Am J Physiol Regul Integr Comp Physiol 303: R580–R589, 2012.
First published July 18, 2012; doi:10.1152/ajpregu.00240.2012.

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

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Submitted 29 May 2012; accepted in final form 16 July 2012

Quick ML, Mukherjee S, Rudick CN, Done JD, Schaeffer AJ, Thumbikat P. CCL2 and CCL3 are essential mediators of pelvic pain in experimental autoimmune prostatitis. *Am J Physiol Regul Integr Comp Physiol* 303: R580–R589, 2012. First published July 18, 2012; doi:10.1152/ajpregu.00240.2012.—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 chemokine ligands 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 5 days after induction of EAP, CCL2 mRNA was elevated 2- to 3-fold, CCL8 by 15-fold, CCL12 by 12- to 13-fold, and CXCL9 by 2- to 4-fold compared with control mice. At 10 days the major chemokines were CXCL13 and CXCL2; at 20 days CCL2 (1- to 2-fold), CCL3 (2- to 3-fold) and CCL11 (2- to 3-fold); and at 30 days, CCL12 (20- to 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.

chronic pain; chemokines; biomarker; inflammation; autoimmunity

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 nonobese 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. Epidemiological 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 Cx_3C 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 (monocyte chemotactic protein-1, MCP-1) and CCL3 (macrophage inflammatory protein-1, MIP-1α) as potential biomarkers of CPPS (8). CCL2 and CCL3 were elevated in expressed prostatic secretions (EPS) of both inflammatory (IIIA) and noninflammatory (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-wk-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}/JB6.129S4-Ccr2^{tm1Ifc}/J (CCR5$^{-/-}$) from Jackson Laboratory (Bar Harbor, ME). 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 100 μg 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).

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Creation of bone marrow chimeras. Male C57BL/6, CCL2$^{-/-}$, and CCR2$^{-/-}$ mice were euthanized, and long bones of the limb were removed, the ends were excised, and the marrow was flushed with MEM plus 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 50-ml conical vial with a 45-μM filter. Cells were spun down at 350 *g* for 10 min and resuspended in 1 ml PBS. The cells were then counted using a hemocytometer and adjusted to a concentration of 8×10^7 cells/ml with a 1:10 ratio of knockout to wild-type bone marrow. CCL2$^{-/-}$ and CCR2$^{-/-}$ mice were then intravenously injected with 100 μl of the bone marrow. Animals were rested for 8 wk before 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 before infection (baseline) and at postinfection days (PIDs) 1, 7, 14, 21, and 28. Mice were tested for pelvic pain in individual Plexiglas chambers ($6 \times 10 \times 12$ 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 before 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 0.04, 0.16, 0.4, 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 Spectophotometer (GE Healthcare, Little Chalfont, Buckinghamshire). Total RNA were reverse-transcribed by RT^2 First Strand Kits (Qiagen) 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, RT^2 SYBR Green qPCR Mastermix (Qiagen) 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 s at 94°C, annealing for 30 s at 60°C and extension for 60 s 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 ΔΔCt method.

Laser capture microdissection. 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 laser capture microdissection (LCM), samples were deparaffinized, stained, dehydrated, and placed for 30 s 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 h with Protinase K. RNA extraction and isolation was preformed with the Arcturus Paradise Extraction and Isolation Kit (Applied Biosystems) according to the manufacture's instructions. The concentration and purity of RNA were determined by measuring absorbance at 260 and 280 nm with a Nanoview Plus Spectophotometer (GE Healthcare). 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 s at 95°C, annealing for 60 s at 55°C, and extension for 10 s at 95°C.

Immunohistochemistry. Prostates were collected from mice immunized for 30 days with EAP, and 5-μm 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, 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 min at 37°C with 1 mg/ml collagenase D (Roche Diagnostics), 10 mM HEPES (Mediatech), and 0.01% DNase I (Sigma) in complete RPMI 1640 (Mediatech). Digested suspensions were passed through a 40-μm 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 min following the digestion protocol above at 37°C. Samples were then passed through a 40-μm nylon mesh filter and centrifuged at 350 *g* for 5 min. 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 Fc€R1α), macrophage/monocytes (CD11b and F4/80), neutrophils (CD11b and Gr1), CD4+ T cells, CD8+ T

Table 1. *Chemokine gene primers*

Chemokine	Forward Primer	Reverse Primer
CCL2	GCTCGCTCAGCCAGATGCAAT	TGGGTTGTGGAGTGAGTGTTC
CCL8	AGAGACAGCCAAAGCTGGAA	CAGGCACCATCTGCTTGTAA
CCL7	AGCTACAGAAGGATCACCAG	CACATTCCTACAGACAGCTC
CCL12	CCTGTGGCCTTGGGCCTCAA	GAGGTGCTGATGTACCAGTTGG
CCL3	ACTGCCTGCTGCTTCTCCTACA	ATGACACCTGGCTGGGAGCAAA
CCL4	ACCCTCCCACTTCCTGCTGTTT	CTGTCTGCCTCTTTTGGTCAGG
CCL6	CACCAGTGGTGGGTGCATCAAG	GTGCTTAGGCACCTCTGAACTC
CCL11	TCCATCCCAACTTCCTGCTGCT	CTCTTTGCCCAACCTGGTCTTG
CXCL9	GGAGTGCAAGGAACCCCAGTA	CTTTTGGCTGACCTGTTTCTC
CXCL2	GCGCCCAGACAGAAGTCATAG	GGCAAACTTTTTGACCGCC
CXCL13	CATAGATCGGATTCAAGTTACGCC	TGCCATCCACAGTCTTGATCGC
Xcl1	CCAGACCTATATCATCTGGGAGG	TGCCATCCACAGTCTTGATCGC
L19	CAACTCCCGCCAGCAGAT	CCGGGAATGGACAGTCACA

See text for abbreviations.

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 means ± SE 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. 1*A*) with chemokine ligands 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- to 3-fold, CCL8 by 15-fold, CCL12 by 12- to 13-fold, and CXCL9 by 2- to 4-fold compared with control NOD mice (Fig. 1, *A* 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- to 2-fold increase in CXCL2 and a 2- to 3-fold increase in CXCL13 mRNA (Fig. 1*B*). At 20 days the predominant chemokines upregulated were CCL2 (1- to 2-fold), CCL3 (2- to 3-fold), and CCL11 (2- to 3-fold). The 30-day time point was characterized by a robust 20- to 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. 2*A*). 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. 2, *B*, *a* and *c*), while CCL12 was associated with

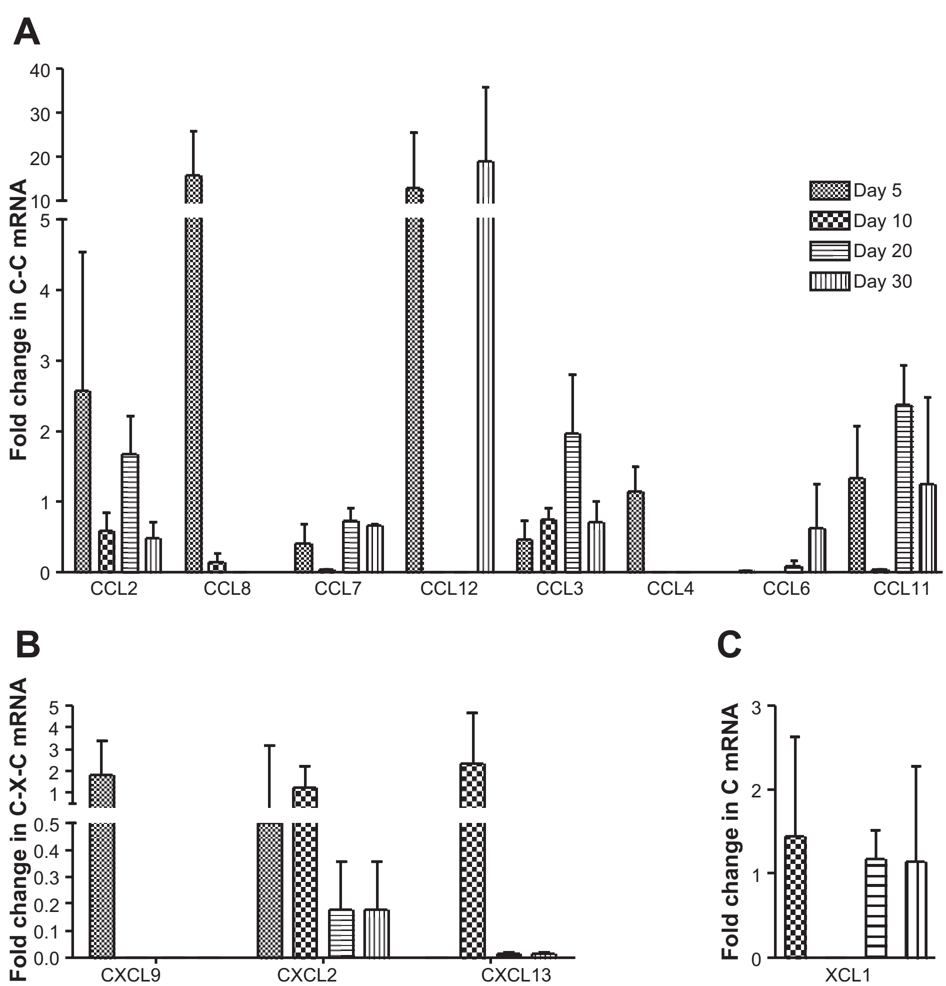

Fig. 1. Kinetics of chemokine expression in the prostates of experimental autoimmune prostatitis (EAP) mice. Prostates from nonobese diabetic (NOD) mice with EAP were collected at 5, 10, 20, and 30 days after EAP onset. Quantitative PCR analysis for select C-C chemokines (*A*), C-X-C chemokines (*B*), and C chemokines (*C*) were performed, and data were expressed as fold change from naïve controls (*A–C*). Data shown are means ± SE of two separate experiments with 3–5 animals per group.

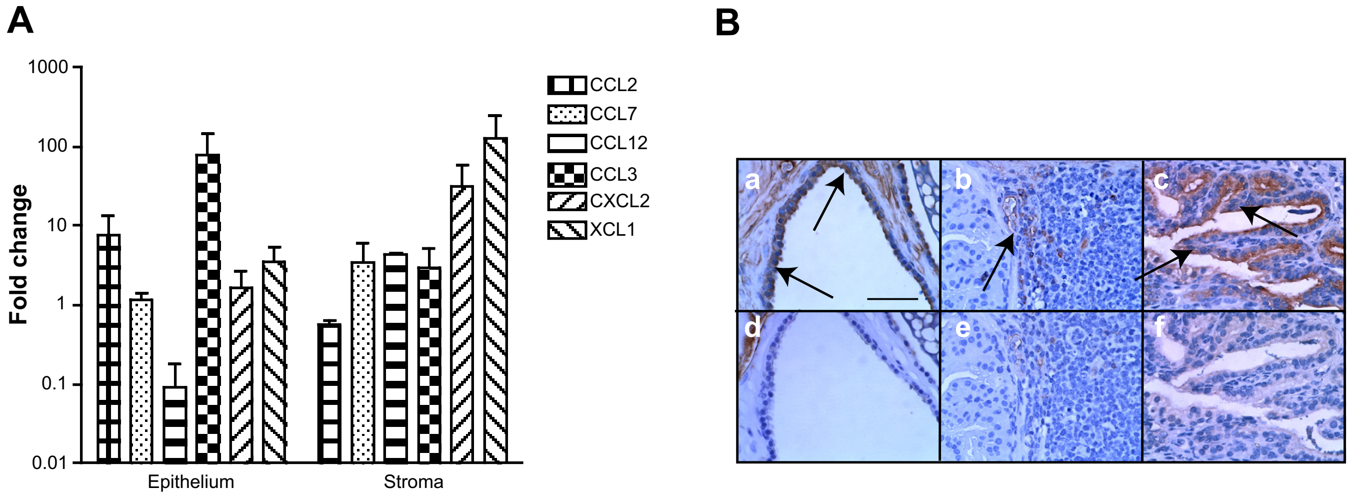

Fig. 2. Sources of chemokine expression in the prostate. *A*: 5-μm sections of the prostates of NOD mice with EAP at *day 30* were subjected to laser capture microdissection to obtain epithelial and stromal cells (~100 cells/sample) separately followed by quantitative RT-PCR analysis for CC, C-x-C, and C chemokines. Data are expressed as fold change from naïve controls. Data are means ± SE 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 (*a*, *b*, and *c*), or isotype control antibody (*d*, *e,* and *f*). Arrows indicate increased CCL2 (compare *a* and *d*), CCL12 (compare *b* and *e*), or CCL3 (*c* and *f*). Scale bar indicates 50 μm.

cells in the prostate stroma (Fig. 2*B*, *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 (Fig. 3). 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 5 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 10 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 after 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 with naïve mice (0.56 ± 0.06 to 1.89 ± 0.06). At 30 days, the immune infiltrate once more was predominantly composed 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 5 ($P < 0.05$) and 10 days ($P < 0.01$) following EAP induction, populations plummeted at 20 and 30 days. Interestingly, the elevated regulatory T cell population was accompanied by a reduction in activated T cells at 10 days ($P < 0.05$) that subsequently expanded at 20 and 30 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 (Figs. 4 and 5). Pelvic pain was measured as tactile allodynia of the suprapubic region and compared with 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 EAP development, before and after 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. 4, *A*–*E*). Pelvic pain expressed as an increase from baseline during 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 h following treatment, but pelvic pain responses returned to pretreatment levels by 3 days after treatment (Fig. 5, *A* 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. 5, *B* and *D*). In both prophylactic and therapeutic antibody administration, no significant changes in threshold responses at the paw

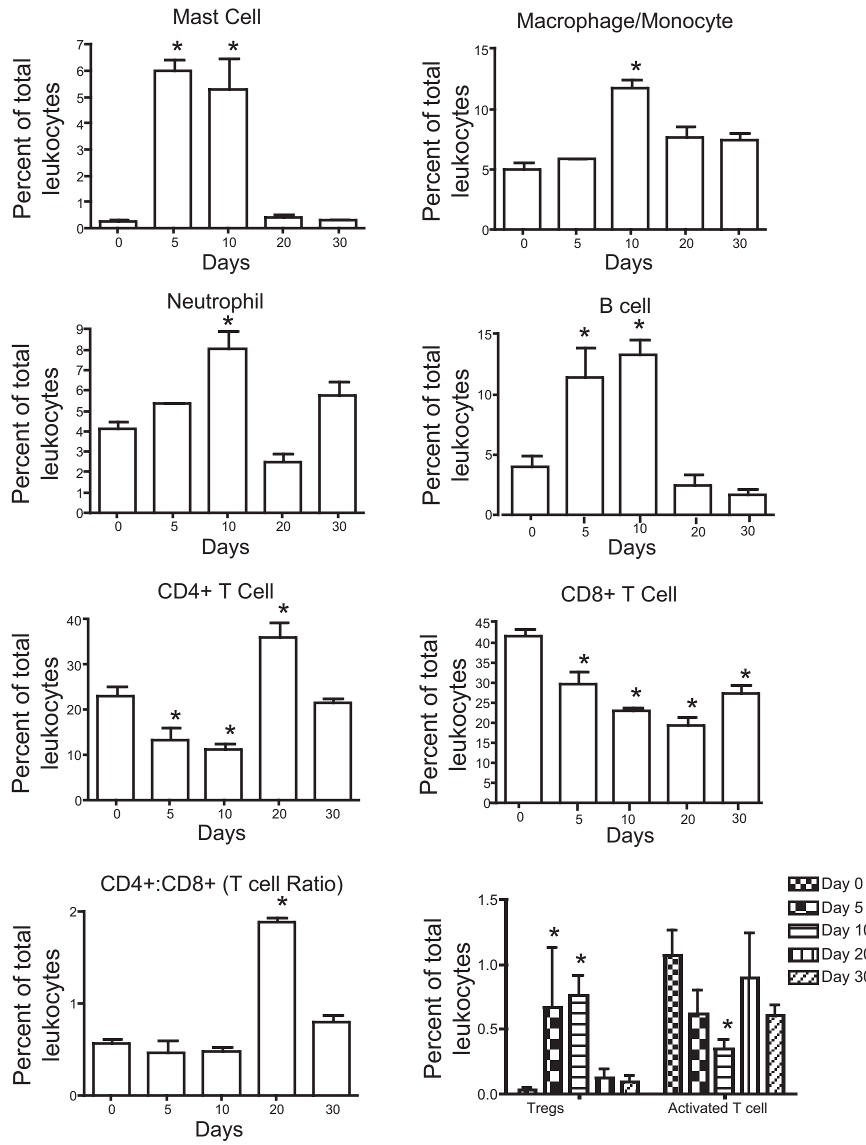

Fig. 3. Kinetics of immune cell recruitment to the prostate in EAP. Prostates of NOD mice with EAP at 0, 5, 10, 20, and 30 days after EAP onset were dissociated into single-cell suspensions, stained with antibodies against individual immune cell types, and analyzed on a flow cytometer using a preset leukocyte gate (CD45). Results are expressed as a percentage of the total leukocyte gate, and 3×10^5 cells were analyzed per prostate. Results shown are means ± SE of 3–6 animals per time point with experiments being repeated at least twice. *Statistical significance at $P < 0.05$.

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. 6, *B* and *C*) and pelvic pain increases that were not significantly different from baseline (Fig. 6*D*). 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. 7, *A* 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. 7, *B* 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. Compared with 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

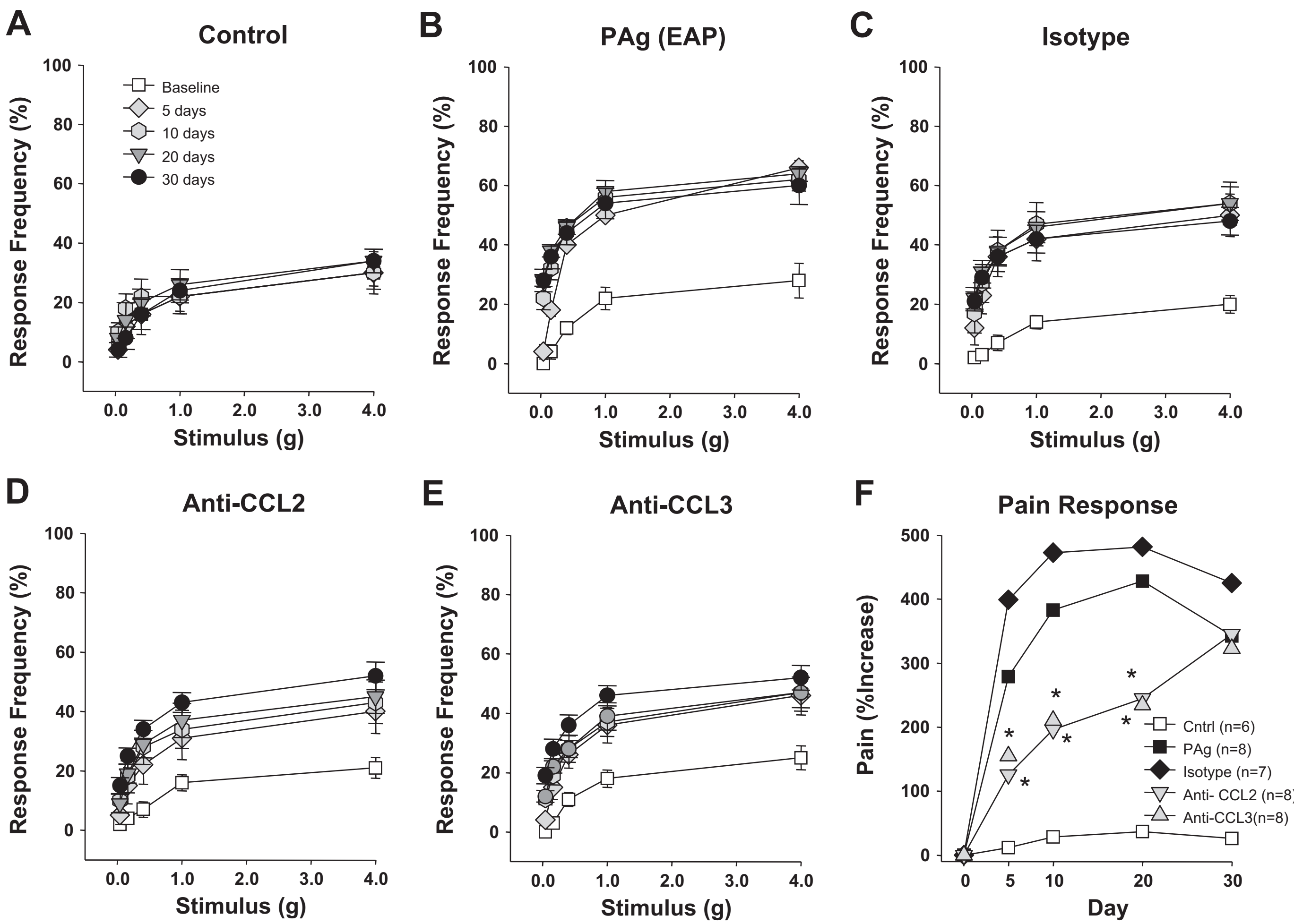

Fig. 4. Anti-CCL2 and anti-CCL3 antibodies inhibit and delay the development of chronic pelvic pain. EAP was initiated using prostate antigen (PAg) in NOD mice alone (*B*) or simultaneously with administration of 100 μg of control goat IgG (isotype, *C*), anti-CCL2 (*D*), or anti-CCL3 (*E*). Referred 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 are shown as the mean percentage of positive response ± SE before instillation of antibody (baseline) and at 5, 10, 20, and 30 days following injection. The symbol key shown in *A* applies to *B*, *C*, *D*, and *E*. Results are also shown as percentage increase in pain from baseline using normalized total frequencies (*F*). *Statistical significance at $P < 0.05$. All experiments were repeated at least two times.

observed in CCR2$^{-/-}$ mice compared with 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 hematopoietic 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. 8*A*) and significantly different from baseline at 10, 20, and 30 days following EAP induction (Fig. 8*B*, $P < 0.05$). 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. 8*D*). These results suggest that hematopoietic 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-α (TNF-α) 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

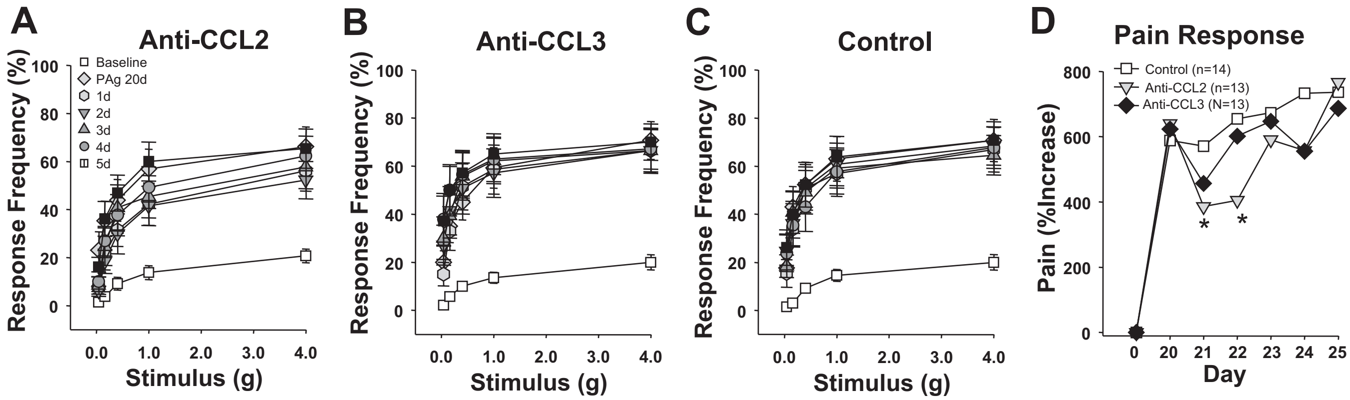

Fig. 5. Anti-CCL2 antibody therapeutically inhibits chronic pelvic pain. EAP was initiated using PAg in NOD mice and allowed to proceed for 20 days at which time 100 μg of anti-CCL2 (*A*), anti-CCL3 (*B*), or control goat IgG (*C*) were administered. Referred visceral hyperalgesia in NOD mice was measured as responses to mechanical stimulation of the pelvic region using von Frey filaments of 5 calibrated forces. Data are shown as the mean percentage of positive response ± SE before instillation of antibody (baseline) and at 1, 2, 3, 4, and 5 days after antibody administration. The symbol key shown in *A* applies to *B*, *C*, and *D*. Results are also shown as percentage increase in pain from baseline using normalized total frequencies. *Statistical significance at $P < 0.05$. All experiments were repeated at least two times.

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 Refs. 4 and 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 before instillation of irritants into visceral organs (allodynia). 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

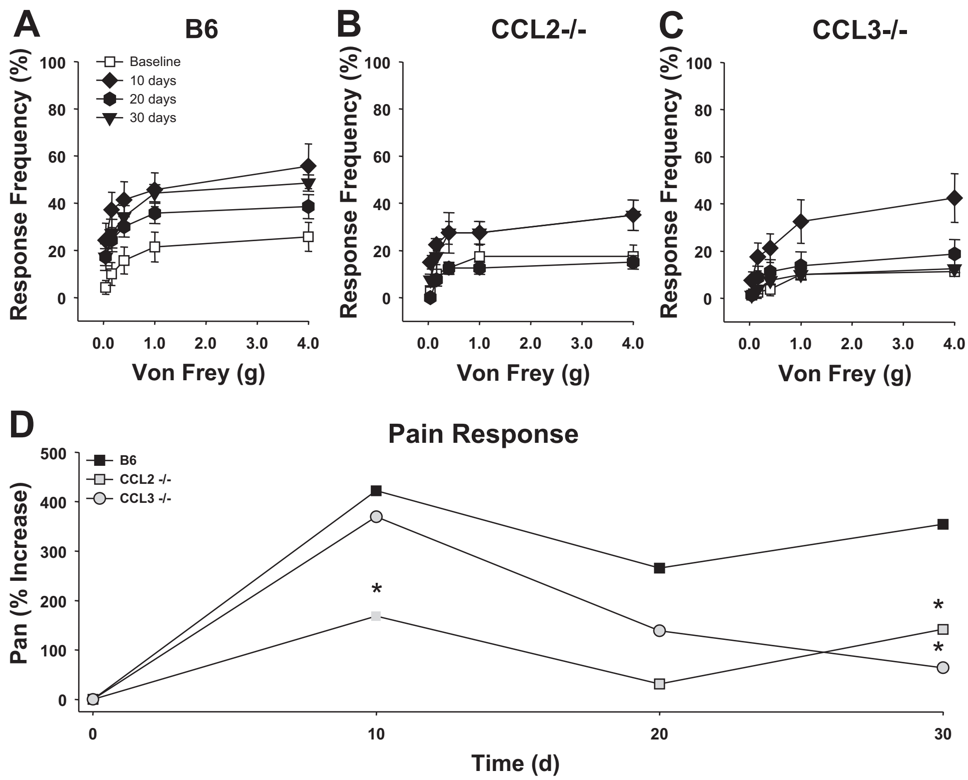

Fig. 6. $CCL2^{-/-}$ and $CCL3^{-/-}$ mice are resistant to chronic pelvic pain. EAP was initiated using PAg in wild-type B6 control (*A*), $CCL2^{-/-}$ (*B*), and $CCL3^{-/-}$ (*C*) mice followed by measurement of referred visceral hyperalgesia. Referred visceral hyperalgesia was measured as responses to mechanical stimulation of the pelvic region using von Frey filaments of 5 calibrated forces. Data are shown as the mean percentage of positive response ± SE before instillation of antigen (baseline) and at 10, 20, and 30 days following injection. The symbol key shown in *A* applies to *B*, *C*, and *D*. Results are also shown as percentage increase in pain from baseline using normalized total frequencies. *Statistical significance at $P < 0.05$. All experiments were repeated at least two times and had 3–5 animals per group.

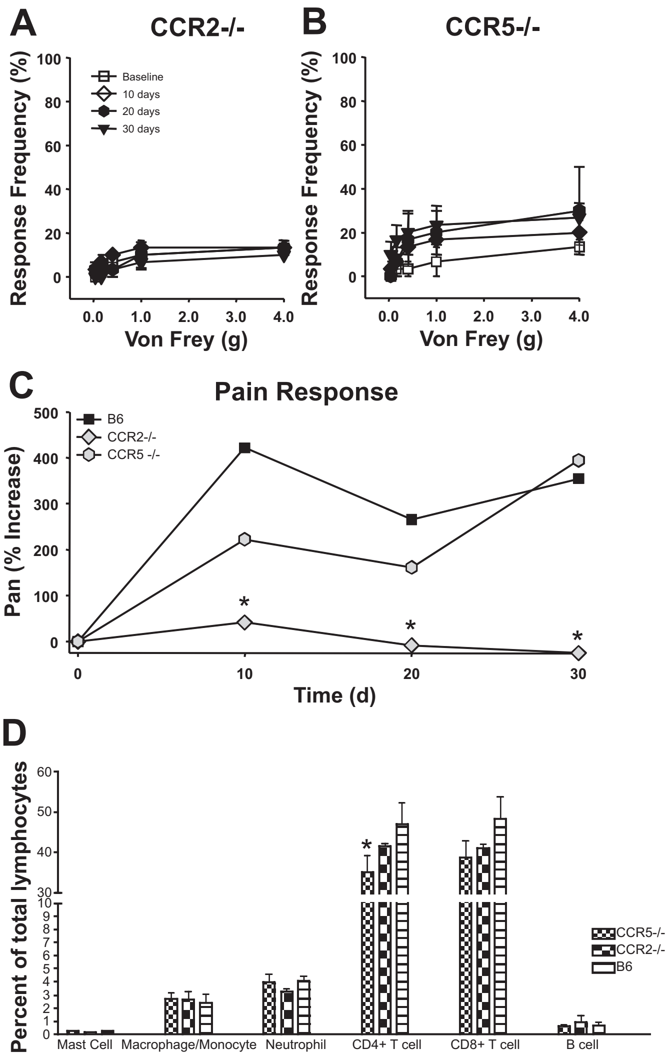

Fig. 7. CCR2$^{-/-}$ but not CCR5$^{-/-}$ mice are resistant to chronic pelvic pain. EAP was initiated using PAg in CCR2$^{-/-}$ (*A*) and CCR5$^{-/-}$ (*B*) mice followed by measurement of referred visceral hyperalgesia. Data are the mean percentage of positive response ± SE before instillation of antigen (baseline) and at 10, 20, and 30 days following injection. The symbol key shown in *A* applies to *B*, *C*, and *D*. 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*). *Statistical significance at $P < 0.05$.

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 IFN-γ-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. Twenty 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 hematopoietic compartment. In contrast to these results, CCL3 knockout mice as well CCR5 knockouts with EAP

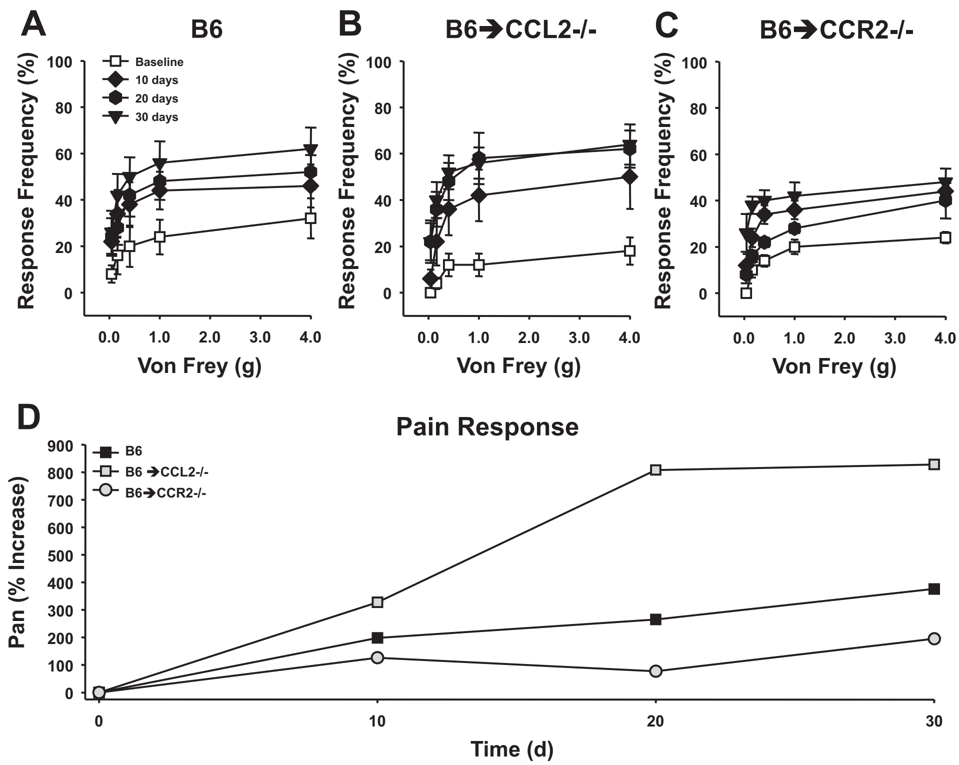

Fig. 8. B6 reconstitution of CCL2$^{-/-}$ and CCR2$^{-/-}$ mice confers pelvic pain susceptibility. CCL2$^{-/-}$ and CCR2$^{-/-}$ mice were reconstituted with 8×10^6 bone marrow cells from B6 mice followed by EAP initiation after 8 wk. Referred visceral hyperalgesia was measured and represented as the mean percentage of positive response ± SE before instillation of antigen (baseline) and at 10, 20, and 30 days following injection. The symbol key in *A* applies to *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.

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 Ref. 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.

GRANTS

The project described was supported by award number R01DK-083609 and K01DK-079019 from the National Institute of Diabetes and Digestive and Kidney Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases or the National Institutes of Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.L.Q., S.M., A.J.S., and P.T. conception and design of research; M.L.Q., S.M., C.N.R., and J.D.D. performed experiments; M.L.Q., S.M., C.N.R., J.D.D., and P.T. analyzed data; M.L.Q., S.M., C.N.R., J.D.D., A.J.S., and P.T. interpreted results of experiments; M.L.Q., S.M., C.N.R., J.D.D., and P.T. prepared figures; M.L.Q., S.M., C.N.R., A.J.S., and P.T. drafted manuscript; M.L.Q., J.D.D., A.J.S., and P.T. edited and revised manuscript; A.J.S. and P.T. approved final version of manuscript.

REFERENCES

1. **Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, DeMartino JA, MacIntyre DE, Forrest MJ.** Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci USA* 100: 7947–7952, 2003.
2. **Alexander RB, Brady F, Ponniah S.** Autoimmune prostatitis: evidence of T cell reactivity with normal prostatic proteins. *Urology* 50: 893–899, 1997.
3. **Alexander RB, Ponniah S, Hasday J, Hebel JR.** Elevated levels of proinflammatory cytokines in the semen of patients with chronic prostatitis/chronic pelvic pain syndrome. *Urology* 52: 744–749, 1998.
4. **Cervero F.** Visceral hyperalgesia revisited. *Lancet* 356: 1127–1128, 2000.
5. **Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL.** Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53: 55–63, 1994.
6. **Collins MM, Stafford RS, O'Leary MP, Barry MJ.** How common is prostatitis? A national survey of physician visits. *J Urol* 159: 1224–1228, 1998.
7. **Dansereau MA, Gosselin RD, Pohl M, Pommier B, Mechighel P, Mauborgne A, Rostene W, Kitabgi P, Beaudet N, Sarret P, Melik-Parsadaniantz S.** Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J Neurochem* 106: 757–769, 2008.
8. **Desireddi NV, Campbell PL, Stern JA, Sobkoviak R, Chuai S, Shahrara S, Thumbikat P, Pope RM, Landis JR, Koch AE, Schaeffer AJ.** Monocyte chemoattractant protein-1 and macrophage inflammatory protein-1alpha as possible biomarkers for the chronic pelvic pain syndrome. *J Urol* 179: 1857–1861; discussion 1861–1852, 2008.
9. **Done JD, Rudick CN, Quick ML, Schaeffer AJ, Thumbikat P.** Role of mast cells in male chronic pelvic pain. *J Urol* 187: 1473–1482, 2012.
10. **Fife BT, Huffnagle GB, Kuziel WA, Karpus WJ.** CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J Exp Med* 192: 899–905, 2000.
11. **Giamberardino MA.** Recent and forgotten aspects of visceral pain. *Eur J Pain* 3: 77–92, 1999.
12. **Haringman JJ, Gerlag DM, Smeets TJ, Baeten D, van den Bosch F, Bresnihan B, Breedveld FC, Dinant HJ, Legay F, Gram H, Loetscher P, Schmouder R, Woodworth T, Tak PP.** A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis. *Arthritis Rheum* 54: 2387–2392, 2006.
13. **Haverkamp JM, Charbonneau B, Crist SA, Meyerholz DK, Cohen MB, Snyder PW, Svensson RU, Henry MD, Wang HH, Ratliff TL.** An inducible model of abacterial prostatitis induces antigen specific inflammatory and proliferative changes in the murine prostate. *Prostate* 71: 1139–1150, 2011.
14. **Hochreiter WW, Nadler RB, Koch AE, Campbell PL, Ludwig M, Weidner W, Schaeffer AJ.** Evaluation of the cytokines interleukin 8 and epithelial neutrophil activating peptide 78 as indicators of inflammation in prostatic secretions. *Urology* 56: 1025–1029, 2000.
15. **Hua VN, Schaeffer AJ.** Acute and chronic prostatitis. *Med Clin North Am* 88: 483–494, 2004.
16. **Huang DR, Wang J, Kivisakk P, Rollins BJ, Ransohoff RM.** Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J Exper Med* 193: 713–726, 2001.
17. **Izikson L, Klein RS, Charo IF, Weiner HL, Luster AD.** Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. *J Exper Med* 192: 1075–1080, 2000.
18. **Karpus WJ, Lukacs NW, McRae BL, Strieter RM, Kunkel SL, Miller SD.** An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. *J Immunol* 155: 5003–5010, 1995.
19. **Laird JM, Martinez-Caro L, Garcia-Nicas E, Cervero F.** A new model of visceral pain and referred hyperalgesia in the mouse. *Pain* 92: 335–342, 2001.
20. **McMahon SB, Abel C.** A model for the study of visceral pain states: chronic inflammation of the chronic decerebrate rat urinary bladder by irritant chemicals. *Pain* 28: 109–127, 1987.
21. **Miller RJ, Jung H, Bhangoo SK, White FA.** Cytokine and chemokine regulation of sensory neuron function. *Handb Exp Pharmacol* 417–449, 2009.
22. **Motrich RD, Maccioni M, Molina R, Tissera A, Olmedo J, Riera CM, Rivero VE.** Presence of INFgamma-secreting lymphocytes specific to prostate antigens in a group of chronic prostatitis patients. *Clin Immunol* 116: 149–157, 2005.
23. **Nadler RB, Koch AE, Calhoun EA, Campbell PL, Pruden DL, Bennett CL, Yarnold PR, Schaeffer AJ.** IL-1beta and TNF-alpha in prostatic secretions are indicators in the evaluation of men with chronic prostatitis. *J Urol* 164: 214–218, 2000.
24. **Nilsson G, Mikovits JA, Metcalfe DD, Taub DD.** Mast cell migratory response to interleukin-8 is mediated through interaction with chemokine receptor CXCR2/Interleukin-8RB. *Blood* 93: 2791–2797, 1999.
25. **Oldham KA, Parsonage G, Bhatt RI, Wallace DM, Deshmukh N, Chaudhri S, Adams DH, Lee SP.** T lymphocyte recruitment into renal cell carcinoma tissue: a role for chemokine receptors CXCR3, CXCR6, CCR5, and CCR6. *Eur Urology* 61: 385–394, 2012.
26. **Ponniah S, Arah I, Alexander RB.** PSA is a candidate self-antigen in autoimmune chronic prostatitis/chronic pelvic pain syndrome. *Prostate* 44: 49–54, 2000.
27. **Pontari MA, Ruggieri MR.** Mechanisms in prostatitis/chronic pelvic pain syndrome. *J Urol* 172: 839–845, 2004.
28. **Rivero VE, Cailleau C, Depiante-Depaoli M, Riera CM, Carnaud C.** Non-obese diabetic (NOD) mice are genetically susceptible to experimental autoimmune prostatitis (EAP). *J Autoimmun* 11: 603–610, 1998.
29. **Rudick CN, Schaeffer AJ, Thumbikat P.** Experimental autoimmune prostatitis induces chronic pelvic pain. *Am J Physiol Regul Integr Comp Physiol* 294: R1268–R1275, 2008.
30. **Tran EH, Kuziel WA, Owens T.** Induction of experimental autoimmune encephalomyelitis in C57BL/6 mice deficient in either the chemokine macrophage inflammatory protein-1alpha or its CCR5 receptor. *Eur J Immunol* 30: 1410–1415, 2000.
31. **Trautmann A, Toksoy A, Engelhardt E, Brocker EB, Gillitzer R.** Mast cell involvement in normal human skin wound healing: expression of monocyte chemoattractant protein-1 is correlated with recruitment of mast cells which synthesize interleukin-4 in vivo. *J Pathol* 190: 100–106, 2000.
32. **White FA, Feldman P, Miller RJ.** Chemokine signaling and the management of neuropathic pain. *Molec Interven* 9: 188–195, 2009.