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Biodegradable Nanoparticles Targeting Circulating Immune Cells Reduce Central and Peripheral Sensitization to Alleviate Neuropathic Pain Following Spinal Cord Injury

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Abstract

Neuropathic pain is a critical source of comorbidity following spinal cord injury (SCI) that can be exacerbated by immune-mediated pathologies in the central and peripheral nervous systems. In this article, we investigate whether drug-free, biodegradable poly(lactide-*co*-glycolide) (PLG) nanoparticle treatment mitigates the development of post-SCI neuropathic pain in female mice. Our results show that acute treatment with PLG nanoparticles following thoracic SCI significantly reduces tactile and cold hypersensitivity scores in a durable fashion. Nanoparticles primarily reduce peripheral immune-mediated mechanisms of neuropathic pain including neuropathic pain-associated gene transcript frequency, TRPA1 nociceptor expression, and MCP-1 (CCL2) chemokine production in the subacute period after injury. Altered central neuropathic pain mechanisms during this period are limited to reduced innate immune cell cytokine expression. However, in the chronic phase of SCI, nanoparticle treatment induces changes in both central and peripheral neuropathic pain signaling, driving reductions in cytokine production and other immune-relevant markers. This research suggests that drug-free PLG nanoparticles reprogram

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Author Contributions:

M.S. performed conceptualization, investigation, data curation, visualization, and wrote the original draft and edits. K.G. performed data analysis, visualization, and original draft writing. I.K. and D.K. both performed investigation and data analysis. D.S. and E.S. performed investigation. B.C. and A.A. performed conceptualization, funding acquisition, and edited draft writing. L.S. performed conceptualization, funding acquisition, visualization, and helped write the original draft and edits. J.P. performed conceptualization, funding acquisition, investigation, data curation, visualization, and both original and edited draft writing.

Conflict of Interest:

L.S. consults for and has financial interests in Cour Pharmaceutical Development, Inc., which has licensed the nanoparticle technology used in this research. These studies were not directly supported by Cour Pharmaceutical Development, Inc.

peripheral proalgesic pathways subacutely after SCI to reduce neuropathic pain outcomes and improve chronic central pain signaling.

Introduction

Neuropathic pain is a debilitating condition affecting up to 80% of individuals who have experienced a spinal cord injury (SCI) [16,43,45]. Pain in the absence of noxious stimuli (allodynia) is mediated by damage to the somatosensory nervous system, differentiating neuropathic pain from nociceptor-mediated pain [47,56,76]. Neuropathic pain can impede rehabilitation and quality of life because traditional pharmacological therapies provide only temporary and often incomplete relief [1,25,38,61,66,70]. SCI-related neuropathic pain is driven by neurological and inflammatory changes that can be categorized into central, peripheral, and supraspinal patterns based on the sites of sensitization [3,18,37,53]. Central mechanisms include damage to spinal cord neurons, often exacerbated by glial activation and production of immune-potentiating cytokines and chemokines [18,19,27,40,49,50,55,60,86]. SCI may also disinhibit distal neurons in the spinal cord and peripheral nervous system, increasing neuronal excitability and hypersensitivity to innocuous stimuli [9,29,57,74,78]. Increased expression of nociceptors, including transient receptor potential ankyrin 1 (TRPA1), on peripheral neurons with nuclei located in the dorsal root ganglia (DRG) contributes to pain responses by driving inflammatory pain signaling [7,8,17,24,42,48,71,87,88]. Satellite cells in the DRG also release inflammatory factors like tumor necrosis factor-α (TNFα) that directly increase neuronal excitability and activate immune cells [89].

Immune modulators are emerging as strategies to reduce inflammatory neuropathic pain. Methylprednisolone has been used to reduce neuropathic pain post-SCI, yet it remains controversial due to treatment-associated adverse effects [12–14,51,62,81]. Nanoparticles (NPs) can be used as drug delivery platforms to distribute anti-inflammatory factors that modulate peripheral neuropathic pain responses through selective immune reprogramming [5,11,63]. Alternatively, poly(lactide-*co*-glycolide) (PLG) NPs, which do not have any active pharmaceutical ingredient, target scavenger receptors on circulating monocytes and neutrophils due to their size and surface charge and reprogram peripheral immune cells through drug-independent mechanisms, ultimately reducing monocyte and neutrophil accumulation at the injury [20,36]. Their direct effects on immune cells are transient, as cessation of drug-free NP delivery results in disease progression [73]. Within the injury, NP-mediated reprogramming induces proregenerative immune cell phenotypes, myelinated axon growth, and enhanced locomotor function [67]. Without crossing the blood brain barrier, PLG NP uptake prompts monocyte and neutrophil splenic sequestration and clearance through apoptosis without exerting broadly immunosuppressive states in therapy recipients.

In this report, we investigated the hypothesis that inflammatory central and peripheral neuropathic pain signaling is reduced by intravenous delivery of drug-free PLG NPs. We found that NP treatment reduces mechanical and cold hypersensitivity, two common metrics of neuropathic pain, in female mice after thoracic SCI. Analysis of central pain mechanisms revealed that microglia activation and neuropathic pain-associated gene and

cytokine expression are only minimally influenced by NP treatment one-week post-injury. At similar timepoints, NP treatment reduces pro-nociceptive gene transcription and neuronal nociceptor expression in the DRG. These results suggest that PLG NPs predominantly modulate the subacute neuropathic pain response through peripheral mechanisms. However, in the chronic phase following SCI, both central and peripheral inflammatory pathways of proalgesic signaling are reduced by NP treatment. Our results indicate that immune modulation through NP treatment holds promise in improving post-SCI neuropathic pain outcomes.

Methods

Nanoparticle and Multichannel Bridge Fabrication

50:50 poly(DL-lactide-*co*-glycolide) (PLG; Evonik Industries, Essen, Germany) with an inherent viscosity of 0.55–0.75 dL/g and a carboxyl end group was used to generate 500–700 nm diameter nanoparticles as previously described [67]. Briefly, PLG was dissolved in dichloromethane at a 20% w/v concentration. A 5x volume of 2% w/v poly(ethylene-*alt*-maleic anhydride) (PEMA) was added to the PLG solution and subsequently sonicated for 30 seconds at 100% amplitude using a CPX 130 Ultrasonic Processor (Cole-Parmer, Vernon Hills, IL). The resulting emulsion was immediately poured into a stirring bath of 0.5% w/v PEMA and left uncovered overnight. Excess PEMA was subsequently removed from the solid PLG NPs using four wash steps involving centrifugation at 5000g for 15 minutes and supernatant aspiration. NPs were then resuspended in a 0.8% w/v sucrose and 0.6% w/v mannitol cryoprotectant solution, lyophilized, and stored at room temperature until use. PLG bridges with longitudinal channels were used to enable visualization and demarcation of regenerating axons within the injury as previously described [67,83]. Briefly, 75:25 PLG (Lakeshore Biomaterials, Birmingham, AL) with an inherent viscosity of 0.76 dL/g was dissolved at 6 wt% in dichloromethane and emulsified with 1% poly(ethylene-alt-maleic anhydride) using a PolyTron 3100 homogenizer (Kinematica AG, Littau, Switzerland). Microspheres with a z-average diameter of 1 μm were generated. Longitudinal channels were created by mixing D-sucrose (Sigma Aldrich), D-glucose (Sigma Aldrich), and dextran (molecular weight 100,000; Sigma Aldrich) at a ratio of 5.3:2.5:1 by mass and caramelizing, cooling, and drawing sugar fibers using a Pasteur pipette. A 1:1 mixture of PLG microspheres and salt was pressed into a salt-lined aluminum mold with longitudinally-placed sugar fibers. After 16 hours of equilibration at high pressure in CO_2 (800 psi) in a custom pressure vessel, adjacent microspheres were fused to create a continuous polymer structure. Pressure release occurred over 40 minutes, after which 1.2 mm bridge sections were cut out and salt porogen was leached out of the polymer structure into water for 2 hours. The bridges were lyophilized and stored in a desiccator until use, at which point they were rehydrated, sterilized with 70% ethanol, and washed with PBS.

Animals: Spinal Cord Injury and Nanoparticle Treatment

6–8 week old (20–25 g) female C57BL/6J mice were used to create a thoracic hemisection spinal cord injury model according to the University of Michigan Animal Care and Use guidelines (The Jackson Laboratories), and all experiments conformed to all relevant ethical and regulatory standards [67]. Female mice were used because male mice have a higher

incidence of urinary tract infections after SCI, which would make it more difficult to interpret inflammatory signaling related to SCI. Briefly, mice were pre-handled 10 times each prior to surgery and assigned randomly to treatment groups. On the day of surgery, a laminectomy was performed at T9/10 in all mice; in sham mice, the spinal cord was subsequently covered with gelfoam, the muscle layer was sutured shut, and the skin was closed with staples. In mice destined for NP or PBS treatment, a 2.2 mm longitudinal segment of spinal cord was removed on the left side. A biomaterial bridge was placed with the ends directly abutting the cord. Gelfoam was used to cover the injury site, after which the muscle was sutured shut and staples were used to close the skin. As previously described, starting two hours after injury, mice received their first intravenous (i.v.) injection of 1 mg of PLG NPs resuspended in 100 μL of PBS, or 100 μL PBS alone, via tail vein injection [67]. Mice received either NPs or PBS every 24 hours for a total of 7 doses. All mice also received daily doses of 2.5 mg/kg prophylactic enrofloxacin to prevent infections through 14 days post-injury.

Mechanical Hypersensitivity

To measure mechanical hypersensitivity, von Frey filaments were used to establish a foot withdrawal threshold as an indicator of mechanical sensitivity [80]. Assessments were performed at 2 weeks following SCI when animals were using their hindlimb for weight support [31,66,68]. A mesh table covered by a plastic box was used for the test environment. Mice were given a 15-minute acclimation period before exposure to testing with a sequence of von Frey filaments (Stoelting, Wood Dale, IL, USA). The filaments were exposed in a perpendicular fashion to both sides of the hindlimb's plantar surface. Filaments were applied for 3 to 5 seconds with the required bending force prior to removal. Von Frey filament gauges of 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, and 2 g were used and given in 3-minute intervals. A positive response was recorded when SCI mice showed quick hindlimb withdrawal, regardless of licking or biting. A lower filament force was used following a positive response. A higher filament force was used if the SCI mice failed to respond. From the previously described method, an average of both hindlimb sides was calculated and converted to millinewtons (mN) to obtain the mechanical threshold [66].

Cold Hypersensitivity

Hypersensitivity to cold stimuli was measured using an acetone-mediated evaporative cooling technique, as described in previous studies [23,66,68]. Using the same environment as the mechanical hypersensitivity tests, 50 μL of 100% acetone was applied to both sides of the ipsilateral hindlimb's plantar surface. The average positive response was recorded as a percentage following acetone application five times per each hindlimb surface using five-minute intervals. Mechanical stimulation was reduced over the course of this procedure. Using a max cutoff of 30 seconds, other pain behaviors resulting from the acetone including lifting, shaking, licking, and biting were documented. To reduce stress on the animals, mechanical hypersensitivity tests were performed before cold hypersensitivity experiments using a 20-minute rest period. Measurements were performed one day prior to SCI to serve as a baseline.

Single Cell RNA Sequencing

PLG bridges and their associated spinal cord tissue were collected from 10 NP- and 9 PBS-treated mice 7 days post-injury. Bridges were minced, dissociated to single cells using Liberase (Roche Diagnostic, Mannheim, Germany) (2 mL per bridge, shaking at 37C for 20 minutes), and passed through a 70 μm filter. Immune cells were selected using CD45+ cell selection kit via magnetic activated cell sorting (Miltenyi Biotec). Pooled samples of single cell solutions were submitted to the University of Michigan Advanced Genomics Core for library preparation and scRNA-seq using 10X Genomics at 20,000 reads per cell on the NovaSeq (S4) 300 cycle sequencer [90]. Post-sequencing data preparation was performed; cells expressing less than 200 genes or more than 5000 genes and more than 10% mitochondrial genes were filtered out. Downstream data analysis was performed using the Seurat package according to their analysis pipeline [41]. Unbiased clustering using uniform manifold approximation and projection (UMAP) (dimensions=20, resolution=1.2) identified 11 cell populations; identification genes can be found in Figure S1 (Fig S1). Cell populations potentially relating to neuropathic pain (neutrophils, dendritic cells, common myeloid progenitors, microglia, macrophages, monocyte progenitors, and monocytes) were considered for further downstream analyses (Table S1). Expression of known neuropathic pain-associated genes (*Dapk1, Ptk2b, Apoe, Msr1, Tlr4, P2ry2, P2ry6*) and cytokines (*Il1b, Tnf, Il6*) was examined in each treatment condition by cell type (Table S2; Table S3) [37,44,53,65,66]. Differential expression between all cells in the PBS and NP conditions was conducted with the Seurat FindMarkers function using the MAST test [32]. Due to low cell numbers, monocyte progenitors were analyzed with the Wilcox test instead of the MAST test. The analysis of the single cell RNA sequencing data was performed using code available at the following link: https://github.com/shea-lab/neu-pain.git.

qRT-PCR

RNA was sequestered from all specimens including spinal cord tissues (5 mm segment centered at injury) and DRGs (L2-L6). Samples were homogenized using a tissue grinder and 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA). Once the RNA was isolated, chloroform extraction and isopropanol precipitation were executed. Next, gene expression over time was measured via quantitative Reverse-Transcriptase PCR (qRT-PCR) using spinal cord tissue and DRGs. cDNA was synthesized with the iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA), and primers were fabricated for qRT-PCR (Table S4) [23,31,65,67]. Fluorescence accumulation in the samples was found by utilizing the manufacturer's protocols for CFX Connect™ Real-Time PCR Detection System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad). With 18s-rRNA expression as a benchmark, gene expression levels were normalized with differences given as fold ratios from the control group. Finally, the following calculations were performed to quantify each sample: $X = 2 - \Delta\Delta C_t$, where $\Delta\Delta C_t = \Delta E - \Delta C_t$ and $\Delta E = C_{t,exp} - C_{t,18s\text{-}rRNA}$, $\Delta Ct = C_{t,control} - C_{t,18s\text{-}rRNA}$ [52]. The sham (laminectomy only) group value of 1 was used as a cut-off value for upregulation.

Immunohistochemistry and Analysis

For immunohistochemistry assays, the lumbar DRGs (L2-L6) were extracted. Tissues were flash-frozen in isopentane and then embedded using Tissue Tek O.C.T compound (Sakura

Finetek, Torrance, CA, USA) with 30% sucrose and transversely cryosectioned using 18 μm slices. Table S2 summarizes the primary and secondary antibodies that were utilized (Table S2). Immune-positive cells from 12 to 15 nonadjacent DRG sections were analyzed per mouse via hand count by blinded reviewers. Using co-staining, multiple markers were analyzed using overlap of different channels in NIH ImageJ (Bethesda, MD, USA). Nociceptive markers were used to label twelve to fifteen nonadjacent DRG (L2-L6) sections in each animal. Next, the number of nociceptor marker-positive cells in the lumbar DRGs was measured against the calculated percentage of Hoechst-positive cells that expressed each specific nociceptive marker. Imaging was performed with an Axio Observer Z1 (Zeiss, Oberkochen, Germany) utilizing a 10x or 20x/0.45 M27 apochromatic objective and an ORCA-Flash 4.0 V2 Digital CMOS camera (C11440–22CU, Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan).

Statistical Analysis

Student's t tests, one or two-way ANOVAs, and Tukey's post hoc test for multiple comparisons were performed for statistical analysis. The Kolmogorov-Smirnov normality test was done with a P-value (alpha) of 0.05 as a cut-off. As such, P-values smaller than 0.05 were considered sufficient for passing. A 0.2 level was used to control type II errors and an alpha value of 0.05 was used to attain sufficient statistical power. An equal variance (ANOVA Model) was assumed for each study after validation. Accounting for other parameters, an effect size of 0.25 was assumed for a suitable sample size in each study. G* Power Software and Prism 9 (GraphPad Software, La Jolla, CA, USA) were used for statistical analysis. Mean ± standard error of the mean (SEM) was used to express every value with $P<0.05$ being labeled statistically significant. The MAST test was used to assess significance in single cell analyses as described above, except with monocyte progenitors wherein the Wilcox test was used due to low cell numbers [32].

Results

Nanoparticles reduce tactile and cold hypersensitivity following thoracic hemisection SCI

Mice were treated with PLG NPs or controls following thoracic SCI and evaluated for tactile and cold hypersensitivity outcomes. C57BL/6J mice underwent sham surgery or a T9/10 hemisection at day 0. Starting 2 hours after surgery, the latter group of mice received 7 daily doses of 1 mg i.v. PLG NPs or an equivalent volume of phosphate buffered saline (PBS) (Table S5). Starting 2 weeks after SCI, NP treatment increased the mechanical force threshold at which injured mice withdrew their paws, an effect that was maintained through 12 weeks post-injury (Fig 1A). While sham mice demonstrated withdrawal at a baseline of approximately 15 mN throughout the evaluated time course, NP-treated mice initially had an aversive response to the application of approximately 7 mN at 2 weeks post-injury. By 4 weeks post-injury, this threshold had risen to approximately 11 mN. NP therapy was similarly protective against cold stimulus withdrawal compared to PBS-treatment (Fig 1B). Sham mice had a withdrawal frequency near 0% throughout the 12-week experiment, while NP-treated mice plateaued at approximately 20%, and PBS-treated mice had a significantly higher withdrawal frequency of approximately 40%. While significant differences between NP and PBS treatment groups in terms of hypersensitivity emerged at 2 weeks post-injury,

final thresholds were reached at approximately 4 weeks for mechanical hypersensitivity and at about 6 weeks for cold hypersensitivity. Together, the observed changes in both mechanical and cold hypersensitivity demonstrate that drug-free PLG NPs induce durable reductions in neuropathic pain, and that these sensory benefits persist into the chronic stages of spinal cord injury.

Nanoparticles minimally alter central mechanisms of neuropathic pain in the subacute period following thoracic SCI

The impact of immediate post-injury PLG NP treatment on central mechanisms of neuropathic pain development were evaluated in subsqeuent experiments. Neuropathic pain-associated gene expression and microglia activation in the spinal cord were examined one week after injury. $CD45^+$ cells were selected via magnetic-activated cell sorting and analyzed via single cell RNA sequencing, and immune cell subtypes were identified based on their relative expression of population-specific genes (Fig S1). Supervised analysis of the known neuropathic-pain associated genes *Dapk1*, *Ptk2b*, *Apoe*, *Msr1*, *P2ry6*, and *P2ry2* (Gene Accession Number GO:0019233) revealed no significant differences between NP- and PBS-treated mice (Fig 2A; Table S2) [37,44,53,65,66,75]. Given recent literature suggesting that microglia activation may play a role in neuropathic pain evolution, we divided our microglia population into activated and inactivated subsets using activation-related genes (Fig 2B) [33,35]. Unexpectedly, our investigation revealed that NP-treated mice had a greater proportion of activated microglia (44%) compared to PBS-treated mice (29%; Fig 2C) [40]. Given these findings, we further evaluated immune populations in the spinal cords of NP- and PBS-treated mice for differential expression of the cytokines *Il1b* and *Tnf*, each of which are implicated in immune-driven neuropathic pain (Fig 3A; Table S3). Transcripts of IL-1β, a cytokine implicated in driving pain-inducing inflammation, were significantly reduced across aggregated neutrophils, dendritic cells, common myeloid progenitors, microglia, macrophages, monocyte progenitors, and monocytes in NP-treated mice compared to PBS-treated controls (Fig 3B) [39,72]. Taken together, the similarity in *Tnf* transcript frequency and neuropathic pain-associated gene expression between NP- and PBS-treated mice one week after injury suggests that NPs predominantly drive the observed changes in subacute neuropathic pain through mechanisms external to the spinal cord.

Subacute responses to nanoparticle treatment involve modulation of peripheral neuropathic pain mechanisms

Nanoparticle modification of peripheral mechanisms of hindlimb sensitivity were evaluated by examining neuropathic pain-associated gene expression in the ipsilateral L2-L6 DRG. These DRG, which are responsible for transmitting sensory afferents from the ipsilateral hindlimb, were isolated from mice treated with NPs or PBS at 1, 2, and 4 weeks post-thoracic SCI. RNA isolated from both $CD45^+$ and $CD45^-$ cells in the DRG revealed a NP-mediated reduction in the expression of *Piezo2*, *Tlr4*, *Scn11a*, and *Trpa1* at each timepoint via qRT-PCR. *Piezo2* is a mechanosensitive ion channel that mediates mechanical hypersensitivity while *Tlr4* is a key immune meditator of the inflammatory pain response [10,30,59]. *Scn11a*, a voltage-gated sodium channel, and *Trpa1*, an ion channel, are both susceptible to inflammation-mediated activation and may cause neuronal hyperexcitability [58,79,85]. Transcript frequency of all four of these were reduced in the DRG of NP-

treated mice (Fig 4A). Interestingly, both *Piezo2* and *Scn11a* also fell between one- and four-weeks post-injury. Further analysis of $IB4^+$ sensory neurons in the DRG 2 weeks after injury revealed a decrease in TRPA1 expression among NP-treated mice relative to PBS-treated mice (Fig 4B). Immunofluorescent staining detected substantially fewer neurons staining positive for the chemokine monocyte chemoattractant protein 1 (MCP-1), also known as C-C motif chemokine ligand 2 (CCL2; Fig 4B). These data suggest that NP administration subacutely reduces peripheral neuropathic pain signaling, including proinflammatory signaling, corresponding to the improvements observed in both mechanical and cold hypersensitivity in Figure 1.

Central and peripheral mechanisms of neuropathic pain are altered in the chronic period following nanoparticle treatment

The durability of the improved mechanical and cold sensitives in NP-treated mice led to an investigation of whether NPs mediate changes in central and peripheral pathways of neuropathic pain at 12 weeks post-injury. The spinal cords of NP- and PBS-treated mice were harvested at 12 weeks post-injury and evaluated via qRT-PCR. Transcripts of the proalgesic cytokines *Il6*, *Il1b*, and *Tnf* were significantly reduced in NP-treated mice, yielding transcript frequencies similar to mice receiving sham surgeries (Fig 5A). Peripheral mechanisms of neuropathic pain were also evaluated for NP-mediated changes. RNA was isolated from ipsilateral L2-L6 DRG, after which qRT-PCR was used to evaluate *Tlr4*, *Mcp1 (Ccl2)*, and *Trpa1* expression. As noted at 1, 2, and 4, weeks after SCI, all three markers were consistently reduced at 12 weeks post-injury (Fig 5B). The number of *Tlr4* and *Mcp1* transcripts, both primarily expressed by resident or infiltrating immune populations within the DRG, as well as transcripts of the nociceptor *Trpa1*, produced by sensory neurons, were reduced following NP treatment. These results suggest that NP administration modulates immune-mediated pathways of both central

Discussion

The studies presented herein reveal that PLG NPs robustly and durably reduce both mechanical and cold hypersensitivity after thoracic SCI, suggesting that NP treatment reprograms neurological pathways responsible for neuropathic pain development and maintenance (Fig 1). These NPs, which are 500 nm in diameter and too large to pass through an intact BBB, primarily associate with monocytes and neutrophils outside of the central nervous system, influencing their trafficking and phenotype to reduce secondary inflammatory damage in the spinal cord [64,67,77]. We investigated whether NP treatment reduces allodynia by altering key central and peripheral immune-linked signaling pathways related to pain development, and our results contribute to a growing body of literature supporting the hypothesis that early modification of the immune response after SCI can significantly modulate neuropathic pain outcomes [12,13,15,81]. These results suggest that our NPs, which are drug-free, biodegradable, and made of FDA-approved PLG, are a translationally-relevant therapy with the potential to significantly alter neuropathic pain outcomes.

A short course of PLG NP therapy immediately following thoracic SCI is sufficient to durably reduce neuropathic pain outcomes through 12 weeks post-injury (Fig 1). These effects differ significantly from most current standard of care medications including opioids, anticonvulsants, and muscle relaxants, which offer only temporary pain relief by transiently targeting dysregulated neuronal circuits [1,25,38,66]. The corticosteroid methylprednisolone has also been applied to reduce secondary immunological injury after SCI, but its use is limited by controversy over the balance between its efficacy and potential consequences relating to induction of a broadly immunosuppressive state [12–14,51,62,81]. In contrast, PLG NPs have selectively reprogrammed monocytes and neutrophils, reducing excessive early immune responses that initiate further neurological damage [67]. Importantly, with treatment cessation after 7 days post-injury, pro-regenerative cytokine-mediated signaling is allowed to proceed without interruption [4,28]. After this time point, NP-treated mice continued to show improvements in mechanical withdrawal thresholds until four weeks post-injury, indicating a dynamic development of neuropathic pain responses in the subacute period after SCI (Fig 1A). Similarly, cold withdrawal frequency changed throughout the first 6 weeks post-injury in NP-treated mice, finally plateauing at a stable level between mice receiving PBS and those receiving sham surgeries (FIG 1B). These results suggest that NPs might operate by affecting different neuropathic pain-related signaling pathways at different times after injury.

Immediate post-injury NP treatment subacutely modified peripheral pathways involved in neuropathic pain responses without significantly affecting neuropathic pain associated gene expression or cytokine production in the spinal cord. The subacute period after SCI is defined by spinal cord neuronal and glial apoptosis, axonal demyelination, Wallerian degeneration, and glial scar evolution, with changes extending into the peripheral nervous system as a result of disrupted neuroimmune interfaces [3,46]. Surprisingly, despite the subacute reduction in tactile and cold hypersensitivity in NP-treated mice compared to PBS-treated negative controls, we did not observe significant reductions in spinal cord gene expression associated with neuropathic pain pathways 7 days after injury (Fig 2A). This time point was chosen for evaluation because it marked a period of initial stabilization in the neutrophil and monocyte responses following SCI. Importantly, the conclusions drawn from our scRNA sequencing studies were limited by the absence of similar data from time-matched DRG. As a result, we primarily took a supervised approach to assessing gene differences between NP- and PBS-treated groups. Among the hypothesized reductions in pain pathway signaling brought about by NP treatment, we examined whether microglia activation, which may contribute to neuropathic pain outcomes in a sex-dependent manner, was altered [2,22,44,82,84]. However, our scRNAseq analysis demonstrated that NP treatment increased the proportion of activated microglia in the spinal cord (Fig 2B–C), which is intriguing and is the focus of further analysis. Across all innate immune populations in the spinal cord, only one of three proalgesic cytokines, *Il1b*, was reduced as a result of NP treatment (Fig 3). While IL-1β induces cellular activation, promotes inflammation, and modulates neuronal excitability, changes in this cytokine alone are unlikely to explain the robust reduction in mechanical and thermal hypersensitivities observed subacutely after injury (Fig 1) [34,37]. Therefore, we investigated expression of known neuropathic pain-associated genes in the ipsilateral DRG, revealing significant

subacute reductions in *Piezo2, Scn11a, Tlr4* and *Trpa1* (Fig 4A). Interestingly, the drop in mechanosensitive ion channel *Piezo2* and sodium-gated ion channel *Scn11a* between 2 and 4 weeks post-injury correlates with the rise in mechanical force tolerance observed in Figure 1A, while the drop in *Tlr4* reflects prior studies demonstrating reduced monocyte and neutrophil frequency in the injury one week after NP treatment [10,30,54,59,67]. NP treatment also led to fewer $TRPA1^+$ and $MCP\text{-}1^+$ sensory neurons in the DRG in the subacute period (Fig 4B). TRPA1 is notable for its role as both a receptor for immune cell-derived proinflammatory agents and as an instigator of downstream inflammatory neuropeptide signaling, while MCP-1 (CCL2) is a potent immune cell-attracting chemokine [7,31,68,69]. Together, these results suggest that NPs predominantly reduce neuropathic pain responses following SCI by altering signaling pathways outside of the spinal cord.

NPs modulate both central and peripheral mechanisms of neuropathic pain in the chronic period after thoracic SCI. The transition from subacute to chronic SCI is demarcated by a maturing glial scar and progressive axonal die-back from the injury, which prevents neuronal regrowth and can further disrupt healthy nociceptive networks [3]. The effect of this plasticity on NP-mediated changes to neuropathic pain development is unclear. Our experiments show that NP treatment immediately post-injury reduces the frequency of spinal cord *Il6*, *Il1b*, and *Tnf* transcripts at 12 weeks post-injury to levels similar to sham surgery recipients (Fig 5A). These cytokines can be produced by glia as well as infiltrating immune cells, reflecting our previous studies demonstrating that NPs reduce immune cell infiltration into the injury and promote proregenerative phenotypes among the remaining spinal cord immune populations [20,21,36,67]. Peripheral neuropathic pain signaling is also reduced at these later timepoints post-injury, as demonstrated by reduced *Tlr4*, *Mcp1*, and *Trpa1* transcript frequency in the DRG of NP-treated mice compared to PBS-treated mice (Fig 5B). Reduced *Mcp1*, a monocyte chemoattractant protein, indicates reduced immune cell entry into the DRG, which is further reflected in the reduced *Tlr4* levels [26]. It is currently unclear exactly how subacutely reprogrammed peripheral neuropathic pain pathways may be influencing the reduction in subsequent central neuropathic pain signaling. Further analysis should focus on the role of bidirectional neuroimmune signaling, in which immune cell mediators directly interact with and promote afferent neuronal signaling that has the capacity to reprogram upstream signaling pathways [6,15]. Regardless, our results show that NP treatment induces robust changes in both central and peripheral neuropathic pain signaling in chronic SCI, which suggests that immunological reprogramming has the capacity to robustly reduce post-SCI neuropathic pain outcomes.

Thoracic SCI is a devastating condition worsened by the spontaneous development of chronic neuropathic pain. While current management strategies are currently limited to symptomatic treatment, biodegradable, drug-free PLG NPs that reprogram both central and peripheral neuropathic pain signaling pathways provide a mechanism to reduce multiple modalities of neuropathic pain. Intravenous injection of NPs acutely after SCI enables reprogramming of circulating monocytes and neutrophils, which themselves are critical to the secondary immune-mediated injury responsible for neuropathic pain induction [67]. Immediate post-injury NP delivery robustly reduced mechanical and cold hypersensitivity through 12 weeks post-injury, with subacute improvement corresponding primarily to reduced peripheral neuropathic pain signaling in the DRG. Chronically, NP-treated mice

displayed reduced central and peripheral neuropathic pain signaling in the spinal cord and DRG, respectively. These results suggest that therapies targeting peripheral neuropathic pain mechanisms subacutely after SCI are efficacious in reducing proalgesic outcomes, leading to improved central pain signaling at chronic time points. These experiments also reveal the need for further work to evaluate how improved subacute peripheral neuropathic pain signaling might impact chronic central neuropathic pain pathways. PLG NPs are a promising therapy for the robust and durable reduction of neuropathic pain outcomes following SCI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability:

The data discussed in this manuscript is available upon request from the co-corresponding authors.

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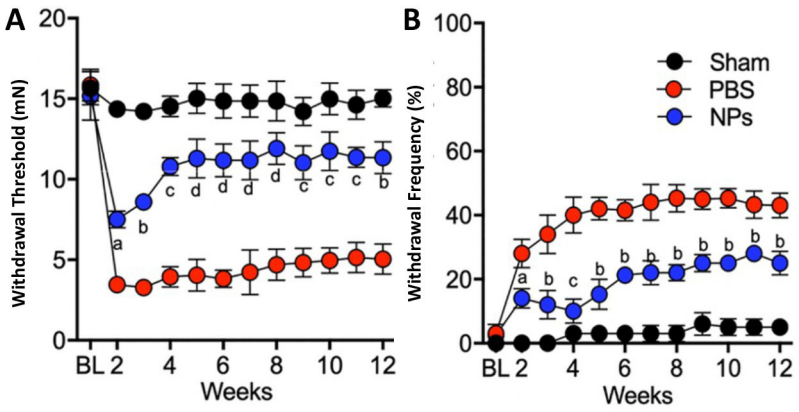

Figure 1: Nanoparticle treatment reduces mechanical- and cold-induced neuropathic pain following thoracic hemisection SCI.

Starting 2 hours after sham surgery or thoracic hemisection with bridge implantation, mice were treated with 7 daily doses of NPs or PBS. NP treatment significantly increased mechanical force-induced withdrawal threshold (A) and cold-induced withdrawal frequency (B) compared to PBS, demonstrating reduced hypersensitivity and neuropathic pain. Both effects were well established at 2 weeks after SCI and were maintained through 12 weeks post-SCI. BL=baseline (pre-injury); error bars indicate mean +/− SEM. Error bars indicate mean +/− SEM; two-way ANOVA with Tukey's post-hoc test was used to determine statistically significant differences between NP- and PBS-treated mice; $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$,$^{d}p<0.0001$ (n=12).

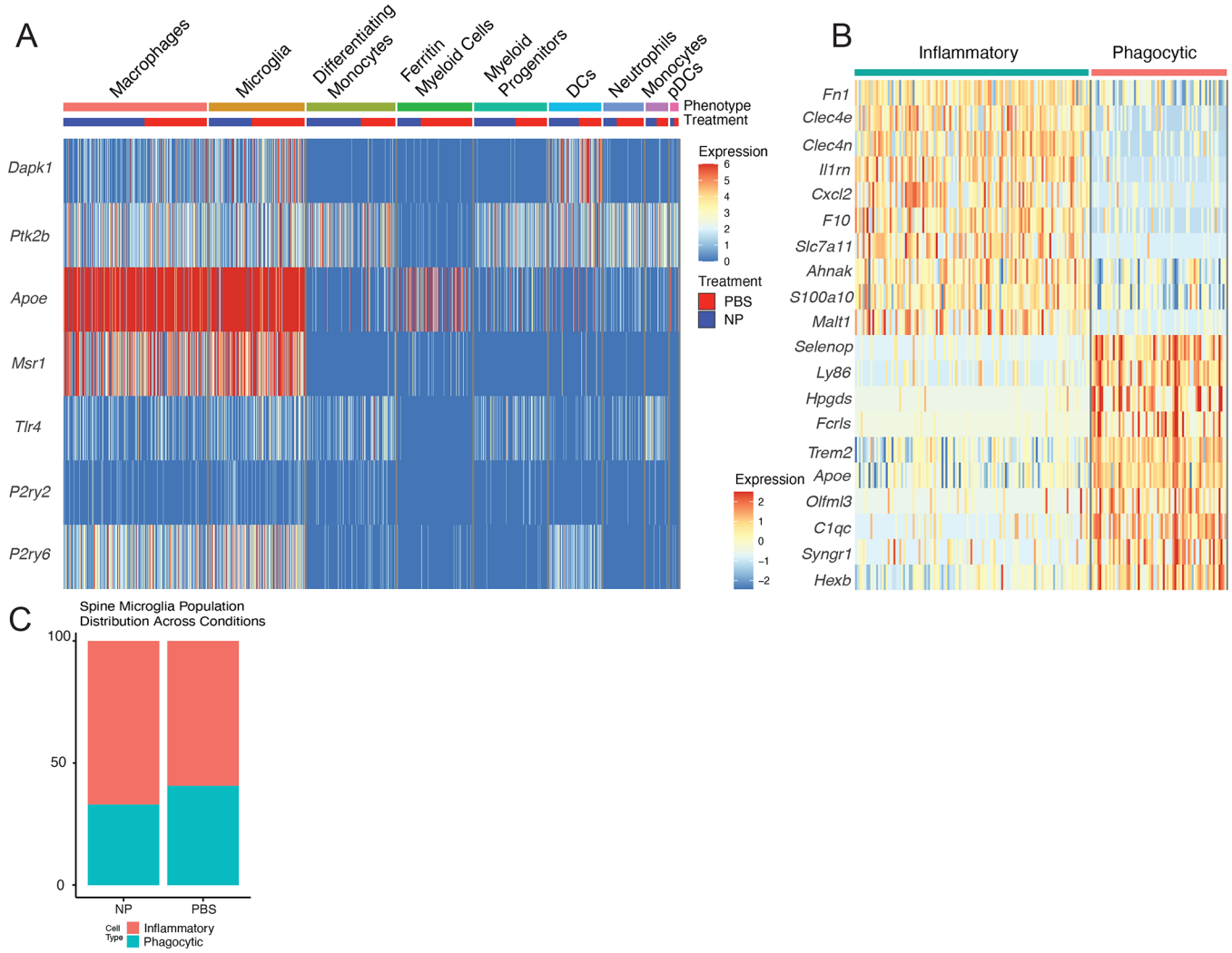

Figure 2: Nanoparticle treatment does not reduce neuropathic pain-associated gene expression or activated microglia frequency in the spinal cord 1 week post-injury.

The spinal cords of thoracic hemisection recipients treated with NPs (n=10) or PBS (n=9) were harvested and processed into single cells, after which $CD45^+$ cells were selected, pooled, and processed for scRNA sequencing. (A) No significant differences were observed between NP- and PBS-treated immune population expression of known pro-nociceptive neuropathic pain-associated genes *Dapk1*, *Ptk2b*, *Il1b*, *Tlr4*, *Apoe*, *P2ry6*, *P2ry2*, *Msr1*, or *Piezo1* after segregation by cell type. (B) Microglia were evaluated for relative expression of genes known to be increased following activation. These results were subsequently used to sort microglia into “Inflammatory” or “Phagocytic” groups. (C) NP treatment altered the percentage of phagocytic and inflammatory microglia in the spine relative to PBS-treated mice.

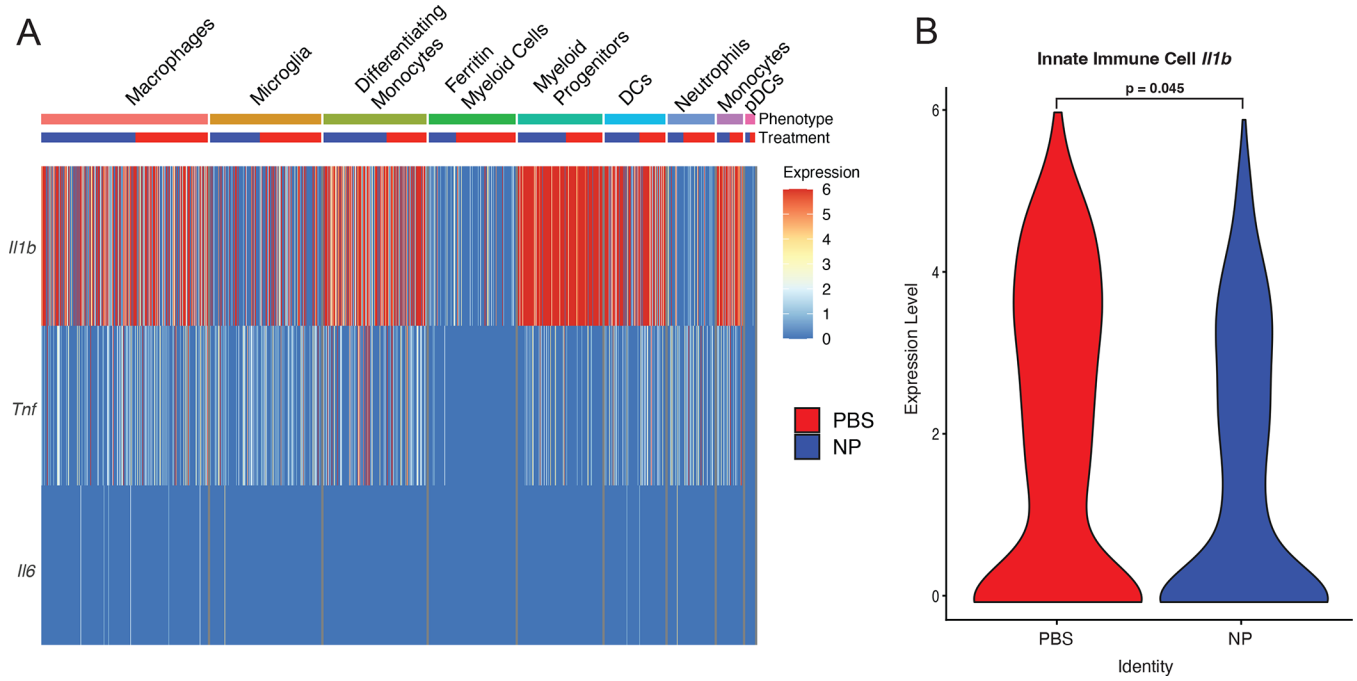

Figure 3: Nanoparticle treatment reduces subacute expression of the pro-nociceptive cytokine *Il1b* in spinal cord macrophages.

(A) scRNA sequencing of NP- (n=10) and PBS-treated (n=9) spinal cords one week after injury enabled evaluation of the frequency of transcripts for neuropathic pain-associated cytokines *Il1b*, *Tnf,* and *Il6*. (B) *Il1b* expression was significantly reduced across all evaluated immune cells in NP-treated mice compared to PBS-treated mice as evaluated by the Wilcoxon test (p=0.045).

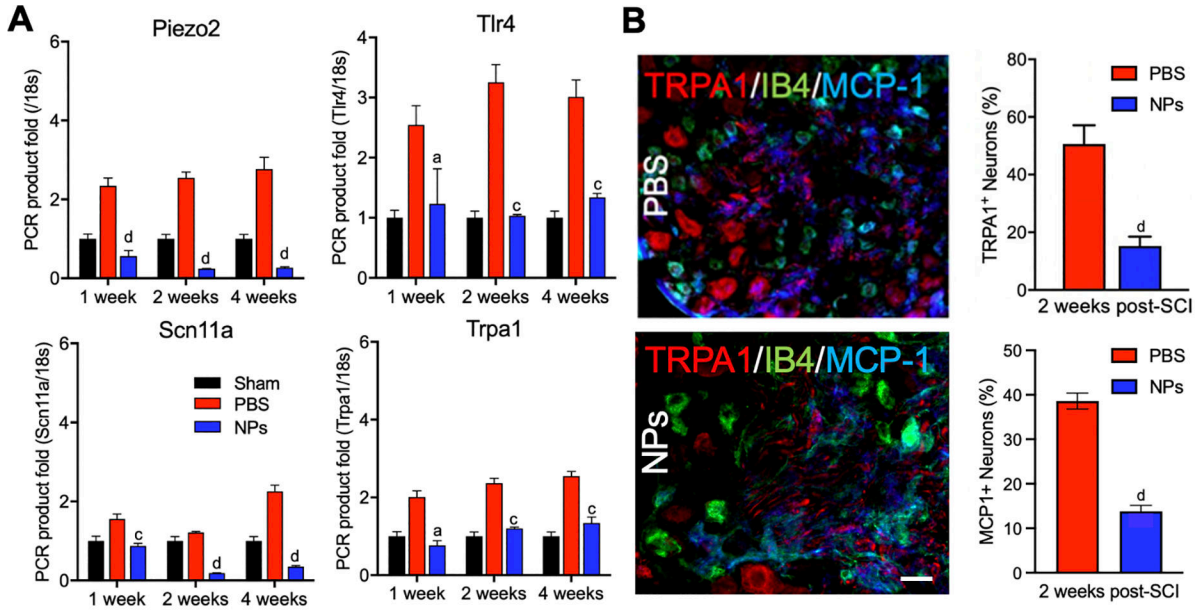

Figure 4: Nanoparticle treatment modulates peripheral mechanisms of neuropathic pain in the acute and subacute period following thoracic SCI.

DRGs were isolated following thoracic SCI at the indicated timepoints and processed for qRT-PCR. (A) NP treatment significantly reduced Piezo2, Tlr4, Trpa1, and Scn11a expression relative to PBS-treated mice between 1 and 4 weeks after injury. (B) The frequency of nociceptor TRPA1-positive neurons and MCP1-positive neurons within the DRGs were significantly decreased among NP recipients compared to PBS recipients at 2 weeks post-SCI. Representative images and quantification of TRPA1+ and MCP1+ neurons as a percent of all neurons are included. Error bars indicate mean +/− SEM; a two-way ANOVA with Tukey's post-hoc test (A) or unpaired t test (B) was used to determine statistically significant differences between NP and PBS treatment conditions; $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ (n=5).

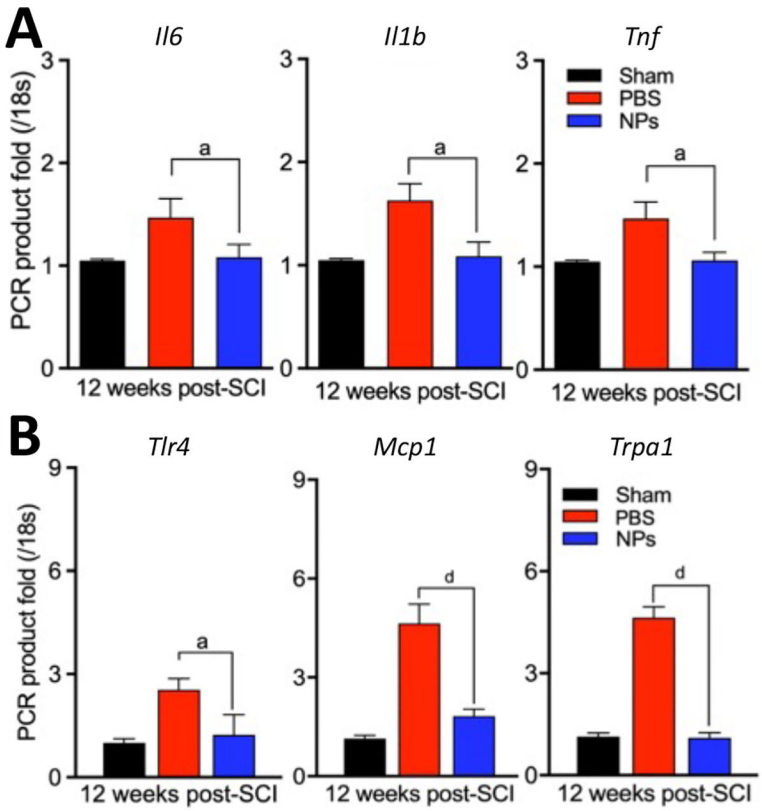

Figure 5: Nanoparticle treatment alters both central and peripheral mechanisms of neuropathic pain in the chronic period following thoracic SCI.

(A) Transcripts of the pro-nociceptive cytokines *Il6*, *Il1b*, and *Tnf* are reduced in the spinal cord at 12 weeks post-injury in NP-treated mice. (B) NP treatment reduced expression of the pro-nociceptive factors *Tlr4*, *Mcp1*, and *Trpa1* in the ipsilateral L2-L6 DRGs during the chronic phase of SCI. These results suggest that NPs reprogram both central and peripheral mechanisms of neuropathic pain in the chronic time frame following thoracic SCI. Error bars indicate mean +/− SEM; one-way ANOVA with Tukey's post-hoc test was used to determine statistically significant differences between NP and PBS treatment conditions; $^{a}p<0.05$, $^{d}p<0.0001$ (n=5).