ANESTHESIOLOGY

Local Sympathectomy Promotes Anti-inflammatory Responses and Relief of Paclitaxel-induced Mechanical and Cold Allodynia in Mice

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Chemotherapy-induced neuropathic pain is a common and difficult-to-treat problem
- Inflammation may support chemotherapy-induced pain by interacting with sensory neurons

What This Article Tells Us That Is New

- Local surgical sympathectomy relieved nociceptive and mechanical sensitization in a mouse model of paclitaxel-induced pain
- Transforming growth factor-β was enhanced in mice after sympathectomy and was capable of reducing paclitaxel-induced mechanical sensitization

ABSTRACT

Background: Patients undergoing cancer treatment often experience chemotherapy-induced neuropathic pain at their extremities, for which there is no U.S. Food and Drug Administration–approved drug. The authors hypothesized that local sympathetic blockade, which is used in the clinic to treat various pain conditions, can also be effective to treat chemotherapy-induced neuropathic pain.

Methods: A local sympathectomy (*i.e.*, cutting the ipsilateral gray rami entering the spinal nerves near the L3 and L4 dorsal root ganglia) was performed in mice receiving intraperitoneal injections every other day of the chemotherapeutic drug paclitaxel. Sympathectomy effects were then assessed in chemotherapy-induced pain-like behaviors (*i.e.*, mechanical and cold allodynia) and neuroimmune and electrophysiologic responses.

Results: Local microsympathectomy produced a fast recovery from mechanical allodynia (mean ± SD: sympathectomy *vs.* sham at day 5, 1.07 ± 0.34 g *vs.* 0.51 ± 0.17g, n = 5, $P = 0.030$ in male mice, and 1.08 ± 0.28 g *vs.* 0.62 ± 0.16 g, n = 5, $P = 0.036$ in female mice) and prevented the development of cold allodynia in both male and female mice after paclitaxel. Mechanistically, microsympathectomy induced transcriptional increases in dorsal root ganglia of macrophage markers and anti-inflammatory cytokines, such as the transforming growth factor-β. Accordingly, depletion of monocytes/macrophages and blockade of transforming growth factor-β signaling reversed the relief of mechanical allodynia by microsympathectomy. In particular, exogenous transforming growth factor-β was sufficient to relieve mechanical allodynia after paclitaxel (transforming growth factor-β 100 ng/site *vs.* vehicle at 3 h, 1.21 ± 0.34g *vs.* 0.53 ± 0.14 g, n = 5, $P = 0.001$ in male mice), and transforming growth factor-β signaling regulated neuronal activity in dorsal root ganglia.

Conclusions: Local sympathetic nerves control the progression of immune responses in dorsal root ganglia and pain-like behaviors in mice after paclitaxel, raising the possibility that clinical strategies already in use for local sympathetic blockade may also offer an effective treatment for patients experiencing chemotherapy-induced neuropathic pain.

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Chemotherapy-induced neuropathic pain is a disabling condition affecting up to 80% of patients during treatment with antineoplastic drugs, including the frontline chemotherapeutic agent paclitaxel.[1,2] There are no U.S. Food and Drug Administration–approved drugs to treat this type of neuropathic pain, and many drugs that are used for the treatment of other neuropathic pain states have shown poor or no analgesic effect on chemotherapy-induced neuropathic pain.[1] Paclitaxel is a common drug for various solid cancers. However, it is often associated with neuropathic pain, which is generally localized in the distal extremities of the body and can persist for months or years after the end of the treatment.[2]

Although the exact mechanisms underlying paclitaxel-induced neuropathic pain remain incompletely known, there are preclinical lines of evidence indicating that immune cells in dorsal root ganglia play critical roles in the development and progression of chemotherapy-induced pain-like behaviors (*i.e.*, mechanical and cold allodynia), which are reminiscent of

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its clinical symptoms.[3] Interestingly, it has been reported that various chemotherapy agents induce the infiltration of monocytes into the dorsal root ganglia and sciatic nerve, where they differentiate into inflammatory macrophages and contribute to pain-like behaviors in several animal models of chemotherapy-induced neuropathic pain,[4–6] including in animals receiving paclitaxel. The association between monocyte/macrophage infiltration and pain-like behaviors in these models of chemotherapy-induced neuropathic pain has been reinforced pharmacologically. Depletion of macrophages using liposome-encapsulated clodronate alleviated paclitaxel-induced mechanical hypersensitivity, *via* the reduction of the paclitaxel-associated increase in macrophages and expression of the proinflammatory tumor necrosis factor alpha in the dorsal root ganglia.[4] Similarly, minocycline, an antibiotic that inhibits monocyte/macrophage infiltration and proinflammatory cytokines alongside other actions, has been shown to prevent paclitaxel-induced mechanical allodynia.[7] Unfortunately, a recent pilot study of minocycline in patients did not support its translation for the prevention of paclitaxel-induced neuropathy in clinic.[8]

Local sympathetic blockade is a well-accepted clinical procedure to treat various inflammatory pain conditions.[9,10] Sympathetic nerves generally promote inflammation in the initial phase of immune responses, whereas they suppress inflammation in the later phase. However, these responses are variable depending on the disease, the involved immune cell receptor, and the local environment in particular tissues.[11] Recent studies have shown that sympathetic nerves drive immune responses in organs such as the thymus, spleen, and lymph nodes, and also regulate macrophage responses and cytokine expression levels in dorsal root ganglia, maintaining inflammatory pain-like behaviors in rats.[11,12] Other animal studies have reported sympathetic nerve sprouting around the dorsal root ganglia neurons after nerve injury, suggesting that sympathetic neurons also contribute to neuropathic pain-like behaviors by facilitating nociceptive transmission.[13,14]

Although various pain conditions seem to be exacerbated or maintained by sympathetic activity, whether and how localized sympathectomy influences the development and progression of chemotherapy-induced neuropathic pain remains to be investigated. We hypothesized that sympathetic nervous system activity also regulates the progression of immune response in dorsal root ganglia and pain-like behaviors in mice treated with paclitaxel. Our preclinical study supports a novel therapeutic approach by which a clinically relevant local sympathectomy can provide relief of chemotherapy-induced neuropathic pain *via* anti-inflammatory responses and transforming growth factor-β signaling.

Materials and Methods

Animals and Procedures

Male and female CD1 mice (8 to 10 weeks of age) from Charles River were used as indicated for behavioral and biochemical experiments. Mice were housed four per cage at 22 ± 0.5°C under a controlled 14/10 h light/dark cycle with free access to food and water. To produce an animal model of chemotherapy-induced neuropathic pain, mice were treated with paclitaxel as previously described.[15] Briefly, 6 mg/ml stock paclitaxel (Sigma-Aldrich, USA) was diluted with Cremophor EL and 95% dehydrated ethanol (1:1 ratio, Sigma-Aldrich) and given at a dosage of 2 mg/kg diluted in saline intraperitoneally every other day for a total of two injections (days 0 and 2 with a final cumulative dose of 4 mg/kg). Control animals received an equivalent volume of the vehicle with proportional amounts of Cremophor EL and 95% dehydrated ethanol diluted in saline. Signs of peripheral neuropathy with a similar phenotype to that in patients have been validated by multiple investigators in this non–tumor-bearing animal model, including a time-dependent development of mechanical and cold allodynia. All experiments and procedures were performed in accordance with the guidelines recommended by the National Institutes of Health, the International Association for the Study of Pain, and the guidelines for Animal Research: Reporting of *In Vivo* Experiments of the National Center for the Replacement, Refinement, and Reduction of Animals in Research and were approved by the Institutional Animal Care and Use Committee at University of Cincinnati (Cincinnati, Ohio). Animals were randomly assigned to experimental groups. Although no statistical power calculation was conducted, the sample size of each experimental group was based on our previous similar studies.[12,16] All of the experimenters were blind to treatment condition.

Drugs and Drug Administration

We purchased the 6-hydroxydopamine (6-OHDA, catalog No. H4381) from MilliporeSigma (USA), transforming growth factor-β (catalog No. TP723438) from Origene (USA), the SB431542 (catalog No. S1067), a transforming growth factor-β inhibitor, from Selleckchem, the liposomal clodronate (catalog No. 283539) from Liposoma (The Netherlands), and C-C chemokine receptor 2 antagonist INCB3344 (catalog No. A3494) from APExBIO (USA). The transforming growth factor-β and transforming growth factor-β inhibitors were administrated intrathecally to deliver reagent into cerebral spinal fluid and dorsal root ganglia tissues, as we have previously described.[16,17] A valid spinal puncture was confirmed by a reflexive tail flick after the needle entry into subarachnoid space. The liposomal clodronate and INCB3344 were administrated intraperitoneally and intravenously, respectively.

Chemical Sympathectomy

Mice were injected intraperitoneally with 6-OHDA (80 mg/kg) in 0.01% ascorbic acid in phosphate-buffered saline at day −3. Control mice received injections of 0.01% ascorbic acid in phosphate-buffered saline.

Local Microsympathectomy

The proximal L3 and L4 spinal nerves and transverse processes on one side were exposed. The spinal nerves (ventral rami) were visualized and freed from surrounding tissue. The gray rami entering the L3 and L4 spinal nerves close to the dorsal root ganglia were identified on the ventral side of the spinal nerve at the position very close to the intervertebral foramen. At this site (Supplemental Digital Content, fig. 1A, http://links.lww.com/ALN/C292), where the gray ramus merges into the spinal nerve just across from the juncture where the dorsal ramus diverges from the ventral ramus, the gray rami and nearby connective tissue were gently dissected away from the nearby blood vessels and cut and disconnected from spinal nerve. Approximately 1 mm of gray ramus was further removed to make a gap and slow regeneration. Both L3 and L4 gray rami were cut in all microsympathectomy surgeries. Sham controls received similar exposure of the spinal nerves, but the gray rami were not cut. Two days after the microsympathectomy, the mice were treated with paclitaxel to mimic chemotherapy-induced neuropathic pain.

Macrophage/Monocyte Depletion

Liposome-encapsulated clodronate was used to deplete phagocytic macrophages.[18] Liposomal clodronate (15 ml/kg, ClodronateLiposomes.com) was intraperitoneally injected on days 5 and 7 after the first paclitaxel injection in mice with microsympathectomy. In response to peer review, INCB3344 was injected intravenously into the tail vein (100 μl of 0.18 mM solution = 18 nmol/injection) on days 5, 6, and 7 after the first paclitaxel injection in mice with microsympathectomy to partially deplete monocytes, as previously reported.[19]

Mechanical and Cold Allodynia

Mechanical allodynia was assessed as the hind paw withdrawal response to von Frey hair stimulation using the up-and-down method, as previously described.[20] Briefly, the mice were first acclimatized (1 h) in individual clear Plexiglas boxes on an elevated wire mesh platform to facilitate access to the plantar surface of the hind paws. Subsequently, a series of von Frey hairs (0.02, 0.07, 0.16, 0.4, 0.6, 1.0, and 1.4 g; Stoelting CO., USA) were applied perpendicular to the plantar surface of hind paw. Testing began with the application of the 0.6 g hair. A positive response was defined as a clear paw withdrawal or shaking. Whenever a positive response occurred, the next lower hair was applied, and whenever a negative response occurred, the next higher hair was applied. The testing consisted of six stimuli, and the pattern of response was converted to a 50% von Frey threshold, using the method described previously.[21] Cold allodynia was also assessed in response to peer review, and performed by the use of the cold plantar assay, as previously described.[22] Briefly, animals were first placed individually into clear acrylic containers separated by black opaque dividers that were set on top of 3/16" borosilicate glass (Stemmerich Inc, USA) and allowed to acclimate for 20 min before testing. A dry ice pellet was applied to the hind paw through the glass. The time until hind paw withdrawal was recorded at 5-minute intervals per mouse for a total of three trials, and the mean withdrawal latency was calculated. Withdrawal latencies were evaluated before and 1 to 14 days after the first injection of paclitaxel.

Real-time Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Mice were terminally anesthetized with isoflurane and lumbar dorsal root ganglia (L3 and L4) were rapidly removed 7 days after paclitaxel treatment. In some experiments, spleen tissue was also isolated. Total RNA was extracted using Direct-zol RNA MiniPrep kit (Zymo Research, USA), which amount and quality were assessed by SimpliNano UV-Vis Spectrophotometer (General Electric, USA) and then converted into cDNA using a High-capacity RNA-to-cDNA kit (Thermo Fisher Scientific, USA). Specific primers for cytokines and markers of monocytes/macrophages, adrenergic and purinergic receptors, as well as glyceraldehyde 3-phosphate dehydrogenase were obtained from PrimerBank.[23] Primer sequences are depicted in Supplemental Digital Content, table 1 (http://links.lww.com/ALN/C293). Real-time quantitative reverse transcriptase–polymerase chain reaction was performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). All samples were analyzed at least in duplicate and normalized by glyceraldehyde 3-phosphate dehydrogenase expression. The relative expression ratio per condition was calculated based on the method described by Pfaffl *et al.*[24]

Immunohistochemical Analysis

Deeply anesthetized mice were perfused through the left ventricle with phosphate-buffered saline solution, followed by 4% paraformaldehyde in phosphate-buffered saline and lumbar dorsal root ganglia (L3 and L4) were collected 7 or 14 days after paclitaxel treatment. All tissues were post-fixed in paraformaldehyde in phosphate-buffered saline overnight and subsequently transferred into 30% sucrose in phosphate-buffered saline for 24 h. For ionized calcium binding adaptor molecule 1 immunohistochemical quantification, dorsal root ganglia tissues were sliced into 12-μm sections and placed on slides, which were then blocked for 1 h at room temperature with 1% bovine serum albumin with 0.2% Triton X-100 in phosphate-buffered saline bovine serum albumin solution. Subsequently, sections were incubated with ionized calcium-binding adaptor molecule 1 primary antibody (goat, 1:1000, catalog No. NB100-1028, Novus) overnight at 4°C, followed by incubation

with the secondary antibody anti-goat Alexa Fluor 555 (1:1,000, Thermo Fisher Scientific) for 1 h at room temperature. DAPI (4',6-diamidino-2-phenylindole, Thermo Fisher Scientific) was used for counterstaining. For the quantification of sympathetic fibers, dorsal root ganglia were sliced into 40-μm sections, and sections were blocked for 1 h at room temperature in the bovine serum albumin solution, incubated with primary antibodies against tyrosine hydroxylase (Th, rabbit, 1:500, catalog No. P40101-0, Pel-Freez Biologicals, USA) for 48 h at 4°C, and followed by incubation with the secondary antibodies anti-rabbit Alexa Fluor® 488 (1:1,000, Thermo Fisher Scientific) for 1 h at room temperature. DAPI was used for counterstaining. For quantification, images from four to five sections of each dorsal root ganglia per group, selected at random, were captured under an Olympus BX63 fluorescent microscope using cellSens imaging acquisition software (Olympus, USA) by an investigator blinded to treatment conditions. Images of all dorsal root ganglia tissue were captured and intensity quantification was performed comparing samples from all experimental groups, prepared with the same staining solutions, then measured using identical display parameters.

Cytokine Array

The mouse cytokine array kits were purchased from R&D (catalog No. ARY006). Animals were terminally anesthetized with isoflurane, and lumbar dorsal root ganglia (L3 and L4) were rapidly removed 7 days after paclitaxel treatment and homogenized in a lysis buffer containing a cocktail of protease inhibitors and phosphatase inhibitors. Concentration of protein was measured using Qubit TM (Invitrogen). Each reaction was performed according to manufacturer's protocol.

Electrophysiology

Intracellular recording in current clamp mode was performed at 36–37°C using microelectrodes in sensory neurons near the dorsal surface of an acutely isolated whole dorsal root ganglia preparation, as previously described for rats.[25] This preparation allows neurons to be recorded without enzymatic dissociation, with the surrounding satellite glia cells and neighboring neurons intact.[26,27] The L4 dorsal root ganglia was isolated from mice 9 days after microsympathectomy and 7 days after paclitaxel treatment. The dorsal root ganglia was secured in the recording chamber and continuously perfused with artificial cerebro-spinal fluid (in mM: NaCl 130, KCl 3.5, NaH_2PO_4 1.25, $NaHCO_3$ 24, Dextrose 10, $MgCl_2$ 1.2, $CaCl_2$ 1.2, 16 mM HEPES, pH = 7.3, bubbled with 95% O_2/5% CO_2). The transforming growth factor-β receptor 1 inhibitor SB431542 (10 ng/ml) or vehicle was included throughout the recording period. Cells were classified as small/likely unmyelinated or large/likely myelinated, based on action potential duration less than 1.5 msec or greater than 1.5 msec duration, respectively, as confirmed by conduction velocity measurements in a subset of cells. Excitability parameters were analyzed as described previously.[28]

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software (USA), and all data were expressed as mean ± SD. No outliers were excluded. Behavioral data were analyzed using two-way repeated-measure ANOVA followed by Bonferroni *post hoc* test. Biochemical and electrophysiologic data were analyzed by two-tailed, unpaired Student *t* test or one-way ANOVA followed by Tukey *post hoc* test, or Mann–Whitney test for electrophysiologic data that were not normally distributed according to the D'Agostino & Pearson omnibus normality test. The criterion for statistical significance was $P < 0.05$.

Results

Systemic Chemical Sympathectomy Delays the Development and Resolution of Paclitaxel-induced Mechanical Allodynia

We hypothesized that chemotherapy-induced neuropathic pain is exacerbated or maintained by sympathetic nervous system activity. We first used a systemic approach to determine the role of the sympathetic nervous system by injecting the chemical 6-hydroxydopamine for a systemic sympathetic denervation or a vehicle control 3 days before paclitaxel treatment in mice (fig. 1A). Systemic sympathectomy had no effect on baseline paw mechanical withdrawal threshold. As previously reported[15,16] and in vehicle control animals, treatment with paclitaxel produced a robust and transient (~2 weeks) mechanical allodynia (fig. 1A), a common readout of chemotherapy-induced neuropathic pain in animals.[4,15] However, animals with systemic sympathectomy showed a delay in both the development and resolution of paclitaxel-induced mechanical allodynia (fig. 1B). This conflicting result is not due to the recovery of sympathetic nervous system activity, as indicated by the sustained loss of tyrosine hydroxylase up to 21 days (fig. 1C), a marker for sympathetic fibers. This result may be due to contrasting systemic *versus* local effects of a global sympathectomy.[11]

Local Microsympathectomy Reduces Paclitaxel-induced Mechanical and Cold Allodynia

Because chemotherapy-induced neuropathic pain generally develops in the distal extremities of the body, we investigated the effects of a local microsympathectomy, in which lumbar gray rami from sympathetic paravertebral ganglia were cut on one side (Supplemental Digital Content, fig. 1, http://links.lww.com/ALN/C292), performed 2 days before paclitaxel treatment (fig. 2A) and resulting in the loss of peripheral sympathetic fibers innervated by those

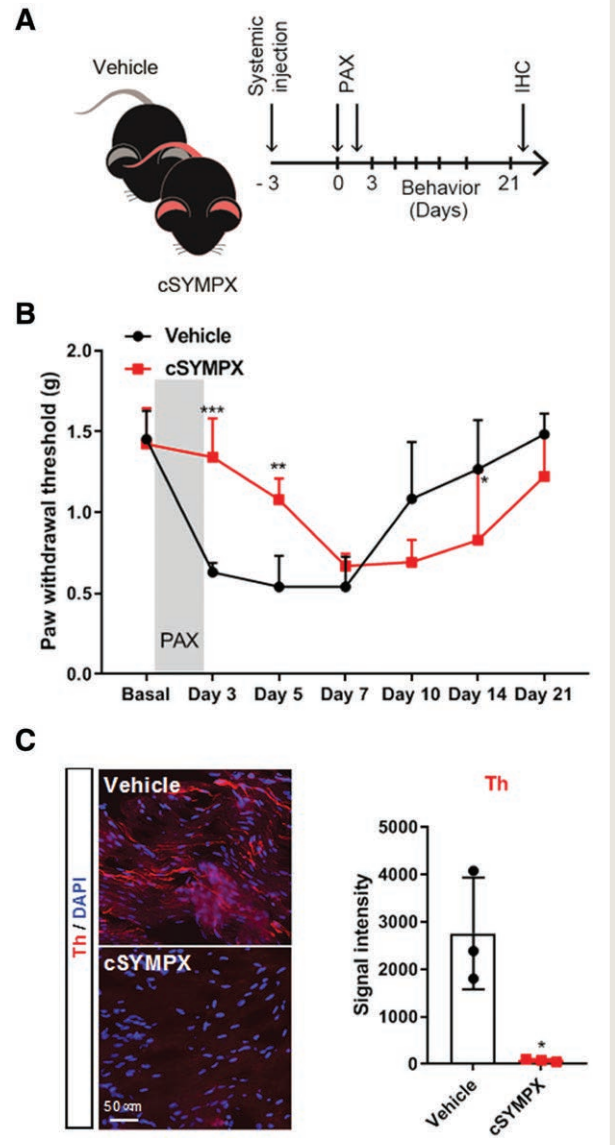

Fig. 1. Systemic chemical sympathectomy delays the development and resolution of paclitaxel-induced mechanical allodynia. (*A*) Schematic illustration of the experiment showing the timeline of systemic chemical sympathectomy (cSYMPX), injections of paclitaxel (PAX), and behavioral and histochemical (IHC) assays. (*B*) Time course of paclitaxel-induced mechanical allodynia in mice (n = 5 male mice per group, two-way ANOVA showed significant difference between cSYMPX and vehicle groups, Group × Time interaction: $F_{6,48} = 12.02$, $P < 0.001$; Bonferroni *post hoc* analysis revealed a significant difference between groups on days 3, 5, and 14; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). (*C*) Representative images and quantification of tyrosine hydroxylase (Th) protein in dorsal root ganglia tissues of vehicle- and cSYMPX-treated mice at day 21 of paclitaxel (n = 3 male mice per group, *t* test; $^{*}P = 0.017$ compared with vehicle).

ganglia for at least 14 days (fig. 2B). Before implementing the paclitaxel-induced animal model, microsympathectomy and sham surgery did not affect baseline mechanical and cold allodynia (fig. 2, C–F). Similar to vehicle control animals, sham control animals developed a marked decrease in the mechanical threshold lasting up to 2 weeks. In contrast, microsympathectomy produced a fast and sustained recovery of mechanical allodynia in the ipsilateral hind paw of male and female mice (mean ± SD: microsympathectomy *vs.* sham at day 5, 1.07 ± 0.34 g *vs.* 0.51 ± 0.17 g, n = 5, $P = 0.030$ in male mice, and 1.08 ± 0.28 g *vs.* 0.62 ± 0.16 g, n = 5, $P = 0.036$ in female mice) after paclitaxel treatment (fig. 2, C and D), whereas no effect was observed in the paw contralateral to the microsympathectomy of these same mice (Supplemental Digital Content, fig. 2A and 2B, http://links.lww.com/ALN/C294). Paclitaxel is well known to increase sensitivity to cold stimuli in patients.[29] Using the cold plantar assay to evaluate the sensitivity to noxious cold stimuli, we demonstrated that microsympathectomy prevented the development of cold allodynia in the ipsilateral hind paw of male and female mice after paclitaxel treatment (fig. 2, E and F). It is worth noting that we have observed similar effects in both males and females of previous microsympathectomy on mechanical behaviors in a nerve injury model and on both mechanical and cold behaviors in a back pain model that induces local inflammation of dorsal root ganglia.[12,13]

Monocytes/Macrophages Are Required for the Relief of Paclitaxel-induced Mechanical Allodynia by Local Microsympathectomy

We have reported that local microsympathectomy alleviated inflammatory pain in a back pain animal model by reducing inflammation and macrophage infiltration in dorsal root ganglia.[12] Infiltration and activation of macrophages has been reported to contribute to pain-like behaviors in animal models of chemotherapy-induced neuropathic pain and their systemic depletion shown to prevent the development of paclitaxel-induced mechanical allodynia.[4] In agreement with previous studies in paclitaxel and vincristine models of chemotherapy-induced neuropathic pain,[4,5] we did not observe transcriptional changes in macrophage markers (data not shown) and the canonical macrophage marker ionized calcium binding adaptor molecule 1 was unaffected in dorsal root ganglia of mice with microsympathectomy compared with mice with sham surgery at day 7 of paclitaxel treatment (fig. 3, A–C). However, transcripts for the macrophage activation marker CD68 (cluster of differentiation 68) and other macrophages markers such as EMR1 (EGF-like module-containing mucin-like hormone receptor-like 1, also known as F4/80) and ITGAM (integrin, alpha X), as well as the monocyte chemoattractant protein CCL2 (chemokine [C-C motif] ligand 2) and its receptor C-C chemokine receptor type 2 were significantly increased in mice with microsympathectomy after paclitaxel (fig. 3C), suggesting that the analgesic effect of microsympathectomy may emerge from the activation and infiltration of macrophages. To assess

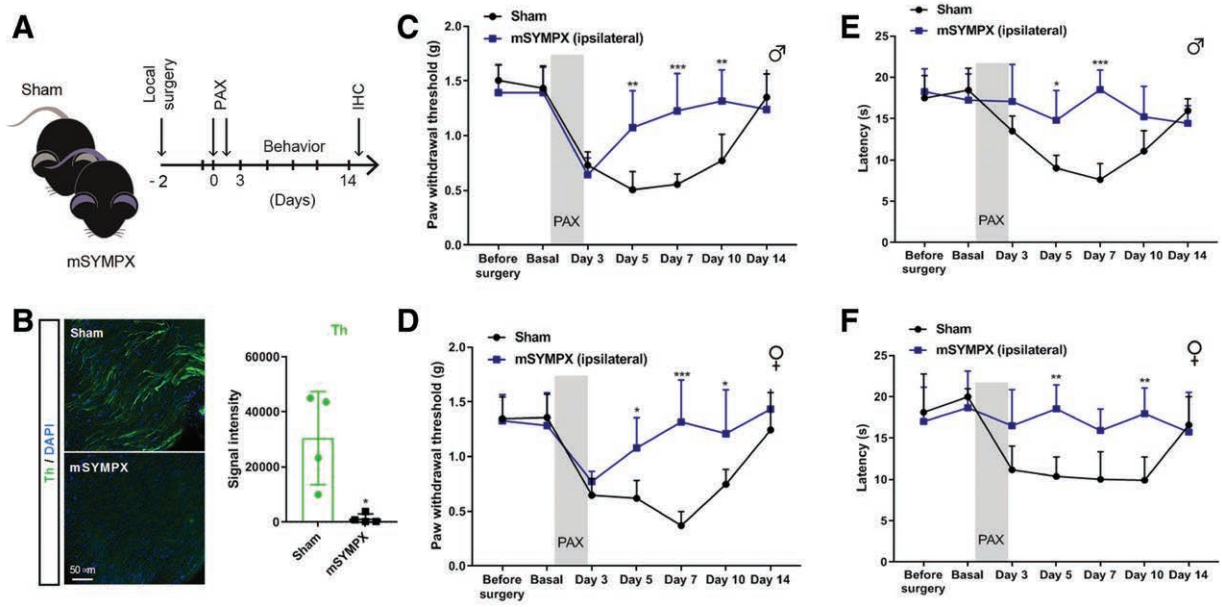

Fig. 2. Local surgical sympathectomy decreases paclitaxel-induced mechanical and cold allodynia. (*A*) Schematic illustration of the experiment showing the timeline of local surgical microsympathectomy (mSYMPX), injections of paclitaxel (PAX), and behavioral and histochemical (IHC) assays. (*B*) Representative images and quantification of tyrosine hydroxylase (Th) protein in dorsal root ganglia tissues of sham- and mSYMPX-treated mice at day 14 of paclitaxel (n = 4 male and female mice per group, *t* test, $^{*}P = 0.013$ compared with sham). (*C* and *D*) Time course of paclitaxel-induced mechanical allodynia tested in ipsilateral hind paw in (*C*) male mice (n = 5 male mice per group, two-way ANOVA showed significant difference between mSYMPX and sham groups, Group × Time interaction: $F_{6,48} = 5.19$, $P < 0.001$; Bonferroni *post hoc* analysis revealed a significant difference between groups on days 3, 5, and 10; $^{**}P < 0.01$, $^{***}P < 0.001$), and (*D*) female mice (n = 5 female mice per group, two-way ANOVA showed significant difference between mSYMPX and sham groups, Group × Time interaction: $F_{6,48} = 5.6$, $P < 0.001$; Bonferroni *post hoc* analysis revealed a significant difference between groups on days 5, 7, and 10. $^{*}P < 0.05$, $^{***}P < 0.001$). (*E* and *F*) Time course of paclitaxel-induced cold allodynia tested in ipsilateral hind paws in (*E*) male mice (n = 5 male mice per group, two-way ANOVA showed significant difference between mSYMPX and sham groups, Group × Time interaction: $F_{6,48} = 6.92$, $P < 0.001$; Bonferroni *post hoc* analysis revealed a significant difference between groups on days 5 and 7; $^{*}P < 0.05$, $^{***}P < 0.001$), and (*F*) female mice (n = 5 female mice per group, two-way ANOVA showed significant difference between mSYMPX and sham groups, Group × Time interaction: $F_{6,48} = 4.91$, $P = 0.001$; Bonferroni *post hoc* analysis revealed a significant difference between groups on days 5 and 10; $^{**}P < 0.01$).

the involvement of infiltrated macrophages in the analgesic effect of microsympathectomy, we used three daily intravenous injections of the C-C chemokine receptor 2 antagonist INCB3344 (fig. 4A), which does not affect CCL2 regulation but is known to reduce the number of circulating monocytes after nerve injury.[19] This treatment significantly negated the analgesic effect of microsympathectomy (fig. 4B), revealing an active inhibition of the paclitaxel-induced mechanical allodynia by C-C chemokine receptor 2 signaling and potentially by infiltrating monocytes/macrophages. Of note, CCL2/C-C chemokine receptor 2 signaling is recognized to participate in microglia activation in the spinal cord and pain hypersensitivity.[30] However, intrathecal injections of INCB3344 did not mitigate the analgesic effect of microsympathectomy (data not shown), suggesting that this effect is independent of local mechanisms. Concordantly, intraperitoneal injections of liposomal clodronate, which is known to mostly deplete circulating monocytes,[18] significantly negated the analgesic effect of microsympathectomy (fig. 4C). Of note, liposomal clodronate minimally affected the expression of ionized calcium binding adaptor molecule 1 in dorsal root ganglia (fig. 4D), but drastically diminished ionized calcium binding adaptor molecule 1 and other monocyte/macrophage markers in the spleen (fig. 4D and Supplemental Digital Content, fig. 3, http://links.lww.com/ALN/C295), a major body reservoir of circulating monocytes. Together, our transcriptional and pharmacologic results suggest a beneficial role in paclitaxel-induced neuropathic pain-like behaviors of circulating monocytes and potentially infiltrating macrophages after local microsympathectomy.

Local Microsympathectomy Promotes Anti-inflammatory Responses in Dorsal Root Ganglia and Relief of Paclitaxel-induced Mechanical Allodynia by Transforming Growth Factor-β1 Signaling

We have previously reported that local microsympathectomy limited the expression of proinflammatory cytokines

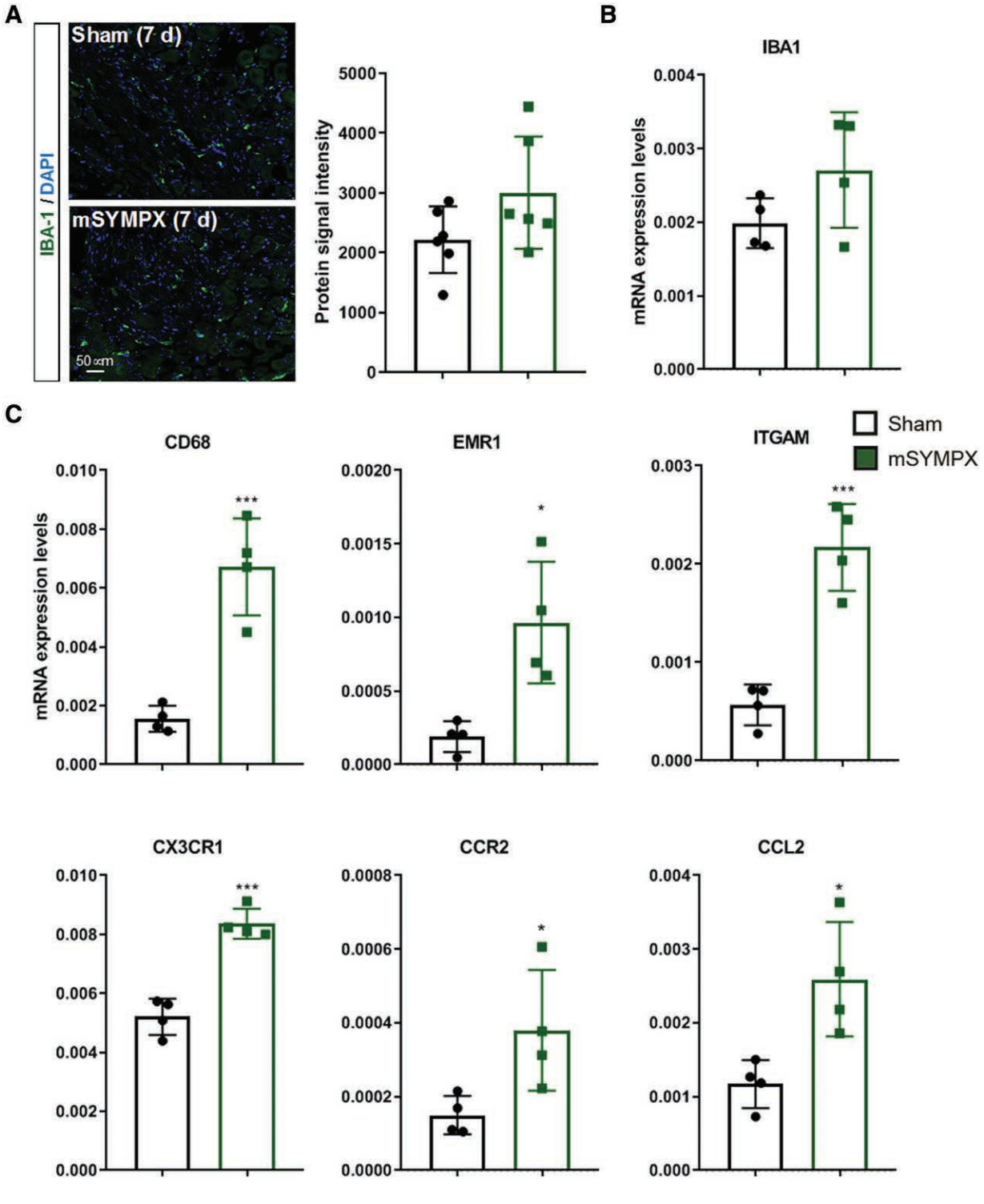

Fig. 3. Transcriptional increases of macrophage markers in DRGs of paclitaxel-treated mice after local sympathectomy. (*A*) Representative images and quantification of ionized calcium binding adaptor molecule 1 (IBA1) protein levels in dorsal root ganglia (DRG) tissues of male mice with sham- and local microsympathectomy (mSYMPX) at day 7 after paclitaxel (n = 6 male mice per group, *t* test, $P = 0.107$ compared with sham). (*B*) The same tissues and conditions were also probed for transcriptional changes and similarly to the protein levels, the IBA1 messenger RNA was unchanged (n = 4 male mice per group, *t* test, $P = 0.141$ compared with sham). (*C*) In contrast, the macrophage markers cluster of differentiation 68 (CD68), EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1), integrin alpha X (ITGAM), and C-X3-C motif chemokine receptor 1 (CX3CR1), as well as the monocyte chemotactic markers chemokine (C-C motif) ligand 2 (CCR2) and C-C chemokine receptor type 2 (CCL2), were significantly increased (n = 4 male mice per group, *t* test, $^{*}P < 0.05$, $^{***}P < 0.001$ compared with sham).

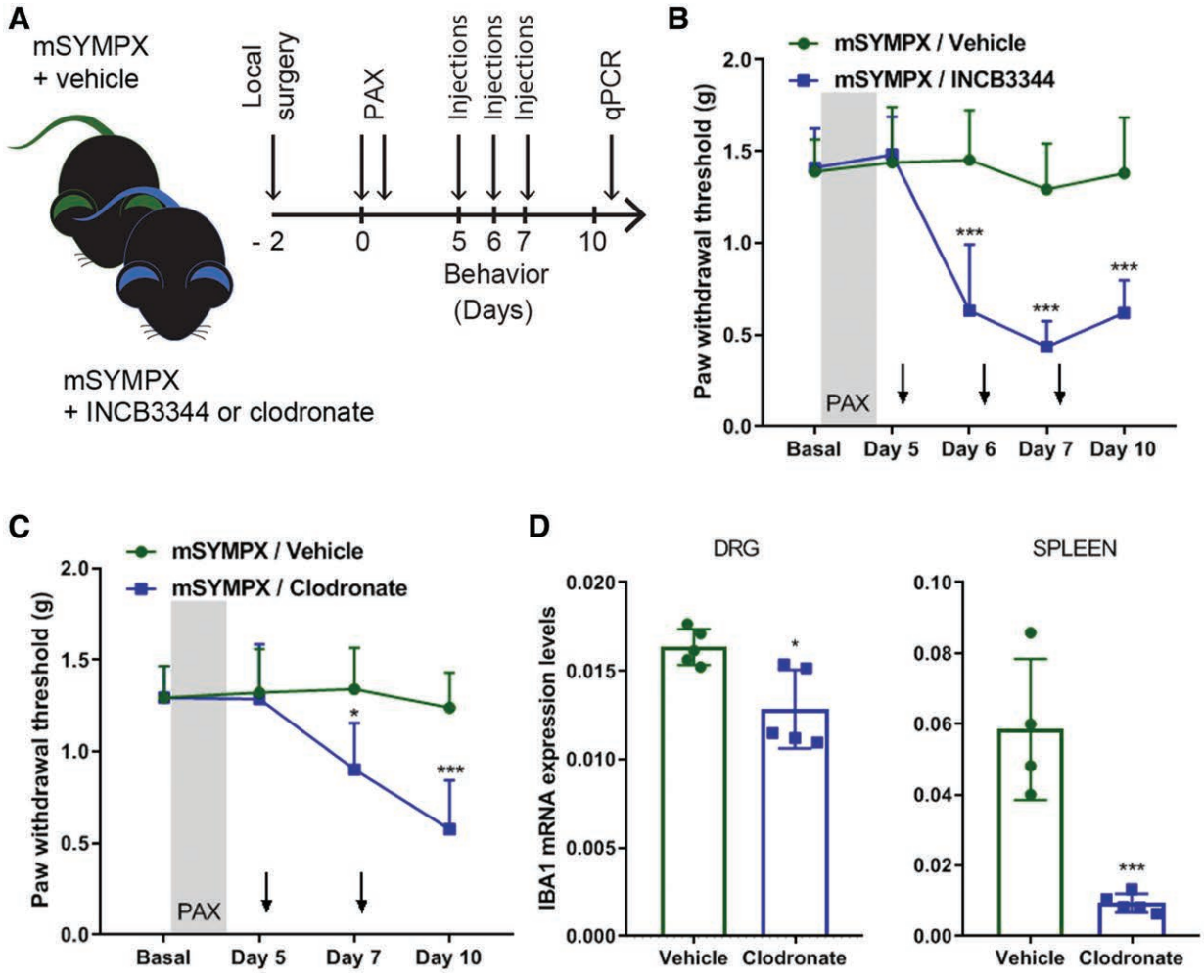

Fig. 4. Depletion of monocytes/macrophages by INCB3344 and liposomal clodronate unmasked the antiallodynic effect of local sympathectomy in paclitaxel-treated mice. (*A*) Schematic illustration of the experiment showing the timeline of local microsympathectomy (mSYMPX), injections of paclitaxel (PAX), injections of INCB3344 (intravenously on days 5, 6, and 7) or liposomal clodronate (intraperitoneally on days 5 and 7), and behavioral and transcriptional (qPCR = real-time quantitative RT-PCR) assays. (*B*) Time course of paclitaxel-induced mechanical allodynia tested in ipsilateral hind paws in male mice treated with a vehicle control and INCB3344 (n = 5 male mice per group, two-way ANOVA showed significant difference between mSYMPX–INCB3344 and vehicle groups, Group × Time interaction: $F_{4,32} = 10.14$, $P < 0.001$; Bonferroni *post hoc* analysis revealed a significant difference between groups on days 6, 7 and 10; ***$P < 0.001$). (*C*) Time course of paclitaxel-induced mechanical allodynia tested in ipsilateral hind paws in male mice treated with a liposomal vehicle control and clodronate (n = 5 male mice per group, two-way ANOVA showed significant difference between mSYMPX-clodronate and vehicle groups, Group × Time interaction: $F_{3,24} = 4.12$, $P = 0.017$; Bonferroni *post hoc* analysis revealed a significant difference between groups on days 7 and 10; *$P < 0.05$, ***$P < 0.001$). (*D*) Liposomal clodronate treatment commonly used to deplete monocytes/macrophages showed a minimal effect on ionized calcium binding adaptor molecule 1 (IBA1) transcriptional expression in dorsal root ganglia (DRGs) (Clodronate × Vehicle groups, $P = 0.012$), but a drastic transcriptional reduction of IBA1 in spleen tissues (Clodronate × Vehicle groups, $P = 0.001$) of mice 10 days after paclitaxel (n = 4–5 male mice per group, *t* test, *$P < 0.05$, ***$P < 0.001$ compared with vehicle).

favoring an anti-inflammatory response in locally inflamed dorsal root ganglia.[12] Transcriptional analyses of dorsal root ganglia from mice with microsympathectomy compared with mice with sham surgery, examined 7 days after subsequent paclitaxel treatment, showed a significant increase of the anti-inflammatory macrophage marker arginase 1, whereas no effect was observed on the proinflammatory macrophage marker nitric oxide synthase 2 (fig. 5A). Concordantly, minimal or no effect was observed on proinflammatory cytokines (fig. 5B). However, transcriptional analyses of anti-inflammatory cytokines revealed that microsympathectomy significantly increased the expression of the interleukin 10, transforming growth factor-β, and its receptor transforming growth factor-β receptor 1 (fig. 5C).

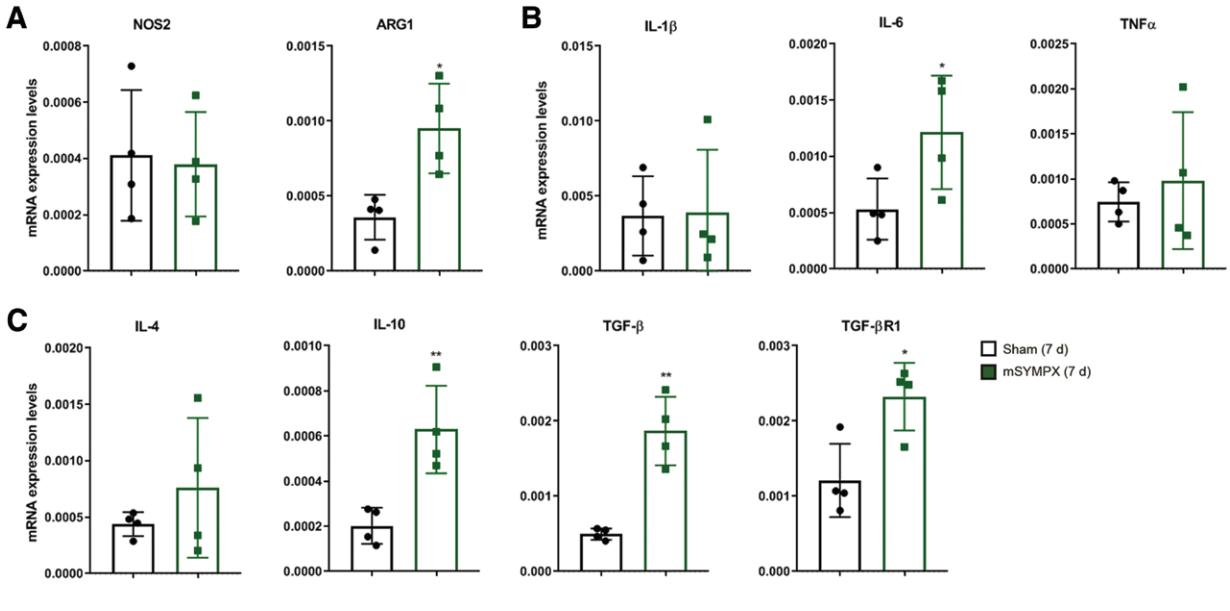

Fig. 5. Transcriptional increases of anti-inflammatory cytokines in dorsal root ganglia (DRGs) of paclitaxel-treated mice after local sympathectomy. Transcriptional expression levels in DRGs 7 days after paclitaxel treatment in male mice with sham surgery or local microsympathectomy (mSYMPX). Transcriptional analyses of (*A*) the macrophage proinflammatory marker nitric oxide synthase 2 (NOS2) and anti-inflammatory marker arginase 1 (ARG1); (*B*) proinflammatory cytokines interleukins (IL) IL-1β, IL-6, and tumor necrosis factor alpha (TNFα); as well as (*C*) anti-inflammatory cytokines IL-4, IL-10, and transforming growth factor-β (TGF-β), and its receptor TGF-βR1. For *A–C*, n = 4 male mice per group, *t* test, $*P < 0.05$, $**P < 0.01$ compared with sham.

Although protein analyses of 40 cytokines (Supplemental Digital Content, fig. 4A, http://links.lww.com/ALN/C296) confirmed the minimal changes in proinflammatory cytokines, it did not validate the transcriptional increase of interleukin 10 (Supplemental Digital Content, fig. 4, B and C, http://links.lww.com/ALN/C296). In contrast, we showed that delivery of exogenous recombinant transforming growth factor-β dose-dependently after 7 days of paclitaxel (fig. 6A) significantly reduced mechanical allodynia (transforming growth factor-β 100 ng/site *vs.* vehicle at 3 h, 1.21 ± 0.34 g *vs.* 0.53 ± 0.14 g, n = 5, $P = 0.001$ in male mice) (fig. 6B), and this reduction was abolished by the co-injection of transforming growth factor-β with the potent and selective transforming growth factor-β receptor 1 inhibitor SB431542 (fig. 6C). Consistently, SB431542 delivered 7 days after paclitaxel (fig. 6D) partially unmasked mechanical allodynia after microsympathectomy (fig. 6E). Microelectrode recordings using vehicle or SB431542, made in an isolated whole dorsal root ganglia preparation 7 d after microsympathectomy and paclitaxel, showed that SB431542 treatment resulted in a significant increase of the number of action potentials that could be evoked in small-sized (fig. 6, F and G) but not large-sized dorsal root ganglia neurons (fig. 6H). Remarkably, SB431542 induced no other changes (*e.g.*, resting potential and spontaneous activity) in the same preparations (Supplemental Digital Content, table 2, http://links.lww.com/ALN/C297). Together these data suggest an essential involvement of transforming growth factor-β1 signaling in neuronal hyperexcitability and the analgesic effect of local microsympathectomy.

Discussion

The prevalence of neuropathic pain is very high after chemotherapy. Currently, there is no U.S. Food and Drug Administration–approved analgesic for chemotherapy-induced neuropathic pain, often leaving clinicians to decrease the dose or duration of an otherwise life-saving therapy. Using the well-characterized paclitaxel-induced animal model of chemotherapy-induced neuropathic pain, we have demonstrated the analgesic effect of a local sympathectomy. This is particularly relevant because local sympathetic blocks (*e.g.*, *via* local anesthetic injections) are standard procedures in the clinic to treat some pain conditions, notably complex regional pain syndrome, but also phantom limb pain, herpes zoster and postherpetic neuralgia, ischemic pain, postmastectomy pain, and cancer pain.[9,10]

The mechanisms by which sympathectomy alleviates neuropathic pain are incompletely known. Because peripheral nerve injury induces sprouting of sympathetic fibers into dorsal root ganglia, many preclinical studies focus on abnormal interactions between these fibers and sensory neurons.[14,31–33] Indeed, it has been found that sympathectomy can reduce the hyperexcitability of sensory neurons

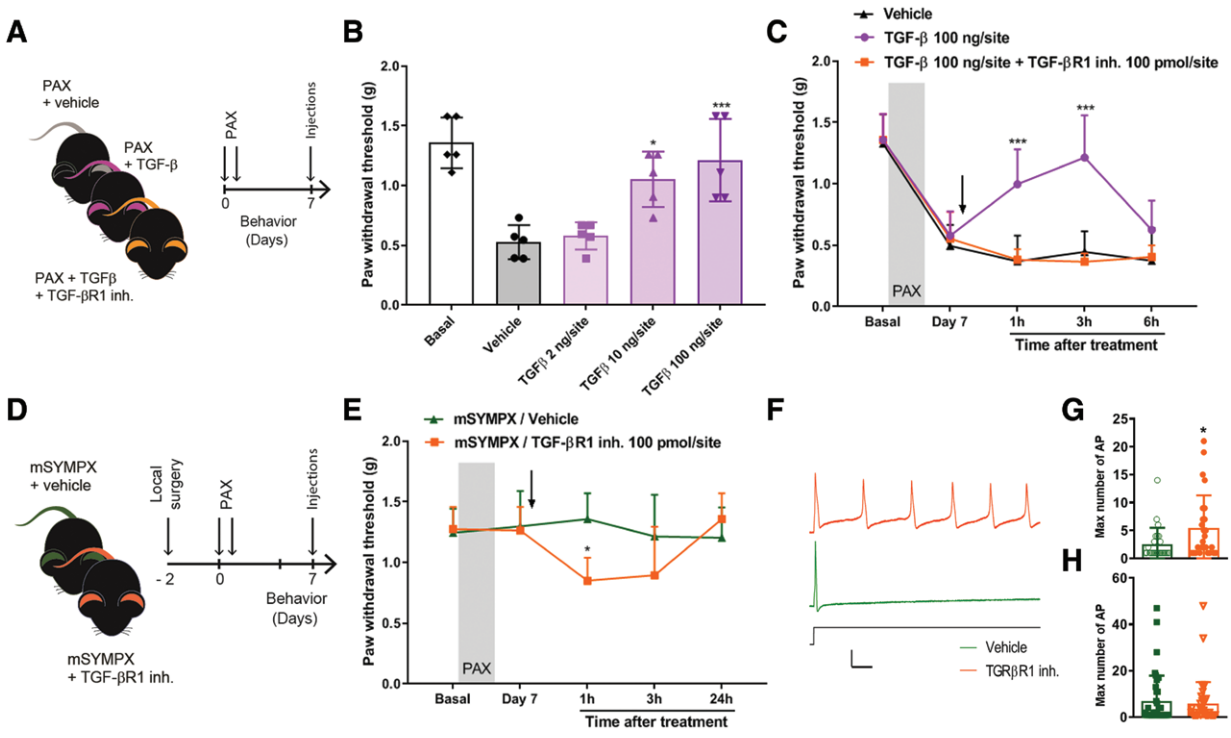

Fig. 6. Local sympathectomy accelerates the resolution of paclitaxel-induced mechanical allodynia through anti-inflammatory transforming growth factor-β (TGF-β) signaling. (*A*) Schematic illustration of the experiment showing the timeline of the injections of paclitaxel (PAX), intrathecal injections of recombinant TGF-β protein or TGF-β + TGF-β receptor 1 inhibitor (TGF-βR1 inh.), and the behavioral assay that was carried out at 7 days after paclitaxel. (*B*) Reversal of paclitaxel-induced mechanical allodynia tested in ipsilateral hind paws in male mice after 3 h of treatment with different doses of recombinant TGF-β (n = 5 mice per group, one-way ANOVA showed significant difference between TGF-β and vehicle groups, Group: $F_{4,20} = 14.02$, $P < 0.001$; Tukey *post hoc* analysis revealed a significant difference at concentrations of 10 ng and 100 ng; *$P < 0.05$, *$P < 0.001$). (*C*) Time course of paclitaxel-induced mechanical allodynia showed that the antiallodynic effect of TGF-β was abolished by the intrathecal co-injection of the TGF-βR1 inhibitor (n = 5 male mice per group, two-way ANOVA showed significant difference between TGF-β and vehicle groups, Group × Time interaction: $F_{8,48} = 5.63$, $P < 0.001$; Bonferroni *post hoc* analysis revealed a significant difference between groups at 1 and 3 h; *$P < 0.001$). (*D*) Schematic illustration of the experiment showing the timeline of local microsympathectomy (mSYMPX), injections of paclitaxel (PAX), injections of TGF-βR1 inh., and the behavioral assay that was carried out at 7 days after paclitaxel. (*E*) TGF-βR1 inhibitor also reverses the antiallodynic effect of mSYMPX on paclitaxel-induced mechanical allodynia in mice (n = 5 male mice per group, two-way ANOVA showed significant difference between mSYMPX-SB431542 and vehicle groups, Group × Time interaction: $F_{4,32} = 3.25$, $P = 0.024$; Bonferroni *post hoc* analysis revealed a significant difference between groups at 1 h; *$P < 0.05$). (*F*) Sample traces of action potential firing in response to suprathreshold currents in small cells isolated after chemotherapy and mSYMPX, with TGFβ1R inhibitor (orange) or vehicle (green) present during the recording session. Shown is response to stimulus of 2.4 nA, which evoked maximum number of action potentials in the TGFβ1R inhibitor cell, and response to stimulus of 3.4 nA in the vehicle treated cell, where it was not possible to evoke more than one action potential. Scale bars = 25 msec, 50 mV. (*G* and *H*) Quantification of the TGF-βR1 inhibitor effect on the maximum number of action potentials evoked by suprathreshold currents in small-size dorsal root ganglia neurons (*G*) and large-size dorsal root ganglia neurons (*H*) isolated from mSYMPX mice treated with paclitaxel (n = 28–29 small neurons per group, or 41–43 large neurons per group; Mann–Whitney test, *$P < 0.05$ compared with vehicle). Additional electrophysiologic details can be found in Supplemental Digital Content, table 2 (http://links.lww.com/ALN/C297).

and pain-like behavior in a lumbar radiculopathy animal model.[34] However, the analgesic effects of sympathectomy in various neuropathic pain animal models are contradictory.[35,36] These contradictions may due to the use of global surgical and chemical methods of sympathectomy, timing of the intervention, and the contrasting effects of the sympathetic denervation of various immune tissues (*e.g.*, thymus, spleen, and lymph nodes).[37,38] We have observed that the use of a systemic chemical sympathectomy results in the delay of both the development and the resolution of paclitaxel-induced mechanical allodynia. Previous studies have demonstrated that distinct T cells and anti-inflammatory cytokines are important for the attenuation and resolution of paclitaxel-induced mechanical allodynia,[15,39] and sympathetic denervation of immune tissues by systemic approaches can potentially interfere with these immune responses. For

instance, sympathetic denervation of the spleen or depletion of the sympathetic neurotransmitter norepinephrine by treatment with reserpine prevents the vagally-stimulated anti-inflammatory responses,[40] reinforcing our rationale for localized sympathetic interventions.

Our preclinical studies have shown that a local microsympathectomy, consisting of cutting the grey rami near the lumbar dorsal root ganglia, is highly effective in reducing neuronal excitability and pain-like behaviors in animal models of neuropathic pain and low back pain.[12,13] Furthermore, this surgery has been shown to mitigate the increase of macrophage and proinflammatory cytokines and decrease of anti-inflammatory cytokines associated with a low back pain model.[12] Although previous studies have found no changes in transcriptional levels of macrophage markers,[4,5] we found that several macrophage markers are increased by microsympathectomy after paclitaxel. Most importantly, similarly to the low-back pain study, we observed a change in the balance between proinflammatory cytokines and decrease of anti-inflammatory cytokines in our paclitaxel-induced animal model, and provided several lines of evidence to demonstrate that this analgesic effect is mediated by monocytes/macrophages and anti-inflammatory transforming growth factor-β signaling. First, local microsympathectomy increased the expression of macrophage marker CD68, as well as the monocyte chemotactic markers CCL2 and C-C chemokine receptor 2 in dorsal root ganglia, suggesting an increase of infiltrating macrophages. Second, monocyte/macrophage depletion significantly diminished the microsympathectomy analgesic effect, suggesting a beneficial role of these cells in this animal model, as previously reported.[41,42] We observed a similar effect with the systemic delivery of the C-C chemokine receptor 2 agonist INCB3344, which is known to partially deplete circulating monocytes.[19] However, intrathecal delivery to spinal and dorsal root ganglia tissues of INCB3344 presented no analgesic effects, suggesting that local C-C chemokine receptor 2 is probably not required for the recruitment of monocytes and attenuation of paclitaxel-induced mechanical allodynia by microsympathectomy. This result may be due to the previously reported observation that the regulation of the monocyte recruiting cytokine CCL2 is unaltered by INCB334.[19] Third, transforming growth factor-β1 levels in dorsal root ganglia dramatically increased after microsympathectomy. Fourth, administration of exogenous transforming growth factor-β1 potently inhibited mechanical allodynia after paclitaxel, and a transforming growth factor-β receptor 1 inhibitor partially blocked both transforming growth factor-β1 and microsympathectomy analgesic effects.

The question arises whether monocytes/macrophages are responsible for the increase in transforming growth factor-β1 in dorsal root ganglia after local microsympathectomy. Depletion of monocytes/macrophages has been shown to delay the resolution of inflammatory pain-like behaviors *via* the impairment of the production of the anti-inflammatory cytokine IL-10 in dorsal root ganglia.[41] However, we were unable to show a similar result or impairment of any anti-inflammatory cytokines, including transforming growth factor-β1, after our depletion of monocytes/macrophages (data not shown). Future studies will attempt to investigate whether there is a link between monocytes/macrophages and transforming growth factor-β1, or whether they are distinct, and transforming growth factor-β1 may indeed be produced by other dorsal root ganglia cells.[43] This may be achieved using cell sorting or other single-cell analysis.[39,44] Another question that arises is: how does transforming growth factor-β1 alleviate paclitaxel-induced mechanical allodynia? Although transforming growth factor-β1 may protect against nerve degeneration resulting from the toxicity of paclitaxel, we showed that blocking transforming growth factor-β1 signaling rapidly unmasked dorsal root ganglia neuronal hyperexcitability. This rapid action is inconsistent with the canonical nerve protective and transcriptional roles of transforming growth factor-β1 signaling. We propose that at least part of the beneficial effect of transforming growth factor-β1 is mediated *via* direct effects on the transforming growth factor-β receptor 1 that is broadly expressed in dorsal root ganglia neurons.[44] We have previously shown that local microsympathectomy reduced the hypersensitivity of large-sized dorsal root ganglia neurons induced by local dorsal root ganglia inflammation.[12] However, we have demonstrated that the analgesia associated with the microsympathectomy in this paclitaxel-induced animal model is mediated, at least in part, by the transforming growth factor-β1 signaling and reduction of ectopic action potentials in small-sized dorsal root ganglia neurons. Consistently, transforming growth factor-β1 was previously reported to result in a rapid block of ectopic action potentials in small sized dorsal root ganglia neurons, and relief of pain-like behaviors in an animal model of nerve injury.[45] Interleukin 10 may also block these ectopic action potentials after paclitaxel.[15] Although we failed to confirm the transcriptional increase of interleukin 10 at the protein level, we should not rule out that interleukin 10 and other anti-inflammatory mediators may be implicated in the prevention and decrease of paclitaxel-induced mechanical and cold allodynia by local microsympathectomy.

Our and other previous studies have shown that systemic depletion of monocytes/macrophages prevents and alleviates paclitaxel-induced mechanical allodynia, but this strategy is unfortunately often accompanied with compromised immune response and higher risks of infection and immune diseases. Alternatively, the detrimental adrenergic communication between sympathetic fibers and macrophages could be avoided by targeting noradrenergic signaling using beta-blockers (*e.g.*, carvedilol), which have been proposed as adjuvants for cancer chemotherapy to protect against cardiotoxicity.[46] However, selective serotonin and noradrenaline reuptake inhibitors (*e.g.*, venlafaxine and duloxetine), which

increase the extracellular levels of noradrenaline and potentiate noradrenergic signaling, seem also to have beneficial effects in reducing paresthesia and pain in cancer patients treated with paclitaxel.[47,48] Although many studies focus on catecholamines, ignoring other sympathetic cotransmitters, a number of additional sympathetic cotransmitters such as adenosine triphosphate have been shown to play a pivotal role in pain and can potentially elicit the activation of neurons, macrophages, and proinflammatory responses in dorsal root ganglia.[13] Catecholamines and adenosine triphosphate may have synergistic effects, and we have previously found that a cocktail of norepinephrine and adenosine triphosphate inhibitors was effective in blocking the effects of stimulation of the dorsal ramus (that also contains sympathetic fibers) in dorsal root ganglia neurons.[13] This is consistent with a high expression of purinergic receptors in dorsal root ganglia tissues and suggests that targeting both adrenergic and purinergic signaling may represent a better therapeutic option for chemotherapy-induced neuropathic pain.

In summary, our results unravel a previously unrecognized link whereby sympathetic fibers obstruct the emergence of anti-inflammatory responses and support the development of paclitaxel-induced mechanical and cold allodynia. Local sympathectomy promotes anti-inflammatory responses and faster resolution of mechanical allodynia, as well as preventing cold allodynia, after paclitaxel treatment. Together these results suggest that sympathetic blocks, already used in the clinic for various pain conditions,[9,10] may represent a novel therapeutic strategy to effectively relieve chemotherapy-induced neuropathic pain.

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Competing Interests

The authors declare no competing interests.

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