CCL2 and CCL3 are essential mediators of pelvic pain in experimental autoimmune prostatitis

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Key words: Chronic pain, chemokines, biomarker, inflammation, autoimmunity.
Abbreviations:

EAP: Experimental autoimmune prostatitis

CP/CPPS: Chronic prostatitis/Chronic pelvic pain syndrome

NOD: Non-obese diabetic mice

B6: C57Bl/6J mice

CCL: Chemokine ligand

CCR: Chemokine receptor
ABSTRACT

Experimental autoimmune prostatitis (EAP) is a murine model of chronic prostatitis/chronic pelvic pain syndrome (CPPS) in men, a syndrome characterized by chronic pelvic pain. We have demonstrated that CCL2 and CCL3 are biomarkers that correlate with pelvic pain symptoms. We postulated that CCL2 and CCL3 play a functional role in CPPS and therefore examined their expression in EAP. Upon examination of the prostate five days after induction of EAP, CCL2 mRNA was elevated 2-3 fold, CCL8 by 15 fold, CCL12 by 12-13 fold and CXCL9 by 2-4 fold compared to control mice. At 10 days the major chemokines were CXCL13 and CXCL2; at 20 days CCL2 (1-2 fold), CCL3 (2-3 fold) and CCL11 (2-3 fold); and at 30 days, CCL12 (20-35 fold) and smaller increases in CCL2, CCL3 and XCL1. Chemokine elevations were accompanied by increases in mast cells and B cells at 5 days, monocytes and neutrophils at day 10, CD4+ T cells at day 20 and CD4+ and CD8+ T cells at day 30. Anti-CCL2 and anti-CCL3 neutralizing antibodies administered at EAP onset attenuated pelvic pain development but only anti-CCL2 antibodies were effective therapeutically. CCL2- and its cognate receptor CCR2-deficient mice were completely protected from development of pain symptoms but assumed susceptibility after reconstitution with wild-type bone marrow. CCL3-deficient mice showed resistance to the maintenance of pelvic pain while CCR5-deficient mice did not show any lessening of pelvic pain severity. These results suggest that the CCL2-CCR2 axis and CCL3 are important mediators of chronic pelvic pain in EAP.
INTRODUCTION

Experimental autoimmune prostatitis (EAP) is a CD4+ T cell-mediated inflammatory disease of the prostate characterized by chronic pelvic pain and serves as a model for chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) in humans (29). The EAP model utilizes rat prostatic antigen injection with adjuvant to induce autoimmune prostatitis in male non-obese diabetic (NOD) mice. This parallels observations in CP/CPPS where the expressed prostatic secretions (EPS) of some patients contain cytotoxic T cells (22), a cell type more commonly associated with autoimmune inflammation and secondary remodeling of injured tissue. Epidemiologic observations indicate that prostatitis conditions are the most frequent urologic diagnosis in young men and the third most frequent urologic diagnosis in men older than 50 yr, representing 8–12% of urology office visits (6). The most common form of prostatitis (Category III) has a prevalence rate in the general population from 5% to 14.2%. CPPS is a poorly understood entity characterized by pelvic or perineal pain, irritative voiding symptoms and sexual dysfunction (15).

Chronic inflammation in EAP is characterized by the presence of inflammatory infiltrates and chemotactic cytokines (chemokines) that are likely to mediate leukocyte accumulation in the prostate. Chemokines can be divided into highly conserved, but distinct, supergene families, the C-x-C, C-C, C and Cx3C families, based on the positions of the first two cysteine residues. Chemokines demonstrate differential chemotactic activity for various leukocyte populations and as such regulate the nature of the
inflammatory reaction in the target tissue. Our laboratory has recently evaluated CCL2 (MCP-1) and CCL3 (MIP-1α,) as potential biomarkers of CPPS (8). CCL2 and CCL3 were elevated in expressed prostatic secretions (EPS) of both inflammatory (IIIA) and non-inflammatory (IIIB) CP/CPPS but not in normal men or men with benign prostatic hyperplasia (BPH) and correlated with clinical pain as determined by the NIH chronic prostatitis symptom index (CPSI). We therefore postulated that CCL2 and CCL3 play a functional role in the pathogenesis of EAP. We assessed the source, nature and kinetics of chemokine expression in the prostate along with phenotyping of the immune infiltrate. Our results indicate an important role for CCL2 and CCL3 in the pathogenesis of pelvic pain in EAP.
MATERIALS AND METHODS

Animals: We used 5 to 7-week-old NOD/ShiLtJ (NOD), C57BL/6J (B6), B6.129P2-Ccl3.tm1Unc/J (CCL3-/-), B6.129S4-Ccl2.tm1Rol/J (CCL2-/-), B6.129S4-Ccr2.tm1Ifc/J (CCR2-/-), B6.129P2-Ccr5.tm1Kuz/J B6.129S4-Ccr2.tm1Ifc/J (CCR5-/-) from Jackson Laboratory (Bar Harbor, Maine). All experiments were done using protocols approved by the Northwestern University Animal Care and Use Committee. EAP was induced in mice using rat prostate antigen (rat prostate lysates) with adjuvant injected subcutaneously in the shoulder as described previously (29). Antibody neutralization was performed using 100ug intraperitoneal injections of polyclonal anti-CCL2/JE (AB479NA, R&D systems), polyclonal anti-CCL3 (AB450NA, R&D systems) or normal goat IgG (AB108C, R&D systems).

Creation of bone marrow chimeras: Male C57BL/6, CCL2-/-, and CCR2-/- mice were sacrificed and long bones of the limb were removed, the ends were excised and the marrow was flushed with MEM + 15% FBS using a syringe attached to a 25 gauge needle. The marrow was broken up by being passed through the syringe and filtered into a 50mL conical vial with a 45uM filter. Cells were spun down at 350g for 10 minutes and resuspended in 1mL PBS. The cells were then counted using a hemocytometer and adjusted to a concentration of 8 x 10^7 cells/mL with a 1:10 ratio of knockout to wild type bone marrow. CCL2-/- and CCR2-/- mice were then
intravenously injected with 100uL of the bone marrow. Animals were rested for 8 weeks prior to EAP induction.

**Behavior testing:** All testing was conducted with the tester blinded to the group, or the treatment that the individual mice received, to eliminate observer bias. Mice were tested prior to infection (baseline) and at post-infection days (PIDs) 1, 7, 14, 21 and 28. Mice were tested for pelvic pain in individual Plexiglas chambers (6 x 10 x 12 cm) with a stainless steel wire grid floor as previously described (29). Referred hyperalgesia indicated by an increase in responsiveness to stimulus intensities that evoked a response under baseline conditions (hyperalgesia) and tactile allodynia indicated by responsiveness to stimuli that provoked no reaction prior to EAP were tested using different calibrated forces of von Frey fibers. Referred hyperalgesia and tactile allodynia were tested using von Frey filaments with forces of .04, .16, .4, 1 and 4 g (Stoelting). Each filament was applied in increasing force order for 1-2 s with at least a 5 s interval between stimulations for a total of 10 times. Stimulation was confined to the pelvic area in the general vicinity of the prostate and care was taken to stimulate different areas within this region to avoid desensitization. Three types of behaviors were considered as positive responses to filament stimulation: 1) sharp retraction of the abdomen; 2) immediate licking or scratching of the area of filament stimulation; or 3) jumping. Response frequency was calculated as the percentage of positive response (out of 10, e.g., 5 responses of 10 = 50%), and data were reported as the mean percentage of response frequency ± SE (5).
Quantitative PCR: Total RNA of tissue and cells was isolated with the QIAGEN RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The concentration and purity of RNA were determined by measuring absorbance at 260 and 280 nm with a Nanoview Plus Spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire). Total RNA were reverse-transcribed by RT² First Strand Kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and single-stranded cDNA was amplified by PCR with specific primers as described in Table 1. For PCR amplification, RT² SYBR Green qPCR Mastermix (Qiagen, Hilden, Germany) was used according to the manufacturer’s protocol. The reaction conditions were as follows: an initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation for 60 sec at 94°C, annealing for 30 sec at 60°C and extension for 60 sec at 72°C with a final melting curve from 50°C to 95°C. The threshold cycle (Ct) values of target genes were normalized with L19 of the same sample and expressed relative to controls. Fold change in gene expression was calculated using the delta delta Ct method.

Laser capture microdissection (LCM): Prostates from NOD/ShiLtJ mice infected with EAP for 30 days or prostates from naïve NOD/ShiLtJ mice were isolated and 5 μm paraffin embedded sections were prepared on PEN foil slides following nuclease free techniques. Immediately before LCM, samples were deparaffinized, stained, dehydrated, placed for 30 secs in Paradise Plus Staining Solution (Applied Biosystems,
Carlsbad, CA) followed by treatment with ethanol and xylene. LCM was performed using the PixCell II Laser Capture Microdissection System (Arcturus Engineering) by melting thermoplastic films mounting on CapSure Macro LCM Caps (Applied Biosystems, Carlsbad, CA). Cells from the epithelium and stroma were separated, collected with LCM Caps, and incubated for 24 hours with Protinase K. RNA extraction and isolation was performed with the Arcturus Paradise Extraction and Isolation Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. The concentration and purity of RNA were determined by measuring absorbance at 260 and 280 nm with a Nanoview Plus Spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire). PCR reaction conditions for chemokine gene assays were as follows: 94°C for 2 min followed by 50°C for 2 min, 45 cycles of denaturation for 15 sec at 95°C, annealing for 60 sec at 55°C, and extension for 10 sec at 95°C.

**Immunohistochemistry:** Prostates were collected from mice immunized for 30 days with EAP and 5μm sections were processed for CCL12, CCL2 and CCL3 staining. Formalin-fixed and paraffin-embedded tissues were examined using anti-mouse CCL2 (MON7035, Cell Science), anti-mouse CCL12 (V-20) (sc-9720, Santa Cruz), CCL3 antibody Goat anti-mouse (M1233, Leinco) or appropriate isotypes or blocking peptides. Positive staining was detected using the ImmunoCruz mouse ABC Staining System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
Cell Separation: Enzymatic methods were utilized for isolation experiments involving single-cell suspensions of the prostate (13). Prostates were rinsed with complete RPMI 1640 containing 10% FBS, cut into small pieces and digested for 40 minutes at 37°C with 1 mg/mL collagenase D (Roche Diagnostics), 10 mM HEPES (Mediatech) and .01% DNase I (Sigma) in complete RPMI 1640 (Mediatech). Digested suspensions were passed through a 40μm nylon mesh and centrifuged. The cell pellet was suspended in FACS staining buffer.

Flow cytometry and immunophenotyping: Prostates were excised and single-cell suspensions of tissues were digested for 40 minutes following the digestion protocol above at 37°C. Samples were then passed through a 40μm nylon mesh filter and centrifuged at 350 x g for 5 minutes. Samples were separated into 96-well plates and washed with FACS staining buffer (PBS + 2% FBS). Cells were then phenotyped for mast cells (CD117 and FcεR1α), macrophage/monocytes (CD11b and F4/80), neutrophils (CD11b and Gr1), CD4+ T cells, CD8+ T cells, B cells (CD45R/B220) and Treg cells (FoxP3) using fluorochrome-conjugated primary antibodies. Stained cells were analyzed on an Accuri C6 flow cytometer and analysis of cell populations was performed using FlowJo 7.6 (Treestar) software.

Statistical analyses. Results were expressed as mean ± SEM and analyzed for statistical significance by unpaired t tests or repeated measures ANOVA using Graphpad statistical software. A value of p < 0.05 was considered statistically significant.
**RESULTS**

**EAP is characterized by elevated chemokines in the prostate.** It has been previously demonstrated that EAP in NOD mice results in the accumulation of an inflammatory infiltrate that presumably was recruited to the prostate during the process of inflammation (28, 29). We therefore examined the prostates of NOD mice over time with and without EAP for differences in expression of chemokine mRNA identified previously as elevated using the Illumina Mouse-6 version 1 Expression BeadChip (data not shown). C-C chemokines represented the largest family of chemokines elevated in the prostate (Fig. 1A) with CCL2, CCL7, CCL8, CCL12, CCL3, CCL4, CCL6 and CCL11 being elevated at various times during EAP. Among the C-X-C chemokines, CXCL2, CXCL9 and CXCL13 were elevated and XCL1 was representative of the C chemokine superfamily (Fig. 1). Five days after induction of EAP, CCL2 was elevated 2-3 fold, CCL8 by 15 fold, CCL12 by 12-13 fold and CXCL9 by 2-4 fold compared to control NOD mice (Fig. 1A and B). At 10 days there were small increases in CCL2 and CCL3 but the major chemokines were of the C-X-C type including a 1-2 fold increase in CXCL2 and a 2-3 fold increase in CXCL13 mRNA (Fig. 1B). At 20 days the predominant chemokines upregulated were CCL2 (1-2 fold), CCL3 (2-3 fold) and CCL11 (2-3 fold). The 30 day time-point was characterized by a robust 20-35 fold increase in CCL12 and smaller increases in CCL2, CCL3 and XCL1 (Fig. 1).

We next examined the site of chemokine expression in the prostate following EAP using microdissection of the epithelial and stromal cells of the prostate followed by
quantitative analysis for chemokine mRNA. These studies, conducted in chronic EAP mice at 30 days following induction, demonstrate that CCL2 and CCL3 are predominantly expressed in the prostate epithelial cells whereas CCL7, CCL12, CXCL2 and Xcl1 are largely produced from the stromal compartment in the prostate (Fig. 2A). To confirm the location as well as production of chemokine proteins in the prostate, sections of the dorsolateral prostate of EAP mice were probed using immunohistochemistry for the representative CC chemokines CCL2, CCL12 and CCL3. CCL2 as well as CCL3 was shown to be strongly associated with epithelial cells lining the prostatic ducts (Fig. 2B, a and c), while CCL12 was associated with cells in the prostate stroma (Fig. 2B, b). These results suggest that EAP induces a robust inflammatory response in the prostate through production of specific chemokines by prostate epithelial and stromal cells.

Quantification of immune infiltrates in EAP. We hypothesized that the pain response in NOD mice could be linked to the types of immune infiltrating cells during EAP development. We therefore utilized single-cell suspensions of the prostates at various time-points to perform flow cytometry and do relative quantification of the immune infiltrates. The prostates of naïve mice were not devoid of immune cells and contain predominantly CD8+ T cells (41.34±1.99%) and CD4+ T cells (23.04±2.4%) with smaller populations of other cell types. At five days following EAP, there were significant reductions (p<0.01) in CD8+ T cell (29.5±3.2%) and CD4+ T cells (13.3±2.8%) coupled with significant increases in mast cells (6.02±0.45%) and B cells (11.4±2.5%) in the
prostate. At ten days, mast cell and B cell numbers stayed significantly elevated (p<0.01), but additionally, macrophage/monocyte populations as well as neutrophils showed a significant increase (p<0.01). Twenty days following EAP induction, CD4+ T cells become the predominant population in the prostate (35.9±3.5%) leading to a reversal of the CD4+:CD8+ T cell ratio compared to naïve mice (0.56±0.06 to 1.89±0.06). At thirty days, the immune infiltrate once more was predominantly comprised of CD4+ and CD8+ T cells with percentages of other cell types not significantly different from naïve mice. We also examined the percentage of activated and regulatory CD4+ T cells at various times during EAP and showed that after an initial expansion of the regulatory T cell population at five (p<0.05) and ten days (p<0.01) following EAP induction, populations plummeted at twenty and thirty days. Interestingly, the elevated regulatory T cell population was accompanied by a reduction in activated T cells at ten days (p<0.05) that subsequently expanded at twenty and thirty days. These results show the development of robust inflammation in the prostate characterized by cell types of regulatory and effector phenotypes.

Neutralization of CCL2 or CCL3 attenuates pelvic pain in EAP. In previous studies in human CPPS, CCL2 and CCL3 have been identified as biomarkers and CCL3 has been correlated with pelvic pain (8). We therefore examined the role of CCL2 and CCL3 in chronic pelvic pain by administering anti-CCL2 or anti-CCL3 antibodies to mice at the onset of EAP or 20 days following the initiation of EAP (Fig. 4 and 5). Pelvic pain was measured as tactile allodynia of the suprapubic region and compared to responses at
the paw region in male mice. Changes in response frequency to von Frey fibers applied
to the pelvic region at EAP onset, during the course of EAP development, prior to and
following anti-CCL2, anti-CCL3 or isotype control antibodies were calculated.
Administration of anti-CCL2 or anti-CCL3 but not an isotype control antibody
prophylactically, at the onset of EAP, resulted in significantly reduced response
frequencies (Fig. 4A-E). Pelvic pain expressed as an increase from baseline during the
course of EAP was shown to be significantly attenuated at 5, 10 and 20 days following
EAP onset in the anti-CCL2 and anti-CCL3 groups but was not significantly different
from isotype antibody or control at 30 days. In a separate series of experiments we
examined the potential of anti-CCL2 and anti-CCL3 neutralization for therapeutic
inhibition of pelvic pain. Mice were treated with anti-CCL2, anti-CCL3 or isotype
control antibodies at 20 days after EAP onset and pain responses were followed daily
for 5 days. Anti-CCL2 treatment resulted in a significantly reduced response frequency
at 24 and 48 hours following treatment but pelvic pain responses returned to
pretreatment levels by 3 days after treatment (Fig. 5A and D). In contrast anti-CCL3
showed a small but statistically insignificant reduction of pain that was not different
from treatment with isotype control antibody (Fig. 5B and D). In both prophylactic and
therapeutic antibody administration, no significant changes in threshold responses at
the paw region or in body weight of the mice were observed (data not shown). These
results suggest that CCL3 and especially CCL2 play important roles in the pathogenesis
of pelvic pain in EAP.
CCL2 and CCL3 deficient mice do not develop chronic pelvic pain in EAP. We examined the development of chronic pelvic pain in B6 mice and mice deficient in either CCL2 or CCL3. Control B6 showed a significant enhancement of pelvic pain at 10 days after initiation of EAP (p<0.01), similar to CCL2-/- (p<0.05) and CCL3-/- (p<0.01) mice. In contrast, 30 days after EAP initiation, both CCL2-/- and CCL3-/- mice showed reduced response frequencies (Fig. 6B and C) and pelvic pain increases that were not significantly different from baseline (Fig. 6D). These results suggest that CCL2 and CCL3 are critical for the maintenance of chronic pelvic pain in EAP.

CCR2 but not CCR5 is critical for development of chronic pelvic pain. We hypothesized that because of the importance of CCL2 and CCL3 in EAP, the absence of the major cognate receptors CCR2 and CCR5 would impact EAP and pelvic pain development. CCR2-/- mice showed a complete absence of pelvic pain development with no significant increase in response frequency from baseline at 10, 20 and 30 days after initiation of EAP (Fig. 7A and C). In contrast, EAP induction in CCR5-/- mice lead to a delayed but significant increase in pelvic pain by 30 days after EAP induction (p<0.05, Fig. 7B and C). These results suggest that while CCR2 is critical for the development of pelvic pain, the CCR5 receptor is less important for chronic pelvic pain development. To examine the impact of the loss of the CCR2 and CCR5 receptors on the ability to recruit immune cells to the prostate we performed single-cell dissociation of the prostates at 30 days after EAP induction and performed immunophenotyping using flow cytometry. In comparison to B6 mice, CCR2-/- and CCR5-/- showed reductions in
CD4+ and CD8+ T cells but approached statistical significance only in CD4+ T cells of CCR5-/- mice. Other immune populations examined were not statistically significant from B6 controls. These results suggest that the differences in immune infiltrates in the prostate do not fully account for the profound differences in pelvic pain responses observed in CCR2-/- mice compared to CCR5-/- and B6 mice.

Reconstitution of CCL2-/- and CCR2-/- mice with B6 bone marrow reinstates susceptibility to chronic pelvic pain. We examined the ability of wild-type haematopoietic cells from B6 mice to reconstitute chronic pelvic pain susceptibility in CCL2-/- and CCR2-/- mice following EAP induction. CCL2-/- mice reconstituted with B6 bone marrow demonstrated EAP-induced increase in response frequencies that was comparable to that observed in B6 mice (Fig. 8A) and significantly different from baseline at 10, 20 and 30 days following EAP induction (Fig. 8B, p<0.05). CCR2-/- mice reconstituted with bone marrow from B6 mice also demonstrated a significant increase in response frequencies from baseline that was statistically significant at 10 and 30 days following EAP induction (p<0.05). Both CCL2-/- and CCR2-/- reconstituted mice demonstrated a time-dependent increase in pelvic pain that was not statistically different from B6 mice (Fig. 8D). These results suggest that haematopoietic expression of CCL2 and its cognate receptor CCR2 are important in the pathogenesis of EAP-induced chronic pelvic pain.
DISCUSSION

CP/CPPS is a disease syndrome with unknown etiology but immune dysfunction in the form of autoimmunity has received considerable support as a potential mechanism from human studies (27) including findings such as demonstration of elevated levels of tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1) in the seminal fluids of men with CP/CPPS (3); the presence of IFN-γ-secreting lymphocytes specific to prostate antigens in some chronic prostatitis patients (22); the ability of CD4+ T lymphocytes in men with CP/CPPS to recognize soluble components in normal semen (2); and the recognition by CD4+ lymphocytes of prostate specific antigen (PSA) (26). A number of studies in humans have identified cytokines and chemokines as elevated in prostatic fluid (14, 23). Two such chemokines, CCL2 and CCL3 have been shown by our laboratory to be potential biomarkers for CPPS with positive correlation to pain symptoms and presence of leukocytes in prostatic secretions (8). We recently demonstrated using the EAP model that the distinguishing feature of CPPS, namely pelvic pain, is observed in EAP along with infiltration of inflammatory cells into the prostate (29). The pelvic pain in this model is represented by referred visceral hyperalgesia of the somatic area, a characteristic that has been previously quantified using electrical and natural stimulation in patients with a variety of different visceral pain states (reviewed in (4, 11)). The referred hyperalgesia has been recapitulated in animal models of visceral pain (19, 20) and is manifested as increased responsiveness to stimuli that provoked no reaction prior to instillation of irritants into
visceral organs (alldynia). In this study, we sought to examine the role of chemokines in the EAP model by systematic identification of chemokines induced, source of chemokines, immune cell types recruited and the consequence of loss of chemokine function on pelvic pain symptoms and immune cell types recruited into the prostate. Our results indicate that CCL2 and CCL3 are critical to the development of chronic pelvic pain.

To understand the pattern and kinetics of leukocyte recruitment and chemokine expression in EAP, we examined the prostates of mice at 0, 5, 10, 20 and 30 days after EAP onset. In our study, mast cells were one of the first series of cells to be activated. These results are in agreement with previous findings that showed increased mast cell numbers in the prostate early on during EAP development (9). Mast cells have been shown to be closely associated with the severity of pelvic pain in EAP and can be induced to undergo chemotaxis by a number of chemokines including the CC chemokines CCL2, CCL7, CCL8, CCL3 and CCL11 (24, 31), all of which are shown to be enhanced 5 days after EAP initiation. CXCL9, an IFNγ induced chemokine known to mediate T cell recruitment, was enhanced at day 5 and did not appear to be associated with an overall increase in T cell infiltration. However regulatory T cells numbers at this time-point were elevated suggesting a role for CXCL9 in regulatory T cell recruitment. A role for CXCL9 along with its receptor CXCR3 has been suggested in regulatory T cell recruitment in human renal cell carcinoma (25). Interestingly, monocyte/macrophage populations that are prime recruits of the CC chemokines appear to be delayed in being
recruited to the prostate until day 10, at which time mast cells, monocyte/macrophages and neutrophils are at their maximal level in the prostate. Two major chemokines elevated at day 10 are CXCL2 and CXCL13, members of the C-×-C-ELR family of chemokines. Neutrophils numbers are significantly elevated in the prostate at day 10 and are likely to be associated with CXCL2, a known inducer of neutrophil recruitment. CXCL13 is a small cytokine that is selectively chemotactic for B cells belonging to both the B-1 and B-2 subsets and is associated at day 10 with a significant elevation in B cells in the prostate. 20 days after EAP onset CD4+ T cells numbers appeared to be maximal either as a result of enhanced recruitment or alternatively, enhanced proliferation. The chemokine milieu in the prostate at the day 10 time-point appeared to have elements of both the Th1 and Th2 type responses with CCL2 and CCL11 representing Th2 responses and CCL3 elevation suggesting a developing Th1 phenotype in the EAP prostate. By 30 days after EAP onset, the leukocytic infiltrate in the prostate appeared to have settled down to levels at or slightly above the normal. However epithelial and stromal cells in the prostate appeared to be producing significant amounts of chemokines including CCL2, CCL3 and CCL12 that are likely to contribute to the continued pathology observed in EAP.

Given our previous studies in human CPPS suggesting a role for CCL2 and CCL3 in chronic pelvic pain (8), we explored the consequence of neutralizing their function in vivo in the EAP model. Concurrent treatment of NOD mice with a single dose of anti-CCL2 or anti-CCL3 antibody at the time of EAP initiation was sufficient to
significantly delay the kinetics of pelvic pain development suggesting either a role for these chemokines in EAP development, chronic pelvic pain development or both. Therapeutic application of the antibodies after development of chronic pelvic pain resulted in a significant but temporary inhibition of pelvic pain with the anti-CCL2 antibody but a less pronounced inhibition with the anti-CCL3 antibody. The temporary nature of the inhibitory response may be attributed to the single dosing and subsequent antibody clearance. In both scenarios, EAP development and chronic pelvic pain are only partially ablated by inhibition of CCL2 and CCL3, suggesting redundancy of these chemokine effects on pain and inflammation and/or partial efficacy using antibody neutralization approaches. Similar studies in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis where CCL2 and CCL3 are important disease mediators have demonstrated the efficacy of CCL2 and CCL3 neutralization on disease severity (18). However, anti-CCL2 antibody-based approaches in early multiple sclerosis human trials have met with less success (12).

In contrast to the partial inhibition observed in the neutralization experiments with EAP, genetic ablation of the CCL2 chemokine or its cognate receptor CCR2 in gene knockout mice appear to be completely protective with regard to the development of chronic pelvic pain. These results are similar to reports in EAE where CCL2 or CCR2 knockout mice are protected from disease pathology (10, 16, 17). Reconstitution of CCL2 and CCR2 knockout mice with B6 bone marrow reconstitutes pelvic pain susceptibility suggesting a role for CCL2 and CCR2 expression in the haematopoietic compartment.
In contrast to these results, CCL3 knockout mice as well CCR5 knockouts with EAP show a mixed pain phenotype. The absence of CCL3 appears to have no effect on the initial kinetics of pain development in EAP but prevents the persistence of pelvic pain. The absence of CCR5 does not appear to have any significant effect on either the onset or continued development of chronic pelvic pain, even though some reduction in CD4+ T cell recruitment to the prostate appears to be present. These results suggest that CCL3 plays an important role in the pathogenesis of chronic pelvic pain but does not appear to be dependent on the CCR5 receptor. Importantly, in contrast to CCL2 that is completely dependent on the CCR2 receptor, CCL3 is known to utilize additional receptors including CCR1. Interestingly, CCL3 in EAE has also been described to utilize a CCR1 mediated mechanism with CCR5-/- mice showing no reduction in disease severity (30). An unexpected feature of EAP in the CCR2 and CCR5 knockout mice is the absence of any profound defects in leukocyte recruitment. We do not rule out the possibility that difference in recruitment may have been more marked at early time-points in EAP, as opposed to the 30 days time-point utilized for our immunophenotyping assays.
PERSPECTIVES AND SIGNIFICANCE

This study describes the chemokine signature and kinetics of expression in the prostate in EAP simultaneously with identification of recruited immune cell types. Furthermore, our study identifies critical roles for CCL2, CCL3 and CCR2 in chronic pelvic pain. An important question raised by these studies is the mechanism mediating pelvic pain induced by CCL2 and CCL3. While showing an association between chemokines and different immune cell types, our studies do not conclusively demonstrate a cause and effect relationship between the chemokine, its target cell type and pelvic pain. On the contrary our results lead us to postulate that the site of action for CCL2 and CCL3 may be at higher centers in the nervous system where CCL2 has been shown to be involved in lowering the threshold of neuronal activation (reviewed in (21)) leading to altered pain neurotransmission (7, 32). These nociceptive responses in inflammatory and neuropathic models of pain have been shown to be dependent on CCR2, through expression both at the site of injury and also in the dorsal root ganglia and spinal cord, resulting in sensitization of primary afferents and spinal cord neurons (1). Future studies will therefore examine the influence of these chemokines and their cognate receptors on peripheral and central neuronal pathways to mediate pelvic pain. These results have important implications in understanding the pathogenesis of human CPPS.
FIGURE LEGENDS

Figure 1. Kinetics of chemokine expression in the prostates of EAP mice. Prostates from NOD mice with EAP were collected at 5, 10, 20 and 30 days after EAP onset. (A) Quantitative PCR analysis for select C-C chemokines, (B) C-X-C chemokines, and (C) C chemokines were performed and data was expressed as fold change from naïve controls (A-C). Data shown is the mean ± SEM of two separate experiments with 3-5 animals per group.

Figure 2. Sources of chemokine expression in the prostate. (A) 5 μm sections of the prostates of NOD mice with EAP at day 30 were subjected to laser capture microdissection to obtain epithelial and stromal cells (approximately 100 cells/sample) separately followed by quantitative RT-PCR analysis for CC, C-x-C and C chemokines. Data was expressed as fold change from naïve controls. Data shown is the mean ± SEM of two independent experiments. (B) Representative CC chemokines were examined using immunohistochemistry of 30 days EAP NOD mice. 5 μm sections of the prostate were probed with anti-CCL2, anti-CCL12, anti-CCL-3 or isotype control antibody (panels d, e and f). Arrows indicate increased CCL3 (compare panels a and d), CCL12 (compare panels b and e), or CCL3 (panels c and f). Scale bar indicates 50 μm.
Figure 3. Kinetics of immune cell recruitment to the prostate in EAP. Prostates of NOD mice with EAP at 0, 5, 10, 20 and 30 days after EAP onset were dissociated into single-cell suspensions, stained with antibodies against individual immune cell types and analyzed on a flow cytometer using a preset leukocyte gate (CD45). Results were expressed as a percentage of the total leukocyte gate and 3×10^5 cells were analyzed per prostate. Results shown are the mean± SEM of 3-6 animals per time-point with experiments being repeated at least twice.

Figure 4. Anti-CCL2 and anti-CCL3 antibodies inhibit and delay the development of chronic pelvic pain. Control mice EAP was initiated using prostate antigen (PAg) in NOD mice alone (B) or simultaneously with administration of 100μg of control goat IgG (isotype, C), anti-CCL2 (D) or anti-CCL3 (E). Referred visceral hyperalgesia in NOD mice was measured as responses to mechanical stimulation of the pelvic region using von Frey filaments of 5 calibrated forces. Data is shown as the mean percentage of positive response ± SEM before instillation of antibody (baseline) and at days 5, 10, 20 and 30 days following injection. The symbol key shown in panel A applies to panels B, C, D and E. Results are also shown as percentage increase in pain from baseline using normalized total frequencies (F). All experiments were repeated at least two times.

Figure 5. Anti-CCL2 antibody therapeutically inhibits chronic pelvic pain. EAP was initiated using prostate antigen (PAg) in NOD mice and allowed to proceed for 20 days at which time 100μg of anti-CCL2 (A), anti-CCL3 (B) or control goat IgG (C) were
administered. Referred visceral hyperalgesia in NOD mice was measured as responses to mechanical stimulation of the pelvic region using von Frey filaments of 5 calibrated forces. Data is shown as the mean percentage of positive response ± SEM before instillation of antibody (baseline) and at 1, 2, 3, 4 and 5 days after antibody administration. The symbol key shown in panel A applies to panels B, C, D and E. Results are also shown as percentage increase in pain from baseline using normalized total frequencies. All experiments were repeated at least two times.

Figure 6. CCL2-/- and CCL3-/- mice are resistant to chronic pelvic pain. EAP was initiated using prostate antigen (PAg) in wild-type B6 control (A), CCL2-/- (B) and CCL3-/- (C) mice followed by measurement of referred visceral hyperalgesia. Referred visceral hyperalgesia was measured as responses to mechanical stimulation of the pelvic region using von Frey filaments of 5 calibrated forces. Data is shown as the mean percentage of positive response ± SEM before instillation of antigen (baseline) and at days 10, 20 and 30 days following injection. The symbol key shown in panel A applies to panels B, C, D and E. Results are also shown as percentage increase in pain from baseline using normalized total frequencies. All experiments were repeated at least two times and had 3-5 animals per group.

Figure 7. CCR2-/- but not CCR5-/- mice are resistant to chronic pelvic pain. EAP was initiated using prostate antigen (PAg) in CCR2-/- (A) and CCR5-/- (B) mice followed by measurement of referred visceral hyperalgesia. Data is shown as the mean
percentage of positive response ± SEM before instillation of antigen (baseline) and at 10, 20 and 30 days following injection. The symbol key shown in panel A applies to panels B, C, D and E. Results are also shown as percentage increase in pain from baseline using normalized total frequencies. All experiments were repeated at least two times and had 3-5 animals per group. Single cell-suspensions of the prostates of CCR2-/- and CCR5-/- mice were subjected to immunophenotyping using flow cytometry and results are expressed as percentage of total leukocytes in the prostate (D).

Figure 8. B6 reconstitution of CCL2-/- and CCR2-/- mice confers pelvic pain susceptibility. CCL2-/- and CCR2 -/- mice were reconstituted with 8 x 10^6 bone marrow from B6 mice followed by EAP initiation after 8 weeks. Referred visceral hyperalgesia was measured and represented as the mean percentage of positive response ± SEM before instillation of antigen (baseline) and at 10, 20 and 30 days following injection. The symbol key in panel A applies to panels B and C. Results are shown as percentage increase in pain from baseline using normalized total frequencies. All experiments were repeated and had 5 animals per group.
Authorship:

MLQ - performed experiments, concept of experiment, analysis, manuscript preparation

SM - performed experiments, concept of experiment, analysis, manuscript preparation

CNR - performed experiments, concept of experiment, analysis

JDD - performed experiments, analysis, manuscript preparation

AJS – Study design, concept of experiment, analysis

PT – Study design, concept of experiment, manuscript preparation
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Figure 1

A

Fold change in C-C mRNA

Day 5
Day 10
Day 20
Day 30

CCL2 CCL8 CCL7 CCL12 CCL3 CCL4 CCL6 CCL11

B

Fold change in C-X-C mRNA

CXCL9 CXCL2 CXCL13

C

Fold change in C mRNA

XCL1
Figure 2
Figure 4

A) Control
B) PAg (EAP)
C) Isotype
D) Anti-CCL2
E) Anti-CCL3
F) Pain Response

Day: 0 5 10 20 30
Pain (% Increase): 0 100 200 300 400 500

Legend:
- Cntrl (n=6)
- PAg (n=8)
- Isotype (n=7)
- Anti-CCL2 (n=8)
- Anti-CCL3 (n=8)
Figure 5

A. Anti-CCL2

B. Anti-CCL3

C. Control

D. Pain Response

Stimulus (g)

Response Frequency (%)

0.0 1.0 2.0 3.0 4.0

Baseline

PAg 20d

1d

2d

3d

4d

5d

A Anti-CCL2

B Anti-CCL3

C Control

Pain (% Increase)

0 200 400 600 800

Control (n=14)

Anti-CCL2 (n=13)

Anti-CCL3 (n=13)

0 20 21 22 23 24 25

Pain Response
Figure 6

A B6

B CCL2-/-

C CCL3-/-

D Pain Response

Von Frey (g)

Response Frequency (%)

Baseline

10 days

20 days

30 days

Pan (% Increase)

Time (d)

0 10 20 30 500

B6

CCL2-/-

CCL3-/-
Figure 7

(A) CCR2-/-

(B) CCR5-/-

(C) Pain Response

(D) Baseline

10 days

20 days

30 days

*
Figure 8

A. **B6**

B. **B6→CCL2−/−**

C. **B6→CCR2−/−**

D. **Pain Response**

- **B6**
- **B6→CCL2−/−**
- **B6→CCR2−/−**

Response Frequency (%) vs. Von Frey (g)

Pan (% Increase) vs. Time (d)
### Table 1. Chemokine gene primers

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<tr>
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