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Chemokine CCL2 and its receptor CCR2 in the dorsal root ganglion contribute to oxaliplatin-induced mechanical hypersensitivity

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Abstract

Activation of innate immune mechanisms within the dorsal root ganglion (DRG) and spinal dorsal horn has been shown to play a key role in the development of neuropathic pain including paclitaxel-related chemotherapy-induced peripheral neuropathy (CIPN). Here we tested whether similar mechanisms are generalizable to oxaliplatin-induced CIPN. After a single intraperitoneal injection of 3mg/kg oxaliplatin, mechanical withdrawal threshold and the expression of C-C chemokine ligand 2 (CCL2) and its receptor, CCR2 in the DRG were measured by behavioral testing and immunohistochemical (IHC) staining, respectively. Mechanical responsiveness increased from the first day after oxaliplatin injection and persisted until day 15, the last day of this experiment. IHC showed that the expression of CCL2/CCR2 started to increase by 4hrs after oxaliplatin treatment, was significantly increased at day 4, and then both signals became normalized by day 15. Co-treatment with intrathecal anti-CCL2 antibodies prevented the development of oxaliplatin-induced mechanical hyper-responsiveness, and transiently reversed established hyperalgesia when given 1 week after chemotherapy. This is the first study to demonstrate CCL2/CCR2 signaling in a model of oxaliplatin-related CIPN; and it further shows that blocking of this signal can attenuate the development of oxaliplatin-induced mechanical hyperalgesia. Activation of innate immune mechanisms may therefore be a generalized basis for CIPN irrespective of the specific class of agent.

Keywords

Neuropathy; CIPN; MCP-1; DRG

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Conflict of Interest Statement

There is no conflict of interest to report.

1. Introduction

Oxaliplatin is a second generation platinum based chemotherapy drug that is commonly used to treat colorectal cancer [42]. Like many other anticancer agents, chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect of oxaliplatin treatment and is the main dose-limiting toxicity of this drug [5]. Up to 90% of all cancer patients treated with chemotherapy are estimated to develop CIPN, minimally manifested as numbness and tingling but at the extreme resulting in spontaneous burning pain [30]. Many oxaliplatin treated patients also experience acute, transient sensory disturbances expressed as cold-triggered paresthesia of the distal extremities. Over 60% of patients completing oxaliplatin chemotherapy complain of persistent neuropathy with chronic pain occurring in 20 to 54% of patients [2,5,38,54] that can last for months after treatment cessation [1,13,63] negatively impacting quality of life [40,53]. Despite recent progress, the mechanism of CIPN is not fully defined; and this has hindered the implementation of fully effective treatment strategies for its relief or prevention.

One mechanism contributing to the generation of CIPN produced by another agent, paclitaxel, is the activation of innate immune mechanisms in the dorsal root ganglion (DRG) and spinal dorsal horn [4,31,64]. Paclitaxel increases the signaling of Toll-like receptor 4 (TLR4) in DRG neurons [34,35] that leads to increased expression of C-C chemokine ligand 2 (CCL2), also called monocyte chemotactic protein 1 (MCP-1) [65]. CCL2 and its receptor, C-C chemokine receptor 2 (CCR2), are key players in the attraction of monocytes to sites of injury and inflammation [3,58]. CCL2 increases the excitability of neurons in chronically compressed DRG, an injury that is also associated with an overexpression of neuronal CCL2 and CCR2 [50,59]. CCL2 was shown to recruit macrophage infiltration to the DRG that was critical in the induction of behavioral signs and reduction in distal epidermal nerve fiber (ENF) density after paclitaxel treatment [67]. The clinical phenotype of CIPN including the reported symptoms, their distribution, the change in quantitative sensory function, and the loss of ENFs is remarkably constant regardless of the provoking agent [9,10,18]. Similarly, the preclinical models of paclitaxel and oxaliplatin CIPN share the same pattern of spinal astrocyte activation [47,68]; and, co-treatment with a non-specific inhibitor of innate immunity, minocycline, prevents both paclitaxel and oxaliplatin induced mechanical hypersensitivity and ENF loss [7,8]. This leads to the hypothesis that CIPN regardless of the provoking agent shares at least some underlying mechanisms. As a first test of this important unifying hypothesis for CIPN, the goal of this work was to test generalizability of a role for CCL2/CCR2 signaling within the DRG in the development of oxaliplatin related CIPN. The development of oxaliplatin-induced mechanical hypersensitivity was assessed by behavior testing, and changes in CCL2/CCR2 expression in DRG neurons was tested using immunohistochemistry (IHC). The expectation was that increased expression of CCL2/CCR2 would accompany mechanical hypersensitivity. Mechanistic linkage between these observations was tested using intrathecal anti-CCL2 antibodies concurrently with oxaliplatin and also following oxaliplatin treatment. Our data show that CCL2/CCR2 in the DRG could be potential targets for the treatment of CIPN.

2. Methods

2.1. Subjects

Sixty-three adult male Sprague-Dawley rats (8–12 weeks old) were used in the study. All animals were housed in a 12 h light/dark cycle (7A–7P) with free access to food and water. The procedures were approved by the Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center and were performed according to the guidelines stated by the National Institutes of Health for Use and Care of Laboratory Animals (NIH publications number 80–23) revised in 2011 and by the committee of research and ethical issues of the International Association for the Study of Pain [72].

2.2. Anesthesia

Animals were anesthetized with isoflurane (Isothesia, 2.5–3%, inhalation; Dublin, OH) before intrathecal injections. Then before being sacrificed for immunohistochemistry studies, they were deeply anesthetized with sodium pentobarbital (Nembutal, 100mg/kg, intraperitoneal; Lundbeck Inc., Deerfield, IL).

2.3. Drug Administration

Oxaliplatin (Tocris) was dissolved in saline to a concentration of 1mg/ml. After all baseline responses were measured (day 0), rats were randomly selected to receive a single intraperitoneal injection of oxaliplatin (3 mg/kg) or an equivalent volume of saline vehicle (day1). Intrathecal injections of anti-CCL2 antibodies were used to test mechanistic linkage between behavioral change induced by oxaliplatin and increased expression of CCL2 using methods previously described [65]. Briefly, 10 µl anti-CCL2 immunoglobulin G (IgG) (500 µg/ml, R&D, Minneapolis, MN) or nonspecific IgG (NS-IgG) were injected in the L5–L6 intervertebral space. Intrathecal drugs were delivered using a 0.5-inch 30-gauge needle connected to a Luer-tipped Hamilton syringe. Accurate positioning of the needle inside the subarachnoid space was verified by the occurrence of a brisk tail-flick on introduction. Rats were randomly divided into 4 groups that were used in two protocols according to the timing of intrathecal injections. The first two groups were used to test the effect of blocking CCL2 in preventing the development of oxaliplatin related CIPN. One group of rats received 4 daily doses of anti-CCL2 antibodies and a second group received non-specific Ig starting 2 days prior to treatment and continuing to day 2 after treatment with oxaliplatin. On the day where both injections were made (day1), the intrathecal injections were given 1 hour prior to oxaliplatin. The remaining two groups were used to test for reversal of established CIPN related mechanical hypersensitivity. Rats with confirmed mechanical hypersensitivity (methods appear in the next section) received 4 daily doses of the antibody (group 3) or non-specific Ig (group 4) beginning 1 week after oxaliplatin treatment (days7 to 10). All injections were done by an investigator blinded to treatment conditions. Animals were observed for any adverse side effects for 2hrs following all injections without occurrence.

2.4. Behavioral testing

Mechanical sensitivity was assessed using the “up-and-down” method as previously described [17]; and used by us in several previous studies [35,65,67]. All behavior testing

was conducted by an investigator blinded to treatment conditions. Environmental factors (i.e. noise level, lighting conditions, and time of testing) were held constant throughout all experiments. Testing was performed in the morning hours (8:00 a.m. – 11:00 a.m.). Animals were placed beneath Plexiglas containers (10 × 10 × 4 in.) set upon elevated wire mesh stands and allowed to acclimate for 15 min. The 50% withdrawal threshold was determined using a series of 8 von Frey monofilaments (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, 15.10 g). Beginning with a bending force of 2.0 g, monofilaments were applied with a force sufficient enough to cause a slight bending against the plantar surface and held for 6–8 seconds without any acceleration in the bending force. Flinching or licking after sharply withdrawing the paw was considered a positive response. Uncertain movement was not counted, and the testing was repeated after allowing the animal to rest from the previous stimuli for at least 1 min. A positive response was followed by another stimulation 1 min later with the next lower force filament. If no response was seen, stimulation with the next higher force monofilament was applied 1 min apart from the previous until three responses for the same filament were obtained.

2.5. Immunohistochemistry

Animals were deeply anesthetized and then perfused through the ascending aorta with warm saline followed by cold 4% Zamboni's fixative in 0.1 M phosphate buffer. The L4 and 5 DRG were removed and post fixed in 4% Zamboni's fixative overnight and then cryoprotected in 30% sucrose solution at 4°C. Longitudinal (20 µm) sections were cut in a cryostat and washed with phosphate buffered saline (PBS) three times for ten minutes. The slices were blocked in 5% normal donkey serum (NDS) and 0.2% Triton X-100 in PBS at room temperature for thirty minutes. DRG sections were incubated overnight at 4°C in 1% NDS containing primary antibodies to determine the cellular co-localization of CCL2 and CCR2 to DRG neuron subtypes: anti-CCL2 (rabbit, 1:500, Millipore, Temecula, CA), anti-CCR2 (goat, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-calcitonin gene-related peptide (CGRP) (guinea pig, 1:2000; Peninsula Labs, San Carlos, CA), anti-Bandeiraea (griffonia) simplicifolia I-isolectin B4 (IB4) (L2895; Sigma-Aldrich), and anti-transient receptor potential cation channel vanilloid 1 (TRPV1) (goat, 1:1000; Neuromics, Edina, MN). On the second day, slices were washed 3 times for ten minutes then incubated with Cy-3 or fluorescein isothiocyanate (FITC) conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) overnight at 4°C. Negative controls for all experiments entailed omission of the primary antibodies. Sections were washed then mounted on glass slides and viewed under a fluorescent microscope (Eclipse E600; Nikon, Japan). IHC images of the DRG were captured using a 20x objective and constant acquisition parameters by investigators blinded to treatment conditions.

2.6. Quantification

Five slices were examined for each animal. All counting was done using NIC Elements Imaging Software (Nikon) by an experimenter blinded to all treatment conditions. The number of CCL2 and CCR2 positively stained neurons along with their diameters were defined. The intensity of positively stained cells was at least 4 times higher than the background by setting a threshold according to each individual slice. Diamidino-2-

phenylindole nucleic acid (DAPI) staining was used to count the total number of neurons in the same slice.

2.7. Statistical Analysis

Data for 50% withdrawal threshold and counts of CCL2 and CCR2 positive cells are presented as means $\pm$ standard error of the mean (SEM). The differences between means were analyzed using Mann-Whitney U test (IHC) or 2-way analysis of variance (ANOVA, behavior) followed by Bonferroni post hoc test. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. Mechanical hypersensitivity was induced by single dose oxaliplatin treatment

Mechanical hypersensitivity was evidenced by a reduction in the 50% withdrawal threshold over several time points (Fig. 1). Oxaliplatin treated rats ($n = 7$) exhibited decreased mechanical withdrawal threshold in comparison to vehicle (saline) treated rats ($n = 8$). The decrease from baseline started on day 2 (24hrs post oxaliplatin injection) where vehicle-treated rats showed a 50% withdrawal threshold of $8.9 \pm 1.6g$ and oxaliplatin treated rats dropped to $4.2 \pm 1.1g$ ($p < 0.001$, Mann-Whitney U test). Oxaliplatin induced mechanical hypersensitivity was sustained through the end of the observation period (day 15) where vehicle-treated rats showed 50% withdrawal threshold of $9.6 \pm 1.7g$, whereas in oxaliplatin rats it was $5.9 \pm 1.4g$ ($p < 0.001$).

3.2. Single dose oxaliplatin induced an increase in expression of CCL2 in small DRG neurons

The expression of CCL2 in the DRG was quantified with IHC (Fig 2a and b). Oxaliplatin treated rats showed a trend to increased expression of CCL2 as early as 4 hrs after treatment compared to vehicle rats. Oxaliplatin treated rats ($n = 4$) showed 8.3% of DRG neurons to be positively stained for CCL2 (207/2490 total) while vehicle treated animals ($n = 4$) showed 5.4% of cells to be CCL2 positive (138/2535 total). On day 4, the expression of CCL2 was significantly higher in oxaliplatin treated rats ($n = 3$), 10.9% of DRG neurons were positive for CCL2 (217/1996 total) while in saline treated rats ($n = 4$) only 3.1% of DRG neurons were positive for CCL2 (115/3736 total; $p < 0.001$, two-way ANOVA). These differences were no longer present by day 15 after treatment as the expression of CCL2 in oxaliplatin treated rats ($N=6$) was found in 4.5% of the DRG neurons (185/4114 total) while in saline treated rats ($N=6$) 3.3% of DRG neurons were CCL2 positive (151/4597 total).

The majority of CCL2 was expressed by small ($< 30 \mu m$) DRG neurons (Fig. 2c). Co-localization further identified these small neurons as nociceptors given that CCL2 was found in cell profiles positive for the nociceptor-specific markers IB4 and CGRP (Fig. 2d). For oxaliplatin treated rats, 4hs after injection, 85.5% of CCL2 positive neurons were small cells, 13.8 % were medium-sized cells (31–44 μm), and only 0.7 % were large cells ($> 45 \mu m$). At day 4 after oxaliplatin, 90.3% of CCL2 positive neurons were small cells and 9.7% were medium-sized cells with no large cells stained positively for CCL2. Finally, on day 15,

86.5% of CCL2 positive neurons were small cells, 13.5% were medium-sized cells, and there were still no CCL2 positive large cells seen at this time point.

3.3. Upregulation of CCR2 positive cells in the DRG following single dose of oxaliplatin

The expression of CCR2 in the DRG after oxaliplatin treatment was measured and quantified in the same fashion as that for CCL2 described above. As shown in Fig 3b, following a single injection with oxaliplatin or saline there was no significant difference in the number of CCR2 positive cells between the two groups at 4hs post injection ($n = 4$ for each group) nor on day 15 ($n = 6$ for each group). In oxaliplatin treated rats, 4.5% (74/1645 total) and 14.0% (219/1565 total) of DRG neurons were positive for CCR-2 at 4hrs post the injection and on day 15, respectively. These results were very close to the vehicle group that had 3.0% (47/1585 total) of neurons stained positively for CCR-2 after 4hs and 13.9% on day 15 (238/1709). However, the number of CCR2 positive neurons was significantly increased at day 4 after oxaliplatin treatment where 28.9% (269/930 total) of DRG neurons were positively stained for CCR2 in oxaliplatin treated rats ($n = 3$) compared to 13.9% (142/1021) of neurons in vehicle treated rats ($n = 4$) ($p < 0.05$, two-way ANOVA).

As in our previous study in paclitaxel CIPN [65], CCR2 was mainly observed in large and medium-sized DRG cells (Fig 3c). Nevertheless, co-localization was found also in some TRPV1 positive neurons (Fig. 3d). After 2hrs of single dose oxaliplatin injection, 21.6% of CCR2 positive neurons were large cells ($> 45\mu\text{m}$), 74.3% were medium-sized cells (30–45 μm) and only 4.1% of CCR2 positive neurons were small cells ($< 30\mu\text{m}$). At 4 days after oxaliplatin treatment, the percentage of CCR2 positive neurons was 50.2% large cells, 42.4% medium-sized cells and 7.4% small cells. Last, on day 15 after oxaliplatin injection, 44.3% of large cells, 35.6% of medium-sized cells and 20.2% of the small cells were CCR2 positive. Therefore, CCR2 was expressed in both large diameter presumably non-nociceptive DRG neurons as well as medium sized neurons likely to be A δ nociceptors but in few small DRG neurons that were likely C-nociceptors.

3.4. Intrathecal anti-CCL2 given before and during oxaliplatin treatment attenuated the development of mechanical allodynia

Intrathecal injection of anti-CCL2 antibodies produced no change in response to von Frey stimulation from the baseline behavioral tests collected before initiating chemotherapy treatment (Fig. 4). Furthermore, there was no significant difference between the 50% withdrawal threshold of intrathecal anti-CCL2 treated rats ($12.8 \pm 1.4\text{g}$, $n = 4$) and those that received intrathecal NS-IgG ($13.0 \pm 1.3\text{g}$, $n = 4$) on day 0. However, the 50% withdrawal threshold of NS-IgG + oxaliplatin treated rats dropped significantly from this baseline at days 2, 4, 6, 8, and 15, respectively. In contrast, the mechanical withdrawal threshold for the anti-CCL2 + oxaliplatin treated rats remained near the baseline values at each measurement time point. Threshold values of anti-CCL2 treated rats were significantly higher than the values in rats treated with NS-IgG specifically on days 6, 8, and 15 ($p < 0.01$). Hence, intrathecal anti-CCL2 reduced the development of oxaliplatin-induced mechanical hypersensitivity.

3.5. Intrathecal anti-CCL2 antibodies transiently reverses pre-established oxaliplatin-induced mechanical allodynia

Both groups of rats showed mechanical hypersensitivity when assessed at day 7 after oxaliplatin (Fig. 5). Rats treated with NS IgG ($n = 6$) showed no improvement in 50% withdrawal threshold over the course of the experiment. On the other hand, treatment with intrathecal anti-CCL2 IgG ($n = 7$) raised the 50% withdrawal threshold value from 1.78 ± 0.2 g before treatment on day 7 to 8.4 ± 1.8 g the next day, after 2 doses of anti-CCL2 on day 8. Mechanical withdrawal threshold showed further improvement with continued anti-CCL2 antibody treatment on days 9 and 10 ($p < 0.001$, two-way ANOVA). The effect faded quickly upon discontinuation of anti-CCL2 antibody treatment such that there was no significant difference between the two groups across days 11 to 16.

4. Discussion

The main findings in this study are that CCL2 and its receptor CCR2 are increased in the DRG following oxaliplatin treatment in parallel to the development of mechanical hypersensitivity. Additionally, intrathecal treatment with an anti-CCL2 antibody during oxaliplatin administration prevents the development of mechanical hypersensitivity; and intrathecal anti-CCL2 antibody treatment also transiently reverses pre-established oxaliplatin-induced mechanical hypersensitivity. These data appear to confirm that there is a generalizability of mechanisms across models of CIPN produced using chemotherapy drugs with very different anti-cancer actions and seem to implicate effects of the innate immune response in these mechanisms.

Models of CIPN using intraperitoneal injection of oxaliplatin have been widely studied [16,26,27]. Here a single injection of 3 mg/kg oxaliplatin induced mechanical hypersensitivity that lasted to 15 days after treatment. This finding is similar though differing in duration from a previous study using a similar single dose approach where mechanical hyper-responsiveness recovered at day 10 after treatment [36]. This difference is most likely due to the different techniques for assessment of mechanical responsiveness. Here, we simply followed the up-down method as established by Chaplan et al [17], while the other study [36] assessed mechanical responsiveness using the method of Tal & Bennett [51]. These two methods differ in the force, frequency, and duration that the stimuli are applied. In Tal & Bennet method, greater diameter von Frey monofilaments (1.479, 2.041, 3.63, 5.495, 8.511, 11.749, 15.136 and 28.84 g) were used with a series of five stimuli for each monofilament applied for 3–5 s. The threshold was then determined as the lowest force that evoked a withdrawal response to one of the five stimuli. While in Chaplan's up and down method, beginning with a bending force of 2.0 g, applications of a series of smaller diameter monofilaments (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.10 g) were applied for 6–8 s each with the 50% withdrawal threshold was determined using the method of Dixon [20].

A strong rationale for studying the effects of a single dose of oxaliplatin on DRG neurons as done here and previously [36] is that the symptoms experienced early in oxaliplatin chemotherapy by patients are strong predictors of long-term CIPN outcomes [44]. Oxaliplatin-related symptoms persisted between dosing cycles and built in intensity with

repeated treatment [44], suggesting that the innate immune mechanisms in the DRG revealed here as engaged by oxaliplatin in animals would similarly be expected to become more exaggerated with repeated dosing. Yet, a clear limitation in the single dose models is that rats only received roughly one-fifth the amount of oxaliplatin that would normally be given to patients. Previous experience with platin-based chemotherapy agents showed that higher doses produced a loss of withdrawal response possibly indicative of the development of numbness [14]. Although patients also develop numbness, this level of neuropathy in rats presents potential difficulty in the interpretation of experimental results and so the lower doses were used as typical in experimental models of both platin- and taxane-induced CIPN [7,8,45,61]. Thus, in considering translation of these findings to the clinic, patients might have more exaggerated innate immune responses than seen here that might require more prolonged or higher dose administration of CCR2 antagonists, by example, to suppress the development of CIPN.

Acute dysesthesia after oxaliplatin infusion occurs in over 70% of patients [46,49]. Symptoms were reported during or immediately after the infusion and would last for a few min to several days and in most cases these symptoms were dose related [22,24,25]. The emergence of CIPN symptoms is often the main reason for poor compliance in patients receiving chemotherapy, forcing dose reduction and leading to sub-optimal therapy [1,16]. Although the mechanism of CIPN is still unclear, many possible pathways have been suggested [11,12,30,55]. Genetic predispositions have been implicated [6,15,60]; and in a recent clinical study [57] subthreshold neuropathy evidenced by deficits in fine touch detection have also been implicated. Other mechanisms such as expression of inflammatory mediators including cytokines and chemokines [4,31,64], DNA damage [39], alterations in ion channels and neurotransmission [41,48], and altered intracellular signaling and cellular function, particularly in mitochondria [21,62,70,71] have all been implicated in the development of CIPN.

Work in our lab has focused on the role of the innate and adaptive immune system in producing CIPN. CCL2/CCR2 signaling between myelinated and unmyelinated neurons of the DRG was suggested to contribute to paclitaxel induced mechanical hypersensitivity [65]. In this study, overexpression of CCL2 in DRG was seen 4hs after a single dose of oxaliplatin, followed by a significant increase in CCR2 on day 4 and this parallels the increase in onset of mechanical hypersensitivity. Co-treatment of rats with CCL2 neutralizing antibodies resulted in an attenuated level of behavioral hypersensitivity suggesting a mechanistic linkage between the behavioral changes and increased CCL2 expression. Yet, of note the co-treatment was not complete indicating that some of the other mechanisms implicated in CIPN as reviewed above had possibly still been engaged. However, on Day 15 the levels of CCL2/CCR2 had decreased to that seen in vehicle treated rats while the mechanical hypersensitivity was still apparent. This would seem to suggest that CCL2/CCR2 signaling participates in the onset of oxaliplatin CIPN, but that other mediators become engaged at later stages of the model to maintain behavioral hypersensitivity. Yet, CCL2 antibody treatment showed an attenuation of pre-established mechanical hypersensitivity suggesting that in spite of the reduced expression level, CCL2/CCR continue to play a key role in maintaining oxaliplatin CIPN. It would seem that

either the signaling of CCR2 is sensitized at these later time points, or there is a non-DRG neuron source of CCL2 that becomes engaged at this later time point.

As in our previous study with paclitaxel CIPN, oxaliplatin-induced increases in CCL2 was found primarily in small CGRP and IB4-positive DRG neurons, while its receptor CCR2 was primarily expressed in medium and large DRG neurons. This implies that both myelinated A β and A δ neurons as well as unmyelinated C neurons in the DRG are involved in oxaliplatin-induced sensory dysfunction [29,32,56]. This would be consistent with the findings that ectopic spontaneous activity (SA) develops in all three cell types with paclitaxel chemotherapy [33,66]. SA in large and medium sized DRG neurons was assayed using a whole DRG explant preparation where factors from neighboring cells were preserved; and CCL2 was shown to evoke calcium influx in both cell types [66], thus CCL2 may be a primary driving factor for altered function in these neurons. As well, SA and altered signaling in large DRG neurons is consistent with numerous reports for a role of these DRG neuron types in contributing to neuropathic pain [19,37]. Small DRG neurons show SA in dissociated cultures and so CCL 2 is unlikely to drive altered activity in these cells [33].

The question could be asked as to whether CCL2/CCR2 signaling in the spinal cord might contribute to oxaliplatin related CIPN. While a role for increased CCL2/CCR2 expression and presumably its signaling are well documented in the DRG [28,52,65,69], the expression of CCR2 in the spinal cord is debated [23,43]. We did not detect CCR2 protein or mRNA in spinal dorsal horn after paclitaxel treatment in our previous study [65]; and so we concluded that the major site of action of CCL2 in paclitaxel CIPN is within the DRG rather than in the spinal cord. Nevertheless, a role for CCL2/CCR2 in the spinal cord in oxaliplatin-related CIPN cannot be excluded.

In conclusion, induction of CCL2/CCR2 signaling in DRG after single dose oxaliplatin contributes to increased mechanical allodynia and blocking this signal could be useful both as prophylaxis against neuropathy and as a salvage measure in patients previously treated with oxaliplatin.

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Fig. 1. Single dose oxaliplatin induces mechanical hypersensitivity manifested by decreased paw withdrawal threshold. Compared to Vehicle-treated rats (n = 8), Oxaliplatin treated rats (n = 7) had a significant decrease in threshold from baseline beginning on day 2 and that persisted until day 15. (* p < .05; ** p < .01).

Fig. 2.

Oxaliplatin induces the expression of CCL2 in small neurons in the DRG. The representative images in (a) shows that the expression of CCL2 (red) is sparse in vehicle-treated rats (left) but becomes prominent by day 4 after oxaliplatin (right). The bar graphs in (b) shows that 4hs after treatment, CCL2 expression in Oxaliplatin rats ($n = 4$, filled bars) had started to increase, but no statistical significance was found when compared to vehicle treated rats ($n = 4$, open bars). This increase reached a significant level on day 4, then was normalized by day 15 ($n = 6$ for all groups). The bar graphs in (c) show that CCL2 was primarily expressed in small neurons of the DRG at all time points, while in (d) the representative images of oxaliplatin rats show that CCL2 (red) was found in both IB4 (top line green) and CGRP (bottom line, green) positive neurons (co-localization is shown in yellow/orange). (***) $p < .001$.

Fig. 3.

Oxaliplatin increases the expression of CCR-2 in medium and large cells in the DRG. The representative images in (a) shows that CCR (red) was expressed at very low levels in vehicle treated rats (left) but became markedly increased by day 4 after oxaliplatin (right). The bar graphs in (b) shows that there was no significant increase in CCR2 expression between oxaliplatin rats (filled bars, n=4) and saline-treated rats (open bars, n=4) at 4hs after treatment or at day 15 (n = 6 for each group), but was significantly elevated at day 4 (n=4 in each group). The bar graphs in (c) show that CCR2 was primarily found in large and medium-sized DRG at all three time points. Finally the representative images in (d) show that even though few small DRG neurons were CCR2+ at day 4 after treatment, when found it was co-localized in TRPV1 (green) positive neurons (co-localization shown in yellow). (* $p < .05$).

Fig. 4.

Treatment with anti-CCL2 immunoglobulin G (IgG) prevents oxaliplatin-induced mechanical hypersensitivity. The 50% withdrawal threshold of anti-CCL2 treated rats (open squares, n = 4) was significantly higher than rats treated with non-sense (NS IgG) (open circles, n = 4). The arrows along the x-axis show the times of IgG treatments. (* $p < .05$; ** $p < .01$).

Fig. 5. Anti-CCL2 immunoglobulin G (IgG) produces transient reversal of oxaliplatin-induced mechanical hypersensitivity. Anti-CCL2 IgG had a potent and cumulative, but impermanent effect, in reversing oxaliplatin-induced mechanical hypersensitivity (open squares, $n = 7$). Rats treated with non-sense IgG ($n = 6$) showed no change in 50% withdrawal threshold over the course of the experiment. The arrows along the x-axis show the times of IgG treatments. (**** $p < .0001$).