Spinal macrophages resolve nociceptive hypersensitivity after peripheral injury

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SUMMARY

Peripheral nerve injury induces long-term pro-inflammatory responses in spinal cord glial cells that facilitate neuropathic pain, but the identity of endogenous cells that resolve spinal inflammation has not been determined. Guided by single-cell RNA sequencing (scRNA-seq), we found that MRC1+ spinal cord macrophages proliferated and upregulated the anti-inflammatory mediator Cdf163 in mice following superficial injury (SI; nerve intact), but this response was blunted in nerve-injured animals. Depleting spinal macrophages in SI animals promoted microgliosis and caused mechanical hypersensitivity to persist. Conversely, expressing Cdf163 in spinal macrophages increased Interleukin 10 expression, attenuated micro- and astrogliosis, and enduringly alleviated mechanical and thermal hypersensitivity in nerve-injured animals. Our data indicate that MRC1+ spinal macrophages actively restrain glia to limit neuroinflammation and resolve mechanical pain following a superficial injury. Moreover, we show that spinal macrophages from nerve-injured animals mount a dampened anti-inflammatory response but can be therapeutically coaxed to promote long-lasting recovery of neuropathic pain.

INTRODUCTION

Peripheral nerve injury induces a pro-inflammatory response in the spinal cord in rodents and humans and can cause chronic neuropathic pain (Albrecht et al., 2018; Costigan et al., 2009; Echeverry et al., 2017). Spinal microglia have traditionally been implicated in this pro-inflammatory response and, in males, promote persistent pain hypersensitivity (Griffin et al., 2007; Lacroix-Fralish et al., 2006b; Sorge et al., 2015; Uttam et al., 2018). However, treatments targeting pro-inflammatory mechanisms, particularly in microglia, were ineffective in patients with neuropathic pain (Eisenach et al., 2010; Ostenfeld et al., 2015; Vanelderen et al., 2015; Wang et al., 2014). These data raise the possibility that additional cell types might influence neuroimmune responses and pain hypersensitivity in neuropathic animals.

Single-cell RNA sequencing (scRNA-seq) studies indicate that the central nervous system (CNS) is composed of highly heterogeneous cell types (Sathyamurthy et al., 2018; Zeisel et al., 2018). Given the cellular complexity of the CNS, of which the spinal cord is a part, we sought to more comprehensively identify additional cellular responses that occur in a model of neuropathic pain (Shields et al., 2003). Here, we hypothesized that a more comprehensive understanding of which spinal cell types respond, or fail to respond, following nerve injury may reveal new regulators of pain hypersensitivity and new therapeutic targets for neuropathic pain.

RESULTS

Classification of spinal cell types in superficially injured and nerve-injured animals using scRNA-seq

The spared nerve injury (SNI) model of neuropathic pain produces long-lasting cellular and molecular changes in the spinal cord as well as mechanical allodynia compared with superficial tissue-injured (SI; also commonly referred to as sham-injured) controls, where mechanical allodynia resolves within 7 days (Figure S1A; Shields et al., 2003). To more comprehensively evaluate long-term cellular changes following superficial tissue injury versus nerve injury, we performed scRNA-seq on lumbar spinal cord segments from adult male mice 14 days post-injury (dpi; SI versus SNI; n = 10 and n = 9 replicates, respectively; Figures 1A and 1B). We sequenced ~20,000 total cells and performed unsupervised cell clustering, which detected 66 cell types that were hierarchically categorized into seven principal cell types (Loo et al., 2019; Figures 1C, 1D, and S1B–S1E). We characterized cell types using marker genes from mousebrain.org (Zeisel et al., 2018) and used binomial testing to identify three (or fewer) genes that molecularly distinguished each of the 66 cell types (Figures S2A and S2B; Table S1). There was a strong correlation between spinal neuron cell types in our dataset and previously published scRNA-seq datasets (Haring et al., 2018; Sathyamurthy et al., 2018; Figure S2C). Our data are accessible as an online web application at https://zylkalab.org/data, which plots
user-directed gene expression across all cell types from SI and SNI animals 14 dpi.

Expansion and proliferation of spinal macrophages are blunted in nerve-injured animals

We then evaluated changes in cell proportions between SI and SNI animals (Figures 1E and 1F). Two of the six cell types significantly reduced in SNI animals were highly similar macrophage cell types (Macro1, Macro2) that express the macrophage mannose receptor (Mrc1; also known as MMR or CD206) and lack expression of microglia-specific genes Tmem119 and Siglech (Bennett et al., 2016; Konishi et al., 2017; Figures S2B and S3A). Unlike spinal microglia, little is known about spinal macrophages in neuropathic pain mechanisms. Macro1 and Macro2 expressed genes associated with border associated macrophages (Figure S3A): antigen-presenting neuroimmune cells that localize along the edges of connective tissue in the CNS (Van Hove et al., 2019). Macro1 expressed higher levels of Ccr2, suggesting that these cells are peripherally derived and may represent macrophages in the dorsal roots (Stratton et al., 2018). Indeed, we detected MRC1+ macrophages in the subdural meninges that surround the spinal cord (meningeal macrophages [MMs]), in the spinal perivascular space (perivascular macrophages [PVMs]), and within the dorsal root (Figures SSB and S3C; Goldmann et al., 2016). The majority of IBA1+ microglia were MRC1+ (Figure S3D), which is consistent with our scRNA-seq data (Figure S3A) and previous studies (Jordão et al., 2019; Ruan et al., 2020). We focused subsequent analyses on spinal MMs and PVMs because they could be reproducibly identified and quantified on the basis of markers and anatomical location (Figure S3B).

The neuroimmune response in the spinal cord following peripheral nerve injury is temporally dynamic in terms of both cellular composition and molecular phenotypes (Echeverry et al., 2017; Zhang and De Koninck, 2006). To investigate whether spinal macrophages undergo a dynamic response following peripheral injury, we quantified the number of MRC1+ MMs and PVMs in naive (non-injured) animals and at 1, 3, 7, and 14 dpi in SI and SNI animals. We detected a significant interaction between time post-injury and both MM and PVM numbers (Figures 2A, 2B, S3E, and S3F). MM expansion was blunted in SNI animals compared with SI animals, whereas PVMs did not differ by type of injury (SI, SNI; Figure 2B). Pairwise Tukey post hoc tests revealed that both MM and PVM populations increase following SI relative to naive animals (7 dpi for MMs, 14 dpi for PVMs), whereas neither cell type expanded from baseline in SNI animals (Figures 2A, S3E, and S3F). We did not detect a difference in
laterality in macrophage populations apart from PVMs in SNI animals, which were more abundant ipsilateral to the side of injury at 14 dpi (Figure S3G). These longitudinal data suggest that spinal macrophage populations expand following a superficial tissue injury and that MM expansion is blunted when the nearby nerve is also injured.

We next injected SI and SNI animals with the thymidine analog 5-ethynyl-2’-deoxyuridine (EdU) to measure the number of MMs and PVMs that entered S phase 3–6 dpi (Figure 2C). Proliferation of MMs, assessed by EdU incorporation, was increased in SI animals relative to naive controls and relative to SNI animals (Figures 2C and 2D). In contrast, EdU labeling of PVMs was not significantly different among naive, SI, and SNI animals (Figure 2D). Together, these findings suggest that a superficial tissue injury promotes expansion of MMs, such as through proliferation and/or migration, and that this expansion is blunted in nerve-injured animals.

Transcriptional responses in spinal macrophages following injury

Hundreds of genes are differentially expressed in bulk spinal cord tissue following peripheral nerve injury (Griffin et al., 2007; Lacroix-Fralish et al., 2006b; Uttam et al., 2018), but these bulk RNA sequencing studies cannot reliably attribute gene expression changes to specific cell types. We thus performed differential gene expression analyses across all 66 cell types, comparing SI with SNI. We detected differentially expressed genes across most of the spinal cell types (a complete list of differentially expressed genes is provided in Table S2). We then used functional Gene Ontology (GO) analyses to gain further insights into how
spinal macrophages responded following nerve injury. None of the upregulated genes in Macro1 or Macro2 cells from SNI animals were associated with a consistent set of pathways. However, many of the downregulated genes in Macro1 and Macro2 cells were associated with cell cycle and proliferation GO terms, consistent with our histological and EdU labeling data above (Figure S3H; complete GO results for Macro1 and Macro2 are provided in Table S3).

Additionally, genes associated with immunoregulatory pathways were reduced in Macro1 and Macro2 cells from SNI animals, including Jak1, Stat3, and various interleukins (Figure S3H; Table S3). Cd163, which encodes a hemoglobin-haptoglobin scavenger receptor that marks anti-inflammatory macrophages and attenuates pro-inflammatory responses (Kim et al., 2006; Philippidis et al., 2004; Yang et al., 2016), was also reduced in Macro1 and Macro2 cells from SNI animals relative to SI controls (Figure 2E). We next histologically quantified CD163/MRC1 colocalization in MMs and PVMs over time. CD163 expression increased over time in both MMs and PVMs, whereas SNI blunted CD163 expression only in MMs (Figures 2F and 2G). Pairwise Tukey post hoc testing revealed that CD163/MRC1 colocalization in MMs increased only in SI animals at 14 dpi compared with baseline levels, whereas colocalization in PVMs increased from baseline at 14 dpi in both injury conditions (Figures S3I). Similar to macrophage expansion, CD163 upregulation was not enriched on either side of the spinal cord (14 dpi data; Figure S3J). Collectively, these data suggest that CD163 is upregulated in MRC1+ spinal macrophages following a superficial injury and that expression of this anti-inflammatory mediator is blunted in MMs when the injury includes the peripheral nerve.

**Spinal macrophage depletion leads to persistent pain and microgliosis**

In SI animals, proliferation and elevated levels of CD163 temporally coincided with resolution of mechanical hypersensitivity (Figure S1A). Therefore, we hypothesized that MRC1+ spinal macrophages might facilitate the resolution of mechanical hypersensitivity. To test this, we depleted MRC1+ spinal macrophages in SI animals and measured changes in mechanical sensitivity (Figure 3A). Selective depletion was achieved by intrathecally injecting mannosylated liposomes containing clodronate (Lip-C), which binds to MRC1 and induces apoptosis when endocytosed (Van Rooijen and Sanders, 1994). We verified that mannosylated liposomes selectively targeted MRC1+ spinal macrophages when loaded with the fluorescent molecule DiI (Lip-DiI; Figures S4A–S4C; note the absence of DiI in spinal microglia labeled with IBA1 and peripheral macrophages of the DRG). Animals intrathecally injected with Lip-C had fewer MMs and PVMs compared with empty liposomes (Lip-E) and saline control animals (Figures 3B and 3C). Lip-E and Lip-C
Figure 4. Targeted CD163 expression in MRC1+ spinal macrophages resolves mechanical hypersensitivity, microgliosis, and astrogliosis in SNI animals

(A) Experimental outline. (B and C) Representative images of GFPf and CD163 colocalization with MRC1 in MMs transfected with mpEmpty or mpCD163 on day 11 post-injection (B), which is 14 dpi (scale bar, 15 μm; see Figure S5A for transfection specificity) and (C) quantification. n = 5 animals per condition averaging two sections per animal. p values, two-sided Student’s t tests. Consecutive datapoints within 1/30th of the range are binned.

(D) Quantification of MMs and PVMs 14 dpi after mpEmpty and mpCD163 treatment. n = 5 and 4 animals per condition from mpEmpty- and mpCD163-treated animals, respectively, averaging two or three sections per animal (see STAR methods). p values, two-sided Student’s t tests. Consecutive datapoints within 1/30th of the range are binned. See Figure S5E for additional cohort data.

(E) Mechanical sensitivity at baseline and 13 dpi following mpEmpty or mpCD163 treatment. n = 5 animals per condition. Mean ± SEM. Bonferroni-Holm-corrected two-sided Student’s t tests between mpCD163 and mpEmpty animals at 13 dpi. *p < 0.05 and ***p < 0.001. See Figure S5D for additional cohort data.

(F) Left: mechanical sensitivity for SNI animals following mpEmpty or mpCD163 treatment at baseline and 1, 3, 7, 14, 21, and 28 dpi. Right: thermal sensitivity measured by Hargreaves assay at baseline and 7, 14, 21, and 28 dpi. n = 10 animals per condition. Mean ± SEM. Bonferroni-Holm-corrected two-sided Student’s t tests. *p < 0.05 and ***p < 0.001.

(G) Representative in situ hybridization images using Mrc1 and Il10 probes in SNI animals following mpEmpty or mpCD163 treatment at 7 dpi. Scale bar, 10 μm.

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administration had no effect on pain thresholds in naive (uninjured) animals (Figure 3D). In contrast, Lip-C-treated SI animals showed profound mechanical hypersensitivity relative to controls (Lip-E or saline injected; Figure 3D). Moreover, Lip-C-treated SI animals exhibited signs of microglial activation, as evidenced by an increase in the total area of spinal IBA1 and the number of IBA1+ cells (Figures 3E and 3F; relative to Lip-E controls). Microglial activation is a hallmark feature of peripheral nerve injury (Echeverry et al., 2017; Eriksson et al., 1993), which we reproduced (Figure S4D). Together, these data indicate that pain fails to resolve in SI animals when MRC1+ spinal macrophages are depleted. Moreover, these findings suggest that the pro- and anti-inflammatory balance between microglia and spinal macrophages, respectively, is dysregulated following nerve injury (Figure S4E).

**Increasing CD163 expression in spinal macrophages resolves neuroinflammation and neuropathic pain**

As spinal macrophages proliferate, upregulate Cd163, and are required for pain resolution in SI animals, we hypothesized that polarizing spinal macrophages toward an anti-inflammatory state in SNI animals might resolve persistent neuroinflammation and mechanical hypersensitivity. To polarize MRC1+ spinal macrophages in vivo, we intrathecally injected mice with mannosylated polyethylenimine nanoparticles (mPEIs) loaded with a mouse Cd163 expression plasmid. Control mPEIs were loaded with an empty expression vector. Experimental and control mPEIs were co-complexed with a farnesylated GFP (GFPf) plasmid to identify transfected cells. Similar to Lip-DiI, GFP transfection was restricted to MRC1+ spinal macrophages (Figure S5A). This mPEI formulation was chosen because (1) mannosse-conjugated nanoparticles target MRC1-expressing spinal macrophages (above), (2) CD163 signaling induces anti-inflammatory IL-10 production and secretion (Philippidis et al., 2004; Yang et al., 2016), (3) expression of CD163 decreases pro-inflammatory cytokine expression in lipopolysaccharide-stimulated human primary macrophages (Alvarado-Vazquez et al., 2017), and (4) CD163 can positively regulate cell proliferation (Chen et al., 2019).

We intrathecally injected SNI animals with mPEI-GFPf-Cd163 (mpCD163) or mPEI-GFPf-empty vector (mpEmpty) at 4 dpi (Figure 4A). SNI animals treated with mpCD163 had higher CD163 expression in MRC1+ spinal macrophages 14 dpi compared with mpEmpty-treated controls (Figures 4B, 4C, and S5B). Additionally, CD163 expression resulted in an expansion of MM and PVM populations (Figure 4D). Although mpEmpty and mpCD163 had no effect on mechanical sensitivity in naive animals (Figure S5C), mpCD163 treatment decreased mechanical sensitivity in SNI animals 13 dpi compared with mpEmpty controls (Figure 4E). The attenuation of mechanical sensitivity began by 7 dpi (3 days post-treatment) and persisted through 28 dpi (Figure 4F). Moreover, mpCD163 treatment alleviated thermal hypersensitivity by 13 dpi compared with mpEmpty controls (Figure 4F). To confirm anti-inflammatory polarization in spinal macrophages, we performed in situ hybridization to probe for the anti-inflammatory cytokine interleukin-10 (Il10). Treatment with mpCD163 increased the number of Il10 transcripts in Mrc1+ cells and increased the proportion of Il10+; Mrc1+ macrophages by 7 dpi (3 days post-treatment; Figures 4G and 4H). Consistent with anti-inflammatory polarization, IBA1 area, IBA1+ cell number, and glial fibrillary acidic protein (GFAP; an astrocyte marker) area were reduced in mpCD163-treated animals (Figures 4I and 4J). We independently replicated the behavioral and neuroimmune effects of targeted mpCD163 treatment in a separate cohort of animals (Figures S5D–S5F). Note that these effects on behavior were dosage dependent, as injecting mice with a higher amount of mpCD163 resulted in an abnormal expansion of MRC1+ macrophages, as predicted (Chen et al., 2019), but no statistically significant change (increase or decrease) in mechanical sensitivity in SNI animals (Figure S5G). Together, these data suggest that targeted elevation of CD163 in MRC1+ spinal macrophages of neuropathic animals can resolve neuroinflammation and attenuate mechanical hypersensitivity.

**DISCUSSION**

The spinal neuroimmune response following nerve injury has traditionally been viewed as unidirectional (pro-inflammatory) (Inoue and Tsuda, 2018), though the cells involved in this response can be coaxed to exhibit anti-inflammatory properties (Chen et al., 2018). Our data suggest a more complex relationship whereby microglia, and possibly other cell types including astrocytes (Figure 4J), are actively restrained by anti-inflammatory spinal MRC1+ macrophages following superficial injury, and this active restraint fails in a pathological nerve injury condition (Figure S4E).

The concept that resident macrophages can activate and resolve inflammation in other regions of the body is not new (Bedoret et al., 2009; McGaha et al., 2011; Miyake et al., 2007; Pinto et al., 2012; Serrats et al., 2010; Winnall et al., 2011). However, this concept has never been explored in the spinal cord in the context of neuropathic pain. Our discovery stems, in part, from a methodological difference in terms of how spinal cells are studied following a nerve injury. It is common for labs to examine the contralateral spinal cord of nerve-injured animals as a control or to compare responses in nerve-injured animals days to weeks after the nerve injury versus sham controls that are either not injured at all or are superficially injured (but examined at a time post-injury that differs from the experimental group). Our data show that MRC1+ macrophages are, in fact, activated in nerve-injured animals (increased number and CD163 expression), but what was unexpected is that this

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(H) Quantification of the mean number of Il10 transcripts in Mrc1+ cells (left) and of the mean proportion of Mrc1+ macrophages expressing >3 Il10 transcripts (right) from SNI animals after mpEmpty or mpCD163 treatment 7 dpi. n = 3 animals per condition averaging two sections per animal. p values, two-sided Student’s t tests. Consecutive datapoints within 1/30th of the range are binned.

(I and J) Representative images of IBA1 and GFAP expression from mpEmpty- and mpCD163-treated SNI animals 14 dpi (I) (left scale bar, 100 μm; right scale bar, 20 μm) and (J) quantification of IBA1 area, IBA1+ cells, and GFAP area. n = 5, 5, and 6 animals per condition for IBA1 area, IBA1 cells, and GFAP area, respectively. IBA1 datapoints generated from averaging two sections per animal. p values, two-sided Student’s t tests. Consecutive datapoints within 1/30th of the range are binned. DH, dorsal horn. See Figure S5F for additional cohort data.
activation response was blunted relative to the SI animals. This blunted activation process would have been overlooked or misclassified had we instead drawn comparisons with naive mice or with the contralateral spinal cord. The mechanisms that prevent spinal MRC1+ macrophages from being fully activated in nerve-injured animals are unknown and will require further study. However, spinal macrophages in nerve-injured animals are not permanently disabled, as targeted expression of Cd163, an anti-inflammatory mediator that MRC1+ macrophages endogenously upregulate in SI animals, increased spinal macrophage Il10 production, limited micro- and astrogliosis, and enduringly resolved neuropathic pain. Our data also point to the importance of increasing CD163 to a level that approximates that of SI animals, as non-physiological levels of CD163 stimulated excessive proliferation/expansion of spinal macrophages and had no effect on pain resolution. We speculate the failure of high-dose CD163 to resolve hypersensitivity may be related to the abnormally large number of MMs in the meningeal space, possibly impeding and/or counteracting MM function.

A variety of pathological pain conditions are associated with neuroinflammation in the spinal cord. Moreover, activated macrophages in the dorsal root ganglia were recently implicated in the initiation and persistence of neuropathic pain (Yu et al., 2020). However, the experimental approaches used in this study non-selectively ablated all resident macrophage subtypes and almost 90% of all mature circulating monocytes. Macrophages that upregulate anti-inflammatory mediators, such as Cd163, would presumably have been ablated as well, which we predict would obscure or eliminate any potential anti-inflammatory macrophage response in the DRG. Although our functional approaches showed greater cell type specificity, we cannot rule out a potential contribution of MRC1+ macrophages in the dorsal roots proximal to the spinal cord, which were also labeled by mannosylated liposomes/nanoparticles (Figures S4B and S5A). Other neuropathic pain models (chronic constriction injury and spinal nerve ligation) (Colburn et al., 1997; Hua et al., 2005) and inflammatory pain models (formalin and carrageenan) (Fu et al., 1999; Hua et al., 2005) induce neuroinflammation in the spinal cord, but whether an opposing anti-inflammatory response in spinal macrophages is present is unknown. Our work has the potential to stimulate further study of anti-inflammatory macrophage responses and the extent to which these responses are dampened in other pathological pain conditions.

Pro-inflammatory spinal microglia are activated in males and females following peripheral nerve injury, and it is increasingly recognized that inflammatory contributions to neuropathic pain differ between males and females (Brings and Zylka, 2015; LaCroix-Fralish et al., 2006a; Sorge et al., 2015). We acknowledge that our experiments were carried out in male animals, and that extensive future experimental work will be needed to evaluate the extent to which anti-inflammatory MRC1+ spinal macrophages participate in pain resolution in females and if these macrophages act via similar or distinct mechanisms.

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2021.02.018.

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AUTHOR CONTRIBUTIONS

J.K.N. and M.J.Z. designed the study. B.T.-B. assisted with histological experiments. L.L. flowed cells on Drop-seq days and provided scRNA-seq advice. J.M.S. established Drop-seq demultiplexing and alignment pipeline and provided scRNA-seq advice. J.K.N. performed experiments and bioinformatic analyses. J.K.N. and M.J.Z. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCE TABLE

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Critical commercial assays

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Experimental models: cell lines

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Experimental models: organisms/strains

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Software and algorithms

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mark J. Zylka (zylka@med.unc.edu).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
The accession number for the raw sequencing data, raw count data, normalized count data, and meta data reported in this paper is GEO: GSE134003. Code for the Shiny web application and histological analyses are available at https://github.com/jkniehaus/Niehaus2021.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice and behavior**
All procedures used in this study were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Mice were maintained three to five per cage on a 12 h:12 h light:dark cycle and given food and water ad libitum. All conditions were assigned randomly and therefore mice of different conditions were co-housed. Three to four-month-old male C57BL/6 mice were used for all experiments. Studies requiring > 10 total animals (e.g., Drop-seq, liposomal depletion, CD163 over-expression) were performed in two cohorts with equal numbers of mice per condition. No statistical methods were used to pre-determine sample size.

**Neuropathic pain model**
Spared nerve injury (SNI) surgeries were performed under a sterile environment as described previously (Shields et al., 2003). Adult male mice were anesthetized with 2% isoflurane and maintained at 1% isoflurane throughout the procedure. SNI and superficial injury (SI) surgeries were performed on the left hind limb of all mice. A 2 cm incision was made in the skin followed by separation of the biceps femoris muscle to expose the sciatic nerve. For SNI animals, the common peroneal and sural nerves were ligated with 6-0 silk suture and transected distal to the ligature. The injury was then closed with forceps and sealed with surgical clips. In SI animals the nerve was exposed but left unharmed. Animals were monitored daily until euthanasia to ensure proper wound healing.

**METHOD DETAILS**

**Mouse behavior**
Mechanical sensitivity measured by von Frey filaments was done using a set of eight filaments that bend at various forces (g): 0.407, 0.692, 1.20, 1.50, 2.04, 3.63, 5.50, 8.50. Each filament was pressed against the hind paw until the pressure either caused the filament to bend (no response) or elicit a withdrawal response. Hindpaw thermal sensitivity was measured using a Plantar Test apparatus (IITC) following the Hargreaves method (Hargreaves et al., 1988). Cutoff time was 20 s. Each animal was measured twice (> 15 minutes between each measurement) and the time to paw withdrawal was averaged. The experimenter was blinded to the animal identity and condition for all behavioral experiments.

**Intrathecal injections and dosing**
Mice were awake and restrained under a thick cloth for all intrathecal (i.t.) injections. Briefly, the injection area was trimmed of all fur and wiped with 70% ethanol. All i.t. injections were performed using a 25 µL Hamilton syringe with a 27-gauge beveled needle.
Cell depletion experiments were performed using the mannosylated macrophage depletion kit (CLD-8914, Encapsula Nano Sciences). Mice were injected i.t. (10 μL) with saline, empty liposomes, or clodronate-containing liposomes. Specificity of liposomal uptake was assessed by administering 10 μL of m-Fluoroliposome®-Dil (CLD-8924, Encapsula Nano Sciences) via i.t. injection. Mannosylated polyethylenimine (mPEI) DNA transfections were performed using the in vivo-jetPEI®-Man kit (Polyplus, 203-10G). mPEI-DNA complexes were made following the manufacturer’s protocol. Briefly, equal parts of plasmid DNA (pDNA) in 5% glucose was mixed with mPEI (N/P ratio of 6) in 5% glucose. The solution was vortexed, incubated at room temperature for 15 minutes, and subsequently administered to mice via i.t. injection. The pDNA composition (by mass) for transfections contained 50% plasmids expressing farnesylated GFP (pGFPf) and 50% plasmids expressing either Flag (pFlag) or the mouse Cd163 ORF (pCD163). All plasmids used human cytomegalovirus (CMV) promoters and were purchased commercially (pGFPf, Clontech; pCD163, Sino Biological, MG51057-CH) or were made in-house (pFlag). We found that freeze-thaw cycles reduced the transfection efficiency of mPEI nanoparticles. To control for lot-to-lot variability and variation in freeze thaw, we stored mPEI nanoparticles in aliquots sufficient for use in 20 mice, and first tested five different concentrations (n = 2 animals per concentration) in vivo to ensure that increased CD163 levels and increased cell number were comparable to that seen in SI injured animals. For cohort 1, animals received a total of 5 μg of pDNA (5 μL total injection; 1 μg/μL). For cohort 2, animals received a total of 1.25 μg of pDNA (5 μL total injection; 0.25 μg/μL).

**Single-cell isolation from spinal cord tissue**

Animals were anesthetized with pentobarbital and perfused with 95% O2/5% CO2 bubbled N-methyl-D-glucamine (NMDG) artificial cerebral spinal fluid buffer (Ting et al., 2014) supplemented with tetrodotoxin (1 μM), 6,7-dinitroquinoxaline-2,3-dione (20 μM), and D-2-amino-5-phosphonovalerate (20 μM, NMDG+) to prevent excitotoxicity during cell dissociation. Lumbar segments of the spinal cord were collected and then quickly chopped into 1 mm pieces and normalized in chilled, bubbling NMDG+ buffer for 15 minutes. Tissue was transferred to digestion buffer (NMDG+ with 2 mg/mL collagenase and 2 mg/mL protease from Streptomyces griseus) prewarmed to 37°C. Tissue was triturated twice (once after 20 minutes and again after 30 minutes) with a 10 mL serological pipette before being spun down at 600 x g for 10 minutes in a prechilled centrifuge (4°C). The supernatant was discarded, and the cell pellet was resuspended and triturated in 1 mL NMDG+ with a fire-polished Pasteur pipette. The cell suspension was added to and mixed with 4 mL of a density gradient solution (1.1 mL Percoll, 2.9 mL NMDG+). Cells were centrifuged at 600 x g for 10 minutes. Cell pellets were resuspended with 1 mL NMDG+, triturated with a fire-polished Pasteur pipette, and passed through a 100 μm filter. A subset of cells were stained with SYTOX Green (Thermo Fisher, 1:250 dilution) and counted manually on a hemocytometer.

**Drop-seq procedure and sequencing**

Methanol fixed cells were rehydrated with PBS. Drop-seq and library preparations were performed as described previously with the exception that the initial PCR amplification was increased to 15 cycles (Loo et al., 2019; Shekhar et al., 2016). Libraries from each sample were tagged with Nextera XT Amplification DNA Library Preparation Kit (Illumina) and sequenced on an Illumina HiSeq 4000. Twenty Drop-seq experiments were performed across four days and batch corrected bioinformatically.

**Histology**

Animals were anesthetized with pentobarbital and then transcranial perfused with pre-chilled PBS followed by freshly made 4% paraformaldehyde (PFA). Lumbar spinal cords were dissected (ensuring meninges remained intact) and post-fixed in PFA overnight. Tissue was then cryoprotected in 30% sucrose for 24 hours before being embedded and frozen in Neg-50 (Thermo Scientific; cat# 25322-68-3). Sections of lumbar segments (L3-L5) were collected and either mounted on Fisher Superfrost Plus slides (cat# 12-7500-02) or kept floating in PBS at 4°C. Sections were washed with PBS three times for five minutes before being blocked with 10% normal donkey serum (NDS) or normal goat serum (NGS) in PBS-T (0.3% Triton) for 30 minutes. Sections were then incubated with primary antibody in 5% NDS/NGS supplemented PBS-T (0.1% Tween) for either two hours (on slide staining) or overnight (floating sections) at room temperature. Sections were washed three times in PBS-T (0.1% Tween) before being incubated in donkey- or goat-conjugated secondary antibodies diluted in PBS for two hours. Sections were washed with PBS, counterstained with DAPI, and mounted (FluoroGel, Electron Microscopy Sciences). For CD163 immunostaining, heat-induced epitope retrieval was performed prior to immunostaining. Briefly, free-floating sections were incubated in 1x citrate buffer (10x Citrate buffer, pH 6.0, Antigen Retriever, Sigma-Aldrich, C9999) at 95°C for 15 minutes before being washed with PBS and subsequent immunostaining.

The following antibodies/concentrations were used: goat polyclonal anti-MRC1/CD206 (R&D Systems, AF25353; 1:200); rabbit monoclonal anti-CD163 (Abcam AB182422; 1:200); rat monoclonal anti-CD31 (PECAM-1) (BD Biosciences, 553370; 1:200); rabbit polyclonal anti-IBA1 (WAKO, 019-19741; 1:400); chicken polyclonal anti-GFP (Aves Labs, GFP-1010; 1:2000); Alexa Fluor 488 donkey anti-goat (Jackson ImmunoResearch Laboratories, 705-545-003; 1:500); Alexa Fluor 488 donkey anti-chicken (Jackson ImmunoResearch Laboratories, 705-545-155; 1:500); Cy3
donkey anti-rat (Jackson ImmunoResearch Laboratories, 712-165-153; 1:500); Alexa Fluor 647 donkey anti-rabbit (Invitrogen, A-31573; 1:500); Alexa Fluor 647 donkey anti-chicken (Jackson ImmunoResearch Laboratories, 703-605-155; 1:500).

To label cycling cells, 80 μg 5-ethyl-2’-deoxyuridine (EdU) in 100 μL of saline was administered by intraperitoneal (i.p.) injection once a day for four consecutive days. Following immunohistochemistry, tissue was treated with an EdU reaction solution (1.6 μM Alexa 488-azide, 4 μM CuSO₄, 100 mM sodium ascorbate, 10 mM Tris pH 8.5 in PBS) for 30 minutes at room temperature before being washed with PBS and mounted.

For in situ hybridization, the RNAscope Fluorescent Multiplex Assay (Advanced Cell Diagnostics, 320851) was performed following target retrieval according to the manufacturer’s protocol. Briefly, slides were dried at 60°C for 30 minutes and post-fixed in cold 4% PFA for 15 minutes. Slides were subsequently dehydrated in 50%, 70%, and 100% ethanol and allowed to air dry for 5 minutes at room temperature. Slides were then submerged in boiling 1x Target Retrieval buffer (Advanced Cell Diagnostics, 322000) for 5 minutes. Slides were briefly washed with room temperature distilled water, rinsed with 100% ethanol, and sections outlined with a hydrophobic pen (ImmEdge, Vector Labs). Finally, slides were pretreated with RNAscope Protease and allowed to incubate for 30 minutes at 40°C in a humidified hybridization oven before continuing with the RNAscope Fluorescent Multiplex Reagent Kit protocol (Document Number 320393-USM) using probes targeting Mrc1 (Advanced Cell Diagnostics, 317261) and II10 (Advanced Cell Diagnostics, 437511-C3). To capture in situ hybridization signal, z stack images were acquired with a 63x oil immersion objective for every cell expressing Mrc1 in a given section.

Image analysis/quantification
Z stack images were acquired with a Zeiss LSM 710 confocal microscope. Microscope settings were consistent between conditions. Images were imported into Fiji for quantification (Schindelin et al., 2012). Prior to quantification, z stacks were flattened to a maximum intensity projection. MMs and PVMs were differentially identified based on anatomical location. For all spinal macrophage analyses, only spinal sections with intact pia mater were included. To assess cell numbers, z stack tile images of the entire section (MMs and PVMs) or the ipsilateral dorsal horn (microglia, astrocytes) were acquired with a 20x objective. A DAPI mask of the stitched maximum intensity projection image was then overlaid on the channel of the protein of interest (e.g., MRC1 or IBA1), and the number of nuclei positive for the protein of interest were counted. For CD163 colocalization experiments, 8-10 z stack images of MRC1+ macrophages were acquired per section in with a 40x oil immersion objective. To quantify CD163 expression in MRC1+ macrophages, masks were generated from the MRC1 channel and the percent of overlapping area with the CD163 channel was calculated. In situ hybridization images were acquired with a 63x oil immersion objective and the signal was quantified following the manufacturer’s guidelines (Advanced Cell Diagnostics, SOP 45-006). Briefly, maximum intensity projections were produced from z stack images. Background intensity was measured and subtracted from each image before region of interests (ROIs) were drawn using based on DAPI channel for cells containing > 3 dots in the Mrc1 channel (a cell’s signal was considered lipofuscin and therefore was not used if its pattern was present across multiple channels). A mask of the II10 channel was then generated using the automatic threshold tool in Fiji (default algorithm), and the number of dots per cell was quantified. All histological quantification data can be found in Table S4. Additional information on image analysis and example code can be found at https://github.com/jkniehaus/Niehaus2021.

Quantification and statistical analysis
Statistical analyses and data visualization were performed in Excel and R. Statistical details (sample number, test used, and comparison details) can be found in figure legends. Sample numbers (n) indicate individual animals with the exception of Figure 2E, where n pertains to cells. For behavioral data, p values were generated by comparing SI and SNI groups at each force (Figures 1B, 3D, 4E, S5C, SSD, and S5G) or time point (Figures 4F and S1A) via two-sided Student’s t tests. The resulting p values were corrected using Holm-Bonferroni multiple comparisons method. For histological experiments where multiple sections were analyzed from each animal, the average was taken to summarize multiple sections. Complete information on histological experiments (both individual sections and average values per animal) can be found in figure legends and Table S4. Two-sided Student’s t tests, Holm-Bonferroni false discovery rate corrections, and two-way ANOVAs with Tukey’s HSD post hoc analysis for pairwise confidence intervals were used as indicated in figure legends. Line graphs are presented as the mean ± standard error of the mean. Boxplot whiskers extend to the highest/lowest value within ± 1.5 x inter quartile range. Example code for scRNA-seq analysis, ANOVAs, and data visualization can be found at https://github.com/jkniehaus/Niehaus2021. Experimental groups were considered significant if p < 0.05.

Processing and alignment of Drop-seq data
Raw reads were processed based on the Drop-seq Toolkit v1.12 (Macoasco et al., 2015; Shekhar et al., 2016) to identify and sort unique cells and unique molecular identifiers (UMIs) with corresponding exon reads. Processed reads were aligned to a mm10-hg19 hybrid genome using STAR (Dobin et al., 2013). Uniquely mapped reads were retained, and short or multi-mapped reads were discarded. Barcoded beads missing a base in their cell barcode were corrected as described in Drop-seq tools v1.12 (http://mccarrollab.org/wp-content/uploads/2016/03/Drop-seqAlignmentCookbook1.2Jan2016.pdf) (Shekhar et al., 2016; Loo et al., 2019).

Cell barcodes were determined to be species-specific if > 90% of the transcripts came from that species or were considered a doublet if neither species achieved 90% specificity. Cell barcodes were not considered if the transcript count sum (mouse+human) was less than 500. Gene expression matrices were then created using mouse-specific UMIs.

**Basic cell/gene filtration and cell clustering**

Cells expressing fewer than 400 unique genes or greater than 10% mitochondrial transcripts were removed. Genes were removed if they were not expressed in at least 30 cells with at least 3 transcripts. Technical batches were corrected in two rounds using ComBat (Johnson et al., 2007) based on Drop-seq day and animal cohort. The resulting batch-corrected gene expression matrix was median-centered and log-transformed to generate our final dataset (termed “Normalized expression” used throughout the text).

Cell clusters were established based on significant principal components (PCs, n = 60), whose eigenvalues were greater than those generated from randomly permuting the dataset (n = 1,000). Louvain clustering was performed with a silhouette score-optimized (Euclidean distance between each cell and the centroid of a given cluster) nearest neighbor parameter (Loo et al., 2019; Shekhar et al., 2016). This resulted in 20 cell clusters.

To resolve cell types with more specificity, the above pipeline was performed a second time on each of the initial 20 clusters. The second clustering iteration was identical to the first with two key cluster refinement exceptions. First, a set of oligodendrocyte-related genes were removed for the second round of clustering (apart from the two initial clusters that were classified as oligodendrocytes) in order to remove signal caused by myelin debris. Second, clusters lacking at least one marker gene (enriched by > 1.0 log10 fold-change compared to other clusters) were merged with the next most similar cluster (Pearson correlation). This resulted in 69 transcriptionally distinct clusters. After annotating each of the 69 clusters, three low-quality clusters were removed based on signs of doublets (expressing genes from two cell types), resulting in 66 final clusters.

The 66 final clusters were classified into seven principal cell types based on their distance from one another. A Pearson correlation distance \(d = 1 - r\) was computed between each cluster where \(d\) is correlation distance and \(r\) is Pearson’s correlation coefficient of the average normalized gene expression between two clusters. Clusters with a distance equal to or less than 1 were classified into the same principal cell type.

**Identification of marker genes and differentially expressed genes**

Binomial tests were used to identify cell markers based on the presence or absence of a given gene’s expression, as described previously (Shekhar et al., 2016). Briefly, this test determines the expression frequency of a given gene across cells in one population (\(N\) cells) compared to a second, reference population (\(M\) cells). As defined previously (Shekhar et al., 2016) the p value for this was computed using the following formula

\[
p_g = \sum_{k=n_0}^{N} \frac{N!}{k!(N-k)!} \gamma^k (1-\gamma)^{N-k}
\]

where \(\gamma = M_g / M\) refers to the presence frequency in the reference population. We determined cell type identities by testing for gene enrichment in one cell type compared to all others and correlating these markers with previously published datasets (Zeisel et al., 2018). We identified cell type-specific genes by comparing gene enrichment in one cell type with others that are hierarchically similar (e.g., Pericytes1 versus Pericytes2 and Pericytes3; hierarchical similarity determined by Pearson’s distance).

Similar to marker genes, differentially expressed genes were obtained using a binomial test (Shekhar et al., 2016) to compare SI and SNI cells within each cell type. To correct for variability and effect size, we required differentially expressed genes to have an FDR-corrected binomial q-value < 0.05 as well as a log2-fold change of > [0.5]. This threshold yielded robust differences in gene expression between SI and SNI animals and is optimal for identifying gene expression differences with zero-inflated data, like scRNAseq data. Functional gene ontology analysis was performed on differentially expressed genes from Macro1 and Macro2 cells using ToppFun using a significance cutoff of 0.05 (Bonferroni & Holm corrected false discovery rate) (Chen et al., 2009).

**Comparisons to other published scRNaseq datasets**

To compare our data to published single-cell/nuclei neuron clusters, average expression values were calculated for every gene from our dataset as well as those made available (Haring et al., 2018; Sathyamurthy et al., 2018). First, we subset the genes used based on variability (standard deviation greater than 0.1). Genes were removed if they were not present in all three datasets. Pearson correlation coefficients were calculated between clusters from our study and neuron clusters from Sathyamurthy et al. 2018 and Haring et al. 2018. The resulting matrix of correlation coefficients were plotted as heatmaps.
Supplemental information

Spinal macrophages resolve nociceptive hypersensitivity after peripheral injury

Jesse K. Niehaus, Bonnie Taylor-Blake, Lipin Loo, Jeremy M. Simon, and Mark J. Zylka
Figure S1 (Related to Figure 1). Mechanical sensitivity changes over time in SI and SNI mice, and benchmarks for scRNAseq viability, data analysis, and cell composition.

(A) Mechanical sensitivity between SI and SNI animals over time using different VF filaments. n = 10 animals per condition. Mean ± s.e.m. Bonferroni-Holm corrected two-sided t-tests compared to baseline (BL). *P < 0.05, **P < 0.01, ***P < 0.001. ns = not significant.

(B) Representative barnyard plot of species-specific scRNAseq with mouse spinal cord and human embryonic kidney cell spike-ins. Species indicated if 90% or more transcripts came from a single species, otherwise classified as “mixed.”

(C) Violin plots showing the number of unique genes and transcripts expressed in cells across each sample. n = 10 and 9 samples from SI and SNI animals, respectively.

(D) Louvain clustering optimization of the initial 20 clusters based on nearest-neighbor (NN) optimization (left) and silhouette score refinement (mid, right). The nearest neighbor parameter resulting in the highest average silhouette score was chosen. Silhouette scores calculated from Euclidean distance to a given cluster’s centroid. Clusters color-coded by principal cell type from Figure 1.

(E) tSNE plots of all cells expressing marker genes of the seven principal cell types.
Figure S2 (Related to Figure 1). Transcriptomic characterization of spinal cord cell types and comparison with published datasets.
(A) (Left) Heatmap of gene expression (average transcripts per million) for neuronal cell types ordered by relatedness (dendrogram, Pearson correlation coefficient). (Right) In situ hybridization of dorsal- and ventral- specific genes of the adult lumbar spinal cord from the Allen Brain Atlas (mousespinal.brain-map.org). Excit = Excitatory. Inhib = Inhibitory.


(C) Heatmap of the average normalized gene expression from our spinal neuron cell types and those generated from Haring et al. 2018 and Sathyamurthy et al. 2018.
Figure S3 (Related to Figure 2). Spinal macrophage transcriptomic characterization, cell marker specificity, and phenotypic changes following SI or SNI.

(A) Dot plots summarizing gene expression of neuroimmune cell markers. nTrans = Average number of transcripts per cell. % Expr = Percentage of cells expressing the corresponding gene. BAM = Border associated macrophage.

(B) Representative images of MMs and PVMs expressing MRC1 in the lumbar spinal cord. Top right scale bar = 100 µm. Bottom right scale bar = 20 µm.

(C) Representative image of MRC1 expression in dorsal root macrophages. Scale bar = 20 µm.

(D) Representative low (far left) and high (three right) magnification images delineating MRC1⁺ macrophages and IBA1⁺ microglia. MG = microglia. Left scale bar = 100 µm. Right scale bar = 20 µm.

(E) Representative images of PVMs in lumbar grey matter at 1, 3, 7, and 14 dpi in SI and SNI animals. Scale bar = 100 µm.

(F) Quantification of MMs (left) and PVMs (right) per section over time separated by condition. Data replotted from Figure 2B. Tukey’s HSD-adjusted p-values. P-values only shown if < 0.05. Consecutive datapoints within 1/30th of the range are binned.

(G) Quantification of MMs (left) and PVMs (right) in SI and SNI animals at 14 dpi ipsilateral (Ips) and contralateral (Cont) to the side of injury. n = 3 animals per condition. P-values < 0.05 are displayed, two-sided t-tests. Consecutive datapoints within 1/30th of the range are binned.
(H) Cell cycle and immunoregulatory gene ontology (GO) terms downregulated in SNI Macro1 and Macro2 cells relative to SI at 14 dpi. Complete list of input genes and GO terms in Table S3.

(I) Quantification of CD163;MRC1 colocation in MMs (top) and PVMs (bottom) over time. Data replotted from Figure 2G. Tukey’s HSD-adjusted p-values. P-values only shown if < 0.05. Consecutive datapoints within 1/30th of the range are binned.

(J) Quantification of CD163;MRC1 colocalization laterality (Ips = Ipsilateral to injury; Cont = Contralateral to injury; Tot = Total) in MMs (left) and PVMs (right) at 14 dpi. Consecutive datapoints within 1/30th of the range are binned.
Figure S4 (Related to Figure 3). Targeted uptake of mannosylated liposomes in MRC1+ macrophages and microgliosis in SI and SNI animals over time.
(A) Representative images of lumbar spinal cord 3 days post-injection of Dil-encapsulated mannosylated liposomes. Arrows, Dil⁺ MMR⁺ IBA1⁺ macrophages. Arrowheads, Dil⁻ MMR⁻ IBA1⁺ microglia. Scale bar = 50 µm.

(B) Representative images from Figure S4A of attached dorsal roots. Dotted line indicates separation of spinal cord and roots. Scale bar = 20 µm.

(C) (Top left) Low magnification image of lumbar DRG 3 days post-injection of Dil-encapsulated mannosylated liposomes. High magnification of DRG (right) and attached roots (left). Top left scale bar = 100 µm. Bottom scale bars in C = 10 µm.

(D) Representative images of IBA1 staining in the dorsal horn of naïve, SI, and SNI animals at 1, 3, 7, and 14 dpi. Scale bar = 100 µm.

(E) Temporal correlation between neuroimmune cell activation (microglia and MRC1⁺ macrophages) and mechanical (mech.) sensitivity following superficial injury and nerve injury. Schematic based on data in our current paper and the literature. Schematic is illustrative, meaning magnitude changes and temporal changes are approximate.
Figure S5 (Related to Figure 4). Mannosylated PEI specificity, additional data supporting targeted CD163 expression, reproducibility of low dose mpCD163 in a second cohort, and effects of high dose mpCD163.

(A) (Left) Representative images of mPEI transfection pattern (GFP) in the spinal dorsal horn 10 days post-treatment. Scale bar = 50 µm. (Right) High magnification image of boxed area. Scale bar = 20 µm.

(B) Representative images of CD163 and GFPf expression in MMs transfected (arrows) and not transfected (arrowheads) with mpCD163. Scale bar = 15 µm.

(C) Mechanical sensitivity in naïve animals before and 10 days after either empty (mpEmpty) or CD163 (mpCD163) treatment. n = 3 animals per condition. Mean ± s.e.m.

(D) Mechanical sensitivity in a second cohort of animals at baseline and 13 days post SNI injury (dpi) after either mpEmpty (n = 5) or mpCD163 (n = 5) treatment. Mean ± s.e.m. Bonferroni-Holm corrected two-sided t-tests between conditions at 13 dpi. *P < 0.05, **P < 0.01.

(E) Quantification of MMs and PVMs 14 dpi in mpEmpty or mpCD163 treated animals. n = 5 animals per condition, 1 section per animal. P-values, two-sided t-tests. Consecutive datapoints within 1/30th of the range are binned.

(F) Quantification of IBA1 area, the number of IBA1+ cells, and GFAP area 14 dpi after mpEmpty or mpCD163 treatments. n = 5 animals per condition, 1 section per animal. P-values, two-sided t-tests. Consecutive datapoints within 1/30th of the range are binned.

(G) (Top) Representative images of MMs 14 dpi after high-dose treatment of mpEmpty (top) and mpCD163 (bottom). Scale bar = 20 µm. (Bottom) Mechanical sensitivity at baseline and 13 dpi after high-dose treatment of either mpEmpty or mpCD163. n = 5
animals per condition. Mean ± s.e.m. No statistically significant differences between mpEmpty and mpCD163 groups.