside down from the cage lid) and reared (standing on hind paws) the same number of times per hour throughout the night (Fig. 3B). By 8 wk, DMM mice covered less distance, climbed less often, as has been reported previously (16), and reared less often than age-matched naïve and sham mice. These decreases continued up to 16 wk postsurgery (Fig. 3B). Decreases in locomotive behaviors could be substantially reversed by indomethacin, suggesting that they are indicative of movement-provoked pain (Fig. S3).

**Ccr2-Null Mice Present Different Pain-Related Behaviors After DMM Surgery.** Ccr2-null mice displayed similar joint-damage scores at 8 wk postsurgery compared with wild-type mice (total joint score, mean ± SEM; wild-type sham = 1.4 ± 1.0; wild-type DMM = 11.5 ± 2.1; Ccr2-null DMM = 8.2 ± 1.5) (Figs. S4 and S5), but they presented a very different sequence of pain-related behaviors. These mice developed marked progressive mechanical allodynia in the operated hind limb over the first 8 wk following DMM, but unlike in wild-type mice, allodynia abruptly began to resolve after 8 wk and was completely resolved by 16 wk (Fig. 4A). Ccr2-null mice were protected from locomotion changes at 8 wk postsurgery: despite the allodynia present at this time point, and despite comparable knee joint damage, Ccr2-null DMM mice traveled, climbed, and reared as much as age-matched naïve Ccr2-

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**Table 1. Number of cells with [Ca\[^{2+}\]]\(_i\) response to MCP-1**

<table>
<thead>
<tr>
<th>Weeks postsurgery</th>
<th>Naïve</th>
<th>Sham</th>
<th>DMM</th>
<th>(\chi^2) test</th>
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<td>631</td>
<td>99</td>
<td>258</td>
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<tr>
<td>+8</td>
<td>165</td>
<td>416</td>
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**Fig. 1.** MCP-1/CCR2 gene and protein expression in DRG is elevated 8 wk after DMM surgery. (A) Real-time RT-PCR of MCP-1 and Ccr2 in sham and DMM wild-type mice normalized to age-matched naïve levels: at 4 wk postsurgery, \(n = 5\), two-tailed \(t\) test, \(P > 0.05\); at 8 wk postsurgery, \(n = 4\) for naïve, \(n = 5\) for sham and DMM, one-way ANOVA with Bonferroni posttest, MCP-1: \(*P < 0.001\) vs. sham and naïve; Ccr2: \(\sim P < 0.001\) vs. naïve, and \(P < 0.01\) vs. sham; at 16 wk postsurgery, \(n = 4\) for naïve, \(n = 7\) for DMM, two-tailed \(t\) test \(*P < 0.01\) for MCP-1 vs. naïve, \(P = 0.003\) for Ccr2 vs. naïve. Results show mean ± SEM. (B) Representative images of in situ hybridization using antisense probes for MCP-1 and CCR2 in DRG sections (L3–L5) taken from DMM wild-type mice at 8 wk postsurgery and age-matched naïve mice. Sense probe control is shown for the DMM condition. Magnification 10x. (Scale bars, 100 \(\mu\)m.) (C) Protein levels of MCP-1 in the supernatants of cells cultured from age-matched naïve, sham, and DMM mice at 4 and 8 wk postsurgery, \(n = 6\) wells, representative of two independent experiments, one-way ANOVA with Bonferroni posttest, \(***P < 0.001\). Results show mean ± 95% confidence interval. (D) Representative traces of individual cells during calcium imaging assay indicating a response to MCP-1. Response to 50 mM potassium solution, used as a positive control, is also shown.
null mice (Fig. 4B). Sixteen weeks after DMM, these mice were still protected from movement-provoked pain behaviors (Fig. 4B). These findings suggest that Ccr2-null mice, despite the initially severe mechanical allodynia, did not progress to develop movement-provoked pain. Interestingly, supernatants from Ccr2-null DMM DRG neurons cultured at 8 wk postsurgery contained levels of MCP-1 below that of wild-type naive mice (Fig. S6), suggesting the presence of a positive auto-feedback loop regulating MCP-1 and CCR2 expression in wild-type mice.

To confirm the key role of CCR2 signaling in development of the observed movement-provoked pain behavior after DMM surgery, we administered a CCR2 receptor antagonist to wild-type DMM mice at 9 wk postsurgery and found that this reversed the decrease in distance traveled (Fig. 4C).

**Discussion**

In a murine destabilization model of knee osteoarthritis, we found that early-onset mechanical allodynia is followed by an up-regulation of MCP-1/CCR2 signaling in the DRG 8 wk after surgery. This finding correlates with the onset of movement-provoked pain behaviors (decreased overnight distance traveled, climbing, and rearing) at 8 wk after DMM surgery. These decreased locomotion behaviors, indicative of pain that is triggered by movement, may be of translational significance in the context of osteoarthritis, where pain is typically triggered by specific activities (8). The significance of mechanical alldynia in the context of clinical osteoarthritis pain is not yet understood. Current evidence confirms that subjects with osteoarthritis have lower pain-pressure thresholds, both at affected and unaffected sites, suggesting that central sensitization contributes to pain in osteoarthritis (reviewed in ref. 11), but it is unclear how these changed thresholds relate to the actual sensation of pain.

These data support the possibility that increased expression of both MCP-1 and its receptor CCR2 may mediate increased pain signaling in osteoarthritis through the direct excitation of DRG neurons, as well as through attracting macrophages to the DRG. It is known that macrophages can express numerous algogenic molecules that may contribute to the development of pain (25). We sought to confirm a role for MCP-1/CCR2 in pain development by assessing pain-related behaviors in Ccr2-null mutant mice. We found that these mice develop structural joint damage and mechanical allodynia during the first 8 wk following DMM surgery, but despite this they are completely protected from the onset of movement-provoked behaviors. Therefore, initiation of decreased locomotion indicative of movement-provoked pain appears to be dependent on MCP-1/CCR2 signaling.

Previous studies using rodent models of neuropathic pain have shown increases in MCP-1 and CCR2 expression in the DRG within 2 wk following peripheral nerve injury. These models included chronic constriction injury (26), unilateral gp120 sciatic...
nerve administration (27), partial ligation of the sciatic nerve (28), and focal nerve demyelination (29). Mechanical allodynia after nerve injury has been reported to be inhibited by treatment with a CCR2 receptor antagonist (27, 29), and Ccr2-null mice were protected from mechanical allodynia after nerve injury but not during acute inflammatory pain tests (21). We find here that Ccr2-null mice initially develop mechanical allodynia (first phase), but it resolves in the absence of MCP-1/CCR2 signaling. Resolution begins at 8 wk in the Ccr2-null mice, which directly correlates with the observed timing of MCP-1/CCR2 expression in wild-type DRG. Therefore, we conclude that the development of allodynia is not dependent on MCP-1/CCR2 signaling but, rather, that the persistence of allodynia is dependent on this signaling.

The pronociceptive effects of chemokines and their receptors include the induction of hyperexcitability of neurons through transactivation of transient receptor potential cation channel subfamily V member 1 (TRPV1) and other ion channels (12). This include the induction of hyperexcitability of neurons through persistence of allodynia is dependent on this signaling. Interestingly, levels of MCP-1 and CCR2 return to baseline or lower by 16 wk when movement-provoked pain behaviors are observed. This finding suggests that the MCP-1/CCR2 pathway is only involved in the initiation of changes in the DRG, but once macrophages are present, the process is no longer dependent on increased MCP-1/CCR2. In addition, although our findings are consistent with a role for MCP-1/CCR2 signaling within the DRG, it is also possible that CCR2 signaling is important in the spinal cord. MCP-1/CCR2 may be acting in the dorsal horn, as has been suggested (26, 35, 36), further contributing to the observed phenotype in the Ccr2-null mice.

This study represents an initial attempt to try and unravel molecular pathways of pain generation in a long-term model of osteoarthritis in a longitudinal fashion. To our knowledge, we are unique in showing that MCP-1/CCR2 signaling is linked to development of functional persistent pain behaviors in the context of a model for a complex disease associated with chronic pain.

There exists a dire need for new targets for the development of analgesics for osteoarthritis pain. Our data strongly implicate MCP-1/CCR2 signaling in the development of joint pain associated with osteoarthritis. In this respect, it is a promising finding that a CCR2 receptor antagonist reversed movement-provoked pain behavior, corroborating the finding that CCR2 signaling is...
involved in pain associated with osteoarthritis. Subsequent work involving detailed studies to determine the effects of CCR2 blockade in long-term studies, both in prophylactic and in therapeutic protocols, may help to resolve whether this pathway constitutes a novel target for the development of tailored analgesics for osteoarthritis.

Materials and Methods

Animals and Surgery. For these studies, a total of 237 mice were used. 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. DMM or sham surgery was performed in the right knee of 10-wk-old male wild-type C57BL/6 mice or male Ccr2 null mice on a C57BL/6 background (Taconic Farms #3736) as previously described (14, 16). 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.

von Frey Testing. Mice were tested using the up-down staircase method of Dixon (37, 38). 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. In both sham and DMM mice, baseline thresholds were assessed before surgery, and thresholds were assessed at the following times postsurgery: weeks 4, 8, 12, and 16.

LABORAS (Assessment of Spontaneous Behavior). Locomotor behaviors [distance traveled, climbing (hanging upside down from wire cage), and rearing (standing on hindlegs)] were assessed overnight by using the LABORAS (Metris) (24). A 5-mg/kg dose of CCR2 receptor antagonist (CCR2-RA) (Tocris Bioscience, RS 504393; DMSO vehicle) or a 50-μg dose of indomethacin (Tocris Bioscience #1708; DMSO vehicle) was administered intraperitoneally immediately before placement on the platform to study CCR2 inhibition effects on behaviors or analgesic reversal of behaviors, respectively.

Histopathology of the Knee. At 8 wk postsurgery, histopathology of the knee was evaluated based on Osteoarthritis Research Society International recommendations (39) (Alison Bendele, Bolder BioPATH, Inc., Boulder CO). Sections of mouse joints were stained with Toluidine blue. Medial and lateral femoral condyles and tibial plateaus were scored for severity of cartilage degeneration on a scale of 0–5, with 5 representing the most damage. Scoring of the osteophytes (largest on tibial or femoral surface under evaluation) and categorization into small, medium, and large was done with an ocular micrometer and scored from 0 to 3, with 3 representing the presence of large osteophytes. Total joint score is the sum of cartilage degeneration scores in the medial tibial, medial femoral, lateral medial, and lateral tibial compartments and the osteophyte scores in the medial and lateral compartments.

RNA Extraction and Quantitative RT-PCR. At 4, 8, or 16 wk postsurgery, ipsilateral innervating DRG, L3–L5, from wild-type DMM mice were collected and ash-frozen in liquid nitrogen. Ipsilateral DRG from sham-operated mice were collected at 8 wk postsurgery only. RNA extraction was performed via a Qiagen RNeasy kit and was reverse-transcribed. Quantitative RT-PCR was performed with the primers: MCP-1 (Qiagen QT00167832) and CCR2 (Qiagen QT02276813) on a Bio-Rad CFX96 machine using SYBR Green reagents (Life Technologies). Relative gene expression for sham and DMM mice was calculated using the 2–ΔΔCt method by using the average of GAPDH levels and the average of ipsilateral and contralateral age-matched naïve levels.

Cell Culture. At 4 and 8 wk postsurgery, cells were acutely isolated from in-ervinating DRG of three to four mice via collagenase 4 (1 mg/mL) and papain (30 U/mL; Worthington Biochemical) digestion. Cells were plated on poly-l-lysine
and laminin- (20 μg/mL) coated glass coverslips (18), and cultured at 37°C with
5% CO2 for 4 d in adult neurogenic medium: F12 with -glutamine, 0.5% FBS,
1x Neurobasal (Life Technologies), penicillin and streptomycin (100 μg/mL and 100
U/mL) (29).

Protein Analysis of Supernatant. Cell culture supernatants were concentrated
via 3-Kd molecular weight cutoff Millipore centrifugal filters. Total protein
levels were determined by BCA assay (Thermo Fisher Scientific), and levels
of MCP-1 protein were determined via ELISA (R&D Systems), following the
manufacturers’ recommendations.

Calcium Imaging. The response of cultured DRG neurons to the chemokine
MCP-1 (200 ng/mL; R&D Systems) was recorded although intracellular Ca2+
imaging, following standard protocols (18). In brief, MCP-1 was applied for 3
min by adding 1 mL of solution directly to the bath chamber. Cells were
washed 3 min before applying positive control solution (potassium, 50 mM)
(21). Three independent experiments each using DRG pooled from three to
four mice were performed. Total number of neurons counted for each
condition: age-matched naïve (4 wk, n = 631 neurons; 8 wk, n = 416), sham
(4 wk, n = 258; 8 wk, n = 339), and DMM mice (4 wk, n = 306; 8 wk, n = 498).

Immunofluorescence. Mice were anesthetized by ketamine and xylazine and
perfused transcardially with PBS followed by 4% (w/vol) paraformaldehyde in
PBS. The spinal cord was dissected and postfixed in 4% paraformaldehyde
overnight followed by cryopreservation in 30% (v/vol) sucrose in PBS. Individual
ipsilateral L3-L5 DRG were embedded with OCT (Tissue-Tek), frozen with dry ice, and cut into 12-μm sections. DRG sections were stained with
the primary antibodies anti-F4/80 (Abcam ab6640) and anti-doublecortin
(Abcam ab77450). Isotype-specific secondary AlexaFluor-488 or AlexaFluor-633
antibodies (Invitrogen) were used. All captured images were exported to
Adobe Photoshop CS 5.1 (Adobe) and adjustments were made to the brightness
and contrast to reflect true colors (40).

In Situ Hybridization. L3-L5 ipsilateral DRG were embedded and sectioned as
above. Mouse-specific CCR2 probes were generated as described previously
(40). For the generation of CCL2 probes, a 500-bp CCL2 cDNA fragment
(nucleotides 51–551 of GenBank no. NM_011333.3) was cloned by PCR by
using mouse brain cDNA. The resulting PCR product was subcloned into a
pCR II-TOPO vector and verified by restriction analysis and automated DNA
sequencing (Perkin-Elmer). The CCL2 template was linearized with XbaI to
generate an antisense probe by using SP6 polymerase. The sense probe was
linearized with HindIII by using T7 polymerase. In situ hybridization histo-
chemistry for MCP-1 and CCR2 was performed by using digoxigenin-labeled
riboprobes (Roche Applied Science), as previously described (40).

Statistical Analysis. We analyzed calcium imaging data by χ 2 test. We ana-
lized all other data using two-tailed t tests or one-way analysis of variance
tests with Bonferroni posttests, as appropriate. Data were tested for nor-
mality by the D’Agostino-Pearson normality test before analysis and trans-
formed if necessary.

Additional Methods. Detailed methodology is described in the SI Materials
and Methods.

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