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Neuronal/astrocytic expression of chemokine (C-C motif) Ligand 2 is associated with monocyte/macrophage recruitment in male chronic pelvic pain

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Keywords

prostatitis; pelvic pain; CPPS; neuroinflammation; microglia; astrocytes; CCL2

1. Introduction

Mice induced with experimental autoimmune prostatitis (EAP) exhibit chronic pelvic pain and increased numbers of immune cells in the prostate, including monocytes and T cells [8; 26]. Proinflammatory Th1 and Th17 T cells play a significant role in prostatitis development while Th2 and regulatory T cells appear to prevent its development [25]. Prostatitis also results in increased expression of several chemokines, including CCL2 and CCL3 [8]. CCL2 and CCL3 have been proposed as biomarkers for patients with prostatitis [7]. Furthermore, mice lacking CCL2, CCL3, or their respective receptors develop prostatitis with blunted tactile allodynia responses compared to control counterparts, suggesting a role for CCL2 and CCL3 as essential chemokines [8] in the development and maintenance of chronic pain. In addition to recruiting immune cells, CCL2 and CCL3 have been reported to play roles in the development of pain in peripheral nerve injury (PNI) models [29]. CCL2 and CCL3 and their respective receptors have also been shown to modulate pain associated signaling in neurons of both the peripheral and central nervous systems [17; 39]. In the dorsal root ganglia (DRG), CCL2 and CCL3 lower the threshold of activation to elicit pain signals to the central nervous system [4; 38]. In the spinal cord, CCL2 can stimulate glial cells to induce the expression of neuromodulators involved in chronic pain, such as brain-derived neurotrophic factor (BDNF) and the P2X4 receptor (P2X4R) [3; 31]. We have previously reported that mice with prostatitis have increased neural activation in their DRGs as well as increased BDNF and P2X4R [34]. Additionally, the spinal cord appears to be in a more activated inflammatory state in mice with prostatitis compared to naïve mice [34].

In this paper, we hypothesized that prostatitis induces chronic pain through mechanisms that are similar to how pain is established in models of PNI. Due to the dual nature of CCL2 as a chemokine and pain modulator [30; 32], we wanted to determine if cells of the immune

system were being recruited to the spinal cord. We demonstrate that CCL2 expression is elevated in the prostate, at the dorsal root ganglia and in lumbar and sacral segments of the spinal cord. At these sites, we also show that monocytes are concomitantly increased early in the disease course, appear to be recruited from the peripheral blood and tend to be of the proinflammatory subtype. Our data suggest that CCL2 plays a critical role in driving neuroinflammation and may contribute to the development or maintenance of chronic pelvic pain.

2. Methods

2.1. Ethics Statement

Animal studies were conducted under protocols 2013-3015 and 2013-2068 approved by the Institutional Animal Care and Use Committee (IACUC) at Northwestern University. Northwestern University conducts its reviews in accordance with the United States Public Health Service (USPHS) regulations and applicable federal and local laws. The composition of the IACUC meets the requirements of the USPHS policy and the Animal Welfare Act Regulations.

2.2. Animal Use

All animal procedures and protocols were approved prior to study commencement and monitored by IACUC at CCM Northwestern. All pain-related animal experiments were conducted in accordance to the IASP Guidelines for the use of animals in research issued on January, 2014. Male 6-8 weeks old C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME) as required. Animals were housed with *ad libitum* access to sterilized food and water. For all experiments, 4 to 5 animals were used per group (unless otherwise stated). Experiments were performed in duplicate or triplicate (N=2/3) with no exclusion of animals.

2.3. Induction of EAP

EAP was induced by subcutaneous injection of rat prostate homogenate at a 1:1 ratio with TiterMax® adjuvant behind the shoulder of the animals (26). Following this, referred allodynia responses were measured by von Frey force filament behavioral testing for pelvic tactile allodynia, as described [23]. Testing was performed prior to EAP induction (baseline) and at day 7, 14, and 28 post-EAP induction unless otherwise stated. Results were calculated as a percentage change above baseline, and for each von Frey filament of increasing force per group individually, values were averaged within experimental groups. Behavioral testing was performed in a blinded manner by a tester who had no knowledge of animal treatment information. For time-course experiments, after 14 and 28 days of EAP induction (unless otherwise stated), mice were sacrificed and prostate, dorsal root ganglia at the lumbar and sacral levels, and the corresponding segments of the spinal cord were removed.

2.4. Adoptive transfer of GFP-positive spleen cells

GFP+ donor mice (C57BL/6-Tg(CAG-EGFP)) were purchased from Jackson Laboratory (Bar Harbor, ME)(Jax stock #006567). Briefly, a single cell suspension from GFP+ donor mice was prepared from the spleens of donor mice with a 40µm strainer, lymphocyte/

monocyte populations were purified using separation medium and one million cells were injected per mouse via the tail vein. GFP+ cells were adoptively transferred into naïve or EAP mice 7 days after induction of prostatitis. 3 days post-adoptive transfer of GFP-positive cells behavioral testing was performed and sacral spinal cords were isolated. GFP+ cells were enumerated by flow cytometry and immunophenotyped.

2.5. Flow cytometry analyses

Spinal cords at the sacral level from naïve and EAP mice were homogenized and passed through a 40µm Cell strainer (Fisher Scientific, Cat No. 22363547) to generate single-cell suspensions. Staining for resident microglia (CD11b, CD39), monocytes (CD11b, Ly6C), and CCL2. For labeling surface marker, fluorescent dyes-conjugated antibodies were incubated for 30 min in the dark at 4°C. For labeling intracellular marker, cells were permeabilized prior to intracellular staining. Intracellular staining was performed by fixation and permeabilization using eBioscience Fix-Perm Intracellular staining buffers (Cat. Num 8222-49 and 8333-56). Staining was performed for 1 hour at room temperature, followed by washing in FACS buffer (2% FCS [HyClone], in PBS [Gibco]). Analyses were performed using FlowJo and data statistically tested using GraphPad Prism™ software, with specific statistical tests described in respective figure legends.

2.6. Mechanical Allodynia.

Mice were tested prior to rat prostate antigen (PAG) injection (baseline) and after development of EAP as previously described [8; 26]. Referred hyperalgesia and tactile allodynia was tested using von Frey filaments applied to the abdomen [15; 16]. Mice were tested in individual Plexiglas chambers (6cm x 10cm x 12cm) with a stainless steel wire grid floor (mouse acclimation period of 20 min prior to testing). Standardized conditions for testing including fixed time-of-day, standard methodology, single experimenter testing of all animals and blinded testing of groups were utilized to combat the limitations of behavior-based pain testing in animal models. Frequency of withdrawal responses to the application of von Frey filaments to the abdomen was tested using five individual fibers with forces of 0.04, 0.16, 0.4, 1.0 and 4.0 grams (Stoelting, USA). Each filament was applied for 1-2s with an inter-stimulus interval of 5s for a total 10 times, and the hairs were tested in ascending order of force. Stimulation was confined to the lower abdominal area in the general vicinity of the prostate. 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 was reported as the mean percentage of response frequency $\pm$ SEM. "Percentage response change is calculated as the change over baseline for response frequencies at each additional time point".

2.7. Immunohistochemistry

On 14 and 28 days after induction of EAP based on pelvic tactile allodynia response, mice were anesthetized with 2.5% isoflurane (Isothesia; Butler) and transcardially perfused with cold PBS followed by ice-cold 4% paraformaldehyde. DRGs, spinal cords of cervical, lumbar and sacral segments and prostates were removed and post-fixed overnight in 4%

paraformaldehyde. Then the tissues were transferred to 30% sucrose solution overnight. Tissues were mounted with ornithine carbamyl transferase embedding medium (Tissue Tek) on dry ice and stored at -80°C for further experiments. The antibodies used in this study were as follows: rabbit anti-TMEM199 (1:1,000, Abcam), goat anti-IBA1 (1:1,000, Novus), rat anti-CD45 (1:500, Abcam), guinea pig anti-NeuN (1:1,000, Synaptic Systems), rabbit anti-PGP9.5 (1:500, Abcam), mouse anti-CCL2 (1:200, Proteintech) and chicken anti-GFAP (1:1,000, Novus). The multiple immunolabelings were visualized respectively by using Cytm2- or Cytm3-conjugated-donkey anti-rabbit IgG, Cytm3-conjugated-donkey anti-mouse IgG, Cytm3 or 5-conjugated-donkey anti-goat IgG, Cytm3-conjugated-donkey anti-rat IgG, Cytm5-conjugated-donkey anti-Guinea Pig IgG, Brilliant Violet 421-conjugated Donkey Anti-Chicken IgG (1:1,000, Jackson ImmunoResearch). Diamond Antifade mounting medium (Invitrogen) with or without DAPI (dependent on wavelength assignment in multicolor labeling).

2.8. CCL2 RNA fluorescence in situ hybridization

Custom RNA FISH Probes were designed against mouse CCL2 mRNA (NM_011333) utilizing the Stellaris® FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner. 10 μm sections of prostate from naive and EAP treated mice were hybridized with the Stellaris FISH Probe set pooled by 14 singly labeled mouse CCL2 probes (tgagccaacacgtggatgct, tggggcgtaactgcacatg, tgggtgaatgagtagcagcag, ctactcattgggatcatctt, tgctgggtgatccctc ttgtag, actacagcttctttgggaca, tctcttgagcttggtgacaa, ttcttggggtcagcacagac, gatctcatttggtccgatc, aaggtgctg aagaccttagg, ttacgggtcaacttcacat, tagtggatgcattagcttca, ctctacagaagtgccttgag, tagttcactgtcacactggg) with TAMRA dye (Biosearch Technologies, Inc.). Following the manufacturer's instructions slides were thawed to room temperature and immersed in 3.7% formaldehyde in 1 X PBS for 10min. Slides were washed twice with 1X PBS for 2-5 minutes and permeabilized by incubation in 70% ethanol for 1 hour at room temperature. Slides were washed in wash buffer A (Biosearch Technologies Cat# SMF-WA1-60), followed by probes hybridization overnight at 37°C . Samples were counterstained with DAPI, rinsed in wash buffer B (Biosearch Technologies Cat# SMF-WB1-20), and coverslipped with Vectashield® Mounting Medium (Vector Laboratories Cat #H-1000).

2.9. Confocal Microscopy and Image Analysis

Images were obtained on a Nikon A1 Confocal Laser Microscope System (Plan Apo 20 $\times$ NA 0.75 and Plan Apo 60 $\times$ NA1.4 Oil, objectives). For quantitative immunofluorescent analysis, the comparable tissues/regions were selected. Images were taken on the same confocal settings as the template for imaging on all groups. After pixel and average lines were selected, laser intensity and gain level on each channel were optimized. For z-series imaging, the same settings were applied to total slice thickness, step value, and the thickness to the surfaces of the section. For image analysis, ImageJ software was used for quantitation after background correction and thresholding for different slices.

2.10. Quantitative immunofluorescence analysis for macrophages in DRG and microglia in DH

DRG sections from L₆-S₂ levels from 3-4 mice per group were immunostained with anti-IBA1 antibody and visualized Cy2-conjugated donkey secondary antibody. The confocal images were obtained on the imaging system mentioned above. After noise was corrected with Image J, the region of interest (ROI) where the afferent neurons distribute was defined. Based on the IBA1-immunoreactivity after thresholding, the parameters of process size and average fluorescence intensity were recorded. The average IBA1-positive process size was statistically analyzed. For analysis of microglia activity under EAP induction, free floating double immunostaining was completed on spinal cord sections at L₆-S₂ level from 3-4 mice per group with anti-NeuN and anti-IBA1 antibodies. After correction of noise and thresholding of the IBA1-immunoreactive channel, the parameters of area, position, and intensity of reactive microglia were recorded and applied to the regions that were defined from the reference of NeuN-immunoreactive distribution in the dorsal spinal cord. Average IBA1-immunoreactivity intensities were statistically analyzed.

2.11. Quantitative immunofluorescence analysis for CCL2 in neurons and astrocytes

Sections from the spinal cord were immunostained with anti-NeuN (synaptic systems 266004), anti-GFAP (Novus, NBP1-05198) and anti-CCL2 (Proteintech, 500-P113) antibodies and visualized respectively by Alexa647-, Cy2- and Cy3-conjugated donkey secondary antibodies (Jackson ImmunoResearch). Triple-labeled confocal images were obtained on the imaging system mentioned above. After noise was corrected for all images of three channels with NIH Image J, the region of interest (ROI) pointed to the dorsal horn of the spinal cord was defined. Based on the threshold NeuN-immunoreactive and GFAP-immunoreactive channels, the parameters were recorded on neurons and astrocyte processes, respectively. Average CCL2 immunofluorescence intensity was recorded and analyzed by applying these parameters to the pre-defined ROI in the dorsal horn of the spinal cord on CCL2-immunoreactive channel, respectively. For quantification of CCL2 immunoreactivity in the primary afferent neuron in DRG, average fluorescence intensity calculated on each pixel was recorded for the pre-defined ROI with the clusters of neurons,

2.12. Intrathecal clodronate injections.

Liposomal clodronate and liposomal PBS control (SKU: CP-010-010) were ordered from clodronateliposomes.org. Mice were kept under anesthesia, and their backs were shaved at the lumbosacral region (L5/L6), and received an injection of 10 µl of liposomal clodronate or liposomal in PBS using a 25 µl Hamilton needle and a 30-gauge, ½ inch needle. Placement of the needle into the spinal column was confirmed by the flick of the tail. The mice were visually observed after each injection to ensure the injection had no adverse effect on the mouse. The mice were behaviorally tested as described above at the indicated time points before, 24, and 48, hours after the injections.

2.13. Statistical Analysis

Statistical analysis was performed using the student's t-test for the comparison between two groups. Multiple comparisons (more than two groups) were carried out using One way or

Two-way analysis of variance (ANOVA) depending on the number of factors followed by Tukey's or Bonferroni's post-tests respectively. All data are presented as mean $\pm$ SD, and the differences are considered statistically significant with a p-value <0.05 . Data analyses and statistical calculations were performed using Microsoft Excel™ and GraphPad Prism™.

Results

CCL2 expression, macrophage recruitment and neuroimmune interaction in the prostate.

To study the effect of EAP induction on neuroinflammation at the level of the prostate (Fig. 1A), we induced EAP and measured tactile allodynia behavioral responses at 0, 7, 14 or 28 days. EAP induced tactile allodynia responses above baseline that were statistically significant at Day 14 and were maintained at Day 28 (Fig. 1B–D). At day 28, CCL2 protein expression was enhanced in the EAP prostates but not in controls (Fig. 1E). Localization of CCL2 expression to particular cell types was difficult given that CCL2 is a secreted factor and could potentially bind receptors expressed on multiple cell types. Using CCL2-specific FISH probes, we show that CCL2 mRNA expression was predominantly localized to the epithelial lining of prostatic ducts in EAP-induced but not naïve mouse prostates (Fig. 1F). We next examined whether increased CCL2 in the prostate was associated with a concomitant increase in macrophage recruitment and activation. Utilizing an antibody against the ionized calcium-binding adaptor molecule 1 (IBA1), a microglia/macrophage-specific cell surface protein [13] associated with cellular activation [12], we examined prostate sections from naïve and EAP-induced mice at day 14 and day 28. IBA1-immunoreactive cells were increased in number in the EAP prostates compared to naïve animals at day 14 and day 28 (Fig. 1G and H) with immunoreactivity predominantly in the stromal cell compartment. We next performed immunofluorescent staining for PGP9.5 to examine neural structures and their interaction with infiltrated IBA-1 positive cells in the prostate. Our data demonstrate an increase in both PGP9.5 expression and IBA1-immunoreactive cells in the prostate of EAP mice compared to naïve counterparts. These increases are observed at both Day 14 and Day 28 but are more pronounced at the later timepoint. Examination of high magnification images demonstrates interaction between the increased numbers of IBA1-positive macrophages and neural cells at Day 14 and Day 28, suggesting crosstalk between these cell types after EAP induction (Fig. 1H). At Day 28 IBA1-positive macrophages exhibited extended processes that lined up with the neuron processes in the prostate stroma (Fig. 1H).

DRG expression of CCL2 and macrophage infiltration.

Enhanced expression of CCL2 in primary sensory neurons and alterations in inflammatory cell recruitment have been reported in neuropathic and inflammatory pain models [5; 14; 27; 30; 33]. Here we evaluated whether increased expression of CCL2 and macrophage recruitment to the prostate also occurs at the level of DRG during EAP (Fig. 2A). To do so, we isolated DRG from mice with and without EAP at Day 14 and Day 28 and examined the expression of CCL2 in these tissues by immunofluorescent staining. Figure 2B and C demonstrates increased expression of CCL2 in the DRG at both time points examined in EAP mice compared to their control counterparts. As CCL2 acts as a recruitment factor for macrophages, we also examined IBA1 immunoreactivity in these tissues as a marker of

myeloid-derived macrophage recruitment and activation[12]. Here we demonstrate a significant difference in recruited IBA1-positive cells at Day14 of EAP that was not evident at Day 28, Fig 2D–F. This is indicative of increased infiltration of macrophages into the DRG or enhanced activation of macrophage or myeloid-derived microglia in response to EAP. Interestingly, the differential observations across time points suggest that macrophage recruitment in response to elevated CCL2 in the DRG is an early event in the peripheral sensitization associated with EAP.

Astrocytes and neurons in the spinal cord show elevated CCL2 expression.

We examined whether increased CCL2 was also detectable at the level of the lower dorsal horn of the spinal cord (Fig. 3A). Immunofluorescent staining was performed on spinal cord tissues from EAP and naïve animals at Day 28. To determine the cellular source of CCL2, we co-stained tissues for NeuN, a marker for neurons, and GFAP, a marker for astrocytes (Fig 3B and C). Quantification of this staining in laminae I and II, regions that contain the majority of second-order afferent neurons and associated regulatory interneurons for visceral nociceptive input (Fig 3B and C), demonstrated significant increases in CCL2 expression in both NeuN and GFAP expressing cells but not in the surrounding tissue (Fig 3D–F). Enhanced expression of CCL2 was not dependent on the alteration of cell numbers of neurons or astrocytes. These results indicate dorsal horn neurons and astrocytes are potential sources of CCL2 in the spinal cord.

Monocyte/macrophage recruitment to the lumbosacral spinal cord.

We next examined whether EAP effects at the spinal cord were associated with an increase in infiltration of monocytes/macrophages and could be localized to regions associated with visceral nociception (Fig. 4A). Spinal cords from EAP and naïve mice were excised at Day 14 and Day 28 and immunofluorescent staining performed for CD45 and TMEM199. Expression levels of these markers differ between infiltrated macrophages (TMEM199 low, CD45 high), and resident microglia (TMEM199 high, CD45 low) (supplementary Fig. 1). Sectioning the spinal cord into vertebral regions allowed us to correlate changes with specific segments of the tissue. Figure 4B shows an overview of the staining pattern for each vertebral section at both Day 14 and Day 28. High power magnification of these images revealed increased expression level of CD45 that was not associated with TMEM199 expression at Day 28 in EAP mice only, compared to Day 14 animals and their control counterparts, Figure 4C. These increases were only observed in the lumbar-sacral regions of the spinal cord (L6-S2) and not in the cervical regions (C4) indicating that increased neural activation at this level potentiates localized immune activation and infiltration. These images were quantified, and the differences were shown to be highly significant at day 28 in EAP (Fig. 4D).

EAP-induced increase in spinal monocytes/macrophages can occur through recruitment from peripheral blood.

To determine whether the source of increased monocytic/macrophage infiltration at the spinal cord could be from peripheral blood, we performed an adoptive transfer experiment with monocytes derived from naïve GFP+ mice. We hypothesized that innate immune cells such as macrophages and monocytes act early during EAP onset; thus, monocytes were

isolated from GFP+ donor mice and adoptively transferred into recipient mice that were naïve or induced with EAP seven days prior to the adoptive transfer (Fig. 5A). Three days later (Day 10), mice were examined by von Frey behavioral testing for tactile allodynia, Figure 5B and showed an increase in responses over baseline. Flow cytometry of excised whole spinal cord tissues revealed a trending increase in GFP+ monocytic cells in mice with EAP compared to control counterparts, Figure 5C. To further delineate the subtype of monocytes, we also stained for the Ly6C marker, which is highly expressed on M1 macrophages (Ly6C^{hi}) and at low levels on M2 macrophages (Ly6C^{lo}). Again, these data revealed a trend towards increased numbers of GFP+ M1 macrophages in the spinal cord tissue of EAP mice, Figure 5D&E. These data suggest that EAP induces a proinflammatory environment in the spinal cord capable of recruiting immune cells.

Activated microglia and macrophages are increased and closely interact in the dorsal horn of the spinal cord in EAP.

Given that IBA1 expression is known to be high on microglia and monocytes/macrophages (supplementary Fig. 1), we utilized IBA1 expression to identify all cells at the spinal cord with this expression pattern at day 28 in EAP. Using the NeuN stain for neurons and IBA1 immunofluorescent staining, we demonstrated a statistically significant increase in IBA1 expression in the dorsal horn that was not associated with neurons, Figure 6 B&C. Enhanced expression of IBA1 could be due to the increased macrophage infiltration and activation, increased microglial activation, or both. To determine this more directly, we performed high-resolution confocal microscopy on dorsal horn tissues from EAP mice using markers for myeloid cells and microglia; CD45, TMEM199, and IBA1. Figure 6D&E indicate close apposition between infiltrated macrophages (CD45 high) and resident microglia (TMEM199 high) and also show enhanced expression of IBA1 on both cell types (Figure 6F). Our results indicate that EAP induces both activated macrophage infiltration and resident microglial activation (Fig. 6D–F).

Depletion of monocytes and microglia in the spinal cord reduces EAP-induced tactile allodynia.

We sought to understand the functional necessity of myeloid cells and microglia for the maintenance of pelvic pain in EAP. Mice with and without EAP were treated intrathecally with PBS or liposomal clodronate ten days after induction of EAP and behavioral testing performed 24 and 48 hours after treatment. No significant difference was observed in EAP mice treated with PBS. However, in EAP mice treated with liposomal clodronate, tactile allodynia was reduced to baseline levels by 48 hours after treatment (Figure 7A & B) a decrease that was shown to be statistically significant (Fig. 7C).

Discussion

CPPS is a chronic condition with unknown etiology and no effective therapy. The primary symptom associated with the syndrome is chronic pain, which has detrimental effects on patient quality of life [28]. While our laboratory and others have focused on the role of the local immune response of the prostate in driving symptomology, there is increasing interest in the role of neuroinflammation in driving pain responses in CPPS [6; 9; 10; 21]. CCL2 has

the potential of influencing nociceptive pathways both through its well-known chemotactic function as well as its role as a neuroimmune mediator. We have previously reported increased expression of CCL2 in the prostate of mice with EAP, indicating a potential role for the CCL2-CCR2 axis in the development of chronic pain [24]. In this study we confirm and extend these findings to show that CCL2 expression occurs in epithelial cells of the prostate and is associated with infiltration of monocytes/macrophages into the prostate stroma. Macrophages identified in this study were closely associated with PGP9.5 labelled nerve fibers in the prostate suggesting a role for neural sensitization in the prostate, as has been demonstrated in other chronic pain conditions such as vulvodynia [2]. We further investigated the role of CCL2 at the level of the DRG at both the onset of peripheral sensitization and nociception and during the transition from the early nociceptive response to development of chronic pelvic pain. Previous studies have shown elevated CCL2 expression in primary sensory neurons in DRG in -experimental DMM-induced osteoarthritis [19] and a model of chronic compression of DRGs[33]. Transgenic over-expression of CCL2 in the urothelium has been shown to induce bladder hypersensitivity, pelvic pain, and voiding dysfunction [36] and global overexpression of CCL2 results in enhanced nociceptive behavior [18]. The expression of CCL2 in the spinal cord has been also shown to promote central sensitization, long-term potentiation, and to regulate CFA-induced persistent inflammatory pain [35]. In this study, we demonstrate increased expression of CCL2 at day 14 and 28 in EAP mice (Figure 2B and C), indicating that CCL2 likely plays an important role in EAP-induced peripheral sensitization. As the critical information integration and processing centers for CPPS-associated nociceptive input, sacral, and lower levels of the lumbar spinal cord were examined for CCL2 expression. Our data showed that CCL2 immunoreactivity increased at day 28 post-EAP induction, but not at day 14. The temporality of this induction suggests that CCL2 plays a distinct role in the transition from acute to chronic tactile allodynia and EAP progression. To further understand the detail of the contribution of CCL2 to EAP, we also demonstrate that increased expression is preferentially localized to GFAP-positive astrocytes and NeuN-positive afferent neurons in the superficial dorsal horn. These data are consistent with a previous report showing that astrocytes and the interaction between astrocytes and neurons contribute to the development of CPPS via the CCL2-CCR2 signaling pathway [20]. Our data are also consistent with reports from a carrageenan-induced model of prostatitis where activated astrocytes are shown to play a role later in disease and are required for maintenance of pain [6].

As an essential player in inflammatory and pain pathologies, CCL2 not only contributes to central and peripheral sensitization but acts as a recruiter for CCR2-positive myeloid-derived macrophages and monocytes. There have also been reports that CCL2 synthesized by metastatic tumors and tissue stroma is the critical signal for the recruitment of CCR2-positive monocytes[22] and that CCR2 deficiency resulted in impaired mechanical allodynia in response to peripheral nerve injury[1; 37]. Macrophage recruitment into the DRG contributes to experimental osteoarthritis-associated pain induced by DMM surgery [19], and CCL2 mediates enhanced transmigration of human HIV-infected monocytes across the blood-brain barrier [11]. Finally, CCR2 expression in either resident microglia or macrophage is sufficient for the development of mechanical allodynia [37].

To demonstrate whether myeloid-derived monocytes or macrophages are recruited in response to enhanced CCL2 at sites along the nociceptive neural pathway, we show increased recruitment of IBA1-positive macrophage during the development of EAP-induced tactile allodynia. Furthermore, neuronal immune crosstalk occurs at every level of EAP-associated sensory /nociceptive input, starting at the peripheral tissue, to the DRG, and finally to well-defined regions of the dorsal spinal cord. Microglial activation as determined by increased IBA1 expression and morphological changes may also be due to CCL2-CCR2 signaling and increased interaction with infiltrated macrophages (Figure 6B–F). Tracking the dynamics of macrophage recruitment to the prostate, peripheral sensory ganglia at lumbar and sacral levels of the dorsal spinal cord, we show increased macrophage recruitment and activation at both day 14 and day 28. We have also shown that it is not associated with neurons but is expressed on the surface of TMEM199-positive microglia and CD45^{high} macrophages. Monocytic infiltration occurs early in the EAP disease course and may drive neural activation in the DRG and spinal cord. Notably, we found that differentiated macrophage recruitment, activation, and the interactions with other cell types in the prostate, DRG, and dorsal spinal cord likely suggest a significant mechanism in EAP-induced CPPS progression. The significance of these findings are heightened by our results demonstrating that ablation of spinal cord macrophage and monocyte cells significantly reduces chronic pain in EAP animals compared to PBS treated controls, indicating a mechanistic role for these cell types in pathophysiological responses.

Taken together, these data demonstrate the neuroimmune role of CCL2 and its association with neurons, astrocytes, microglia and macrophages in the periphery and CNS in EAP. Our results suggest that neuroimmune mechanisms such as macrophage-microglial interactions may be key to the induction and maintenance of pelvic pain responses in CPPS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Tactile allodynia, CCL2 immunoreactivity and macrophage recruitment to the prostate (A) The diagram indicates the pathway of EAP-induced nociceptive input from autoimmune injury in the prostate to lower lumbar/sacral spinal cord. (B) Average response change to von Frey fiber application to the pelvic region of Naïve and EAP treatment at day 7, 14 and 28 days. Naïve vs EAP. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, based on two-way ANOVA with Bonferroni post-tests for multiple comparisons. $n = 4/5$ mice per group, $N = 2$ representative experiments for behavioral experiment, error bars are SD. (C-D) Pelvic tactile allodynia response frequencies, in control and EAP treated mice at day 7, 14, 28. Responses are depicted as increased percentage response frequency above baseline. (E) Enhanced CCL2 (red) immunoreactivity was observed in epithelial layer induced by EAP as compared with control. Note, high power images were shown in inlays a' and b'. DAPI labels the nucleus

(blue). n=3 prostate samples, per group (F) RNA FISH for probe specific to CCL2 depicting higher CCL2 RNA fluorescence intensity in epithelium layer in EAP compared to naïve. n=3 prostate samples, per group (G) Representative images prostate sections showing IBA1-labeled macrophage (red) in naïve mice and after EAP treatment. PGP9.5-immunoreactive afferent fibers in the stroma of prostate were visualized by Cy2-conjugated secondary antibody (green). DAPI stained the nucleus. n=3 prostate samples, per group (H) High power images showing infiltrated macrophages morphologically and the interactions between PGP9.5-positive sensory fibers and activated macrophages in prostatic stroma under EAP induction.

Figure 2. CCL2 immunoreactivity and recruitment of myeloid-derived macrophages to the DRG (A) CCL2 expression and macrophage cells were examined in the DRG. (B) In DRG frozen sections images CCL2 immunoreactivity were evaluated in Naïve and 14 and 28 days after EAP treatment. (C) Quantification of CCL2 immunoreactivity in DRG at day 14 and 28. (D) EAP treatment induced recruitment of IBA1-positive macrophages (green) in DRG in Naïve (Top) and EAP (Bottom) treated mice at day 14 and 28. Scale bars=10μm. (E and F) Quantification of IBA1-positive cell processes referred as both size and area in DRG at day 14 and 28. For all quantification analysis, A two-way ANOVA was used. * $p < 0.05$, *** $p < 0.001$, $n = 3-4$ per group.

Figure 3. CCL2 expression in neurons and astrocytes in the lower dorsal spinal cord. CCL2 expression in the spinal cord (A) In upper panel (B and C) representative images show NeuN (white), GFAP (green) and CCL2 (red) immunoreactivities in dorsal spinal cord in Naïve (B) and EAP (C) treated C57BL6 mice at day 28. Lower panels present colocalization by combining CCL2 and NeuN-positive neurons or GFAP-positive astrocytes. The regions with yellow dashed lines were designated as the superficial dorsal horn for CCL2 immunoreactivity qualification. Scale bar=50µm. (C-E) Analysis of quantitative immunofluorescence for CCL2 fluorescence intensities in neuron (D), astrocyte (E) and the area outside neuron and astrocyte (F) in the defined regions. *** $p < 0.001$, $n = 5$ per group.

Figure 4. Myeloid-derived macrophage recruitment to the dorsal spinal cord.

Sections spanning cervical to sacral segments of the spinal cord were labeled with TMEM199 (green) specific for microglia, CD45 (red) for myeloid-derived macrophage and DAPI (blue) for nuclear counterstain. (A) Stacks of representative multiple-channel confocal images were obtained on different levels, including C4, L2, 3, 4, 5, 6, S1 and S2, from Naïve and EAP treated mice to examine location of macrophage recruitment to central nervous system at day 14 and 28 after EAP treatment. Sections from the cervical spinal cord segment were selected as experimental controls. Red dashed line rectangles indicate high power images followed in (C). (C) In representative images, macrophages with an activated morphology were observed in the dorsal horn of spinal cord induced by EAP at day 28. High power insets in the squares with white dashed lines show the differential macrophage recruitment and morphological activation in lower levels of lumbar and sacral spinal cord. Scale bar=100µm, left and middle panels; scale bar=20µm, right panel. (D) Cell counting indicates that more macrophage were recruited to the lower level of dorsal spinal cord at day 28 post EAP treatment, but not at day 14. Student's t test, ***p<0.001, n=3-4 per group.

Figure 5. Periphery-derived myeloid-derived monocytes are recruited to the central nervous system under EAP induction.

(A) Leucocytes isolated from GFP⁺ transgenic donor mice were adoptively transferred into Naïve and EAP treated C57BL/6 mice. 3 days post transfer, pelvic tactile allodynia responses were evaluated by von Frey filament test (B). Single cells separated from sacral level of spinal cord, percentages of GFP⁺ cells in total cells, Ly6C^{high} (D) and Ly6C^{low} (E) in GFP⁺ population were quantified by flow cytometry. ** $p < 0.01$, $n = 5$ per group.

Figure 6. Activated microglia and macrophages interact closely and are increased in the spinal cord

(A). NeuN-labeled neurons (white) were taken as a reference for the definition of the region of interest under EAP induction. (B) In an enlarged area including the whole superficial dorsal horn defined with red dash lines, IBA1 (green) immunoreactivities were measured at day 28 in Naïve and EAP treated C57Bl6 mice. Scale bar=50μm. and shown to be statistically elevated in EAP (C)*** p<0.001, as assessed by students T-test, n=13-25 low power images from 3 animals per group. (D) Representative image shows EAP-induced infiltrated macrophage-resident microglia interaction occurred in the dorsal spinal cord of sacral segment at day 28 post EAP induction. Note that a and b point to the direct contacts of these two kinds of cells, which prompted the separation of CD45 and TMEM199 signals in (E) by plot profile analysis for the line that spanned over the two types of cells. (F) High magnification confocal image shows analyses of colocalization of the common marker-IBA1, microglia marker-TMEM199 and monocyte marker-CD45. Scale bar=5μm in (D and F).

Figure 7: Depletion of monocytes and microglia in the spinal cord reduces EAP-induced tactile allodynia.

Naïve and mice with EAP were treated intrathecally with clodronate 10 days after induction of EAP. Tactile allodynia was measured by behavioral testing at days 0 and 10 before treatment and 24 and 48 hours after treatment. Data is representative of 3 independent experiments with 5 mice per group. (A) Graphical representation of percent response change above baseline at Day 10, Day 11 and Day 12 using Day 0 as baseline. (B) Graphical representation of percent response change at Day 11 and Day 12 using Day 10 as a baseline (post-treatment response). (C) Graphical representation of altered tactile allodynia responses at Day post-treatment. ** $p < 0.01$.