Published in final edited form as:

J Pain. 2016 July ; 17(7): 775–786. doi:10.1016/j.jpain.2016.02.011.

Dorsal root ganglion infiltration by macrophages contributes to paclitaxel chemotherapy induced peripheral neuropathy

Hongmei Zhang¹, Yan Li¹, Marianna de Carvalho-Barbosa¹, Annemieke Kavelaars², Cobi J. Heijnen², Phillip J. Albrecht³, and Patrick M. Dougherty¹

¹The Department of Anesthesia and Pain Medicine Research, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

²Department of Symptom Research, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

³Center for Neuropharmacology & Neuroscience, Albany Medical College, Albany, NY 12208

Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is a disruptive and persistent side-effect of cancer treatment with paclitaxel. Recent reports showed that paclitaxel treatment results in the activation of Toll-like receptor 4 (TLR4) signaling and increased expression of monocyte chemoattractant protein 1 (MCP-1) in dorsal root ganglion cells. In this study, we sought to determine whether an important consequence of this signaling and also a key step in the CIPN phenotype was the recruitment and infiltration of macrophages into dorsal root ganglia (DRG). Here, we show that macrophage infiltration does indeed occur in a time course that matches the onset of the behavioral CIPN phenotype in Sprague-Dawley rats. Moreover, depletion of macrophages by systemic administration of liposome-encapsulated clodronate (clodosome) partially reversed behavioral signs of paclitaxel-induced CIPN as well as reduced TNF α expression in DRG. Intrathecal injection of MCP-1 neutralizing antibodies reduced paclitaxel-induced macrophage recruitment into the DRG and also blocked the behavioral signs of CIPN. Intrathecal treatment with the TLR4 antagonist LPS-RS blocked mechanical hypersensitivity, reduced MCP-1 expression, and blocked the infiltration of macrophages into the DRG in paclitaxel treated rats. Finally, the inhibition of macrophage infiltration into DRG following paclitaxel treatment with clodronate or LPS-RS prevented the loss of intra-epidermal nerve fibers (IENFs) observed following paclitaxel treatment alone. Taken altogether, these results are the first to indicate a mechanistic link such that activation of TLR4 by paclitaxel leads to increased expression of MCP-1 by DRG neurons resulting in macrophage infiltration to the DRG that express inflammatory cytokines and the combination of these events results in IENF loss and the development of behavioral signs of CIPN.

Corresponding Author: Patrick M. Dougherty, PhD., Department of Pain Medicine, The University of Texas M.D. Anderson Cancer Center, 1400 Holcombe Blvd. Unit 110, Houston, TX 77030, Tel: 713-745-0438, Fax: 713-794-4590, pdougherty@mdanderson.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure:

There is no conflict of interest among the authors.

KeywordsCancer; Neuropathic pain; TLR4; MCP-1; TNF α **1. INTRODUCTION**

Chemotherapy induced peripheral neuropathy (CIPN) represents a dose-limiting adverse effect of cancer treatment which affects as many as half of cancer patients treated with single agents, and over 75% when combination therapies are utilized^{30,36}. CIPN is observed following the administration of several types of drugs commonly used for the treatment of many of the most common solid and hematologic malignancies, including vinca alkaloids, taxanes, platinum derivatives, and bortezomib^{62,17,30}. Furthermore, CIPN represents a clinical problem that is steadily on the rise as the number of long-term cancer survivors increases. CIPN most often presents as a sensory neuropathy with complaints of burning and shooting pains, tingling, and numbness, and observed as a length-dependent neuropathy with a “stocking and glove” distribution; and common analgesics aimed at reducing the painful symptoms are often ineffective^{8,9,16,20,26,25,40}. The anti-cancer modes of action for the various chemotherapeutic drugs are largely understood, but the neurotoxic mechanisms contributing to the selectivity of the damage to sensory neurons alone and the clinical severity of CIPN remain unclear^{62,17,30}. Currently, there are no pharmacologic or other means available to inhibit the occurrence of CIPN. Hence, dose reduction and ultimately withdrawal of the offending agent is the only option to slow the development of CIPN, potentially impacting optimal treatment^{62,17,30}.

Paclitaxel is one of the most effective chemotherapeutic drugs widely used for the treatment of solid tumors such as ovarian, breast, and non-small cell lung carcinoma; and also associated with the development of CIPN³⁶. Although the specific mechanisms underlying the development of paclitaxel CIPN remain undefined, there are several lines of evidence indicating that engagement of innate immunity plays a key role^{48,37,43,42,41}. For example, application of minocycline, an inhibitor of pro-inflammatory cytokine release, prevents mechanical allodynia induced by paclitaxel^{15,44}, and we have demonstrated that intrathecal treatment with the TLR4 antagonist lipopolysaccharide-RS (LPS-RS) transiently reversed pre-established CIPN mechanical hypersensitivity and prevented the development of any behavioral signs of CIPN when given as a protective agent during chemotherapy⁴³. Further, it was shown that paclitaxel treatment induces increased expression of monocyte chemoattractant protein-1 (MCP-1) in DRG and spinal cord and blockade of MCP-1/CCR2 signaling by anti-MCP-1 antibody or CCR2 antisense oligodeoxynucleotides significantly attenuated paclitaxel induced mechanical hypersensitivity, as well as the loss of distal intra-epidermal nerve fibers (IENF)⁶⁴. MCP-1/CCL2 is a potent chemokine that regulates migration and infiltration of monocytes/macrophages²², and macrophages have been observed in DRG and the spinal dorsal horn in models of paclitaxel-induced CIPN^{48,44}. Since a characteristic role of innate immunity involves monocyte/macrophage secretion of pro-inflammatory mediators, including TNF- α , IL-1 β , IL-6, MIP-1 α , MIP-1 β and MCP-1, that are widely recognized to contribute to an array of persistent pain states^{27,46,35}, we hypothesized that paclitaxel treatment activates innate immunity resulting in macrophage

recruitment to DRG and that these then drive the induction and maintenance of paclitaxel-induced peripheral hypersensitivity.

2. MATERIALS AND METHODS

2.1 Animals

Adult male Sprague-Dawley rats (weighing 250–300g, Harlan, Houston, TX, USA) housed in a 12 h light/dark cycle with free access to food and water were used in all experiments. The studies were approved by the Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center and were performed in accordance with the National Institutes of Health Guidelines for Use and Care of Laboratory Animals.

2.2 Paclitaxel CIPN model

Animals were treated with paclitaxel as previously described^{49,23,14,7,43}. Briefly, 6 mg/ml stock pharmaceutical grade paclitaxel (TEVA Pharmaceuticals, Inc. USA) was diluted with sterile 0.9% saline to 1 mg/ml and given at a dosage of 2 mg/kg intraperitoneally (i.p.) every other day for a total of four injections (days 1, 3, 5, and 7). Control animals received an equivalent volume of the vehicle only, which consisted of equal amounts of Cremophor EL and ethanol diluted with saline to reach a concentration of vehicle similar to the paclitaxel concentration. Rats were observed carefully for any abnormal behavioral changes every other day following the treatment. Signs of peripheral neuropathy with a similar phenotype as in patients have been validated in this non-tumor-bearing animal model of paclitaxel CIPN by multiple investigators^{49,23,14,7,43}.

2.3 Intrathecal treatment

Intrathecal drug delivery was performed by lumbar puncture as previously described^{21,63,43}. Rats were anesthetized with isoflurane (2.5%) and injected between the L4–L5 intervertebral space using a 0.5-inch 30-gauge needle connected to a luer-tipped Hamilton syringe. Correct subarachnoid positioning of the tip of the needle was verified by tail-flick. TLR4 antagonist LPS derived from *Rhodobacter sphaeroides* (LPS-RS, 20 µg in 20 µL PBS; InvivoGen, San Diego, CA) or anti-MCP-1 neutralized antibody (200 µg/mL, 20 µL per application; AbD Serote, Raleigh, NC) or equal amount of nonspecific IgG (Rabbit IgG, Jackson ImmunoResearch, West Grove, PA) were delivered intrathecally 24 hours prior to the first injection of paclitaxel and was continued once daily for the next 7 days for a total of 8 injections (day 0–7). Intrathecal injection was given 30 minutes prior to paclitaxel when both drugs were administered on the same day.

2.4 Intravenous injection of clodronate

The macrophage toxin clodronate in liposomes (clophosome-A, 7mg/ml clodronate disodium) or control liposome (FormuMax, Sunnyvale, CA) were intravenously administered to 24 rats (12 verses 12) with the volume of 0.8 ml on day 7 and day 10 in paclitaxel treated rats.

2.5 Mechanical withdrawal threshold

Mechanical withdrawal threshold was tested before, during and following paclitaxel treatment by an experimenter blinded to treatment groups. The 50% paw withdrawal threshold in response to a series of eight von Frey hairs (0.41 to 15.10 g.) was examined by the up-down method as described previously¹⁸. Animals were placed under clear acrylic cages atop a wire mesh floor. Beginning with a filament with a bending force of 2.0 g., the filaments were applied to the paw just below the pads with no acceleration at a force just sufficient to produce a bend and held for 6–8 sec. A quick flick or full withdrawal was considered a response, in which case the next lower filament was applied. If no response was observed then the next higher filament was applied. This continued until three responses to a single filament were observed. The test will be performed three hours after drugs application on those days when both happened.

2.6 Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p., Lundbeck, Inc., Deerfield, IL) at days 3, 7, 14 and 21 after paclitaxel treatment. Then they were perfused through the ascending aorta with warm saline followed by cold 4% paraformaldehyde in 0.1 M PBS. The L4 and L5 DRG, spinal cord, the spleen and the hindpaw foot pad were removed, fixed in 4% paraformaldehyde for 6 hours, and then cryo-protected in 30% sucrose solution. Tissue blocks were then submerged in optimal cutting temperature (OTC) medium and frozen. Transverse spleen and spinal cord sections (20 μ m), longitudinal DRG sections (8 μ m), and hindpaw epidermal cross-sections (14 μ m) were cut in a cryostat, mounted on gelatin-coated glass slides (Southern Biotech, Birmingham, AL), and processed for immunofluorescent labeling. After blocking in 5% normal donkey serum and 0.2% Triton X-100 in PBS for 1 hour at room temperature, DRG sections were incubated overnight at 4°C in 1% normal donkey serum and 0.2% Triton X-100 in PBS containing primary antibodies against CD68 to visualize macrophages (mouse, 1:500; Abcam, Cambridge, MA), MCP-1 (rabbit, 1:500; AbD Serotec, Raleigh, NC) or anti-TNF α (goat, 1:200; Santa Cruz, Dallas, TX). The skin sections were similarly blocked and then incubated with primary antibodies directed at PGP9.5 (rabbit polyclonal, 1:1000; CedarLane), or GAP43 (rabbit polyclonal, 1:300; Abcam #ab16053) to visualize intra-epidermal nerve fibers. After washing, sections were then incubated with the species-appropriate FITC or Cy3-conjugated secondary antibodies overnight at 4°C, or at room temperature for 2 hours. Slides were then mounted in glycerol and coverslipped for microscopic analysis.

2.7 Quantification

DRG, spinal cord and splenic sections were viewed under a fluorescent microscope (Eclipse E600; Nikon, Japan) and all quantifications were made with NIC Elements imaging software (Nikon, Japan). All images were taken by an investigator blinded to treatment conditions using identical acquisition parameters and the final representative figures are presented as the original images without further modification. CD68-positive cells were not evenly distributed through the DRG and so these were counted over the entire DRG using a 20 $\times$ objective and then expressed as cells per unit area. TNF α and MCP-1 positive cells were

evenly distributed over the DRG. The numbers of total and positive neurons from 3 sections of DRG of 3 rats were counted; data from 3 sections of the same rat were averaged and then mean values used for Mann-Whitney U test comparisons. The intensity of positively stained neurons was at least 4 times higher than the background by setting a threshold according to individual slice, and the number of positively stained neurons was counted. The number of total neurons in the same area was counted. The percentages of positive neurons to total neurons were calculated and statistically compared³³ (Huang et al., 2014a)(Huang et al., 2014a)(Huang et al., 2014a). Intraepidermal nerve fiber (IENF) analysis for PGP/GAP43 was performed on images captured utilizing a Zeiss Axio Imager M2 microscope equipped with standard Zeiss objectives (20×) and fluorescent filters for Cy2, alexa488, and DAPI fluorophores, motorized stage control, and a Hamamatsu ORCA Falsh4.0 digital camera attached to a Dell Precision T3600 PC running Win7 and Neurolucida Software to operate the scope system. Epidermal contours and endings were mapped from individual full depth (3D) deep focused images utilizing Neurolucida and NeuroExplorer software (MBF Biosciences, Essex, VT). Once mapped, the number of IENF found crossing the basement membrane and fragments per unit length of epidermis, and the percentage that were double labeled with GAP43 were calculated.

2.8 Statistical analysis

All results are presented as means $\pm$ SEM and analyzed with GraphPad Prism 5. Differences between means were tested for significance using *t*-test or one-way ANOVA. The cell counts for percentage of immune-positive neurons were analyzed using 1-way analysis of variance. An alpha value of $P < 0.05$ was considered significant.

3. RESULTS

3.1 Paclitaxel induces mechanical hyper-responsiveness coincident to DRG infiltration by macrophages

Consistent with our previous studies^{65,64,43}, rats treated with paclitaxel (2mg/kg, 4 ×) showed a significant decrease in mechanical withdrawal threshold by day 7, which became more pronounced by day 14 and was sustained through at least day 21 (Figure 1A). In contrast, rats treated with vehicle showed no significant change in withdrawal threshold throughout the experimental period (Figure 1A). Cells positively labeled for CD68, a lysosomal protein present in activated macrophages³², were significantly increased in the L4–5 DRG as early as 3 days after the initiation of chemotherapy treatment. The number of CD68-positive cells continued to increase at day 7, reached a maximum at day 14, and remained significantly elevated at day 21 (Figure 1B). Representative images of CD68 immunolabeling in L4–5 DRG from a vehicle treated animal at day 14 (Figure 1C) compared with those taken from paclitaxel-treated animals at days 3, 7, 14, and 21 (Figures 1D, 1E, 1F, 1G) demonstrates the infiltration of CD68-positive macrophages following paclitaxel treatment.

3.2 Clodronate reduces macrophage numbers and TNF α expression and partially reverses mechanical hypersensitivity in paclitaxel CIPN

The role of DRG macrophages in paclitaxel-induced CIPN was tested using the macrophage toxin clodronate in liposomes (clodrosome, 7mg/ml) or liposomes alone (vehicle) intravenously administered (1.0 ml) on day 7 and day 10 of treatment. As shown in Figure 2A, the mechanical withdrawal threshold was significantly increased from day 10 through day 14 of paclitaxel injection in clodronate co-administrated rats. In contrast, animals co-treated in the same fashion with control liposomes developed full mechanical hypersensitivity (n = 6). Animals treated with clodronate plus vehicle showed no change in mechanical withdrawal threshold (not shown). The spleen and DRG were collected on day 14 and the tissues were used to assess the impact of clodronate treatment on CD68 immunoreactivity. Clodronate treatment markedly reduced the immunolabeling for CD68 in the spleen of paclitaxel treated rats compared with control liposome treated rat spleen (Figures 2B, 2C). Clodronate treatment also significantly reduced the number of cells expressing CD68 in the DRG of paclitaxel treated rats compared to vehicle-paclitaxel treated rats (Figures 2D, 2E, 2F). The phenotype of these macrophages was further explored by measuring the expression of TNF α in DRG. The expression of TNF α was increased on day 14 in paclitaxel treated DRG (Fig. 2G and 2H) versus the vehicle controls. This increase was abolished by clodronate treatment (Fig. 2G and 2I).

3.3 Anti-MCP-1 blocks the mechanical hypersensitivity of paclitaxel CIPN and reduces the macrophage infiltration in DRG

Previous studies showed that activation of MCP-1/CCR2 signaling in DRG neurons plays a critical role in the development of paclitaxel CIPN⁶⁴. The involvement of MCP-1 in provoking macrophage infiltration in paclitaxel CIPN was investigated by intrathecal injection of anti-MCP-1 neutralizing antibody. Anti-MCP-1 or control IgG (200 μ g/ml, 20 μ l per application) was delivered once daily prior to and during paclitaxel treatment by lumbar puncture between the L4–L5 intervertebral space. As shown in Figure 3A, at day 14 after paclitaxel treatment, animals co-treated with non-specific IgG developed mechanical hypersensitivity (paw withdrawal threshold of 4.32 ± 1.1 g; n = 6), compared with animals co-treated with anti-MCP-1 who maintained near baseline measures (paw withdrawal threshold of 14.32 ± 0.96 g; n=6). Animals did not show any change in paw withdrawal threshold in the groups co-treated with Cremophor vehicle and intrathecal non-specific IgG or anti-MCP-1 (Figure 3A). Consistent with the behavior data, anti-MCP-1 treatment markedly reduced macrophage infiltration in the DRG of paclitaxel-treated rats (Figures 3B, 3C, 3D). CD68 immunoreactivity in the DRG did not increase in the negative controls treated with Cremophor vehicle treated rats receiving non-specific IgG or anti-MCP-1 (Figures 3E, 3F).

3.4 LPS-RS prevents mechanical hypersensitivity of paclitaxel CIPN and reduces macrophage infiltration to the DRG

Since we have recently demonstrated that TLR4 plays an important role in paclitaxel CIPN⁴³, the involvement of TLR4 in macrophage infiltration was investigated by intrathecal injection of a TLR4 receptor antagonist. LPS-RS was injected twice daily prior to and

during paclitaxel treatment by lumbar puncture between the L4–L5 intervertebral space (1 µg/ml, 20 µl per injection). Macrophage infiltration at day 14 was significantly inhibited by LPS-RS co-treatment (Figures 4A, 4B, 4F), and behavioral hypersensitivity was also reduced (4E). In order to clarify the relationship of TLR4, MCP-1, and macrophage infiltration, we further tested the expression of MCP-1 in the DRG of PBS or LPS-RS treated animals after 14 days of paclitaxel. LPS-RS treatment significantly decreased the expression of MCP-1 in the DRG (Figures 4C, 4D, 4G). These results indicate that blocking the activation of TLR4 receptor reduces the expression of MCP-1 in the DRG which subsequently limits the infiltration of macrophages.

3.5 Clodronate or LPS-RS prevents the loss of epidermal nerve fibers induced by paclitaxel

Paclitaxel treatment has been previously shown to induce a loss of IENFs⁷. Here, we confirm that at day 14 of paclitaxel treatment there was a significant reduction in PGP-labeled IENFs, and newly show that IV treatment with clodronate or intrathecal treatment with LPS-RS protected against this paclitaxel-induced loss (Figure 5E). Double label images of PGP and GAP43 demonstrate a 100% co-localization of markers among the IENF from control and treatment groups, indicating that the small caliber epidermal innervation is constantly remodeling (Figures 5A, 5B, 5C, 5D), as we have previously documented^{2,3,6,47}. Importantly, fainter PGP immunolabeling was consistently detected among the paclitaxel-treated specimen compared with the control or those paclitaxel-treated rats that also received clodronate or LPS-RS also. (Figure 5B vs 5A, 5C, 5D, respectively). For the paclitaxel group images, the PGP labeling needed to be captured with longer camera exposure settings compared with the other groups, likely due to an apparent decrease in the PGP content of axons following chemotherapeutic treatment, which we have previously noted⁶.

4. DISCUSSION

In the present study, we present for the first time data that mechanistically links a number of previously disparate findings that have been shown involved in the generation of paclitaxel related CIPN. Paclitaxel is shown to induce the infiltration of macrophages into the DRG in a time course matching the onset of behavioral hypersensitivity to mechanical stimuli. Depletion of macrophages by intravenous liposome encapsulated clodronate (clodronate) partially reversed paclitaxel-induced mechanical hyperalgesia and reduced the expression of the pro-inflammatory cytokine TNFα. Intrathecal injection of MCP-1 neutralizing antibody similarly reduced mechanical allodynia and paclitaxel-induced macrophage recruitment into the DRG. Additionally, the TLR4 antagonist LPS-RS attenuated the mechanical hypersensitivity, reduced MCP-1 expression and blocked the infiltration of macrophages into the DRG normally observed in paclitaxel treated rats. Finally, we demonstrated that consistent with previous findings, paclitaxel resulted in a loss of IENF endings which corresponds with increased hindpaw sensitivity⁷, but importantly the IENF endings were preserved in the clodronate or LPS-RS co-treatment groups.

Neuropathic pain is known to involve an important role of glial cells and pro-inflammatory immune responses in the underlying basic pathophysiology^{12,4,58}. An emerging body of data

suggests that paclitaxel also exerts effects on the immune system by stimulating anti-tumor and anti-autoimmunity effects^{11,57}. For example, previous reports showed that a higher dose of paclitaxel than used here (cumulative dose 24 mg/kg, $3 \times 8\text{mg/kg}$) also induced a persistent mechanical allodynia that was accompanied by an infiltration of macrophages into the DRG of rats and this was prevented by co-treatment with minocycline during chemotherapy^{44,34}, and application of minocycline, an inhibitor of pro-inflammatory cytokine release, prevents mechanical allodynia and IENF loss induced by paclitaxel using the doses of paclitaxel as in this study⁷. Activation of macrophages has been reported to contribute to experimental neuropathic pain states by releasing potent pro-inflammatory mediators, including tumor necrosis factor- α (TNF α), interleukin-1 β (IL-1 β), MCP-1, nerve growth factor (NGF), nitric oxide (NO) and prostanooids⁵². Furthermore, when macrophages are depleted from, or recruited into, peripheral tissues experimentally, they have been shown to either attenuate or exacerbate, respectively, inflammatory pain produced by zymosan or acetic acid⁵¹. As well, it has been shown that the supernatant from macrophages activated *in vitro* by LPS is hyperalgesic due to the cytokine content⁵⁵. Here, we demonstrate that a relatively low dose of paclitaxel also induced DRG infiltration by macrophages in a time course that matched the development of mechanical hyperalgesia. Macrophage infiltration was evident beginning at day 3 and reached a maximum at day 14 of chemotherapy. The numbers of macrophages in the DRG declined by day 21 though mechanical hyperalgesia was maintained at the same level as day 14. This result indicates that macrophage recruitment is engaged in the induction, but possibly not the maintenance, of paclitaxel CIPN. Depletion of macrophages with clodronate attenuated the development of mechanical allodynia induced by paclitaxel and also reduced the TNF α levels in the DRG, reinforcing the hypothesis that macrophage infiltration plays a role in the development of mechanical hypersensitivity.

It has been recognized that inflammatory mediators released from immune cells contribute to persistent pain states. For instance, it has been observed that MCP-1 mRNA expression is markedly increased in the injured rat sciatic nerves in parallel with an increase of macrophage recruitment⁵⁶. However, the development of mechanical allodynia is totally abrogated in CCR2 (MCP-1 receptor) knockout mice while macrophage recruitment is only attenuated¹. A recent publication from our group demonstrated that paclitaxel induced an increase in the expression of MCP-1 in DRG and spinal cord, and that blockade of MCP-1/CCR2 signaling by anti-MCP-1 antibody or CCR2 antisense oligodeoxynucleotides significantly attenuated paclitaxel-induced mechanical hypersensitivity⁶⁴. In the current study, we show that intrathecal anti-MCP-1 blocked mechanical allodynia induced by paclitaxel and also significantly reduced macrophage infiltration into the DRG. Another chemokine CX3CL1 up-regulation and macrophages infiltration into the DRG in paclitaxel treated has been reported in another group³⁴. They also found that intrathecal or systemic injection of CX3CL1 neutralizing antibody blocked paclitaxel-induced macrophage recruitment in the DRG and attenuated paclitaxel-induced allodynia. Furthermore, depletion of macrophages by systemic administration of clodronate inhibited paclitaxel-induced allodynia, which is consistent with our report here. Importantly, this study moves the field forward by lining all these observations and also showing that all this signaling in the DRG

results in the distal loss of IENFs that have also been shown as involved in the overall CIPN phenotype.

TLR4 is an important receptor in the innate immune system activated by LPS and by endogenous molecules such as the damage associated molecular pattern HMGB1. TLR4 antagonists reduce nerve injury-induced hyperalgesia in mice and rats^{5,35}, and knockout of either TLR4 or its signaling co-factor CD14 results in abbreviated post-inflammatory hyperalgesia, reduced spinal glial responses to inflammation, and reduced neuropathic pain^{54,12,19}. Macrophages secrete a variety of pro-inflammatory factors following LPS stimulation, and although there are no obvious structural similarities to LPS, paclitaxel exerts LPS-mimetic properties in primary macrophages and macrophage cell lines¹⁰. Paclitaxel and LPS appear to share a TLR4/MyD88-dependent pathway in generating inflammatory mediators, but also share a TLR4- dependent/MyD88-independent pathway which leads to the activation of microtubule-associated protein kinase (MAPK) and nuclear factor (NF)-kappa B¹⁰. Our recent publication showed that the expression of TLR4 is up-regulated in DRG and spinal cord after the treatment of paclitaxel, while intrathecal application of TLR4 antagonist lipopolysaccharide-RS (LPS-RS) prevented any behavioral signs of CIPN when given as a protective agent during chemotherapy⁴³. Consistent with the behavior result, we found that macrophage recruitment is minimal when LPS-RS is co-administered during chemotherapy. Moreover, intrathecal LPS-RS also prevented the increase in expression of MCP-1 normally observed with paclitaxel treatment. Furthermore, we also importantly demonstrate that the decrease in macrophage recruitment to DRG by clodronate or LPS-RS, and subsequent decrease in pro-inflammatory cytokine expression, may contribute to the preservation of IENF following paclitaxel treatment. Although the direct causal link between small caliber nociceptor IENF decreases and chronic pain are inconsistent³⁸, in nearly every chronic neuropathic pain condition examined, a loss of IENF is observed. Therefore, the preservation of these IENF with clodronate or LPS-RS may also be contributing to the reversal of paclitaxel-induced behavioral hypersensitivity.

An important consideration in these findings is that activation of TLR4 by paclitaxel in rodents is widely accepted^{24,28,37}, but this effect is controversial for human TLR4. Cytokine production from human macrophages and other tissues by paclitaxel has been reported supporting activation of human TLR4^{61,13,31,60}, but others have not observed this^{45,66}. LPS binds the TLR4 accessory protein MD-2 that results in activation of murine macrophages^{28,37}; but it is claimed that paclitaxel binds human MD-2 in a fashion that precludes activation of TLR4^{66,50,39}. This discrepancy has obvious importance in the context of the mechanisms of CIPN as well as in the broader scope of the utility of paclitaxel as a chemotherapeutic. A link between chemoresistance and even the promotion of aggressiveness by paclitaxel has been found in many human cancer types when TLR4 is expressed^{29,59,53}. A possible explanation may be that paclitaxel's binding to microtubules enhances its signaling via TLR4²⁸. As well, it remains unclear in human tissues whether the full canonical signaling path of TLR4 is engaged to produce CIPN, or whether it is only sufficient that TLR4 is engaged to the extent of activation the non-canonical MAP kinase pathway is sufficient⁴².

In summary, the results from our current study indicate that paclitaxel treatment activates the TLR4 receptor which in turn induces increased expression of MCP-1 (and perhaps other chemokines and pro-inflammatory cytokines) to promote the infiltration of macrophages into the DRG where they play a key role in the generation of the CIPN phenotype. It remains unknown what specific mechanism is engaged by macrophages to produce the CIPN phenotype or the loss of IENF endings.

Acknowledgments

This work was supported by grants from the National Institutes of Health (NS046606) and the H.E.B. Professorship in Cancer Research

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*. 2003; 100:7947–7952. [PubMed: 12808141]
2. Albrecht PJ, Hines S, Eisenberg E, Pud D, Finlay DR, Connolly MK, Pare M, Davar G, Rice FL. Pathologic alterations of cutaneous innervation and vasculature in affected limbs from patients with complex regional pain syndrome. *Pain*. 2006; 120:244–266. [PubMed: 16427199]
3. Albrecht PJ, Rice FL. Role of small-fiber afferents in pain mechanisms with implications on diagnosis and treatment. *Curr Pain Headache Rep*. 2010; 14:179–188. [PubMed: 20425194]
4. Austin PJ, Moalem-Taylor G. The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. *J Neuroimmunol*. 2010 Dec 15;229:26–50. [PubMed: 20870295]
5. Bettoni I, Comelli F, Rossini C, Granucci F, Giagnoni G, Costa B. Glial TLR4 receptor as new target to treat neuropathic pain: Efficacy of a new receptor antagonist in a model of peripheral nerve injury in mice. *GLIA*. 2008; 56:1312–1319. [PubMed: 18615568]
6. Bowsher D, Geoffrey WC, Nicholas AK, Carvalho OM, Haggett CE, Tedman B, Mackenzie JM, Crooks D, Mahmood N, Twomey JA, Hann S, Jones D, Wymer JP, Albrecht PJ, Argoff CE, Rice FL. Absence of pain with hyperhidrosis: a new syndrome where vascular afferents may mediate cutaneous sensation. *Pain*. 2009 Dec 15;147:287–298. [PubMed: 19836135]
7. Boyette-Davis J, Xin W, Zhang H, Dougherty PM. Intraepidermal nerve fiber loss corresponds to the development of Taxol-induced hyperalgesia and can be prevented by treatment with minocycline. *Pain*. 2011; 152:308–313. [PubMed: 21145656]
8. Boyette-Davis JA, Cata JP, Driver LC, Novy DM, Bruel BM, Mooring DL, Wendelschafer-Crabb G, Kennedy WR, Dougherty PM. Persistent chemoneuropathy in patients receiving the plant alkaloids paclitaxel and vincristine. *Cancer Chemother Pharmacol*. 2013; 71:619–626. [PubMed: 23228992]
9. Boyette-Davis JA, Cata JP, Zhang H, Driver LC, Wendelschafer-Crabb G, Kennedy WR, Dougherty PM. Follow-up psychophysical studies in bortezomib-related chemoneuropathy patients. *J Pain*. 2011; 12:1017–1024. [PubMed: 21703938]
10. Byrd-Leifer CA, Block EF, Takeda K, Akira S, Ding A. The role of MyD88 and TLR4 in the LPS-mimetic activity of taxol. *Eur J Immunol*. 2001; 31:2448–2457. [PubMed: 11500829]
11. Cao L, Sun D, Cruz T, Moscarello MA, Ludwin SK, Whitaker JN. Inhibition of experimental allergic encephalomyelitis in the Lewis rat by paclitaxel. *J Neuroimmunol*. 2000 Aug 1;108:103–111. [PubMed: 10900343]
12. Cao L, Tanga FY, DeLeo JA. The contributing role of CD14 in Toll-like receptor 4 dependent neuropathic pain. *Neuroscience*. 2009; 158:896–903. [PubMed: 18976692]
13. Cassidy P, Moos P, Kelly R, Fitzpatrick FA. Cyclooxygenase-2 induction by paclitaxel, docetaxel and taxane analogs in human monocytes and murine macrophages: structure-activity relationships and their implications. *Clin Cancer Res*. 2002; 8:846–855. [PubMed: 11895918]

14. Cata JP, Weng HR, Chen JH, Dougherty PM. Altered discharges of spinal wide dynamic range neurons and down-regulation of glutamate transporter expression in rats with paclitaxel-induced hyperalgesia. *Neuroscience*. 2006; 138:329–338. [PubMed: 16361064]
15. Cata JP, Weng HR, Dougherty PM. The effects of thalidomide and minocycline on taxol-induced hyperalgesia in rats. *Brain Res*. 2008 Sep 10;1229:100–110. [PubMed: 18652810]
16. Cata JP, Weng H-R, Burton AW, Villareal H, Giralt S, Dougherty PM. Quantitative sensory findings in patients with bortezomib-induced pain. *J Pain*. 2007; 8:296–306. [PubMed: 17175202]
17. Cavaletti G, Marmiroli P. Chemotherapy-induced peripheral neurotoxicity. *Nat Rev Neurol*. 2010; 6:657–666. [PubMed: 21060341]
18. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Meth*. 1994; 53:55–63.
19. Christianson CA, Dumalo DS, Stokes JA, Dennis EA, Svensson CI, Corr M, Yaksh TL. Spinal TLR4 mediates the transition to a persistent mechanical hypersensitivity after the resolution of inflammation in serum-transferred arthritis. *Pain*. 2011; 152:2881–2891. [PubMed: 22019135]
20. de Carvalho Barbosa M, Kosturakis AK, Eng C, Wendelschafer-Crabb G, Kennedy WR, Simone DA, Wang XS, Cleeland CS, Dougherty PM. Quantitative sensory analysis of peripheral neuropathy produced by colorectal cancer and its exacerbation by cumulative dose of oxaliplatin chemotherapy. *Cancer Res*. 2014; 74:5955–5962. [PubMed: 25183707]
21. De la Calle JL, Paino CL. A procedure for direct lumbar puncture in rats. *Brain Res Bull*. 2002 Nov 30;59:245–250. [PubMed: 12431755]
22. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res*. 2009; 29:313–326. [PubMed: 19441883]
23. Dina OA, Chen X, Reichling D, Levine JD. Role of protein kinase C[ϵ] and protein kinase A in a model of paclitaxel-induced painful peripheral neuropathy in the rat. *Neuroscience*. 2001 Dec 14;108:507–515. [PubMed: 11738263]
24. Ding AH, Porteu F, Sanchez E, Nathan CF. Shared actions of endotoxin and taxol on TNF receptors and TNF release. *Science*. 1990 Apr 20;248:370–372. [PubMed: 1970196]
25. Dougherty PM, Cata JP, Burton AW, Vu K, Weng HR. Dysfunction in multiple primary afferent fiber subtypes revealed by quantitative sensory testing in patients with chronic vincristine-induced pain. *J Pain Symptom Manage*. 2007; 33:166–179. [PubMed: 17280922]
26. Dougherty PM, Cata JP, Cordella JV, Burton A, Weng H-R. Taxol-induced sensory disturbance is characterized by preferential impairment of myelinated fiber function in cancer patients. *Pain*. 2004; 109:132–142. [PubMed: 15082135]
27. Dray A. Inflammatory mediators of pain. *Br J Anaesth*. 1995; 75:125–131. [PubMed: 7577246]
28. Fitzpatrick FA, Wheeler R. The immunopharmacology of paclitaxel (Taxol®) docetaxel (Taxotere®), and related agents. *Int Immunopharmacol*. 2003; 3:1699–1714. [PubMed: 14636822]
29. Geller MA, Bui-Nguyen TM, Rodgers LM, Ramakrishnan S. Chemotherapy induces macrophage chemoattractant protein-1 production in ovarian cancer. *Int J Gynecol Cancer*. 2010; 20:918–925. [PubMed: 20683396]
30. Grisold W, Cavaletti G, Windebank AJ. Peripheral neuropathies from chemotherapeutics and targeted agents: diagnosis, treatment, and prevention. *Neuro-Oncol*. 2012; 14:iv45–iv54. [PubMed: 23095830]
31. Hashiguchi T, Kobayashi H, Tosaka T, Libet B. Two muscarinic depolarizing mechanisms in mammalian sympathetic neurons. *Brain Res*. 1982; 242:378–382. [PubMed: 6288171]
32. Holness CL, Simmons DL. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood*. 1993 Mar 15;81:1607–1613. [PubMed: 7680921]
33. Huang ZZ, Li D, Liu CC, Cui Y, Zhu HQ, Zhang WW, Li YY, Xin WJ. CX3CL1-mediated macrophage activation contributed to paclitaxel-induced DRG neuronal apoptosis and painful peripheral neuropathy. *Brain Behav Immun*. 2014; 40:155–165. [PubMed: 24681252]
34. Huang ZZ, Li D, Liu CC, Cui Y, Zhu HQ, Zhang WW, Li YY, Xin WJ. CX3CL1-mediated macrophage activation contributed to paclitaxel-induced DRG neuronal apoptosis and painful peripheral neuropathy. *Brain Behav Immun*. 2014; 40:155–165. [PubMed: 24681252]
35. Hutchison MR, Zhang Y, Brown K, Coats BD, Shridhar M, Sholar PW, Patel SJ, Crysedale NY, Harrison JA, Maier SF, Rice KC, Watkins LR. Non-stereoselective reversal of neuropathic pain by

- naloxone and naltrexone: Involvement of toll-like receptor 4 (TLR4). *Eur J Neurosci.* 2008; 28:20–29. [PubMed: 18662331]
36. Jaggi AS, Singh N. Mechanisms in cancer-chemotherapeutic drugs-induced peripheral neuropathy. *Toxicology.* 2012; 291:1–9. [PubMed: 22079234]
 37. Javeed A, Ashraf M, Riaz A, Ghafoor A, Afzal S, Mukhtar MM. Paclitaxel and the immune system. *Eur J Pharmaceut Sci.* 2009; 38:283–290.
 38. Kalliomaki M, Kieseritzky JV, Schmidt R, Hagglof B, Karlsten R, Sjogren N, Albrecht P, Gee L, Rice F, Wiig M, Schmelz M, Gordh T. Structural and functional differences between neuropathy with and without pain? *Exp Neurol.* 2011; 231:199–206. [PubMed: 21683699]
 39. Kawasaki K, Gomi K, Nishijima M. Cutting edge:Gln²² of mouse MD-2 is essential for species-specific lipopolysaccharide mimetic action of taxol. *J Immunol.* 2001; 166:11–14. [PubMed: 11123270]
 40. Kosturakis AK, He Z, Li Y, Boyette-Davis JA, Shah N, Thomas SK, Zhang H, Vichaya EG, Wang XS, Wendelschafer-Crabb G, Kennedy WR, Simone DA, Cleeland CS, Dougherty PM. Subclinical peripheral neuropathy in patients with multiple myeloma before chemotherapy is correlated with decreased fingertip innervation density. *J Clin Oncol.* 2014 Oct 1.32:3156–3162. [PubMed: 25154818]
 41. Li Y, Adamek P, Zhang H, Tatsui CE, Rhines LD, Mrozkova P, Li Q, Kosturakis AK, Cassidy RM, Harrison DS, Cata JP, Sapire K, Zhang H, Kennamer-Chapman RM, Jawad AB, Ghetti A, Yan J, Palecek J, Dougherty PM. The Cancer Chemotherapeutic Paclitaxel Increases Human and Rodent Sensory Neuron Responses to TRPV1 by Activation of TLR4. *J Neurosci.* 2015 Sep 30.35:13487–13500. [PubMed: 26424893]
 42. Li Y, Zhang H, Kosturakis AK, Cassidy RM, Zhang H, Kennamer-Chapman RM, Jawad AB, Colomand CM, Harrison DS, Dougherty PM. MAPK signaling downstream to TLR4 contributes to paclitaxel-induced peripheral neuropathy. *Brain Behav Immun.* 2015 Jun 9.
 43. Li Y, Zhang H, Zhang H, Kosturakis AK, Jawad AB, Dougherty PM. Toll-like receptor 4 signaling contributes to paclitaxel-induced peripheral neuropathy. *J Pain.* 2014; 15:712–725. [PubMed: 24755282]
 44. Liu CC, Lu N, Cui Y, Yang T, Zhao ZQ, Xin WJ, Liu XG. Prevention of paclitaxel-induced allodynia by minocycline: Effect on loss of peripheral nerve fibers and infiltration of macrophages in rats. *Mol Pain.* 2010; 6:76. [PubMed: 21050491]
 45. Mantey CL, Qureshi N, Stutz PL, Vogel SN. Lipopolysaccharide antagonists block taxol-induced signaling in murine macrophages. *J Exp Med.* 1993; 178:695–702. [PubMed: 8101863]
 46. Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. *Nat Rev Neurosci.* 2005; 6:521–532. [PubMed: 15995723]
 47. Pare M, Albrecht PJ, Noto CJ, Bodkin NL, Pittenger GL, Schreyer DJ, Tigno XT, Hansen BC, Rice FL. Differential hypertrophy and atrophy among all types of cutaneous innervation in the glabrous skin of the monkey hand during aging and naturally occurring type 2 diabetes. *J Comp Neurol.* 2007 Apr 1.501:543–567. [PubMed: 17278131]
 48. Peters CM, Jimenez-Andrade JM, Jonas BM, Sevcik MA, Koewler NJ, Ghilardi JR, Wong GY, Mantyh PW. Intravenous paclitaxel administration in the rat induces a peripheral sensory neuropathy characterized by macrophage infiltration and injury to sensory neurons and their supporting cells. *Exp Neurol.* 2007; 203:42–54. [PubMed: 17005179]
 49. Polomano RC, Mannes AJ, Clark US, Bennett GJ. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. *Pain.* 2001; 94:293–304. [PubMed: 11731066]
 50. Resman N, Gradisar H, Vasi J, Keber MM, Pristovsek P, Jerala R. Taxanes inhibit human TLR4 signaling by binding to MD-2. *FEBS Lett.* 2008 Nov 26.582:3929–3934. [PubMed: 18977229]
 51. Ribeiro RA, Vale ML, Thomazzi SM, Paschoalato AB, Poole S, Ferreira SH, Cunha FQ. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *Eur J Pharmacol.* 2000 Jan 3.387:111–118. [PubMed: 10633169]
 52. Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci.* 2007; 10:1361–1368. [PubMed: 17965656]

53. Szajnik M, Szczepanski MJ, Czystowska M, Elishaev E, Mandapathil M, Nowak-Markwitz E, Spaczynski M, Whiteside TL. TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. *Oncogene*. 2009; 28:4353–4363. [PubMed: 19826413]
54. Tanga FY, Natile-McMenemy N, DeLeo JA. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A*. 2005 Apr 19; 102:5856–5861. [PubMed: 15809417]
55. Thomazzi SM, Ribeiro RA, Campos DI, Cunha FQ, Ferreira SH. Tumor necrosis factor, interleukin-1 and interleukin-8 mediate the nociceptive activity of the supernatant of LPS-stimulated macrophages. *Mediators Inflamm*. 1997; 6:195–200. [PubMed: 18472820]
56. Toews AD, Barrett C, Morell P. Monocyte chemoattractant protein 1 is responsible for macrophage recruitment following injury to sciatic nerve. *J Neurosci Res*. 1998 Jul 15; 53:260–267. [PubMed: 9671983]
57. Tsavaris N, Kosmas C, Vadiaka M, Kanelopoulos P, Boulamatsis D. Immune changes in patients with advanced breast cancer undergoing chemotherapy with taxanes. *Br J Cancer*. 2002 Jul 1; 87:21–27. [PubMed: 12085250]
58. Vallejo R, Tilley DM, Vogel L, Benyamin R. The role of glia and the immune system in the development and maintenance of neuropathic pain. *Pain Pract*. 2010; 10:167–184. [PubMed: 20384965]
59. Volk-Draper L, Hall K, Griggs C, Rajput S, Kohio P, DeNardo D, Ran S. Paclitaxel therapy promotes breast cancer metastasis in a TLR4-dependent manner. *Cancer Res*. 2014; 74:5421–5434. [PubMed: 25274031]
60. Wang J, Kobayashi M, Han M, Choi S, Takano M, Hashino S, Tanaka J, Kondoh T, Kawamura KI, Hosokawa M. MyD88 is involved in the signalling pathway for Taxol-induced apoptosis and TNF- α expression in human myelomonocytic cells. *British Journal of Haematology*. 2002 Aug 1; 118:638–645. [PubMed: 12139759]
61. White CM, Martin BK, Lee L-F, Haskill JS, Ting JP-Y. Effects of paclitaxel on cytokine synthesis by unprimed human monocytes, T lymphocytes, and breast cancer cells. *Cancer Immunol Immunother*. 1998; 46:104–112. [PubMed: 9558006]
62. Wolf S, Barton D, Kottschade L, Grothey A, Loprinzi CL. Chemotherapy-induced peripheral neuropathy: prevention and treatment strategies. *Eur J Cancer*. 2008; 44:1507–1515. [PubMed: 18571399]
63. Xu JJ, Walla BC, Diaz MF, Fuller GN, Gutstein HB. Intermittent lumbar puncture in rats: a novel method for the experimental study of opioid tolerance. *Anesth Analg*. 2006; 103:714–720. [PubMed: 16931686]
64. Zhang H, Boyette-Davis JA, Kosturakis AK, Li Y, Yoon SY, Walters ET, Dougherty PM. Induction of monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR2 in primary sensory neurons contributes to paclitaxel-induced peripheral neuropathy. *J Pain*. 2013; 14:1031–1044. [PubMed: 23726937]
65. Zhang H, Yoon S-Y, Zhang H, Dougherty PM. Evidence that spinal astrocytes but not microglia contribute to the pathogenesis of paclitaxel-induced painful neuropathy. *J Pain*. 2012; 13:293–303. [PubMed: 22285612]
66. Zimmer SM, Liu J, Clayton JL, Stephens DS, Snyder JP. Paclitaxel binding to human and murine MD-2. *J Biol Chem*. 2008; 283:27916–27926. [PubMed: 18650420]

Perspective

This paper shows that activation of innate immunity by paclitaxel results in a sequence of signaling events that results in the infiltration of the dorsal root ganglia by activated macrophages. Macrophages appear to drive the development of behavioral hypersensitivity and the loss of distal epidermal nerve fibers and hence play an important role in the mechanism of paclitaxel related neuropathy.

Highlights

- Innate immunity is engaged by paclitaxel treatment to generate peripheral neuropathy.
- Blockade of TLR signaling attenuates the development of all signs of CIPN.
- Blockade of MCP-1 signaling prevents infiltration of macrophages, behavioral signs and loss of skin IENFs.
- Elimination of macrophages attenuates the behavioral and skin changes associated with CIPN.

Figure 1.

The time course of mechanical allodynia and macrophage infiltration in DRG after systemic administration of paclitaxel (Pac). **A:** mechanical withdrawal threshold (in grams) for vehicle (n=6) (open circles) and paclitaxel treated rats (n=6) (filled circles). **B:** Bar graph summarize the group data and indicate the number of macrophage infiltration into DRG at day 3, day 7, day 14 and day 21 following paclitaxel treatment (black bars) compared to vehicle treatment (open bars). Representative images of CD68+ macrophage immunostaining in L4–5 DRG from a vehicle treated animal at day 14 (**C**) compared with those taken from paclitaxel-treated animals at days 3, 7, 14, and 21 (**D**, **E**, **F**, and **G**, respectively). * = $p < 0.05$, *** = $p < 0.001$.

Figure 2.

The macrophage toxin clodronate (Clo) prevented the development of mechanical hypersensitivity induced by paclitaxel (Pac), depleted macrophages in spleen and DRG, and prevented an increase in DRG TNF α . **A:** Clodronate treated rats developed significantly less mechanical hypersensitivity following paclitaxel treatment (filled circles) compared to paclitaxel rats that were treated with liposome vehicle (V) alone (open circles). **B:** Normal CD68+ immunolabeling of macrophages in spleen after 14 days of paclitaxel treatment. **C:** CD68+ immunolabeling in spleen 14 days after paclitaxel in rats treated with i.v. clodronate.

D: The bars summarize the grouped data showing that clodronate treatment (black bars) significantly reduced the infiltration of macrophages into the DRG induced by paclitaxel treatment compared to rats receiving paclitaxel and liposome vehicle (open bars). **E:** CD68+ immunolabeling of macrophages in DRG after 14 days paclitaxel-liposome treatment and **F:** following paclitaxel and clodronate. **G:** Clodronate treatment (right bar) significantly reduced the expression of TNF α in DRG that is significantly increased with paclitaxel (center bar) compared to the paclitaxel vehicle (left hand bar). **H:** Immunostaining of TNF α in DRG after 14 days of paclitaxel plus inert liposomes and **I:** following i.v. clodronate. *= p<0.05, **= p<0.01, ***= p<0.001.

Figure 3.

Intrathecal treatment with anti-MCP-1 antibodies (Anti) prevented behavioral hypersensitivity and macrophage infiltration after 14 days of paclitaxel (Pac) treatment. **A:** Mechanical hypersensitivity did not develop in rats treated anti-MCP-1 antibodies (black bar) during paclitaxel treatment whereas rats treated with rabbit non-specific IgG (NS-IgG) and paclitaxel did (open bar). Rats treated with the paclitaxel vehicle (V) and NS-IgG (light gray bar) or vehicle and anti-MCP antibodies (dark gray bar) showed no change in mechanical withdrawal threshold. **B:** Anti-MCP-1 treatment (black bar) significantly inhibits the paclitaxel-induced increase in CD68+ immunolabeling for macrophages in DRG after 14 days of paclitaxel treatment (open bar), while the V-IgG (light gray bar) and V-Anti treated rats (dark gray bar) showed no macrophage infiltration. Representative images of CD68+ macrophage immunolabeling in L4-5 DRG from paclitaxel + control IgG treatment is shown in **C**, while that for the paclitaxel + anti-MCP-1 treatment group is shown in **D**, the vehicle and control IgG treatment is in **E**, and the vehicle + anti-MCP-1 treatment is in **F**. ***= $p < 0.001$.

Figure 4.

The effect of intrathecal LPS-RS on DRG macrophage infiltration, the expression of MCP-1, and behavioral hypersensitivity. Representative images of CD68+ immunolabeling in the DRG following paclitaxel (Pac) + PBS treatment is shown in **A** while that for paclitaxel + LPS-RS treatment is shown in **B**. The representative image in **C** shows that paclitaxel + PBS treatment increases the expression of MCP-1 while in **D** this expression is greatly reduced following paclitaxel + LPS-RS treatment. The bar graphs in **E** show that rats treated with paclitaxel (P) + PBS (black bars) have a significantly lower mechanical withdrawal

threshold than the rats that received paclitaxel + LPS-RS (L) (open bars). The groups treated with paclitaxel vehicle (V) + LPS-RS (light gray bar) or vehicle + PBS (dark gray bar) showed no change in mechanical withdrawal from baseline. The bar graphs in **F** show the macrophage counts in DRG for the paclitaxel + PBS treated rats (open bar) and for the paclitaxel-LPS-RS treated rats (black bar). The bar graphs in **G** show the percentage of MCP-1+ cells in the paclitaxel + PBS treated rats (open bars) and the paclitaxel + LPS-RS treated rats (black bars). *= p<0.05, **= p<0.01, ***= p<0.001.

Figure 5.

Hindpaw skin tissue from vehicle + vehicle (Control), paclitaxel + vehicle (Paclitaxel), paclitaxel + clodronate, and paclitaxel + LPS-RS, animals were sectioned and immunolabeled for PGP (green), GAP-43 (red) and DAPI (blue). Hindpaw skin tissue from control (A, A', A''), paclitaxel-treated with vehicle (B, B', B''), or with clodronate (C, C', C''), or LPS-RS (D, D', D'') demonstrate that GAP43 has a 100% coincidence labeling with PGP fibers among the IENF (white arrows depicted in half size separate channel splits) across all conditions. Mag bar in (A) = 25μM. Summarized intraepidermal nerve fiber

(IENF) counts in **E** confirm that paclitaxel-treatment significantly reduces IENF, and importantly demonstrate that clodronate or LPS-RS treatment prevents IENF loss. * $p < 0.02$

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript