More than Clearing the Clutter: The Imperative Role of Efferocytosis in Repair and Immune Reprogramming in the Damaged Nervous System

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABCA1	ATP binding cassette subfamily A member 1
AC	Apoptotic cell
AD	Alzheimer's disease
ADAM17	A disintegrin and metalloprotease 17
AKT	Ak strain transforming
AP-1	Activator protein 1
Arg	Arginase
Arp2/3	Actin related protein 2/3
AS	Atherosclerosis
ATF3	Activating transcription factor 3
ATP	Adenosine tri-phosphate
Aβ	Amyloid beta
BAI1	Brain angiogenesis inhibitor 1
BDNF	Brain-derived neurotrophic factor
C1q	Complement component 1q
C3	Complement component 3
Calr	Calreticulin
CaMK	Calcium/calmodulin-dependent protein kinases
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CEBP-1	CCAAT/enhancer-binding protein 1
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CR3	Complement receptor 3
CSPG	Chondroitin sulfate proteoglycans
CTB	Cholera toxin beta
CXCR	C-X-C motif chemokine receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DHA	Docosahexaenoic acid
DLK	Dual leucine zipper kinase
DNA	Deoxyribonucleic acid

DRG	Dorsal root ganglion
EC	Endothelial cells
ELMO	Engulfment and cell motility 1
ERK	Extracellular-signal-regulated kinase
Fb	Fibroblasts
GABA	Gamma-aminobutyric acid
GAP43	Growth-associated protein 43
Gas6	Growth arrest-specific protein 6
GC	Granulocyte
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GLUT	Glucose transporter 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GULP	Engulfment adaptor protein 1
GvHD	Graft-versus-host disease
ICAM	Intercellular Adhesion Molecule 1
IFN	Interferon
IgM	Immunoglobulin M
IL	Interleukin
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoproteein
LIF	Leukemia inhibitory factor
LPC	Lysophosphatidycholine
LRP1	Low density lipoprotein receptor-related protein 1
LXR	Liver X receptor
Ly6C	Lymphocyte antigen 6
Mac	Macrophage
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein-1
MES	Mesenchymal progenitor cells
MFG-E8	Milk fat globulin protein E8
MG	Myasthenia gravis
MHC	Major histocompatibility complex
Mo	Monocytes
MoDC	Monocyte-derived dendritic cell
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide

NaMN	Nicotinamide adenine dinucleotide phosphate
NCAM	Neural cell adhesion molecule
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK	Natural killer cell
NKG2D	Natural killer group 2d
NMDAR	N-methyl-D-aspartate receptor
NMJ	Neuro-muscular junction
NMN	Nicotinamide mononucleotide
NMNAT	NMN/NaMN adenylyltransferase
NO	Nitrous oxide
Nogo	Neurite outgrowth inhibitor
P0	Myelin protein zero
P75NTR	p75 neurotrophin receptor
PGF	Placental growth factor
PKC	Protein kinase C
PLCg2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2
PM	Plasma membrane
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
Rac1	Rac family Small GTPase 1
RAE1	Ribonucleic acid export 1
RAG	Regeneration-associated gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
SARM1	Sterile alpha and toll/interleukin-1 receptor motif-containing 1
SC	Spinal cord
SCG10	Superior cervical ganglion 10
SCI	Spinal cord injury
SIRPα	Signal regulatory protein alpha
SLE	Systemic lupus erythematosus
SNI	Sciatic nerve injury
SOCS	Suppressor of cytokine signaling
SPM	Specialized pro-resolving mediators
SPRR1	Small proline-rich repeat protein 1
STAT	Signal transducer and activator of transcription

TGFß	Transforming growth factor beta
TIM	Translocase of the inner membrane
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cells
Trem2	Triggering receptor expressed on myeloid cells 2
tRNA	Transfer ribonucleic acid
UCP2	Uncoupling protein 2
VEGF	Vascular endothelial growth factor
WAVE1	WASP-family verprolin homologous protein 1
WD	Wallerian Degeneration
YAP	Yes-associated protein 1

ABSTRACT

Evolutionarily, the nervous system and immune system have been intertwined for hundreds of millions of years. In healthy conditions, these systems work diligently to maintain homeostasis and proper functioning. In summation, they keep our bodies moving, our organs operating, our minds thinking, and our bodies safe from foreign pathogenic invaders. However, in the event of a challenge to homeostasis, like a traumatic injury, both systems engage complex signaling cascades to degenerate parts of cells that can't be saved, protect those that can, remove harmful debris, and regenerate and repair to again obtain homeostasis. A common system to study these complex response mechanisms is that of a peripheral nerve injury.

My research over the past several years has been focused around fully understanding the complex immune-nerve communication and consequences that occurs following peripheral nerve injury. The work herein keenly elaborates on the time course and content of the immune response after peripheral nerve crush injury. We show that granulocytes are the first to respond with infiltrating monocytes entering a few days later and finally dendritic cells about a week after injury. We however show little evidence of significant immune infiltration into dorsal root ganglia of the sciatic nerve and rather DRG-resident immune cell morphological changes. It is also demonstrated that mesenchymal progenitor cells are key in shaping the inflammatory milieu after injury. The requirement of *Csf2* for conditioning-lesion-induced dorsal column axon regeneration is evidenced as well as its role in skewing the inflammatory response. The dynamicity of the immune non-immune responses to nerve injury in wild-type an SARM1 knockout animals at multiple timepoints is compared and contrasted. Finally, we are the first group to show the occurrence of efferocytosis (the phagocytosis of apoptotic cells) in the injured nerve, identify a specific transcriptomic identity for macrophages engaged in this action, and investigate the anti-inflammatory signaling this process propagates.

CHAPTER 1:

Introduction

The Dynamic Interplay Between the Immune and Nervous Systems

1 Introduction: They Dynamic Interplay Between the Immune and Nervous Systems

1.1 Abstract

There exists a litany of cells types in the peripheral nerve environment including fibroblasts, endothelial cells, resident immune cells, myelinating Schwann cells (SCs), and most importantly for the function of the nerve, neuronal extensions called axons. In the case of the sciatic nerve, theses axons extend from their cells bodies in dorsal root ganglia (DRGs) near the spinal cord all the way to the muscles they innervate in the leg and foot. Many forms of injury can occur in the nerve including chronic constriction, complete transection, and crush. Following a crush injury one can break down the damaged axon into three portions: the injury site where the insult occurred, the proximal stump that is still attached to the cell body in the DRG, and the distal stump that is cut off from cell body by the injury **(Figure 1.1)**. The proximal stump will seal its ruptured membrane and begin to upregulate several pro-regenerative genes and proteins that will aid in its extension back toward its muscular targets. The distal stump however will undergo an active destructive process known as Wallerian degeneration. Following injury, SCs trans-differentiate into repair SCs and circulating immune cells infiltrate through the ruptured blood-nerve barrier. These cells act to remove cellular debris from the distal segment, create a cellular bridge through the hypoxic injury site, and release pro-growth factors to aid proximal segment regeneration. If all goes well, the peripheral nerve has the amazing capability to significantly regenerate and return function. Though, the repair process is not always complete and novel therapies to improve regeneration efficacy are needed.

Within the peripheral nerve there may be a yet unexplored biological phenomenon called efferocytosis, or the phagocytosis of apoptotic cells (ACs). This process occurs frequently in our bodies, every second of every day, to maintain homeostasis and prevent tissue damage that would result from secondary necrosis. To accomplish this, an AC releases several chemotactic “find me” signals into its environment to attract patrolling phagocytes. An AC also expresses several “eat me” signals—not involved in normal pathogen phagocytosis—that bind to specific efferocytic receptors, differentiating the two processes. Once bound, the AC is ingested and its many cellular components catabolized and either exported or utilized by the phagocyte for anabolic processes and to promote an anti-inflammatory and pro-resolution environment. Efferocytosis has received

much attention in other systems and diseases including atherosclerosis, auto-immune disorders, areas of immune cell development (spleen and thymus), and only recently stroke, but not in the peripheral nerve. Deciphering the role efferocytosis plays in the nerve injury response will not only further elaborate our understanding of immune function following injury, but also provide key insights into potential routes for effective therapies.

1.2 Tissue and Cellular Responses to PNS Injury

The ability of the peripheral nervous system (PNS) to spontaneously regenerate injured fibers has long been evidenced. Following injuries including sciatic nerve crush/injury (SNC/I), chronic constriction, and transection, peripheral neurons are able to initiate pro-growth and regenerative pathways that enable them to extend axons across the injury site and reconnect with their peripheral targets. For this to proceed properly, a litany of events needs to occur: 1) degradation of nerve fibers in the distal nerve stump; 2) subsequent removal of fragmented axons and myelin debris by glia and immune cells; 3) the activation of pro-regenerative signaling pathways in the surviving cell soma and axonal segment; 4) extension of the axonal growth cone past the injury site and toward target tissues; and 5) reconnection of the neuron to its target tissue and rebuilding of normal synaptic function. The first two stages will be discussed here and the following three in Introduction Section 1.3.

1.2.1 Distal stump degeneration

The sheer force of a neuronal injury causes immediate damage and necrosis to surrounding cells of the injury site including glia, cells associated with the vasculature, tissue-resident fibroblast-like and immune cells, and others. Neuronal extensions (axons) are damaged as well and depending on injury severity, the distal portions of injured axons may separate from their cell bodies. At this point, the axon can be separated into three distinct regions: the portion remaining in-tact with the soma is termed the proximal segment; the separated axonal portion is called the distal stump; and finally, the injury site where the direct insult has occurred **(Figure 1.1)**. In the peripheral nervous system (PNS), the surviving neuron and its proximal segment have the ability to regenerate (an event that will be discussed later), though first the distal segment must degenerate and be cleared away. Surprisingly, even though cut-off from its source of nutrients from the soma

(Gaudet et al., 2011a; Gordon, 2016; Gordon & Borschel, 2017; Lieberman, 1971), the distal segment appears relatively normal for up to 24hrs after transection injury (Coleman, 2005). Though quickly after this period the distal stump begins an active process of self-destruction termed Wallerian degeneration (WD) (Cattin & Lloyd, 2016), which is distinct from classic cell death and apoptosis pathways (Gerdts et al., 2016; Wang et al., 2012; Waller, 1850). Central to this process are several molecular initiators, they include elevation of intra-axonal calcium, activation of calcium-dependent proteases calpain, degradation of the nicotinamide adenine dinucleotide (NAD+)-synthesizing enzyme nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2), reduction in axonal metabolites NAD+ and adenosine triphosphate (ATP), and activation of executioner molecules including sterile alpha and TIR motif containing 1 (SARM1), dual leucine kinase (DLK), and axundead (Axed).

Immediately after injury there is a rapid influx of calcium which activates proteases like calpain that begin to digest the cytoskeleton (Adalbert et al., 2012; M. Ma, 2013; Stirling et al., 2014; P. Tang et al., 2015; Williams et al., 2014; J. N. Zhang et al., 2016a). Loss of cytoskeletal integrity and the rise in intracellular calcium (Avery et al., 2012; R. Villegas et al., 2014) activate one of the first key initiators of WD, the sensor molecule DLK. At the same time there is a loss of bioenergetic enzymes like NMNAT2 (J. Wang et al., 2005) and essential metabolites NAD+ (Belenky et al., 2007; Chiarugi et al., 2012; J. Wang et al., 2005) (which NMNAT2 produces from NMN or NaMN) and ATP. DLK senses abnormal cytoskeletal structure, an event that occurs following injury (Bounoutas et al., 2011; Hammarlund et al., 2009b; Marcette et al., 2014; J. E. Shin et al., 2012b; Valakh et al., 2013a; Watkins et al., 2013; Yan et al., 2009). Though DLK can also be regulated by changes in calcium (Yan et al., 2012), activity of ubiquitin ligases (Collins et al., 2006; Nakata et al., 2005), and phosphorylation states of other proteins (Watkins et al., 2013). DLK promotes further loss of NMNAT2 by promoting its turnover (Shin et al., 2012; Summers et al., 2018.; Walker et al., 2017). DLK also activates other downstream executions of axonal degeneration like c-Jun N-terminal kinase (JNK) and SARM1. Mutant mice lacking DLK that experience sciatic nerve crush showed delayed axonal degeneration (B. R. Miller, Press, Daniels, Sasaki, Milbrandt, & Diantonio, 2009).

JNK regulates microtubule dynamics and promotes axon fragmentation. Genetic disruption of all three JNKs (JNK1-3) protects axons from degenerating after injury (Bennett et al., 2001; B. R. Miller, Press, Daniels, Sasaki, Milbrandt, & DiAntonio, 2009). SARM1 interacts with axundead

and further promotes loss of NAD+ through enzymatic degradation (Gerdts et al., 2015; Essuman et al., 2017; Neukomm et al., 2017). Further loss of NAD+ leads to extended depletion of ATP and energetic failure with the distal stump becoming unable to continue normal cellular processes. ATP depletion also contributes to loss of mitochondrial membrane potential via a now defective Na^{+}/Ca^{2+} exchanger and calcium channels which leads to a secondary calcium wave (Gerdts et al., 2015; Gerdts et al., 2013; Loreto et al., 2015; Rishal & Fainzilber, 2014) and release of reactive oxygen species (ROS). Collectively, activated SARM1, elevated calcium, and reduced NMNAT2 (Jonathan Gilley & Coleman, 2010) and NAD lead to increased protease calpain activity which carries out degradation and collapse of the cytoskeletal elements of the distal stump.

1.2.1.1 Sterile Alpha and TIR Motif Containing 1 (SARM1)

Since the 1980s we have known that axonal degeneration after injury is an active rather than passive process. This was first realized with the serendipitous discovery of the Wallerian degeneration slow (Wld^{S}) mutant mouse whose axons would be spared following sciatic nerve injury for weeks (Lunn et al., 1989). Genetic analyses of these mice found they had a highly stable fusion protein of the bioenergetic enzyme NMNAT1 and a fragment of the ubiquitination factor UBE4B (Conforti et al., 2000; Mack et al., 2001). The Wld^{S} gene not only protects from axotomy, but also glaucoma, peripheral neuropathy, and moto neuron diseases (Beirowski et al., 2008; Ferri et al., 2003; Mi et al., 2005; Sajadi et al., 2004; M. S. Wang et al., 2002). Some have suggested a pro-survival role for Wld^{S} in the nucleus, where it predominates and interacts with SIRT1 (Araki et al., 2004; Mack et al., 2001). Though, later studies verified that it was the long-lived, and aberrantly-located, NMANT1 which substituted for the rapidly depleted NMNAT2 in the distal axon following injury that conferred axoprotection (Araki et al., 2004; Beirowski et al., 2009; M. S. Cohen et al., 2012; J Gilley & Coleman, 2010a; Mack et al., 2001; Sasaki & Milbrandt, 2010). NMNAT1 was able to maintain distal segment NAD+ levels and thus inhibit downstream degeneration mechanisms including activation of SARM1.

Years later a forward genetic screen in flies identified dSarm (the fly homologue of SARM1) as an essential gene for WD and solidified its role in axon degeneration in mammals (Osterloh et al., 2012). Intriguingly, genetic deletion of SARM1, akin to Wld^{S}, in the mouse led to prolonged distal stump survival up to 14 days post-injury (Gerdts et al., 2013b; Osterloh et al., 2012). Though SARM1 knockout mice, compared to Wld^{S} mice, seem to be a more useful tool in

studying WD mechanisms as Wld^S mice begin to show signs of denervation and neuromuscular abnormalities with age (Jonathan Gilley et al., 2017). *In vitro,* SARM1 knockout dorsal root ganglia (DRGs) are also protected from axon degeneration induced by withdrawal of nerve growth factor (Gerdts et al., 2013a). Further, activation of SARM1 in the absence of injury is sufficient to promote axon degeneration (Gerdts et al., 2015b; Essuman et al., 2017; Neukomm et al., 2017). Activated SARM1 not only promotes neuronal death following injury, but also in response to a variety of stressors including mitochondrial toxins, oxygen/glucose deprivation, and viral infection (P. Mukherjee et al., 2013; Summers et al., 2014).

A more recent body of evidence suggests that SARM1 is the main executioner of axon degeneration primarily through the enzymatic NADase activity of its TIR domain (Gerdts et al., 2015b; Horsefield et al., 2019). Accordingly, reduction in NMNAT2 and NAD+ occurs around 2-3 hours after axotomy, around the same time that SARM1 becomes active. The loss of NAD+ is drastically repressed in *Sarm1*-/- and catalytically dead SARM1 axons (Gerdts et al., 2015b; Essuman et al., 2017). Expectedly, as an essential biosynthetic enzyme, knocking out NMNAT2 in mice is embryonically lethal. Though NMNAT2 and SARM1 or Wld^S double knockout mice are completely healthy and live into adulthood (J Gilley & Coleman, 2010b; Jonathan Gilley et al., 2015; Milde et al., 2013). Though primarily thought to be triggered by DLK, another potential mechanism of SARM1 activation has been recently supported, focusing on the ratio of NMN to NAD+. This hypothesis postulates that NMN can activate SARM1 through its ARM domain and competes with NAD+ for this binding site. The loss of NMNAT2, which keeps NMN at low levels during homeostasis, leads to a buildup of its metabolite NMN which overwhelms NAD+ and activates the NADase activity of the SARM1 TIR domain (Loreto et al., 2020).

1.2.2 Removal of cellular debris

Following sciatic nerve injury, the environment of the distal nerve and injury site is laden with degenerating and fragmented axonal segments, myelin debris shed by Schwann cells (SCs), and other debris from surrounding supporting cells (endothelial cells, pericytes, vascular smooth muscle cells, and endoneurial fibroblast-like cells). This debris, including neurite outgrowth inhibitor (Nogo), myelin-associated glycoprotein (MAG), and chondroitin sulfate proteoglycans (CSPGs), is inhibitory for regeneration and must be cleared if the surviving axons are to regenerate (Case & Tessier-Lavigne, 2005; S. Chen & Bisby, 1993; Gordon, 2020; Martini et al., 2008a; M.

E. Schwab & Strittmatter, 2014; Yiu & He, 2006). This act is carried out primarily by surviving SCs, tissue-resident macrophages, and infiltrating myeloid cells (Mi et al., 2005; Vaquié et al., 2019a; Vargas & Barres, 2007; K. M. Wong et al., 2017). Injury signals prompt SCs to begin a complex reprogramming process into repair Schwann cells (rSCs). In this process, myelinating SCs downregulate several pro-myelination genes (MBP, P0, EEgr2, Krox20, Pmp22, and MAG) (Kristjan R. Jessen & Mirsky, 2008; Nocera & Jacob, 2020a) and upregulate immature/progenitor SC factors including p75NTR, GFAP, ErbB2, p38, Erk1/2, MAP, and NCAM (Arthur-Farraj et al., 2017; Z. L. Chen et al., 2007a; Gordon, 2009; Guertin et al., 2005; Kristjan R. Jessen & Mirsky, 2008; D. P. Yang et al., 2012). In preparation for the digestion of myelin, SCs also upregulate lysosomal/autophagic digestion genes (Gomez-Sanchez et al., 2015; Jang et al., 2016).

SC reprogramming is orchestrated by the master transcription factor c-Jun (Arthur-Farraj et al., 2012; Monje et al., 2010; Parkinson et al., 2008). Deactivation of c-Jun prevents the de-differentiation process and impedes both myelin removal and axon regeneration (Arthur-Farraj et al., 2012; Fontana et al., 2012; Hantke et al., 2014a; Klein et al., 2014a; Parkinson et al., 2004, 2008). Other signaling cascades have been implicated in SC trans-differentiation including Ras/Raf/MEEK/ERK, p38-MAPK, JNK-MAPK, Notch, YAP, Notch, and specific epigenetic modifications (Agthong et al., 2006; Arthur-Farraj et al., 2012; Mindos et al., 2017; Monje et al., 2010; Napoli et al., 2012; Parkinson et al., 2008; Woodhoo et al., 2009). Activation of the transcription factor STAT3 is also needed for long-term survival of rSCs (Benito et al., 2017). Damage associated molecular patters (DAMPs) and/or alarmins like DNA, cytochrome C, and H_2O_2 released by damaged mitochondria contribute to SC reprogramming via sustained upregulation of Erk-MAP signaling (Duregotti et al., 2015a). rSCs also leave quiescence, re-enter the cell cycle, and begin to proliferate (D. P. Yang et al., 2008). Interestingly in the distal nerve stump of Wld^S mice, SCs do not trans-differentiate into rSCs (Arthur-Farraj et al., 2012) suggesting a potential reason for the lack of clearance of the distal segment in these mice.

The adult mouse sciatic nerve harbors two physically separated populations of nerve-resident macrophages. Epineurial macrophages are located in the epineurium, the connective tissue surrounding the nerve, while endoneurial macrophages are located within the nerve fascicle in close contact with axons (M. Mueller et al., 2003; Ydens et al., 2020a). Following injury-induced reprogramming, rSCs begin to phagocytose myelin debris and axonal fragments alongside tissue-resident endoneurial macrophages. This occurs within 2 days post-injury before the peak influx of

circulating monocytes (M. Mueller et al., 2001), though infiltrating immune cells contribute to nerve repair in following days as discussed below. Clearance of myelin debris by rSCs and immune cells is thought to occur primarily through TAM receptor tyrosine kinases (RTKs) like Axl and MER (Lutz et al., 2017), other phosphatidylserine receptors like Tim-1/4 and CD300a, and various scavenger receptors including CR3, AI/II, TREM2, and Fc receptors (Cabral da Costal et al., 1997; Dejong & Smith, 1997; Safaiyan et al., 2021). Macrophage release of nitric oxide (NO) has also been implicated as one of the methods through which they aid the continual breakdown of myelin (D. Levy et al., 2001; Panthi & Gautam, 2017).

While local SCs and macrophages could likely complete the clearance process on their own, the process is expedited by recruitment of circulating monocytes that differentiate into phagocytic macrophages. Following injury, the blood-nerve-barrier is breached and allows for ample access to the injury site. To recruit blood-borne immune cells, several cytokines and chemokines that promote chemotaxis are released by SCs, endoneurial fibroblasts, tissue-resident macrophages, and fast-responding neutrophils. The main chemoattractant drawing in monocytes is C-C motif chemokine ligand 2 (CCL2), which binds monocyte C-C motif chemokine receptor 2 (CCR2) and CCR4 and promotes chemotaxis (Abbadie et al., 2003a; Charo & Ransohoff, 2006; Deshmane et al., 2009; Mack et al., 2001). Studies show many of the cell types listed above release CCL2, though SCs seem to be a primary source (Subang & Richardson, 2001) and may be triggered by rising levels of tumor-necrosis factor alpha (TNFα), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), or SARM1-JUN signaling (Subang & Richardson, 2001; Sugiura et al., 2000; Tofaris et al., 2002; Q. Wang et al., 2018). CCL2 mRNA is expressed in the injury site and proximal stump adjacent to the injury site, while the distal stump has widespread CCL2 (Cheepudomwit et al., 2008). This occurs as early as 12 hours post-injury and peaks between 1-3 days post-injury. Application of CCL2-neutralizing antibodies after nerve injury prevented macrophage accumulation in the sciatic nerve and reduced myelin clearance (Lindborg et al., 2017a; Perrin et al., 2005). Similar to the absence of SC response, there is not an increase in macrophage infiltration in Wld^S or CCR2 knockout mice after injury (Niemi et al., 2013).

SCs have also been shown to release TNFα, monocyte chemoattractant protein-1 (MCP-1), placenta growth factor (PGF) (Chaballe et al., 2011; Deshmane et al., 2009; Gaudet et al., 2011b; Martini et al., 2008b), IL-6, ciliary neurotrophic factor (CNTF) (He & Jin, 2016), IL-1ß, IL-1α, and LIF (Rigoni & Negro, 2020) which contribute to monocyte recruitment. Endoneurial

fibroblasts secrete CSF1 to attract monocytes (Groh et al., 2015) and injury site ROS (likely from axonal mitochondria and other sources) recruit leukocytes (Rigoni & Negro, 2020). Once circulating monocytes enter the injury site, the microenvironment instructs their differentiation into mature phagocytes which bolster the clearance of cellular and myelin debris through similar mechanisms as in SCs (Klein & Martini, 2016). How these cells exit or are removed from the nerve environment is still under investigation, but studies suggest they may either traffic back to local lymph nodes or undergo apoptosis and subsequent clearance (Kuhlmann et al., 2001). Ablation of macrophages via genetic toxicity or liposome delivery has shown to be detrimental to WD (Barrette et al., 2008b; T. Liu et al., 2000).

While macrophages are the primary professional phagocyte with the highest capacity for fiber debris clearance, neutrophils also play a significant role following nerve injury (Lindborg et al., 2017b). They infiltrate the injury site within hours and peak around 24h. Neutrophils are recruited by binding of their G-protein-coupled receptors (GPCRs), including C-X-C motif chemokine receptor 2 (CXCR1) and CXCR2, by DAMP molecules released after injury like DNA, histones, high mobility group protein B1 (HMGB1), N-formyl peptides, ATP, interleukin-1α, TNFα, CXCL1/2, and many others (G. Y. Chen & Nuñez, 2010; de Filippo et al., 2013; Eash et al., 2010; Nadeau et al., 2011a; Rajarathnam & Desai, 2020). Neutrophils work alongside SCs and macrophages to phagocytose debris by recognizing opsonized targets with complement receptor 3 (CR3) or Fc receptors like FcγRIIa (Daniele Notarangelo et al., 2017). They also secrete several pro-inflammatory factors that recruit additional circulating immune cells including CCL2. Further, neutrophils release several pro-growth factors and can promote phagocytic macrophages to release anti-inflammatory factors like transforming growth factor beta (TGFß) and IL-10 (Robertson et al., 2014a; Soehnlein & Lindbom, 2010a). Interestingly, when monocyte-derived macrophages are lacking, neutrophils become long-lived and take over a large portion of the phagocytic load in their stead (Lindborg et al., 2017b).

1.3 Intrinsic and Extrinsic Growth Mechanisms

If the distal axon segment has successfully undergone Wallerian degeneration and Schwann cells and immune phagocytes have cleared away debris and myelin and axon fragments, the axon stump connected to the neuronal cell soma can begin the regeneration process. Depending on the type and severity of injury, some axons will reconnect with their postsynaptic targets and

re-form synapses. Within the environment of a regenerating neuron, there are complex intrinsic and extrinsic growth mechanisms that come into play. Intrinsically the surviving neuron upregulates numerous regeneration-associated genes (RAGs), seals the terminus of the damaged proximal stump, restructures its cytoskeleton to form a growth cone, and initiates extension towards the injury site. Meanwhile extrinsically, tissue-resident and infiltrating immune cells, repair Schwann cells, fibroblasts, endothelial cells, and other elements work in synchrony to promote the extension of the injured peripheral nerve. These two broad mechanisms should not be thought of as separate entities, as close and frequent cross-talk between all cell types involved is required for proper and complete regeneration.

1.3.1 Intrinsic responses to injury

Immediately following PNS injury, the proximal stump retracts up to the nearest node of Ranvier and begins to re-seal its exposed cytoplasm and form a growth cone (Ertürk et al., 2007a; Fishman & Bittner, 2003; Kamber et al., 2009; Martin Kerschensteiner et al., 2005; Knöferle et al., 2010; Koley et al., 2019; Spira et al., 1993; J. N. Zhang et al., 2016b). Vesicles originating from the cell body endoplasmic reticulum and Golgi being anterogradely transported toward the axon are captured by a net of microtubules and fuse with the remaining proximal stump plasma membrane to accomplish this feat (Erez et al., 2007; Erez & Spira, 2008; Kamber et al., 2009; Spira et al., 2003). The sudden influx of intracellular calcium plays a paramount role in membrane sealing and growth (Bradke et al., 2012a; Czogalla & Sikorski, 2005; McNeil, 2005; Tuck & Cavalli, 2010; Yoo et al., 2003) and activates kinases like mitogen-activated protein kinase (MAPK) and calcium/calmodulin-dependent protein kinase (CaMK) (Ghosh-Roy et al., 2010; Mahar & Cavalli, 2018), phosphatases including LAR, PTP, and BEM-1 (Haworth K et al., 1998) and proteases like calpain to affect gene expression and remodel the cytoskeleton to a more mailable state (Bradke et al., 2012a; Ertürk et al., 2007a; Ghosh-Roy et al., 2010; Kamber et al., 2009; Martin Kerschensteiner et al., 2005; Spira et al., 1993; Verma et al., 2005a). Calpain restructures microtubules and neurofilaments as well as cleaves spectrin at the plasma membrane (PM), thus reducing the PMs rigidity (Gitler & Spira, 1998, 2002; Kamber et al., 2009; Spira et al., 2003). Superior cervical ganglion 10 (SCG10) has also been shown to play a role in the proximal stump as it promotes microtubule dynamicity and supports growth cone formation (M. R. J. Mason et al., 2002; E. Shin et al., 2014).

While the surviving neuron is attempting to reinstate homeostasis, there are numerous changes in gene and protein expression that initiate the process of regeneration. Sensibly, there is a reduction in synaptic transmission proteins including ion channels and synaptic proteins (Tedeschi et al., 2016; Y. Zhou et al., 2001) and increase in hundreds of regeneration-associated genes (RAGs) and proteins like GAP43, tubulin, actin, SCG10, CAP23, SCG10, SPRR1, galanin, gp130, ATF3, mTOR, STAT3, and others (Abe et al., 2012; Bonilla et al., 2002a; Bosse et al., 2006; Gumy et al., 2011; Holmes et al., 2000a; N. Lee et al., 2004a; K. Liu et al., 2010, 2011; M. R. J. Mason et al., 2002; K. K. Park et al., 2008; Y. Zhou et al., 2001; Zigmond, 2001, 2012). Increases and decreases have been documented in the expression of several particular mRNAs (Boeshore et al., 2004; Costigan et al., 2002). Many of these RAGs are expressed strongly during development as well, prompting some to suggest many regeneration programs are a reactivation of these developmental signaling systems. Retrograde signals transported from the injury site to the nucleus promote expression of many of these pro-regenerative genes and inhibit expression of pro-degenerative genes to initiate and sustain axon regeneration (Ben-Yaakov et al., 2012; Cavalli et al., 2005; Michaelevski et al., 2010; Rishal & Fainzilber, 2014b; J. E. Shin et al., 2012b; Terenzio et al., 2017; Xiong et al., 2010a). Additionally, several transcription factors; like c-Jun, STAT3, and ATF (Ben-Yaakov et al., 2012; Chandran et al., 2016; R. P. Smith et al., 2011), growth factors; like NGF, and cytokines; like LIF (Holmes et al., 2000a; Zigmond, 2001), have been identified to trigger expression or de-repression of several RAGs.

Growth-associated protein 43 (GAP43) has been shown to regulate actin cytoskeleton dynamics during regeneration and also acts as a substrate for protein kinase C (PKC) to promote axonal growth (Larsson, 2006; Laux et al., 2000). Some RAGs like activating transcription factor 3 (ATF3) have regulatory roles and instead of directly affecting regeneration mechanisms, they activate other RAGs like small proline-rich protein 1 (SPRR1), Galanin, and growth-associated protein 43 (GAP43). STAT3, potentially by working with mTOR (W. Chen et al., 2016; Gey et al., 2016), is necessary for the initiation of growth but not for growth cone elongation (Bareyre et al., 2011a; N. Lee et al., 2004b). At the injury site there is a reduction in phosphatase and tensin homolog (PTEN) and corresponding increase in mTOR expression (Terenzio et al., 2018). SCG10 is upregulated in motor and dorsal root ganglion neurons after sciatic nerve crush (Voria et al., 2006) and its signal prolonged if successful regeneration is delayed (M. R. J. Mason et al., 2002). It also localizes in the growth cone of regenerating fibers and its overexpression enhances neurite

outgrowth (Grenningloh et al., 2004). SCG10 not only promotes regeneration in the PNS, it is also upregulated in neurons following injury to the olfactory bulbs (Ronique Pellier-Monnin et al., 2001). SPRR1A increases over 60-fold following sciatic nerve injury and localizes to axonal compartments. Overexpression of SPRR1A improves neurite outgrowth while reducing its signaling detriments outgrowth (Bonilla et al., 2002b). Galanin has shown importance as pro-survival signal in DRGs following peripheral nerve injury. Genetic mutation of galanin prompted a nearly 3-fold increase in DRG apoptosis and a 35% reduction in regenerating peripheral neurons (Holmes et al., 2000b). In a different injury system, peripheral diabetic neuropathy, regular administration of galanin was found to improve sciatic nerve regeneration and reduce neuropathic pain (X. F. Xu et al., 2016).

Interestingly many proteins and pathways have dual roles in both regeneration and degeneration. For example, axon transport, mitochondrial and cytoskeletal dynamics, and microtubule-associated CRMPs show this dichotomous relationship (Blanquie & Bradke, 2018; L. H. Forbes & Andrews, 2017; Frati et al., 2017; Gitler & Spira, 1998; Pease & Segal, 2014; Prior et al., 2017; G. M. Smith & Gallo, 2018). Previously discussed factors including DLK, JNK, calpain, SCG10, and NMNAT2 also maintain this relationship (Y. Fang et al., 2012; J Gilley & Coleman, 2010c; Hammarlund et al., 2009a; B. R. Miller, Press, Daniels, Sasaki, Milbrandt, & DiAntonio, 2009; J. E. Shin et al., 2012a; Y. Suzuki et al., 2003; Watkins et al., 2013; Yan et al., 2009). DLK specifically, as a sensor of axonal integrity (Hammarlund et al., 2009a; Valakh et al., 2013b; Xiong et al., 2010b; Yan et al., 2009), has the ability to activate pro-regenerative and degenerative pathways (Hammarlund et al., 2009a; B. R. Miller, Press, Daniels, Sasaki, Milbrandt, & DiAntonio, 2009; Yan et al., 2009). In the proximal stump after nerve injury, activated DLK is retrogradely transported with JNK to the cell body where it can activate activator protein 1 (AP-1) and transcription factors like c-Jun (Cavalli et al., 2005; Drerup & Nechiporuk, 2013; Kenney & Kocsis, 1998; Lindwall & Kanje, 2005) that act to promote RAG expression including GAP43, PARG, CEBP-1, and shy-2 (Rishal & Fainzilber, 2014c; J. E. Shin et al., 2012a; Watkins et al., 2013). As mentioned previously in the distal stump, DLK can activate the primary executioner of Wallerian degeneration SARM1, leading to the fragmentation and degeneration of nerve fibers in the distal stump.

Mitochondria also have a key role in promoting regeneration by buffering local damage signals and regulating the high-energy task of axon extension (Q. Han et al., 2016; Rawson et al.,

2014). Reduction in mitochondrial transport suppresses regeneration (Cartoni et al., 2016, 2017; Q. Han et al., 2016), while increased motility or local injection of mitochondria into the injured nerve enhances neuroprotection (B. Zhou et al., 2016). Additionally, upon injury, mitochondria release another set of important signaling molecules, reactive oxygen species (ROS). Some evidence suggests ROS are primarily harmful molecules which damage cell function and integrity, inhibition of which improves recovery after injury (Caillaud et al., 2018; M. Kim et al., 2019; Y. Qian et al., 2018; Ullah et al., 2018). Though recent studies admonish these claims as ROS seem to be important in tissue healing and regeneration (Gough & Cotter, 2011; Holmström & Finkel, 2014; Niethammer et al., 2009; Rieger & Sagasti, 2011). Reduced levels of ROS actually impair regeneration (Love et al., 2013; Niethammer et al., 2009) and H_2O_2 can act as a chemoattractant for leukocytes imperative for clearing axonal and myelin debris (Gauron et al., 2016; Klyubin et al., 1996; Love et al., 2013; E. W. Miller et al., 2010; Romero et al., 2018). H_2O_2 can interact with regeneration-promoting Hedgehog (Gauron et al., 2016; Klyubin et al., 1996; Love et al., 2013; Meda et al., 2016; E. W. Miller et al., 2010; Romero et al., 2018) and activates MAPKs and other RAGs like Src (Foley et al., 2004). Additionally, H_2O_2 has been shown to aid in the trans-differentiation of Schwann cells (Negro et al., 2018), a process imperative for proper regeneration.

After the proximal axon stump is re-sealed, cytoskeletal elements come together and promote dynamic extension toward the injury site. Growth cones form within hours following a PNS crush injury (Ertürk et al., 2007b; Pan et al., 2003) and are composed of microtubules at its most proximal portion with actin extending distally to form lamellipodia and filopodia (Dent & Gertler, 2003). Much cytoskeletal remodeling must occur for formation of the growth cone including microtubules, neurofilaments, actin, spectrin, and ankyrin (Bradke et al., 2012b; Kamber et al., 2009; Verma et al., 2005b). Much of this is driven by the activity of calpain (Gitler & Spira, 1998, 2002; Spira et al., 2003). To extend, growth cone F-actin is continuously polymerized at the distal front and binds to existing and repaired extracellular matrices. This traction, with the help of actin-myosin motors, pulls the regenerating axon forward toward the site of injury (Dent & Gertler, 2003). Local translation of mRNA is also imperative for successful axon regeneration and provides local access to proteins that are critical for growth cone reformation and motility (Verma et al., 2005b; Vogelaar et al., 2009; Yan et al., 2009). As the growth cone extends toward the injury site, it actively senses and utilizes many extrinsic signals from repair Schwann cells, immune cells, and fibroblast-like cells that promote regeneration.

1.3.2 Extrinsic responses to injury

While activation of intrinsic growth capacities of surviving neurons is essential for axon regeneration, extending growth cones and re-growing axons would make almost no headway if it were not for the many extrinsic factors present to facilitate. The removal of cellular and myelin debris is an important first step in this process, though the regenerating axons now must cross the tumultuous terrain of the injury site and extend further distally to reach their target tissues. A variety of cells aid in this process including repair Schwann cells that guide and provide substrate for growth cone extension, macrophages that stimulate blood vessel formation, fibroblasts and epithelial cells that stimulate frequent cross-talk amongst these different populations to help build a bridge across the injury site, and finally the neuromuscular junction that provides chemotactic guidance ques. Most of these cells have multiple roles throughout the regeneration process, each of which will be described in detail below.

1.3.2.1 Schwann cells

As touched upon briefly when removal of debris from the injury site was discussed above, following injury Schwann cells (SCs) undergo a reprogramming process into repair Schwann cells (rSCs). Their pro-myelination genes are downregulated while pro-growth and proliferative genes are upregulated. The master regulator identified in this process is the transcription factor c-Jun (Arthur-Farraj et al., 2012; Fontana et al., 2012; I. S. Han et al., 2007; Harrisingh et al., 2004). Alterations of c-Jun through deactivation or genetic deletion significantly impairs rSC myelin clearance, trophic factor release, and regeneration (Arthur-Farraj et al., 2012; Fontana et al., 2012; Hantke et al., 2014b; Klein et al., 2014b; Parkinson et al., 2004, 2008; Zanazzi et al., 2001). Other important factors for the trans-differentiation process and maintenance of the rSC state include extracellular signal-regulated kinase (ERK) (Arthur-Farraj et al., 2012; Fontana et al., 2012; Harrisingh et al., 2004; Napoli et al., 2012), STAT3 (Benito et al., 2017), JNK (Monje et al., 2010; Parkinson et al., 2004, 2008; Yamauchi et al., 2008, 2011), sonic hedgehog (Hashimoto et al., 2008), and even epigenetic modifications including histone (de)methylation and (de)acetylation (Fuhrmann et al., 2018; Kristjan R Jessen & Mirsky, 2019; K. H. Ma & Svaren, 2016, 2018; Nocera & Jacob, 2020b).

One of the primary roles for rSCs in promoting repair is the formation of a cellular bridge extending through the damaged, hypoxic space of the injury (Parrinello et al., 2010a; Rosenberg et al., 2014; Y. Xiao et al., 2015) rSCs release basal lamina factors that deposit in the extracellular matrix of the injury site (Chernousov & Carey, 2000; Namgung, 2014) and mediate interactions between the axonal growth cone and rSC integrins (Bunge & Bunge, 1994; Carr & Johnston, 2017; Namgung, 2014). They also express Netrin 1 which has been shown to interact with the neuronal CDC receptor and guide axons across the bridge (Dominici et al., 2017; X. P. Dun & Parkinson, 2020; Jaminet et al., 2013; Rosenberg et al., 2014; Webber et al., 2011). rSCs increase expression of SRY-box transcription factor 2 (SOX2) and ephrin receptor EphB2 which interact directly with ephrin B ligands on fibroblasts (Coulthard et al., 2012; Parrinello et al., 2010a; Roberts et al., 2017). This signaling prompts an increase and re-localization of N-CAM and N-cadherin to the cell surface which prompts a signaling switch from contact-inhibition to attraction. This promotes rSCs to form extended cords and migrate collectively across the injury site (Arthur-Farraj et al., 2012; Parrinello et al., 2010a). Though they can be halted if they come into contact with any remaining debris, emphasizing the necessity for effective myelin removal beforehand (Parrinello et al., 2010a).

In addition to providing substrate for regenerating axons, rSCs release several pro-growth and survival factors necessary for successful regeneration. rSCs release exosomes containing RNAs, p75NTR, and other proteins which are ingested by re-growing axons and stimulate positive growth cone dynamics (Lopez-Verrilli et al., 2013). They also upregulate neuronal pro-survival and growth factors like c-Jun, GDNF, NT3, BDNF, NGF, and VEGF (Arthur-Farraj et al., 2012; Z. L. Chen et al., 2007b; Fontana et al., 2012; Kristjan R Jessen & Mirsky, 2019; Vaquié et al., 2019b) which foster a pro-regenerative environment. Released rSC cytokines including LIF, IL-6, and CNTF can bind gp130 receptors on neurons and activate JAK-STAT pathways to initiate upregulation of axonal growth systems (Bareyre et al., 2011b; Cafferty et al., 2001; Kristjan R Jessen & Mirsky, 2019; Madduri & Gander, 2010; Müller et al., 2007; P. D. Smith et al., 2009). Interestingly, rSCs release differential factors based on the type of neuron (sensory vs. motor) they myelinate (Bolívar et al., 2020; Brushart et al., 2013; Höke et al., 2006). Additionally, there are specialized perisynaptic SCs present at the neuromuscular junction where the axon meets innervated muscle fibers (Sanes & Lichtman, 1999; Son & Thompson, 1995). Following injury,

they maintain their placement and help guide regenerating axons to re-innervate motor endplates (Auld & Robitaille, 2003; Kang et al., 2014; Y. Xiao et al., 2015).

Once the axonal growth cones have extended across the bridge they reach another set of SCs in the distal segment that survived injury, maintained their basal laminae, and formed structures call the "Bands of Büngner." (Arthur-Farraj et al., 2012; Burnett & Zager, 2004; Fontana et al., 2012; Kristján R Jessen et al., 2015). Here, SCs express high levels of laminin, NCAM, N-cadherin, and other adhesion molecules all while adopting a lengthened, "band-like" morphology. These bands act as guiding tubes for regenerating axons to growth through and prevent them from following aberrant routes (Gomez-Sanchez et al., 2017; Hoffman-Kim et al., 2010; Kristján R Jessen et al., 2015; Parrinello et al., 2010a). Interestingly the transcriptome of these SCs differs from injury site rSCs as they show less of an increase in genes that promote cell motility, plasticity, migration, and proliferation (Arthur-Farraj et al., 2017; Clements et al., 2017a; Kristjan R. Jessen & Arthur-Farraj, 2019).

1.3.2.2 Immune cells

The sciatic nerve is full of tissue-resident macrophages that maintain immune surveillance. Their primary role is to preserve homeostasis by clearing regularly turning over cell populations, patrolling for pathogens, and support normal cell-cell interactions. Though, upon injury, they become activated and first shift to clearing away cellular debris and myelin. Though they have several additional parts to play in promoting regeneration including repairing injury-site vasculature, releasing pro-growth factors for neurons, and providing directional cues for both axons and SCs. As these tissue-resident immune cells quickly respond to injury, they are accompanied by infiltrating neutrophils which aid in debris removal. These, in partnership with SCs, and endoneurial and epineural macrophages, release several chemotactic factors (CCL2, TNFα, and CSF-1) to recruit circulating monocytes. Infiltrating monocytes then differentiate into macrophages and dendritic cells which further aid in removal of cellular debris and later release of trophic factors. Recent evidence has suggested natural killer cells (NKs) infiltrating a few days after peripheral nerve injury also play a role. Through the interaction of NK natural killer group 2D (NKG2D) receptor and injured axon ribonucleic Acid export 1 (RAE1), NKs release granzyme B to activate axon degeneration programs (Davies et al., 2019).

Though there are many immune cell types involved, macrophages seem to be one of the most important contributors to the injury and repair process. Accordingly, lack of macrophages due to pharmacological or genetic manipulation is quite detrimental to both Wallerian degeneration and regeneration (Barrette et al., 2008b; T. Liu et al., 2000). Comparatively, a delay in the recruitment of macrophages into the injured nerve, as in mice lacking common chemotactic molecules like Nos2, IL-1ß, or TNFα, led to a similar phenotype (D. Levy et al., 2001; Liefner et al., 2000; Perrin et al., 2005). In line with their varying roles, macrophages have also been known to exhibit a wide spectrum of phenotypes (S. J. Forbes & Rosenthal, 2014; Murray et al., 2014; Novak & Koh, 2013; Wynn et al., 2013) a point discussed in introduction section 1.4.

The injury site of a peripheral nerve becomes highly hypoxic due to the damaging of local blood vessels that would normally supply a steady stream of oxygen. This hypoxic environment encourages macrophages to secrete vascular endothelial growth factor (VEGF) to stimulate angiogenesis of new blood vessels and to aid in the initial formation of the tissue bridge that spans the injury site and later allows for a stable platform for rSC cords (Cattin et al., 2015). Macrophage slit guidance ligand 3 (Slit3) and VEGF work in unison to promote bridge formation across the injury site (Cattin et al., 2015; B. Chen et al., 2020; X. P. Dun & Parkinson, 2020; X. peng Dun et al., 2019). As many macrophages are responding locally at the site of injury, remaining outer layer macrophages in the epineurium work to provide a barrier to rSC joint migration and aids in their pathfinding across the injury site and toward the "Bands of Büngner" (Cattin et al., 2015). These macrophages express high levels of Slit3 (X. peng Dun et al., 2019) which interacts with rSC roundabout guidance receptor 1 (Robo1) that is upregulated due to increased Sox2 expression. This interaction acts as a repulsive signal to keep SCs on the right path (Blockus & Chédotal, 2016).

There are several factors expressed by immune cells that could putatively promote axon regeneration including arginase 1, oncomodulin, and NGF (DeFrancesco-Lisowitz et al., 2015; Gilad et al., 1996; Kurimoto et al., 2013; S. K. Lee & Wolfe, 2000; Leon et al., 2000; Sas et al., 2020; Schreiber et al., 2004; Y. Yin et al., 2003, 2006, 2009). Indirectly, macrophages promote regeneration by encouraging the expression of neuronal RAGs (Kwon et al., 2013; Niemi et al., 2013, 2016). While canonically thought to amplify the inflammatory response, neutrophils may also play a significant role in promoting repair. As mentioned previously they contribute early on to the phagocytosis of myelin fragments at the injury site, they are also known to release several

growth factors like NGF (Sas et al., 2020) and angiogenic factors like VEGF (Christoffersson et al., 2010; Dalli, Montero-Melendez, et al., 2013; Gong & Koh, 2010). They are too known to release macrovesicles containing nucleic acids and proteins that drive a pro-resolving phenotype in other myeloid cells (Dalli, Montero-Melendez, et al., 2013; Gasser & Schifferli, 2004). Additionally, their eventual apoptosis and subsequent phagocytosis by macrophages pushes these macrophages toward an anti-inflammatory phenotype by promoting release of TGFß and IL-10 (Robertson et al., 2014b; Soehnlein & Lindbom, 2010b), a topic that will be discussed later in the final introduction section on efferocytosis.

1.3.2.3 Fibroblasts and epithelial cells

While a majority of focus is placed on the crucial roles of SCs and immune cell in the injured PNS, they paint an incomplete picture. One must also consider additional surrounding cells including endothelial cells and fibroblasts (including fibroblast-like mesenchymal cells). These cells populate the nerve environment, cross-talk with SCs and macrophages, and are imperative in the formation of the tissue-bridge. In response to VEGF and other angiogenic factors, endothelial cells proliferate and migrate to generate new blood vessels, reduce the hypoxic state, and work to form the tissue bridge between the proximal and distal nerve stumps. As mentioned earlier, these vessels in turn provide a substrate and directionality for rSCs to migrate (Bergert et al., 2015; Cattin et al., 2015; Y. J. Liu et al., 2015; Potente et al., 2011; Tozluoğlu et al., 2013). Without endothelial cells, rSC migration is inhibited and they are unable to guide regenerating axons (Cattin et al., 2015). Once new blood vessels are solidified, they upregulate several survival factors like VEGF which activates PI3K/AKT signaling. This not only promotes endothelial cell survival (Warren & Iruela-Arispe, 2010) but VEGF is also known to have beneficial effects on regenerating fibers (Z. Fang et al., 2020; Kilic et al., 2006; Storkebaum et al., 2004). Another survival factor. Fibroblast growth factor (FGF), is also known to have similar effects on endothelial cells and nerve fibers (Beenken & Mohammadi, 2009; Grothe et al., 2006; Guzen et al., 2012; Jungnickel et al., 2006). Finally, endothelial cells have been extensively evidenced to interact with and direct the immune response to damage and pathological circumstances (Danese et al., 2007; Y. Shao et al., 2020).

Fibroblast-SC interactions are essential for regeneration (Cattin et al., 2015; Parrinello et al., 2010b). Fibroblast-released TGFß not only shifts the inflammatory milieu toward a pro-

resolving phenotype, but also promotes shifts in SC gene expression in their reprogramming toward rSCs (Clements et al., 2017b). These cells also promote collective SC migration across the injury site through interactions of Tenascin C and ß-1 integrin (Z. Zhang et al., 2016). They also aid in the degradation of myelin in the sciatic nerve (Goodrum et al., 1994; Schubert & Friede, 1981). Further, fibroblasts can play a role in regulating the inflammatory response in a variety of tissues (Buechler & Turley, 2018). They can upregulate factors like IL-6, CCL20, and IL-1ß under inflammatory conditions or following injury to magnify the immune response and/or recruit circulating immune cells (Hideya Kitamura et al., 2011; H. N. Nguyen et al., 2017; Richard et al., 2012). Contrastingly, they too can suppress inflammation and maintain/promote homeostasis by release of IL-33, IDO1, and cultivation of inhibitory regulatory T cells (Tregs) (Haniffa et al., 2007; Kolodin et al., 2015; Mahapatro et al., 2016; Tykocinski et al., 2017).

1.3.2.4 Neuromuscular junction

Intriguingly, PNS axons that are able to regenerate after injury can sometimes find their original post-synaptic partners with surprising accuracy (Q. T. Nguyen et al., 2002). However, this is not always the case (Lingappa & Zigmond, 2013) and the number of axons that reach their targets can be quite partial (Gordon et al., 2009). Degenerating nerve terminals release several DAMPs including ATP, and mitochondrial DNA and cytochrome c which activate ERK1/2 in SCs and promote their activation though calcium-dependent pathways (Duregotti et al., 2015b; Negro et al., 2016). Evidence also suggests some factors released at the nerve terminal like CXCL12α (also called SDF-1) can act as a chemoattractant for regenerating and extending axons via binding to axonal CXCR4. The administration of recombinant CXCL12α *in vivo* and *in vitro* enhances axonal growth after neurotoxin synapse degeneration and quickens motor recovery. Sensibly, inhibition of CXCL12α with a neutralizing antibody significantly delays recovery (Negro et al., 2017). Direct pharmacological agonism of CXCR4 also quickens the rescue of neurotransmission after injury, while antagonism is preventative (Negro et al., 2019). Zanetti et al., further verified the significance of this receptor-ligand interaction in regeneration following sciatic nerve crush on top of toxin-induced synaptic degeneration (Zanetti et al., 2019).

1.4 “Bad” Inflammation and “Good” Inflammation

Upon injury, insult, or infection to any biological system, there is inevitably an immune response. This is triggered primarily by factors released by injured cells into the blood and surrounding area. These factors recruit first-responding immune cells (often neutrophils), which begin phagocytosis of debris and subsequent release of additional inflammatory and chemotactic signaling molecules. These proceed to recruit more specialized innate immune cells like monocytes/macrophages and dendritic cells, which can activate the adaptive arm of the immune system (B and T cells) if needed. While this is the stereotypical story of the immune system's recruitment and activation, instilled within this formula are vast nuances and distinct specializations that have crucial and long-lasting consequences to human health, tissue repair, and homeostatic function.

1.4.1 The harms of immunity in the nervous system

In most popular and scientific culture, a commonly relayed message is that inflammation is inherently bad. This is indeed true in some cases. Evidence shows that excessive inflammation is deleterious through production of toxic cytokines, free radicals, neurotransmitters, and proteases (Czeh et al., 2011; Glass et al., 2010; M. Kerschensteiner et al., 2009; Takeuchi, 2010; Wee Yong, 2010). When an inflammatory response persists, continued production of inflammatory cytokines and ROS can result in synapse loss, cell death, and functional impairment in the nervous system (Amor et al., 2010; Bao et al., 2009; Hein & O'Banion, 2009; Horn et al., 2008; Kigerl et al., 2009a; Rao et al., 2012). Chronic inflammation is also associated with neurodegenerative disorders like Alzheimer's disease (AD) (Sokolova et al., 2009; Walter et al., 2007). Amyloid beta (Aβ), a key feature of AD, induces inflammatory astrocyte and microglia phenotypes that can contribute to neurodegeneration (S. Liu et al., 2012; Meraz-Ríos et al., 2013a; Wirz et al., 2013). Aβ can directly bind and activate microglial pro-inflammatory receptors like toll-like receptor 2 (TLR2), TLR4, cluster of differentiation 14 (CD14), and TLR6 (Landreth & Reed-Geaghan, 2009; S. Liu et al., 2012; Stewart et al., 2010). Expectedly, microglia surrounding Aβ plaques exhibit increased inflammatory signaling (W. S. T. Griffin et al., 1995). Forming a positive feedback loop, inflammation can also increase Aβ species by enhancing expression of its precursor protein and activating necessary cleavage enzymes (Karran et al., 2011). Another CNS disease with chronic inflammation, multiple sclerosis (MS), is commonly thought to be propagated by autoreactive B cells and certain subsets of T cells which cause white matter loss and subsequent

neurodegeneration (Jadidi-Niaragh & Mirshafiey, 2011; Linker et al., 2002; Lovas et al., 2000; Reboldi et al., 2009; samoilova, EB; Horton, JL; Hilliard, B; Liu, TST; Chen, 1998). The inflammation they propagate leads to axonal demyelination in part by toxic signaling to oligodendrocytes (Tanner et al., 2011).

Another set of afflictions effected by inflammation include spinal cord injury (SCI) and neuropathic pain. SCI is followed by a large influx of inflammatory cells which, if persistent, can contribute to further neuronal death on top death from the initial insult (Ankeny et al., 2006; Bastien et al., 2015; David et al., 2012; Hansen et al., 2013; L. Yang et al., 2004). It was found that preventing infiltration of neutrophils and/or macrophages into the spinal cord can improve recovery (Bao et al., 2004, 2008; Popovich et al., 1999). While these cells may not be fundamentally destructive, their surrounding pro-inflammatory environment containing pro-inflammatory factors like TNFα, IL-1ß, and free radicals which may drive them toward a more damaging phenotype (Bao et al., 2004, 2008; David & Kroner, 2011; Genovese et al., 2008; A. Kumar et al., 2013; Moskowitz et al., 2010; Zong et al., 2012). Metalloproteinases and pro-inflammatory cytokines like TNFα released by granulocytes, macrophages, and epithelial cells may also make neurons more susceptible to excitotoxicity (Love, Louis, & Ellison, 2008). Though, macrophages and spinal cord-resident microglia are relatively plastic. If the environment were to change with a reduction of these factors, they can shift and take on a pro-resolving phenotype and begin to alleviate pathology (Davis et al., 2013; Guerrero et al., 2012; Shechter et al., 2013).

A few weeks after peripheral nerve injury (PNI) inflammation normally subsides and returns to homeostatic levels, though if there is no resolution it can lead to chronic neuropathic pain (Huh et al., 2017; Ji et al., 2016; Littlejohn, 2015). Activation of spinal cord microglial purinergic receptor P2 purinoceptor 7 and TLRs can lead to release of inflammatory mediators IL-1ß and TNFα through nuclear factor-κB (NF-κB), all known to be crucial for promotion of neuropathic pain (Chessell et al., 2005; Clark et al., 2010; Heneka et al., 2014; Inoue, 2006; Ji et al., 2014; K. Kobayashi et al., 2011; Peng et al., 2016; Tanga et al., 2005). IL-1ß acts on spinal cord neurons and increases their excitatory synaptic transmission by regulating N-methyl-d-aspartate receptor (NMDARs) and gamma-aminobutyric acid (GABA)-release (Clark et al., 2015; Kawasaki et al., 2008; Reeve et al., 2000; Viviani et al., 2003). TNFα alters synaptic plasticity and pushes astrocytes toward a reactive phenotype (Kronschläger et al., 2016; Liddelow et al., 2017). PNI leads to increased brain-derived neurotrophic factor (BDNF) release from spinal cord

microglia which binds TrkB receptors on surrounding neurons which downregulates the potassium-chloride transporter channel KCC2, leading to an increase in intracellular Cl^- ions. Increased Cl^- converts GABAergic inhibition in these neurons to excitation, shifting the balance toward synaptic over-excitation and contributing to neuropathic pain (Coull et al., 2005; Guan et al., 2016; Hildebrand et al., 2016; Keller et al., 2007; Trang et al., 2009; Tsuda et al., 2003; Tsuda, Kuboyama, et al., 2009; Ulmann et al., 2008). Accordingly, specific deletion of BDNF from microglia reduces PNI-induced pain (Sorge et al., 2015). Preventing microglial proliferation also improved neuropathic pain following PNI (Gu et al., 2016).

Patients with neuropathy have two to three times higher IL-2 and TNF and lower IL-10 and IL-4 mRNA compared to healthy patients (Üçeyler et al., 2007). IL-4 was 20-fold higher and TNF much lower in patients with painless neuropathies compared to those with painful conditions (Doupis et al., 2009). In animal models of neuropathic pain, IL-4 has a significant analgesic effect (Hao et al., 2006; Kiguchi et al., 2015; S. Sun et al., 2016; Vale et al., 2003). IL-4 knockout mice with peripheral nerve injures have higher pain levels along with increased inflammation in their spinal cords pain (¨ C ¸eyler et al., 2011). Other factors known to be involved in promoting nerve hypersensitivity following PNI include CX3CR/L1 (Clark et al., 2009; Staniland et al., 2010; Zhuang et al., 2007), CCR5 (Matsushita et al., 2014), IFNγR1 (Tsuda, Masuda, et al., 2009), CSF1R (Guan et al., 2016; Okubo et al., 2016), Trem2 (M. Kobayashi et al., 2016), complement (Carroll, 2004; R. S. Griffin et al., 2007), prostaglandins (Galli et al., 2005; Kalinski, 2012), IL-6 (Kawasaki et al., 2008), purinergic receptors (Barragán-Iglesias et al., 2014; K. Kobayashi et al., 2011; Tsuda et al., 2003), and CCR/L2 (Abbadie et al., 2003b; Echeverry et al., 2011; Thacker et al., 2009).

Inflammation not only has a negative connotation in the CNS, but in the PNS as well including diabetic neuropathy. Most patients with diabetes (60-70%) are also afflicted by some form of neuropathy (Edwards et al., 2008; Sinnreich, M; Taylor, BV; Dyck, 2005; Smith, AG; Singleton, 2012; Tracy et al., 2009). Advanced neuropathy eventually results in segmental demyelination and axon degeneration (Dyck & Giannini, 1996). Some evidence suggests increased levels of long-chain fatty acids and oxidized cholesterol penetrate the blood-nerve barrier and initiate neurogenic inflammation and recruit innate and adaptive immune cells (Nowicki et al., 2010; O'brien et al., 2014). Diabetic patients with neuropathies often have heightened levels of several inflammatory factors like TNFα, IL-6, ROS, and master inflammatory transcription factor

NF-κB (Cameron & Cotter, 2008; Hussain et al., 2013; Salmenniemi et al., 2005; Shoelson et al., 2006; Timmins et al., 2009; Vincent, AM; Callaghan, CC; Smith, AL; Feldman, 2011; Y. Wang et al., 2006). In animal models of diabetes, many pro-inflammatory mediators are increased and dysregulated in early and late disease (Hinder et al., 2018; Hur et al., 2015; Lupachyk et al., 2012; O'brien et al., 2015). Adipocytes, fat cells with increased accumulation in diabetics, release inflammatory mediators like TNFα, leptin, and adiponectin (Shimomura et al., 2002).

Myasthenia gravis (MG) is an auto-immune disorder characterized by degenerating neuromuscular junctions (NMJ) and resulting muscle weakness and fatigue (Gilhus & Verschuuren, 2015). The focal point of MG pathology is dysfunction in the thymus, where structural and functional abnormalities prevent developing auto-reactive T cells from being eliminated (Berrih-Aknin et al., 1987; Cavalcante et al., 2011). Normally germinal centers—where B cells mature—are rarely present in the thymus, though in MG many germinal centers can be found and are surrounded by acetylcholine receptor-expressing myeloid cells (Roxanis et al., 2002; Sims et al., 2001). Auto-antibodies and complement factor opsonization of NMJ nicotinic acetylcholine receptor, muscle-specific tyrosine kinase, and/or lipoprotein receptor-related protein 4 facilitate the destruction of the NMJ (Conti-Fine et al., 2006; Dalakas, n.d.; Tüzün & Christadoss, 2013). Many chemokines, cytokines, and lymphocytes are increased in the MG thymus including CCL17, CXCL10, IFNs, MHC-II, CCL5, CCL21, IL-17, IL-32, CXCL12, IL-6, and CCL19 (Aricha et al., 2011; Berrih-Aknin et al., 2009; Cordiglieri et al., 2014; Feferman et al., 2005; le Panse et al., 2006; Meraouna et al., 2006; Poëa-Guyon et al., 2005; Roche et al., 2011; A Uzawa et al., 2014; Akiyuki Uzawa et al., 2016; Z. Wang et al., 2012; Xie et al., 2016). Further, the number of circulating inflammatory Th17 T cells is increased in patients with MG (Z. Wang et al., 2012) which secrete inflammation-propagating factors like IL-17, INFg, and GMCSF, while reducing expression of anti-inflammatory IL-10 (Cao et al., 2016; Masuda et al., 2010; J. A. Villegas et al., 2019; Z. Wang et al., 2012).

1.4.2 The benefits of immunity in the nervous system

Though there is abundant evidence and several contexts in which inflammation causes harm and destruction, a significant body of work highlights the necessity and benefit of an inflammatory response. As stated previously, the clearance of cellular debris after injury is essential for returning to homeostasis and for any potential of regeneration after nervous system

injury. Tissue-resident macrophages and infiltrating neutrophils and monocyte-derived macrophages play significant roles in this process. Inflammatory responses can also have beneficial effects on promoting neuroprotection and regeneration (Bollaerts et al., 2017; Morganti-Kossmann et al., 2002; Schwartz et al., 1999; Stoll et al., 2002). Interestingly in some conditions, preventing inflammation has been shown to increase cell death and secondary tissue damage (Allan & Rothwell, 2001; Morganti-Kossman et al., 1997). Importantly, certain forms of immune signaling are essential for normal nervous system function. In response to peripheral infection or insult, there is a transient level of CNS inflammation which activates microglia and astrocytes to encourage an evolutionary "sickened behavior" including increased temperature, lethargy, and hypophagia which allows the body to reallocate resources toward healing (Bluthé et al., 2000; Dantzer et al., 2008; Henry et al., 2009; Imeri & Opp, 2009; Serrats et al., 2010). This response is quelled quickly and does not involve entrance of circulating immune cells or neuropathology (Dantzer et al., 2008; Norden & Godbout, 2013). Further, hippocampal neurogenesis promoted by learning and cognitive performance is regulated by effective T cell signaling (Derecki et al., 2010; Kipnis et al., 2012; Ziv et al., 2006). Additionally, co-culture of pro-resolving macrophages with neural stem cells activates peroxisome proliferator activated receptor gamma (PPARγ) and TLRs to enhance differentiation into neurons and oligodendrocytes while promoting neurite outgrowth (Lei et al., 2016; S. F. Ma et al., 2015; Rolls et al., 2007). Other inflammatory factors like IFNγ, TNFα, IL-4 IL-6, and others have known roles in regulating neurogenesis, successful long-term potentiation, and learning (Baron et al., 2008; Bosak et al., 2018; del Rey et al., 2013; Elmariah et al., 2005; McAfoose & Baune, 2009; Schneider et al., 1998).

While chronic or aberrant activation can propagate diseases like AD and MS, proper immune cell functioning can act to prevent or recover from these conditions. Myeloid cells are major players in clearing Aβ plaques and act to delay onset of the disease (Simard et al., 2006). CCR2 knockout mice that have impaired monocyte/macrophage recruitment show accelerated AD progression, likely due to lack of Aβ clearance (Michaud et al., 2013; Naert & Rivest, 2011). Inflammatory astrocyte and microglia phenotypes early in AD may also be beneficial as they aid in clearing of Aβ peptides (Meraz-Ríos et al., 2013b). Endogenous cytokines released by anti-inflammatory T cells and macrophages, like IL-4 and IL-10, can also improve the phagocytosis of Aβ (Koenigsknecht-Talboo & Landreth, 2005; Michelucci et al., 2009). Outside administration of these factors can induce phagocytosis and are similarly beneficial for neurodegenerative diseases

(Sokolowski & Mandell, 2011). Further, mice lacking anti-inflammatory factors like IL-4, IL-33, or IL-10 show significantly worsened MS-like pathology (Jiang et al., 2012; Ponomarev et al., 2007; Jingxian Yang et al., 2009).

In addition to their roles in phagocytosis in the CNS and normal brain function, immune cells also play key positions in tissue regeneration and nervous system remyelination following injury. Injection of the fungal wall extract zymosan promotes optic nerve regeneration after crush injury (Baldwin et al., 2015) via macrophage release of oncomodulin (Y. Yin et al., 2006, 2009) and/or neutrophil-released growth factors like NGF (Sas et al., 2020). IL-4 or IL-13-exposed, anti-inflammatory macrophages can suppress deleterious inflammation, promote angiogenesis, axon regeneration, oligodendrogenesis, and functional recovery after traumatic brain injury (Kigerl et al., 2009b; Schonberg et al., 2007; B. Zhang et al., 2015). Injection of therapeutic microglia following spinal cord injury induces axonal regeneration (Barrette et al., 2008a). T cells have even been shown to facilitate axonal regeneration and functional recovery after injury (Hauben et al., 2000; Ishii et al., 2012; Walsh et al., 2015).

As mentioned previously, leukocytes and also microglia are significant sources for neurotrophic factors like EGF, PDGF, FGF, CNTF, GDNF, IFG-1, osteopontin, BDNF, and oncomodulin (Sousa-Victor et al., 2018; Yong & Rivest, 2009). Several of these factors are also beneficial for the proliferation and differentiation of oligodendrocytes which can then begin remyelination (Higashiyama et al., 1991; Miron et al., 2013a; O'Donnell et al., 2002; Scafidi et al., 2014; Yuen et al., 2013). Broadly, immune cells aid remyelination (Goldstein et al., 2016; Rawji et al., 2016) through myelin debris clearance. Depletion of monocytes and macrophages through clodronate liposomes reduces remyelination potential (Kotter et al., 2001; Triarhou & Herndon, 1985). Mice lacking inflammatory factors IL-1ß or TNFα had delayed remyelination after injury (Arnett et al., 2001; J. L. Mason et al., 2001). Further, stimulation of immune cells with TLR ligands (Glezer et al., 2006; Setzu et al., 2006), IL-4 (Butovsky et al., 2006), or M-CSF (Laflamme et al., 2018) is able to indirectly enhance remyelination.

1.4.3 Layered complexity in immune phenotypes

As shown by the examples and studies above, much of our thinking regarding inflammation has been dichotomous, the "bad" and the "good." For decades much of the work examining inflammation used this distinction with common annotations of "X1" and "X2," where is "X" is

the cell of interest, "1" designates classically activated/pro-inflammatory, and "2" designates alternatively-activated/anti-inflammatory/pro-resolving. This was initially and most commonly used when examining M1 and M2 macrophages (Mills et al., 2000; Orecchioni et al., 2019; Shapouri-Moghaddam et al., 2018; Yunna et al., 2020). The inspiration for the scheme originated from T cell nomenclature in which Th1 cells activate macrophages and Th2 cells inhibit macrophage activation and instead promote antibody production by mature B cells. The primary factors released by these T cell subtypes, IFNγ by Th1 and IL-4/10 by Th2, can skew macrophages toward a pro-inflammatory "1" or pro-resolution "2" phenotype (F. O. Martinez & Gordon, 2014; Wynn & Vannella, 2016). These two macrophage phenotypes have been studied extensively over the years in many injury/disease models and across several organisms. Following injury there is a preferential influx of M1 macrophages that are a few days later replaced with M2 macrophages (Nadeau et al., 2011b). Intriguingly, *in vitro* studies have shown that macrophages can shift between M1 and M2 phenotypes (Davis et al., 2013; Khallou-Laschet et al., 2010a; van den Bossche et al., 2016). Other *in vitro* studies often indicate that M2-like, but not M1-like, macrophages promote neurite growth in DRG neurons, characteristic of regeneration (Kigerl et al., 2009c). *In vivo,* a nerve conduit releasing IL-4, an M2-skewing factor, was placed between the two stumps of an axotomized sciatic nerve. There it significantly increased the number of regenerating fibers, suggestive that M2 macrophages are indeed pro-regenerative (Mokarram et al., 2012).

This naming notation soon spread to other cells including A1 and A2 astrocytes (Clarke et al., 2018; Liddelow et al., 2017; Neal et al., 2018; X. Xu et al., 2018), M1 and M2 microglia (X. Liu et al., 2016; Miron et al., 2013b; Orihuela et al., 2016; Y. Tang & Le, 2016), and N1 and N2 neutrophils (Fridlender et al., 2009; García-Culebras et al., 2019; Y. Ma et al., 2016; Masucci et al., 2019), among others. While this characterization of cells was useful in determining cell function and polarization, with access to novel and more powerful tools, such as single cell RNA sequencing (scRNA-seq), we are now able to more fully examine and characterize these cells and begin to understand the truly nuanced and complex identities of these cellular subsets. For example, through scRNA-seq, our lab has been able to identify at least five different macrophage populations following sciatic nerve crush. This is in line with and adds upon research from other researchers (Burl et al., 2018; Gubin et al., 2018; Qie et al., 2020; Ydens et al., 2020b). Others have found further nuances in differeent immune populations, with multiple distinct subtypes of

brain microglia identified in recent studies (Kubick et al., 2020; Q. Li et al., 2019; Syage et al., 2020). As we begin to utilize new tools, we can begin to further our understanding of these different cellular populations and break away from the simple distinction of "bad" pro-inflammatory and "good" anti-inflammatory cells. Findings ways to harness and direct these inflammatory responses in the direction we desire can be an invaluable tool, not only in reducing its negative effects as in AD, peripheral neuropathy, and neuropathic pain but in its capacity to promote growth, regeneration, and repair.

1.5 Efferocytosis and the Catabolism of Apoptotic Cells

In the human body, the turnover rate for different cell types is highly variable. The range spans from highly stable neurons that can last a life time to neutrophils in the immune system that perish daily (200 billion per day). Hundreds of billions of cells die in the human body every day (Kinchen & Ravichandran, 2008; Nagata, 2018) and millions every second (C. Gregory, 2009). In order to maintain homeostasis, they must be quickly and effectively cleared in a process called efferocytosis, the phagocytosis of apoptotic cells (ACs). While seemingly a semantic difference, the efferocytosis of dying cells involves a myriad of signaling molecules, receptors, and intracellular mediators that are quite distinct from those of phagocytosis of pathogens. Further, while phagocytes engage in both processes, their activation states and the paracrine signaling molecules they release differ greatly. Below I will touch upon the three distinct phases of efferocytosis, the signaling molecules involved, and what happens when this process goes awry.

Efferocytosis is composed of three main stages: Apoptotic cell finding, cell binding, and internalization and degradation. Upon apoptosis, a cell releases several molecules termed "find me" signals which promote the efferocyte's (phagocytic cell "eating" the apoptotic cell) chemotaxis to the apoptotic cell. These include several lipid mediators (lysophosphatidycholine, sphingosine-1-phosphate), nucleotides (ATP, UTP), cytokines (CX3CL1), and other molecules (RP S19, EMAP II, ttRS, ICAM-1). Each with their own families of receptors, LRP1, P2Y2, CX3CR1, and various scavenger receptors, respectively. Once the efferocyte has found the AC, it then binds to various "eat me" signals displayed on the AC cell surface. The key "eat me" signal is phosphatidylserine (PS), which is normally retained on the cytoplasmic portion of the plasma membrane by flippases. This externalized orientation is mediated by scramblases in a calcium- and caspase-dependent manner upon induction of apoptosis. The efferocyte can bind PS directly

through receptors like stabilin-1, TIM-4, and BAI1 or indirectly via bridging molecules like Gas6, protein S, and MFG-E8. Additionally, components of complement like C1q, C3, IgM bind ACs and act as "eat me" signals when bound to efferocyte receptors LRP1, CRT, and FcγRIIA. Activation of theses efferocytic receptors causes downstream, Rac1-dependent actin polymerization, efferocytic cup formation, and ingestion of the AC. Finally, the AC is internalized and undergoes processing through the endolysosomal compartment and its components catabolized.

1.5.1 Apoptotic cell finding

Many cells are capable of acting as efferocytes including macrophages, dendritic cells, fibroblasts, and epithelial cells. While these cells share extra- and intra-cellular signaling cascades in this process, their capacity and voraciousness differ quite greatly (Lars Peter Erwig et al., 2006; S. Kumar & Birge, 2016; Parnaik et al., 2000). Often these cells are split into two camps: professional and non-professional phagocytes, a characterization that is maintained when discussing efferocytosis. Professional phagocytes include macrophages, dendritic cells, neutrophils while non-professional phagocytes include fibroblasts, endothelial cells, epithelial cells, and most other cells. As macrophages are the most abundant and most frequently involved in efferocytosis, they will be the primary focus moving forward. Professional phagocytes have the capacity to efferocytose multiple apoptotic cell targets nearly simultaneously and can catabolize and process apoptotic cell components relatively quickly (25-95min). On the other hand, non-professional phagocytes are only able to eat one cell at a time and take much longer (5-9hr) to process cellular components (Parnaik et al., 2000). While this may seem highly ineffective and suggest a build-up of ACs overtime, it has been exemplified that mice lacking macrophages are still able to effectively—though delayed—clear ACs (C. S. Lee et al., 2016; Jenifer Monks et al., 2008; Wood et al., 2000).

Macrophages are arguably the most abundant and active efferocyte throughout the body. There are approximately 200 billion macrophages spread out through the average adult human, constantly sensing their environment for ACs (Lukens, Lee, Bithell, Foeerster, & Athens, 1993). They are uniquely positioned with their cellular machinery to bind AC targets and dispose of them quickly. Remarkably, some macrophages have been observed eating/digesting up to 20 ACs at one time (Church et al., 2016a; Dransfield et al., 2015a; Firdessa et al., 2014b; Lam et al., 2009; Nakaya

et al., 2006a; Schlam et al., 2015a). Interestingly, macrophages seem to preferably ingest ACs through one side of its cell surface to bring them through their digestive machinery, almost like an assembly line (Nakaya et al., 2008). Within macrophages are multiple, primed homeostatic and reactive signaling cascades ready to upregulate their digestive machinery and process the massive intake of AC-derived cellular contents including cholesterol, calcium, lipids, glucose, membrane components, and others. The details of these signaling mechanisms will be covered in-depth in later sections.

Sensibly, microglia and macrophages are highly abundant in areas of high levels of apoptosis. The dentate gyrus of the brain, where there is high neuronal precursor cell turnover, has an abundance of microglia (Luo et al., 2016). Elsewhere in the intestine, lung, bone marrow, testes, and thymus, macrophages are known to be ever-present and actively eating the continual supply of apoptotic cells (R. W. Bailey et al., 2002; de Paepe et al., 2004; DeFalco et al., 2015; Elliott & Ravichandran, 2016; C. S. Lee et al., 2016; D. Park et al., 2011; Pittet & Weissleder, 2011; Sunaga et al., 2013; Surh & Sprent, 1994). Non-professional phagocytes are used more often by the body to clear dead cells in areas that are scarce in professional phagocyte presence such as lung alveoli and intestinal epithelium (Burstyn-Cohen et al., 2012; Dini et al., 2002; Elliott et al., 2010; Juncadella et al., 2013; Kevany, BM.; Palczewski, 2010; C. S. Lee et al., 2016; Z. Lu et al., 2011; Lysiak et al., 2000; J Monks et al., 2005; Jenifer Monks et al., 2008; Wood et al., 2000). They tend to eat dying cells in much later stages of apoptosis, suggesting they may need a stronger summation of "eat-me" signals to carry out the act (Parnaik et al., 2000). Further, while professional phagocytes ingest apoptotic cells upon first interaction, non-professional cells often delay eating of the dying cell for hours after first contact (Lööv et al., 2015; Parnaik et al., 2000). Interestingly, it has been shown that macrophages release IGF-I which binds IGF-IR on epithelial cells and reduces their appetite for apoptotic cells (C. Z. Han et al., 2016). This can help prevent an epithelial cell-initiated inflammatory response as non-professional phagocytes are less capable in maintaining tolerance than professional cells.

1.5.1.1 AC-released "find me" signals and associated efferocyte receptors

Apoptotic cells are not merely standing by awaiting their eventual phagocytosis, they are active participants in their clearance. To attract phagocytes, ACs release several "find me" signals into the extracellular space that act as chemoattractants for efferocytes. Signals released include

lipids (lysophosphatidycholine & sphingosine-1-phosphate), chemokines (CX3CL1), nucleotides (ATP/UTP), and others (Elliott et al., 2009; Gude et al., 2008; Lauber et al., 2003; Medina & Ravichandran, 2016; R. B. Mueller et al., 2007; Peter et al., 2008; Truman et al., 2008). Lysophosphatidycholine (LPC) was one of the first "find me" signals to be discovered and some argue it is the most important (Elliott et al., 2009; Hochreiter-Hufford & Ravichandran, 2013; Lauber et al., 2003; Peter et al., 2008). LPC is a lipid produced by the cleavage of phosphatidylcholines by activity of phospholipase A2 (Peter et al., 2008, 2010, 2012). Upon secretion, LPC acts as an attractant by binding the G-protein coupled receptor (GPCR) G2A on macrophages (Peter et al., 2008). Another lipid "find me" signal is sphingosine-1-phosphate (S1P). S1P is generated from sphingosine by sphingosine kinase and can bind to phagocyte GPCRs like S1P receptors 1-5 to promote efferocyte chemotaxis (Gude et al., 2008).

Another key "find me" signal is the cytokine CX3CL1/fractalkine which mainly acts as a ligand for CX3CR1 (Peter et al., 2010; Truman et al., 2008). In a specific *in vivo* example, many B cells die during maturation and were found to release CX3CL1 upon apoptosis which bound to phagocyte CX3CR1 and influenced phagocyte migration (Truman et al., 2008). A final set of "find me" signals includes nucleotides, specifically ATP and UTP (Elliott et al., 2009) which can be released through pannexin-1 channels (Chekeni et al., 2010; Elliott et al., 2009). These can attract efferocytes by binding to purinergic receptors like the P2Y purinoceptor 2 (Elliott et al., 2009). While those discussed above have received the most attention, there are several additional "find me" signals including thromobospondin-1 (Moodley et al., 2003), tRNA synthetase (Wakasugi & Schimmel, 1999), and even altered electrical activity (Pethig & Talary, 2007; Weihua et al., 2005).

1.5.2 Apoptotic cell binding

Upon arrival of an efferocyte to an AC, it next must decide to "eat" or "not eat" the dying cell. It does this by weighing the summation of positive "eat me" and negative "don't eat me" signals (Elliott & Ravichandran, 2010; L. P. Erwig & Henson, 2008; Hanayama et al., 2002; Hoffmann et al., 2001; Kojima et al., 2017; J. Martinez, 2017). If the "eat me" signals overwhelm opposing signals, the efferocyte will proceed with AC ingestion and degradation. Far and away the most powerful "eat me" signal is the lipid mediated phosphatidylserine (PS). PS is produced by phosphatidylserine synthase 1 and 2 (Arikketh et al., 2008; Kay & Grinstein, 2013). In a healthy cell PS is important for the function of several intracellular proteins and aids in tethering proteins

to the plasma membrane. Some of these include E3 ubiquitin-ligase NEDD4, isoforms of protein kinase C, isoforms of phospholipase C and D, several phosphatases, a number of synaptotagmins, and several annexins that have roles in membrane-cytoskeletal anchoring and membrane trafficking (Lemmon, 2008).

In homeostatic conditions, choline-containing phospholipids like phosphatidylcholine are predominantly maintained in the outer plasma membrane leaflet, and amino-phospholipids including PS are predominately on the inner leaflet (Leventis & Grinstein, 2010). In these conditions, flippase transporters (primarily ATP11A and ATP11C) maintain PS on the intracellular portion of the plasma membrane (Segawa et al., 2014, 2016). Upon initiation of apoptosis, rising levels of intracellular calcium active scramblases (mainly the XKR family) via caspase activity (Elmore, 2007) which flip PS onto the extracellular leaflet of the plasma membrane (Valerie A. Fadok et al., 1998; J. Suzuki et al., 2013, 2014, 2016). Caspases also work to inactivate flippases to prevent PS from flipping back to the intracellular leaflet (Valerie A. Fadok et al., 1998; Segawa et al., 2014, 2016; Tajbakhsh et al., 2020). A secondary and recently recognized AC ligand is calreticulin (Calr) which functions similarly to PS. Calr is upregulated on the surface of ACs and is recognized by LDL receptors like LRP1 on phagocytes to aid in the induction of engulfment (Gardai et al., 2005b; Gold et al., 2010; Reddy et al., 2002; Tajbakhsh et al., 2018). Additional "eat me" signals include ICAM-3, oxidized LDL, annexin I, thrombospondins, components of complement like C1q, and altered plasma membrane glycosylation states (Arur et al., 2003; Lauber et al., 2004). Interestingly, autophagy has been shown to be necessary for generation of find-me (lysophosphatidycholine) and eat-me (PS) signals (Qu et al., 2007).

Efferocytes employ a multitude of diverse receptors in order to perceive the many "eat me" signaling molecules expressed by ACs. Most signal through PS including single-pass transmembrane receptors like TIM-4, stabilin-2 and CD300f, G protein-coupled receptors like BAI1, integrins such as αvβ3/5, immunoglobulins like Trem2, and TAM family receptor tyrosine kinases (RTKs) like Mer, Axl, and Tyro3 (Matthew L Albert et al., 2000; Burstyn-Cohen et al., 2012; S. Das et al., 2011; Dransfield et al., 2015b; Lemke & Burstyn-Cohen, 2010; Lu, Q.; Gore, M.; Zhang, Q.; Camenisxh, T.; ...; Goff, SP.; Leemke, 1999; Miyanishi et al., 2007; Nishi et al., 2014; D. Park et al., 2007; S. Y. Park et al., 2008; Rothlin et al., 2015; Scott, RS.; McMahon, EJ.; Pop, SM.; ...; EEarp, HS.; Matsushima, 2001; Seitz et al., 2007; Linjie Tian et al., 2014a; Y. Wu

et al., 2005; P. G. Zagórska et al., 2014). Efferocyte TIM-4 directly binds PS (Miyanishi et al., 2007; Thornley et al., 2014) but is unable to signal intracellularly (N. Kobayashi et al., 2007; Miyanishi et al., 2007). It has a very high affinity for PS and thus primarily acts as a tethering protein, maintaining the connection with the AC (Freeman et al., 2010; K. Wong et al., 2010; Yanagihashi et al., 2017). Stabilin-2 binds PS directly and interacts with GULP and αvβ3/5 to activate the ELMO (engulfment and cell motility) family of proteins which inevitably activate Rac1 (Akakura et al., 2004a; Matthew L Albert et al., 2000; S. Kim et al., 2012; Kinchen et al., 2005a). CD300f is another direct PS-binding receptor which promotes efferocytosis, mice deficient for CD300f had an accumulation of ACs and increased chance to develop lupus-like disease (L Tian et al., 2016). BAI1 can also directly bind PS and signals downstream to activate ELMO (D. Park et al., 2007).

While most receptors are activated by PS-binding, some receptors are unable to connect to PS directly and instead rely on bridging molecules. Integrin αvβ3/5 relies on the lactadherin bridging molecule milk fat globule-EGF factor 8 protein (MFG-E8) which binds PS (Akakura et al., 2004a; Matthew L Albert et al., 2000; M. H. Andersen et al., 1997; Mikkel H. Andersen et al., 2000; Hanayama et al., 2002; Poon et al., 2010a; Ravichandran & Lorenz, 2007; Shi et al., 2004). MFG-E8 is secreted by macrophages and immature DCs (Hanayama et al., 2006). TAM RTKs MER, Tyro3, and Axl rely on bridging molecules Gas6, protein S, tubby-like protein 1, and MFG-E8 to bind with PS (Burstyn-Cohen et al., 2012; Caberoy et al., 2010; J. Chen et al., 1997; Hall et al., 2005; C. Y. Hu et al., 2009a; Kawano & Nagata, 2018; Lemke, 2013; Lew et al., 2014; Scott, RS.; McMahon, EJ.; Pop, SM.; ...; EEarp, HS.; Matsushima, 2001; Stitt et al., 1995; Tibrewal et al., 2008; Tsou et al., 2014; Y. Wu et al., 2005; A. Zagórska et al., 2014). Interestingly some bridging molecules bind with higher affinity than others, with MGF-E8 binding more tightly than protein S or Gas6 (Hanayama et al., 2002)

Triggering receptor expression on myeloid cells-2 (Trem2) is expressed on myeloid cells including macrophages and dendritic cells (Daws et al., 2001; Seno et al., 2009; Turnbull et al., 2006; K. Wu et al., 2015) and, when working with DAP12, has known roles in proliferation, phagocytosis and anti/pro-inflammation (Bouchon et al., 2001; Chu et al., 2008; M. Colonna & Wang, 2016; Hsieh et al., 2009; Poliani et al., 2015; Quan et al., 2008; Takahashi et al., 2005). A definitive ligand for Trem2 has been elusive, but there is some evidence it binds phospholipids like PS and phosphatidylcholine and lipoproteins like ApoE (Atagi et al., 2015; C. C. Bailey et al.,

2015; J. P. Cannon et al., 2012; Yeh et al., 2016). One group found that infiltrating macrophages in mice fed a high-salt diet and subjected to a transient middle cerebral occlusion injury to induce stroke were skewed toward an inflammatory and anti-efferocytic phenotype. Enhancement of Trem2 signaling improved efferocytic capacity and reduced inflammation (M. Hu et al., 2021a).

PS can come in a few different flavors, each of which having different effects on classic PS signaling. One major modification generated in a caspase-dependent manner is oxidation, creating oxidized-PS (oxPS). oxPS can act as a stronger "eat me" signal for phagocytes (Kagan et al., 2002; John Savill et al., 2002a; Tyurin et al., 2014). Some of the PS-binding molecules described above, including Gas6, MFG-E8, and Tim-1, bind oxPS with higher affinity than PS (Tyurin et al., 2014) and thus enhance efferocytosis. Blocking of oxPS with neutralizing antibodies reduced efferocytic effectiveness by macrophages (M. I. K. Chang et al., 1999; Greenberg et al., 2006). An additional PS modification is hydrolyzation into lysophosphatidylserine (lyso-PS). Rather than classic PS receptors, lyso-PS binds GPCRs and can act as an endogenous anti-inflammatory mediator (Frasch et al., 2013; Frasch & Bratton, 2012; Hajime Kitamura et al., 2012).

While these efferocytic receptors and bridging molecules vary greatly in their make-up and binding partners, intriguing evidence suggests there may be a master regulator for many of these molecules, ERK5. ERK5 is in the family of MAPKs and GPCRs. Heo found that macrophages lacking ERK5 had reduced expression of many efferocytosis bridge molecules and receptors including MER, Gas6, and MFG-E8 among others. Thus, Heo indicated ERK5 activation is required for AC clearance via upregulation of these efferocytic signaling molecules and also shifting of macrophages toward a pro-resolution "M2" phenotype. *In vivo*, these knockout mice showed reduced clearance of ACs and enhanced progression of atherosclerosis due to ineffective efferocytosis and buildup of necrotic cells (Heo et al., 2014).

1.5.2.1 Rac1 activation and efferocyte cup formation

While there is generous diversity in the number and types of efferocytosis receptors, nearly all of them culminate into the activation of a singular pathway. Perhaps the simplest example of this is activation of the efferocyte receptor BAI1 by PS. Activated BAI1 stimulates engulfment and cell motility 1 (ELMO1). ELMO1 in turn recruits the guanine nucleotide exchange factor (GEF) Dock180 to the plasma membrane for activation which switches out GDP for GTP on the

RHO family GTPase Rac1 (Brugnera et al., 2002; D. Park et al., 2007). Rac1, via WASP-family verprolin homologous protein 1 (WAVE1) and Arp2/3 (I. R. Evans et al., 2013; Kinchen et al., 2005b; Miki et al., 1998), promote actin polymerization to form an efferocytic cup which the efferocyte utilizes to ingest the AC. The set of efferocyte receptors Stabilin-2 and LRP1 both interact with GULP to elicit Rac1 activity (S. H. Lee et al., 2008; S.-Y. Park et al., 2008; Su et al., 2002). Integrins αvβ3/5 also signal through the ELMO-Dock180-Rac1 pathway (Akakura et al., 2004b; M L Albert et al., 2000). TAM RTK binding to PS through Gas6 and protein S promotes their dimerization and phosphorylation which activates PLCg2. PLCg2 then recruits $p130^{CAS}$ to activate the CrkII-ELMO-Dock180 module (M L Albert et al., 2000; Brugnera et al., 2002; Y. Wu et al., 2005). Quite beautifully, all of these diverse AC ligands, free-floating bridging molecules, and efferocyte receptors come together to activate one simple and common pathway and initiate the complex task of efferocytosis.

As efferocytes are constantly surveying their environment and are prepared to eat potential apoptotic cells, healthy cells need a mechanism to ward off these hungry efferocytes. The primary technique employed here is expression of "don't eat me" signals. The most potent inhibitor of efferocytosis is CD47, or integrin associated protein (IAP) (Elward et al., 2005; Oldenborg et al., 2000; Poon et al., 2010b; Ravichandran, 2010). AC plasma membrane-associated CD47 binds to SIRPα receptors on efferocytes (Gardai et al., 2005a; Nilsson & Oldenborg, 2009) which leads to activation of phosphatases SHP-1 and SHP-2 and attenuation of downstream activation of Rac signaling (Blazar, BR.; Lindberg, FP.; Taylor, 2001; Okazawa et al., 2005; Oldenborg et al., 2000), which is necessary for phagocytic/efferocytic cup formation. In order to induce apoptosis, expression levels of CD47 are reduced as caspase activation leads to shedding of the molecule (Azuma et al., 2011). An additional "don't eat me" signal is CD31 (Brown et al., 2002; Poon et al., 2010b), or platelet endothelial cell adhesion molecule (PECAM-1), which binds homophilically to CD31 and causes detachment of phagocytes from ACs (Brown et al., 2002). CD300a is another potent "don't eat me" signal (Voss et al., 2015). Interestingly the cellular microenvironment plays a key role in deciding if efferocytosis occurs or does not. In inflammatory conditions TNFα is often present, this cytokine can upregulate the expression of CD47 to reduce efferocytosis (Kojima et al., 2016). Another inflammatory factor, HMGB1, can block integrin αvß3 and PS signaling, thus blocking efferocytosis (Friggeri et al., 2010). In addition to membrane-

bound signals, some cells even express “keep out” signals like lactoferrin to impair homing of efferocytes like neutrophils and eosinophils (Green et al., 2016; Seung Yoon Park & Kim, 2017).

1.5.3 Apoptotic cell internalization and degradation

After an efferocyte has contacted stronger and/or more “eat me” signals than “don’t eat me” signals, the next stage of efferocytosis begins, internalization and degradation. The efferocyte plasma membrane envelopes the AC and brings it into an early endosome. As the endosome matures, the protein Rab5 is recruited which initiates fusion with a phagosome (Kitano et al., 2008). Rab5 is exchanged for Rab7 which mediates fusion with late endosomes and lysosomes into a phagolysosome, where cellular contents are digested (Cantalupo et al., 2001; Elliott et al., 2010; Epp et al., 2011; Harrison et al., 2003; Johansson et al., 2007; Rink et al., 2005). Classically, if the phagocytosed cell was infected or the phagocyte had ingested a pathogen, the phagolysosome would fuse with MHC-II loading compartments that would be loaded with peptides from the ingested cell/pathogen and presented to activate other immune cells (Chakraborty et al., 2005; Martinez-Pomares & Gordon, 2007; Meier et al., 2003; Saric et al., 2016). Though in efferocytosis, instead of fusing with MHC-II loading compartments, the mature phagolysosome is shunted toward recycling endosomes (C. Yin et al., 2016, 2019). This unique step in efferocytosis avoids presentation of “self” peptides form the AC to other immune cells and avoids initiating autoimmunity.

Though an AC has carried out much self-digestion and compartmentalization before being ingested by an efferocyte, it still contains significant levels of cellular components the efferocyte must process. Ingestion of an AC results in a doubling of intracellular content (Kiss et al., 2006). The efferocyte takes on lethal amounts of excess lipids, cholesterol, glucose, and other molecules. AC-derived cholesterol is processed by the cholesterol acyl transferase ACAT which forms cholesterol esters (Cui et al., 2007). This helps prevent the membrane-damaging effects free, unmodified cholesterol would have on the efferocyte. Once ACAT has done its work, the modified cholesterol is either used by the cell or exported from the efferocyte via the ATP-binding cassette transporter (ABCA1) (Kiss et al., 2006; Yvan-Charvet et al., 2010). Interestingly, efferocytes begin to upregulate their expression of ABCA1 even before ingestion via signaling through efferocyte PS receptors like LRP1 and nuclear receptors like PPARγ (Chawla et al., 2001; Kiss et al., 2006; Venkateswaran et al., 2000; Xian et al., 2017). Signaling through efferocyte receptor

BAI1 and AC-derived sterols also promote ABCA1 expression (Fond et al., 2015). AC-derived glucose and lactate are exported from the cell by various glucose and lactate transporters including GLUT1 through upregulation of of *Sgk1* and MCT1 (Galván-Peña & O'Neill, 2014; Morioka S, et al., 2018). A recent group also suggested a major role for the solute carrier (SLC) family of proteins which are increased during efferocytosis and are involved in the release of lactate, shifting of efferocyte metabolic pathways, and enhancement of anti-inflammatory signaling (Morioka S, et al., 2018).

In order to keep up with large numbers of ACs and efferocytic events, efferocytes can engage several pathways to ensure continual AC clearance is maintained (Ortega-Gómez et al., 2013). Efferocyte activation of PPARγ/δ and LXR α/β aids in upregulating and replenishing phagocytic receptors (A-Gonzalez et al., 2009; Mukundan et al., 2009) as well as upregulating lipid metabolism pathways (Mukundan et al., 2009; Rőszer et al., 2011). Loss of PPARδ reduces expression of efferocytosis factors like C1q, MFG-E8, and MER which reduces efferocyte effectiveness (Mukundan et al., 2009).

Though, efferocytes do have their limit. Once they reach capacity, there is a drastic decline in AC clearance (Zent & Elliott, 2017). There have been reports that macrophages can be digesting 10-20 cells at a time (Church et al., 2016b; Firdessa et al., 2014a; Lam et al., 2009; Miyanishi et al., 2007; Nakaya et al., 2006a; D. Park et al., 2011; Schlam et al., 2015b)**.** However excess carbohydrates (glucose/sucrose) can cause a macrophage to become vacuolated, lose phagocytic capacity, and potentially face death as a result of too much energy production (G. J. Cannon & Swanson, 1992; D. Park et al., 2011). One mechanism efferocytes employ to avoid this is upregulating mitochondrial uncoupling protein 2 (UCP2) to reduce mitochondrial membrane potential and ATP production (D. Park et al., 2011). Interestingly, reduced UCP2 reduced efferocytosis while overexpression enhances it (Blanc et al., 2003; D. Park et al., 2011). Though, macrophages do not eat cells to the point of lysis and tend to reach capacity around 4 hours of continuous eating (Church et al., 2016b; Grandjean et al., 2016). Prolonged exposure (24 hours) to apoptotic cells can dampen further efferocytic activity due to fatigue (Church et al., 2016b). This could be due to multiple factors including lack of sufficient plasma membrane to surround the AC, depletion of efferocytosis surface receptors, or unavailability of intracellular signaling molecules like plasma membrane-associated Rac. Contrastingly, short-term exposure (4 hours) followed by a break period and then re-exposure can actually enhance their efficiency with

increased signaling efficiency and endosome transport as macrophage efferocytic machinery is primed after their first exposure (A-Gonzalez et al., 2009).

1.5.4 The immune-modulating effects of efferocytosis

As stated above, normal phagocytosis of a pathogen leads to further activation of the immune system through peptide presentation via MHC-II to non-phagocytic immune cells. Further, a pro-inflammatory cascade is initiated that aids the immune system in mobilizing quickly and efficiently to remove the invading threat. When an efferocyte is ingesting an apoptotic "self" cell, like a dying neuron, skin cell, or hepatocyte, the aforementioned cascade is the last thing one wants. Instead, the AC and efferocyte have multiple mechanisms to instead drive an anti-inflammatory and regulated pro-resolving response to avoid the breaking of self-tolerance. Upon AC ingestion there is a small burst in pro-inflammatory signaling, mainly TNFα (Lucas et al., 2003). This trend is quickly squashed by PPARγ and D6 signaling which act to suppress inflammatory mediators like TNFα, IL-6, INF-1, and CCL5 primarily via inhibition of the master inflammatory transcription factor NF-κB (Am et al., 2006; A. Das et al., 2014; C. D. Gregory & Devitt, 2004; Pashover-Schallinger et al., 2012; von Knethen et al., 2013; Yoon et al., 2015). Congruently the efferocyte enhances anti-inflammatory signaling by promoting release of TGFß and IL-10 (de Paoli et al., 2014; Kleinclauss et al., 2006; F. O. Martinez et al., 2009; Zent & Elliott, 2017; Shuang Zhang et al., 2019). Components of the ingested AC, including sterols, actually feed into this process by activating sterol receptors like PPARγ/d and LXRα which stimulate IL-10 and TGFß. They also prevent removal of a corepressor on the promoter sequence of TNFα and IL-1ß and inhibit their transcription. Many of these factors go on to promote anti-inflammatory phenotypes in other cells like T cells. TGFß and IL-10 signaling leads to differentiation into regulatory T cells (Tregs) and Th2 cells which go on to promote pro-resolution (A-Gonzalez et al., 2009; A-González & Castrillo, 2011; Hsu et al., 2015; Mukundan et al., 2009; Oh & Li, 2013; Proto et al., 2018). Tregs also enhance efferocytosis efficiency via secretion of IL-13 which promotes macrophage upregulation of IL-10 to enhance downstream Rac1-dependent actin assembly of phagosomes (Proto et al., 2018).

There is indeed an entire category of these anti-inflammatory factors termed specialized pro-resolving mediators (SPMs). These include lipoxins, resolvins, protectins, and maresins (Bannenberg et al., 2005; Claria & Serhan, 1995; Dalli, Zhu, et al., 2013; J, Dalli; C, 2016; B. D.

Levy et al., 2001; Mitchell et al., 2002; Serhan et al., 2000, 2002, 2009, 2015). Lipoxins, or lipoxygenase interaction products, are metabolites of arachidonic acid and signal the resolution of acute inflammation (Samuelsson et al., 1987; Serhan, 2005). Interestingly they can act as chemoattractants for mononuclear cells but do not stimulate pro-inflammatory chemokine release (Serhan, 2007). Resolvins are derived from omega-3 fatty acids and contain two common families, E (RvE) and D (RvD) series (J. M. Schwab et al., 2007; Serhan et al., 2002). Resolvins are potent blockers of inflammation and can halt neutrophil infiltration (Y. P. Sun et al., 2007) and reduce cytokine expression (S. Hong et al., 2003). Protectins are produced by the oxygenation of docosahexaenoic acid (DHA) and have anti-inflammatory and protective effects (S. Hong et al., 2003). One of the most well-known members, protectin D1, has a significant role as an anti-inflammatory, anti-apoptotic, and neuroprotective molecule (S. Hong et al., 2003; Marcheselli et al., 2003; P. K. Mukherjee et al., 2004; Serhan et al., 2006). Maresins, derived from the phrase "macrophage mediator in resolving inflammation" are also DHA metabolites with potent anti-inflammatory effects (Deng et al., 2014; Serhan et al., 2009). Interestingly the administration of Aspririn can "jump-start" resolution by quickening production of several SPMs (Arita et al., 2005; Chiang et al., 2004).

As mentioned earlier, after injury neutrophils act as a rich source of ACs after injury and can polarize efferocytic macrophages to be more efficient efferocytes (Horckmans et al., 2017). For example, binding of MER by Gas6 and PS activates 5-LOX which enhances production of SPMs (B. Cai et al., 2016). As a potential therapy, omega-3 fatty acids are known to increase presence of several SPMs (Serhan, Charles N., Chiang, 2013). Intriguingly, SPMs play dual roles here, in addition to acting as anti-inflammatory mediators they also promote apoptosis of neutrophils after they have completed their immune function. They do this by enhancing caspase activation and suppressing ERK/Akt pro-survival signaling (el Kebir et al., 2007; el Kebira et al., 2012). Certain AC-derived molecules act as precursors for SPMs like n-3/6 fatty acids including arachidonic, eicosapentaenoic, and docosapentaenoic acid (Claria & Serhan, 1995; Serhan et al., 2002, 2009, 2015). Additionally, these SPMs help create a positive feedback cycle by increasing efferocytosis capacity of macrophages which in turn produce more SPMs (Chiang et al., 2015; Dalli & Serhan, 2012; Freire-de-Lima et al., 2006; Godson et al., 2000; Ohira et al., 2010; Prieto et al., 2015; J. M. Schwab et al., 2007; Serhan et al., 2002).

In addition to increasing SPMs, efferocytosis promotes many other signaling pathways to resolve inflammation. Signaling through efferocyte receptors like TAM RTKs is known to stimulate production of IL-10, TGFß, prostaglandin e2, platelet-activating factor (Valerie A Fadok et al., 1998; Grimsley & Ravichandran, 2003; Peter M. Henson et al., 2001; Michalski, Megan N, et al., 2016; Ren & Savill, 1998; Rothlin et al., 2007; John Savill, 1997; John Savill et al., 2002b). Activation of nuclear hormone receptors like PPARγ and LXR also have direct inflammatory effects by inhibiting pro-inflammatory IL-23, IL-17, and G-CSF (C. Hong et al., 2012; Johann et al., 2006; Mukundan et al., 2009; Rőszer et al., 2011; Stark et al., 2005). *In vitro* efferocytosis studies with human PLB-985 cells that do not express PS during apoptosis failed to induce TGFß upon ingestion. Though when PS was exogenously induced in these cells the secretion of TGFß by efferocytes was restored (Huynh et al., 2002). As these anti-inflammatory factors are released, macrophages take on a more pro-resolving phenotype (Schif-Zuck et al., 2011) and increase expression of immunoregulatory signaling like 12/15-lipoxygenase (Uderhardt & Krönke, 2012) which creates pro-resolving lipid mediators and encourages immune cell emigration back to lymphoid organs and cessation of inflammation (Schif-Zuck et al., 2011).

In addition to increasing production of anti-inflammatory factors, activation of many of the same receptors also acts to reduce pro-inflammatory signaling. TAM RTKs act to broadly suppress TLR, INFR, IL-1, and IL-12 signaling (Cvetanovic & Ucker, 2004; Sunjung Kim et al., 2004; Rothlin et al., 2007). Mechanistically it is thought the JAK-STAT signaling of these pro-inflammatory factors is inhibited by RTK-dependent activation of SOCS1 and SOCS3 (Rothlin et al., 2007). Further, activation of MER is known to suppress pro-inflammatory NF-κB (Camenisch et al., 1999; Cui et al., 2007; V A Fadok, Savill, et al., 1992; V A Fadok, Voelker, et al., 1992; Scott, RS.; McMahon, EJ.; Pop, SM.; ...; EEarp, HS.; Matsushima, 2001). Even some of the bridging molecules alone are able to regulate immune responses like MFG-E8 (Ait-Oufella et al., 2007; Dai et al., 2014; Jinushi et al., 2007; Kojima et al., 2017; G. Sun et al., 2017). Other downstream receptors like PPARγ/δ, when activated by sensation of increased cholesterol, can suppress inflammatory responses (Ipseiz et al., 2014; Kidani & Bensinger, 2012; Mukundan et al., 2009).

Some groups have sought to employ the inherent downstream activation of anti-inflammatory pathways through effective efferocytosis by adding apoptotic cells to a highly inflammatory environment. Indeed, therapeutic injection of apoptotic cells is able to induce

immunosuppression and improve conditions of graft versus host disease (GvHD), diabetes, arthritis, and others (Bonnefoy et al., 2016; Gatza et al., 2008; Mevorach et al., 2014; Morelli & Larregina, 2010, 2016; Mougel et al., 2012; Perruche et al., 2009; Xia, Chang-Qing, 2007). Interestingly the route of administration makes a key difference with ACs injected intravenously primarily producing immune tolerance and ACs injected subcutaneously more often leading to immunogenicity. This is in part due to the fact that ACs in the blood traffic to the spleen, thought to be a primarily tolerogenic organ, while ACs in the skin are engulfed by skin-derived dendritic cells and trafficked to lymph nodes, considered a more immunogenic organ (Battisto & Bloom, 1966; Chaput et al., 2007; Conlon et al., 1980; Ferguson et al., 2002; C. F. Scott et al., 1983). Encouragingly this therapy is currently in Phase I/IIa clinical trials for preventing GvHD during stem cell transplantation (Mevorach et al., 2014).

1.5.4.1 Induction of tolerance and avoidance of auto-immunity

In order to avoid auto-inflammatory conditions brought on by presentation of self-peptides to other immune cells, efferocytes have several clever techniques they employ while engulfing and digesting ACs. One employed mechanism mentioned earlier is shunting of some efferocytosed cargo toward recycling endosomes instead of the MHC-II loading compartments of phagolysosomes (C. Yin et al., 2016, 2019). This minimizes the potential contact of self-peptides with MHC-II molecules. As a parallel safety measure, phagosomes in pro-resolving macrophages-those more likely to carry out efferocytosis-undergo acidification more efficiently (Canton et al., 2014). This acts to advance the digestion of ingested peptides into fragments too small to load onto MHC-II molecules. Outside of efferocyte, the immune system has derived additional methods to avoid auto-immunity and induce tolerance. Dendritic cells that have engulfed dead or dying cells present antigens to CD8+ and CD4+ T cells while DCs that have efferocytosed apoptotic cells present only to CD8+ T cells. This lack of dual activation causes the cytotoxic T cells to upregulate TRAIL once they are exposed to their activating antigen which acts to inhibit further immune activation (Janssen et al., 2003, 2005; Joseph C. Sun & Bevan, 2003). Further, the anti-inflammatory factors released from successful efferocytosis like IL-10 and TGFß act to promote tolerance (John Savill et al., 2002b; Voll et al., 1997). Interestingly when portions of the efferocytic pathway are altered, including MFG-E8, MER, C1q, AC-efferocyte bridging molecules, and PPARδ, it can lead to auto-immune conditions (Botto et al., 1998; Hanayama et al., 2004; R. S.

Scott et al., 2001). The harmful consequences of defective efferocytosis will be discussed further in the next section.

1.5.5 When efferocytosis goes awry

Efferocytosis often carries on unnoticed, clearing away dying cells and maintaining homeostasis. Though when this process goes awry, the consequences can be dire. As stated above, millions of cells die every second in the human body, and in the case of an injury hundreds of millions can die in an instant. A vast majority of these cells are quickly cleared through successful efferocytosis and prevents the spewing of intracellular contents into the extracellular space (Peter M. Henson et al., 2001; Kurosaka et al., 2003; Maderna & Godson, 2003; Peter et al., 2010; J Savill & Fadok, 2000; John Savill et al., 2002b; Voll et al., 1997). If an AC is stagnant for a time, a second wave of clearance mechanisms kicks in, opsonization. Surrounding immune cells release molecules like C1q (L. Colonna et al., 2016; Liang et al., 2014), IgM (L. Colonna et al., 2016; S. J. Kim et al., 2002; Liang et al., 2014), ficolins (Schmid et al., 2012), pentraxins (Bijl et al., 2003; Janko et al., 2011; van Rossum et al., 2004), and even nuclear materials (Zirngibl et al., 2015) to tag and opsonize the late apoptotic cell (Gaipl et al., 2001; Mevorach et al., 1998; Ogden et al., 2001, 2005; Poon et al., 2010b; Quartier et al., 2005; Taylor et al., 2000). This added layer of signaling highlights the AC and engages additional phagocytic receptors to ensure its clearance including FcγRIIA, C1q receptor, CR1, CD91, and calreticulin (Franz et al., 2007; Hart et al., 2004; Ogden et al., 2001).

Though, if an AC escapes both these layers of targeting, they can become secondarily necrotic which results in an explosion of the cell and release of all intracellular contents—also termed damaged associated molecular patterns (DAMPs)—including genetic material (nucleotides), free-floating calcium, and pro-inflammatory factors (HMGB1) among others into the environment (Linton et al., 2016; Scaffidi et al., 2002; Schrijvers et al., 2005a; Thorp & Tabas, 2009; Yamasaki et al., 2008). Now, instead of AC clearance occurring in its normal immunologically silent fashion (Peter M. Henson et al., 2001; Hoffmann et al., 2001; Ravichandran & Lorenz, 2007), a large inflammatory response can occur. This process can have highly damaging effects, leading to pathological inflammation and even breaking of self-tolerance and initiation of autoimmunity (Baumann et al., 2002a; A. L. Evans et al., 2017; Fink & Cookson, 2005; Poon et al., 2010b; Ren et al., 2003; John Savill et al., 2002b). Comfortingly even if a cell

becomes necrotic, phagocytes can employ certain machinery to clean up the debris. Phagocyte DNase1 and DNase1L3 can digest extracellular DNA released from necrotic cells (Napirei et al., 2005; Sisirak et al., 2016) and their scavenger receptors including MSR1, MARCO, and SCARF1 can be used to clear other released cellular components (Ramirez-Ortiz et al., 2013; Shichita et al., 2017). Though, this process is not perfect and sometimes cellular contents escape capture do promote an inflammatory response, especially if there are any defects in efferocytic machinery.

1.5.5.1 Alterations in efferocytic pathways lead to pathology

In an effort to understand the complexities of efferocytic machinery, several groups have genetically or pharmacologically altered different components and assessed outcomes on health and disease. With the large number of efferocytic ligands and receptors, one would expect the system would be highly redundant. Surprisingly, loss of a single receptor in many cases significantly reduces efferocytic efficiency in mice. Alterations of MER (P. L. Cohen et al., 2002b; Garbin et al., 2013b; Thorp et al., 2008), CD36, LRP1 (Garbin et al., 2013b), SR-BI (Tao et al., 2015), Tim-1/4 (Miyanishi et al., 2007; S. Xiao et al., 2012), SCARF1 (Ramirez-Ortiz et al., 2013), or CD300f (Linjie Tian et al., 2014b) resulted in reduced clearance of ACs (Ait-Oufella et al., 2008; Doran et al., 2017; Thorp et al., 2008). Even more, double or triple receptor knockouts in mice leads to exaggerated advanced onset and/or more severe disease than single knockouts (Q. Lu & Lemke, 2001; Miyanishi et al., 2012). This suggests that perhaps PS receptors act in a complex together and all portions are needed. As efferocytosis is impaired in these conditions, there is also a concordant increase in pro-inflammatory cytokines that are normally inhibited in this process (Ait-Oufella et al., 2008; Kimani et al., 2014; Linjie Tian et al., 2014b). Further, reduction is efferocyte receptor signaling directly leads to pathologies in mice including auto-immunity, advanced atherosclerosis, and other diseases. These diseases will be discussed in more detail in following sections.

Interestingly some receptors, like MER, can be endogenously altered and prompted to inhibit efferocytosis. MER can be cleaved by metalloproteinases like ADAM17 to produce a shed form of the protein (sMer) (Sather et al., 2007a; Thorp, Vaisar, et al., 2011; Wan et al., 2013). sMer can act in a dominant negative fashion and compete for binding with essential bridging molecules like Gas6 and protein S, reducing effective efferocytic signaling (Doran et al., 2017; Wan et al., 2013). ADAM17 also cleaves the scavenger receptor CD36 and reduces efferocytosis

(Driscoll et al., 2013). Accordingly, employing antagonists against ADAM17 can result in increased efferocytosis, a potential therapy for disorders with an efferocytic etiology (Boersma et al., 2005; Kenis et al., 2006). Finally, other forces of nature including viruses and cancers have been able to hijack the efferocytosis system to their advantage. Viruses and cancers cleverly increase their expression of surface PS to broadly reduce inflammation and promote immune tolerance (Chiba et al., 2012; Ferris et al., 2014; Graham et al., 2014; Kelleherjr et al., 2015; Morganti-Kossmann et al., 2002). Viruses also use this technique to invade cells of the immune system through their efferocytosis of infected ACs (Amara & Mercer, 2015; Y. H. Chen et al., 2015; Czuczman et al., 2014; Feng et al., 2013; Mercer & Helenius, 2008, 2010; Morizono & Chen, 2014). Potential PS-blocking strategies have been employed as potential therapies in both these cases with promising results (Bondanza, Zimmermann, Rovere-Querini, et al., 2004; Dowall et al., 2015; Frey et al., 2009; Huang et al., 2005; Moody et al., 2010; Shibata et al., 2014; Soares et al., 2008).

1.5.5.2 Atherosclerosis

An area of disease where efferocytosis is heavily studied is atherosclerosis (AS). AS cardiovascular disease is a major cause of death in men and women across the world (Mozaffarian et al., 2015; H. Wang et al., 2016). In healthy blood vessels, macrophages digest any small cholesterol and lipid plaques that may form. Though once an individual has entered advanced stages of AS, macrophages can become overwhelmed through ingestion of oxidized low-density lipoproteins (LDL), other modified lipoproteins, and fat plaques transforming them into “foamy” macrophages gorged with lipids (S. G. Chen et al., 2010). These “foamy” macrophages have a reduced efferocytic capacity of around 20-fold compared to those in healthy tissues (Kockx, 1998; Schrijvers et al., 2005b). In AS lesions there is an accumulation of extracellular debris and increased numbers of ACs, suggesting a defect in efferocytosis (M. I. K. Chang et al., 1999; Ravichandran, 2010). Macrophages make up >40% of ACs in AS lesions (Frank D. Kolodgie et al., 2000) and contribute to plaque necrosis and deleterious cardiovascular events (Hansson et al., 2015; Y. Li et al., 2013; Liao et al., 2012; Martinet et al., 2011; Y. N. Qian et al., 2014). Here they have reached capacity and are unable to continue efferocytosis of additional plaques, propagating the disorder. Indeed, the level of AC accumulation is correlated with AS progression (Frank D. Kolodgie et al., 2000).

Interestingly in early stages of AS macrophage apoptosis is beneficial as it reduces the cellularity of the lesion (Liao et al., 2012; Tabas, 2005) and maintains an anti-inflammatory environment as they eat and are eaten by other immune cells, promoting IL-10 production (Babaev et al., 2008; Caligiuri et al., 2003; Y. Liu et al., 2006). Though in late stages of AS, as the necrotic core forms and efferocytosis is reduced, these apoptotic macrophages become necrotic (Ball et al., 1995; F D Kolodgie et al., 2004; Schrijvers et al., 2007; Tabas, 2005), activate detrimental inflammatory pathways (via IL-6, IL-12, TNFα, etc.), and release damaging proteases (Apostolakis & Spandidos, 2013; S. G. Chen et al., 2010; Ohayon et al., 2008; Shah, 2007). Advanced AS "foamy" macrophages accumulate more ROS (Chistiakov et al., 2016), activating 12/15-lipoxygenase which further inhibits efferocytosis (Y. I. Miller, Viriyakosol, et al., 2003; Y. I. Miller, Worrall, et al., 2003; Schrijvers et al., 2005b; Swarnakar et al., 1999).

Increasing ROS in lesion sites also leads to the formation of oxidized lipids (Song et al., 2016) that not only further promote the formation of "foamy" macrophages, but they actively compete with ACs for efferocyte recognition (Aprahamian et al., 2004; Bird et al., 1999; M. I. K. Chang et al., 1999; Oka et al., 1998; Schrijvers et al., 2005b). These molecules compete for immune receptors CD14 and SR-BI, scavenger receptors, and can increase Rho kinase activity in AS lesions to impair macrophage actin polymerization (Y. I. Miller, Viriyakosol, et al., 2003; Nakaya et al., 2006b; Ogden et al., 2005; Randolph, 2014; Schrijvers et al., 2007; Song et al., 2016). Macrophages aren't the only cell type that lose their efferocytic capacity with disease progression, as blood vessel smooth muscle cells (Vengrenyuk et al., 2015) and dendritic cells (Thorp, Subramanian, et al., 2011) interact with oxidized lipids they too are less able to clear accumulating ACs. Oxidized LDL (oxLDL) promotes the accumulation of inedible ACs in AS plaques (Schrijvers et al., 2005b). oxLDL also induces the production of phospholipid antibodies which may cause the shedding of AC "eat me" ligands (M. I. K. Chang et al., 1999; Y. I. Miller, Viriyakosol, et al., 2003). When LPC (a major component of oxLDL) was continuously infused in AS mice, it impaired efferocytosis and worsened disease (Aprahamian et al., 2004). This could also be due to disturbance in the LPC "find me" chemoattractant gradient released by ACs.

While excess modified lipids seem to the primary culprit for promotion of AS, alterations in several efferocytic receptors and signaling pathways have also been implicated. Some groups have found a reduction in efferocytosis mediators including MGF-E8 (Ait-Oufella et al., 2007), LRP1 (Gorovoy et al., 2010), and MER (Thorp, Vaisar, et al., 2011), as well as increased "don't

eat me" signal CD47 (Boucher & Herz, 2011) in the necrotic AS core. These alterations often lead to the accumulation of ACs and acceleration of atherosclerotic disease (Ait-Oufella et al., 2008; Tajbakhsh et al., 2018; Thorp et al., 2008). Experimental alterations of factors like Gas6 (Maree et al., 2007), MER (Ait-Oufella et al., 2007; P. L. Cohen et al., 2002a; Thorp et al., 2008), MFG-E8 (Asano et al., 2004; Hanayama et al., 2004), C1q (van Vré et al., 2012), LRP1 (Boucher et al., 2003; Lillis et al., 2008; Yancey et al., 2010), SR-BI (Tao et al., 2015), and Calr (Gardai et al., 2005a) reduce efferocytic capacity, increase inflammation (TNFα and MMP-9), and enhance lesion formation (Ait-Oufella et al., 2007, 2008; Bhatia et al., 2007; Thorp et al., 2008; Yancey et al., 2010). In human GWASs, AS patients were shown to have reduced expression of the "eat me" signal Calr (Kojima et al., 2014). Weakened Calr-LRP1 signaling may reduce the magnitude of ABCA1 upregulation macrophages need to export excess cholesterol, thus propagating the disease.

Most arterial defects of efferocytotic signaling are tied to the heightened inflammation found there (Y. I. Miller, Viriyakosol, et al., 2003; Tao et al., 2015). TNFα promotes expression of the potent "don't eat me" signal CD47 on ACs (Kojima et al., 2016). As mentioned previously, defective efferocytosis often leads to heighted inflammation, thus increasing levels of TNFα and creating a powerful positive feedback loop (Zhu et al., 2016). TLR signaling can also reduce the expression of bridge molecule MFG-E8 and prevent proper formation of MER, LRP1, and SR-BI (A-Gonzalez et al., 2009; Costales et al., 2013). ADAM17, which as mentioned earlier can cleave MER, is abundantly present in AS necrotic cores and acts to impede efferocytosis (Garbin et al., 2013a; Gorovoy et al., 2010; Sather et al., 2007b; Thorp, Vaisar, et al., 2011; Wan et al., 2013). Interestingly in AS, macrophages were more inclined toward a pro-inflammatory M1 phenotype instead of an M2 resolution phenotype (Yamamoto et al., 2011). M2 macrophages have higher efferocytic capacity than M1 macrophages (W. Xu et al., 2006) and tend to reduce the severity of AS (H. Y. Chang et al., 2015) as they reduce pro-inflammatory mediators like MCP-1 and TNFα while upregulating pro-resolution factors including mannose receptor and CD163 (Feig et al., 2011). Arginase 1 is an M2 marker associated with efferocytosis and is expressed in early AS where efferocytes are effectively clearing ACs (Leitinger & Schulman, 2013). Contrastingly, arginase 2 is an M1 marker and is expressed more highly in late, progressive AS (Khallou-Laschet et al., 2010b). Many potential therapies for treating AS employ a reduction in inflammatory signaling and/or an increase in efferocytic effectiveness (Arai et al., 2005; Bories et al., 2013;

Boucher & Herz, 2011; Caligiuri et al., 2003; Gautier et al., 2009; Kojima et al., 2016; S. Li et al., 2009; Y. Liu et al., 2006; Tao et al., 2015).

1.5.5.3 Lupus and auto-immunity

When a phagocyte does arrive and attempt to clean up the remnants of a cell that has undergone secondary necrosis, some self-peptides (like DNA) can be ingested, processed, and presented via MHC-II molecules to activate the inflammatory arm of the immune system that then produces autoreactive antibodies (Bondanza, Zimmermann, Dell'Antonio, et al., 2004; Bondanza, Zimmermann, Rovere-Querini, et al., 2004; Kuenkele et al., 2003; Muñoz et al., 2010; Rovere et al., 2000; Silva et al., 2008). When released into circulation, these antibodies can bind to other "self" cells and propagate an autoimmune response (Muñoz et al., 2009, 2010) leading to disorders like systemic lupus erythematosus (SLE). SLE is an autoimmune disease affecting several organs including the lungs, skin, kidneys, heart, nervous system, and other sysstems (Cancro et al., 2009). SLe patients have circulating auto-antibodies that tag nuclei, DNA, phospholipids, and other cellular contents which propagates the disease (Rahman et al., 2008).

It is known that clearance of ACs in humans with SLE is reduced, pointing toward defective efferocytosis (Baumann et al., 2002b; Hepburn et al., 2007; Herrmann et al., 1998; Kuhn et al., 2006; Muñoz et al., 2010). As further evidence for this, AC challenged macrophages derived from SLE patients cannot carry out efferocytosis as effectively as healthy patient macrophages (Baumann et al., 2002b; Herrmann et al., 1998; W. H. Shao & Cohen, 2011). Mechanistically it is though that, similar to atherosclerosis, there are endogenous alterations in efferocytic machinery that reduce the capacity for AC clearance (Herrmann et al., 1998; Muñoz et al., 2010). Indeed, humans with deficient C1q expression (Botto & Walport, 2002) or higher levels of shed TAM RTKs (Ballantine et al., 2015; J. Wu et al., 2011) in their serum are more prone to developing SLE. Genetic polymorphisms in bridging molecule MFG-E8 are also associated with patients being afflicted with SLE (C. Y. Hu et al., 2009b). Genetic deletion of several efferocytic signaling proteins in animals like MGF-E8, BAI1, TIM-4, MER, and C1q led to reduced efferocytosis and SLE-like disorders (Asano et al., 2004; Hanayama et al., 2004; Q. Lu & Lemke, 2001; Rodriguez-Manzanet et al., 2010; Schweigert et al., 2014; Scott, RS.; McMahon, EJ.; Pop, SM.; ...; EEarp, HS.; Matsushima, 2001).

SLE targets multiple tissues and has widespread accumulation of ACs, but interestingly in more targeted inflammatory disease like cystic fibrosis, asthma, and COPD, the accumulation of ACs occurs predominantly in the lung (P M Henson & Tuder, 2008). This points to the local role efferocytosis plays in health and disease. While atherosclerosis and SLE have gained the most attention regarding defective efferocytosis, links to several other disorders have been posited. Evidence exits that links other auto-immune disorders including rheumatoid arthritis, diabetes, and others (Green et al., 2016; Heimberg et al., n.d.; S. Li et al., 2009; B. A. O'Brien et al., 2002; Bronwyn A. O'Brien et al., 2006; Ravichandran, 2010; Waterborg et al., 2018, 2019) to impaired AC clearance.

1.5.6 Efferocytosis in the nervous system

While efferocytosis has received an abundance of attention in other areas like atherosclerosis, lupus, diabetes, and others, its function in the nervous system is only recently being explored. There are just a handful of publications investigating the role of efferocytosis following strokes in the CNS, almost all in the last six years. As microglia are the primary immune cell of the CNS, they carry out the vast majority of efferocytosis there (Damisah et al., 2020; Fu et al., 2014), though peripheral immune cells do infiltrate and aid in the process following injury (W. Zhang et al., 2019). Interestingly macrophages *in vitro* were shown to respond to apoptotic neurons more quickly and with greater phagocytic capacity than microglia (Iadecola & Anrather, 2011; Ritzel et al., 2015). Though microglia are imperative for the later stages of healing as they become the major efferocyte around seven days after ischemic stroke, as monocytes leave (Ritzel et al., 2015). Non-professional phagocytes participate in CNS efferocytosis as well including endothelial cells (Grutzendler et al., 2014; Saha et al., 2018), pericytes (Shibahara et al., 2020), oligodendrocytes (Ludwin, 1990; K. B. Nguyen & Pender, 1997), and astrocytes (Damisah et al., 2020; Sloan et al., 2017).

Several common efferocyte signaling molecules found in the peripheral are also active in the CNS. Microglial Tim-4 and BAI1 are important for phagosome formation and stabilization around dying neurons (Mazaheri et al., 2014). Brain microglia and macrophage utilize the IL-4-STAT6- PPARγ-Arg1 signaling axis to carry out efferocytosis in the ischemic brain (Szanto et al., 2010). PPARγ activation regulates several efferocytic gene targets (like CD36) and is induced during annexin 1-mediated efferocytosis of apoptotic neurons (da Rocha et al., 2019; Flores et al.,

2016). Astrocytes and microglia employ MER, GULP1, complement, and MGEF-10 to engulf synapses and apoptotic neurons as well as ABCA1 to facilitate release of ingested cholesterol (Chung et al., 2013; Damisah et al., 2020; Iram et al., 2016; Konishi et al., 2020; Morizawa et al., 2017; Stevens et al., 2007). Ischemia following stroke elicits reprogramming of phagocytes toward an efferocytic phenotype with upregulation of efferocyte receptors and bridging molecules, alterations of their cytoskeleton, and activation of anti-inflammatory regulators (W. Cai et al., 2019; Perego et al., 2011; Shengxiang Zhang, 2019).

Similar to the peripheral systems, abolishment or reduction in efferocyte signaling can lead to disease and worsened outcomes after injury. A deficiency in STAT6 leads to larger infarct volumes and impaired neurological behavior in an animal stroke model (W. Cai et al., 2019). Defects in Axl or MER also led to reduced efferocytosis and worsening of outcomes after stroke (C. F. Chang et al., 2018). Interestingly it was recently revealed that a high-salt diet can lead to a reduction in macrophage efferocytic receptor Trem2, reducing efferocytosis and impairing recovery after stroke (M. Hu et al., 2021b). Arginase 1 (Arg1), known to designate pro-resolving immune cells, has shown to play a main role in CNS efferocytosis. Efferocytic molecules like annexin 1 and IL-4 as well as AC-derived L-arginine increase Arg1 expression (Gray et al., 2005; Yurdagul et al., 2020). Arg1, through L-arginine catabolism, activates Rac1 to polymerize actin for phagocytic cup formation (Yurdagul et al., 2020). Deletion of Arg1 impairs an efferocyte's ability to ingest multiple ACs (C. Ma et al., 2021; Yurdagul et al., 2020), impairs lysosomal activity (C. Ma et al., 2021; Shen et al., 2016), and de-represses inflammatory nitric oxide and superoxide production (Fouda et al., 2018).

Culmination of research in the CNS and other tissues has identified a few potential avenues for treatments following stroke and other CNS disorders. Injection of IL-4 enhances microglial efferocytosis and improves recovery after stroke (Zhao et al., 2015). Introduction of a PPARγ agonist before and after a rat cerebral artery occlusion improves neurologic function and reduces infarct size (Sundararajan et al., 2005; Zhao et al., 2007). Treatment of hemorrhagic stroke with the retinoid bexarotene, which activates retinoid X receptors (RXRs), results in increased expression of efferocytosis receptors like Axl and CD36 which improve neurological outcomes (Certo et al., 2015; C. F. Chang et al., 2020). Further, several specialized pro-resolving mediators (SPMs) discussed earlier can reduce severity of stroke and improve recovery by reducing immune infiltration and inflammation, enhancing efferocytosis, and reducing neuronal injury (P. Yin et al.,

2018). Interestingly some literature reveals a potential detriment of CNS efferocytosis where damaged neurons that could potentially survive and regenerate are cleared by efferocytes as they express “eat me” signals (Fricker et al., 2012). In fact, inhibition of phagocytosis following transient ischemia can reduce neuronal loss (Bellizzi et al., 2016; Neher et al., 2013; Jin Yang et al., 2021). Though crucially one must consider the timing of these therapies to achieve optimal success. Immediately following stroke, infiltrating immune cells are actually skewed toward a reparative phenotype, though it is replaced by a pro-inflammatory and non-efferocytic phenotype in later phases. Thus, it is important to reduce the ingestion of damaged neurons that could survive early on, but increase efferocytosis in later stages to enhance post-stroke recovery (Ting et al., 2020).

In addition to ischemic and hemorrhagic stroke, frequent observations are made of increased and accumulating ACs in neurodegenerative disorders including Parkinson’s, Alzheimer’s, and Huntington’s disease (Mattson, 2000). This in part may be due to increased rates of cell death, but importantly loss of “find me” CX3CL1 signaling was shown to worsen these diseases (Cardona et al., 2006). While this work has been encouraging, there remain several unanswered questions regarding the role of efferocytosis in the nervous system. Is efferocytosis involved in injury and disease states of the peripheral nervous system? If so, are similar signaling mechanisms employed including the many “find me,” “eat me,” and “don’t eat me” molecules and receptors? What are the specific cell types involved in the process, both the ACs and efferocytes? How quickly after injury does efferocytosis engage? Does the process last only during degeneration and debris clearance or is it sustained throughout the regeneration process? What are the consequences of successful and unsuccessful efferocytosis both in terms of debris clearance and immune regulation? Do defects in efferocytosis contribute to conditions like diabetic neuropathy, myasthenia gravis, and neuropathic pain? While the work described below answers some of these questions, large advancements must still be made to understand the key principles governing effective efferocytosis in peripheral nervous tissues. Once these mechanisms are more fully understood, they can be better employed as potential therapies for the treatment of debilitating and deadly nervous system injuries and diseases.

1.6 Figures

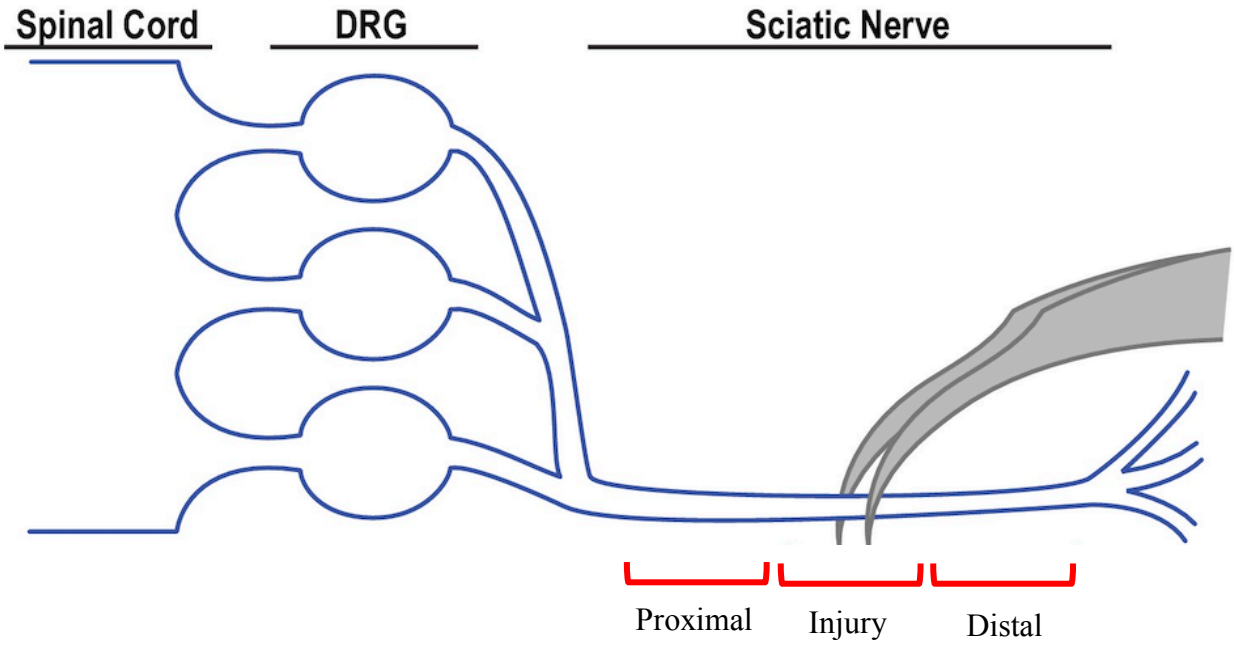

Figure 1-1 Anatomy of lumbar spinal cord, DRGs, and injured sciatic nerve.
Diagram displaying the relation of the spinal cord, DRGs, and sciatic nerve within the nervous system. After sciatic nerve crush using forceps, the sciatic nerve can be broken down into three segments: proximal segment, injury site, and distal stump. The distal stump will undergo Wallerian degeneration and be cleared away by Schwann and immune cells. The proximal stump will seal its ruptured membrane, form a growth cone, and extend through the injury site after endothelial cells, fibroblasts, and Schwann cells have formed a cellular bridge. The proximal stump will then reach the surviving Schwann cells in the Bands of Büngner which will guide it toward its terminal muscle targets.

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CHAPTER 2:
Analysis of the Immune Response to Sciatic Nerve Injury: Efferocytosis as a Key Mechanism of Nerve Debridement

2 Analysis of the Immune Response to Sciatic Nerve Injury: Efferocytosis as a Key Mechanism of Nerve Debridement

2.1 Abstract

Sciatic nerve crush injury triggers sterile inflammation within the distal nerve and axotomized dorsal root ganglia (DRGs). Granulocytes and pro-inflammatory Ly6C^{high} monocytes infiltrate the nerve first, and rapidly give way to Ly6C^{negative} inflammation-resolving macrophages. In axotomized DRGs, few hematogenous leukocytes are detected and resident macrophages acquire a ramified morphology. Single-cell RNA-sequencing of injured sciatic nerve identifies five macrophage subpopulations, repair Schwann cells, and mesenchymal precursor cells. Macrophages at the nerve crush site are molecularly distinct from macrophages associated with Wallerian degeneration. In the injured nerve, macrophages "eat" apoptotic leukocytes, a process called efferocytosis, and thereby promote an anti-inflammatory milieu. Myeloid cells in the injured nerve, but not axotomized DRGs, strongly express receptors for the cytokine GM-CSF. In GM-CSF deficient (*Csf2*$^{-/-}$) mice, inflammation resolution is delayed and conditioning-lesion induced regeneration of DRG neuron central axons is abolished. Thus, carefully orchestrated inflammation resolution in the nerve is required for conditioning-lesion induced neurorepair.

2.2 Introduction

In the injured adult mammalian CNS, the regenerative capacity of severed axons is very limited. However, regeneration of dorsal column axons in the rodent spinal cord can be augmented if preceded by a conditioning lesion to the sciatic nerve (McQuarrie et al., 1977; Neumann and Woolf, 1999; Richardson and Issa, 1984). This seminal observation has been exploited extensively to identify mechanisms that promote axon regeneration (Abe and Cavalli, 2008; Blesch et al., 2012; Chandran et al., 2016). Traumatic PNS injury leads to sterile inflammation at the site of injury and within the distal nerve stump where axons undergo Wallerian degeneration (Kim and Moalem-Taylor, 2011; Perry et al., 1987). In addition, a remote inflammatory response is observed in axotomized dorsal root ganglia (DRGs) (Hu and McLachlan, 2003; Lu and Richardson, 1991) and the lumbar spinal cord (Guan et al., 2016; Hu et al., 2007; Zhang et al., 2007). The innate arm of the immune system is important for peripheral nerve regeneration, as well as conditioning-

lesion-induced dorsal column axon regeneration (Kwon et al., 2015; Niemi et al., 2013; Salegio et al., 2011; Zigmond and Echevarria, 2019). Very recent studies employed single cell RNA sequencing (scRNA-seq) to describe gene expression in naïve and injured peripheral nervous tissue at cellular resolution (Wang et al., 2020; Wolbert et al., 2020; Ydens et al., 2020). A comparative analysis of immune cell profiles within the injured sciatic nerve and axotomized DRGs, however, has not yet been carried out.

The sciatic nerve trunk is covered by the epineurium, a protective connective tissue sheath that harbors fibroblasts, macrophages, and blood vessels. The more delicate perineurium covers nerve bundles and the endoneurium is a tube-like structure wrapped around individual myelinated fibers. The endoneurium contains macrophages and fibroblast-like mesenchymal cells (MES) (Carr et al., 2019; Ydens et al., 2020). Following PNS injury, Schwann cells (SC) reprogram into repair cells and together with MES and nerve-resident macrophages produce chemokines and cytokines to promote entry of hematogenous immune cells (Arthur-Farraj et al., 2012; Muller et al., 2010; Richard et al., 2012; Ydens et al., 2020). Repair SC, together with innate immune cells, contribute to nerve debridement, formation of new blood vessels, and release of growth promoting molecules, thereby creating a microenvironment conducive for long-distance axon regeneration and tissue repair (Barrette et al., 2008; Clements et al., 2017; DeFrancesco-Lisowitz et al., 2015; Hoke et al., 2000; Martini et al., 2008). Despite recent progress, it remains unclear which cell types in the injured nerve contribute to tissue debridement and there is a paucity in our understanding of the underlying molecular mechanisms (Brosius Lutz et al., 2017; Klein and Martini, 2016).

Sciatic nerve injury leads to a remote and strong cell body response in axotomized DRG neurons (Chandran et al., 2016). This includes induction of neuron-intrinsic growth programs, neuronal release of cytokines and chemokines, activation of intra-ganglionic tissue-resident macrophages, immune-like glia, and entry of hematogenous leukocytes (Cafferty et al., 2004; McLachlan and Hu, 2014; Richardson and Lu, 1994; Richardson et al., 2009; Wang et al., 2018; Zigmond and Echevarria, 2019). Experimentally induced intra-ganglionic inflammation, triggered by injection of *C. parvum* bacteria into DRGs, increases axon regeneration following dorsal root injury (Lu and Richardson, 1991). Intra-ganglionic expression of recombinant CCL2 leads to increased macrophage staining, enhanced DRG neuron outgrowth *in vitro* (Niemi et al., 2016), and regeneration of DRG neuron central projections following spinal cord injury (Kwon et al., 2015).

Here we employed a combination of flow cytometry, mouse reporter lines, and immunofluorescence labeling to describe the leukocyte composition in the injured sciatic nerve and axotomized DRGs. We used parabiosis to show that upon sciatic nerve crush injury (SNC), the origin, magnitude, and cellular composition of immune cell profiles is very different between the nerve and DRGs. For a comparative analysis, we carried out bulk RNA sequencing of DRGs and single cell RNA sequencing (scRNA-seq) of injured nerves. We report the cellular make up, cell-type specific gene expression profiles, and lineage trajectories in the regenerating mouse PNS. Computational analysis revealed cell-type specific expression of engulfment receptors and bridging molecules important for eating of apoptotic cell corpses, a process called efferocytosis (Henson, 2017). We show that within the injured nerve, monocytes (Mo) and macrophages (Mac) eat apoptotic leukocytes, and thus, contribute to inflammation resolution. Strikingly, Mac at the nerve injury site are molecularly distinct from Mac in the distal nerve stump. *Csf2ra* and *Csf2rb*, obligatory components of the GM-CSF receptor (Hansen et al., 2008), are strongly expressed by myeloid cells in the injured nerve, but not in axotomized DRGs. Functional studies with *Csf2*$^{-/-}$ mice, deficient for GM-CSF, show that this cytokine regulates the inflammatory milieu in the injured nerve and is important for conditioning lesion elicited dorsal column axon regeneration. Taken together, our work provides novel insights into a rich and dynamic landscape of injury-associated cell states, and underscores the importance of properly orchestrated inflammation resolution in the nerve for neural repair.

2.3 Results

2.3.1 Quantitative analysis of immune cell profiles in the injured sciatic nerve

Despite recent advances in our understanding of PNS injury-induced inflammation, a comparative analysis of the leukocyte subtypes within the injured sciatic nerve and axotomized DRGs does not yet exist. For identification and quantification of immune cell profiles at different post-injury time points, adult mice were subjected to a mid-thigh sciatic nerve crush (SNC) injury. SNC leads to axon transection, but preserves the surrounding epineurium (**Figure 2.1A**). Flow cytometry was used to assess the composition of injury-mobilized immune cell profiles in the nerve and DRGs (gating strategy is illustrated in **Figure 2.2**). To minimize sample contamination

with circulating leukocytes, mice were perfused with physiological saline prior to tissue collection. The nerve trunk was harvested and divided into a proximal and distal segment. The distal segment included the injury site together with the distal nerve stump (**Figure 2.1A**). For comparison, the corresponding tissues from naïve mice were collected. In naïve mice, ~300 live leukocytes ($CD45^+$) are detected within a ~5 mm nerve segment. At day 1 following SNC (d1), the number of $CD45^+$ cells in the distal nerve increases sharply, peaks around 23,100± 180 cells at d3, and declines to 14,000± 200 at d7 (**Figure 2.1B**). Further analysis shows that granulocytes (GC), identified as $CD45^+CD11b^+Ly6G^+CD11c^-$ cells, are absent from naïve nerve, but increase to 7,800± 300 at d1. By d3, the number of GC dropped below 1,000 (**Figure 2.1C**). A robust and prolonged increase of the Mo/Mac population ($CD45^+CD11b^+Ly6G^-CD11c^-$) is observed, reaching 7,300± 120 cells at d1, peaking around 13,200± 240 at d3, and declining to 3,200± 90 at d7 (**Figure 2.1D**). Monocyte-derived dendritic cells (MoDC), identified as $CD45^+CD11b^+Ly6G^-CD11c^+$ cells, increase more gradually. They are sparse at d1, reach 1,100± 30 at d3, and 3,400± 60 at d7 (**Figure 2.1E**). Few $CD11b^-$ conventional DC (cDC), identified as $CD45^+CD11b^-Ly6G^-CD11c^+$ cells, are present at d1 and d3 and cDC increase to 600± 20 at d7 (**Figure 2.1F**). The total number of lymphocytes ($CD45^+CD11b^-CD11c^-Ly6G^-$) is low, but significantly elevated at d1, d3, and d7 post-SNC (**Figure 2.1G, 1H**). In marked contrast to the distal nerve stump, flow cytometry of the proximal nerve stump shows that SNC does not significantly alter immune cells number or composition (**Figure 2.3A-K**). The sharp divide in myeloid cell distribution within the injured nerve is readily seen in longitudinal sections stained with anti-F4/80 (**Figure 2.3L**). The distal nerve stump was identified by anti-GFAP staining, a protein upregulated in repair Schwann cells (**Figure 2.3L**). In sum, SNC-elicited inflammation in the nerve is confined to the crush site and the distal nerve stump where severed fibers undergo rapid Wallerian degeneration. GC increase sharply and peak within 24h, followed by Mo/Mac, MoDC, and few lymphocytes.

2.3.2 Quantitative analysis of immune cell profiles in axotomized DRGs

Immunofluorescence staining of DRG sections shows that SNC causes a transient increase in Iba1 and F4/80 immunolabeling, peaking around d3 and declining at d7 (**Figure 2.4A**). Flow cytometric analysis of DRGs from naive mice identifies on average ~600 live leukocytes per ganglion, including GC, Mo/Mac, MoDC, cDC, and lymphocytes (**Figure 2.4B-G**). At d1, no significant change in intra-ganglionic immune cell profiles is observed. At d3, there is a ~2-fold increase in

leukocytes, however a significant increase is only observed for Mo/Mac (**Figure 2.4C**). At d7, the Mo/Mac population is significantly reduced compared to d3. The MoDC and cDC populations are elevated at d7 when compared to DRGs from naïve mice (**Figure 2.4D-E**). Lymphocytes are present in naïve DRGs but do not significantly increase during the first week post-SNC (**Figure 2.4F-G**). The presence of $CD3^+$ T cells in DRGs was validated by immunofluorescence labeling of L5 DRG sections (**Figure 2.5**). For an independent assessment of the kinetics and magnitude of SNC-induced inflammation in the nerve trunk and DRGs, we used Western blotting to carry-out a 3-week time-course analysis. Probing tissue lysates with anti-CD11b shows that the injury-induced increase in myeloid cells in the nerve trunk exceeds the one in axotomized DRGs by an order of magnitude (**Figure 2.4H-I**). Taken together, these studies show that SNC induces a remote immune response in axotomized DRGs that is strikingly different in magnitude and cellular composition from injured nerve tissue.

2.3.3 Sciatic nerve injury triggers massive infiltration of immune cells into the injured nerve, but not axotomized DRGs

Endoneurial Mac in the sciatic nerve and DRGs respond to injury (Mueller et al., 2003; Muller et al., 2010), however, there are no reliable cell surface markers to distinguish between tissue-resident and injury-mobilized hematogenous immune cells that enter the nerve or axotomized DRGs. To examine cell origin, we employed parabiosis, that is conjoined wildtype (WT) and tdTomato (tdTom) reporter mice that share blood circulation. We chose parabiosis over bone marrow transplantation because of potential confounding effects caused by irradiation (Guimaraes et al., 2019). One month after parabiosis surgery, both parabionts were subjected to unilateral SNC. Sciatic nerves, DRGs, and spinal cords were harvested at different post-injury time points (**Figure 2.6A**). Shared blood circulation was assessed by flow cytometry of the spleen, and revealed a myeloid cell ($CD45^+CD11b^+$) chimerism of 27.3± 1.5 (**Figure 2.7**). At d3 following SNC, flow cytometric analysis of nerves isolated from WT parabionts identifies 28.4± 6.7% $tdTom^+$ myeloid ($CD45^+CD11b^+$) cells (**Figure 2.6B**). Fractionation of myeloid cells into Mo/Mac ($CD45^+CD11b^+Ly6G^-CD11c^-$) and MoDC ($CD45^+CD11b^+Ly6G^-CD11c^+$) further revealed that 27.1± 6.9% of Mo/Mac and 30± 5.6% of MoDC are $tdTom^+$ in the injured WT parabiont (**Figure 2.6C**). When coupled with ~27% chimerism (**Figure 2.7C**), this suggests that blood-borne cells make up the vast majority of immune cells in the injured nerve. Histological analysis of injured

nerves from WT parabionts identified numerous tdTom$^+$ cells (**Figure 2.6D**). During the first 24h, tdTom$^+$ cells are confined to the injury site (data not shown). At d3 and d7, tdTom$^+$ cells are preferentially found at the injury site but also present within the distal nerve stump where fibers undergo Wallerian degeneration (**Figure 2.6D and Figure 2.19C**). In the proximal nerve, very few tdTom$^+$ cells are detected at any post-SNC time point (**Figure 2.6D**). A two-week time course analysis of axotomized DRGs harvested from WT parabionts identified a modest and transient increase of tdTom$^+$ cells (**Figure 2.6E**). DRG sections from naïve mice revealed that the number of tdTom$^+$ cells per field-of-view (4,000 μm^2) is very low. Following SNC, there is a modest, but statistically significant increase in tdTom$^+$ cells at d3 and d7, but not at 14d, suggesting that only a small number of hematogenous leukocytes enter axotomized DRGs (**Figure 2.6F**). Together these studies show that SNC-elicited intra-ganglionic increase of Iba1$^+$ and F4/80$^+$ immune profiles (**Figure 2.4A**) primarily occurs through mechanisms that involve DRG-resident macrophages, rather than hematogenous immune cells. Of note, during the first two weeks post-SNC, no tdTom$^+$ cells were detected in the lumbar spinal cord (data not shown), suggesting that hematogenous immune cells do not significantly contribute to SNC-triggered spinal cord inflammation.

2.3.4 Sciatic nerve injury triggers significant macrophage morphological changes in axotomized DRGs

In tissue sections of axotomized DRGs, there is a rapid increase in Iba1 and F4/80 immunoreactive profiles (**Figure 2.4A**), yet in DRGs of parabiotic mice the number of blood-derived tdTom$^+$ immune cells is modest (**Figure 2.6E-F**). This raises questions regarding the underlying cellular basis of increased Iba1 immunoreactivity. Previous studies reported that upon sciatic nerve injury, DRG-resident Mac undergo limited proliferation (Leonhard et al., 2002; Yu et al., 2020). To examine whether altered macrophage morphology may contribute to increased Iba1 staining, axotomized DRGs were subjected to whole-mount immunofluorescence labeling with anti-Iba1 (**Figure 2.6G**). Three-dimensional projection analysis of Mac profiles, in the absence of nerve injury (intact) and at 3d post-SNC, revealed a 2.3-fold increase in the total volume occupied by Iba1$^+$ cells (**Figure 2.6I**). Two distinct Mac morphologies were observed in intact DRGs, a majority (84± 2%) of amoeboid cells and a smaller population (16± 2%) of elongated cells (**Figure 2.6H-J**). SNC triggers Mac morphological changes in axotomized DRGs (**Figure**

2.6H). Many Iba1$^+$ cells acquire a more complex, stellate morphology and exhibit enveloping extensions. At d3, Mac with amoeboid (60± 2%), elongated (10± 3%), and stellate morphologies (30± 4%) are identified. And at d7, amoeboid (40± 3%), elongated (5± 1%), and stellate (55± 4%) shaped Mac are detected (**Figure 2.6J**). While the SNC-triggered Mac morphological changes are quite striking, they do not alter the average volume of individual cells (**Figure 2.6K**). Based on these studies we conclude that local proliferation and morphological changes, rather than infiltration of blood-borne cells, contribute to increased Iba1 immunoreactivity in axotomized DRGs.

2.3.5 Immune-associated co-expression networks in axotomized DRGs

To gain insights into SNC-triggered genome wide transcriptional changes in DRGs, we carried out bulk RNA sequencing of ganglia harvested from naïve, d1, d3, and d7 injured mice. To understand the modular network structure associated with peripheral axotomy, we carried out weighted gene co-expression network analysis (WGCNA) at different post-injury time points (Geschwind and Konopka, 2009; Zhang and Horvath, 2005). WGCNA permits identification of modules of highly co-expressed genes that likely function together. Focusing on prominently regulated gene modules, we find a previously described module (pink module (Chandran et al., 2016)), enriched for regeneration associated gene (RAG) products, including *Jun, Fos, Stat3, Smad1, Atf3,* among other genes. In addition, WGCNA identifies a large turquoise module (**Figure 2.8A-B**), which along with the pink module, is stably upregulated following SNC (**Figure 2.9A-B**). To annotate module function, we applied gene ontology (GO) enrichment analyses, which showed enrichment (Benjamini-corrected p values < 0.05) for several GO categories associated with immune system function in the turquoise module. The enrichment plot for GO regulation shows a strong upregulation for *immune system processes* (**Figure 2.8C**). The most significantly upregulated GO terms include *cell activation*, *immune effector process*, and *defense response* (**Figure 2.9**). Ingenuity pathway analysis (IPA) identified several upstream activators, including cytokines and growth factors (IFNγ, TNF, IL1b, IL6, TGFβ1, IL10, IL4, IFNβ1, IL2) and the transcription regulators STAT1, STAT3, IRF7, RELA (**Figure 2.9D**). The upregulation of immune system processes in axotomized DRGs correlates with a modest ~1.5-fold increase of gene products encoding the canonical macrophage markers *Itgam* (CD11b), *Aif1* (Iba1), and *Adgre1* (F4/80) (**Figure 2.8D-F**). In comparison, expression levels and fold-upregulation

of *Atf3, Jun*, and *Stat3* are very robust (**Figure 2.8G-I**). Expression of the chemokine receptor *Ccr2* and the receptor subunits for the GM-CSF receptor (*Csf2ra* and *Csf2rb*) are elevated in axotomized DRGs, however expression levels are low, especially for *Csf2rb* (~1 fpkm) (**Figure 2.8J-L**). Moreover, some of the immune gene activity observed in axotomized DRGs may involve non-hematopoietic cells. Collectively, RNA-seq provides independent evidence that SNC triggers a remote inflammatory response in DRGs, however this does not result in a massive increase in transcripts encoding canonical Mac markers. This conclusion is consistent with flow cytometry (**Figure 2.4B-G**), Western blot analysis (**Figure 2.4H-I**), and 3D reconstruction of Mac (**Figure 2.6G-K**) in axotomized DRGs.

2.3.6 The cellular landscape of injured peripheral nerve tissue

To de-convolute the cellular complexity of injured sciatic nerve tissue in an unbiased manner, we applied scRNA-seq to capture the transcriptional landscape at single cell resolution. Because injury-induced expansion of the immune compartment peaks around d3 (**Figure 2.1B**), we chose this time point to dissect and process whole nerves for single cell capture, using the 10x Genomics platform. A total of 17,384 cells was sequenced with 16,204 used for downstream analysis after removing cells with fewer than 200 genes, more than 7,500, or mitochondrial content greater than 25%. Median unique genes per cell was 2,507. More than 20 different cell clusters were identified using shared nearest neighbor clustering algorithm. Results are visualized using Uniform Manifold Approximation and Projection (UMAP) for dimension reduction (**Figure 2.10A**). The top 100 genes enriched in each cluster (**Table 2.1**) were used to assign cluster specific cell identities. Most prominently featured are immune cells, identified by their strong expression of *Ptprc* (encoding CD45). Innate immune cells (*Itgam*/CD11b) make up a median 42.22% (± 1.39%), and lymphocytes less than 1.73%(± .27%), of the cells in the injured nerve (**Figure 2.10B-C**). Other abundantly featured cell types include mesenchymal progenitor cells (MES). We identify three distinct MES subpopulations (**Figure 2.10A**), reminiscent of a recent study examining the nerve response to digit tip amputation (Carr et al., 2019). In the injured sciatic nerve, MES make up 18.49%(±.98%) of cells and differentially express the markers *Pdfgra* and *Sox9* (**Figure 2.10D-E**). MES are a rich source of extracellular matrix (ECM) molecules, including collagens (*Col1a, Col3a, Col5a, Col6a), Fn1/*fibronectin*, Fbn1/*fibrillin-1*, Lamb2/*laminin-b2, and numerous proteoglycans (**Figure 2.11A).** Individual MES clusters are identified as perineural

MES (pMES) (*Slc2a1/*Glut1*, Itgb4/*integrin-β4*, Stra6/*stimulated by retinoic acid 6*, Sfrp5/*secreted frizzled related protein 5), endoneurial MES (eMES) (*Wif1/*Wnt inhibitory factor 1*, Bmp7*), and differentiating MES (dMES) *(Gas1/*Growth arrest-specific 1*, Ly6a/*SCA-1*, Tnc/*tenascin*, Sfrp1/*secreted frizzled-related protein 1). The dMES cluster is fused to a small population of fibroblasts (Fb) (**Figure 2.10A**). STRING Reactome pathway analysis for MES clusters identifies *extracellular matrix organization* as top hit (**Figure 2.12**). Further analysis revealed that cells in eMES, but not in clusters pMES and dMES, are neural crest derived (Carr et al., 2019; Gugala et al., 2018).

Three clusters of Schwann cells (SC1-3) represent 17.48% (±1.53%) of cells in the injured nerve (**Figure 2.10A**). Cluster SC1 contains proliferating cells marked by *Mki67/*Ki67 expression (**Figure 2.10J**) and many cells that strongly express *Ncam1, Chl1/*cell adhesion molecule L1-like*, Erbb3, Epha5, Thbs2/*thrombospondin-2*, Tnc, Hbegf,* and the BMP antagonist *Sostdc1* (**Figure 2.10F, Figure 2.13, and Table 2.1)**. SC1 enriched transcription regulators (SC1-TR) include *Zfp706, Tead1, Sox6, Nr2f1/COUP-TF* (**Figure 2.10K**). SC3 cells express high levels of *Ngfr/*p75*, Nrcam, Gfra1/*GDNF family receptor alpha 1*, Btc/*betacellulin*, Gjb1/*connexin-32*, Cryab/*crystallin alpha B*, Tnfrsf12a/*Fn14*, Gadd45b* (**Figure 2.10G, Figure 2.13, and Table 2.1)**. SC3-TR include *Sox4, Runx2, Hmga1, Jun,* and the POU family member *Pou3f1*, a repressor of BMP and Wnt signaling, associated with a pro-myelinating cell state (**Figure 2.10K**). Cluster SC2, flanked by SC1 and SC3, expresses *nes/*nestin and *Cryab*. UMAP splits the SC2 cluster and places a subset of cells near MES cells, likely because of relatively higher expression in ECM encoding genes (*Bgn, Dcn,* and *Fn1*) compared to clusters SC1 and SC3. SC2 cells have a median 584 (± 22) genes per cell and may have a higher degree of technical variation. STRING identified *axon guidance* and *integrin cell surface interactions* as top REACTOME pathways for SC1. *Axon guidance*, *gap junction assembly*, and *microtubule-dependent trafficking* are top hits for SC3 (**Figure 2.13**).

Cells associated with the nerve vasculature make up 14.2% (± 3.19%). They include three clusters of endothelial cells (EC1-3), strongly expressing *Pecam*/CD31, representing 9.92% (± 2.69%) of cells (**Figure 2.10H and Figure 2.14**). There are two pericyte cell clusters (PC1 and PC2) enriched for the pericyte markers (*Pdgfrb, Rgs5*) and vasculature-associated smooth muscle cells (*Acta2, Des, Myl9, Mylk),* representing 4.2% (± .44%) (**Figure 2.10I and Figure 2.15**). A small cluster of chondrocyte-like cells (CL: *Comp/cartilage oligomeric matrix protein, Col27a1,*

Jun) represents 0.5% (± 0.44%). A cell cluster (3.09% (±1.08%)), designated Hyb, harbors few erythrocytes (*Hba, Hbb*) and some cell hybrids (Hyb). These cells had a median 521 (± 27) expressed genes which was the lowest of any cell cluster and no clear identity could be assigned (**Figure 2.10B**).

Of relevance for neuronal regeneration, ECM components and numerous extracellular molecules known to regulate axon growth and regeneration are expressed by different cell types in the injured nerve (**Figure 2.11A**). MES and Fb are rich sources of gene products with neurotrophic and neurotropic properties, and thus may act in a paracrine fashion to regulate neuronal survival and direct axonal growth (**Figure 2.11B**). dMES express (*Igf1, Ogn/*osteoglycin*, Nid1/*Nidogen-1, *Ntn1/*netrin-1, *Postn/*periostin, *Gdf10/*BMP3b*, Cxcl12/*SDF1*, Dcn/*decorin*, Grn/*progranulin*, Sparc/*osteonectin*, lamb2/*laminin-b2*, Serpinf1*), eMES (*Spp1, Dcn, Nid1/*nidogen-1*, Sparc, Serpine2/*glia-derived nexin*, Lum/*lumican*, Gpc3/*glycpican-3), and pMES (*Ntn1*, *Cldn1/*claudin-1*, Efnb2/*ephrin-b2, *Mdk*/midkine*, Nid1, Sdc4/*syndecan-4*, Thbs4/*thrombospondin-4*, Gpc3*). Repair Schwann cells in clusters SC1 and SC3 express high levels of cytokine receptor like factor 1 *(Crlf1),* and SC3 highly express cardiotrophin-like cytokine factor 1 *(Clcf1). Crlf1 and Clcf1* are both members of the CNTF ligand family that signal through gp130. In addition, SC1 (*Chl1, Ncam1, Nrn1/*neuritin-1, *Ptn/pleiotrophin, Sema3e, Sema7a, Reln/*reelin), and SC3 (*Reln*, *Dag1/*dystroglycan, *Gdnf*, *Nrcam, Sema3b*) express numerous membrane-bound and soluble factors with known roles in axon growth and guidance. Subpopulations of myeloid cells exhibit high expression of the osteopontin-encoding gene, *Spp1* and progranulin (*Grn*), powerful neurite outgrowth promoting factors (**Figure 2.11B**) (Altmann et al., 2016; Wright et al., 2014). Taken together, scRNA-seq of injured nerve reveals that multiple cell types contribute to a large repertoire of extracellular molecules with neurotrophic and axon growth promoting properties.

2.3.7 Mesenchymal progenitor cells in the injured nerve shape the inflammatory milieu

Non-hematopoietic cells in the injured nerve, including structural cells such as MES and Fb, show high immune gene activity and likely play a major role in shaping the inflammatory milieu (**Figure 2.16**). In comparison, repair SC exhibit low immune gene activity, suggesting they play a less important role in shaping nerve inflammation (**Figure 2.16**). In the 3d injured nerve,

eMES express several chemokines (*Ccl2, Ccl7, Ccl9, Ccl11/*Eotaxin)*, Mif/*Macrophage migration inhibitory factor*, Spp1, Thbs4/*Thrombospondin-4*,* and *Il33.* Cells in dMES express *Mif*, *Csf1, Cxcl14* and the complement components *C1s1, C1ra, C3, C4b.* Cells in pMES express *Ccl11, Cfh/*Complement factor h*, Mdk,* and *Thbs4*. Moreover, MES in the injured nerve likely contribute to wound healing and fibrosis, since they express several WNT pathway antagonists, including *Wfi1, Sfrp1/*Secreted frizzled related protein 1*, Sfrp2, Sfrp4,* and *Sfrp5 (***Figure 2.12 and Table 2.1**). In the injured heart for example, blocking of WNT signaling was found to be critical to limit fibrosis and to promote differentiation of Mo into Mac (Meyer et al., 2017).

2.3.8 The immune repertoire of injured sciatic nerve

The mononuclear phagocyte system (MPS) is comprised of Mo, Mac, and DC, cell types that are readily detected in the injured nerve by flow cytometry (**Figure 2.1**). UMAP, overlaid with Seurat-based clustering of scRNA-seq datasets, identified a connected continuum of 7 cell clusters in the MPS (Mo, Mac1-5, and MoDC), characterized by strong expression of *Itgam/CD11b* (**Figure 2.10C**) and various degrees of the commonly used myeloid cell markers *Adgre1*/F4/80, *Aif1/*Iba1*, Cd68, Cx3cr1* and *Cd209a/*DC-SIGN (**Figure 2.17A-E**). Cells in the MPS strongly express the myeloid lineage-defining transcription factor PU.1 (*Spi1*). The C/EBP family member TF (*Cebpb*) is expressed by Mo/Mac, but not dendritic cells (**Figure 2.17F**). Myeloid cells are a rich source of fibronectin, extracellular proteases, and hydrolases (*Fn1, Tgfbi, Adam15, CtsC, CtsS, Gusb*) and likely play a major role in ECM remodeling, cell adhesion, and fibrosis. Monocytes strongly express *Ly6c2/*Ly6C*, Chil3/*chitinase-like 3*, Ifitm6/*interferon-induced transmembrane protein 6*, Itgal/*integrin αL*, Gsr/*glutathione reductase*, Hp/*haptoglobin (**Figure 2.18**). In addition, they express the TRs *Hif1a, Trps1,* and *Cebpb*/C-EBPβ, a bZIP TR important for Mo survival (**Figure 2.17F**).

In the UMAP plot, the Mo cluster is flanked by three macrophage subpopulations (Mac1-Mac3) (**Figure 2.10A**). Mac1 cells express (*Fcgr2b/*Fc gamma receptor 2b*, Arg1/*arginase-1*, Ltc4s/*leukotriene C4 synthase*, Lpl/*lipoprotein lipase*, Camkk2*). Mac2(*Cx3cr1, Ccr2, Csf1r)* and Mac3 (*Cx3cr1, Mrc1/*CD206*, Ccr2, Adgre1/*F4/80*, Csf1r, Cd38*) express overlapping, yet distinct, sets of surface receptors (**Figure 2.18**). Of note, individual Mac subpopulations often co-express markers traditionally associated with M1-like and M2-like cells, indicating that these markers are of limited use to describe the more complex physiological states of Mac subpopulations in the

injured nerve. Mac4 cells are characterized by high levels of *Trem2/* triggering receptor expressed on myeloid cells 2*, Arg1/*arginase-1*, Pf4/*CXCL4*, Stab1/*stabilin-1*, Cd68* (**Figure 2.18**) and express the TRs *Cebpa, Mafb, Mef2a* (**Figure 2.17K**). Cluster Mac5 is small, 239 cells, and harbors dividing (*Mki67*) myeloid cells with "stem-like" features (*Stmn1/ Stathmin-1, Top2a, Hmgb2, Tupp5)* (**Figure 2.10J**, **Figure 2.18, and Table 2.1)**. In addition, a smaller group of dividing cells (*Mki67, Top2a*) is embedded in the MPS and located between clusters Mac2 and MoDC (**Figure 2.19A-B**). To distinguish between dividing nerve resident myeloid cells and dividing blood-derived myeloid cells, we subjected WT-tdTom parabionts to SNC (**Figure 2.6A**). At 3d post-SNC, WT nerves were analyzed for $tdTom^+$ cells that co-stain with anti-Ki67 and anti-F4/80 (**Figure 2.19C**). $TdTom^+F4/80^+Ki67^+$ cells were identified, indicating that blood-borne, stem-like myeloid cells are present in the injured sciatic nerve. Mac2 cells express high levels of MHCII genes (*H2-Aa, H2-Ab1, H2-Eb1, M2-DM*) and the CD74 invariant chain of MHCII (*Cd74*), typically associated with antigen presentation to $CD4^+$ T cells. The MPS harbors monocyte-derived dendritic cells (MoDC), professional antigen presenting cells, characterized by high level expression of MHCII genes, *Itgax/*CD11c*, Itgb7/*integrin-β7*, Napsa/*Napsin-A, and *Cd209a*/DC-SIGN (**Figure 2.17E**, **Figure 2.18**). Mac2 and MoDC express *Ciita* (**Figure 2.17F**), a class II transactivator, that promotes MHCII gene expression (Accolla et al., 2019). Few plasmacytoid DCs (pDC) (*Siglech, Ly6d*) and conventional DCs (cDC) (*Clec9a, Xcr1, Itgae, Tlr3, Ifi205, Cd24a, Btla/*CD272*)* are detected in the MPS (**Figure 2.19D-E**).

cDC show enriched expression of the TRs *Batf3, Id2, Irf5, Irf8, Mycl, Srebf2* (**Figure 2.17F**). DC clusters can readily be distinguished from other myeloid cells, based on their expression of *Bcl11a*, a TR that determines DC fate (Ippolito et al., 2014). Cells in the MoDC cluster show high expression of the TRs *Nfkb1, Pou2f2*, *Runx1, Rel*/c-Rel, and *Ikbkb*/IKKβ (**Figure 2.17F**). The GC cluster in the d3 nerve is small, 314 cells, and mainly includes neutrophils (*S100a8, S100a9, Mmp9, Retnlg/*Resistin-like gamma), intermingled with few eosinophils (*Siglecf*) (**Figure 2.10A**, **Figure 2.18**). Overall, the Seurat cluster analysis is in good agreement with the abundance and identity of immune cell profiles detected by flow cytometry and also reveals the presence of a large and connected continuum of cell states in the myeloid compartment (**Figure 2.10A**). To infer the most probable differentiation trajectories from Mo toward their descendants, we used Slingshot, a method for pseudo-time trajectory analysis (Street et al., 2018). The analysis reveals a bifurcated trajectory and provides independent evidence that blood-borne

Mo that enter the nerve where they give rise to different Mac subpopulations as well as MoDC. The predicated differentiation trajectory indicates that Mo first give rise to Mac3, and cells in cluster Mac3 then differentiate either into Mac1, Mac2, or Mac4 cells. Furthermore, Mac2 cells are predicted to differentiate into MoDC (**Figure 2.20).**

The "connected continuum" of Mo/Mac in the injured nerve, as revealed by scRNA-seq, was independently verified by flow cytometry. The Mo/Mac population ($CD45^+CD11b^+Ly6G^-CD11c^-$) is highly plastic and can be subdivided based on surface levels of the lymphocyte antigen 6C (Ly6C). Ly6C is expressed at high levels on proinflammatory, circulating monocytes and is downregulated as they infiltrate tissues and mature into macrophages and dendritic cells (King et al., 2009). As expected, scRNA-seq of injured nerve shows that *Ly6c2*, the gene encoding Ly6C, is strongly expressed by Mo, but much less by Mac subpopulations (**Figure 2.17G**). Flow cytometry shows that naïve nerve tissue harbors a small Mac population, mostly comprised of $Ly6C^-$ (70%) cells and few $Ly6C^{int}$ (16%) and $Ly6C^{hi}$ (14%) cells (**Figure 2.17H**). At d1 post-SNC, the number of Mo/Mac increases sharply and Ly6C distribution is skewed toward classically activated $Ly6C^{hi}$ cells (50%), with fewer $Ly6C^{int}$ (41%) and $Ly6C^-$ (9%) cells (**Figure 2.17I**). At d3, $Ly6C^{hi}$ (28%), $Ly6C^{int}$ (47%), and $Ly6C^-$ (25%) cells are detected (**Figure 2.17J**) and at d7, the majority of Mo/Mac are non-classical $Ly6C^-$ (65%) and intermediate $Ly6C^{int}$ (25%), with few $Ly6C^{hi}$ cells (10%) (**Figure 2.17K**). This shows that $Ly6C^{hi}$ Mo migrate into the injured nerve in large numbers and increase inflammation during the acute phase. Later, as nerve inflammation resolves, the Mo/Mac number and polarization gradually return back to pre-injury homeostatic levels (**Figure 2.17L-M**). Noteworthy, the Mo/Mac population in axotomized DRGs shows an opposite response with regard to surface Ly6C distribution. In naïve DRGs, Mo/Mac are comprised of $Ly6C^-$ (30%), $Ly6C^{int}$ (27%), and $Ly6C^{hi}$ (43%) cells. Upon SNC, the distribution shifts to 75%, 16%, and 9% on d1, to 53%, 20%, and 27% on d3, and 52%, 23%, and 25% on d7 (**Figure 2.21**). Together, these data show that SNC-triggered inflammation in the nerve is massive and characterized by a short pro-inflammatory phase that rapidly transitions to a resolving state. A similar immune response is not observed in axotomized DRGs.

2.3.9 Identification of macrophage subpopulations with distinct functions and distribution patterns in the injured nerve

Mac subpopulations show overlapping, yet distinct, expression patterns of the canonical markers *Adgre1*(F4/80), *Aif1*(Iba1), *Cd68,* and *Cx3cr1* (**Figure 2.17A-D**). Moreover, cells in Mac4 and some cells in clusters Mac1 and Mac3 express high levels of *Arg1*, while other Mac subpopulations do not (**Figure 2.17N**). To explore tissue distribution of *Arg1*$^+$ cells relative to F4/80$^+$ and CD68$^+$ cells in naïve and injured nerves, we subjected *Arg1-YFP* reporter mice to SNC. In naïve mice, no YFP$^+$ cells are observed (**Figure 2.17O**) while few F4/80$^+$ and CD68$^+$ are detected (**Figure 2.22**). At d1, few YFP$^+$ cells accumulate near the injury site (data not shown) and at d3 many more are present (**Figure 2.17P**). Unexpectedly, YFP$^+$ cells are confined to the nerve crush site and largely absent from the distal nerve stump. This stands in contrast to F4/80$^+$ and CD68$^+$ macrophages, found at the injury site and the distal nerve (**Figure 2.22**). At d7, only few *Arg1-YFP*$^+$ cells are found at the injury site and none in the distal nerve stump (**Figure 2.17Q**). F4/80$^+$ Mac, on the other hand, are more uniformly distributed within the injury site and distal nerve stump (**Figure 2.22**). This shows the existence of different immune compartments in the injured nerve. A subpopulation of *Arg1*$^+$ macrophages (including cells in cluster Mac4) is preferentially localized to the crush site, whereas F4/80$^+$ macrophages (including cells in cluster Mac2 and Mac3) are abundant in the distal nerve where fibers undergo Wallerian degeneration. Pathway analysis of cell clusters in the innate immune compartment reveals common functions in phagocytosis, phagosome, and endolysosomal digestion, but also highlights important differences (**Figure 2.18**). KEGG pathways specific for Mo include *cytokine signaling* and *leukocyte trans-endothelial migration*, providing independent evidence for their hematogenous origin. Mo are highly plastic and predicted to give rise to monocyte-derived Mac subpopulations in the injured nerve (**Figure 2.20**). Top KEGG pathways for Mac3 are *chemokine signaling pathway*, *complement and coagulation cascades*, and *cytokine-cytokine receptor interaction* (**Figure 2.18**). For Mac1 cells, *complement and coagulation cascades*, suggesting that Mac1 and Mac3 play roles in opsonization and blocking of endoneurial bleeding. For Mac2 cells, KEGG pathway analysis identified *Leishmaniasis* and *Tuberculosis* as top hits (**Figure 2.18**). For Mac4 cells, pathway analysis identified *negative regulation of immune system processes* and *cholesterol metabolism*. Cholesterol metabolism in Mac4 cells includes gene products that regulate reverse *cholesterol transport* (*Abca1/* ATP-binding cassette subfamily A1, *Abcg1*/ATP-binding cassette subfamily

G1 , *Ctsd/*Cathepsin-D, *Ctsb*/Cathepsin-B*), cholesterol and lipid storage (Plin2/*perilipin*), formation of cholesterol esters (Soat1), cholesterol ester hydrolysis and lipoprotein metabolism* (*Lipa/*lipase-A*, Nceh1/*Neutral cholesterol hydrolase 1*, Apoe/*Apolipoprotein E*)* and *intracellular cholesterol transport (Npc2/*Niemann-Pick C2 and *Scarb2/*Scavenger receptor class B member 2*)* (**Figure 2.23A-I**). The abundance of gene products that protect from cholesterol overloading (Haidar et al., 2006; Viaud et al., 2018; Wu et al., 2018), suggests that this cluster is comprised of cholesterol laden cells. Importantly, tissue-resident macrophages in naïve nerves (Wang et al., 2020), either do not express cholesterol regulatory gene products, or express them at significantly lower levels (**Figure 2.23J-S**).

2.3.10 Cell-type-specific expression of engulfment receptors in the injured nerve

In the injured nerve, blood-borne phagocytes and repair SC collaborate in myelin removal. Repair SC use the receptor tyrosine kinases AXL and MER for myelin phagocytosis (Brosius Lutz et al., 2017). Clusters SC1 (Axl^{hi}, $Mertk^{-}$) and SC3 (Axl^{low}, $Mertk^{int}$) exhibit differential expression of these two receptors (**Figure 2.25**). Interestingly, *Axl* and *Mertk* expression in myeloid cells is very low, suggesting that innate immune cells and repair SC employ different mechanisms for myelin phagocytosis. Mac subclusters strongly express the myelin binding receptors *Lrp1* (low density lipoprotein receptor-related protein 1), *Pirb* (paired Ig-like receptor B), *Cd300lf* (sphingomyelin receptor), and several scavenger receptors (*Msr1, Cd36, Cd68*), including high levels of opsonic receptors (*Fcgr1, Fcgr3, Fcgr4, Fcer1g*) that may contribute to phagocytosis of antibody marked myelin debris (**Figure 2.25, and Table 2.1**) (Atwal et al., 2008; Grajchen et al., 2018; Izawa et al., 2014; Kuhlmann et al., 2002; Stiles et al., 2013). Compared to Mo/Mac of injured nerves, phagocytosis receptor expression is much lower in naïve nerve Mac (**Figure 2.25**).

In addition to debris phagocytosis, myeloid cells participate in removal of apoptotic cells (AC), primarily dying neutrophils and other leukocytes. Phagocytic uptake of AC, called efferocytosis, is mediated by a range of specialized engulfment receptors and mechanisms for ingestion (Boada-Romero et al., 2020). AC are selectively recognized due to phosphatidylserine (PS) or calreticulin (*Calr*) accumulation on their surface; both function as strong "eat me" signals (**Figure 2.24A**). Conversely, healthy cells display the "don't eat me" signal CD47 that binds to the cell surface receptor SIRPα (signal regulatory protein α) encoded by *Sirpa*, to block efferocytosis

(Kourtzelis et al., 2020). *Calr and Cd47* are boadly expressed by cells in the injured nerve, while *Sirpa* is largely confied to myeloid cells (**Figure 2.24B**). PS is directly recognized by cell surface receptors such as CD300 family members (*Cd300a, Cd300lb, Cd300lf*), stabilin-1 (*Stab1*), and oxidized-PS by the scavenger receptor *Cd36*, molecules that are expressed by phagocytes in the injured nerve (**Figure 2.24C**). Alternatively, PS binds indirectly, via bridging molecules, to engulfment receptors (Voss et al., 2015). Interestingly, in the injured sciatic nerve, numerous cell types express specific sets of bridging molecules, indicating that they may contribute in an autocrine or paracrine manner to AC removal. Bridging molecules prominently expressed include complement C1q components (*C1qa, C1qb, C1qc, C1ra*), annexins (*Anxa1-5*), pentraxin (*Ptx3*), thrombospondin 1 (*Thbs1*), collectin kidney protein 1 *(Colec11),* soluble collectin placenta 1 (*Colec12*), galectin-3/MAC-2 (*Lgals3*), growth arrest-specific 6 (*Gas6*), protein S *(Pros1*), milk fat globule-EGF factor 8 (*Mfge8*), and apolipoprotein E (*Apoe*) (**Figure 2.24B**). Bridging molecules that bind to PS are recognized by a large and diverse set of engulfment receptors on phagocytes, including Lrp1, Trem2, Dap12 (*Tyrobp*), C1q receptor (*C1qr/Cd93*), C3a receptor 1 (*C3ar1*), integrin αMβ2, (*Itgam, Itgb2*), integrin αv (*Itgav*), integrin β3 (*Itgb3*), CD14, and members of the scavenger receptor family (*Cd68* and *Msr1*/Mac scavenger receptor 1) (Doran et al., 2020; Erriah et al., 2019; Korns et al., 2011). Strikingly, many of these engulfment receptors are expressed by myeloid cells, and are particularly abundant in cluster Mac4 (**Figure 2.24C**). Indirect evidence that Mac4 cells eat AC corpses, is the strong expression of gene products that regulate lipid metabolism and mechanisms that protect cells from excessive cholesterol loading, such as reverse cholesterol transport and cholesterol esterification (**Figure 2.23**). To assess whether expression of gene products involved in efferocytosis are upregulated following nerve injury, we took advantage of recently published scRNA-seq data sets generated from naïve mouse sciatic nerve tissue (Wang et al., 2020; Ydens et al., 2020). Importantly, bridging molecules and engulfment receptors are either not expressed by macrophages in the naïve nerve, or expressed at much lower levels than in Mac4 cells in the injured nerve (**Figure 2.26).**

2.3.11 Efferocytosis of leukocytes in the injured sciatic nerve

To directly test whether efferocytosis takes place in the injured nerve, we first examined the presence of AC corpses. Viability-dye labeling, combined with flow cytometry, identified an increase in AC at d3 and d7 post-SNC (**Figure 2.24D**). During nerve debridement, degenerated

nerve fibers and AC corpses are removed. In order to distinguish between efferocytosis of dying leukocytes and phagocytosis of nerve fiber debris, we generated $WT^{CD45.1}$-$tdTom^{CD45.2}$ parabiotic mice (**Figure 2.24E**). Both mice in the parabiosis complex were subjected to bilateral SNC. At d3 post-SNC, live cells in the injured $WT^{CD45.1}$ nerve were analyzed by flow cytometry (gating strategy is illustrated in **Figure 2.7**). All $tdTom^+$ cells in the injured nerve of the $WT^{CD45.1}$ parabiont are blood-borne immune cells. Moreover, cells that are $CD45.1^+tdTom^+CD45.2^-$ represent $tdTom^+$ leukocytes that were eaten in the nerve by $CD45.1^+$ phagocytes. In non-parabiotic (single) tdTom mice, ~95% of myeloid cells ($CD11b^+$) in the 3d injured nerve are $tdTom^+$ (**Figure 2.24F**) and in the $WT^{CD45.1}$ parabiont ~39% are $CD11b^+tdTom^+$ (**Figure 2.24G**). Importantly, in the $WT^{CD45.1}$ parabiont, $CD45.1^+tdTom^+CD45.2^-$ (Q3) cells are readily detected in the injured nerve and such cells are not present in tdTom (single) mice (**Figure 2.24H-I**). This indicates that efferocytosis of apoptotic leukocytes takes place in the injured nerve. To determine which immune cell types eat apoptotic leukocytes, we analyzed $CD45.1^+tdTom^+$ cells for surface levels of Ly6C and CD11c to distinguish between maturing Mo/Mac ($Ly6C^{hi}$ to $Ly6C^-$) and MoDC ($CD11c^+$). Mo/Mac have the biggest appetite for $tdTom^+$ apoptotic leukocytes, more so than MoDC, suggesting they remove the bulk of dying leukocytes (**Figure 2.24K-M and Figure 2.27**). As negative controls, non-parabiotic $tdTom^{CD45.2}$ mice were processed in parallel (**Figure 2.24J-L and 2.27**). Collectively, these studies show that efferocytosis of dying leukocytes takes place in the injured sciatic nerve, and thus, serves as an important mechanism to clear the nerve of AC corpses.

2.3.12 *Csf2* deficiency skews the immune response in the injured nerve toward classically activated $Ly6C^{hi}$ monocytes

While PNS injury elicited inflammation is important for axon regeneration, it is not clear whether inflammation in the nerve or axotomized DRGs is a primary driver of peripheral axon regeneration, or conditioning-lesion-induced central axon growth (**Figure 2.28A**). Bulk RNA-seq of axotomized DRGs and scRNA-seq of injured nerve identified chemokine and cytokine ligand-receptor systems preferentially expressed in the injured nerve. GM-CSF signaling is of interested because this cytokine is present in the injured nerve and has been implicated in neuroprotection and axon repair (Be'eri et al., 1998; Franzen et al., 2004; Legacy et al., 2013). Moreover, GM-CSF increases surface expression of galectin-3 (Saada et al., 1996) and in non-neural tissues galectin-3 functions as a bridging molecule for efferocytosis of apoptotic immune cells (Erriah et al., 2019;

Wright et al., 2017). Transcripts for the GM-CSF receptor subunits (*Csf2ra* and *Csf2rb*) are abundantly expressed by myeloid cells in the injured nerve (**Figure 2.28B-C)**, but not in axotomized DRGs (**Figure 2.8K-L**). To assess the role in nerve injury triggered inflammation, we employed *Csf2*$^{-/-}$ mice (**Figure 2.29**) and subjected them to SNC. Flow cytometry was used to quantify immune cell profiles in naïve nerves and at 1d, 3d, and 7d post-SNC. In naïve WT and *Csf2*$^{-/-}$ mice, the number of endoneurial Mac is comparable, and the majority of them are Ly6C^{-} or Ly6C^{int} cells (**Figure 2.28D-E**). In the d3 injured nerve, there is a strong increase in the Mo/Mac population, in both, WT and *Csf2*$^{-/-}$ mice (**Figure 2.28F-G**). However, when analyzed for surface Ly6C expression, significantly fewer Ly6C^{-} cells are present in *Csf2*$^{-/-}$ mice. Conversely, the population of Ly6C^{hi} cells is significantly elevated in *Csf2*$^{-/-}$ mice when compared to WT mice (**Figure 2.28H**). This indicates that Mo/Mac maturation and inflammation resolution in the injured nerve of *Csf2*$^{-/-}$ mice is significantly delayed. Delayed maturation is only observed in the Mo/Mac population, since analysis of surface Ly6C expression on MoDC is comparable between WT and *Csf2*$^{-/-}$ mice (**Figure 2.28I**).

2.3.13 *Csf2* is required for CL-induced dorsal column axon regeneration

To assess whether proper Mo/Mac maturation in the injured nerve is important for conditioning-lesion-induced regeneration of central axon projections, adult WT and *Csf2*$^{-/-}$ mice were either subjected to bilateral SNC or sham operated. Seven days later, a dorsal column lesion (DCL) was placed at cervical level 4 of the spinal cord. Five weeks following DCL, cholera-toxin B (CTB) traced dorsal column axons were analyzed in longitudinal spinal cord sections (**Figure 2.28A**). DCL causes axon "die-back" (Horn et al., 2008). In WT mice without conditioning lesion, there is a 600± 80 μm gap between the lesion center, and the most proximal, CTB labeled axons (**Figure 2.28J-K**). In WT mice that received a conditioning lesion, traced axons grew close to the spinal cord injury site (**Figure 2.28J-K**). In parallel processed *Csf2*$^{-/-}$ mice, without conditioning lesion, there is a 720± 120 μm gap between the lesion center, and the most proximal CTB labeled axons (**Figure 2.28J-K**). However, in *Csf2*$^{-/-}$ mice subjected to a conditioning lesion, dorsal column axon regeneration is not significantly enhanced (**Figure 2.28J-K**). This shows that *Csf2* is important for conditioning-lesion-induced central axon regeneration.

GM-CSF has pleiotropic functions and its receptors are found on hematopoietic cells, glial cells, and subsets of neurons (Donatien et al., 2018; Franzen et al., 2004). SNC leads to

upregulation of GM-CSF in the nerve (Mirski et al., 2003) and acute administration of GM-CSF following SNC leads to a transient increase in PNS axon regeneration (Bombeiro et al., 2018). To assess whether loss of *Csf2* attenuates neurite outgrowth *in vitro*, we cultured DRG neurons from adult WT and *Csf2*$^{-/-}$ mice. After 20 h, many neurons with axons were identified in both WT and *Csf2*$^{-/-}$ cultures (**Figure 2.28L**). Quantification of axon growth did not identify *Csf2* dependent differences in total axon length or the longest axon (**Figure 2.28M**). A second cohort of WT and *Csf2*$^{-/-}$ mice was subjected to a conditioning lesion 3d prior to harvesting of axotomized DRGs. In both WT and *Csf2*$^{-/-}$ cultures, neurite outgrowth is significantly increased when compared to DRGs prepared from naive mice (**Figure 2.28M**). Collectively, this shows that reduced axon regeneration in the dorsal columns of *Csf2*$^{-/-}$ mice is not due to loss of conditioning-lesion-induced activation of neuron-intrinsic growth programs and indicates that *Csf2* promotes regeneration through cell non-autonomous, extrinsic mechanisms.

2.4 Discussion

We show that compression injury to the sciatic nerve triggers massive infiltration of blood-borne immune cells into the nerve. Granulocytes enter first, closely followed by Ly6C^{hi} monocytes. After a short pro-inflammatory phase, the immune milieu rapidly transitions toward resolution and is dominated by Ly6C^{-} Mac. Analysis of axotomized DRGs revealed upregulation of immune-associated gene co-expression networks, however infiltration of blood-borne immune cells was very limited. DRG-resident macrophages downregulate surface Ly6C upon nerve injury and undergo striking morphological changes. Single-cell RNA-seq identified 10 immune cell clusters in the injured nerve. Monocytes and their descendants, Mac1-Mac5 subpopulations and MoDC are abundantly present. The immune compartment includes a population of blood-derived, proliferating myeloid cells (Mac5) with stem-like features. Mononuclear phagocytes in the injured nerve form a connected continuum of 8 cell clusters, including a subpopulation of Arg1^{+} Mac localized to the nerve crush site. In contrast, F4/80^{+} Mac are more evenly distributed in the nerve and associated with Wallerian degeneration. Apoptotic cell corpses rapidly accumulate in the injured nerve. Experiments with parabiotic mice show that Mo/Mac and MoDC contribute to nerve debridement by "eating" apoptotic leukocytes. In *Csf2*$^{-/-}$ mice, pro-inflammatory Ly6C^{hi} Mo/Mac are elevated in the injured nerve, while the number of anti-inflammatory Ly6C^{-} cells is reduced. This exacerbation of inflammation correlates with loss of conditioning-lesion induced central axon

regeneration. Collectively, a comparative analysis of the immune response to PNS injury reveals striking differences in the inflammatory landscape between the nerve injury site, the degenerating nerve stump, and axotomized DRGs. Efferocytosis of apoptotic leukocytes is identified as a key mechanism of nerve debridement and inflammation resolution. Perturbed resolution of nerve inflammation, as observed in *Csf2*$^{-/-}$ mice, blocks conditioning-lesion-induced central axon regeneration.

2.4.1 Evidence for specific immune compartments within the injured nerve

Traumatic PNS injury causes necrosis of SC, MES, and vasculature-associated cells at the nerve injury site. Disruption of the vasculature leads to endoneurial bleeding and tissue hypoxia. Necrosis is a violent form of cell death that disrupts the plasma membrane and leads to the release of intracellular damage-associated molecular patterns (DAMPs) into the extracellular milieu. Release of intracellular content, in any tissue, causes a strong pro-inflammatory response (Frank and Vince, 2019; Vannella and Wynn, 2017). Distal to the nerve crush site, transected nerve fibers undergo Wallerian degeneration and release DAMPs as they disintegrate. However, in the distal nerve the abundance and composition of DAMPs, such as the absence of double-stranded DNA and nuclear proteins, is very different from the nerve crush site (Bortolotti et al., 2018). Thus, depending on where Mo enter the injured nerve, they may encounter very different microenvironments and adapt site specific phenotypes (Cane et al., 2019). The strong accumulation of *Arg1-YFP*$^{+}$ cells at the nerve injury site, but not along degenerating fibers, supports the idea that Mo/Mac adapt microenvironment specific phenotypes. Studies with chimeric mice show that hematogenous leukocytes first accumulate at the injury site and later along severed fibers that undergo Wallerian degeneration. The density of blood-derived leukocytes is highest at the injury site and correlates with the extent of tissue damage. We speculate that F4/80^{+} Mac associated with Wallerian degeneration function in phagocytosis of myelin debris and degenerated axons, whereas *Arg1*$^{+}$ Mac near the injury site primarily function in removal of apoptotic cell corpses. In support of this idea, *Arg1*$^{+}$ Mac, highly enriched in cluster Mac4, express the highest levels of engulfment receptors and gene products important for reverse cholesterol transport, a strong indicator for ongoing efferocytosis (Yvan-Charvet et al., 2010).

2.4.2 Efferocytosis of apoptotic leukocytes in the injured sciatic nerve

Studies with chimeric mice show that upon sciatic nerve injury, Mo/Mac, and to a lesser extent MoDC, participate in nerve debridement by eating dying leukocytes. Bridging molecules that facilitate recognition of AC are abundantly expressed by immune and non-immune cells in the injured nerve. Compared to Mac from naïve PNS tissue, cells in subcluster Mac4 of the injured nerve show highly elevated expression of engulfment receptors. Some engulfment receptors, including *Lrp1, Axl*, and the scavenger receptor class B member 2 (*Scarb2)*, are expressed by MES and repair SC, suggesting that immune and non-immune cells participate in nerve debridement, possibly including efferocytosis. Whether the large and diverse array of engulfment receptors expressed in the injured nerve reflects eating of specific debris, AC corpses, or a high degree of functional redundancy is unknown and requires further investigation. The most likely prey eaten by Mo/Mac and MoDC are dying neutrophils. Neutrophils are very abundant at early post-injury time points, have a short life span, and spontaneously die by apoptosis (Greenlee-Wacker, 2016; Lindborg et al., 2017). In non-neural tissues, efferocytosis of neutrophils triggers anti-inflammatory responses in Mo, Mac, and DC, a prerequisite for inflammation resolution (Greenlee-Wacker, 2016). Thus, efferocytosis is not simply a mechanism for garbage removal, but also a key driver to reprogram professional phagocytes from a pro-inflammatory to an anti-inflammatory state (Boada-Romero et al., 2020; Eming et al., 2017; Ortega-Gomez et al., 2013). In a similar vein, efferocytosis in the injured sciatic nerve may drive inflammation resolution and wound healing. In humans, dysregulation of efferocytosis can cause chronic inflammatory and autoimmune diseases, including asthma, systemic lupus erythematous, and atherosclerosis (Kawano and Nagata, 2018). Additional studies are needed to determine whether defective efferocytosis and impaired inflammation resolution in the PNS contribute to excessive tissue damage and neuropathic pain.

2.4.3 The immune compartment of the 3-day injured sciatic nerve exhibits an immunosuppressive character

Rapid removal of AC corpses protects from secondary necrosis and is closely associated with the induction of immunological self-tolerance. Commensurate with this, the low presence of lymphocytes and Natural killer cells in the nerve indicates that the microenvironment is immunologically “cold” and dominated by immunosuppressive mechanisms. We propose that

efferocytosis in the injured nerve is key to switch from a pro-inflammatory environment to resolution and restoration of tissue integrity (Kourtzelis et al., 2020; Ortega-Gomez et al., 2013). At 3d post-SNC, expression of the pro-inflammatory cytokines and chemokines (*Ifng, Il1a, Il1b, Tnf*) is very low. Most myeloid cells express high levels of anti-inflammatory *Cd52*, a glycoprotein that binds to HMGB1 to suppress T cell function (Bandala-Sanchez et al., 2018; Rashidi et al., 2018). *Trem2*$^{+}$*Arg1*$^{+}$ cells are strongly enriched in cluster Macs4 and show gene signatures suggestive of myeloid suppressive cells (Katzenelenbogen et al., 2020; Yurdagul et al., 2020). Further evidence for an immunosuppressive environment is the strong expression of *Pirb* by myeloid cells, a type 1 membrane protein with four cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that inhibit immune cell activation (van der Touw et al., 2017). Myeloid inhibitory C-type lectin-like receptor (*Clec12a), Lair1* (leukocyte-associated Ig-like receptor-1)*, Fcgr2b* (low affinity immunoglobulin gamma Fc region receptor IIb), and the CD300 family receptors *Cd300a* and *Cd300lf*, all of which contain ITIMs (Rozenberg et al., 2018), are strongly expressed, and thus, may reduce nerve inflammation. TGFβ is expressed by efferocytotic Mac in the lung (Yoon et al., 2015). In the injured sciatic nerve, *Tgfb1* is expressed by myeloid cells and is important for axon regeneration (Clements et al., 2017; Kourtzelis et al., 2020). Cells in clusters Mac1, Mac2, and Mac3 express high levels of *Rbpj*, a TR that restrains ITAM (immunoreceptor tyrosine-based activation motif) signaling and promotes a, resolving Mac phenotype (Foldi et al., 2016). Mac4 cells express the transmembrane glycoprotein NMB (*Gpnmb*), a negative regulator of inflammation that has protective effects following tissue injury (Zhou et al., 2017). Of interest, in the 3d injured nerve, Mac1, Mac3, and Mac4 strongly express the TRs *Maf*/c-Maf and *Mafb*/MafB. MafB promotes reprogramming of macrophages into an M2-like, resolving phenotype (Kim, 2017) and c-Maf is a checkpoint that programs Mac and is critical for the acquisition of an immunosuppressive phenotype (Liu et al., 2020).

2.4.4 *Csf2* deficiency alters nerve inflammation and blocks conditioning-lesion-induced axon regeneration

Parabiosis revealed massive infiltration of blood-borne immune cells into the injured nerve but not axotomized DRGs. This finding was independently confirmed by flow cytometry, Western blotting, 3D reconstruction of Iba1^{+} cells, and RNA-seq of axotomized DRGs. The small increase in hematogenous leukocytes in axotomized DRGs was unexpected, since infiltration of Mo/Mac

is thought to be a key driver of conditioning-lesion-induced axon regeneration (Kwon et al., 2015; Richardson and Issa, 1984; Zigmond and Echevarria, 2019). Consistent with previous reports, sciatic nerve injury causes a strong increase in Iba1 immunoreactivity in DRGs. We provide evidence that increased Iba1 immunoreactivity is, at least in part, a reflection of macrophage morphological changes triggered by nerve injury. Additional mechanisms may include local myeloid cell proliferation (Yu et al., 2020) and infiltration of a small number of blood-borne myeloid cells.

SNC triggers an inflammatory response in the nerve and in axotomized DRGs, although quantitatively and qualitatively very different, it remains unclear which immune compartment is important for conditioning lesion elicited axon regeneration. To revisit this question, we took advantage of RNA-seq datasets generated from DRGs and nerves and searched for immune signaling pathways preferentially upregulated in the injured nerve, but not axotomized DRGs. Focusing on GM-CSF signaling, a cytokine that rapidly accumulates in the distal nerve stump (Mirski et al., 2003), we observed strong expression of both GM-CSF receptor subunits (*Csf2ra* and *Csf2rb*) in nerve macrophages but not axotomized DRGs. GM-CSF is known to promote Mo migration and Mac polarization (Ijaz et al., 2016; Vogel et al., 2015; Wicks and Roberts, 2016). Of interest, GM-CSF upregulates surface expression of galectin-3 on SC and Mac (Saada et al., 1996) and galectin-3 is thought to promote phagocytosis of myelin debris and participate in re-programming of Mac toward an anti-inflammatory phenotype (Erriah et al., 2019; Rotshenker, 2009). Recent evidence shows that galectin-3 promotes efferocytosis of neutrophils and promotes inflammation resolution (Quenum Zangbede et al., 2018; Wright et al., 2017). Following SNC in *Csf2*$^{-/-}$ mice, the ratio of Ly6C^{hi} to Ly6C^{-} Mo/Mac is significantly skewed toward the former. Functional studies with *Csf2*$^{-/-}$ mice highlight a critical role for conditioning-lesion-induced regenerative growth of severed dorsal column axons. Neurite outgrowth studies with primary DRG neurons suggest that the regenerative failure in *Csf2*$^{-/-}$ mice is not due to failed activation of DRG neuron-intrinsic growth programs, but due to changes in extrinsic, environmental influences. Because *Csf2* receptor expression is very low in axotomized DRGs, this suggests that *Csf2* dependent accumulation of Ly6C^{-} Mac in the nerve is important for conditioning-lesion-induced axon regeneration. We speculate that *Csf2* functions non-cell autonomously in the injured nerve to generate an extracellular milieu capable to sustain neuron-intrinsic growth programs activated by injury. In a similar vein, axotomy to corticospinal neurons is sufficient for the induction of

neuron-intrinsic growth programs, but not maintenance. However, neuron-intrinsic growth programs in corticospinal neurons can be maintained by environmental cues released from stem cells grafted near the injury site (Kumamaru et al., 2018; Poplawski et al., 2020). While our studies demonstrate an important role for *Csf2* in conditioning-lesion-induced axon regeneration, we cannot rule out potential contributions by DRG macrophages. However, the small number of hematogenous macrophages detected in axotomized DRGs suggests that potential pro-regenerative immune mechanisms would need to be exerted by tissue-resident macrophages. We acknowledge that axon regeneration was examined in *Csf2* global knock-out mice, and thus, it is possible that *Csf2* deficiency affects immune cells before they enter the injured sciatic nerve (Hamilton, 2019) or within the injured spinal cord (Choi et al., 2014; Huang et al., 2009).

Taken together, we provide a comparative analysis of SNC-induced inflammation in the nerve and axotomized DRGs and identify two very different immune compartments, the former primarily comprised of hematogenous leukocytes and latter of tissue-resident endoneurial Mac. Mac subpopulations in the injured nerve are not uniformly distributed, indicating the existence of specific immune microenvironments. Efferocytosis of dying leukocytes is observed in the injured nerve, and thus, contributes to nerve debridement and inflammation resolution. If this process is curtailed, conditioning-lesion induced regeneration of DRG neuron central axons is impaired.

2.5 Materials and Methods

2.5.1 Animals

All procedures involving mice were approved by the Institutional Animal Care and Use Committee at the University of Michigan and Weill Cornell Medicine, and performed in accordance with guidelines developed by the National Institutes of Health. Adult (8-16 week-old) male and female mice on a C57BL/6 background were used throughout the study. Mice were housed under a 12 h light/dark cycle with standard chow and water ad libitum. Mouse lines included, *Csf2*$^{-/-}$ (Jackson Laboratories, Stock No: 026812), *ROSA26-tdTom,* constitutively expressing membrane bound tdTomato in all cells (Jackson Laboratories, Stock No. 007576), CD45.1 (Jackson Laboratories, Stock No: 002014), and *Arg1-eYFP* reporter mice (Jackson Laboratories, Stock No: 015857).

2.5.2 Genotyping of *Csf2* mice

Genomic (g) DNA was isolated from adult WT or *Csf2*$^{-/-}$ mice. Briefly, tissue samples were harvested and digested in lysis buffer (10 mM TrisHCl pH8, 25 mM EDTA, 0.1 M NaCl, 1% SDS) with Proteinase K overnight at 55°C. The following day, gDNA was extracted and resuspended in water. The following PCR primers were used: Csf2 forward 5'-GTGAAACACAAGTTACCACCTATG-3', Csf2 reverse 5'-TTTGTCTTCCGCTGTCCAA-3'; neomycin forward 5'-CTTGGGTGGAGAGGCTATTC-3', neomycin reverse 5'-AGGTGAGATGACAGGAGATC-3'. PCR parameters: 95°C for 2 min, (95°C for 1 min, 55°C for 30 s, 72°C for 20 s) repeated for 35 cycles, 72°C for 5 min.

2.5.3 Surgical procedures

All surgeries were carried out under aseptic conditions. Mice were deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) or with isoflurane (5% induction, 2-3% maintenance, SomnoSuite Kent Scientific). Buprenorphine (0.1 mg/kg) was given pre-emptively and post-operatively.

2.5.3.1 Sciatic nerve crush injury

For sciatic nerve surgery, thighs were shaved and disinfected with 70% ethanol (Covidien, 6818) and iodine (PDI Healthcare, B40600 A small incision, at mid-thigh, was made on the skin, underlying muscles separated, and the sciatic nerve exposed. For sham operated mice, the nerve was exposed but not touched. For SNC, the nerve was crushed for 15 seconds, using fine forceps (Dumont #55, Roboz Surgical Instruments, RS-5063). Skin was closed with 7mm reflex wound clips (Cell Point Scientific, 203-1000).

2.5.3.2 Dorsal column lesion

Spinal cord surgery was carried out as described previously (Yoon et al., 2013). Briefly, the C4 lamina was removed using micro-rongeurs (Roboz Surgical Instruments, RS-8306) under a stereomicroscope. The spinal column was exposed, and McPherson-Vannas Micro Dissecting Spring Scissors (Roboz Surgical Instruments, RS-5600) were inserted 1 mm deep. A hemisection

of the dorsal spinal cord was carried out to transect all axons in the dorsal columns. The lesion was confirmed by probing with fine forceps. Next, dorsal muscle layers were closed using Perma-Hand Black sutures (5-0, Ethicon) and skin incisions were closed using coated Vicryl sutures (5-0, Ethicon, J463G).

2.5.3.3 Axon tracing

For tracing of ascending sensory axons in the dorsal columns, tracer was injected into the sciatic nerve 5 weeks after SCI (Yoon et al., 2013). Briefly, the sciatic nerve was exposed at mid-thigh level and held in place using dumont #7 curved forceps (Fine Science Tools, 11271-30) and Miltex halsted mosquito forceps (Integra LifeSciences, 12460-174) to provide tension for the injection. Cholera toxin B (CTB, List Biological Laboratories, #104, 1.5 µl of 1% solution in water) was injected into sciatic nerves using a Nanofil 10 µL syringe with a 36 gauge beveled needle (World Precision Instrument, NF36BV-2). The needle was removed ~30 sec after injection to prevent backflow of fluid. Mice were sacrificed 3 days after tracer injection, spinal cords sectioned and stained as described (Yoon et al., 2013). Dorsal column lesion completeness was confirmed by absence of traced axons in transverse spinal cord sections rostral to the lesion. The distance between the lesion epicenter and the tip of traced axons was quantified by an investigator blinded with respect to mouse genotype and whether a conditioning lesion was applied or not.

2.5.3.4 Parabiosis

Isochronic, same sex mice were housed in the same cage for at least 2 weeks prior to surgery. Mice were deeply anesthetized and their left or right sides shaved from just above the shoulder to below the knee. Eye ointment was applied to both mice to prevent drying. The skin was cleaned 3 times using ethanol and iodine pads before a unilateral skin-deep incision was made from the elbow to the knee on each animal. Skin fascia adjacent to the incision was peeled back using a pair of blunt forceps. Mice were joined at the knee and elbow joints using non-absorbable sutures by running the suture needle through the muscle just under each joint in both animals and completing the suture. Absorbable sutures were used to join the skin of each mouse around the shoulder and hindlimbs. 7mm reflex wound clips were used to join the remainder of skin between the mice. Mice were allowed to recover for 3-4 weeks before further surgery

2.5.4 DRG cultures

Unilateral SNC was performed on adult mice 3 days prior to culture. The uninjured side was used as control. The dorsal spinal column from adult mice was exposed and the identity of lumbar DRGs established by counting vertebras from the hipbone (Sleigh et al., 2016). L3-L5 DRGs were dissected and harvested into L-15 with N2 (Gibco, 17502048) or N1 (Sigma-Aldrich, N6530) supplement on ice. DRGs were rinsed 5 times in L-15 with Penicillin/ Streptomycin (Life Technologies, 15140-122) and minced in growth media (DMEM Ham's F-12, 10% FBS, 1X N2 or N1 supplement and 16 nM Cytosine arabinoside (Sigma-Aldrich, C1768) with McPherson-Vannas Micro Dissecting Spring scissors. DRGs were digested in collagenase type 2 (10 mg/ml, Worthington Biochemical, LS004176) in Ca^{2+}, Mg^{2+} free PBS (Gibco, 100010023) at 37°C for 20 minutes. Ganglia were dissociated by trituration using a fire polished Pasteur pipette, followed by centrifugation (5 minutes, 160 x g) and trituration in wash buffer (DMEM Ham's F-12, Gibco, 10565-018; 10% FBS, Atlanta Biologicals, S11550; 1% Penicillin/Streptomycin, Life Technologies, 15140-122) twice. Cells were plated in growth media at a density of 0.5 DRG per well in a 24-well plate (flat bottom plates, Corning, 3524) coated with poly-L-lysine 0.01% (MW 70,000-150,000) (Sigma-Aldrich, P4707) for 45 minutes at 37°C, followed by wash in dH_20, dried and coated with 0.2 mg/mL laminin (Sigma-Aldrich, L2020). Cells were placed in a humidified incubator at 37°C, 5% CO_2 for 20 hours.

2.5.5 Immunofluorescence staining

Primary DRG neuron cultures were fixed in 4% paraformaldehyde (PFA) in 1x PBS (Sigma-Aldrich, 158127) for 15 minutes at RT, followed by 2 brief rinses in PBS. Cells were permabilized in 0.3% Triton-X100 (Sigma, T8787) in PBS for 5 minutes at RT. Cells were incubated in blocking buffer, 2% FBS, 2% heat shock fraction V BSA (Fisher Scientific, BP1600), 0.3% Triton-x-100 in PBS for 1 hour. Cells were incubated with anti-Neurofilament heavy chain (NFH, 1:100; Aves Lab, NFH) in blocking buffer overnight at 4°C and rinsed 3x in 0.3% triton-x-100 in PBS, 5 minutes each. Donkey anti-chicken Cy3 (1:200, Jackson Immunoresearch, 703-165-155) in blocking buffer was added for 45 minutes at room temperature. Cells were rinsed in PBS for 5 minutes. Hoechst 33342 (1:50,000 in PBS; Invitrogen, H3570) was added for 10 minutes at RT, followed by 2 washes in PBS. Cells were imaged on a Zeiss Axio Observer Z1 fitted with a

Zeiss Axiocam 503 mono camera using the EC PlnN 10x objective. Single plane, tile scans were randomly acquired for each well. For immunofluorescence staining of neural tissues, mice were killed and perfused transcardially with ice-cold PBS for 2 min followed by ice-cold, freshly prepared 4% paraformaldehyde for 10 min. Spinal cord, sciatic nerves, and L4-L5 DRGs were collected and post-fixed in perfusion solution overnight. After that the solution was switched to 30% sucrose in PBS and tissues were kept at 4ºC degrees for at least 12 h. Tissues were covered with tissue Tek (Electron Microscopy Sciences, 62550-01) and stored at -80 ºC. Spinal cord sections and longitudinal sciatic nerve sections were cut at 12 µm and DRGs at 10 um thickness using a cryostat (Leica Biosystems, CM3050S). Sciatic nerve and DRG sections were mounted on Superfrost^{+} microscope slides (Fisher Scientific, 12-550-15) and air dried for at least 12h. Spinal cord sections were stained in 24-well plates as free floating sections. The following primary antibodies were used, anti-Iba1 (1:500; WAKO, 019-19741), anti-F4/80 (1:500; Thermo Fisher Scientific, MA1-91124), anti-CD68 (1:500, Abcam, ab125212), anti-GFAP (1:500, DAKO, Z0334), anti-SCG10 (1: 2,000, Novus Biological, NBP1-49461), anti-CTB (1: 10,000, List Biological Laboratories, #703).

2.5.6 Quantification of neurite outgrowth

Neurite lengths was quantified as described previously(Robak et al., 2009). Briefly, neurofilament-H stained cultures were used for neurite growth analyses. Only cells with neurites ≥ 30 µm were included in the analyses from randomly acquired tile scans using WIS-Neuromath (Kalinski et al., 2019).

2.5.7 Whole mount DRG analysis

2.5.7.1 Staining

Mice were subjected to unilateral SNC as described above. L4 DRGs from the uninjured (intact) and injured side were dissected and post-fixed in 4% PFA/PBS overnight at 4˚C. For tissue clearing of DRGs, we used the iDISCO technique (Bray et al., 2017; Renier et al., 2014). Briefly, post-fixed samples were washed in 1x PBS and then dehydrated at room temperature with a series of 15 minute washes with methanol in 0.05x PBS (20%, 40%, 60%, 80% and 100% vol/vol). Samples were bleached overnight with 5% H_2O_2 in 100% methanol at 4˚C. The next day samples

were rehydrated with a series of 15 minute washes of methanol in 0.05x PBS + 0.2% Triton x-100 (80%, 40%, 20%, and 0% vol/vol). Samples were permeabilized in 1xPBS with 0.2% Triton X-100, 20% DMSO, and 0.3*M* Glycine at 37ºC for 4 hours, followed by blocking with overnight incubation at 37˚C in 1xPBS with 0.2% Triton X-100, 10% DMSO, and 6% donkey serum. Samples were then washed twice for 1 hour in room temperature 1xPBS with 0.2% Tween 20 and 10 μg/ml heparin (PTwH). Then, samples were incubated with goat anti-Iba1 (1:200, Novus Biologicals, NB100-1028) in PTwH plus 5% DMSO and 3% donkey serum at 37˚C for 3 days. Samples were washed 6 times in PTwH: 3 washes for 15 minutes at room temperature, followed by 2 washes for 1 hour at 37˚C and last wash overnight at 37˚C. Incubation with donkey anti-goat Alexa Fluor 488 (1:200, Jackson ImmunoResearch, 705-545-147) and the pan-nuclear stain TOPRO3 (Thermo Fisher Scientific, T3605) was performed in PTwH solution plus 3% donkey serum for 2 days at 37˚C. Then, the 6 washes in PTwH were repeated as above, and the next day samples were processed for clearing. Samples were dehydrated in methanol/water series of 20%, 40%, 60%, and 80% vol/vol for one hour each at room temperature followed by two washes in 100% methanol for 30 minutes each. Samples were then incubated in 66% dichloromethane (DCM) and 33% methanol, followed by two incubations in 100% DCM for 30 minutes each. Finally, samples were cleared and stored in dibenzylether (DBE).

2.5.7.2 Morphological analysis

For each cleared DRG, three different regions of interest were acquired on an inverted Nikon C1 confocal microscope at 60X using 0.25μm z-steps. Image stacks were processed in ImageJ software for background subtraction (rolling ball radius of 10 pixels for Iba1 channel, and 20 for Topro3 signal), followed by mean filtering (1.5-pixel radius for Iba1 signal, and 2.0 for Topro3). Filtered images were then processed in Imaris software (Bitplane) to perform 3D surface rendering, and extraction of morphological characteristics (e.g number of structures, cell, and processes volume). Iba1 immunoreactive cells were categorized based on morphological parameters: somal shape, branch number, and branch extension. Amoeboid cells were defined as having rounded somata of variable size with occasional short ramifications. Elongated cells exhibited an extended and regular rod shaped or arced somal morphology with only rare short

branches. Stellate cells were clearly distinguished from the other cell types by having three or more elongated and curved branches.

2.5.7.3 Density analysis

For estimation of total Iba1 density, whole cleared DRGs were imaged using 3D tile scanning at 20X on a Leica Sp8 confocal microscope. Alignment and stitching were performed with the Leica Application Suite X (LAS X). Images were pre-processed using LAS X Lightning detection package, and subsequently processed using Imaris software. To estimate the total density of Iba1 labeling within DRGs, 3D surface rendering of Iba1 was used, and the volume of reconstructions was normalized against the total volume of the corresponding whole DRG. Group size was based on previously published work (Hollis et al., 2015).

2.5.8 Western blot analysis

Sciatic nerves and L3-L5 DRGs were dissected and lysed separately in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 3.5 mM sodium dodecyl sulfate, 12 mM sodium deoxycholate, pH 8.0) supplemented with 50 mM β-glycerophosphate (Sigma-Aldrich, G9422-100G), 1 mM Na_3VO_4 (Sigma-Aldrich, S6508-10G), and protease inhibitor cocktail (1:100, Sigma-Aldrich, P8340-5ML). Tissues were kept on ice, briefly homogenized with a motorized tissue homogenizer (RPI, 299200), and subjected to sonication (Fisher Scientific Sonic Dismembrator, Model 500) at 70% amplitude for 3 seconds. Tissue lysates were centrifuged at 15,000 rpm at 4°C for 10 minutes (Eppendorf, 5424R). The supernatant was transferred to a new tube and protein concentration was measured with a *DC* Protein Assay Kit (Bio-Rad, 5000111) using a photospectrometer at 750 nm (Molecular Devices, SpectraMax M5e). Samples were diluted with 2x Laemmli sample buffer (Bio-Rad, 1610737) containing 5% β-mercaptoethanol (EMD Millipore, 6010), boiled for 10 minutes at 100°C, and stored at -80°C for analysis. For SDS-PAGE, equal amounts of total protein (5-10 μg) were loaded per lane of a 15% gel. Separated proteins were transferred onto PVDF membrane (EMD Millipore, IPVH00010) for 2.5 hours at 200 mA in cold transfer buffer (25 mM TrisHCl, 192 mM Glycine, 10% Methanol). Membranes were blocked in 5% blotting-grade blocker (BioRad, 1706404) prepared in 1x TBS-T (TBS pH 7.4, containing 0.1% Tween- 20) for 1 hour at room temperature, and probed overnight at 4°C with the following primary antibodies diluted in 1x TBS-T with 3%

BSA (Fisher Scientific, BP1600): α-CD11b (1:1000, Abcam, ab133357), α-ERK1/2 (1:5000, Cell Signaling Technologies, 9102). Horseradish peroxide (HRP)-conjugated α-rabbit secondary IgG (EMD Millipore, AP182P) were used. All HRP-conjugated secondary antibodies were diluted at half the dilution of the corresponding primary antibody in 3% BSA in 1x TBS-T, and the HRP signal was developed with various strengths of chemiluminescent substrates from Thermo Fisher Scientific (Pico Plus, 34580 or Femto, 34095) or from Li-COR Biosciences (926-95000). Protein band intensity was visualized and quantified in the linear range using LI-COR C-Digit (CDG-001313) and Image Studio Software (Version 5.2.5).

2.5.9 Cell isolation for flow cytometry

Adult mice, naïve and at d1, d3, and d7 post-SNC were deeply anesthetized with a mixture of Xylazine and Ketamine and perfused transcardially with ice-cold phosphate-buffered saline (PBS) for 5 minutes. DRGs at lumbar levels L3-L5 were harvested and pooled in ice-cold PBS. Injured and uninjured sciatic nerves were dissected. From injured nerves, the proximal stump and the distal stump (including the injury site) were harvested and pooled separately. Similar sized segments from uninjured nerves were collected for comparison. In addition, spleen was harvested.

2.5.9.1 Flow cytometry

To analyze immune cell profiles in dorsal root ganglia (DRG), sciatic nerves (SN), and spleen, mice were transcardially perfused for 5 min with ice-cold PBS to flush out all blood cells in circulation. The spleen was dissected, and splenocytes were passed through a 70 μm Falcon cell strainer (Corning, 352350). Red blood cells were lysed with Ammonium-Chloride-Potassium (ACK) lysing buffer. DRG and SN were harvested bilaterally. For analysis of DRGs (6 DRGs per mouse X 3-4 mice= 18-24 DRGs) and SN from 2-3 mice (2 SN per mouse x 2-3 mice = 4-6 SN) were pooled separately and used for one run. The collected nerve segments were cut into small pieces with microscissors and incubated in 1 ml collagenase (4mg/ml Worthington Biochemical, LS004176) and dispase (2mg/ml, Sigma-Aldrich, D4693) in PBS for 30-45 min at 37°C degrees in a 15mL conical tube. Tissues were gently triturated with a P1000 pipette every 10 min. Next, samples were rinsed in DMEM with 10% FBS and spun down at 650 g for 5 min. This step was repeated three times and the resulting pellet gently re-suspended in 1 mL of 27% Percoll (Sigma

Aldrich, P4937) in PBS. Then 3 ml of 27% Percoll were added to bring the final volume to 4 ml. Samples were spun at 900g for 20 min in a clinical centrifuge (Beckman Coulter Allegra 6R). The top layers (with myelin and other debris) were carefully aspirated. The final 100 µl were resuspended in 1 ml of PBS with 2% FBS and filtered through a pre-washed 40 µm Falcon filter (Corning, 352340). Cells were pelleted at 650 g for 5 min at 4°C. Cells were labeled with fixable viability dye (Thermo Fisher Scientific, 65086614), blocked with αCD16/32 (BD Pharmingen, 553141), and stained with fluorescent antibodies and isotype controls. Immune cells ($CD45^+$) were further classified as myeloid ($CD45^+CD11b^+$), cDC ($CD45^+CD11b^-CD11c^+Ly6G^-$), MoDC ($CD45^+CD11b^+Ly6G^-CD11c^+$), GC ($CD45^+CD11b^+Ly6G^+CD11c^-$), and Mo/Mac ($CD45^+CD11b^+Ly6G^-CD11c^-$). Data were acquired using a FACSCanto II (BD Biosciences) flow cytometer and analyzed with FlowJo software (Treestar) as described previously (Baldwin et al., 2015).

2.5.9.2 Antibodies

CD11b-PE-Cy7 (Thermo Fisher Scientific, 25-0112-82), Rat IgGk Isotype Control-PE-Cy7 (Thermo Fisher Scientific, 25-4031-82) CD45-e450 (Thermo Fisher Scientific, 48-0451-82), Rat IgG2b Isotype Control-e450 (Thermo Fisher Scientific, 48-4031-82), CD45.1-e450 (Biolegend, 110721), Mouse IgG2ak Isotype Control-e450 (Biolegend, 400235), CD45.2-APC (Biolegend, 109813), Mouse IgG2ak Isotype Control-APC (Biolegend, 400221), Ly6G-APC-Cy7 (BD Biosciences, 560600), Rat IgG2a Isotype Control-APC-Cy7 (BD Biosciences, 552770), CD11c-PerCP-Cy5.5 (Thermo Fisher Scientific, 45-0114-82), Arm Ham IgG Isotype Control-PerCP-Cy5.5 (Thermo Fisher Scientific, 45-4888-80), Ly6C-FITC (BD Biosciences, 561085), Rat IgM Isotype Control-FITC (BD Biosciences, 553942). All antibodies were used at a working concentration of 1:100 except for CD11b (1:200).

2.5.9.3 Statistics

Statistical analysis was performed in GraphPad Prism (v7) using paired or un-paired 2-tailed Student's t test, or 1-way or 2-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test, as indicated in the figure legends. A p value < 0.05 (*) was considered significant. $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

2.5.10 Transcriptomics analysis, bulk RNA-seq of DRGs, and scRNA-seq of sciatic nerves

For gene expression analysis of naïve and axotomized DRGs, we carried out bulk RNA sequencing of L3-L5 ganglia harvested from naïve mice (n=3), d1 (n=3), d3 (n=3), and d7 (n=3) following bilateral SNC. For each data point, 18 ganglia were collected form 3 mice, pooled, flash frozen and lysed in Trizol solution for RNA extraction (Chandran et al., 2016). RNA-sequencing was carried out using TrueSeq RiboZero gold (stranded) kit (Illumina). Libraries were indexed and sequenced over 2 lanes using HiSeq4000 (Illumina) with 75-bp paired end reads. Quality control (QC) was performed on base qualities and nucleotide composition of sequences, to identify potential problems in library preparation or sequencing. Sequence quality for the dataset described here was sufficient that no reads were trimmed or filtered before input to the alignment stage. Reads were aligned to the latest Mouse mm10 reference genome (GRCm38.75) using the STAR spliced read aligner (version 2.4.0). Average input read counts were 58.8M per sample (range 53.4M to 66.2M) and average percentage of uniquely aligned reads was 86.3% (range 83.8% to 88.0%). Raw reads were filtered for low expressed genes and normalized by variance stabilization transformation method. Unwanted variation was removed by RUVSeq (1.20.0) with k=1. Differentially expressed genes were identified using the bioconductor package limma (3.42.2) with FDR<0.1 and the resulting gene lists were used as input for Ingenuity pathway analysis (Qiagen). Weighted gene co-expression network analysis was conducted using WGCNA R-package (ver 1.69). Soft thresholding power of 18 was used to calculate network adjacency. CutHeight of 0.3 was used to merge similar co-expression modules. Enrichment analysis for gene set was performed with GSEA (ver 2.2.2) using MsigDB (ver 7.0). Normalized enrichment score (NES) was used to assess enrichment of gene sets.

2.5.11 Preparation of cells for scRNA-seq

Mice were transcardially perfused with ice-cold PBS for 5 min to flush out all blood cells in circulation. The sciatic nerve trunk was harvested and a segment that contains the injury site and the distal nerve stump, up to the branch point of the tibial nerve, used for further processing. A minimum of 3 mice (6 nerves) was used to obtain sufficient cells for analysis using the 10x Genomics platform. The collected nerve segments were cut into small pieces with microscissers

and incubated in 1 ml PBS supplemented with collagenase (4mg/ml Worthington Biochemical, LS004176), dispase (2mg/ml, Sigma-Aldrich, D4693), and actinomycin D (45 µM, Sigma Aldrich, A1410) for 30-45 min at 37°C degrees in a 15mL conical tube. Tissues were gently triturated with a P1000 pipette every 10 min. Next, samples were rinsed in DMEM with 10% FBS and spun down at 650 g for 5 min before removing supernatant. The resulting pellet was gently re-suspended in 1 mL of 27% Percoll (Sigma Aldrich, P4937) in PBS. Then 3 ml of 27% Percoll were added to bring the final volume to 4 ml. Samples were spun at 900g for 20 min with no brake in a clinical centrifuge (Beckman Coulter Allegra 6R). The top layers (with myelin and other debris) were carefully aspirated. The final 100 µl were resuspended in 1 ml of PBS with 2% FBS and filtered through a pre-washed 40 µm Falcon filter (Corning, 352340) with an additional 5 ml of PBS with 2% FBS. Cells were pelleted at 650 g for 5 min at 4°C. The supernatant was removed and the cell pellet resuspended in 180 µl of MACS buffer (Miltenyi, 130-091-376) diluted 1:20 in PBS (final bovine serum albumin [BSA] was 0.5%) and 10 µl of myelin removal beads were added (Miltenyi, 30-096-731). To remove all myelin debris, cells were incubated with myelin depletion beads for 15 min at 4°C with intermitted tapping. Cells were rinsed in 5 ml of MACS buffer, gently inverted several times and spun at 300g for 10 min. Cells were separated from myelin beads using the MidiMACS separator (Miltenyi, 130-042-302) and LS columns (Miltenyi, 130-042-401). The flow through solution with the cells was centrifuged and the cells resuspended in 50 µl of Hanks balanced salt solution (Gibco, 14025092) supplemented with 0.04% BSA (Fisher Scientific, BP1600). The cell number and live/dead ratio was determined using propidium iodine labeling and a hemocytometer.

2.5.12 10x Genomics single cell RNA-seq library preparation

For encapsulation of single cells with microbeads into nanodroplets, the Chromium Next GEM Single Cell 3' GEM Library & Gel Bead Kit v3.1 and Chromium Next GEM Chip G Single Cell Kit were used. Approximately 12,000 cells in a final volume of 43 µl were used for barcoding, using the 10X Genomics Chromium Controller. The library preparation of barcoded cDNAs was carried out in a bulk reaction, following instructions provided by the manufacturer. A small aliquot of the library was used for quality control with a bioanalyzer followed by library sequencing at the Advanced Genomics Core of the University of Michigan. The NovaSeq Illumina 6000 was used

with an S4 flowcell, yielding 1.05 Billion reads (7-11% of the flowcell) (Individual samples ranging from 290 to 424 million reads). NovaSeq control software version 1.6 and Real Time Analysis (RTA) software version 3.4.4 were used to generate binary base call (BCL) formatted files.

2.5.13 Data analysis and availability

Raw scRNAseq data were processed using the 10x Genomics CellRanger softeware version 3.1.0. The CellRanger "mkfastq" function was used for de-multiplexing and generating FASTQ files from raw BCLs. The CellRanger "count" function, with default settings was used with the mm10 reference supplied by 10x Genomics, to align reads and generate single cell feature counts. Per sample, approx. 5,800 cells with median genes per cell of 2,507 were obtained. CellRanger filtered cells and counts were used for downstream analysis in Seurat version 3.1.2 implemented in R version 3.6.2. Cells were excluded if they had fewer than 200 features, more than 7500, or the mitochondrial content was more than 25%. Reads from multiple samples were merged and normalized following a standard Seurat SCTransform integration pipeline (Hafemeister and Satija, 2019); mitochondrial mapping percentage was regressed out during the SCTransform normalization step. Principal component analysis was performed on the top 3000 variable genes and the top 30 principle components were used for downstream analysis. A K-nearest neighbor graph was produced using Euclidean distances. The Louvain algorithm was used with resolution set to .5 to group cells together. Non-linear dimensional reduction was done using UMAP. The top 100 genes for each cluster, determined by Seurat's FindAllMarkers function and the Wilcoxon Rank Sum test, were submitted to version 11 of the string-db.org to determine functional enrichment; referred to as STRING analysis.

To model developmental trajectories of cells that comprise the mononuclear phagocyte system (MPS), the Bioconductor package, slingshot version 1.4.0 was used. The integrated Seurat object was subset to include only MPS cells and slingshot was instructed to start from monocytes. The pseudo-time from the three slingshot constructed lineages were used in random regression forest to reveal the most influential genes, on pseudo-time. Random forests were implemented with the Ranger package of R from 1400 trees, 200 genes at each node, and the Gini index,

"impurity", measure for gene importance. The bulk RNA-seq and scRNA-seq data is available online in the Gene Expression Omnibus (GEO) database (GSE153762).

Cell identities, as defined above, were saved for the 3d injured nerve. Single-cell transcriptomes from YFP.pos and YFP.neg macrophage populations identified in naïve peripheral nerve tissue (Wang et al., 2020), were downloaded and given the label Mac_Naive. The log2 transformed raw counts of the 3d injured Mac1-5 and Mo as well as the Mac_Naive cells were subjected to batch correction using the ComBat function from the Bioconductor "sva" package (Leek et al., 2012). Injured nerve Mo/Mac and naïve Mac made up the two batches and the following arguments were passed to ComBat: mod=NULL, par.prior=TRUE, mean.only=FALSE, prior.plots=FALSE. After batch correction each cell type and gene had a highly repeated minimum number near 0. To aid in plotting and determining "percent expressed" this value was replaced with 0. The average expression for each gene and each cell type was calculated for the purpose of making dotplots. Any cell type with more than 85% zeros was not given a dot. The dots represent percent expressed by radius and average expression, scaled across cell type, by color.

2.6 Acknowledgements

We thank Richard Zigmond and members of the Giger lab for critical reading of the manuscript. This work was supported by NIH T32 NS07222, Training in Clinical and Basic Neuroscience (AK), NIH T32 GM113900, Training Program in Translational Research (LH), the New York State Department of Health Spinal Cord Injury Program C33267GG (EH and RG), the Wings for Life Foundation (CY), the National Eye Institute (NEI), National Institutes of Health, R01EY029159 and R01EY028350 (BMS and RG), the Stanley D and Joan H Ross Chair in Neuromodulation fund (BMS), and the Dr Miriam and Sheldon G Adelson Medical Research Foundation (RK, DG, RG).

2.7 Author Contribution

Lucas D Huffman (LDH), Ashley L Kalinski (ALK), Choya Yoon (CY), and Patrick C Duncker (PCD) all contributed equally to the completion of this project. LDH, ALK, CY, PCD, Craig Johnson (CJ), Riki Kawaguchi (RK), Daniel H Geschwind (DHG), and Benjamin M Segal (BMS)

contributed to data curation. LDH, ALK, CY, PCD, Rafi Kohen, CJ, RK, Juan Sebastian Jara (JSJ), Edmund Hollis (EH), DHG, BMS, and Roman J Giger (RJG) contributed to formal analysis. LDH, ALK, CY, EH, DHG, BMS, and RJG contributed to funding acquisition. LDH, ALK, CY, PCD, Rafi Kohen, Ryan Passino, Hannah Haffner, Kevin S Carbajal (KSC), JSJ, and RJG contributed to investigation. LDH, ALK, PCD, and CJ contributed to visualization. LDH, ALK, CY, PCD, CJ, KSC, and RJG contributed to methodology, LDH, ALK, and RJG contributed to writing – review and editing. CJ, RK, DHG, BMS contributed to software. RK, EH, DHG, and BMS contributed resources. EH, DHG, BMS, and RJG contributed to supervision. RJG contributed to conceptualization.

2.8 Tables

Table 2-1. List of top five cluster enriched genes for immune cell clusters identified in the 3d post-SNC nerve.
First column (P_val), probability of getting the "elevated" expression values in these cells under the null hypothesis that all cells have the same expression of the gene. Second column (avg_logFC), average log2 Fold-Change between cells in this cluster relative to cells in all other clusters. Third column (pct.1), percent of the cluster's cells which express the gene. Fourth column (pct.2), percent of non-cluster cells which express the gene. Fifth column (p_val_adj), p_val adjusted so that 5% of the list is expected to have false positives. Sixth column (cluster), associated cell cluster. Sixth column, enriched gene. The full sheet of the top 100 genes for each cell cluster including immune cells can be found at https://cdn.elifesciences.org/articles/60223/elife-60223-fig5-data1-v3.xlsx

p_val	avg_logFC	pct.1	pct.2	p_val_adj	cluster	gene
0	1.924008117	0.781	0.163	0	Mo	Chil3
0	1.415313464	0.65	0.043	0	Mo	Ly6c2
0	1.153372957	0.826	0.176	0	Mo	Ifitm6
0	1.106439147	0.765	0.105	0	Mo	Hp
0	0.78868658	0.589	0.079	0	Mo	Itgal
0	1.426942886	0.98	0.799	0	Mac1	Fn1
0	1.384737266	0.665	0.204	0	Mac1	Lpl
0	1.38230321	0.663	0.212	0	Mac1	Tppp3
0	1.348837008	0.712	0.115	0	Mac1	Ltc4s
0	1.319261319	1	0.65	0	Mac1	Lyz2
0	1.625349821	1	0.513	0	Mac2	Cd74
0	1.595721667	0.994	0.401	0	Mac2	H2-Aa
0	1.449877797	0.997	0.401	0	Mac2	H2-Ab1
0	1.426145592	0.985	0.323	0	Mac2	H2-Eb1
0	1.249222779	0.993	0.597	0	Mac2	Ccr2
0	1.474636321	1	0.688	0	Mac3	Lgmn
0	1.370771892	0.862	0.342	0	Mac3	Mrc1
0	1.273570576	1	0.662	0	Mac3	Ctsc
0	1.246990162	0.955	0.541	0	Mac3	Dab2
0	1.207764266	0.898	0.36	0	Mac3	F13a1
0	2.148740377	1	0.867	0	Mac4	Ctsd
0	1.841925501	0.923	0.206	0	Mac4	Pf4
0	1.828630466	0.994	0.544	0	Mac4	Plin2
0	1.770593779	0.683	0.084	0	Mac4	Gpnmb
0	1.713387024	0.878	0.213	0	Mac4	Arg1
0	1.783862084	0.987	0.143	0	Mac5	Mki67
0	1.572105286	0.883	0.101	0	Mac5	Ube2c
0	1.527519926	0.95	0.127	0	Mac5	Pclaf

p_val	avg_logFC	pct.1	pct.2	p_val_adj	cluster	gene
0	1.488688587	0.979	0.119	0	Mac5	Birc5
0	1.199634233	0.841	0.061	0	Mac5	Nusap1
0	2.523326685	0.971	0.341	0	MoDC	H2-Eb1
0	2.519169438	0.992	0.527	0	MoDC	Cd74
0	2.448597919	0.976	0.418	0	MoDC	H2-Ab1
0	2.419285305	0.976	0.418	0	MoDC	H2-Aa
0	1.800974778	0.608	0.09	0	MoDC	Ifitm1
0	1.940823494	0.977	0.135	0	cDC	Wdfy4
0	1.70903806	0.954	0.055	0	cDC	Ifi205
0	1.503330336	0.87	0.026	0	cDC	Sept3
0	1.317664948	0.949	0.053	0	cDC	Flt3
0	1.27754791	0.852	0.006	0	cDC	Clec9a
0	3.958020268	0.838	0.019	0	GC	S100a8
0	3.928895789	0.812	0.007	0	GC	S100a9
0	2.730951696	0.882	0.096	0	GC	Mmp9
0	2.28273958	0.78	0.059	0	GC	Il1b
0	2.17383976	0.557	0.023	0	GC	Cxcl2
0	3.163420231	0.742	0.01	0	T/NK	Ccl5
0	2.647859172	0.554	0.001	0	T/NK	Gzma
0	2.206176013	0.852	0.128	0	T/NK	AW112010
0	2.059157123	0.738	0.001	0	T/NK	Nkg7
0	1.808445286	0.797	0.001	0	T/NK	Il2rb

2.9 Figures

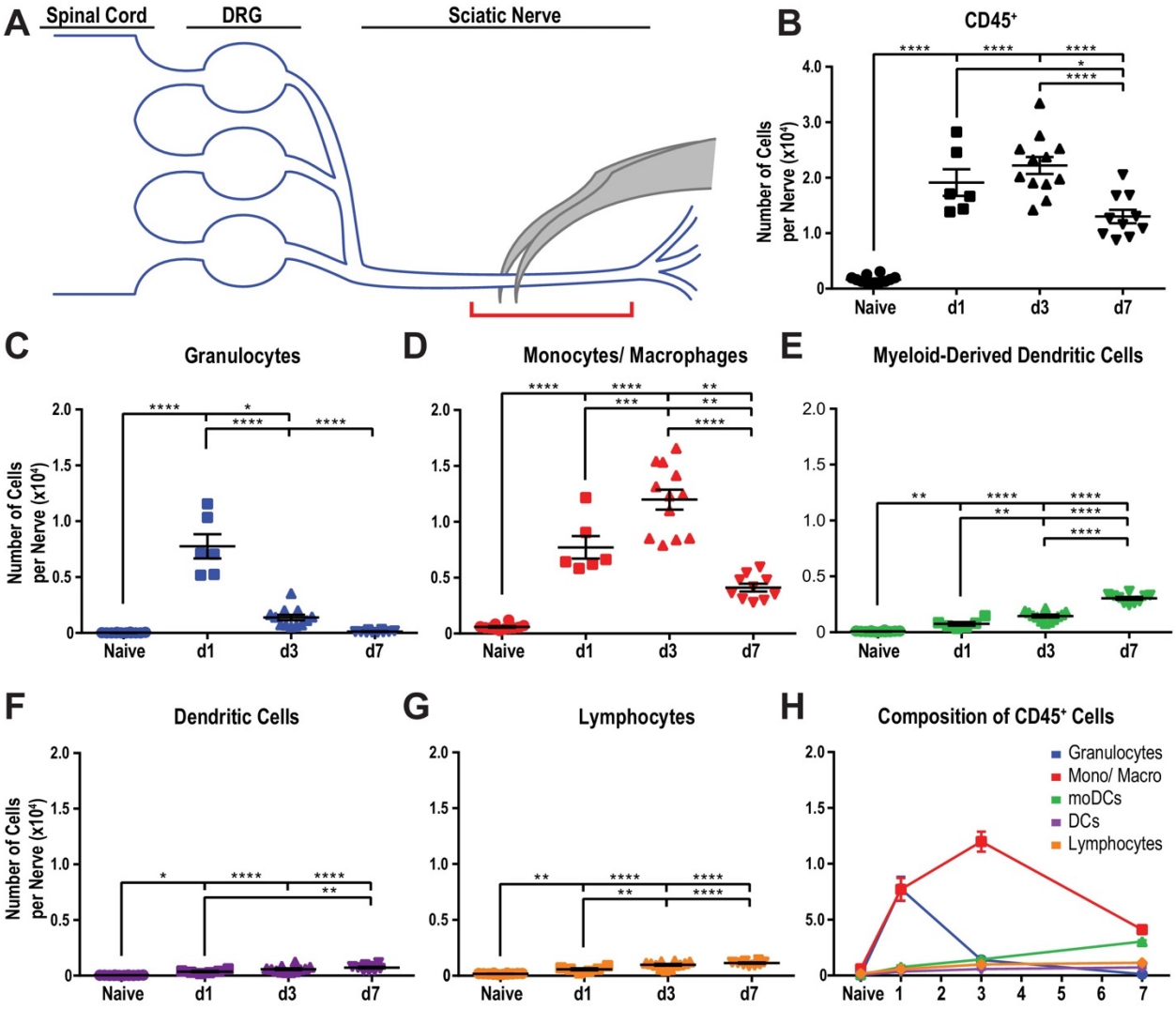


Figure 2-1 Immune cell profiles in the injured murine sciatic nerve.
A. Anatomy of lumbar spinal cord and DRGs connected to the sciatic nerve. The location of the crush site within the nerve trunk and the tissue segment collected for flow cytometry (red bracket) are shown. B. Quantification of live, $CD45^+$ leukocytes, normalized per sciatic nerve trunk. Flow cytometry of nerve tissue collected from naïve mice (n= 10 biological replicates, with 6 nerves per replicate), day 1 (d1) post-SNC (n= 7), d3 (n= 12), and d7 (n= 12). C. Quantification of granulocytes ($CD45^+$, $CD11b^+$, $Ly6G^+$) per nerve trunk. D. Quantification of Mo/Mac ($CD45^+$, $CD11b^+$, $CD11c^-$, $Ly6G^-$) per nerve trunk. E. Quantification of MoDC ($CD45^+$, $CD11b^+$, $CD11c^+$, $Ly6G^-$) per nerve trunk. F. Quantification of cDC ($CD45^+$, $CD11b^-$, $CD11c^+$, $Ly6G^-$) per nerve trunk. G. Quantification of lymphocytes ($CD45^+$, $CD11b^-$) per nerve trunk. H. Composition of $CD45^+$ leukocytes in the nerve trunk at different post-injury time points. Flow data are represented as mean cell number ± SEM. Statistical analysis was performed in GraphPad Prism (v7) using 1-way or 2-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test. For B-G, unpaired two-tailed t-test with Welch's correction. A p value < 0.05 (*) was considered significant. $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

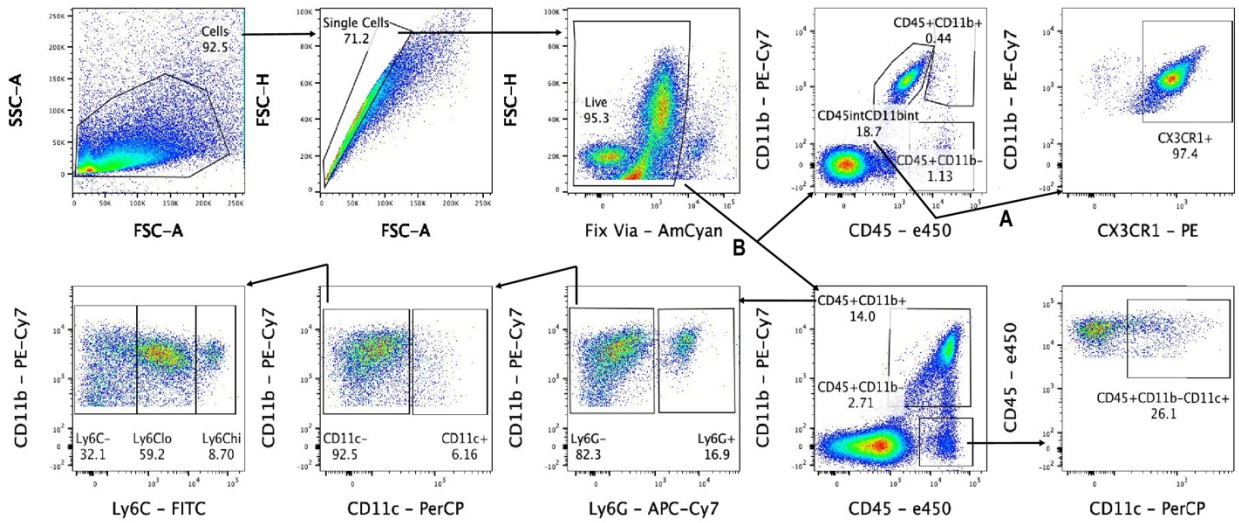


Figure 2-2 Gating scheme for flow cytometry.

Cells were first gated with forward scatter (FSC-A) and side scatter (SSC-A) to exclude debris. Cells were then gated with forward scatter height (FSC-H) and FSC-A to find single cells and to exclude doublets. Live cells were isolated by negative staining for fixed viability dye (Fix Via). In nerves, DRGs, spinal cord, and spleen, leukocytes were analyzed as follows: lymphocytes were isolated as $CD45^+$, $CD11b^-$ cell and then further separated based on CD11c positivity as cDC or as $CD45^+$, $CD11b^-$, $CD11c^-$ lymphocytes. Myeloid cells ($CD45^+$, $CD11b^+$) were further separated into $Ly6G^+$ granulocytes. The remaining cells ($CD45^+$, $CD11b^+$, $Ly6G^-$) were characterized as MoDC ($CD45^+$, $CD11b^+$, $CD11c^+$, $Ly6G^-$), Mo/Mac ($CD45^+$, $CD11b^+$, $CD11c^-$, $Ly6G^-$), and Microglia ($CD45^{int}$, $CD11b^{int}$, $CX3CR1^+$).

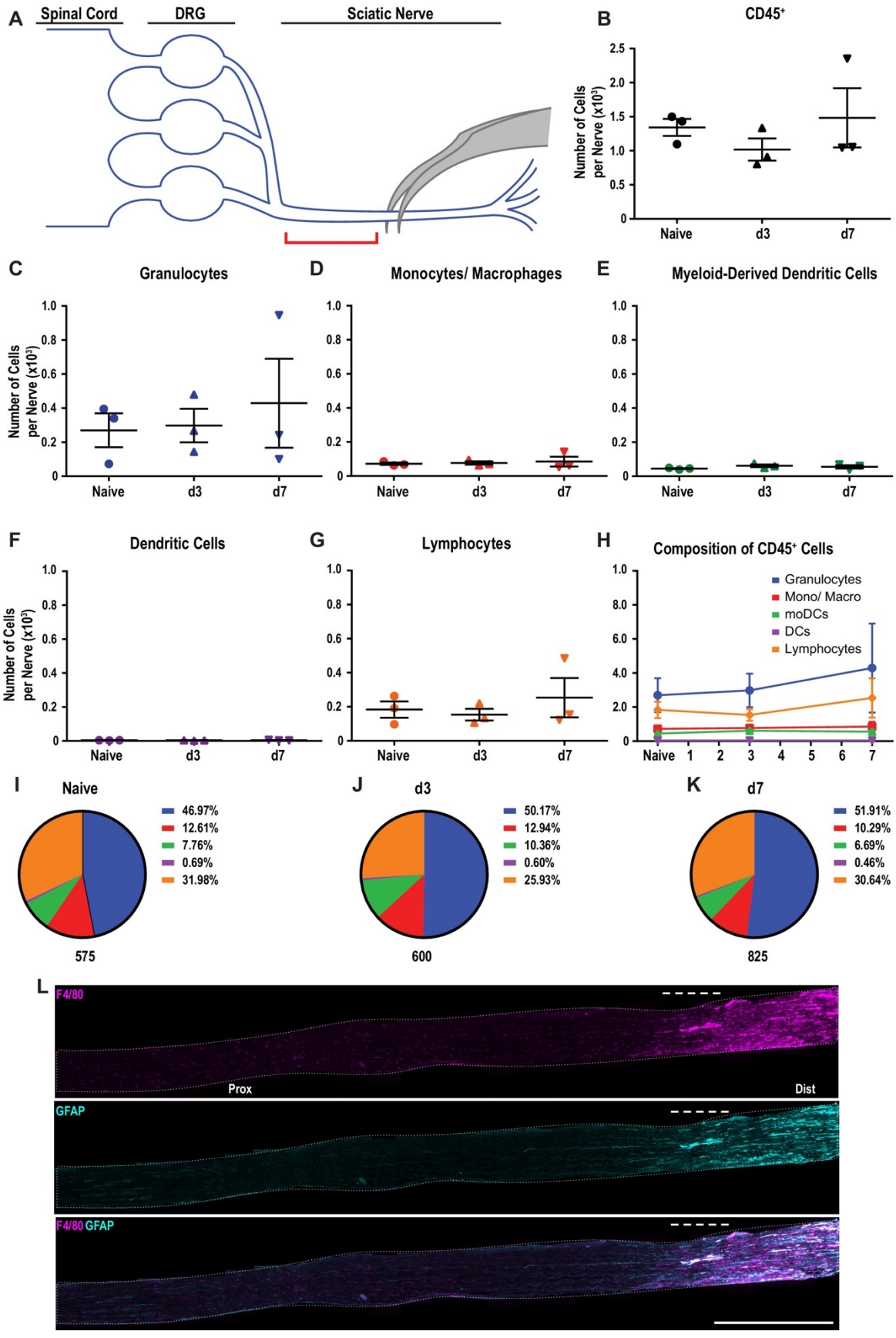

A
Spinal Cord
DRG
Sciatic Nerve
B
CD45+
Number of Cells per Nerve (x10³)
Naive
d3
d7
C
Granulocytes
D
Monocytes/ Macrophages
E
Myeloid-Derived Dendritic Cells
F
Dendritic Cells
G
Lymphocytes
H
Composition of CD45+ Cells
Granulocytes
Mono/ Macro
moDCs
DCs
Lymphocytes
I
Naive
46.97%
12.61%
7.76%
0.69%
31.98%
575
J
d3
50.17%
12.94%
10.36%
0.60%
25.93%
600
K
d7
51.91%
10.29%
6.69%
0.46%
30.64%
825
L
F4/80
Prox
Dist
GFAP
F4/80 GFAP

Figure 2-3 Immune cell profiles in the sciatic nerve proximal to the injury site.
A. Anatomy of lumbar spinal cord and DRGs connected to the sciatic nerve. The location of the crush site within the nerve trunk and the tissue segment collected for flow cytometry (red bracket) are shown. B. Quantification of live, $CD45^+$ cell in the proximal nerve, per ~ 5mm segment. Flow cytometry of nerve tissue collected from naïve mice (n= 3), d3 (n= 3), and d7 (n= 3) following SNC. C. Quantification of granulocytes ($CD45^+$, $CD11b^+$, $Ly6G^+$) per nerve segment. D. Quantification of Mo/Mac ($CD45^+$, $CD11b^+$, $CD11c^-$, $Ly6G^-$) per nerve segment. E. Quantification of MoDC ($CD45^+$, $CD11b^+$, $CD11c^+$, $Ly6G^-$) per nerve segment F. Quantification of cDC ($CD45^+$, $CD11b^-$, $CD11c^+$, $Ly6G^-$) per nerve segment. G. Quantification of lymphocytes ($CD45^+$, $CD11b^-$) per nerve segment. H. Composition of $CD45^+$ leukocytes in the proximal nerve stump at different post-SNC time points. I-K. Percentile of each cell type at different post-injury time points. For flow cytometry, data are represented as mean cell number ± SEM. Statistical analysis was performed in GraphPad Prism (v7) using 1-way or 2-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test. For B-G, unpaired two-tailed t-test with Welch's correction. A p value < 0.05 (*) was considered significant. $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). L. Longitudinal sections of sciatic nerve trunk at d3 following SNC, stained with anti-F4/80 (red) to label macrophages and anti-GFAP (green) to label repair Schwann cells. The proximal (Prox) and distal (Dist) sides of the nerve, relative to the crush site (dashed line), are indicated. Consistent with flow cytometry, nerve inflammation is not significantly elevated proximal to the nerve crush site. Scale bar, 1000 μm.

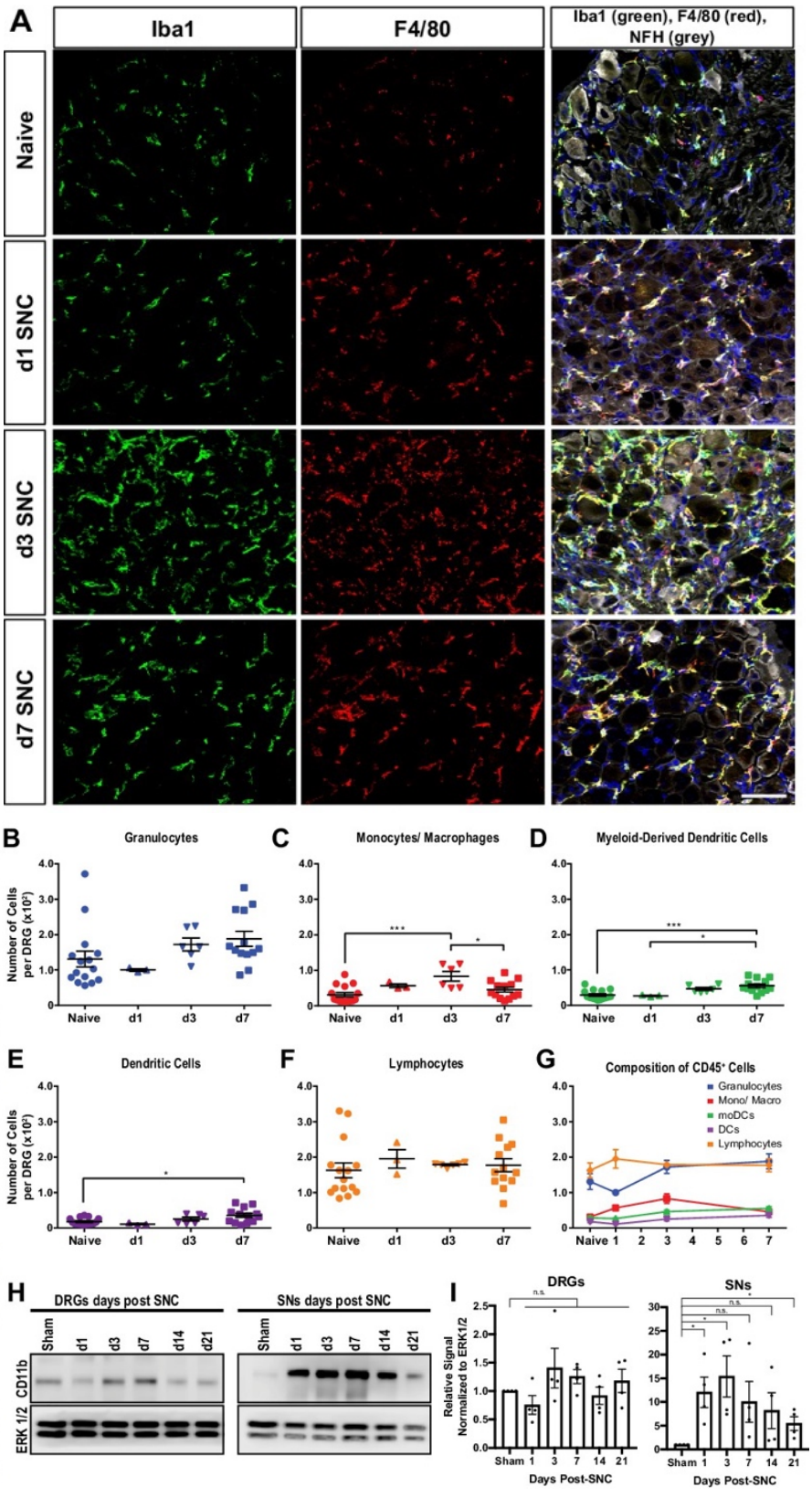

A
Iba1
F4/80
Iba1 (green), F4/80 (red), NFH (grey)
Naive
d1 SNC
d3 SNC
d7 SNC
B
Granulocytes
Number of Cells per DRG (x10²)
Naive
d1
d3
d7
C
Monocytes/ Macrophages
D
Myeloid-Derived Dendritic Cells
E
Dendritic Cells
F
Lymphocytes
G
Composition of CD45⁺ Cells
Granulocytes
Mono/ Macro
moDCs
DCs
Lymphocytes
H
DRGs days post SNC
SNs days post SNC
Sham
d1
d3
d7
d14
d21
CD11b
ERK 1/2
I
DRGs
SNs
Relative Signal Normalized to ERK1/2
n.s.
Sham
1
3
7
14
21
Days Post-SNC

Figure 2-4 Immune cell profiles in axotomized DRGs.

A. Representative images of L4 DRG cross sections from naïve mice, d1, d3, and d7 post-SNC. Macrophages were stained with anti-Iba1 and anti-F4/80. Neurons were stained with anti-NFH. Scale bar, 50 μm. B. Quantification of granulocytes per DRG detected by flow cytometry. For flow cytometry of DRGs, naïve mice (n= 14 biological replicates), d1 (n= 3), d3 (n= 5), and d7 (n= 12) post-SNC mice were used. Granulocytes ($CD45^+$, $CD11b^+$, $Ly6G^+$) per DRG are shown. C. Quantification of Mo/Mac ($CD45^+$, $CD11b^+$, $CD11c^-$, $Ly6G^-$) per DRG. D. Quantification of MoDC ($CD45^+$, $CD11b^+$, $CD11c^+$, $Ly6G^-$) per DRG. E. Quantification of cDC ($CD45^+$, $CD11b^-$, $CD11c^+$, $Ly6G^-$) per DRG. F. Quantification of lymphocytes (CD45+, $CD11b^-$) per DRG. G. Composition of $CD45^+$ leukocytes in lumbar DRGs identified by flow cytometry. Flow data are represented as mean cell number ± SEM. Each data point represents L3-L5 DRGs pooled from 3-4 animals (18-24 DRGs), biological replicates, n= 3-14. Statistical analysis was performed in GraphPad Prism (v7) using 1-way or 2-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test. For B-F, unpaired two-tailed t-test with Welch's correction. A p value < 0.05 (*) was considered significant. $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). H. Western blots analysis of DRGs and sciatic nerves (SNs) prepared from sham operated mice and mice at different post-SNC time points (d1-d21), probed with anti-CD11b and anti-ERK1/2 as loading control. I. Quantification of CD11b signal in DRGs and SNs. Unpaired two-tailed Student's t-test compared to sham operated. n.s. not significant, *$p<0.05$, biological replicates n= 4 (with 4 mice for each time point).

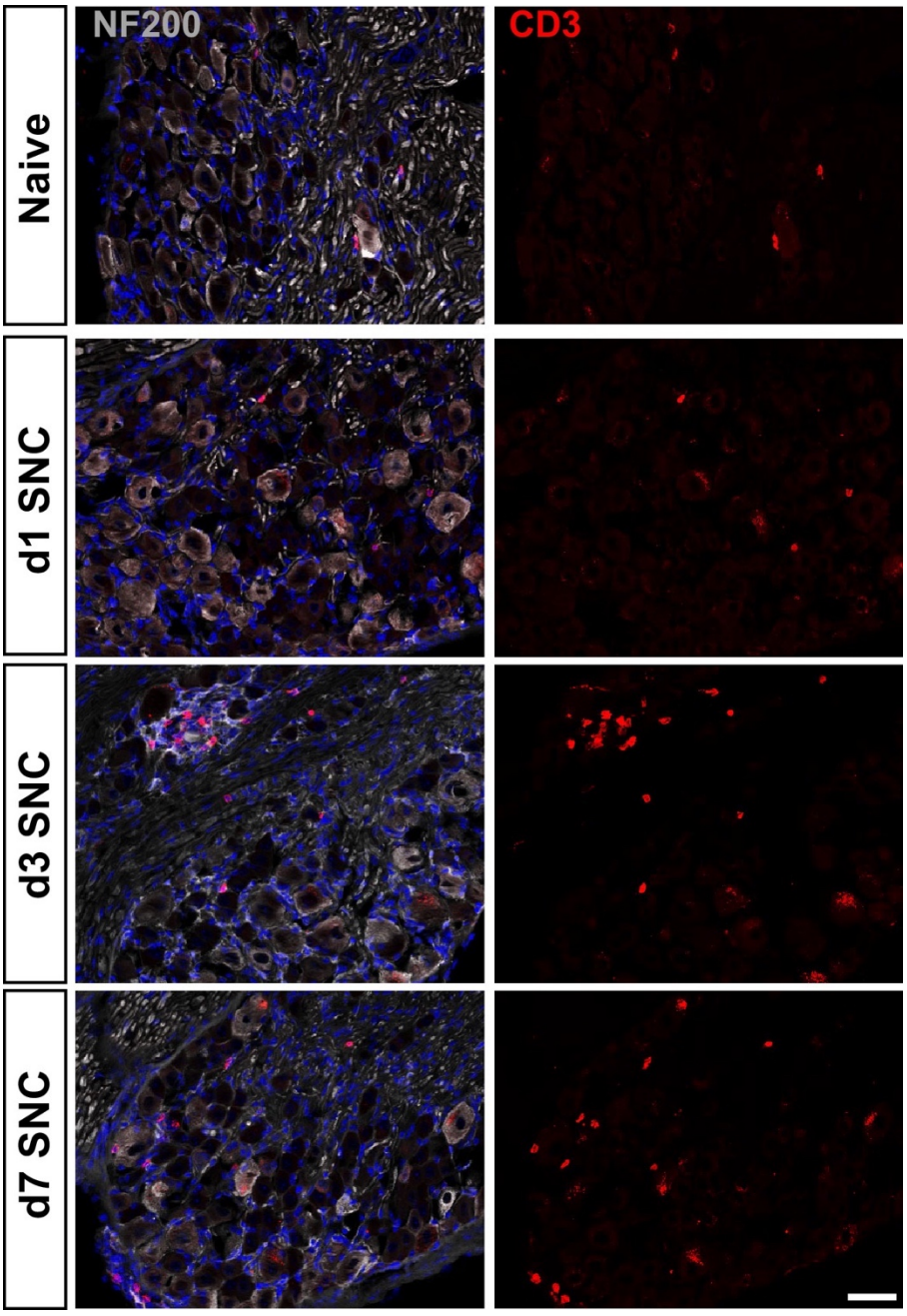


Figure 2-5 T cells in naïve and axotomized DRGs.
Representative images of L4 DRG cross sections from naïve mice, d1, d3, and d7 post-SNC. T cells were labeled with anti-CD3 (red), neurons with anti-NF200 (white). DAPI (blue) was used for nuclear staining (blue). Scale bar, 50 μm.

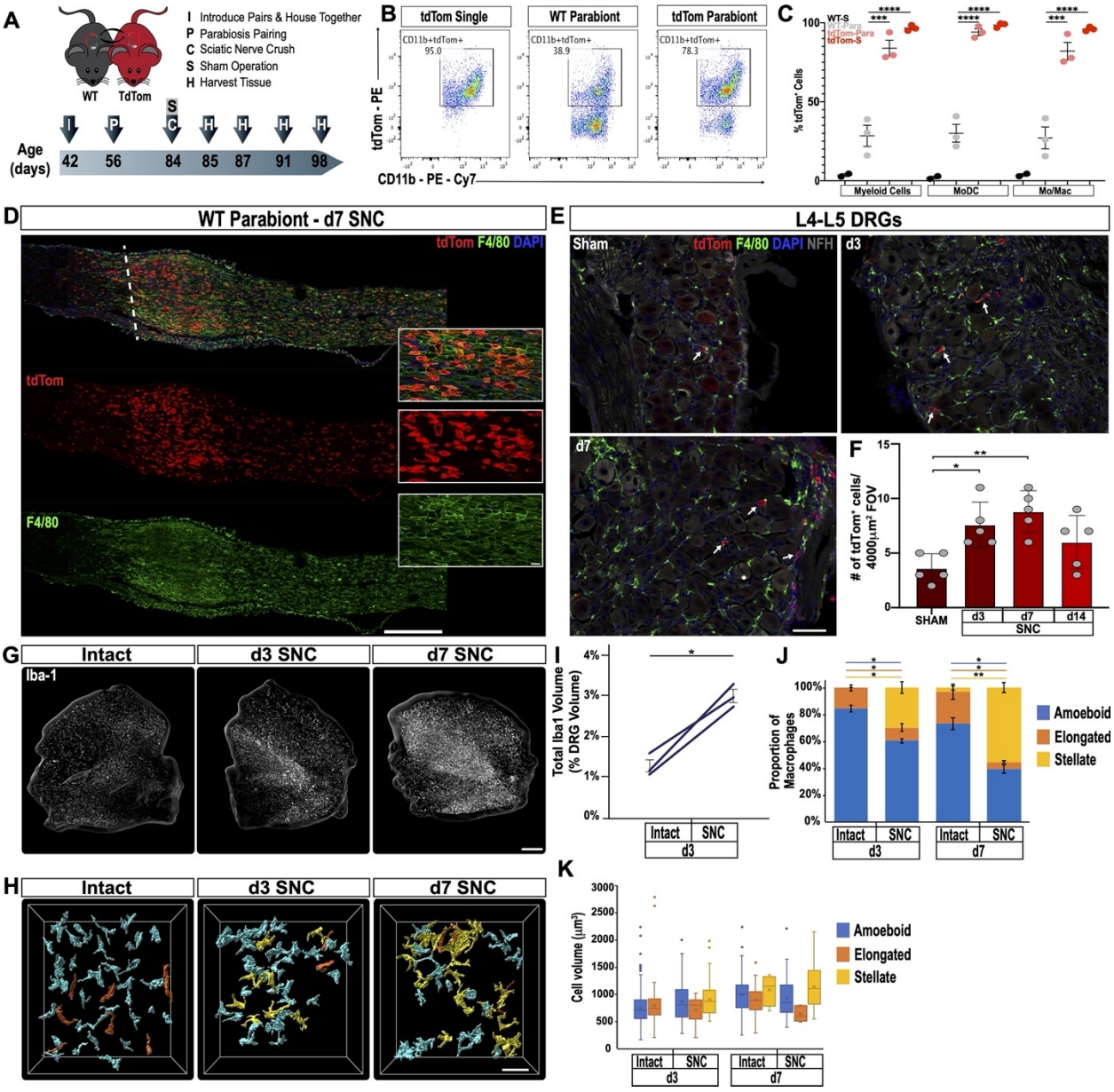


Figure 2-6 Sciatic nerve injury triggers massive accumulation of hematogenous leukocytes in the injured nerve but not axotomized DRGs.

A. Parbiosis complex of a wildtype (WT) and a tdTomato (tdTom) mouse. Mice were surgically paired at postnatal day 56. The timeline of the experiment is shown. B. Flow cytometric analysis of sciatic nerve trunks collected from non-parabiotic (single) tdTom mice, WT parabionts, and tdTom parabionts. Dotplot of live ($CD11b^+$, $tdTom^+$) cells in the d3 post-SNC nerve. C. Quantification of $tdTom^+$ myeloid cells in the 3d injured nerve of WT single mice (WT-S), WT parabiont (WT-para), tdTom parabiont (tdTom-para), and tdTom single (tdTom-S) mice. The fraction of $tdTom^+$ myeloid cells ($CD45^+$, $CD11b^+$), MoDC ($CD45^+$, $CD11b^+$, $CD11c^+$, $Ly6G^-$), and Mo/Mac ($CD45^+$, $CD11b^+$, $CD11c^-$, $Ly6G^-$) is shown. For quantification of $tdTom^+$ immune cells, nerves from the WT parabiont and the tdTom parabiont were harvested separately (3 mice per data point) with n= 2-3 biological replicates. Flow data are represented as fraction of $tdTom^+$ cells ± SEM. Statistical analysis was performed in GraphPad Prism (v8) using 1-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test. p value of < 0.001 (***) and $p < 0.0001$ (****). D. Longitudinal sciatic nerves sections of the WT parabiont at d7 post-SNC. The nerve crush site

is marked with a white dotted line, proximal is to the left, distal to the right. Anti-F4/80 (green) and $tdTom^+$ cells (red) staining is shown. Scale bar, 500 µm, for insets, 20 µm. E. Lumbar DRG cross sections of WT parabionts harvested from sham operated mice, at d3, and d7 post-SNC. Sections were stained with anti-F4/80 (green) and anti-NF200 (white). Hematogenous ($tdTom^+$) leukocytes are marked with white arrows. Scale bar, 50 µm. F. Quantification of $tdTom^+$ cells per field of view (FOV = 4000 μm^2) in DRG sections of the WT parabiont. Data are shown as number of $tdTom^+$ cells ± SEM, n= 3-5 mice per time point. Student's t test with $p < 0.5$ (*) considered statistically significant, $p < 0.01$ (**). G. Whole mount anti-Iba1 immunofluorescence staining of L4 DRGs from intact, d3, and d7 post-SNC time points. Scale bar, 200 µm. H. Morphological reconstruction of $Iba1^+$ cells in DRGs with Imaris. Analysis of DRG-resident macrophages revealed amoeboid (cyan) and elongated (orange) morphologies if the nerve was not injured. At d3 and d7 post-SNC, a subpopulation of $Iba1^+$ cells with stellate (yellow) morphology was observed in DRGs. Scale bar, 50 µm. I. Quantification of total volume of $Iba1^+$ structures in DRGs, rendered by Imaris. The total volume of $Iba1^+$ structures per DRG was quantified on the intact side and the injured side of the same mouse at d3 post-SNC (n= 3 mice). Paired Students t test, p value < 0.05 (*), was considered significant. J. Quantification of $Iba1^+$ cells with amoeboid, elongated, and stellate morphologies. K. Quantification of cell volume of individual $Iba1^+$ cells with amoeboid, elongated, and stellate morphologies. At d3 post-SNC, a total of 416 cells were reconstructed on the intact side and a total of 234 cells on the injured side. At d7 post-SNC, a total of 136 cells were reconstructed on the intact side and a total of 93 cells on the injured side. The distribution of morphological categories ± SEM (J) and cell volumes ± SEM (K) are shown. Paired, two-tailed Student's t test, a p value < 0.05 (*) was considered significant. $p < 0.01$ (**).

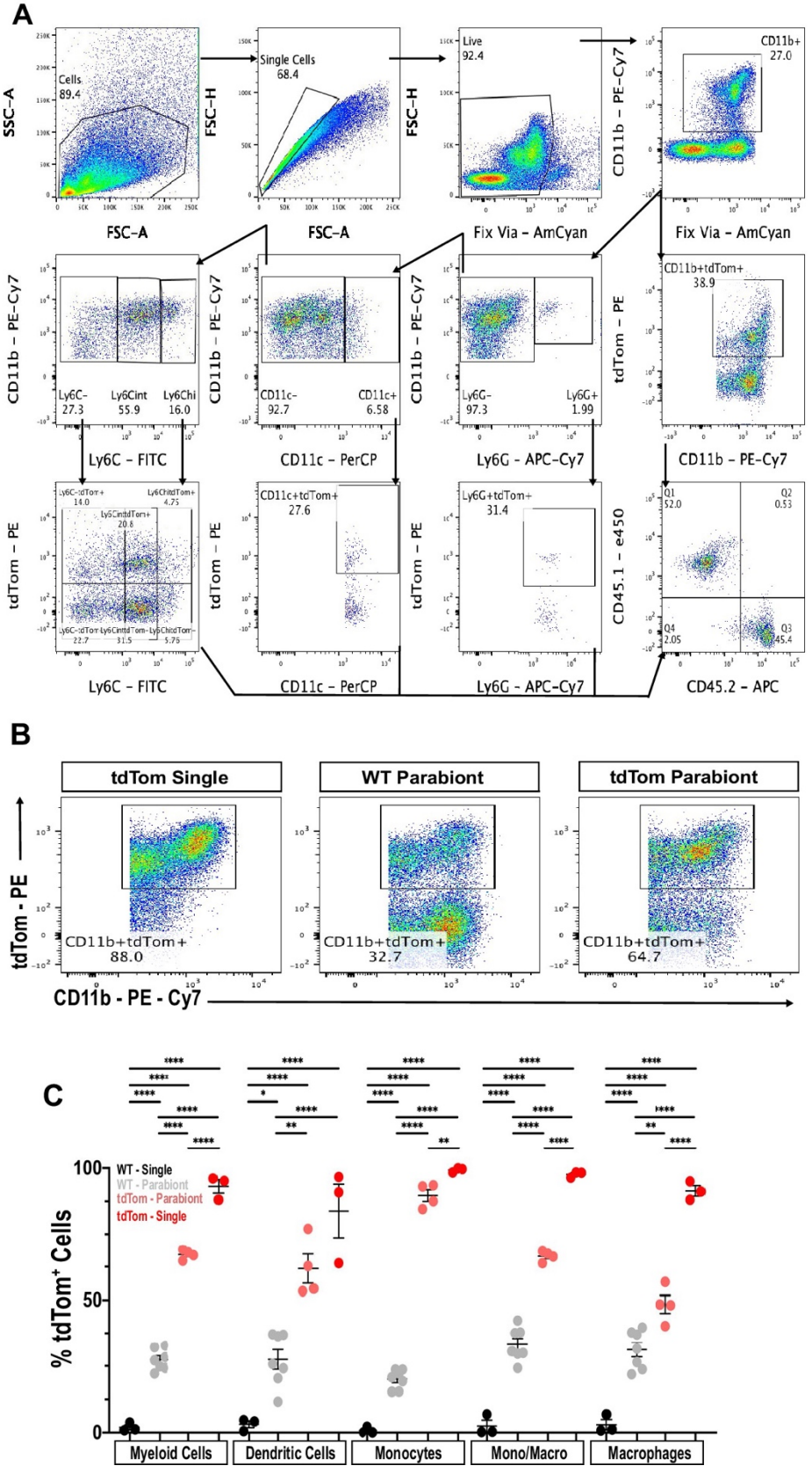

A
Cells
89.4
SSC-A
FSC-A
Single Cells
68.4
FSC-H
FSC-A
Live
92.4
FSC-H
Fix Via - AmCyan
CD11b+
27.0
CD11b - PE-Cy7
Fix Via - AmCyan
CD11b - PE-Cy7
Ly6C-
27.3
Ly6Cint
55.9
Ly6Chi
16.0
Ly6C - FITC
CD11b - PE-Cy7
CD11c-
92.7
CD11c+
6.58
CD11c - PerCP
CD11b - PE-Cy7
Ly6G-
97.3
Ly6G+
1.99
Ly6G - APC-Cy7
tdTom - PE
CD11b+tdTom+
38.9
CD11b - PE-Cy7
tdTom - PE
Ly6C - FITC
tdTom - PE
CD11c+tdTom+
27.6
CD11c - PerCP
tdTom - PE
Ly6G+tdTom+
31.4
Ly6G - APC-Cy7
CD45.1 - e450
Q1
52.0
Q2
0.53
Q4
2.05
Q3
45.4
CD45.2 - APC
B
tdTom Single
WT Parabiont
tdTom Parabiont
tdTom - PE
CD11b+tdTom+
88.0
CD11b+tdTom+
32.7
CD11b+tdTom+
64.7
CD11b - PE - Cy7
C
WT - Single
WT - Parabiont
tdTom - Parabiont
tdTom - Single
% tdTom+ Cells
100
50
0
Myeloid Cells
Dendritic Cells
Monocytes
Mono/Macro
Macrophages

Figure 2-7 Flow cytometry gating scheme to assess chimerism of parabiotic mice.
A. Cells were first gated with forward scatter (FSC-A) and side scatter (SSC-A) to exclude debris. Cells were then gated with forward scatter height (FSC-H) and FSC-A to find single cells and to exclude doublets. Live cells were isolated by negative staining for fixed viability dye (Fix Via). After live cell gating, myeloid cells were isolated by CD11b positivity and assessed for the percentage of $tdTom^+$ cells. Following gating for granulocytes ($CD11b^+$, $Ly6G^+$), MoDC ($CD11b^+$, $CD11c^+$, $Ly6G^-$), and Mo/Mac ($CD11b^+$, $Ly6G^-$, $CD11c^-$) were identified. Mo/Mac were further subdivided into $Ly6C^{hi}$, $Ly6C^{int}$ and $Ly6C^-$ cells. The fraction of $tdTom^+$ cells for each cell type was determined. B. Representative flow cytometry dot plots of splenic myeloid cells ($CD11b^+$, $tdTom^+$) in WT (CD45.1) parabiont and non-parabiotic (tdTom single) and parabiotic mice. C. Quantification of chimerism in the spleen. The percentile of $tdTom^+$ myeloid cells, MoDC, Mo/Mac that are ($Ly6C^{hi}$), ($Ly6C^{int}$), and Mac ($Ly6C^-$) is shown (n= 3-7 biological replicates). Flow data are represented as mean ± SEM. Statistical analysis was performed in GraphPad Prism (v8) using 1-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test. A p value < 0.05 (*) was considered significant. $p < 0.01$ (**), and $p < 0.0001$ (****).

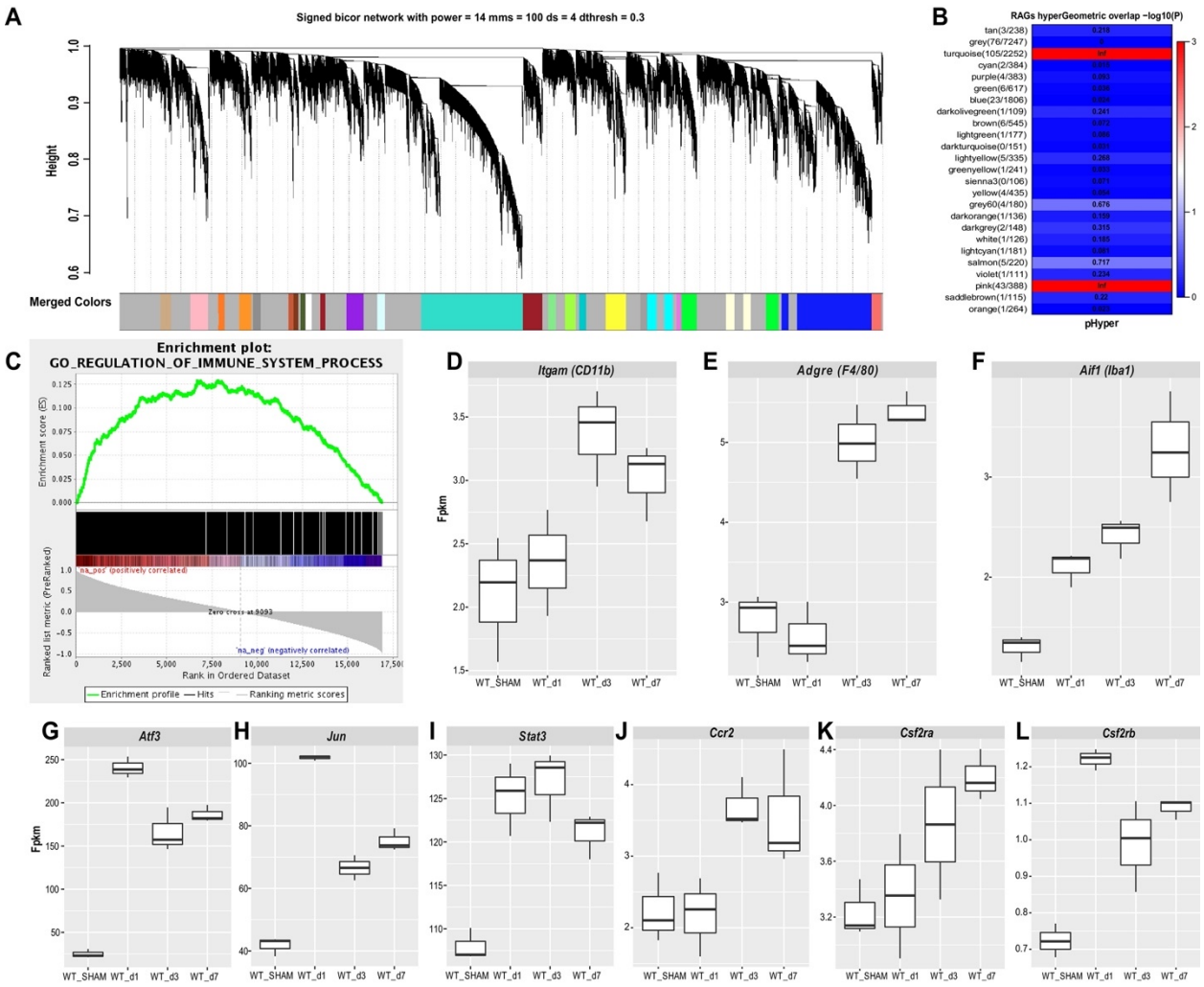


Figure 2-8 Stable up-regulation of immune function associated gene co-expression networks in axotomized DRGs.

Analysis of bulk RNAseq data from naïve and axotomized DRGs. DRGs were harvested from sham operated mice, d1, d3, and d7 post-SNC. A. Network analysis of whole transcriptomes from naïve and axotomized DRGs. Gene dendrogram identifies several co-expression modules. B. Gene ontology (GO) analysis revealed significant and stable upregulation of the pink and turquoise modules. C. Gene set enrichment analysis. Shown is the enrichment plot for GO terms of the turquoise module with overrepresentation of immune system processes. D-F. Quantification of SNC-induced upregulation of commonly used macrophage markers *Itgam* (CD11b), *Adgre1* (F4/80), and *Aif1* (Iba1) in axotomized DRGs. G-I. Quantification of SNC-induced upregulation of the RAGs *Atf3, Jun,* and *Stat3* in DRGs. J-L. Quantification of SNC-induced upregulation of the chemokine receptor *Ccr2*, and the GM-CSF receptor subunits *Csf2ra* and *Csf2rb* in DRGs. Gene expression levels are shown as Fpkm (fragments per kilobase of transcript per million mapped reads).

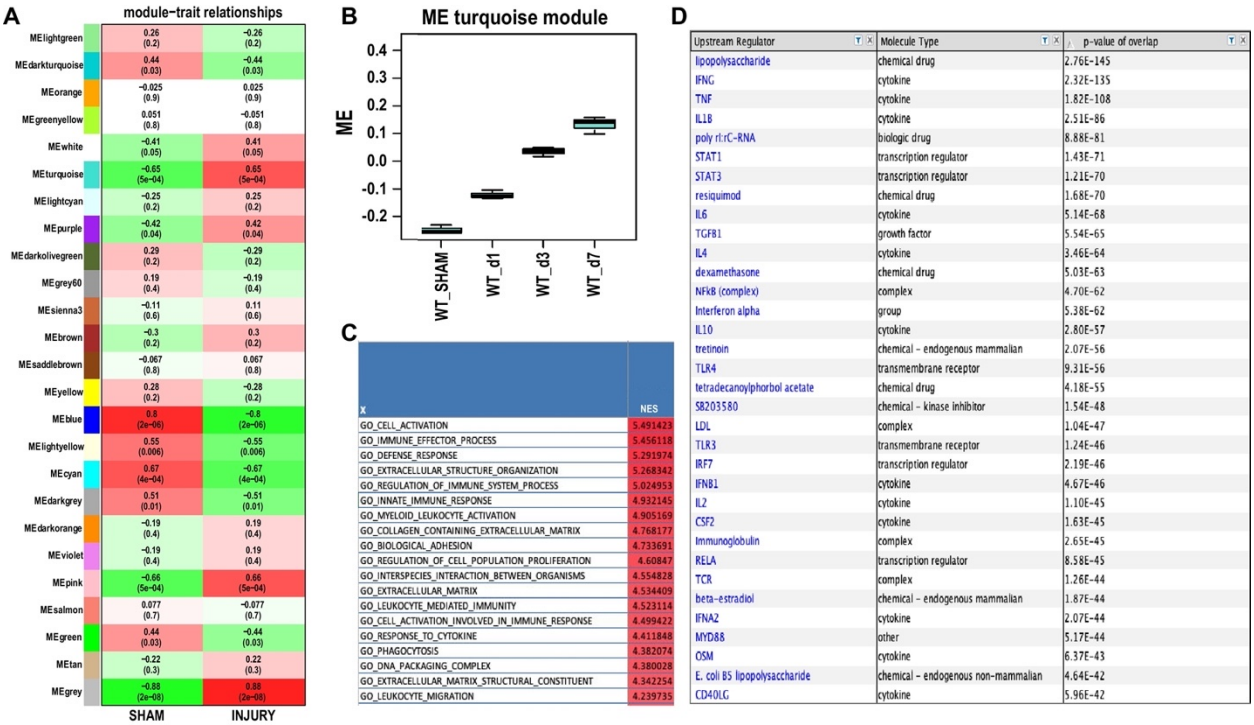


Figure 2-9 Module – The trait relationships in axotomized DRGs and analysis of the immune turquoise module.

A. Heatmap of the correlation of WGCNA modules with indicated experimental conditions (sham operated mice and following nerve injury). The values in each cell are Pearson's correlation co-efficient and Student asymptotic p-values (parenthesis). The green to red color represents strong negative to positive-correlation of experimental condition and Module Eigen (ME) gene expression. B. Induction of turquoise gene co-expression module in axotomized DRGs of wildtype (WT) mice following SNC at d1, d3 and d7 (ME gene expression). C. GO terms enriched in the turquoise module. Normalized enrichment scores (NES) are shown. D. Ingenuity pathway analysis (IPA) predicted upstream regulators of injury-induced immune pathways in axotomized DRGs. GSEA's core enrichment genes in immune pathways obtained from differentially expressed genes comparing d1 to sham DRGs; FDR<0.1 were used as input for IPA.

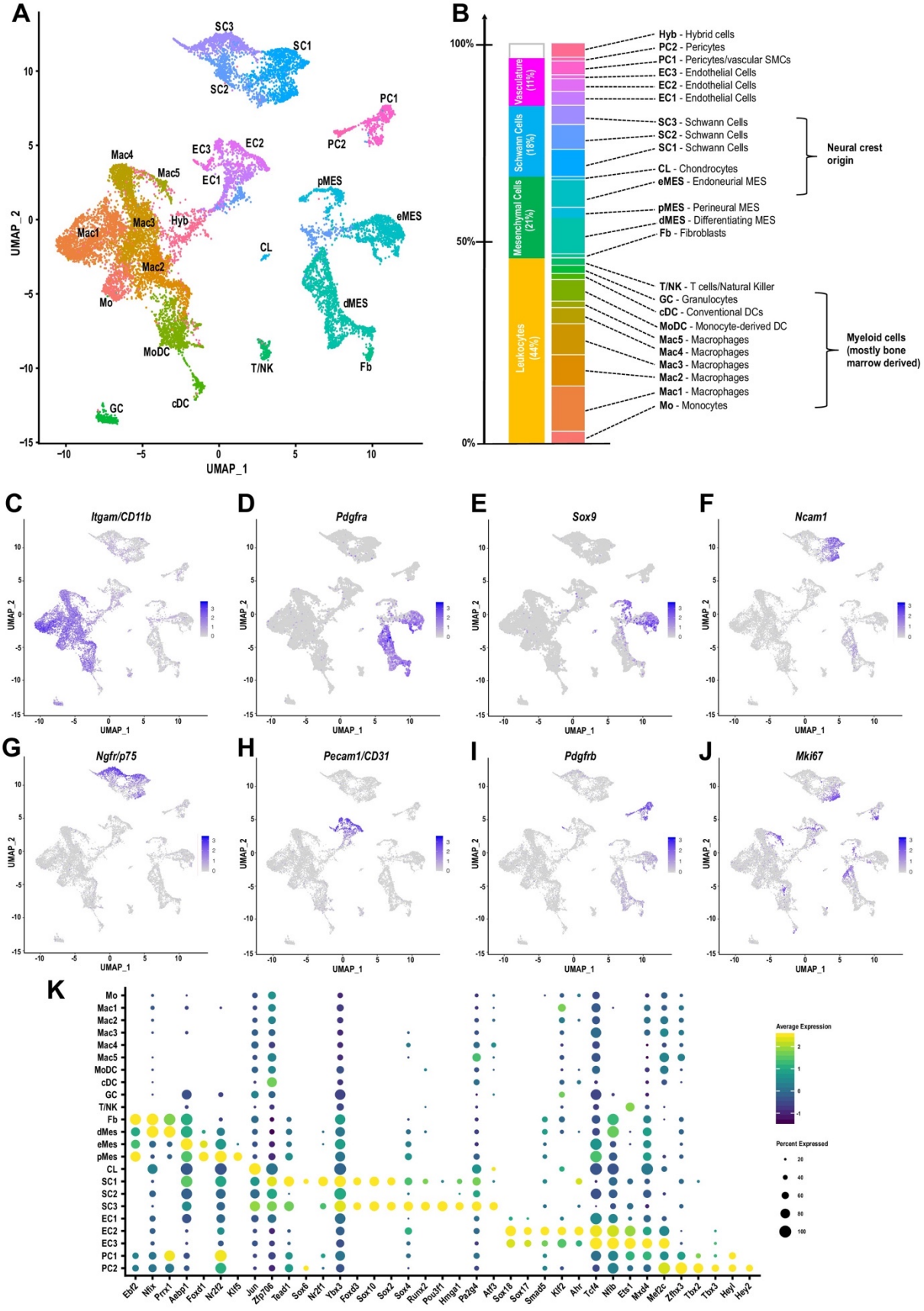

A
SC3
SC1
SC2
PC1
PC2
EC3
EC2
EC1
Mac4
Mac5
Mac3
Hyb
Mac1
Mac2
Mo
CL
pMES
eMES
dMES
MoDC
T/NK
Fb
cDC
GC
UMAP_2
UMAP_1
B
100%
50%
0%
Vasculature (11%)
Schwann Cells (18%)
Mesenchymal Cells (21%)
Leukocytes (44%)
Hyb - Hybrid cells
PC2 - Pericytes
PC1 - Pericytes/vascular SMCs
EC3 - Endothelial Cells
EC2 - Endothelial Cells
EC1 - Endothelial Cells
SC3 - Schwann Cells
SC2 - Schwann Cells
SC1 - Schwann Cells
CL - Chondrocytes
eMES - Endoneurial MES
pMES - Perineural MES
dMES - Differentiating MES
Fb - Fibroblasts
T/NK - T cells/Natural Killer
GC - Granulocytes
cDC - Conventional DCs
MoDC - Monocyte-derived DC
Mac5 - Macrophages
Mac4 - Macrophages
Mac3 - Macrophages
Mac2 - Macrophages
Mac1 - Macrophages
Mo - Monocytes
Neural crest origin
Myeloid cells (mostly bone marrow derived)
C
Itgam/CD11b
D
Pdgfra
E
Sox9
F
Ncam1
G
Ngfr/p75
H
Pecam1/CD31
I
Pdgfrb
J
Mki67
K
Mo
Mac1
Mac2
Mac3
Mac4
Mac5
MoDC
cDC
GC
T/NK
Fb
dMes
eMes
pMes
CL
SC1
SC2
SC3
EC1
EC2
EC3
PC1
PC2
Ebf2 Nfix Prrx1 Aebp1 Foxd1 Nr2f2 Klf5 Jun Zfp706 Tead1 Sox6 Nr2f1 Ybx3 Foxd3 Sox10 Sox2 Sox4 Rumx2 Pou3f1 Hmga1 Pa2g4 Atf3 Sox18 Sox17 Smad5 Klf2 Ahr Tcf4 Nfib Etv1 Mxd4 Mef2c Zfhx3 Tbx2 Tbx3 Heyl Hey2
Average Expression
Percent Expressed

Figure 2-10 The cellular landscape of injured peripheral nerve.
A. Singe-cell transcriptome of injured mouse sciatic nerve at d3 post-SNC, n= 3 biological replicates. Unsupervised Seurat-based clustering identifies 24 cell clusters. Cell type identity for each cluster was determined by expression analysis of established markers. B. List of all cell types identified by scRNA-sequencing. The size (percentile) of cell clusters and lineage relationships are shown. Abbreviations for cell cluster identities are indicated and used throughout the manuscript. C-J. Feature plots of established cell markers used for identification of major cell types in the injured nerve. Shown are UMAP plots with markers for myeloid cells (*Itgam/*CD11b), fibroblast-like/mesenchymal cells (*Pdgfra, Sox9*), repair Schwann cells (*Ncam1, Ngfr/*p75), endothelial cells (*Pecam/*CD31), pericytes /smooth muscle vascular cells (*Pdgfrb*), and mitotically active cells (*Mki67/*Ki67). Expression levels are color coded and calibrated to average gene expression. K. Dotplot shows cell type-specific expression of the most abundant transcription regulators (TRs) in Fb, dMES, eMES, pMES, CL, SC1-3, EC1-3, PC1, and PC2 clusters identified by scRNA-seq of 3d injured sciatic nerve. Dotplot analysis shows the average gene expression (color coded) and percent of cells (dot size) that express the listed TRs in each cluster.

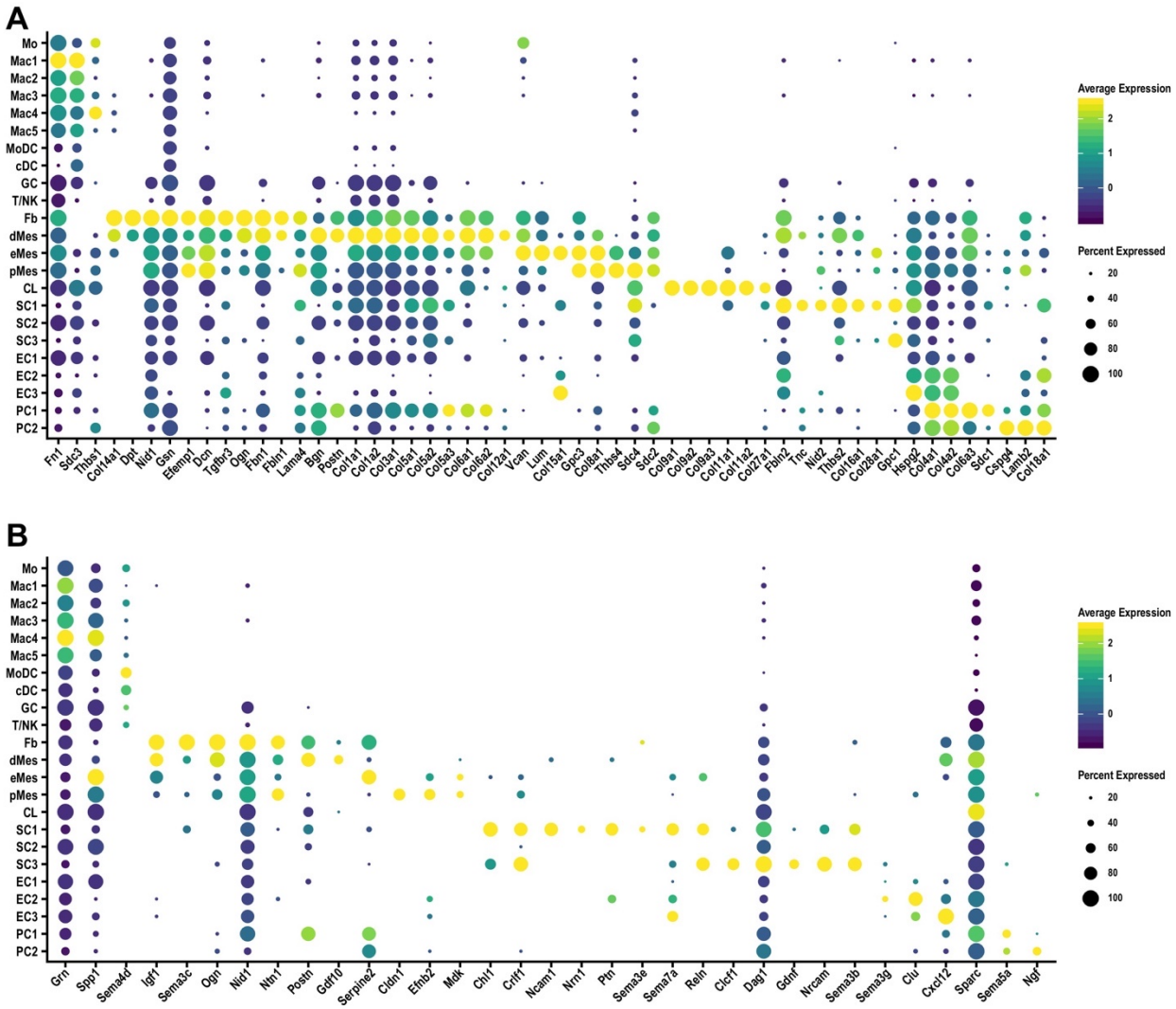


Figure 2-11 Cell cluster specific expression of ECM components and molecules that regulate axon growth in the injured sciatic nerve.

A. Dotplot analysis of extracellular matrix molecules prominently expressed in the d3 post-SNC nerve. B. Dotplot analysis of gene products implicated in axon growth, guidance, and regeneration in the d3 post-SNC nerve. Expression levels, normalized to average gene expression (color coded) are shown. For each cell cluster the percentile of cells expressing a specific gene is indicated by the dot size.

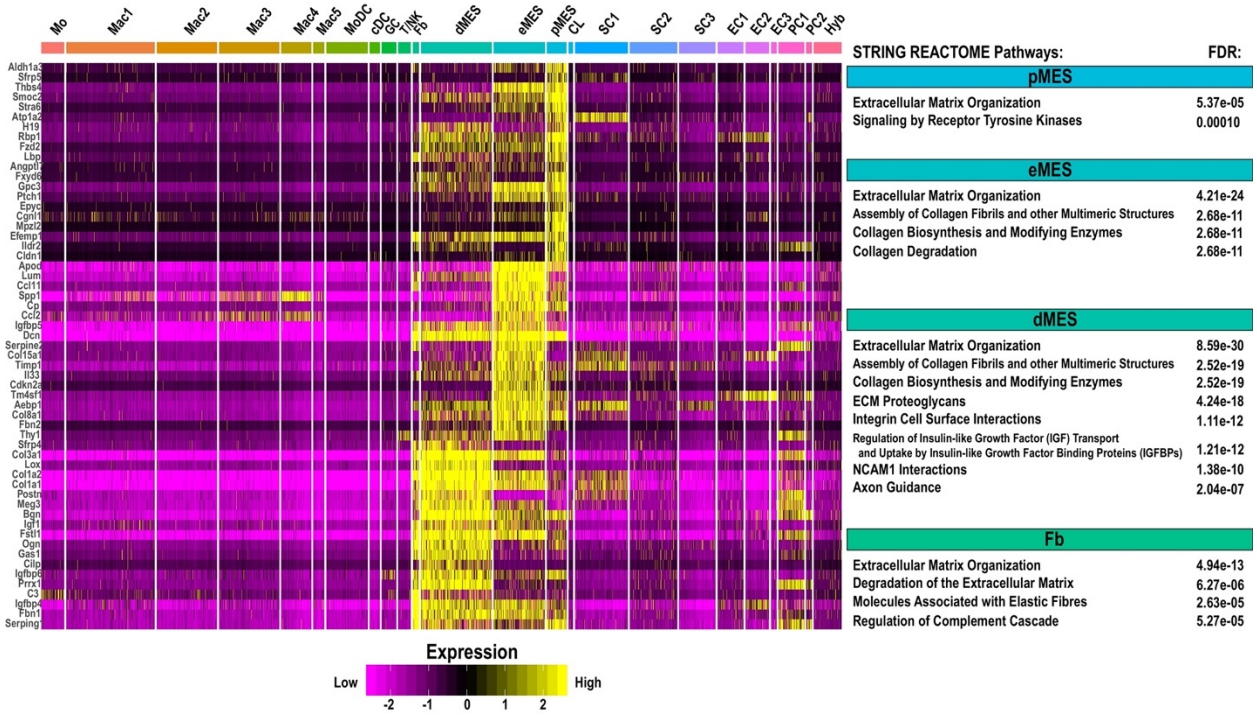


Figure 2-12 Analysis of single cell gene expression in mesenchymal cell and fibroblast clusters in the injured sciatic nerve.

Heatmap of top genes enriched in perineural mesenchymal cells (pMES), endoneurial mesenchymal cells (eMES), differentiating mesenchymal cells (dMES), and epineural fibroblasts (Fb) in the d3 post-SNC nerve. Expression levels are calibrated to median gene expression. STRING REACTOME Pathways are listed. FDRs (false discovery rates) are shown.

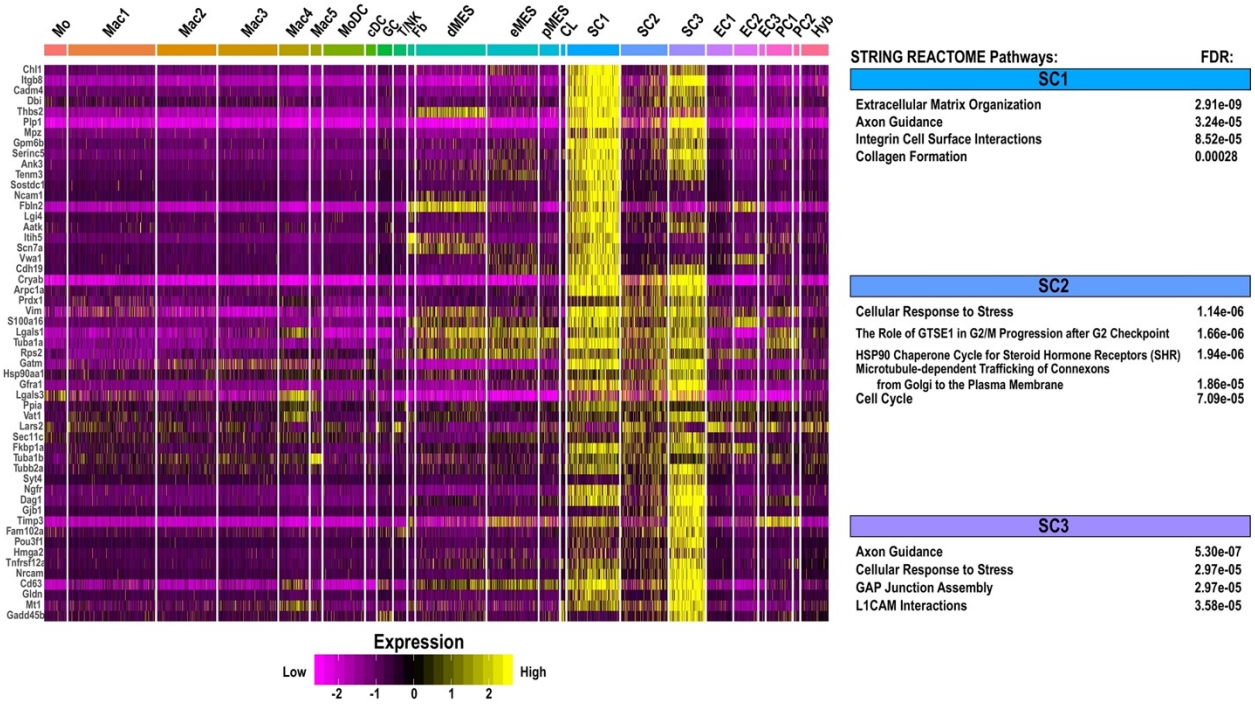


Figure 2-13 Single cell gene expression in Schwann cell clusters in injured sciatic nerve.
Heatmap of top genes enriched in repair Schwann cell clusters (SC1, SC2, and SC3) in the d3 post-SNC nerve. Expression levels are calibrated to median gene expression. STRING REACTOME Pathways are listed. FDRs (false discovery rates) are shown.

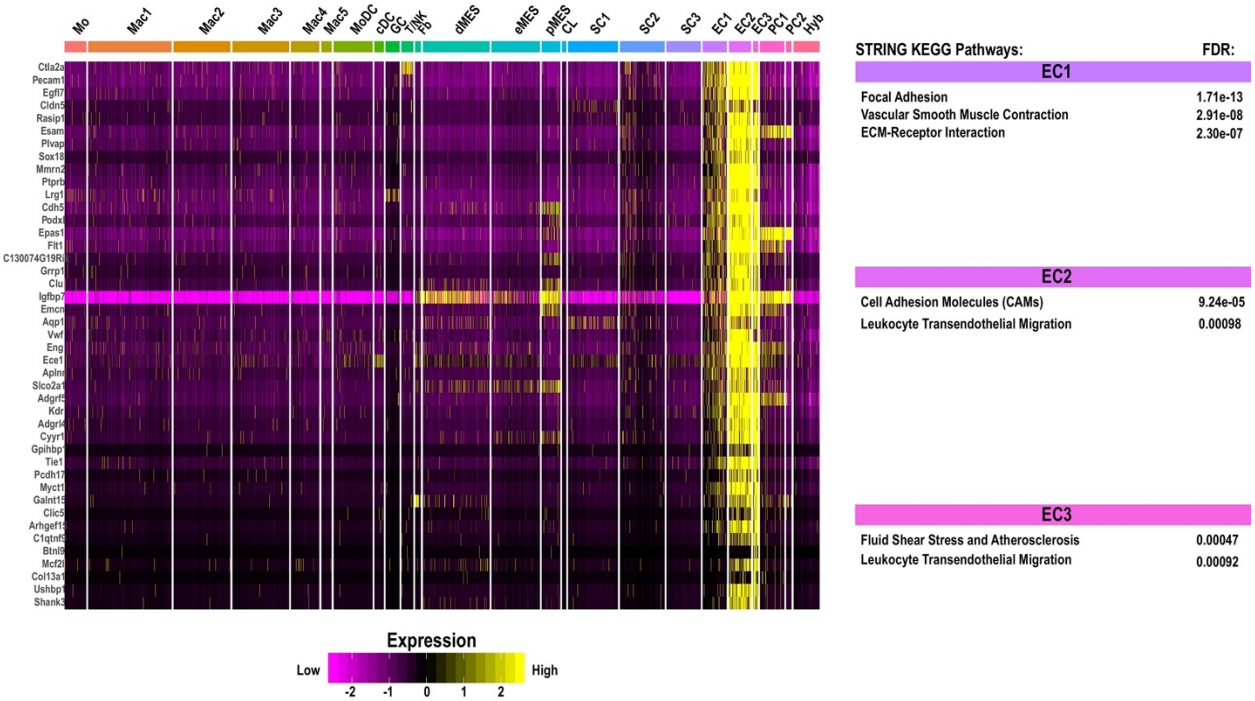


Figure 2-14 Single cell gene expression in endothelial cell clusters in injured sciatic nerve.
Heatmap of top genes enriched in endothelial cell clusters (EC1, EC2, and EC3) in the d3 post-SNC nerve. Expression levels are calibrated to median gene expression. STRING KEGG Pathway analysis and FDRs (false discovery rates) are shown.

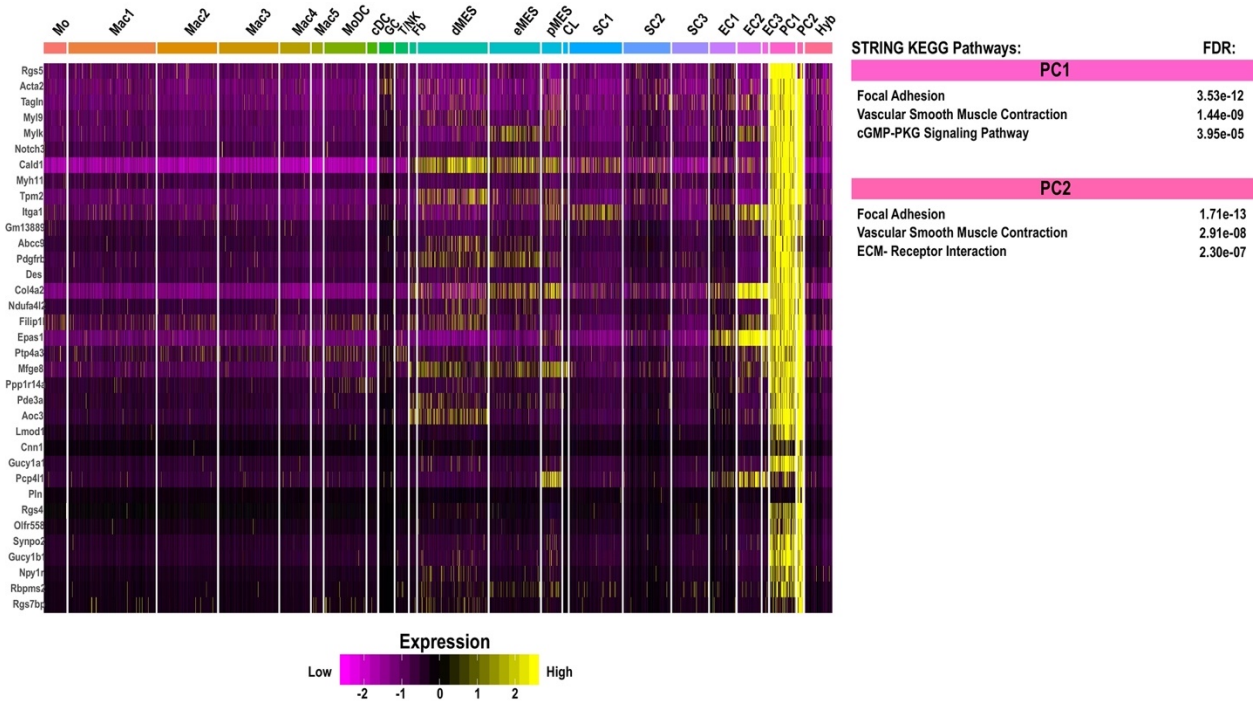


Figure 2-15 Single cell gene expression in pericyte cell clusters in injured sciatic nerve.
Heatmap of top genes enriched in pericyte clusters (PC1 and PC2) in the d3 post-SNC nerve. Expression levels are calibrated to median gene expression. STRING REACTOME Pathways are listed. FDRs (false discovery rates) are shown.

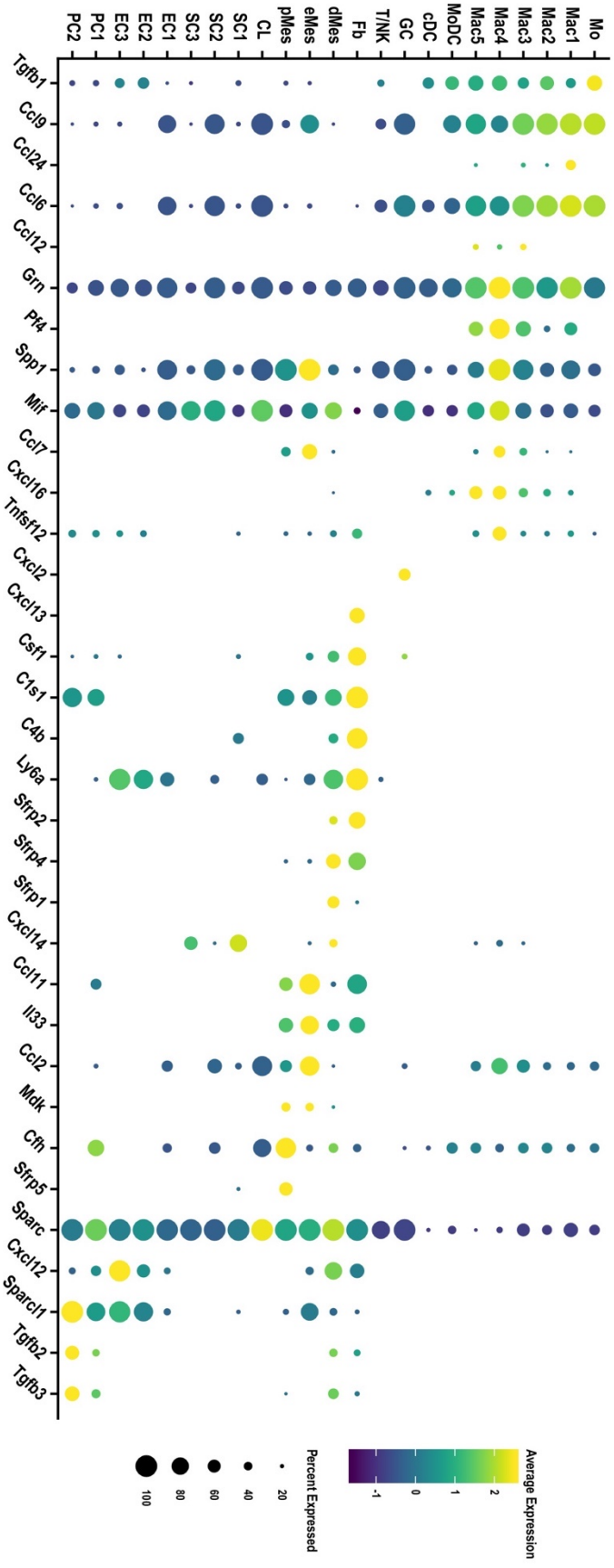

PC2
PC1
EC3
EC2
EC1
SC3
SC2
SC1
CL
pMes
eMes
dMes
Fb
T/NK
GC
cDC
MoDC
Mac5
Mac4
Mac3
Mac2
Mac1
Mo
Tgfb1
Ccl9
Ccl24
Ccl6
Ccl12
Grn
Pf4
Spp1
Mif
Ccl7
Cxcl16
Tnfsf12
Cxcl2
Cxcl13
Csf1
C1s1
C4b
Ly6a
Sfrp2
Sfrp4
Sfrp1
Cxcl14
Ccl11
Il33
Ccl2
Mdk
Cfh
Sfrp5
Sparc
Cxcl12
Sparcl1
Tgfb2
Tgfb3
100
80
60
40
20
Percent Expressed
-1
0
1
2
Average Expression

Figure 2-16 Single cell gene expression of immune modulatory molecules in the injured peripheral nervous system tissue.
Dotplot analysis of gene products with immune modulatory function in the d3 post-SNC nerve. Expression levels, normalized to average gene expression (color coded). For each cell cluster the percentile of cells that express the listed gene (dot size) is shown.

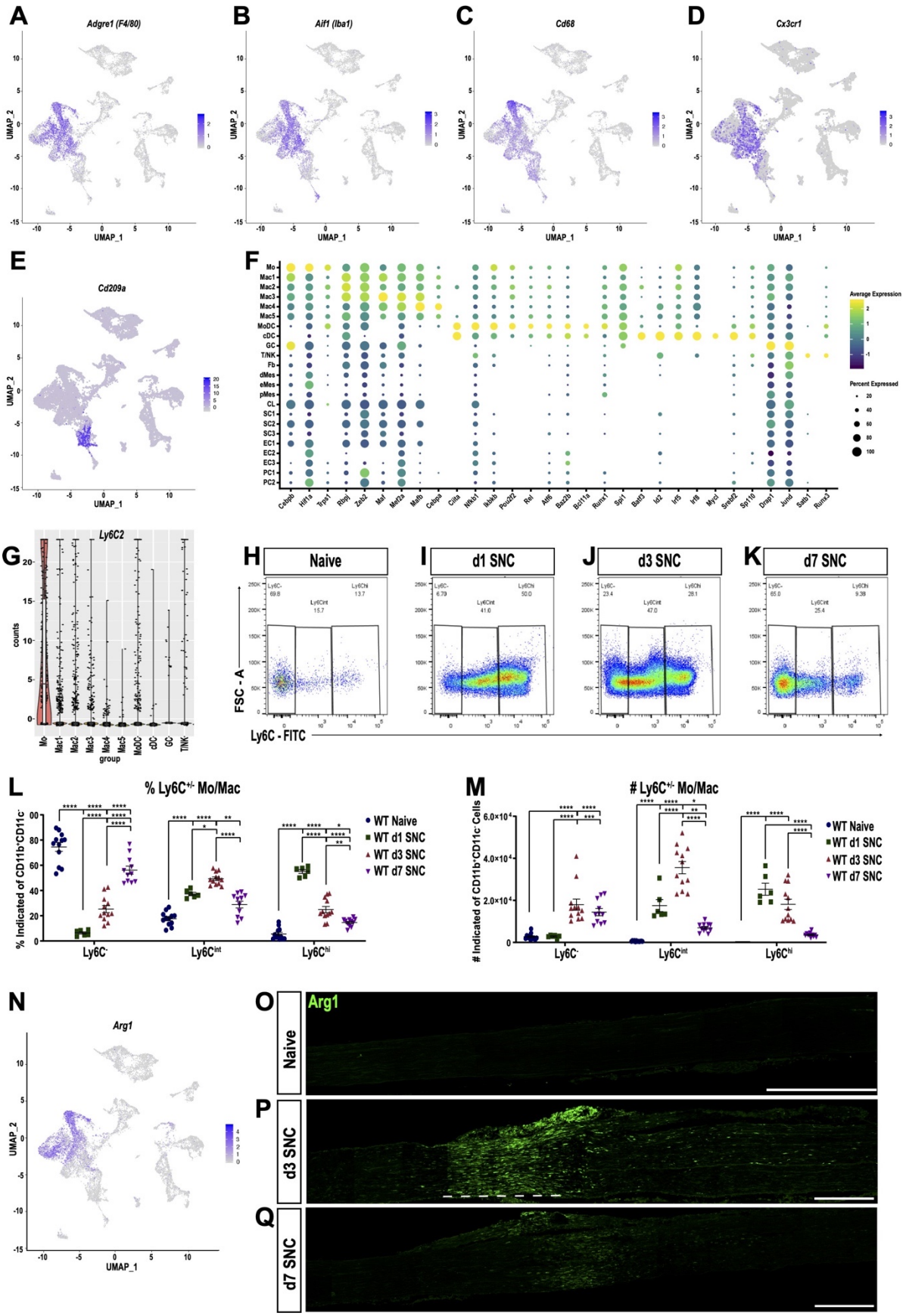

A
Adgre1 (F4/80)
B
Aif1 (Iba1)
C
Cd68
D
Cx3cr1
E
Cd209a
F
Mo
Mac1
Mac2
Mac3
Mac4
Mac5
MoDC
cDC
GC
T/NK
Fb
dMes
eMes
pMes
CL
SC1
SC2
SC3
EC1
EC2
EC3
PC1
PC2
Cebpb
Hif1a
Trps1
Rbpj
Zeb2
Maf
Mef2a
Mafb
Cebpa
Ciita
Nfkb1
Ikbkb
Pou2f2
Rel
Atf6
Baz2b
Bcl11a
Runx1
Spi1
Batf3
Id2
Irf5
Irf8
Myc
Srebf2
Syk1b
Dnajb1
Jund
Satb1
Runx3
Average Expression
Percent Expressed
G
Ly6C2
counts
group
H
Naive
I
d1 SNC
J
d3 SNC
K
d7 SNC
FSC - A
Ly6C - FITC
L
% Ly6C+/- Mo/Mac
% Indicated of CD11b+CD11c-
WT Naive
WT d1 SNC
WT d3 SNC
WT d7 SNC
Ly6C-
Ly6Cint
Ly6Chi
M
Ly6C+/- Mo/Mac
Indicated of CD11b+CD11c- Cells
N
Arg1
O
Naive
P
d3 SNC
Q
d7 SNC

Figure 2-17 Macrophage subpopulation in the injured nerve are functionally distinct and localize to specific sites.

A–E. Feature plots of *Adgre1* (F4/80), *Aif1* (Iba1), *Cd68* (Scavenger receptor class D*), Cx3cr1* (Fractalkine receptor), and *CD209a* (DC-SIGN) expression in the d3 post-SNC nerve. F. scRNAseq dot plot analysis of transcription regulators (TRs) enriched in leukocytes. Average gene expression and percentage of cells expressing the TR are shown. G. Violin plot of *Ly6c2* (Ly6C) expression in immune cells of the d3 post-SNC nerve. H-K. Flow cytometric analysis of sciatic nerve Mo/Mac ($CD45^+$, $CD11b^+$, $Ly6G^-$, $CD11c^-$) in naïve mice, d1, d3, and d7 post-SNC mice. Mo/Mac maturation was assessed by Ly6C surface staining. L, M. Quantification of Ly6C distribution on Mo/Mac in naïve nerves and at different post-SNC time points (n= 11 biological replicates per time point); (L) Percentile of $Ly6C^-$, $Ly6C^{int}$, and $Ly6C^{hi}$ Mo/Mac and (M) number of $Ly6C^-$, $Ly6C^{int}$, and $Ly6C^{hi}$ Mo/Mac. Flow data are represented as mean ± SEM. Statistical analysis was performed in GraphPad Prism (v7) using 1-way or 2-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test. A p value < 0.05 (*) was considered significant. $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). N. Feature plot showing *Arg1* (Arginase-1) expression in the 3d post-SNC nerve. O-Q. Longitudinal sciatic nerve sections of *Arg1-YFP* reporter mice, from naïve mice (O), d3 (P) and d7 (Q) post-SNC mice. YFP^+ cells (green) are localized to the injury site (underlined with a dashed line), proximal is to the left. Representative example of n= 4 biological replicates. Scale bar, 200 μm.

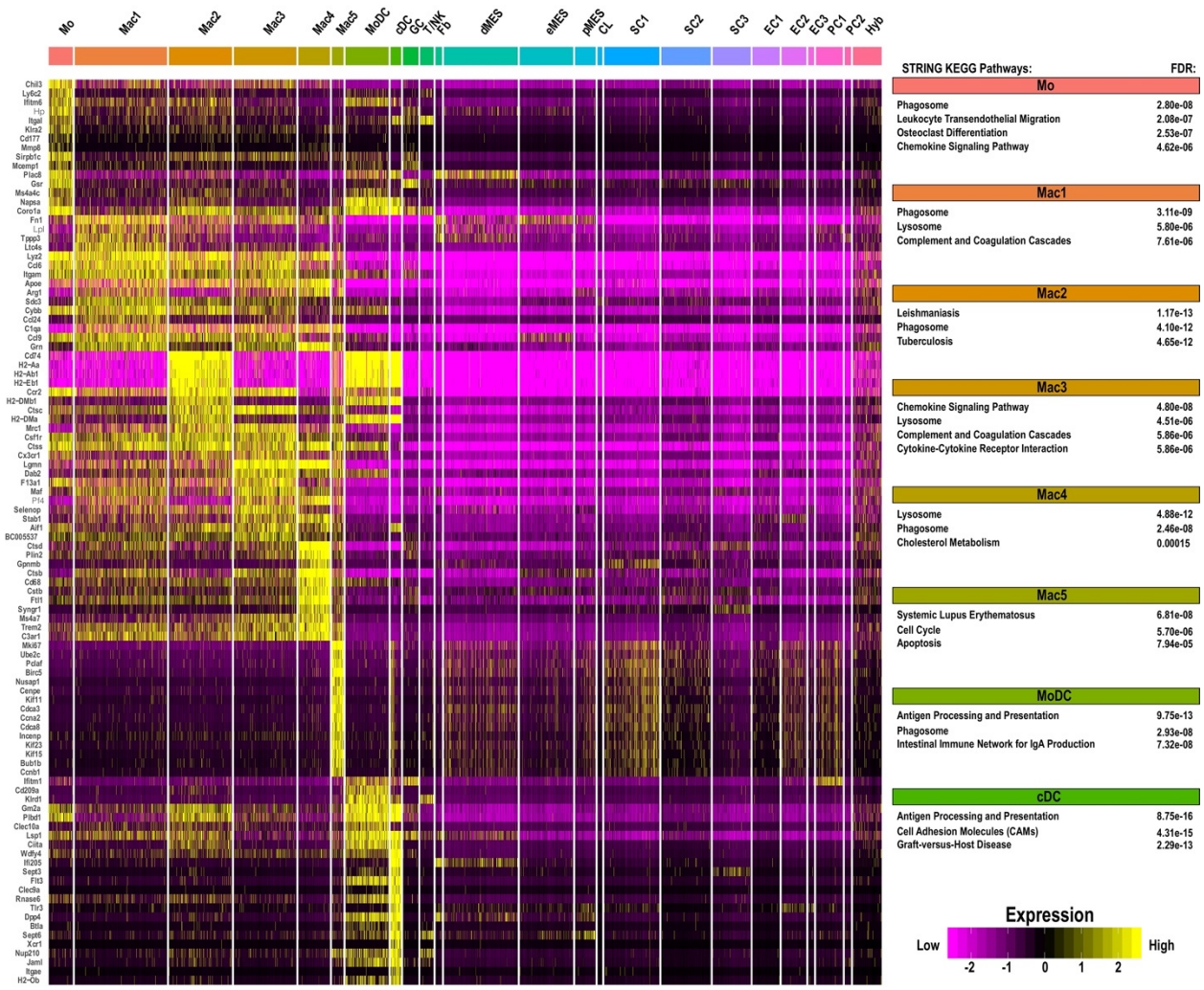


Figure 2-18 Single cell gene expression in myeloid cell clusters of injured sciatic nerve.
Heatmap of top genes enriched in monocytes (Mo), macrophage clusters 1-5 (Mac1-5), MoDC, and cDC in the d3 post-SNC nerve. Expression levels are calibrated to median gene expression. STRING KEGG Pathway analysis and FDRs (false discovery rates) are shown.

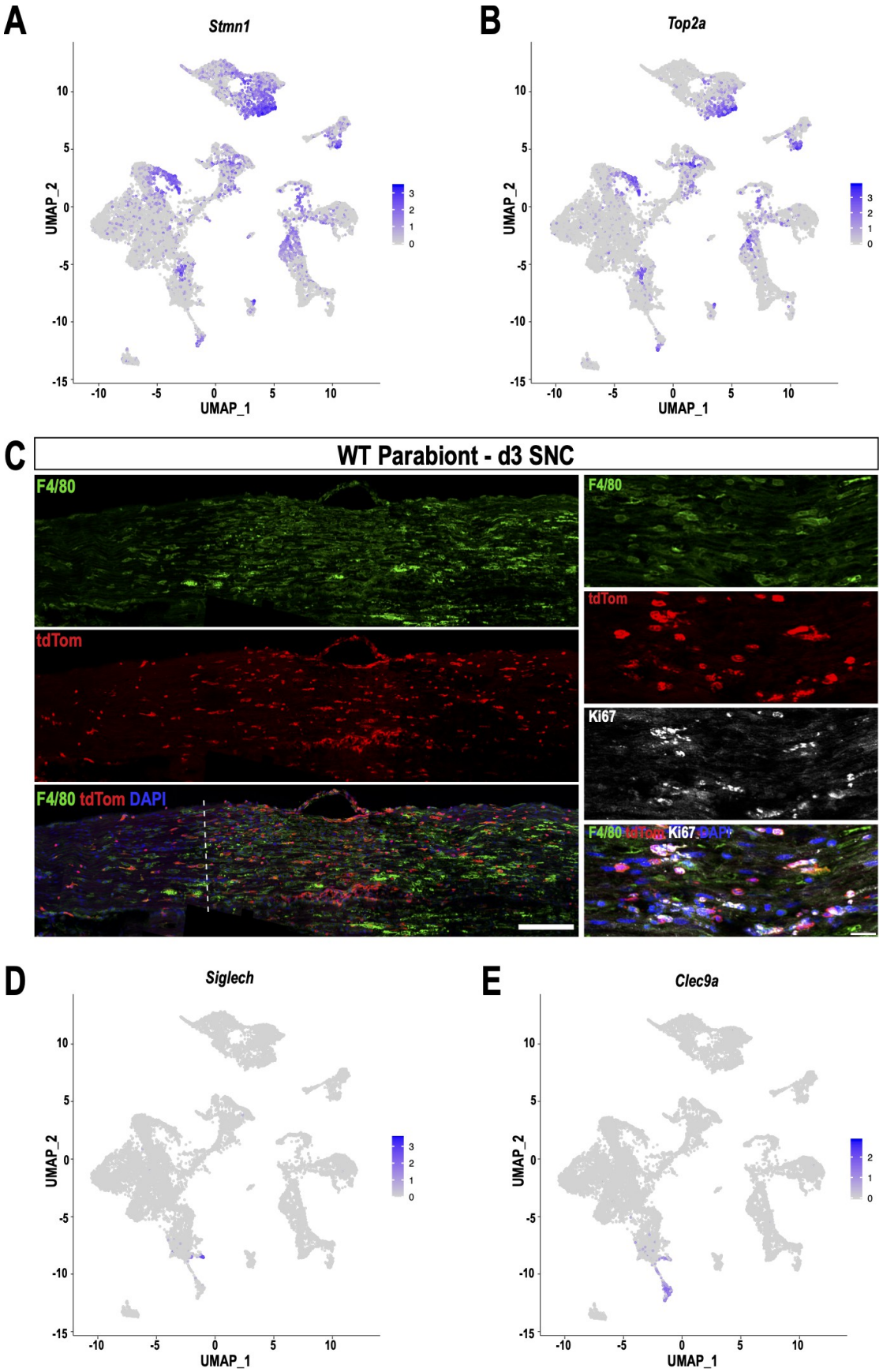

A
Stmn1
B
Top2a
UMAP_1
UMAP_2
C
WT Parabiont - d3 SNC
F4/80
tdTom
F4/80 tdTom DAPI
Ki67
D
Siglech
E
Clec9a

Figure 2-19 Identification of blood-borne, stem-like myeloid cells in the sciatic nerve following crush injury.

A. Feature plots of *Stmn1* (Stathmin-1) and B. *Top2a* (DNA topoisomerase II alpha) highlight proliferating cells, including Mac5 cells and a small group of myeloid cells located between clusters Mac2 and MoDC. C. Longitudinal sciatic nerve section of 3d WT parabiont, the dotted line marks the injury site, proximal is to the left. $TdTom^+$ cells are blood-borne immune cells originating from the tdTom parabiont. Macrophages are stained with anti-F4/80 (green), scale bar, 100 µm. Higher magnification images of the injury site. Some $tdTom^+F4/80^+$ macrophages are stained with anti-Ki67 (white). Scale bar, 50 µm. D. Feature plots of *Siglech*, a marker for pDC and E. *Clec9a*, a marker for cDC, in the 3d injured nerve.

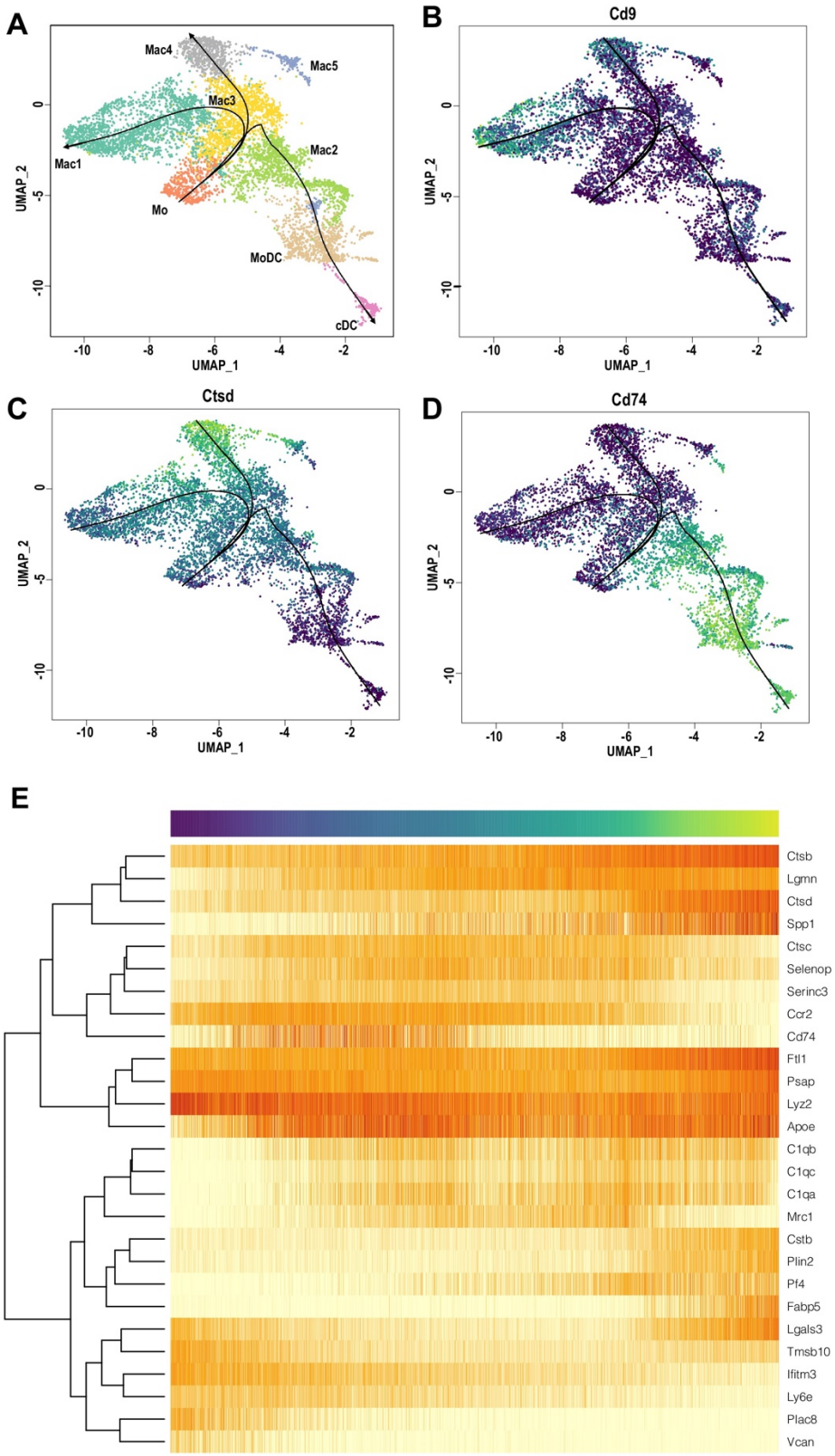

A
Mac4
Mac5
Mac3
Mac2
Mac1
Mo
MoDC
cDC
UMAP_1
UMAP_2
B
Cd9
C
Ctsd
D
Cd74
E
Ctsb
Lgmn
Ctsd
Spp1
Ctsc
Selenop
Serinc3
Ccr2
Cd74
Ftl1
Psap
Lyz2
Apoe
C1qb
C1qc
C1qa
Mrc1
Cstb
Plin2
Pf4
Fabp5
Lgals3
Tmsb10
Ifitm3
Ly6e
Plac8
Vcan

Figure 2-20 Infiltrating monocyte to macrophage differentiation pathway based on single cell expression modeling.

Pseudo time trajectory analysis of cell differentiation in the d3 post-SNC nerve A. Predicted cell differentiation in the myeloid cell compartment. Slingshot analysis was used to predict how Mo (monocytes) differentiate into specific Mac subpopulations and MoDC. Feature plots for *Cd9* (Tetraspanin), *Ctsd,* and *Cd74* are shown as representative examples. B. Pseudo time gene expression changes of top genes used for trajectory analysis.

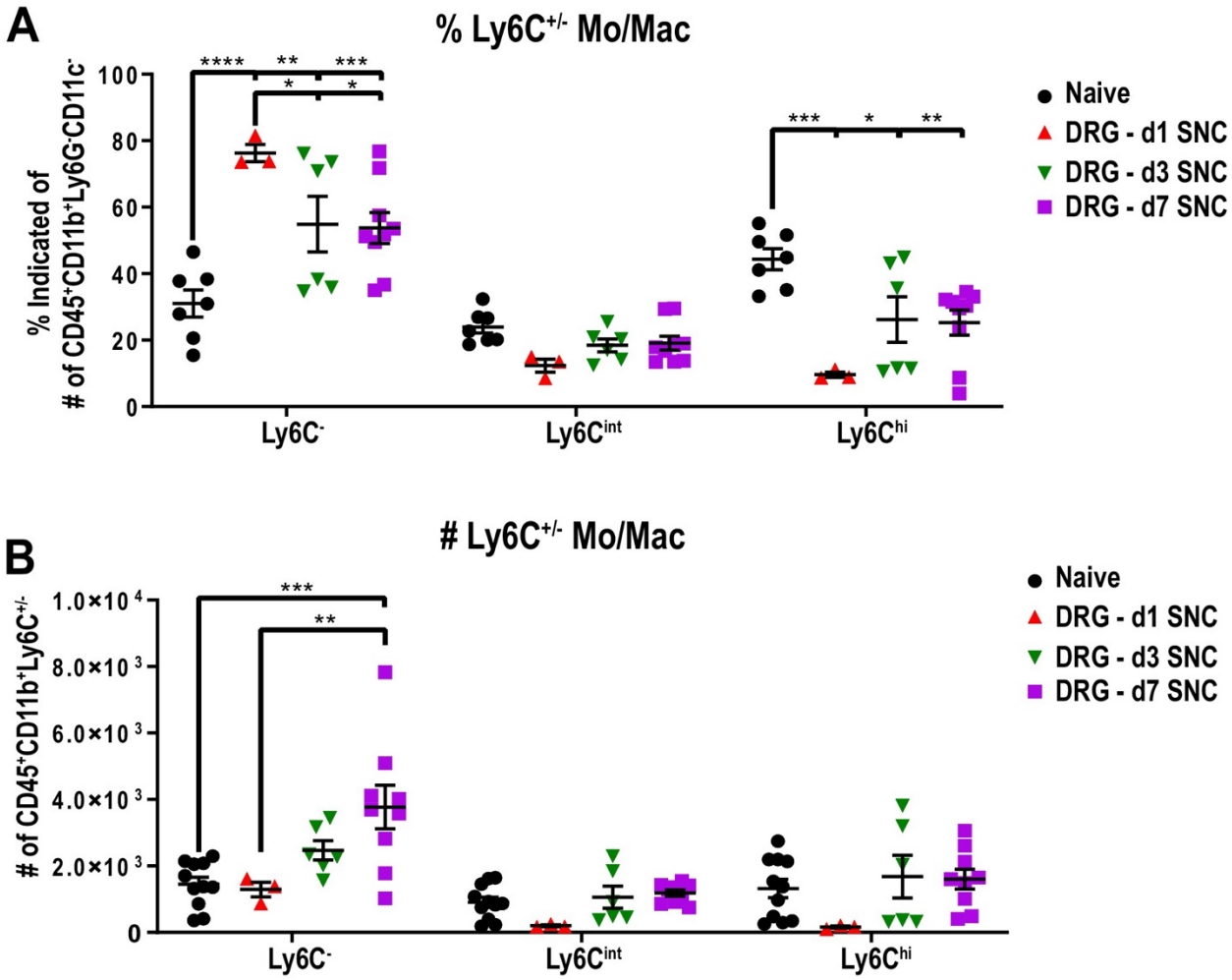


Figure 2-21 Monocye and macrophage maturation in axotomized DRGs, assessed by the surface expression of the marker Ly6C.

Flow cytometric analysis of Mo/Mac in naive and axotomized DRGs. The surface distribution of Ly6C on Mo/Mac ($CD45^+$, $CD11b^+$, $Ly6G^-$, $CD11c^-$) is shown. A. The percentile of Ly6C cells and B. the number of Ly6C cells in naive DRGs, d1, d3, and d7 post-SNC. Mo/Mac were binned into $Ly6C^{hi}$, $Ly6C^{int}$, and $Ly6C^-$ cells. Flow data are represented as mean ± SEM. Each data point represents L3-L5 DRGs pooled from 3-4 animals (18-24 DRGs), biological replicates, n= 3-9. Statistical analysis was performed in GraphPad Prism (v7) using 1-way or 2-way ANOVA with correction for multiple comparisons with Tukey's post-hoc test. A p value < 0.05 (*) was considered significant. $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

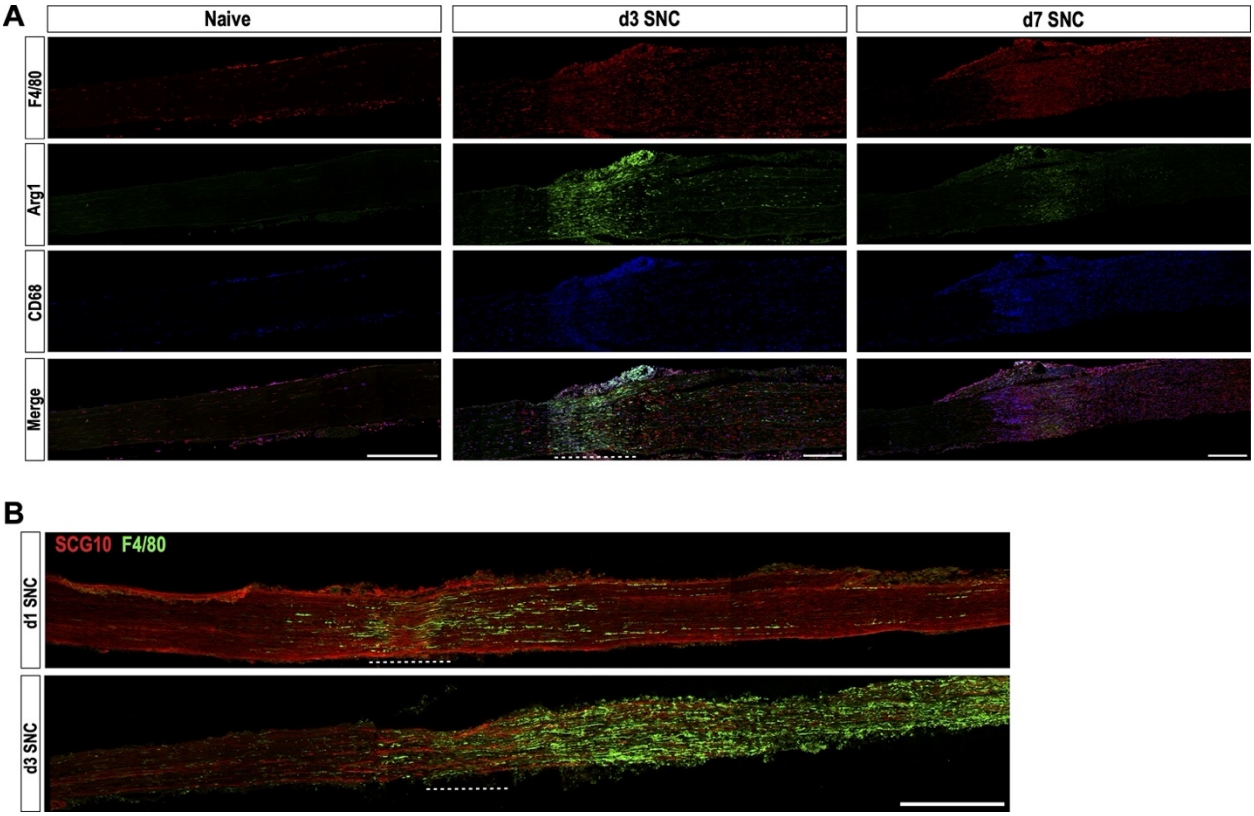


Figure 2-22 Evidence for distinct immune compartments in the injured sciatic nerve.
A. Longitudinal sections of naïve and injured (3d and 7d) sciatic nerves from *Arg1-YFP* reporter mice. The injury site is underlined with a dotted line. Proximal is to the left. Distribution of F4/80^{+}, *Arg1-YFP*$^{+}$, and CD68^{+} macrophages is shown. Scale bar, 200 µm. B. Longitudinal sciatic nerve sections of adult mice at d1 and d3 post-SNC. The crush site is marked with a dotted line, proximal is to the left. Nerves were stained with anti-SCG10 to visualize regenerating sensory axons and anti-F4/80 to stain for a subpopulation of macrophages. At d1, F4/80^{+} macrophages are found near the crush site. At d3, F4/80^{+} macrophages are abundantly present throughout the distal nerve stump, adjacent to SCG10^{+} regenerating sensory axons. Scale bar, 500 µm.

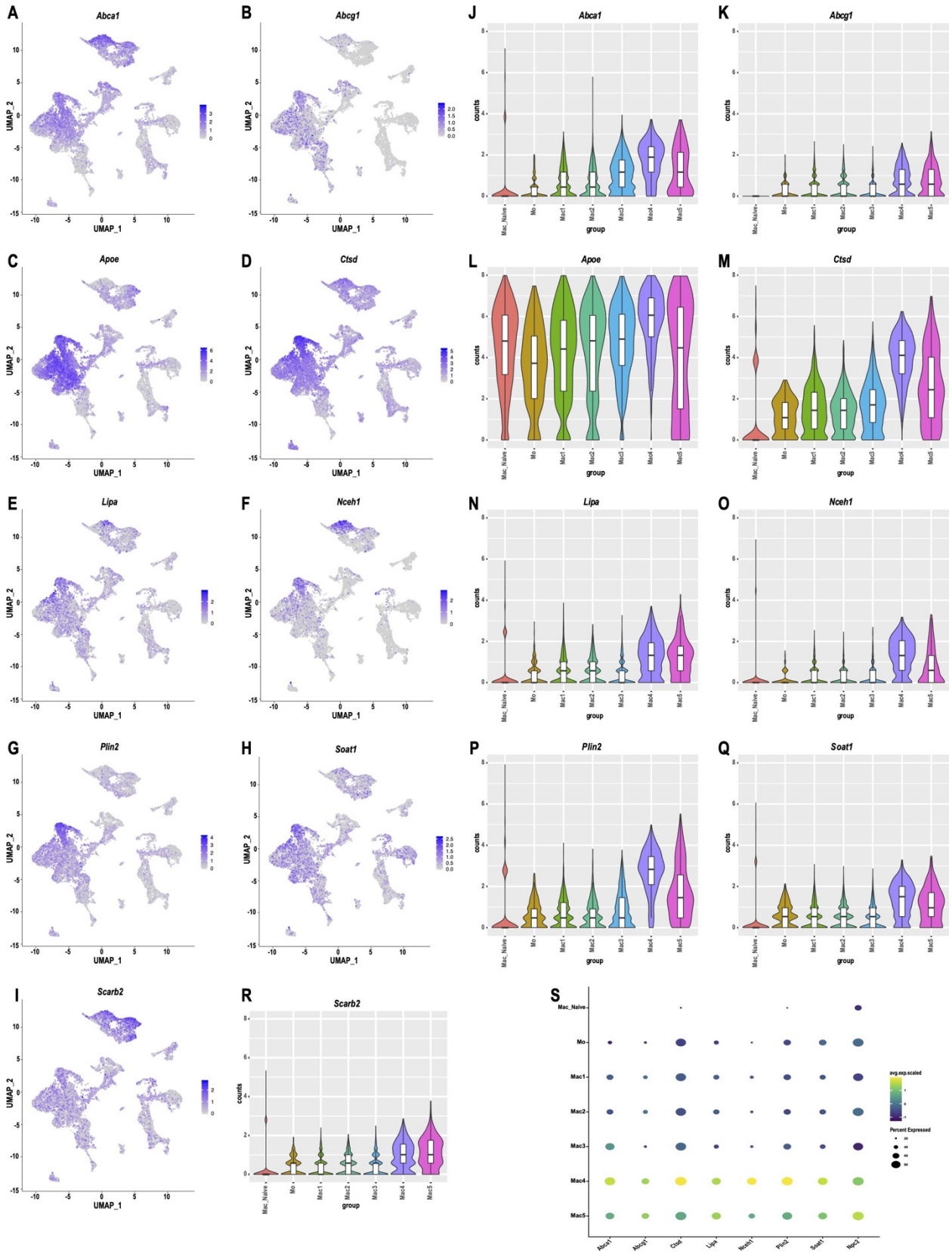

A
Abca1
B
Abcg1
C
Apoe
D
Ctsd
E
Lipa
F
Nceh1
G
Plin2
H
Soat1
I
Scarb2
J
Abca1
K
Abcg1
L
Apoe
M
Ctsd
N
Lipa
O
Nceh1
P
Plin2
Q
Soat1
R
Scarb2
S
UMAP_1
UMAP_2
counts
group
Mac_Naive
Mo
Mac1
Mac2
Mac3
Mac4
Mac5
avg.exp.scaled
Percent Expressed

Figure 2-23 Expression of gene products that regulate cholesterol transport and metabolism in macrophages of injured and naïve nerve.

Feature plots of gene products implicated in cholesterol transport and lowering of cellular cholesterol levels. A. *Abca1/CERP* (ATP-binding cassette subfamily A1). B. *Abcg1* (ATP-binding cassette subfamily G1). C. *Apoe* (Apolipoprotein E). D. *Ctsd* (Cathepsin-D). E. *Lipa* (Lipase A). F. *Nceh1* (Neutral cholesterol ester hydrolase 1). G. *Plin2* (Perilipin 2). H. *Soat1* (Sterol O-acyltransferase 1), I. *Scarb2* (Scavenger receptor class B 2). Calibration of gene expression for feature each plot is shown. J-R. Violin plots for cholesterol regulatory gene products in naïve Mac, Mo, and Mac1-5 subpopulations. S. Dot plot showing expression of cholesterol transporters and metabolic enzymes in naïve nerve Mac in comparison to Mo and Mac1-5 in injured nerve. Expression levels are normalized to average gene expression (color coded). For each cell cluster the percentile of cells that express the listed gene (dot size) is shown.

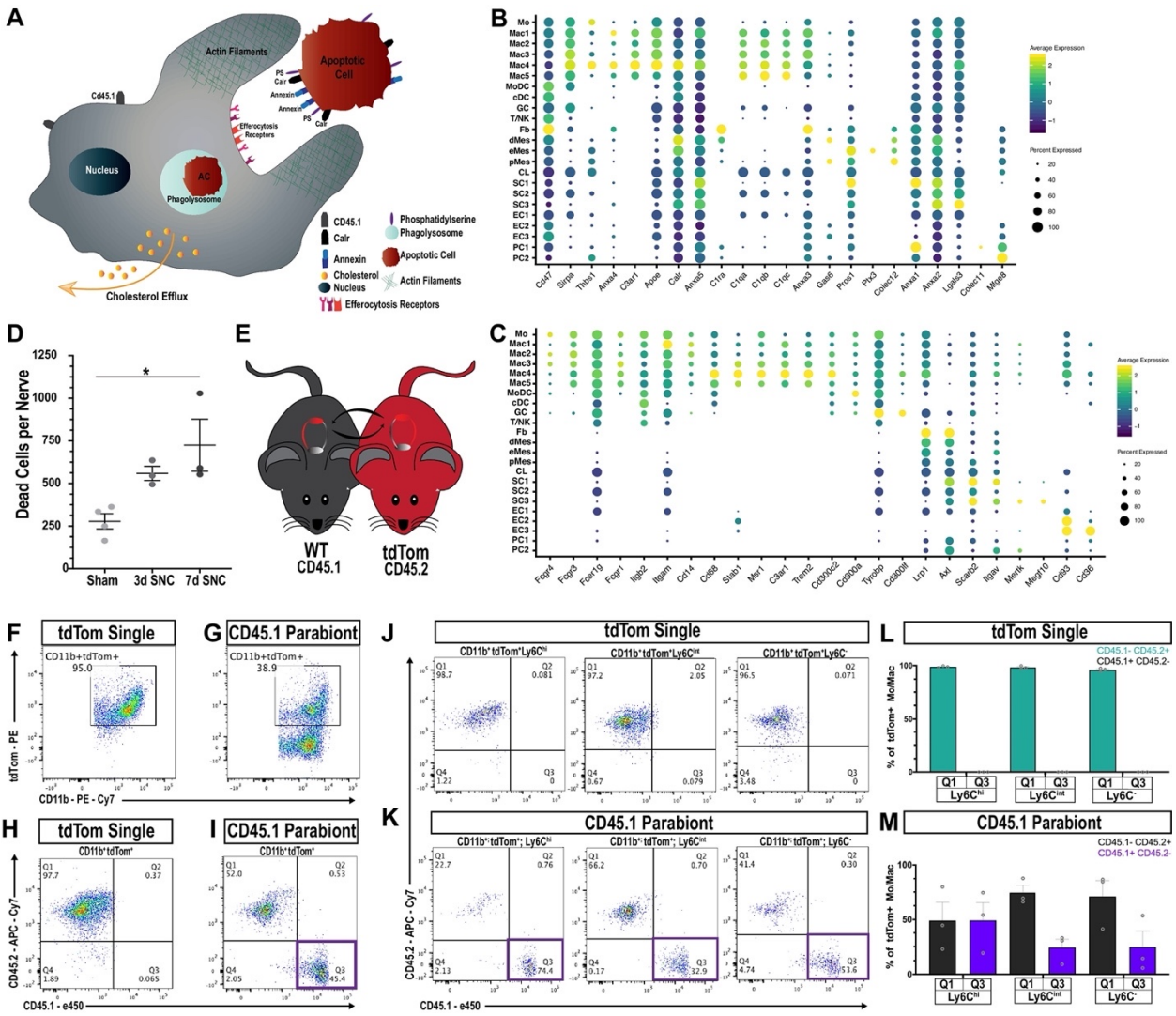


Figure 2-24 Macrophages "eat" dying leukocytes in the injured nerve.

A. Cartoon of phagocyte with actin rich phagocytic cup eating a tdTom$^+$ apoptotic cell (AC). "Eat me" signals displayed on the surface of AC allow direct or indirect recognition via engulfment receptors. Following engulfment by phagocytes, AC are digested in the phagolysosome. Cellular cholesterol levels are controlled by upregulation of specific efflux mechanisms. B. scRNAseq dotplot analysis of "don't eat me" molecules (*Cd47, Sirpa*) and bridging molecules prominently expressed across cell types in the d3 post-SNC nerve. Average gene expression and percentage of cells expressing the gene are shown. C. scRNAseq dotplot analysis of engulfment receptors in the d3 post-SNC nerve. Average gene expression and percentage of cells expressing the gene are shown. D. Flow cytometric analysis of dead cells accumulating in the d3 and d7 nerve (n= 3 biological replicas per time point). Data are represented as mean ± SEM. E. Parabiosis complex of WT (CD45.1) mouse with a (CD45.2) tdTom reporter mouse. F. Flow cytometry dot plot showing tdTom$^+$ myeloid cells (CD45.2$^+$, CD11b$^+$) in the sciatic nerve of non-parabiotic (tdTom single) mice. G. Flow cytometry dot plot showing tdTom$^+$ myeloid cells (CD11b$^+$) in the sciatic nerve of the WT CD45.1 parabiont. H. Flow cytometry dot plot of CD11b$^+$, tdTom$^+$ gated cells from non-parabiotic (tdTom$^+$ single) mice, analyzed for CD45.1 and CD45.2 surface staining. I. Flow cytometry dot plot of CD11b$^+$, tdTom$^+$ gated cells from the CD45.1 parabiont, analyzed for CD45.1 and CD45.2 surface staining. Quadrant 3 (Q3) identifies CD45.1$^+$, tdTom$^+$, CD45.2$^-$ myeloid cells, indicative of ongoing efferocytosis. J. Flow cytometry dot plots of Mo/Mac in the injured nerve of non-parabiotic (tdTom single)

mice. Mo/Mac maturation was assessed by Ly6C surface staining. Shown are monocytes ($Ly6C^{hi}$), Mo/Mac ($Ly6C^{int}$), and Mac ($Ly6C^{-}$). K. Flow cytometry dot plots of Mo/Mac in the injured nerve of the CD45.1 parabiont. Shown are monocytes ($Ly6C^{hi}$), Mo/Mac ($Ly6C^{int}$), and Mac ($Ly6C^{-}$). The quadrant with $CD45.1^{+}$, $tdTom^{+}$, $CD45.2^{-}$ cells (Q3) is highlighted. Biological replicates n= 3, with 3 parabiotic pairs per replica. L, M. Quantification of $CD45.1^{+}$, $tdTom^{+}$, $CD45.2^{-}$ cells in quadrant Q3 and $CD45.2^{+}$, $tdTom^{+}$, $CD45.1^{-}$ cells in Q1. (L) In the injured nerve of (tdTom single) mice, no $CD45.1^{+}$ cells are detected. (M) In the injured nerve of the WT CD45.1 parabiont, $CD45.1^{+}$, $tdTom^{+}$, $CD45.2^{-}$ Mo ($Ly6C^{hi}$), Mo/Mac ($Ly6C^{int}$), and Mac ($Ly6C^{-}$) are found; n= 3 biological replicates, with 3 parabiosis pairs pooled per replicate.

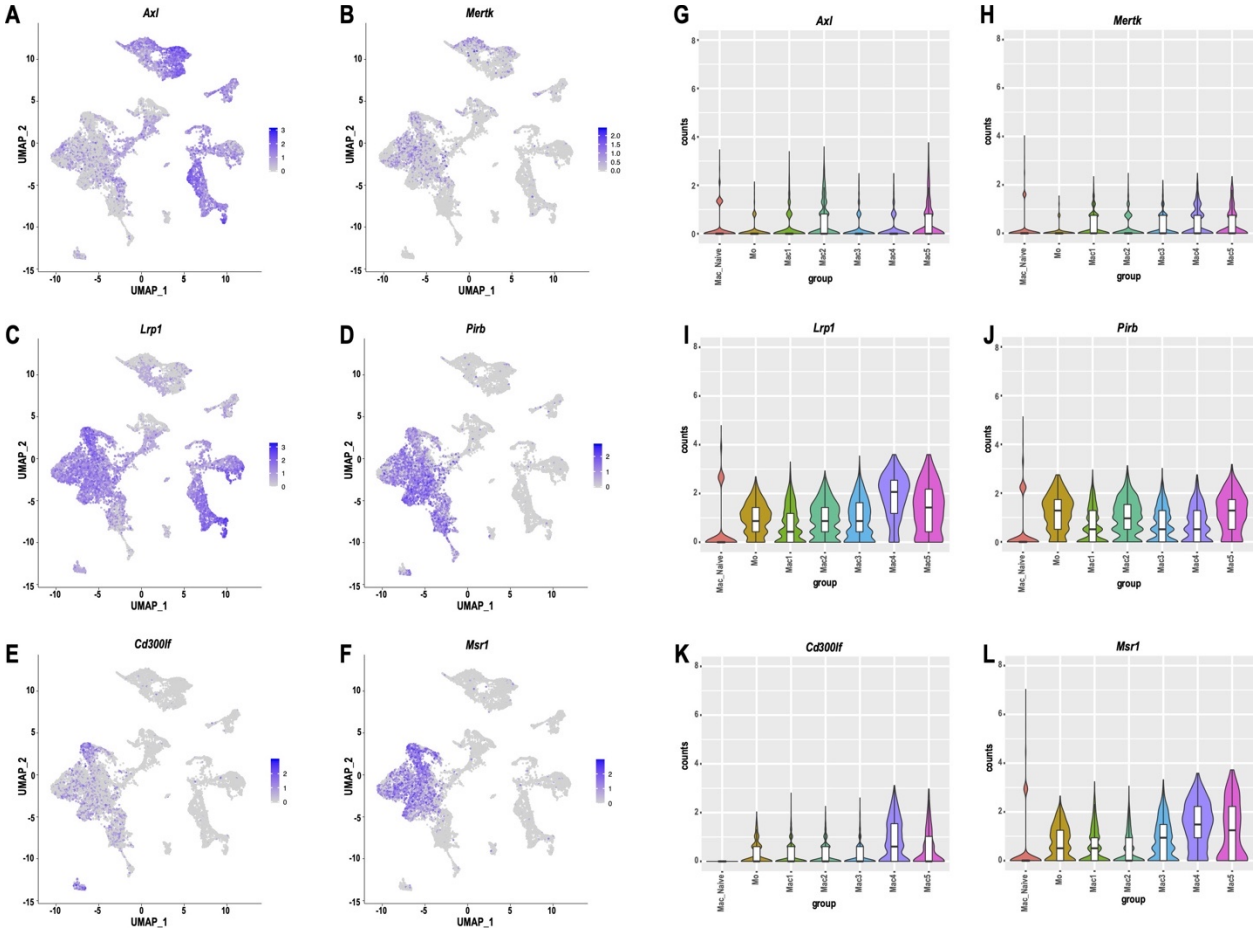


Figure 2-25 Expression of gene products implicated in myelin binding and phagocytosis.
A-F. Feature plots and G-L. Violin plots of *Axl* (TAM receptor tyrosine kinase AXL), *Mertk* (TAM receptor tyrosine kinase MER), *Lrp1* (Low density lipoprotein receptor-related protein 1), *Pirb* (Paired Ig-like receptor B), *Cd300lf* (CD300 molecule like family member F), and *Msr1* (Macrophage scavenger receptor 1) expression in the d3 post-SNC nerve. Violin plots in G-L show relative expression in Mac from naïve nerve and 3d injured Mo and Mac1-5 subpopulations.

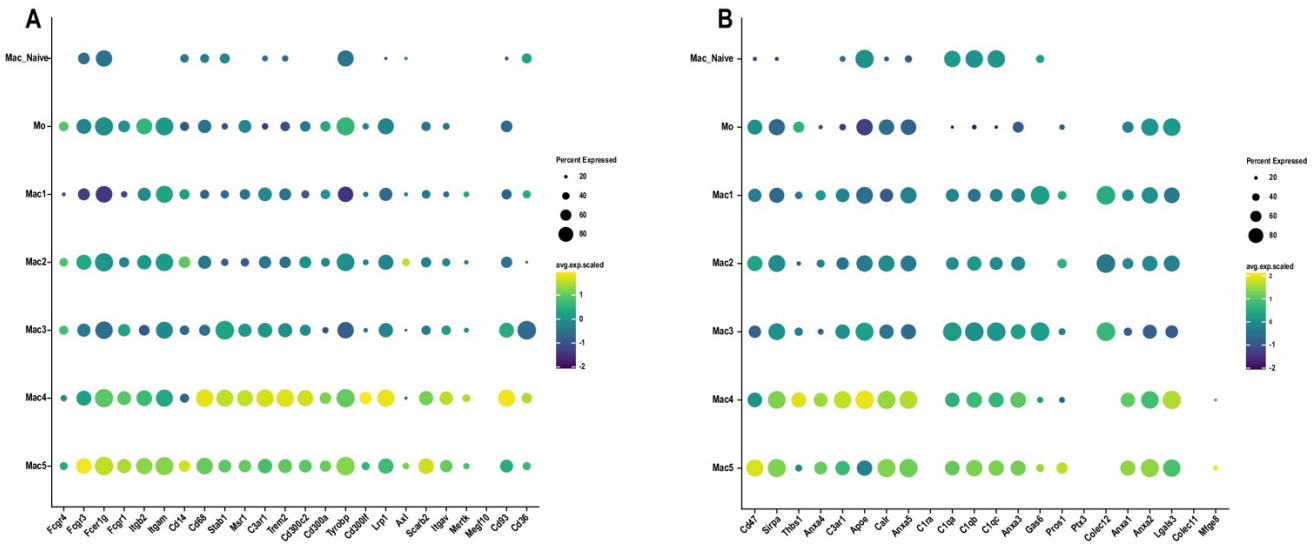


Figure 2-26 Expression analysis of bridging molecules and engulfment receptors in macrophages of naïve nerve and injured nerve.

A. Dotplot showing expression of engulfment receptors in in naïve Mac in comparison to Mo and Mac1-5 in injured nerve. B. Dotplot showing expression of "don't eat me" (*Cd47* and *Sirpa*) signals, as well as bridging molecules, in in naïve Mac in comparison to Mo and Mac1-5 in injured nerve. Expression levels are normalized to average gene expression (color coded). For each cell cluster the percentile of cells that express the listed gene (dot size) is shown.

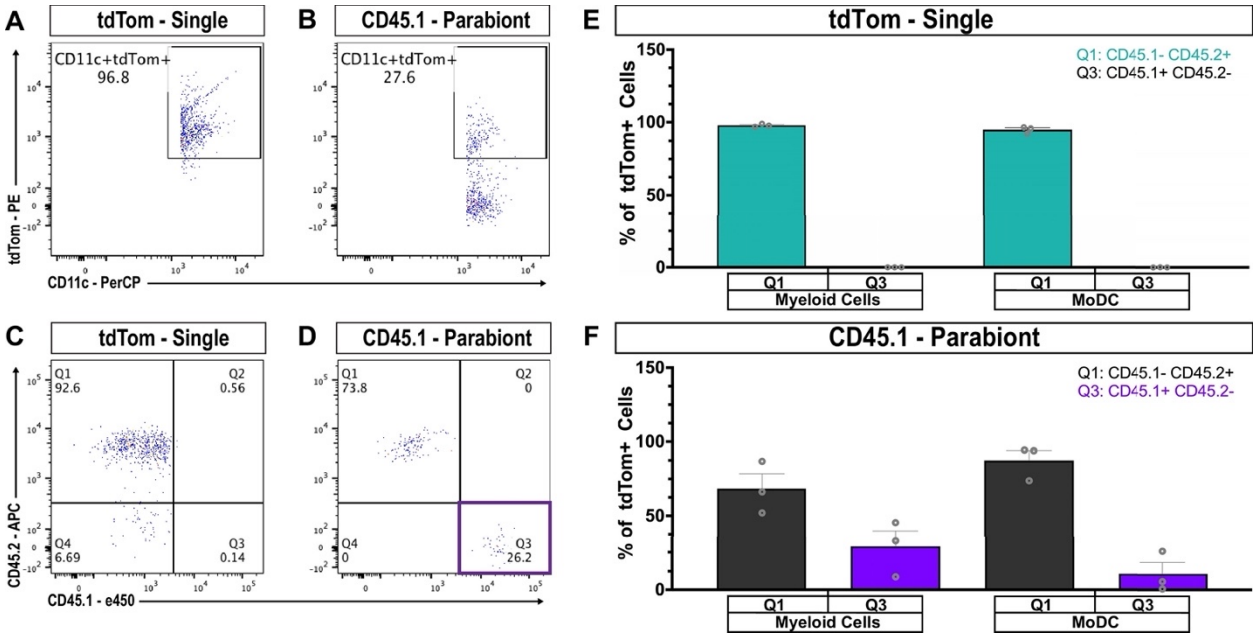
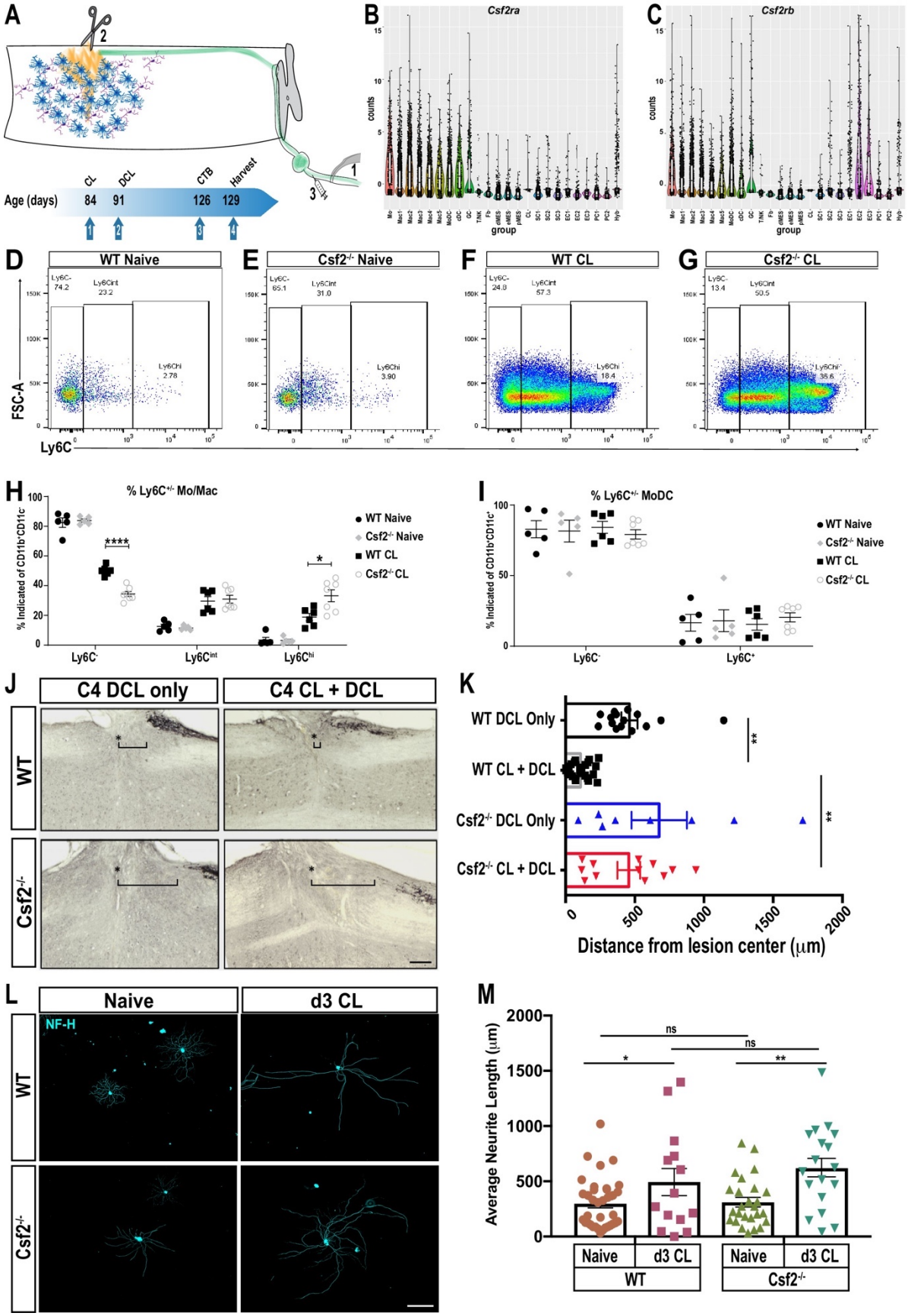


Figure 2-27 Contribution of MoDC to efferocytosis in the injured sciatic nerve.
A. Flow cytometric analysis of 3d sciatic nerve from non-parabiotic (tdTom single) mice. Dot plot shows that 96.8% of MoDC ($CD11b^+$, $CD11c^+$, $Ly6G^-$) are $tdTom^+$ B. Flow cytometric analysis of 3d sciatic nerves from CD45.1 parabionts. Dot plot shows that 27.6% of MoDC are $tdTom^+$ C. In the 3d sciatic nerves of non-parabiotic (tdTom single) mice, no $tdTom^+$, $CD45.1^+$ MoDC are present. D. In the 3d sciatic nerve of the CD45.1 parabiont, $CD45.1^+$ and $CD45.2^+$ MoDC are found. E. Quantification of CD45.1 and CD45.2 myeloid cells ($CD11b^+$) and MoDC in the 3d sciatic nerve of non-parabiotic (single) tdTom mice. As expected, $CD45.1^+$ cells are not detected. F. Quantification of CD45.1 and CD45.2 myeloid cells ($CD11b^+$) and MoDC in the 3d sciatic nerve of the CD45.1 parabiont. The presence of $tdTom^+$, $CD45.1^+$, $CD45.2^-$, $CD11b^+$, $CD11c^+$, $Ly6G^-$ cells, indicates that MoDC participate in efferocytosis.


A
2
1
3
CL
DCL
CTB
Harvest
Age (days)
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91
126
129
1
2
3
4
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Csf2ra
counts
group
C
Csf2rb
counts
group
D
WT Naive
Ly6C-
74.2
Ly6Cint
23.2
Ly6Chi
2.78
FSC-A
Ly6C
E
Csf2-/- Naive
Ly6C-
65.1
Ly6Cint
31.0
Ly6Chi
3.90
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WT CL
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WT
Csf2-/-

Figure 2-28 The factor GM-CSF is required for conditioning injury-induced dorsal column axon regeneration after lesioning injury.

A. Schematic showing conditioning lesion to the sciatic nerve (1) followed by dorsal column lesion (2) and tracer injection in the nerve (3). Experimental time line of conditioning lesion (CL), dorsal column lesion (DCL), cholera-toxin B (CTB) injection, and time of tissue harvest are shown. B, C. Violin plots of *Csf2ra* and *Csf2rb* expression in the d3 post-SNC sciatic nerve, as assessed by whole nerve tissue scRNAseq analysis. D-G. Flow cytometry dot plots of WT and *Csf2*$^{-/-}$ nerves from naive mice and 3d following conditioning lesion (CL) to the sciatic nerve. Ly6C surface staining was used to assess maturation of the Mo/Mac population. Ly6C^{hi} (immature), Ly6C^{int}, and Ly6C^{-} (mature) cells are shown. H. Quantification of percentage of Mo/Mac (CD45^{+} CD11b^{+} CD11c^{-} Ly6G^{-}) that are Ly6C^{-}, Ly6C^{int} and Ly6C^{hi} in WT and *Csf2*$^{-/-}$ mice without (naïve) and with CL. I. Quantification of surface Ly6C on MoDC (CD45^{+} CD11b^{+} CD11c^{+}) in WT and *Csf2*$^{-/-}$ mice without (naïve) and with CL. Unpaired t-test with correction for multiple comparisons using Holm-Sidak method, * $p < 0.05$; **** $p < 0.0001$. J. Sagittal sections through cervical spinal cords of wild-type (WT) and *Csf2*$^{-/-}$ mice, five weeks following bilateral DCL at cervical level 4 (C4). The spinal cord lesion site is labeled with a star (*), rostral is to the left and caudal is to the right. To enhance dorsal column axon regeneration, a CL to the sciatic nerve was performed 7d prior to DCL (CL + DCL). Dorsal column axons were visualized by CTB injection in the sciatic nerve. The brackets indicated the distance between the lesion center and the rostral tip of CTB labeled axons. K. Quantification of axon regeneration. The distance between CTB labeled axon tips and the center of the spinal lesion was measured; 0 μm marks the injury site, the gap between the lesion center and traced axons (= retraction) is shown for WT and *Csf2*$^{-/-}$ without CL. For each genotype and experimental condition $n \geq 8$ biological replicates. One-way ANOVA with Tukey posthoc correction. ** $p<0.01$. Scale bar, 200 μm. L. Representative images primary DRG neurons isolated from WT and *Csf2*$^{-/-}$ mice, with and without a d3 CL. Cultures were stained with of anti-neurofilament H (NF-H) M. Quantification of neurite length. Neuromath was used to quantify neurite length, neurites less than 30 μm in length were excluded from the analysis. $n \geq 114$ neurons, n= 2 biological replicates. Two-tailed Student's t-Test with Tukey posthoc correction was used. *$p<0.05$; ** $p<0.01$. Scale bar, 500 μm.

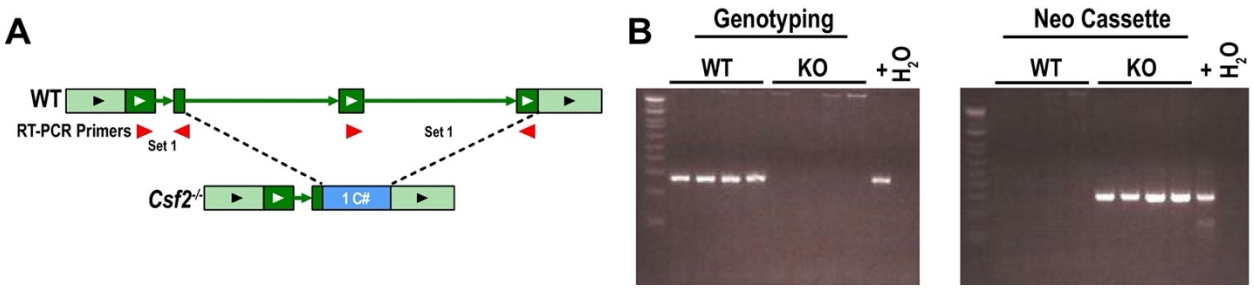

Figure 2-29 Locus and PCR genotyping of *Csf2-/-* mice.
A. Schematic of *Csf2* gene locus of WT and germline *Csf2*$^{-/-}$ mice. Primer sets were designed both inside (set 2) and outside (set 2) of the deleted region. B. Agarose gel shows PCR genotyping results from naïve brains of WT and *Csf2*$^{-/-}$ (KO) animals against primer set 2 (left) or against the neomycin cassette (right). In the absence of genomic DNA (water (H_2O) control), no PCR product is observed.

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CHAPTER 3:
The Immune Response to Wallerian Degeneration Identifies Compartment Specific Reprogramming of Macrophages

3 Immune Response to Wallerian Degeneration Identifies Compartment Specific Reprogramming of Macrophages

3.1 Abstract

The nervous system and immune system stand in constant dialogue and this interaction is particularly strong following nervous system injury or disease. In the mouse, a sciatic nerve crush injury (SNC) causes a rapid inflammatory response comprised of a highly heterogeneous population of myeloid cells, T cells, and Natural killer cells. Micro-dissection of the nerve injury site and distal nerve stump followed by flow cytometry and single cell RNA-sequencing, identified distinct immune compartments comprised of overlapping, yet distinct, macrophage subpopulations. To disentangle the immune response to nerve tissue compression from inflammation associated with WD, *Sarm1*$^{-/-}$ mice were subjected to SNC. In both WT and *Sarm1*$^{-/-}$ mice, a robust and comparable immune response is observed at the nerve injury site. Inflammation in the distal nerve stump of *Sarm1*$^{-/-}$ mice is reduced compared to WT mice, however monocytes are elevated. We used parabiosis of *Sarm1*$^{-/-}$ and tdTomato mice and found that blood-borne Mo enter the distal nerve in *Sarm1*$^{-/-}$ mice prior to WD. In the distal nerve stump of *Sarm1*$^{-/-}$ mice, endoneurial mesenchymal cells upregulate the chemokine *Ccl2* prior to WD. Flow cytometric analysis of injured *Ccr2*$^{-/-}$ mice revealed a significant reduction of Mo, both at the injury site and the distal nerve. Together our studies identify different immune compartments within the injured sciatic nerve, show that inflammation occurs prior to WD, and identify CCL2-CCR2 signaling as an important mechanism of Mo chemotaxis and nerve inflammation.

3.2 Introduction

Axonal injury caused by trauma or metabolic imbalances can trigger a biochemical program that results in axon self-destruction, a process known as Wallerian degeneration (WD) (Coleman and Hoke, 2020). In the peripheral nervous system (PNS) WD is associated with nerve fiber disintegration, accumulation of myelin debris, and nerve inflammation (Rotshenker, 2011). Denervated Schwann cells (SC) undergo reprogramming into repair (r)SC. Fragmented axons and myelin debris are rapidly cleared by rSC and professional phagocytes of the innate immune system (Jang et al., 2016; Klein and Martini, 2016). The timely clearance of degenerated fibers in the PNS

stands in contrast to the injured central nervous system (CNS), where clearance of degenerated axons and myelin debris is protracted and accompanied by prolonged inflammation (Bastien and Lacroix, 2014; Vargas and Barres, 2007).

The molecular mechanisms that underlie WD are evolutionarily conserved. Nerve injury results in a rapid loss of axonal NMNAT2, an enzyme in the nicotinamide adenosine dinucleotide (NAD+) biosynthetic pathway (Coleman and Hoke, 2020). The drop in axonal NAD+ and fragmentation of the distal axon is delayed in Wallerian degeneration slow (Wld^s) mice, overexpressing a more stable protein with NAD+ biosynthetic activity (Coleman and Freeman, 2010). In wild-type mice, the injury induced drop in axonal NAD and simultaneous increase in nicotinamide mononucleotide (NMN) triggers activation of the metabolic sensor Sarm1. Activated Sarm1 has NADase catabolic function, leading to a further drop in axonal NAD+ and rapid fiber disintegration (Essuman et al., 2017; Figley et al., 2021; Jiang et al., 2020; Osterloh et al., 2012). The importance of Sarm1 for WD was originally demonstrated by loss-of-function studies and protection of severed axons from WD (Osterloh et al., 2012). Similar to Wld^s mice, severed axons in *Sarm1-/-* mice are protected from WD and remain intact for weeks (Coleman and Hoke, 2020).

Large peripheral nerves, such as the sciatic nerve, are protected by the epineurium, a tough collagen-rich structure that harbors fibroblasts and epineurial macrophages (Mac^{epi}), forming a first line of defense. A distinct population of macrophages, called endoneurial macrophages (Mac^{endo}) resides within nerve fascicles and stands in close contact with myelinated and non-myelinated fibers (Ydens et al., 2020). Scatted between nerve fibers lay endoneurial mesenchymal cells (eMES), fibroblast-like cells with a neural crest origin (Carr et al., 2019; Chen et al., 2021; Joseph et al., 2004; Richard et al., 2014). A peripheral nerve compression injury triggers a highly orchestrated immune response, composed of Mac^{endo} and infiltrating, blood-derived leukocytes. Nerve trauma not only results in fiber transection and WD of severed axons, but also causes necrotic cell death at the injury site and release of damage associated molecular patterns (DAMPs). Nerve trauma can disrupt the nerve vasculature, resulting in endoneurial bleeding, hypoxia, and breakdown of the blood-nerve-barrier. Thus, the micromilieu near the nerve injury site is expected to be different than in the distal nerve, where physical trauma is not directly experienced. Nerve compression results in the release of chemotactic factors that promote extravasation and nerve infiltration of circulating leukocytes. Neutrophils arrive within hours, followed by monocytes

(Mo) that, upon nerve entry, differentiate into macrophages (Mac) and monocyte-derived dendritic cells (MoDC) (Kalinski et al., 2020; Lindborg et al., 2017; Ydens et al., 2020). Immune cells in the injured nerve exert a plethora of functions; the complement system (Ramaglia et al., 2007) and Natural killer cells (NK) promote WD of damaged axons (Davies et al., 2019). Macrophages, and possibly neutrophils, phagocytose nerve debris, including degenerating myelin and axon remnants (Kuhlmann et al., 2002). Macrophages protect the injured nerve from secondary necrosis by eating apoptotic leukocytes and thereby contribute to inflammation resolution (Kalinski et al., 2020). In addition to nerve debridement, macrophages promote angiogenesis, release factors that stimulate axon regeneration and wound healing (Cattin et al., 2015; Pan et al., 2020). Importantly, inflammation may play a central role in nerve injury inflicted pain syndromes. Recent work shows that sensory neuron-associated macrophages regulate neuropathic pain (Silva et al., 2021).

Despite the well-established role of the immune system in the nerve injury response and tissue repair, a comparative analysis of the immune milieu at the nerve compression site, versus the immune response associated with WD, has not yet been carried out. To address this void, we subjected mice to mid-thigh sciatic nerve crush injury, micro-dissected the injury site and distal nerve segment for a comparative analysis by flow cytometry, single cell transcriptomics, immunofluorescence staining, immunoblotting blotting, and cytokine ELISA at different post-SNC time points. Our studies identified highly dynamic and distinct immune compartments in the injured PNS. Specific macrophage subpopulations preferentially localize to the injury site versus the distal nerve stump. Where they participate in nerve debridement by eating apoptotic leukocytes and myelin ovoids. Experiments with chimeric mice show that monocytes enter the distal nerve in *Sarm1-/-* parabionts prior to WD. This shows that physical disintegration of axons is not required to trigger entry of blood-borne leukocytes. Single cell transcriptomics identified upregulation of Ccl2 in the distal nerve and in *Ccl2* mice, monocyte entry into the nerve is significantly reduced.

3.3 Results

Non-hematopoietic cells in injure nerve tissue, including vascular cells, SC, and structural cells such as MES, show immune gene activity, indicating they play major roles in shaping the inflammatory milieu. To resolve the cellular complexity of injured PNS tissue in an unbiased manner, we applied scRNA-seq to capture the transcriptional landscape at single cell resolution. Seven days (d7) following SNC, the sciatic nerve trunk, including the nerve crush site and the

distal nerve up to the trifurcation, was harvested (**Figure 3.1A**). A total of 36,508 cells were sequenced with 32,967 cells used for downstream analysis after removing cells with fewer than 500 genes, more than 7500, or mitochondrial content greater than 15%. The median unique genes per cell was 2,568. Nearest neighbor clustering algorithm identified a total of 30 cell clusters using the first 30 principle components and a resolution parameter of 0.5. The 30 principle components are visualized using Uniform Manifold Approximation and Projection (UMAP) (**Figure 3.1B**). The top 100 genes enriched in each cluster were analyzed and used to assign cluster-specific cell identities (Kalinski et al., 2020). Immune cells, identified by their strong expression of *Ptprc* (encoding CD45), form the largest cell population in the d7 nerve. Consistent with flow cytometric studies (Kalinski et al., 2020), the myeloid cell population (*Itgam*/CD11b) is large and dominated by macrophages (Mac 22.7% of cells in the injured nerve), monocytes (Mo 2%), and monocyte-derived dendritic cells (MoDC 9.1%). In addition, conventional dendritic cells (cDC, *Clec9a, Cd24a, Wdfy4,* 1.7%), plasmocytoid dendritic cells (pDC, *Siglech/*sialic acid binding Ig-lectin h*, Itgae*/CD103, 0.8%), Mast cells (MC, *Ms4a2, Cpa3, Mcpt4*, 0.4%), and few granulocytes (GC, *S1009a, S1008a, Mmp9, Cxcr2* <0.1%) are present. The lymphocyte population is comprised of T cells (TC, *Cd3g, Cd8b*, 4.6%), natural killer cells (NK, *Ncr1, Il2rb, Gzma,* 3.2%), and T/NK (0.8%). Only few B cells are detected <0.1%. Additional cell types include Schwann cells (SC 17.7%), fibroblast-like (FB) and mesenchymal cells (MES) that form the epineurium, perineurium and endoneurium (FB and MES, 25.4%), and several cell types associated with the nerve vasculature, including endothelial cells (EC, 3%), pericytes (PC, 5.9%), and vascular smooth muscle cells (vSMC, 2%).

In the UMAP plot macrophages form a connected continuum of multiple clusters, indicating a high degree of heterogeneity. All macrophages strongly express complement C1q (*C1qa, C1qb, C1qc*), the C3a anaphylaxtoxin chemotactic receptor (*C3ar1*), the lysosomal protease legumain (*Lgmn*), the membrane spanning 4-domain protein *Ms4a7*, and the selenoprotein P *(Selenop)* (data not shown). Macrophages in Cluster 8 express high levels of *Retnla*/FIZZ1, *Mgl2*, *Cd163*, gene products previously shown to be enriched in epineurial Mac (Wang et al., 2020; Ydens et al., 2020). Additional Mac subpopulations include clusters 4, 5, 9, 23 and 28 (**Figure 3.1B**). Cluster 5, and to a lesser extent cells in cluster 4, express high levels of *Cd68*, *Trem2, Tyrobp, Spp1*, *Apoe,* and cathespins (*Ctsb, Ctsd, Ctsz*) that function as lysosomal proteases. Mac in cluster 4, but not cluster 5, express high levels of MHCII genes (*H2-Aa, H2-*

Ab1, H2-Eb1, M2-DM) and the CD74 invariant chain of MHCII (*Cd74*), typically associated with antigen presentation to $CD4^+$ T cells. Expression of MHCII gene products in cluster 4 is shared with cells in clusters 3 and 19, including MoDC (*Itgax*/CD11c, *Cd209a*/DC-SIGN, *Napsa*/Napsin-A), cDC in cluster 22 (*Clec9a, Cd24a, Wdfy4*), and pDC in cluster 26 (*Siglech*/sialic acid binding Ig-lectin h*, Itgae*/CD103). Mo in cluster 20 express high levels of *Ly6C2*, *Chil3, Gsr, Plac8, Hp.* To infer the most probable differentiation trajectories from Mo toward their descendants, we used Slingshot, a method for pseudo-time trajectory analysis (Street et al., 2018). The analysis revealed a bifurcated trajectory and provides independent evidence that blood-borne Mo entering the nerve give rise to Mac in cluster 4 and subsequently Mac in clusters 9 and 5. A second predicted line of differentiation indicates that Mo are precursors for MoDC (cluster 3) (**Figure 3.1**).

3.3.1 Comparative analysis of cellular composition between 3d and 7d injured sciatic nerve

Flow cytometric analysis of naïve and injured sciatic nerve tissue identified a rapid expansion in the immune compartment (Kalinski et al., 2020; Lindborg et al., 2017). Neutrophils enter the injured nerve within hours, followed by blood-borne Mo that upon nerve entry differentiate into their respective derivatives. For a comparative analysis of the cellular make-up of 3d and 7d injured nerve, we generated a 3d nerve scRNA-seq dataset of the injury site and distal nerve up to the trifurcation. The 3d injured nerve dataset included previously reported scRNA-seq datasets (Kalinski et al., 2020) and newly sequenced 3d injured nerve tissue. Altogether, a total of 28,370 cells were sequenced from 3d injured nerve, and after applying the same exclusion criteria as described above, 24,672 cells with a median unique feature count of 2,364 were used for downstream analysis. Nearest neighbor clustering algorithm identified 24 cell clusters using the first 30 principle components and a resolution parameter of 0.5. The 30 principle components of the 3d injured nerve are visualized using UMAP (**Figure 3.2**). Similar to the 7d injured nerve (**Figure 3.1**), the myeloid cell compartment in the 3d injured nerve forms a connected continuum comprised of multiple clusters, indicating a high degree of heterogeneity (**Figure 3.2**). In addition to Mo (*Ly6C2, Chli3*) 3%, five subpopulations of Mac (clusters 0, 2, 4, 10, and 16) 33.2%, MoDC (cluster 6) 5.7%, pDC (cluster 17) 0.3%, cDCs (cluster 18) 1.5%, and GC (cluster 19) 1.5% are identified. Few lymphocytes are detected in the 3d injured nerve. Cluster 15 (2.4%) harbors TC and NK cells. Similar to the 7d injured nerve, non-immune cell types in the 3d injured nerve

include SC (clusters 3 and 8) 12.7%, Fb/MES (clusters 1, 5, 9, 11 and 13) 28%, and several cell types associated with the nerve vasculature, EC (clusters 7 and 21) 5.8%, and PC (clusters 12 and 20) 4.7%. Obvious differences in immune cell composition between the 3d and 7d injured nerve are the rapid drop in neutrophils, expansion of lymphocytes and the separation of the TC/NK population (cluster 15) in the 3d nerve (**Figure 3.2**) into distinct clusters harboring TC (cluster 6), NK cells (cluster 12), and T/NK (cluster 27) in the 7d nerve (**Figure 3.1**). While cells in the mononuclear phagocyte system (MPS), including Mo, Mac, and MoDC, are readily identified in the 3d and 7d injured nerve, scRNA-seq provides a unique opportunity to assess gene expression changes at different post-injury time points, offering insights into "cellular plasticity," and by inference immune cell phenotype and function.

To track how different cell clusters change in size and gene expression as a function of time, we used the 3d dataset as a "reference" and projected its PCA structure onto "query" datasets from different time points as defined by (Stuart et al., 2019) and implemented through Seurat v3. This technique finds anchor cells between the reference and query datasets, then uses a weighted vote classifier based on the known reference cell labels to yield a quantitative score for each cell's predicted label in the query dataset. A prediction score of 1 means max confidence, all votes, for the predicted label and a score of 0 means no votes for that label. To assess the similarity between 3d and 7d cells we focused on two statistics: the median prediction score for the 7d cells and the squared Pearson correlation between the overlapping, top 3,000 variable, genes in each dataset (2,284 genes). For most of the non-immune cell types, expression of the top variable genes did not change globally between 3d and 7d post-SNC. For example, the cells predicted as Fb in the 7d nerve had a median prediction score (PS) of 0.99 and an R-squared of 0.82. In a similar vein, the fibroblast-related eMES (PS = 1.00, R2 = 0.89), dMES (PS = 0.90, R2 = 0.91) and pMES (PS = 0.94, R2 = 0.89) exhibit a high degree of similarity between 3d and 7d. Cells associated with the nerve vasculature in the 3d and 7d nerve, including EC show very similar scores (PS = 1.00, R2 = 0.93) and PC (PS = 1.00, R2 = 0.92), indicating that in the injured nerve these cells show a limited degree of plasticity and can readily be identified. This stands in marked contrast to cells in the MPS and Schwann cells. In the 3d nerve, Mac in cluster 0 form the largest subpopulation (Figure 2), when assigned to cells in the 7d injured nerve, they had a median prediction score of 0.77 (R2=0.86) and no 7d cluster had more than 48% of cells given this label (cluster 8^{7d} - 48%, cluster 9^{7d} - 47%, cluster 23^{7d} - 24%, cluster

28^{7d} - 25%). This suggests that the Mac subpopulation in cluster 0^{3d} is highly plastic and marks a transient cell state that is no longer exists in the 7d injured nerve. Noteworthy, not all cells in the MPC show a similarly high degree of plasticity. For example, 7d cells assigned Mac from cluster 2^{3d} had a median prediction score of 0.91 (R2=0.94) and made up 70% of cluster 4^{7d}. Similarly, 7d cells assigned as Mac cluster 10^{3d} had a median prediction score of 0.91 (R2=0.86) and show highest similarity to cells in cluster 5^{7d} (80%). Mo are very similar between the 3d and 7d nerve, presumably because they recently entered the injured nerve and did not yet adapt their gene expression to the micro-environment (PS=0.99, R2=0.95). Dendritic cells (DC) are present in the 3d nerve, however many more MoDC, cDC and pDC are found in the 7d nerve. MoDC at 7d had a prediction score of 0.98 (R2=0.95) relative to MoDC at cluster 6^{3d} and make up 92% of cluster 3^{7d}. In a similar vein, cDC at cluster 18^{3d} and ^{7d} cells had a median prediction score of 1 (R2=0.97) and made up 92% of cells in Cluster 22^{7d} and all cells in cluster 31^{7d}. pDC had prediction score of 0.90 (R2=0.94) and made up 97% of cluster 26^{7d} and 89% of cluster 29^{7d}. Comparative analysis of DC, reveals that gene expression for specific DC subpopulations is more stable between 3d and 7d post-injury when compared to Mac subpopulations.

3.3.2 Identification of different immune compartments within the injured sciatic nerve

For a comparative analysis of the cellular composition around the sciatic nerve injury site, where trauma is experienced, versus the distal nerve stump, where axons undergo WD, we micro-dissected nerve segments that contain the injury site (~ 5 mm in length) or the distal nerve stump (~ 5 mm in length) from the same animals (**Figure 1.1**). To assess immune cell composition, tissues were analyzed by flow cytometry (Kalinski et al., 2020). Compared to sham operated mice, the number of myeloid cells in the nerve segment that contains the injury site and the distal nerve segment, were significantly increased. The injury site of the 3d nerve contained 2,437 (± 973) myeloid cells per nerve segment while the 3d distal segment contained 953 (± 215) cells per nerve (**Figure 3.3F**). In a similar vein, macrophages 242 (± 43) cells in sham operated mice, 1,778 (± 792) cells per nerve in the 3d injury site, and 700 (± 171) cells per nerve in the 3d distal segment (**Figure 3.3E-F**). A comparison of single cell transcriptomes in the 7d injury site to 7d whole nerve single cell transcriptomics shown in **Figure 3.1**, revealed that all major cell types are present at the injury site, yet their relative abundance appears to be cell type dependent (**Figure 3.4**). This

stands in contrast to 7d distal nerve tissue, where Mac in clusters 5 are missing and Mac in cluster 9 are greatly reduced (**Figure 3.5 and 3.1**). This suggests that these two Mac subpopulations are preferentially localized to the injury site and largely absent from the distal nerve. Gene products strongly expressed by Mac enriched at the injury site (clusters 5 and 9), include *Gpnmb, Fabp5, Syngr1*. Conversely, Mac in clusters 5 and 9 are devoid of *Ccr2* and the MHCII components *H2-Aa, H2-Ab* and *Cd74,* while other Mac subpopulations (clusters 4, 23, and 28) express these gene products (data not shown). Mo are more abundant in the 7d distal nerve than the injury site, while MoDC are abundantly found in both compartments. Notably, injury associated TC and NK appear to be homogenously distributed between the injury site and distal nerve, as no significant differences in cell numbers were detected (**Figure 3.6)**. Together these studies identify distinct immune compartments within the injured sciatic nerve and show that select Mac subpopulations are strong enriched at the injury site, while others are more uniformly distributed.

3.3.3 Immune response to sciatic nerve crush injury in *Sarm1-/-* mice

The immune response to sciatic nerve compression is composed of cells that respond to the tissue wound inflicted by trauma and cells that respond to WD of severed nerve fibers in the distal nerve stump. Because *Sarm1* deficiency delays to onset of WD by approximately 2 weeks following SNC, we subjected cohorts of WT and *Sarm1-/-* mice to SNC to distinguish between the immune response triggered by nerve trauma in the presence or absence of WD. Consistent with previous studies, in WT mice severed nerve fibers in the distal stump are fully disintegrated 7d post-SNC and myelin ovoids abundantly present. In parallel processed *Sarm1-/-* nerves, fibers in the distal nerve remain intact and myelin ovoids are not detected (data not shown). Nerves from WT and *Sarm1-/-* were dissected (including the injury site and distal stump up to the nerve trifurcation), and subjected to flow cytometry to identify the main immune cell types. In sham-operated WT and *Sarm1-/-* mice, Mac ($CD45^+CD11b^+CD11c^-Ly6G^-$) are present at comparable numbers (**Figure 3.3A-B**). This was independently verified by anti-F4/80 immunostaining of longitudinal sciatic nerve sections (**Figure 3.7**). In 3d and 7d whole nerves, total counts of myeloid immune cells were significantly elevated in WT and KO mice (**Figure 3.3B and 3.7**). However, compared to WT 3d—3130 (± 466) cells per nerve—and WT 7d—2476 (± 486) cells per nerve—the total number of myeloid cells per nerve was significantly reduced in KO mice at 3d—1585 (± 375) cells per nerve—but not at 7d—1546 (± 233) cells per nerve (**Figure 3.3B**). Significantly

fewer Mac are found in the injured *Sarm1-/-* nerve at 3d—933 (± 223.6) than in WT nerve 2476 (± 353). Mac in the 7d *Sarm1-/-* nerve 1223 (± 185) are reduced to WT nerves 1990 (± 341) (**Figure 3.3A-B**). A significant difference in neutrophil accumulation was found between genotypes with 64 (± 15) found per WT nerve and 16 (±4) per KO nerve. Though neurtrophil numbers are quite low in the 7d injured nerve and are likely not contributing significantly at this timepoint. Other immune cell types were comparable and did not significantly differ between WT and KO nerves with 261 (± 103) DCs, and 668 (± 17) lymphocytes found per WT nerve and 158 (± 20) DCs, and 515 (± 25) lymphocytes found per KO nerve.

To specifically assess inflammation in nerve tissue distal to the injury site, the 3d nerve trunk from WT and *Sarm1-/-* mice was harvested and micro-dissected into "injury site" and "distal nerve" and analyzed by flow cytometry. In WT distal nerve, significantly more myeloid cells are present—953 (± 215) per segment—than in KO distal nerve—479 (± 212) per segment (**Figure 3.3F**). More Mac are detected 700 (± 171) per distal nerve segment in WT mice compared to *Sarm1-/-* mice 344 (± 158) (**Figure 3.3E-F**). High magnification imaging of the proximal stump, injury site and distal stump, confirmed that there is a reduction in F4/80+ Mac in the distal nerve, but there is no difference at the injury site (**Figure 3.3G**). While the Mac population is reduced in *Sarm1-/-* mice compared to WT, it is clearly elevated compared to sham-operated mice (**Figure 3.3B**). This suggests that WD independent mechanisms contribute to nerve inflammation, however WD is required for full blown nerve inflammation. For a time course analysis, sciatic nerves and DRGs at 1d, 3d, 7d, 14d, and 21d post-SNC were harvested and analyzed by Western blotting (**Figure 3.3H-I**). Additionally, nerves from 3d and 7d SNC were divided into proximal, injury and distal segments and analyzed separately by Western blotting. In the injury region, where mechanical nerve compression leads to necrotic cell death, inflammation in WT and *Sarm1-/-* nerves is elevated when compared to proximal nerve (**Figure 3.3J-K**). In WT mice, the distal nerve showed an increase in CD11b at 3d that peaked around 7d. This increase was not observed in the distal nerve of *Sarm1* mice. As an independent verification of the flow cytometry and Western blotting data, longitudinal nerve sections were stained for $F4/80^{+}$ macrophages at 3d and 7d after SNC (**Figure 3.3C-D**). In nerves of sham-operated mice, morphology and number of macrophages is similar between WT and *Sarm1-/-* mice (**Figure 3.7**). At 3d, accumulation of large numbers of Mac was observed at the injury site in both, WT and *Sarm1-/-* mice (**Figure 3.3C).**

However, at 7d, Mac in WT nerves fill the injury site and distal stump, and in *Sarm1-/-* nerves the number of Mac in the distal nerve is reduced (**Figure 3.3D**).

Next, we subjected 7d *Sarm1-/-* nerves (whole nerves) to scRNA-seq and in addition split injured nerves into "injury site" and "distal nerve" as described above for WT nerves (**Figure 1.1**). For cell type identification the top 200 cell type enriched genes were used and compared to cell types identified in the 7d injured WT nerve. Analysis of the 7d injury site of WT and *Sarm1-/-* nerves, revealed that all major cell types are present. Importantly, cells in the immune compartment of WT and *Sarm1-/-* show a high degree of similarly (**Figure 3.8**). Mo are 99% identical, Mac in clusters 5 (97%), cluster 9 (93%), cluster 4 (91%), epiMac (96%), and MoDC (99%). In addition, lymphocytes including TC (98%) and NK (99%) near the injury site are very similar in WT and *Sarm1-/-* mice. This shows, the in *Sarm1-/-* mice, the immune response to nerve tissue compression is very similar at the cellular and molecular level to WT mice. Consistent with flow cytometry, the "distal nerve" of *Sarm1-/-* mice is inflamed and harbors Mo and a smaller population of Mac and MoDC (**Figure 3.9**). These findings indicate that Mo enter the distal nerve stump of *Sarm1-/-* long before axons undergo WD and suggests that Mo chemotactic compounds are released either from severed axons, denervated SC, nerve resident Mac, or MES long before axon fragmentation occurs.

3.3.4 In injured *Sarm1-/-* mice, blood-borne leukocytes enter the distal nerve stump prior to WD

Flow cytometry and scRNA-seq indicate that immune cells enter the distal nerve of *Sarm1-/-* mice before severed nerve fibers start to disintegrate. This shows that WD is not required to trigger nerve inflammation in the distal nerve stump. Because our studies were carried out with *Sarm1* global knock-out mice, potential confounding effects due to *Sarm1* deficiency in immune cells that influence cell phenotype or trafficking cannot be ruled out. To assess whether WT leukocytes enter the distal nerve stump of *Sarm1-/-* mice prior to WD, we used parabiosis. Age-matched adult *Sarm1-/-* and tdTom reporter mice were parabiotically fused and one month later the *Sarm1-/-* parabiont was subjected to bilateral sciatic nerve crush. Parallel processed WT/tdTom parabionts were used for comparison. Histological staining of the 7d injured nerve in the *Sarm1-/-* parabiont revealed tdTom+ leukocytes readily enter the injury site, comparable to WT mice (Kalinski et al., 2020). In the distal nerve stump of *Sarm1-/-*, tdTom+ leukocytes were present, however they were fewer than in parallel processed WT parabionts and they were F4/80 negative

(data not shown). Thus, parabiosis experiments independently show that WD is not necessary for immune cell entry into the distal nerve, however nerve fiber disintegration is necessary for the full-blown immune response associated with WD.

3.3.5 Monocytes enter the distal nerve prior to WD

Analysis of scRNA-seq data generated from *Sarm1-/-* 7d distal nerve tissue, identified Mo as the main immune cell type (**Figure 3.9**). They comprise 30% of the immune cells in the distal nerve, but only 6% of the immune cells at the injury site. In comparison, the contribution of Mac^{epi} to the immune cell population is 13% in the distal nerve and 9% for the injury site, further underscoring the expansion of Mo in the distal nerve stump of *Sarm1-/-* mice (Figure 3.10). In *Sarm1-/-* nerves, the two most abundant Mac populations at the injury site (clusters 4 and clusters 9) make up 23% and 24%, respectively, while the corresponding Mac in the distal nerve cluster 4 (0.3%) and cluster 9 (4%) are greatly reduced. Mac in cluster 23 make up 11% in the distal nerve and 6% of the injury site. Lymphocytes, TC (5%) and NK (4%) are found at the injury site and the distal nerve, TC (7%) and NK (7%) (**Figure 3.10**).

Strong chemotactic CCR2-CCL2 signaling is a well-known mechanism used to recruit circulating monocytes that express CCR2 to tissue sites that release CCL2 upon injury (Pan et al., 2020). When we examined our *Sarm1-/-* 7d distal nerve scRNA-seq data for CCL2 expression, we found the highest transcript levels in eMES and to a lesser but still significant extent throughout the Mo population (**Figure 3.11**). Circulating monocytes express high levels of the protein Ly6C, levels of which remain high for a short time following extravasation. However, upon nerve entry Mo receive signals from the nerve micro-environment and begin to differentiate and reduce expression of Ly6C to become a Mo/Mac Ly6C "intermediate" population and later a $Ly6C^{-}$ population once they become mature Mac (Kalinski et al., 2020). We employed flow cytometry to assess differences in infiltration or maturation of Mo to Mac in the sciatic nerve following SNC in WT and *Ccr2* KO mice. Nerves from these mice were crushed and 3d post-injury harvested and divided into injury site and distal nerve segments for further analysis. As previously observed, $Ly6C^{hi}$ Mo were recruited to the WT nerve and at 3d post-injury began to shift toward a $Ly6C^{int}$ population (Kalinski et al., 2020). Similar to WT and *Sarm1-/-* mice, we found more Mac in *Ccr2-/-* injury site than in the distal nerve (**Figure 3.12**). Though strikingly, in both injury site and distal nerve of the *Ccr2-/-* nerve we found a distinct lack of $Ly6C^{hi}$ Mo at 3d post-injury. Mo in the WT

injury site numbered 924 per segment compared to 148 per segment in the *Ccr2-/-* nerve. This was recapitulated in the distal stump with 851 Mo per segment found in WT samples and 47 Mo per segment in *Ccr2-/-* samples. These data indicate that *Ccr2-/-* mice lack recruitment of circulating Mo into the injured nerve. Intriguingly in the injury site of the *Ccr2-/-* nerve, there are more Ly6C^{int} cells—6,076 per segment—than in the WT injury site—4,079 per segment (**Figure 3.12**). Suggesting that a compensatory reaction from tissue-resident Mac substitutes for Ly6C^{int} cells that would be sourced from CCL2-CCR2-recruited Ly6C^{hi} Mo.

3.3.6 In the injured sciatic nerve macrophages "eat" apoptotic neutrophils

We previously showed that blood-borne leukocytes that enter the injured sciatic nerve are eaten by Mo/Mac and to a lesser extent by MoDC in a process termed efferocytosis. Efferocytosis is a key mechanism for inflammation resolution, as apoptotic cell corpses are eaten before they burst and release DAMPs into the environment. Because different immune cell types accumulate in large numbers in the injured nerve, it is not clear what cells form the "prey" eaten my macrophages. In order to identify the apoptotic cell types that are "food" for efferocytic macrophages, we employed genetic labeling of immune cells. We previously used parabiosis to label all blood-borne immune cells that enter the injured nerve (Kalinski et al., 2020). We generated R26$^{LSL\text{-}Tomato}$ reporter mice that express tdTom under the transcriptional control of the *Ly6g* promoter to selectively label granulocytes. The most abundant granulocyte population that infiltrates the injured sciatic nerve are neutrophils. They peak around 1d post SNC and are preferentially found at the injury site. Neutrophils have a short half-live time and are a well-known prey for efferocytic macrophages in non-neural tissues (Horckmans et al., 2017). We first validated the effectiveness of the neutrophil reporter with preliminary studies identify many tdTom$^+$ cells at the injury site in the 1d injured nerve (**Figure 3.13A**). Next, we analyzed a cohort of 3 reporter mice by flow cytometry. Whole sciatic nerves at 1d and 3d post-SNC were dissected and examined for the presence of F4/80$^+$ macrophages that are also tdTom$^+$ cells. Because neutrophils do not express F4/80, cells that are F4/80$^+$tdTom$^+$ mark macrophages that have eaten apoptotic (tdTom$^+$) neutrophils. At 1d post-injury, 184 F4/80$^+$tdTom$^+$ cells per nerve are detected and 128 F4/80$^+$tdTom$^+$ cells per nerve at the 3d timepoint (**Figure 3.13B**). Taken together, these data indicate that neutrophils are eaten in the injured nerve by macrophages, whereby they will promote inflammation resolution.

3.4 Discussion

Common causes of PNS injury are physical trauma, diabetes, and chemotherapy, all of which can trigger nerve fiber damage, functional deficits, and pain syndromes. Focusing on sciatic nerve compression injury, we carried out a detailed analysis of injury induced nerve inflammation at the nerve compression site and within the distal nerve stump. The study identified two distinct immune compartments comprised of overlapping, yet distinct immune cell populations. The injury site harbors two prominent macrophage subpopulations that are largely absent from the distal nerve stump where axons undergo WD. We propose that Mac subpopulations specific to the injury site respond to physical trauma induced necrotic cell death and release of DAMPs that trigger a pro-inflammatory immune response. In the distal nerve stump, severed axons undergo WD in the absence of necrotic cell death, thus creating a micromilieu distinct from the nerve injury site. The immune compartment in the 3d and 7d injured nerve is dominated by cells in the MPS, including 5 Mac subpopulations in the 3d nerve and 7 Mac subpopulations at 7d, as identified by scRNA-seq. Compared to other immune cell types, Mac are highly plastic and show global changes in gene expression, suggesting they change their phenotype, and by extension their function. The wide-ranging changes in Mac gene expression make it difficult to assign subcluster identifies in the 3d injury nerve to the corresponding Mac subclusters in the 7d nerve. Compared to Mac, other cell populations in the MPS are less plastic and show similar gene expression profiles in the 3d and 7d injured nerve, these include Mo, MoDC, cDC and pDC. In a similar vein, gene expression profiles of lymphocytes, including T cells and NK are more stable at 3d and 7d.

The immune response to nerve trauma and WD overlap and are difficult to untangle. To temporally separate the immune response to nerve trauma from WD, we subjected *Sarm1-/-* mice to SNC and micro-dissected nerve tissue the contains the injury site or distal nerve tissue. Flow cytometry and scRNA-seq revealed that the immune response to SNC is remarkably similar between WT and *Sarm1-/-* mice. The same immune cell types are present at the injury site at comparable numbers. Moreover, scRNA-seq identified the same Mac subpopulations and they exhibit highly similar gene expression profiles as in the injured WT nerve. This further underscore that immune cells at the injury site respond to tissue wounding and not to WD. Surprisingly, the distal nerve in *Sarm1-/-* is inflamed 7d post-SNC, a time point long before any physical signs of axon degeneration are observed. This shows that axonal transection causes a stress response within the distal axon that leads to the release of chemotactic molecules long before axon fragmentation

and myelin disintegration. Upon closer examination we identified Mo as the main cell population in the distal nerve of *Sarm1* KO mice, while macrophages are reduced compared to injured WT nerves. To demonstrated that blood-borne leukocytes enter the distal nerve of injured *Sarm1-/-* mice, we employed *Sarm1/tdTomato* parabiosis and detected Tdtom+/F4/80- immune cells. This suggests that Mo chemotactic signal are released from the distal nerve in *Sarm1-/-* mice. Analysis of scRNA-seq datasets generated from*Sarm1-/-* distal nerve identified high levels of *Ccl2* expression by eMES, suggesting that CCL2 via CCR2 expressed by Mo, promotes entry of circulating Mo into the distal nerve stump. While CCL2-CCR2 is known to function in Mo chemotaxis, the role of this ligand-receptor pair in Mo entry into the injury site and the distal nerve stump has not yet been examined. Experiments with *Ccr2-/-* mice show that entry of CD11b+Ly6C+ Mo into the nerve injury site as well as the distal nerve stump is significantly reduced in *Ccr2* mice. However, in *Ccr2-/-* mice the injured nerve is still inflamed and harbors many CD11b+Ly6C^{int} cells, indicating that additional chemotactic mechanisms are at play, including proliferation of endoneurial macrophages.

3.5 Materials and Methods

3.5.1 Animals

All procedures involving mice were approved by the Institutional Animal Care and Use Committee at the University of Michigan and Weill Cornell Medicine, and performed in accordance with guidelines developed by the National Institutes of Health. Adult (8–16 week-old) male and female mice on a C57BL/6 background were used throughout the study. Mice were housed under a 12 hr light/dark cycle with standard chow and water ad libitum.

3.5.2 Surgical procedures

All surgeries were carried out under aseptic conditions. Mice were deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) or with isoflurane (5% induction, 2–3% maintenance, SomnoSuite Kent Scientific). Buprenorphine (0.1 mg/kg) was given pre-emptively and post-operatively.

3.5.2.1 Sciatic nerve crush injury

For sciatic nerve surgery, thighs were shaved and disinfected with 70% ethanol (Covidien, 6818) and iodine (PDI Healthcare, B40600). A small incision, at mid-thigh, was made on the skin, underlying muscles separated, and the sciatic nerve exposed. For sham operated mice, the nerve was exposed but not touched. For SNC, the nerve was crushed for 15 s, using fine forceps (Dumont #55, Roboz Surgical Instruments, RS-5063). Skin was closed with 7 mm reflex wound clips (Cell Point Scientific, 203–1000).

3.5.3 Immunofluorescence staining

For immunofluorescence staining of neural tissues, mice were killed and perfused transcardially with ice-cold PBS for 2 min followed by ice-cold, freshly prepared 4% paraformaldehyde for 10 min. Spinal cord, sciatic nerves, and L4-L5 DRGs were collected and post-fixed in perfusion solution overnight. After that the solution was switched to 30% sucrose in PBS and tissues were kept at 4°C for at least 12 hr. Tissues were covered with tissue Tek (Electron Microscopy Sciences, 62550–01) and stored at −80°C. Longitudinal sciatic nerve sections were cut at 12 μm thickness using a cryostat (Leica Biosystems, CM3050S). Sciatic nerve sections were mounted on Superfrost+ microscope slides (Fisher Scientific, 12-550-15) and air dried for at least 12 hr before staining. Anti-F4/80 was the only antibody used (1:500; Thermo Fisher Scientific, MA1-91124).

3.5.4 Western blot analysis

Sciatic nerves were dissected and lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 3.5 mM sodium dodecyl sulfate, 12 mM sodium deoxycholate, pH 8.0) supplemented with 50 mM β-glycerophosphate (Sigma-Aldrich, G9422-100G), 1 mM Na3VO4 (Sigma-Aldrich, S6508-10G), and protease inhibitor cocktail (1:100, Sigma-Aldrich, P8340-5ML). Tissues were kept on ice, briefly homogenized with a motorized tissue homogenizer (RPI, 299200), and subjected to sonication (Fisher Scientific Sonic Dismembrator, Model 500) at 70% amplitude for 3 s. Tissue lysates were centrifuged at 15,000 rpm at 4°C for 10 min (Eppendorf, 5424R). The supernatant was transferred to a new tube and protein concentration was measured with a DC Protein Assay Kit (Bio-Rad, 5000111) using a photospectrometer at 750 nm (Molecular Devices, SpectraMax M5e). Samples were diluted with

2x Laemmli sample buffer (Bio-Rad, 1610737) containing 5% β-mercaptoethanol (EMD Millipore, 6010), boiled for 10 min at 100°C, and stored at −80°C for analysis. For SDS-PAGE, equal amounts of total protein (5–10 μg) were loaded per lane of a 15% gel. Separated proteins were transferred onto PVDF membrane (EMD Millipore, IPVH00010) for 2.5 hr at 200 mA in cold transfer buffer (25 mM TrisHCl, 192 mM Glycine, 10% Methanol). Membranes were blocked in 5% blotting-grade blocker (BioRad, 1706404) prepared in 1x TBS-T (TBS pH 7.4, containing 0.1% Tween- 20) for 1 hr at room temperature, and probed overnight at 4°C with the following primary antibodies diluted in 1x TBS-T with 3% BSA (Fisher Scientific, BP1600): α-CD11b (1:1000, Abcam, ab133357), α-ERK1/2 (1:5000, Cell Signaling Technologies, 9102). Horseradish peroxide (HRP)-conjugated α-rabbit secondary IgG (EMD Millipore, AP182P) were used. All HRP-conjugated secondary antibodies were diluted at half the dilution of the corresponding primary antibody in 3% BSA in 1x TBS-T, and the HRP signal was developed with various strengths of chemiluminescent substrates from Thermo Fisher Scientific (Pico Plus, 34580 or Femto, 34095) or from Li-COR Biosciences (926-95000). Protein band intensity was visualized and quantified in the linear range using LI-COR C-Digit (CDG-001313) and Image Studio Software (Version 5.2.5).

3.5.5 Flow cytometry

3.5.5.1 Tissue harvest and isolation

Adult mice, naïve and at d3 and d7 post-SNC were deeply anesthetized with a mixture of Xylazine and Ketamine and perfused transcardially with ice-cold phosphate-buffered saline (PBS) for 5 min. Injured and uninjured sciatic nerves were dissected. In some cases from injured nerves, the proximal stump, distal stump, and injury site were separately harvested and multiple samples pooled. Similar sized segments from uninjured nerves were collected for comparison.

3.5.5.2 Tissue processing and flow cytometry

To analyze immune cell profiles in sciatic nerves (SN), mice were transcardially perfused for 5 min with ice-cold PBS to flush out all blood cells in circulation. SN were harvested bilaterally. For analysis of SN from 2 to 3 mice (2 SN per mouse x 2–3 mice = 4–6 SN) were pooled separately and used for one run. The collected nerve segments were cut into small pieces

with microscissors and incubated in 1 ml collagenase (4 mg/ml Worthington Biochemical, LS004176) and dispase (2 mg/ml, Sigma-Aldrich, D4693) in PBS for 30–45 min at 37°C degrees in a 15 mL conical tube. Tissues were gently triturated with a P1000 pipette every 10 min. Next, samples were rinsed in DMEM with 10% FBS and spun down at 650 g for 5 min. This resulting pellet gently re-suspended in 1 mL of 27% Percoll (Sigma Aldrich, P4937) in PBS. Then 3 ml of 27% Percoll were added to bring the final volume to 4 ml. Samples were spun at 900 g for 20 min in a clinical centrifuge (Beckman Coulter Allegra 6R). The top layers (with myelin and other debris) were carefully aspirated. The final 100 µl were resuspended in 1 ml of PBS with 2% FBS and filtered through a pre-washed 40 µm Falcon filter (Corning, 352340). Cells were pelleted at 650 g for 5 min at 4°C. Cells were labeled with fixable viability dye (Thermo Fisher Scientific, 65086614), blocked with αCD16/32 (BD Pharmingen, 553141), and stained with fluorescent antibodies and isotype controls. Immune cells (CD45+) were further classified as myeloid (CD45+CD11b+), classic dendritic cells (CD45+CD11b-CD11c+Ly6G-), monocyte-derived dendritic cells (CD45+CD11b+Ly6G-CD11c+), granulocytes (CD45+CD11b+Ly6G+CD11c-), and monocyte/macrophages (CD45+CD11b+Ly6G-CD11c-). Data were acquired using a FACSCanto II (BD Biosciences) flow cytometer and analyzed with FlowJo software (Treestar) as described previously (Baldwin et al., 2015).

3.5.5.3 Antibodies

CD11b-PE-Cy7 (Thermo Fisher Scientific, 25-0112-82), Rat IgGk Isotype Control-PE-Cy7 (Thermo Fisher Scientific, 25-4031-82) CD45-e450 (Thermo Fisher Scientific, 48-0451-82), Rat IgG2b Isotype Control-e450 (Thermo Fisher Scientific, 48-4031-82), Ly6G-APC-Cy7 (BD Biosciences, 560600), Rat IgG2a Isotype Control-APC-Cy7 (BD Biosciences, 552770), CD11c-PerCP-Cy5.5 (Thermo Fisher Scientific, 45-0114-82), Arm Ham IgG Isotype Control-PerCP-Cy5.5 (Thermo Fisher Scientific, 45-4888-80), Ly6C-FITC (BD Biosciences, 561085), Rat IgM Isotype Control-FITC (BD Biosciences, 553942). All antibodies were used at a working concentration of 1:100 except for CD11b (1:200).

3.5.5.4 Statistics

Statistical analysis was performed in GraphPad Prism (v7) using paired or un-paired 2-tailed Student's t test, or 1-way or 2-way ANOVA with correction for multiple comparisons with

Tukey's post-hoc test, as indicated in the figure legends. A p value < 0.05 (*) was considered significant. p<0.01 (**), p<0.001 (***), and p<0.0001 (****).

3.5.6 Preparation of sciatic nerve samples for scRNA-seq

Mice were transcardially perfused with ice-cold PBS for 5 min to flush out all blood cells in circulation. The sciatic nerve trunk was harvested and a segment that contains the injury site and the distal nerve stump, up to the branch point of the tibial nerve, used for further processing. A minimum of three mice (six nerves) was used to obtain sufficient cells for analysis using the 10x Genomics platform. The collected nerve segments were cut into small pieces with microscissers and incubated in 1 ml PBS supplemented with collagenase (4 mg/ml Worthington Biochemical, LS004176), dispase (2 mg/ml, Sigma-Aldrich, D4693), and actinomycin D (45 µM, Sigma Aldrich, A1410) for 30–45 min at 37°C in a 15-mL conical tube. Tissues were gently triturated with a P1000 pipette every 10 min. Next, samples were rinsed in DMEM with 10% FBS and spun down at 650 g for 5 min before removing supernatant. The resulting pellet was gently re-suspended in 1 mL of 27% Percoll (Sigma Aldrich, P4937) in PBS. Then 3 ml of 27% Percoll were added to bring the final volume to 4 ml. Samples were spun at 900 g for 20 min with no brake in a clinical centrifuge (Beckman Coulter Allegra 6R). The top layers (with myelin and other debris) were carefully aspirated. The final 100 µl were resuspended in 1 ml of PBS with 2% FBS and filtered through a pre-washed 40 µm Falcon filter (Corning, 352340) with an additional 5 ml of PBS with 2% FBS. Cells were pelleted at 650 g for 5 min at 4°C. The supernatant was removed and the cell pellet resuspended in 180 µl of MACS buffer (Miltenyi, 130-091-376) diluted 1:20 in PBS (final bovine serum albumin [BSA] was 0.5%) and 10 µl of myelin removal beads were added (Miltenyi, 30-096-731). To remove all myelin debris, cells were incubated with myelin depletion beads for 15 min at 4°C with intermitted tapping. Cells were rinsed in 5 ml of MACS buffer, gently inverted several times and spun at 300 g for 10 min. Cells were separated from myelin beads using the MidiMACS separator (Miltenyi, 130-042-302) and LS columns (Miltenyi, 130-042-401). The flow through solution with the cells was centrifuged and the cells resuspended in 50 µl of Hanks balanced salt solution (Gibco, 14025092) supplemented with 0.04% BSA (Fisher Scientific, BP1600). The cell number and live/dead ratio was determined using propidium iodine labeling and a hemocytometer.

3.5.7 10x genomics scRNA-seq library preparation

For encapsulation of single cells with microbeads into nanodroplets, the Chromium Next GEM Single Cell 3' GEM Library and Gel Bead Kit v3.1 and Chromium Next GEM Chip G Single Cell Kit were used. Approximately 12,000 cells in a final volume of 43 µl were used for barcoding, using the 10X Genomics Chromium Controller. The library preparation of barcoded cDNAs was carried out in a bulk reaction, following instructions provided by the manufacturer. A small aliquot of the library was used for quality control with a bioanalyzer followed by library sequencing at the Advanced Genomics Core of the University of Michigan. The NovaSeq Illumina 6000 was used with an S4 flowcell, yielding 1.05 Billion reads (7–11% of the flowcell) (Individual samples ranging from 290 to 424 million reads). NovaSeq control software version 1.6 and Real Time Analysis (RTA) software version 3.4.4 were used to generate binary base call (BCL) formatted files.

3.5.8 Sequencing data analysis

Raw scRNAseq data were processed using the 10x Genomics CellRanger softeware version 3.1.0. The CellRanger 'mkfastq' function was used for de-multiplexing and generating FASTQ files from raw BCLs. The CellRanger 'count' function, with default settings was used with the mm10 reference supplied by 10x Genomics, to align reads and generate single cell feature counts. Per sample, approximately 5800 cells with a median of 2507 genes per cell were obtained. CellRanger filtered cells and counts were used for downstream analysis in Seurat version 3.1.2 implemented in R version 3.6.2. Cells were excluded if they had fewer than 200 features, more than 7500, or the mitochondrial content was more than 25%. Reads from multiple samples were merged and normalized following a standard Seurat SCTransform integration pipeline (Hafemeister and Satija, 2019); mitochondrial mapping percentage was regressed out during the SCTransform normalization step. Principal component analysis was performed on the top 3000 variable genes and the top 30 principle components were used for downstream analysis. A K-nearest neighbor graph was produced using Euclidean distances. The Louvain algorithm was used with resolution set to. five to group cells together. Non-linear dimensional reduction was done using UMAP. The top 100 genes for each cluster, determined by Seurat's FindAllMarkers function and the Wilcoxon Rank Sum test, were submitted to version 11 of the string-db.org to determine functional enrichment; referred to as STRING analysis.

To model developmental trajectories of cells that comprise the mononuclear phagocyte system (MPS), the Bioconductor package, slingshot version 1.4.0 was used. The integrated Seurat object was subset to include only MPS cells and slingshot was instructed to start from monocytes. The pseudo-time from the three slingshot constructed lineages were used in random regression forest to reveal the most influential genes, on pseudo-time. Random forests were implemented with the Ranger package of R from 1400 trees, 200 genes at each node, and the Gini index, 'impurity', measure for gene importance. The bulk RNA-seq and scRNA-seq data is available online in the Gene Expression Omnibus (GEO) database (GSE153762).

Cell identities, as defined above, were saved for the 3d injured nerve. Single-cell transcriptomes from YFP.pos and YFP.neg macrophage populations identified in naïve peripheral nerve tissue (Wang et al., 2020), were downloaded and given the label Mac_Naive. The log2 transformed raw counts of the 3d injured Mac1-5 and Mo as well as the Mac_Naive cells were subjected to batch correction using the ComBat function from the Bioconductor 'sva' package (Leek et al., 2012). Injured nerve Mo/Mac and naïve Mac made up the two batches and the following arguments were passed to ComBat: mod = NULL, par.prior = TRUE, mean.only = FALSE, prior.plots = FALSE. After batch correction each cell type and gene had a highly repeated minimum number near 0. To aid in plotting and determining 'percent expressed' this value was replaced with 0. The average expression for each gene and each cell type was calculated for the purpose of making dotplots. Any cell type with more than 85% zeros was not given a dot. The dots represent percent expressed by radius and average expression, scaled across cell type, by color.

3.6 Acknowledgements

We thank Dr. Kiwook Kim at the University of Illinous at Chicago for donating Ly6g$^{tm2621(cre)Arte}$ mice for use in identifying the prey for efferocytic macrophages in the sciatic nerve.

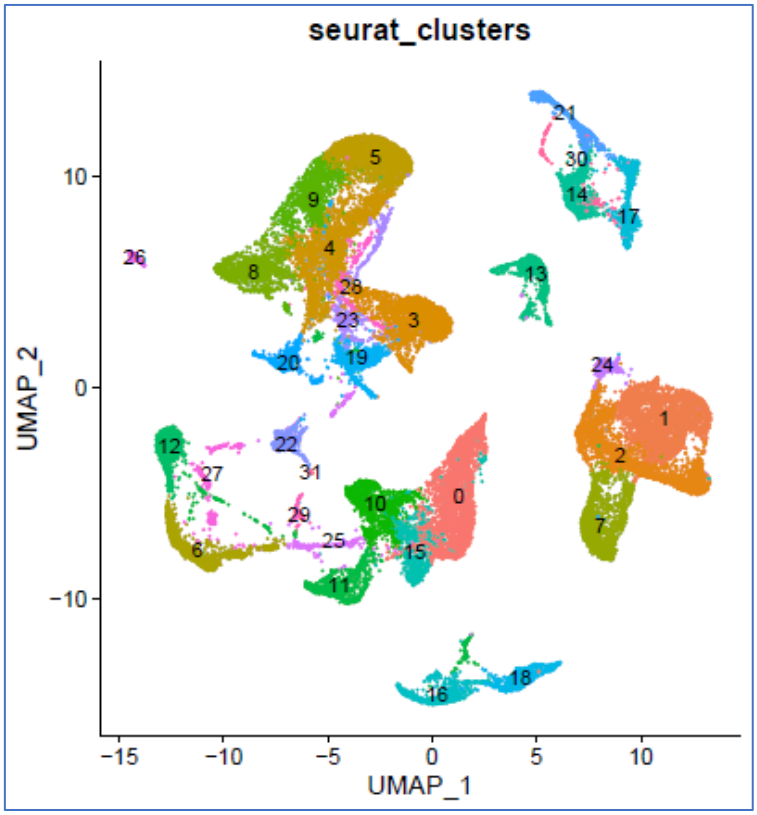
3.7 Author Contribution

Lucas D Huffman contributed to data acquisition, formal analysis, investigation, methodology, and writing. Ashley L Kalinski contributed to data acquisition, formal analysis, investigation, visualization, and methodology. Craig Johnson contributed to data curation, formal

analysis, and software. Hannah Hafner and Rafi Kohen contributed to data acquisition and visualization. Roman J Giger contributed to formal analysis, funding acquisition, investigation, methodology, supervision, and conceptualization.

3.8 Figures

A



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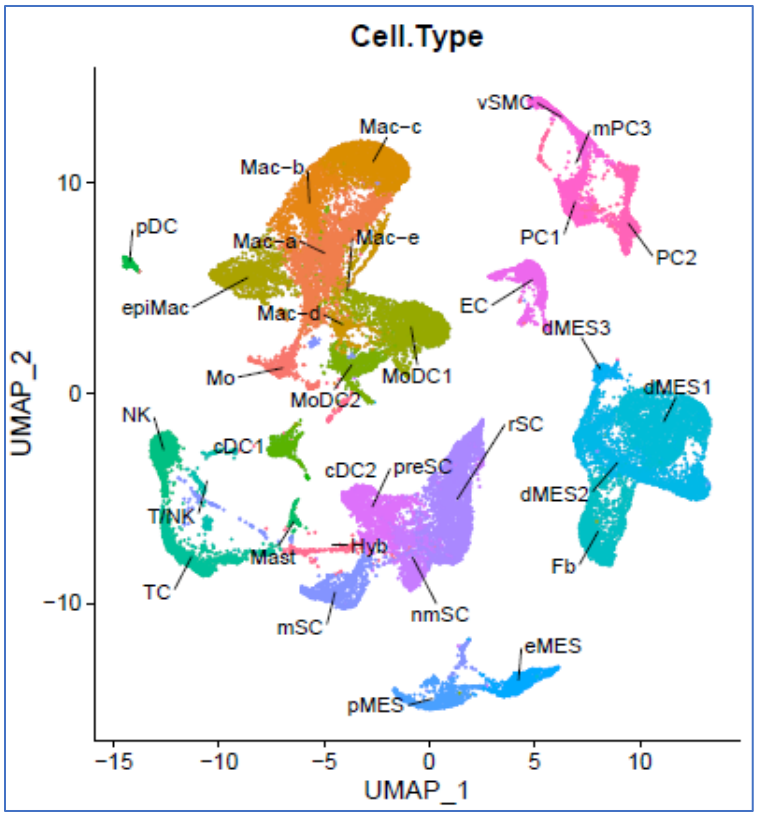
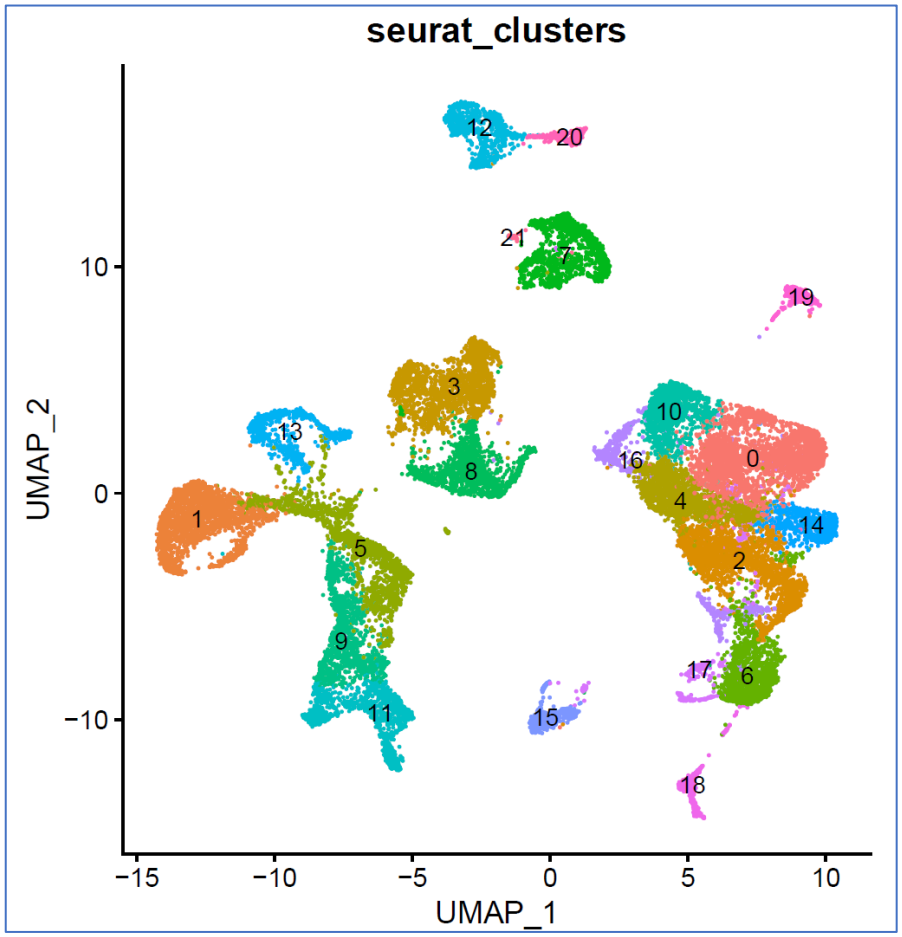


Figure 3-1 Distribution of cell clusters in the whole 7d post-injury sciatic nerve.
Singe-cell transcriptome of injured mouse sciatic nerve at d7 post-SNC, n = 5 biological replicates. A. Unsupervised Seurat-based clustering identifies 30 cell clusters. B. Cell clusters are labeled by numbers and cell type identity for each cluster, as determined by expression analysis of established markers.



Cluster	Cell Identity	Cluster	Cell Identity
0	Mac 1	11	Fb
1	eMES	12	PC1
2	Mac2	13	pMES
3	SC1	14	Mo
4	Mac3	15	T/NK
5	Mitotic MES	16	Mac5
6	MoDC	17	pDC
7	EC1	18	cDC
8	SC2	19	GC
9	dMES	20	PC2
10	Mac4	21	EC2

Figure 3-2 Distribution of cell clusters in the whole 3d post-injury sciatic nerve.
Singe-cell transcriptome of injured mouse sciatic nerve at d3 post-SNC, n = 6 biological replicates. Unsupervised Seurat-based clustering identifies 21 cell clusters (top). Cell clusters are labeled by numbers (bottom) and cell type identity for each cluster, as determined by expression analysis of established markers, is shown on the right..

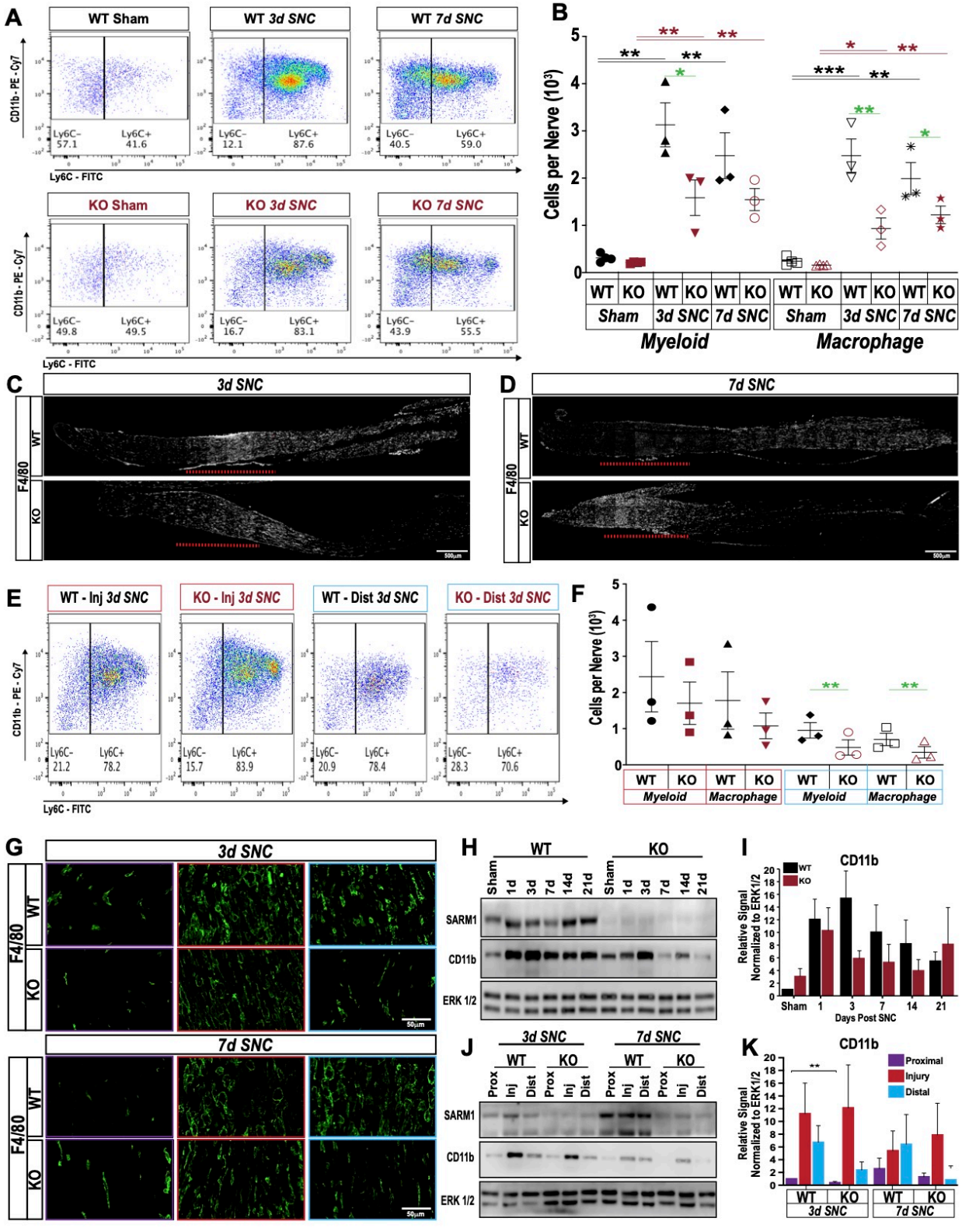
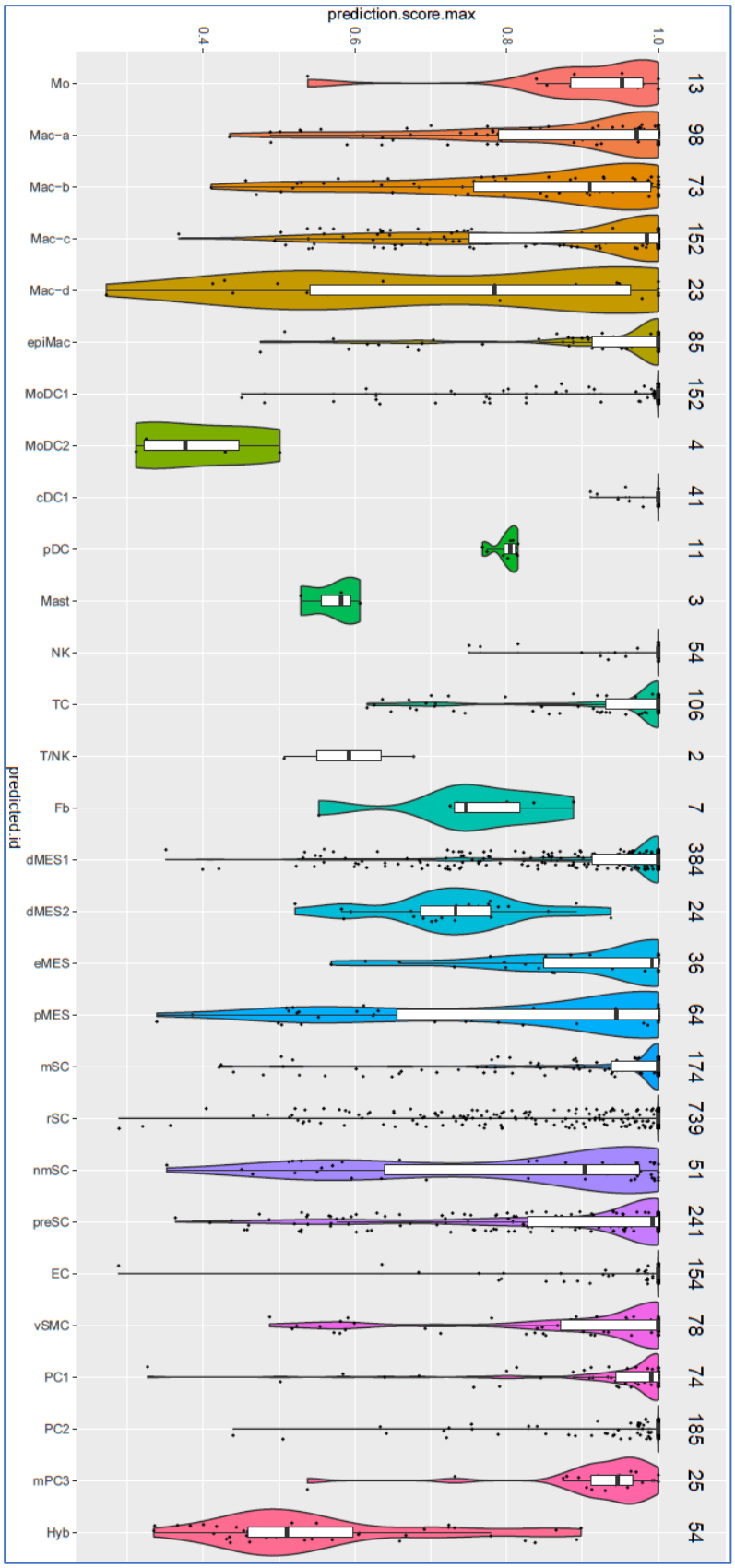

A
WT Sham
WT 3d SNC
WT 7d SNC
KO Sham
KO 3d SNC
KO 7d SNC
CD11b - PE - Cy7
Ly6C - FITC
Ly6C− 57.1
Ly6C+ 41.6
Ly6C− 12.1
Ly6C+ 87.6
Ly6C− 40.5
Ly6C+ 59.0
Ly6C− 49.8
Ly6C+ 49.5
Ly6C− 16.7
Ly6C+ 83.1
Ly6C− 43.9
Ly6C+ 55.5
B
Cells per Nerve (10³)
WT KO WT KO WT KO WT KO WT KO WT KO
Sham 3d SNC 7d SNC Sham 3d SNC 7d SNC
Myeloid
Macrophage
C
3d SNC
D
7d SNC
F4/80
WT
KO
E
WT - Inj 3d SNC
KO - Inj 3d SNC
WT - Dist 3d SNC
KO - Dist 3d SNC
Ly6C− 21.2
Ly6C+ 78.2
Ly6C− 15.7
Ly6C+ 83.9
Ly6C− 20.9
Ly6C+ 78.4
Ly6C− 28.3
Ly6C+ 70.6
F
Cells per Nerve (10³)
WT KO WT KO WT KO WT KO
Myeloid Macrophage Myeloid Macrophage
G
3d SNC
7d SNC
50μm
H
WT
KO
Sham 1d 3d 7d 14d 21d Sham 1d 3d 7d 14d 21d
SARM1
CD11b
ERK 1/2
I
CD11b
WT
KO
Relative Signal Normalized to ERK1/2
Sham 1 3 7 14 21
Days Post SNC
J
3d SNC
7d SNC
WT KO WT KO
Prox Inj Dist Prox Inj Dist Prox Inj Dist Prox Inj Dist
SARM1
CD11b
ERK 1/2
K
CD11b
Proximal
Injury
Distal
Relative Signal Normalized to ERK1/2
WT KO WT KO
3d SNC 7d SNC

Figure 3-3 Crush-induced inflammation and accumulation of immune cell profiles.
A. Flow cytometry dot plots showing monocyte/macrophage populations. Macrophages were categorized as CD45+CD11b+Ly6G-CD11c-Ly6C-. B. Quantification of myeloid (CD45+CD11b+) and macrophage populations in WT and SARM1 KO whole nerves from 3d sham-operated and 3d or 7d post-injury mice following sciatic nerve crush. C-D. Whole sciatic nerve sections from 3d and 7d post-injury mice stained with the macrophage marker F4/80. E. Flow cytometry dot plots of monocyte/macrophage populations in WT and SARM1 KO sciatic nerves 3d post-injury. The injury site and distal segment were micro-dissected and analyze separately. F. Quantification of myeloid cells and macrophages from WT and SARM1 KO nerves separated by injury site and distal segment. G. Magnified immunofluorescent images of 3d and 7d post-injury sciatic nerve segments. Images in purple boxes taken from the proximal stump; images in red boxes taken from the injury site; images in blue boxes taken from the distal stump. H-K. Western blot membranes and analyses of whole nerves across multiple injury timepoints or micro-dissected nerves 3d and 7d post-injury assessing SARM1 and CD11b expression. Two-tailed t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.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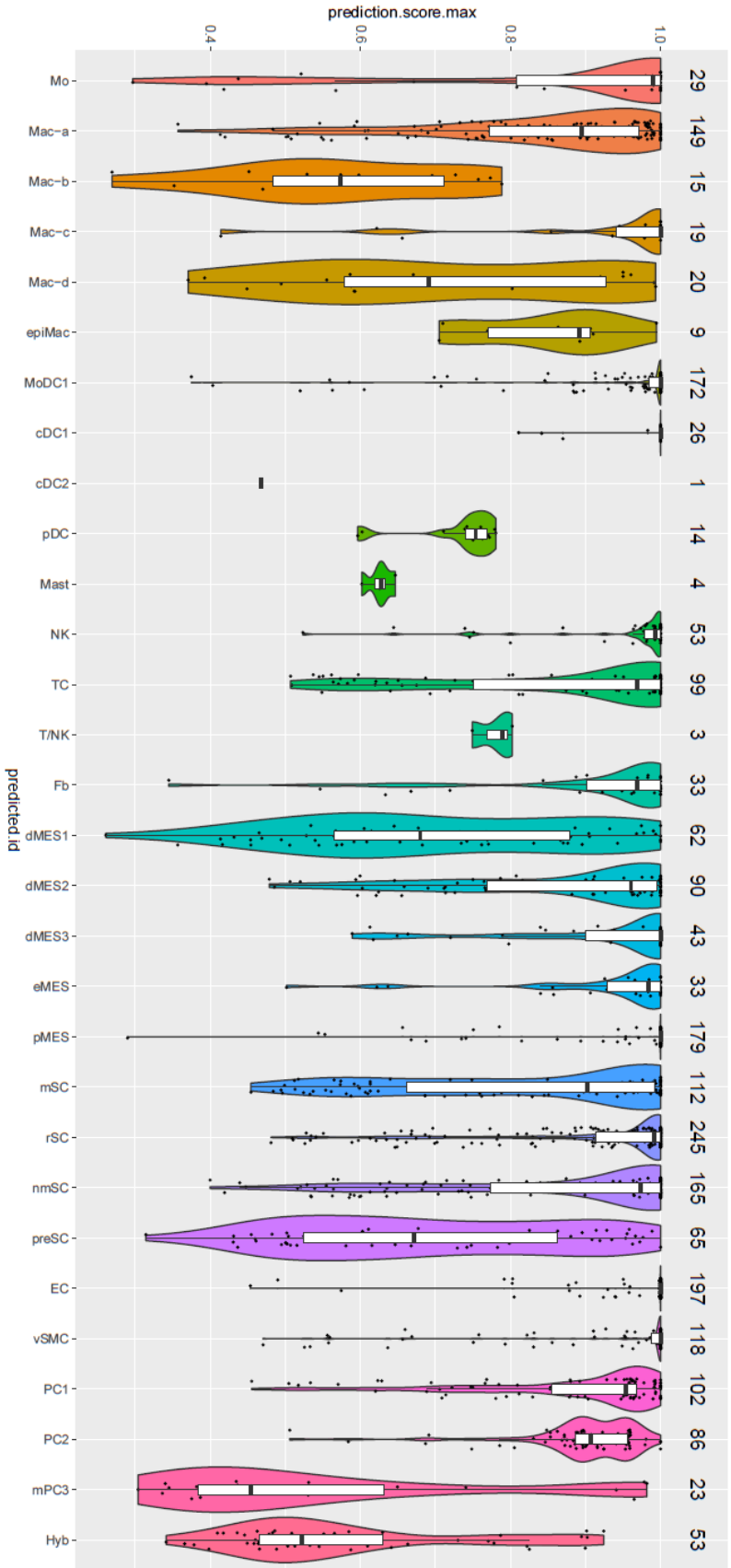
41
11
3
54
106
2
7
384
24
36
64
174
739
51
241
154
78
74
185
25
54

Figure 3-4 Cell cluster similarity between WT 7d injury site and whole nerve.
Single cell RNA-seq data of whole nerve, 7d post-SNC, was used as a reference and compared to cells identified by scRNA-seq of the microdissected injury site. The top 200 cell cluster defining genes were used for comparison in a violin plot and the percentile of similarity is shown on the y-axis. Cell cluster identity is shown on the x-axis. The number of cells assigned to each cell type is shown on top of the violin plot.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preSC
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mPC3
Hyb
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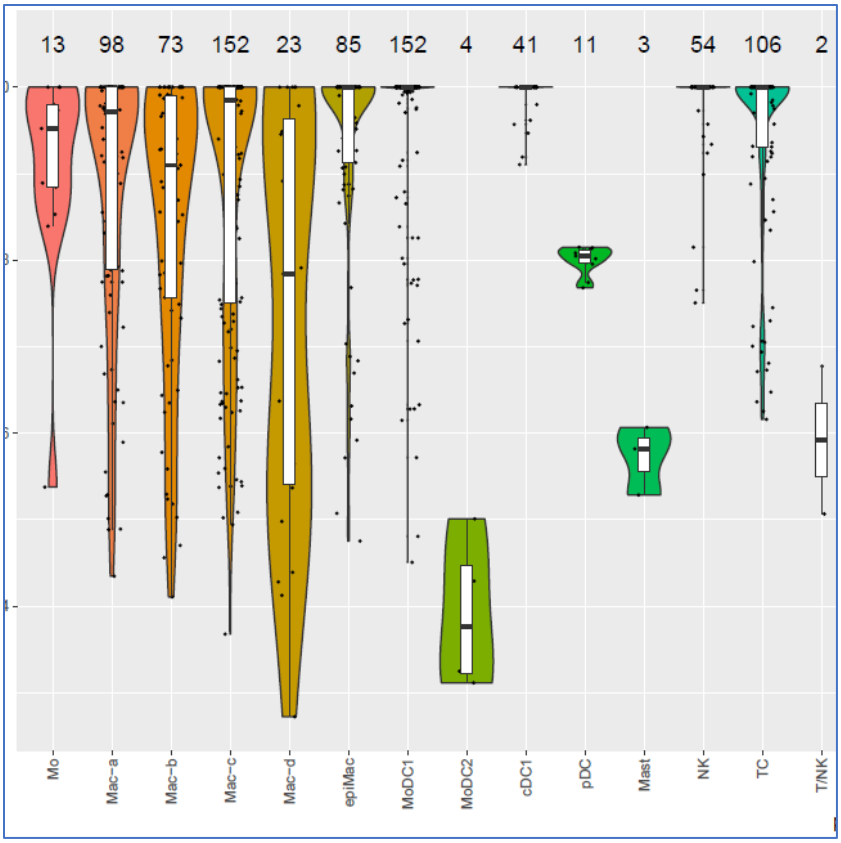
15
19
20
9
172
26
1
14
4
53
99
3
33
62
90
43
33
179
112
245
165
65
197
118
102
86
23
53

Figure 3-5 Cell cluster similarity between WT 7d distal segment and whole nerve.
Single cell RNA-seq data of whole nerve, 7d post-SNC, was used as a reference and compared to cells identified by scRNA-seq of the microdissected distal nerve. The top 200 cell cluster defining genes were used for comparison in a violin plot and the percentile of similarity is shown on the y-axis. Cell cluster identity is shown on the x-axis. The number of cells assigned to each cell type is shown on top of the violin plot.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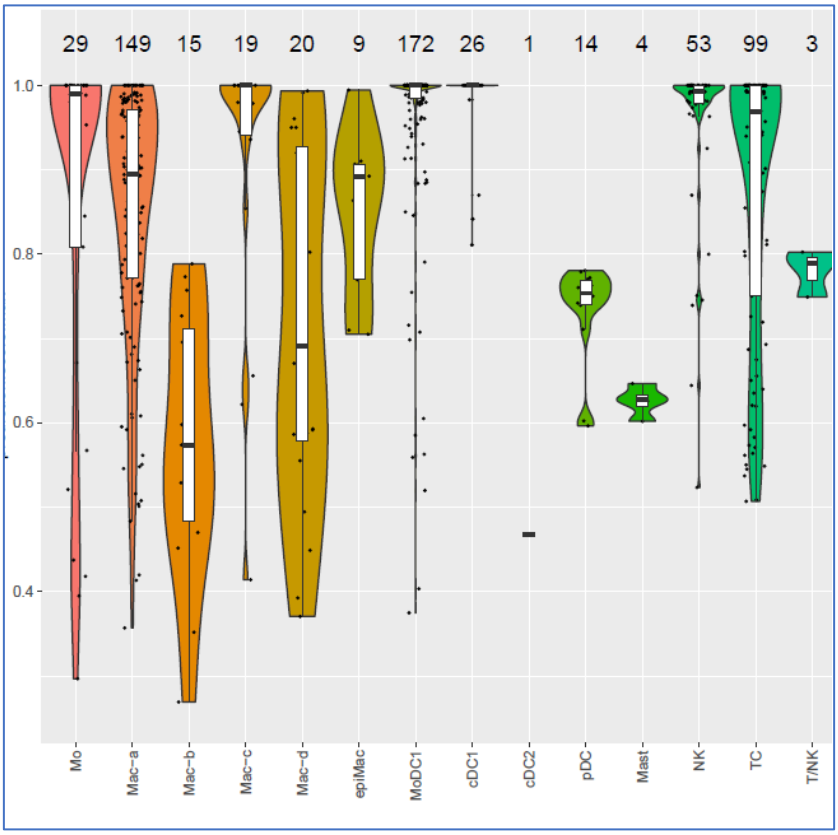
TC
T/NK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MoDC1
cDC1
cDC2
pDC
Mast
NK
TC
T/NK

Figure 3-6 Comparison between WT 7d injury and distal segments to whole nerve.
Immune cells identified by scRNA-seq of the micro-dissected injury site (top) and immune cells identified by scRNA-seq of the micro-dissected distal nerve (bottom) aligned to the whole nerve 7d reference data set. Note the immune cell number and composition is different between the injury site and the distal nerve.

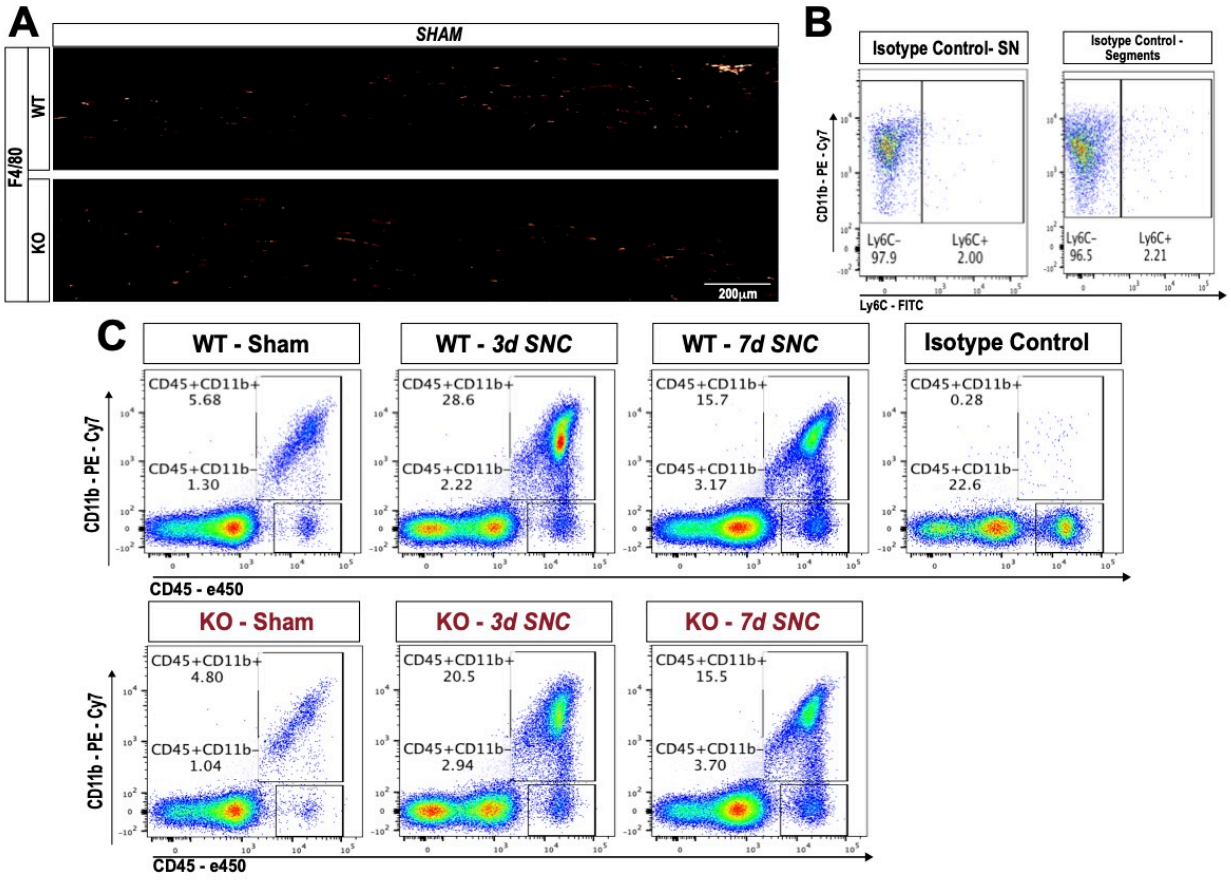
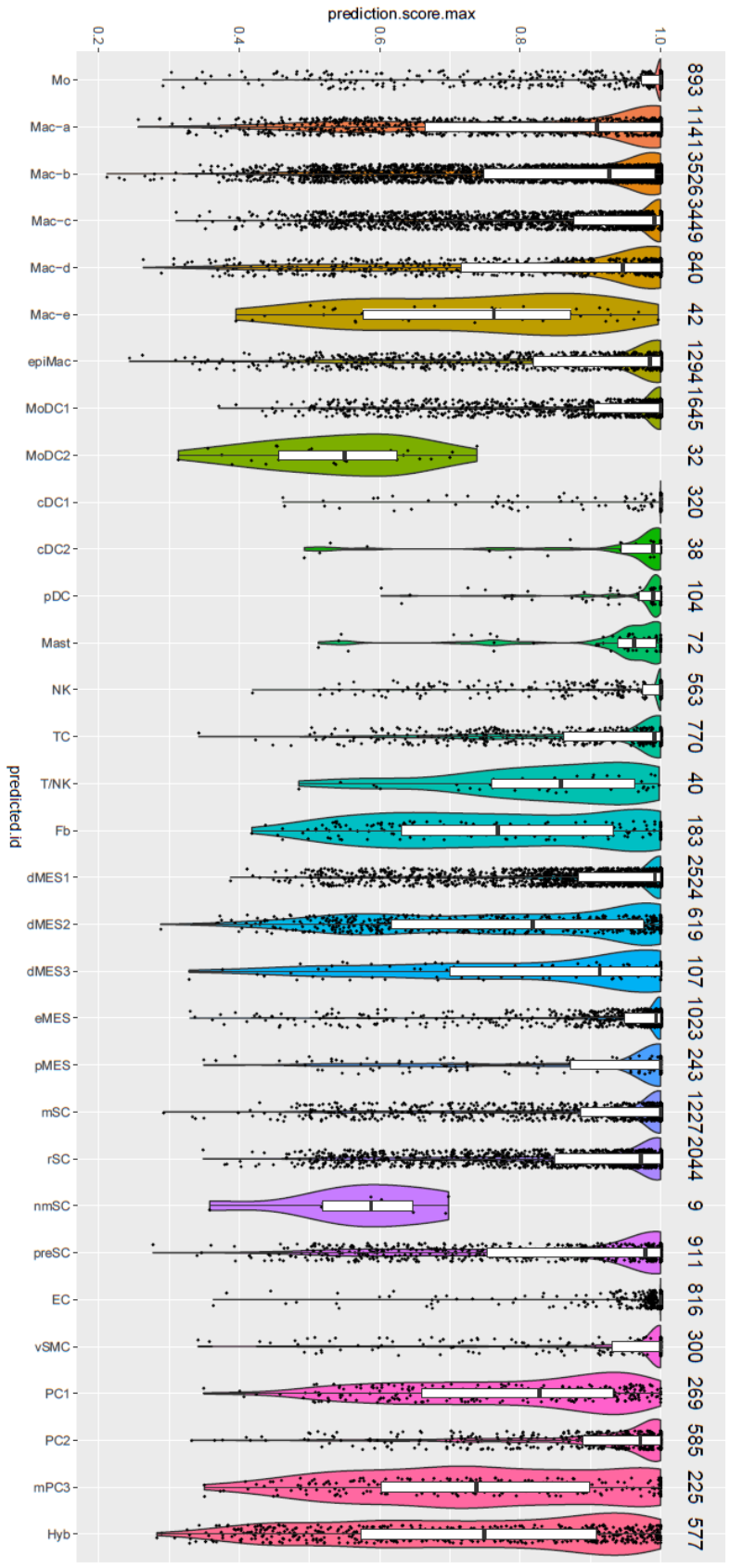


Figure 3-7 Assessment of changes in immune cells in sham and injury conditions.
A. Sciatic nerves taken from 3d sham-operated animals stained with macrophage marker F4/80. B Isotype control flow cytometry dot plots for Ly6C expression. C. Flow cytometry dot plots assessing CD45 and CD11b expression in whole sciatic nerves across multiple timepoints and between WT and KO mice.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dMES1
dMES2
dMES3
eMES
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mSC
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nmSC
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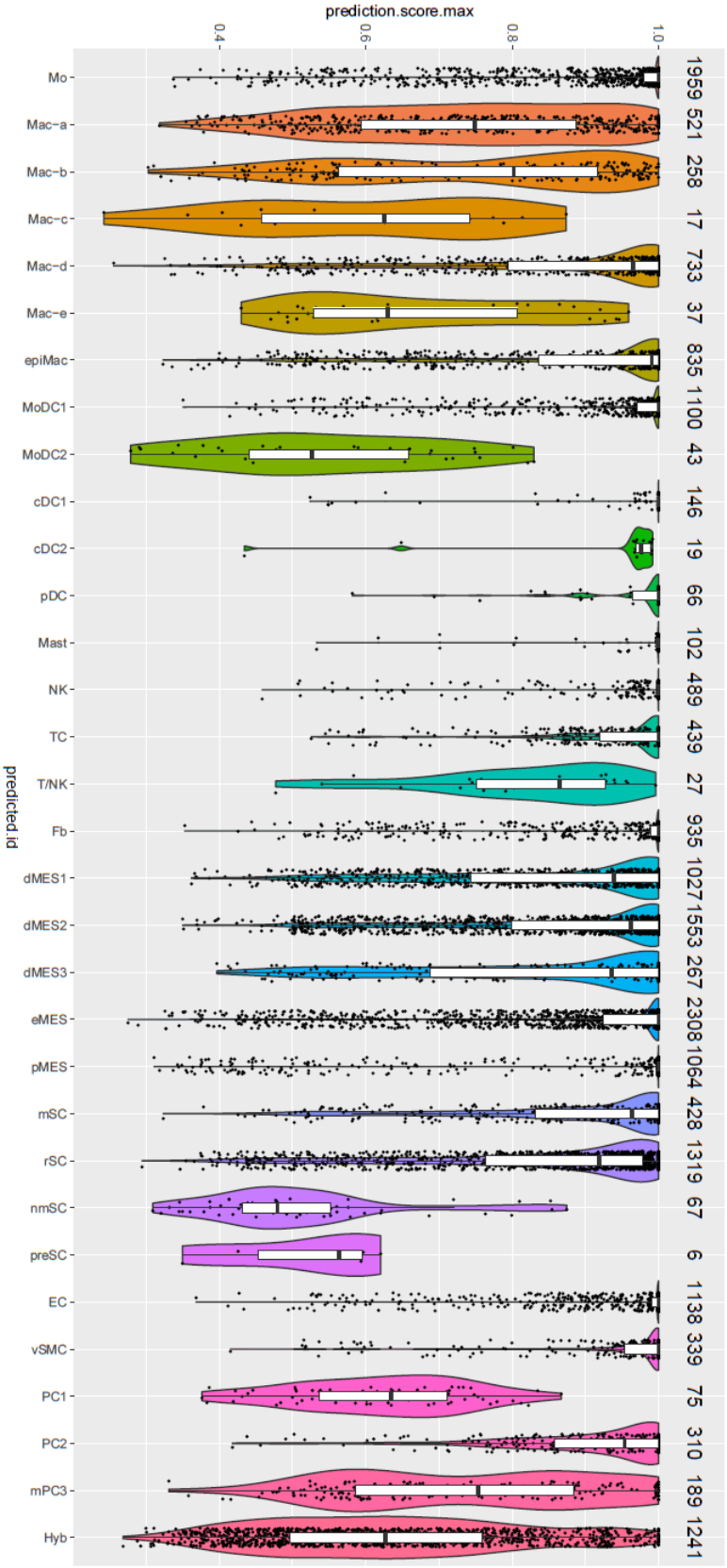
PC1
PC2
mPC3
Hyb
893
1141
3526
3449
840
42
1294
1645
32
320
38
104
72
563
770
40
183
2524
619
107
1023
243
1227
2044
9
911
816
300
269
585
225
577

Figure 3-8 Cell cluster similarity between Sarm1-/- 7d injury site and whole nerve.
Single cell RNA-seq data of whole nerve, 7d post-SNC, was used as a reference and compared to cells identified by scRNA-seq of the micro-dissected injury site from 7d injured Sarm1-/- mice. The top 200 cell cluster defining genes were used for comparison in a violin plot and the percentile of similarity is shown on the y-axis. Cell cluster identity is shown on the x-axis. The number of cells assigned to each cell type is shown on top of the violin plot.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Hyb
1959
521
258
17
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37
835
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43
146
19
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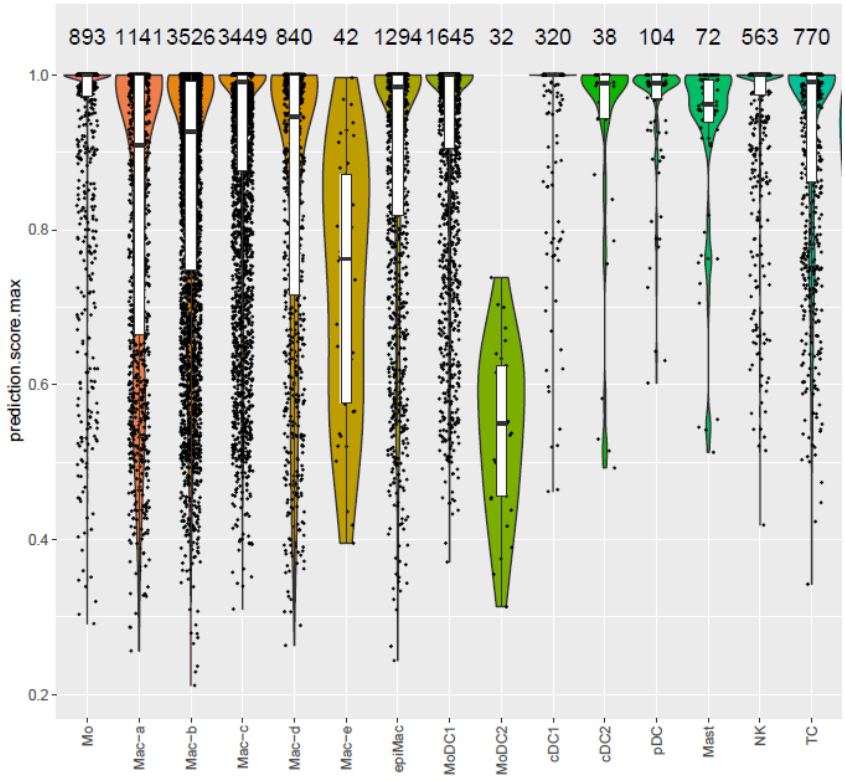
439
27
935
1027
1553
267
2308
1064
428
1319
67
6
1138
339
75
310
189
1241

Figure 3-9 Cell cluster similarity between Sarm1-/- 7d distal segment and whole nerve.
Single cell RNA-seq data of whole nerve, 7d post-SNC, was used as a reference and compared to cells identified by scRNA-seq of the micro-dissected distal nerve from 7d injured Sarm1-/- mice. The top 200 cell cluster defining genes were used for comparison in a violin plot and the percentile of similarity is shown on the y-axis. Cell cluster identity is shown on the x-axis. The number of cells assigned to each cell type is shown on top of the violin plot.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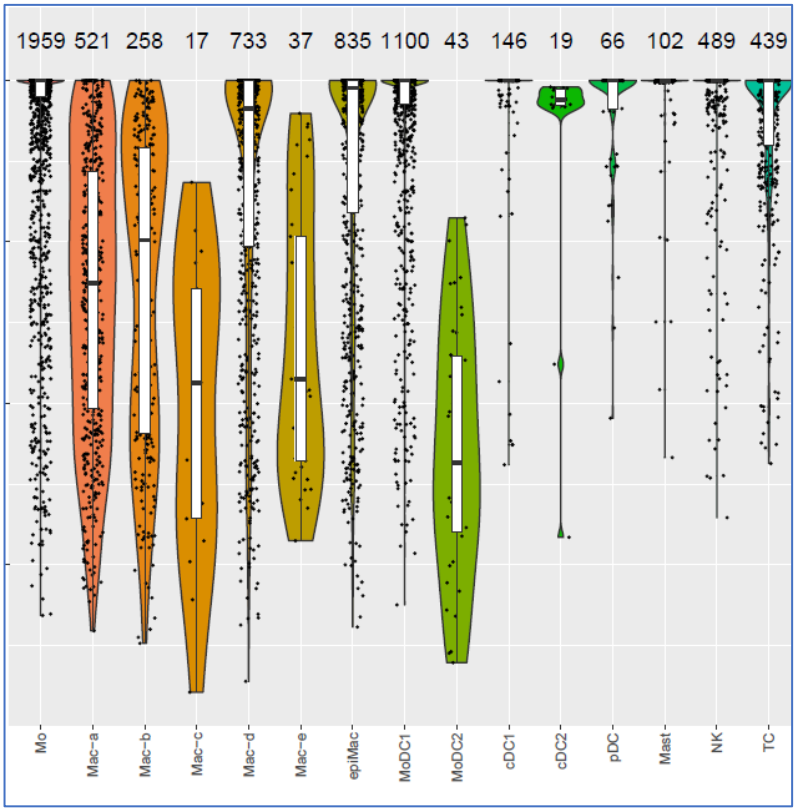
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MoDC2
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cDC2
pDC
Mast
NK
TC

Figure 3-10 Comparison of Sarm1-/- 7d injury and distal segments to whole nerve.
Immune cells identified by scRNA-seq of the micro-dissected injury site of Sarm1-/- mice (top) and immune cells identified by scRNA-seq of the micro-dissected distal nerve of Sarm1-/- mice (bottom), aligned to the whole nerve 7d WT nerve reference data set. Not the immune cell number and composition are different between the injury site and the distal nerve.

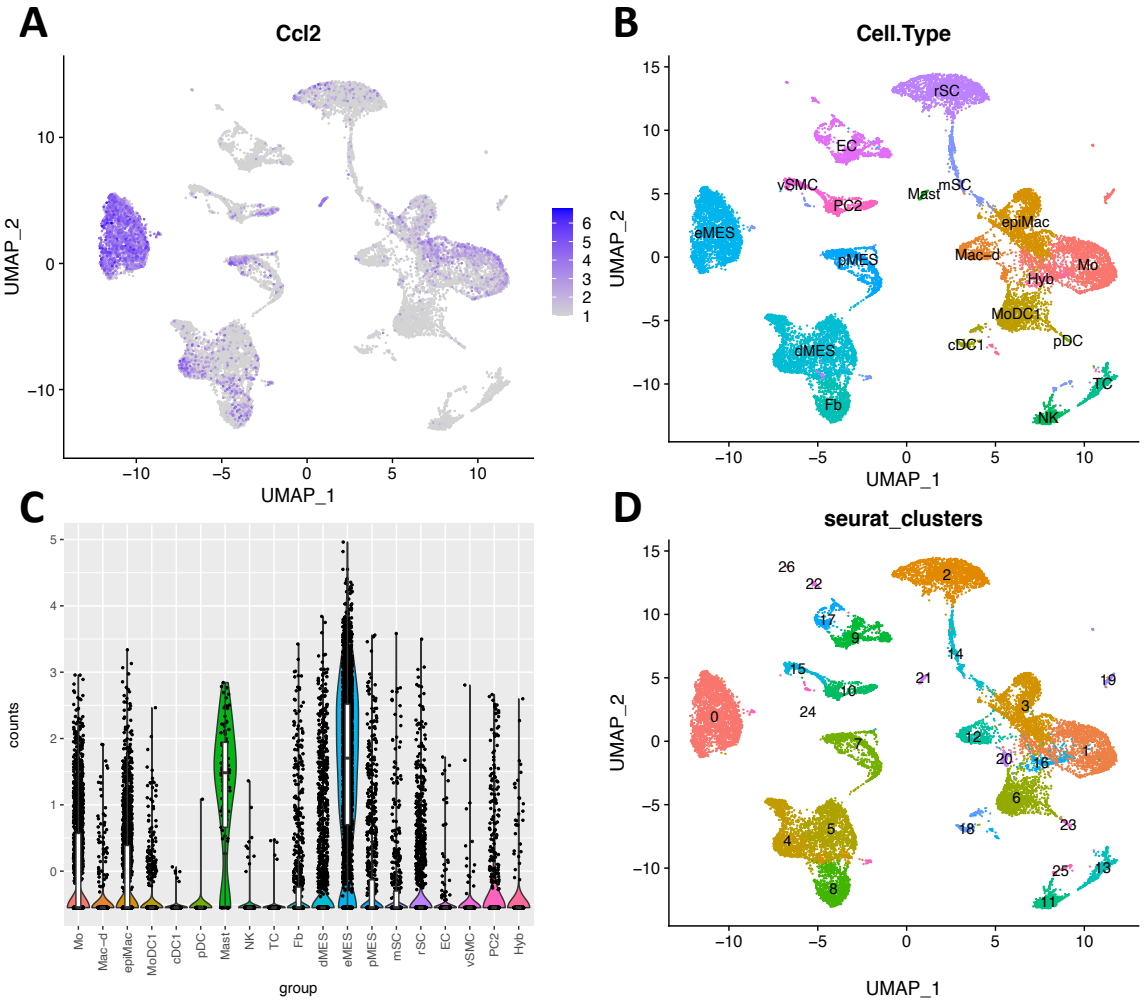

Figure 3-11 CCL2 expression in Sarm1-/- sciatic nerve 7d post-injury.
A. Feature plot for Ccl2 of 7d distal nerve stump of Sarm1-/- mice shows strong expression in eMES (endoneurial mesenchymal cells) and to a lesser extent in Mo (monocytes). B. UMAP plot with cluster identities. C. Violin plot showing Ccl2 expression in different cell populations identified in the 7d distal nerve stump of Sarm1-/- mice. D. Seurat clusters with numbered cell clusters.

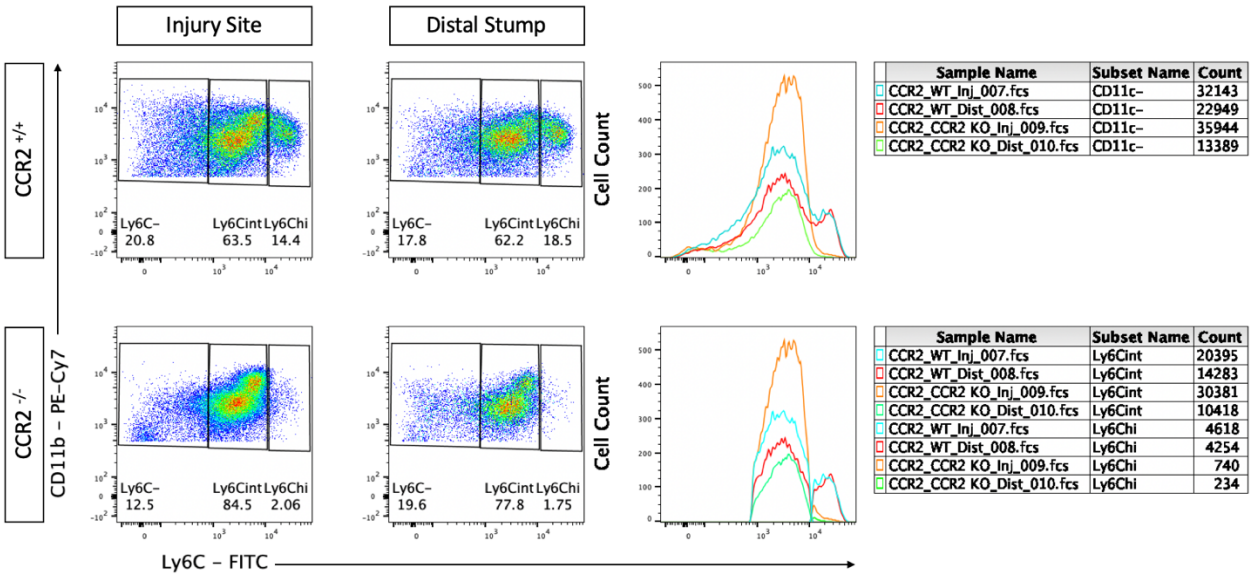

Figure 3-12 Lack of Ly6Chi monocytes in crushed sciatic nerves from Ccr2-/- mice.
Flow cytometry dot plots and quantitative histograms of Ly6C expression in 3d post-injury sciatic nerves separated into injury site and distal segments from WT and CCR2 KO mice (n=5 bilateral-crushed mice pooled per genotype). The top-right histogram shows all CD45+CD11b+Ly6G-CD11c- monocytes and macrophages. The bottom-right histogram displays the Ly6Cint mono/mac and Ly6Chi monocyte populations for comparison of absolute numbers.

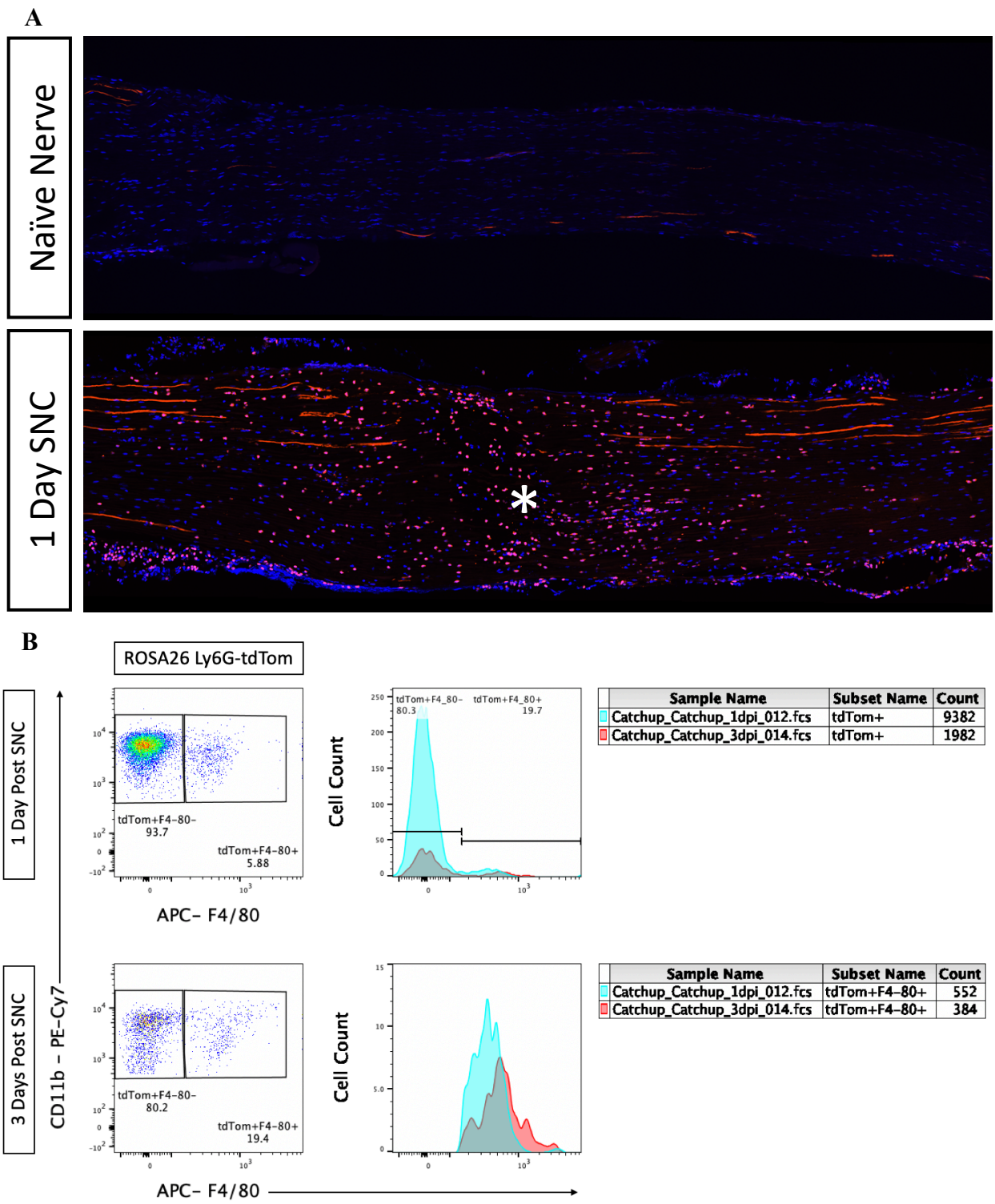

Figure 3-13 tdTom$^+$ neutrophils are efferocytosed by F4/80$^+$ macrophages after SNI.
A. Representative immunofluorescent image of a naïve 1d post-injury whole sciatic nerve from a R26LSL-Tomato fluorescent mouse with tdTom$^+$ neutrophils. The asterisk marks the injury site. B. Flow cytometry dot plots and quantitative histograms of of F4/80$^+$ sciatic nerve macrophages that have ingested tdTom$^+$ neutrophils at 1d and 3d post-injury. Cells represented here are CD45$^+$CD11b$^+$tdTom$^+$. The bottom-right histogram is a magnified inset of the tdTom$^+$F4/80$^+$ population from the histogram above.

3.9 References

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CHAPTER 4:
Discussion and Future Directions

4 Discussion and Future Directions

4.1 Abstract

Comprehensively, the work presented in this dissertation provides key insights into the multicellular and spatial complexity of the immune and non-immune response to peripheral nerve injury. Most impactful to the fields of neuroinflammation and neuro-regeneration is our (1) descriptive analysis of the time-course and spatial differences in reaction to peripheral nerve injury, (2) identification of unique subsets of macrophage, Schwann cell, endothelial cell, and other cell clusters with distinct transcriptomic and functional significances, (3) contribution to the understanding of the differences in inflammatory and pro-regenerative response in the DRG and local nerve environments, and (4) robust evidence for the role of efferocytosis and clearance of apoptotic cells in promoting resolution of inflammation and pro-regenerative signaling in the injured peripheral nerve. These findings, thoughts on critical next steps, and implications for therapeutic potential are discussed below.

4.2 Comprehensive Depiction of the Injured Nerve Environment

Others have characterized portions of the response to sciatic nerve injury, usually focusing on certain subsets of cells or systems. Immunologically, Kigerl et al., identified distinct subsets of macrophages that dichotomously promote either neuronal death or regeneration in the injured spinal cord (Kigerl et al., 2009). Neuronally, therapeutic potential for removing PTEN inhibition of mTOR in DRGs to promote sciatic nerve regeneration was suggested by Steward & Gallaher (Gallaher & Steward, 2018). Through implantation of a synthetic oxygen carrier, Luo et al., improved Schwann cells survival and enhanced sciatic nerve regeneration (Ma et al., 2018). Phillips et al., recently found implantation of a scaffold containing pre-aligned endothelial cells allowed for improved axon regrowth across a transected nerve (Muangsanit et al., 2021). Though, few groups have formally collected and demonstrated the complex response described in one narrative.

We are one of the first groups to fully sequence and characterize the cellular response to sciatic nerve injury. Other groups have employed bulk RNA-seq to investigate transcriptional changes in DRGs and sciatic nerve after injury (Hinder et al., 2017), in pain conditions (Sun et al., 2017), and after various therapies like acupuncture (Lv et al., 2020) and spinal cord stimulation (Stephens et al., 2018). More recent analyses have shifted preference toward scRNA-seq as it

allows for more powerful conclusions about the roles of specific cell types in the injured nerve and associated DRGs (Chen et al., 2021; Hu et al., 2016; Renthal et al., 2020). Though we believe our extended and in-depth analysis of data collected from single cell sequencing, flow cytometry, protein quantification, immunofluorescent labeling, mouse genetics, and novel surgical paradigms contributes a unique and holistic perspective of an organism's response to peripheral nerve injury.

Through flow cytometry and protein analysis we have identified the stereotyped time course of immune infiltration into the sciatic nerve, importantly we found key differences between infiltration into the proximal nerve stump and that of the injury and distal segment. In the former, our data show there is nearly no change in immune cell composition at 3dpi or 7dpi compared to naïve conditions. Contrastingly, at 1dpi the distal stump and injury site experience a significant influx of granulocytes—primarily neutrophils—which taper off at 3dpi. These cells likely respond within hours of injury and begin to release chemoattractants to promote infiltration of circulating monocytes (Niemi et al., 2020). Congruently there is an increase in the number of monocytes and macrophages at 1dpi that peak at 3dpi and wane at 7dpi. While many of these macrophages may be tissue-resident, our utilization of parabiotic mice allowed us to show that a significant portion of these are entering from the blood. There is also a modest increase in the number of myeloid-derived dendritic cells, peaking at 7dpi.

Though, immune infiltration into the nerve is only a small snapshot of the story. Our employment of scRNA-seq allowed us to identify the complex milieu of cells responding to injury. Through this method we identified 12 different populations of cells, many comprised of multiple subtypes with distinct transcriptional profiles. Speaking specifically to this variety, we identified five unique macrophage clusters, three Schwann cell clusters, three mesenchymal stem cell populations, three endothelial cell clusters, and two pericyte populations in addition to other singular cell types (myeloid-derived/conventional dendritic cells, granulocytes, T/natural killer cells, fibroblasts, chondrocytes, and a hybrid immune/endothelial cell population). This data not only shows the many cell types involved in an injury response, but underscores the magnitude of diversity within each population that points to distinct properties and functions.

To dive deeper into this theme, we performed an in-depth analysis of the five macrophage subpopulations (Mac1-5). Here we discovered that each group was functionally distinct, exemplified their developmental trajectory, and also their localization to specific sites of the injured nerve. Circulating monocytes infiltrate the blood-nerve barrier and, depending on their

microenvironment, take on a specific phenotype (Canè et al., 2019; Ydens et al., 2020). We showed that monocytes mature into the Mac3 population which gives rise to the Mac1/2/4 populations. Further, we found that Mac2/3 localize preferentially to the distal segment of the nerve and likely participate in Wallerian degeneration, while the Mac1/4 populations were found preferentially at the injury site and have roles in opsonization and efferocytosis of apoptotic cells. In addition to this variety we exemplified that mesenchymal progenitor cells of three clusters (perineural, endoneurial, and differentiating) are rich sources of neurotrophic factors essential for regeneration as well as several chemokines that can contribute to immune chemotaxis.

4.3 Differences in Sciatic Nerve and DRG Inflammation

For decades there has existed a battle between two camps: those that believe dorsal root ganglia (DRG) inflammation drives sciatic nerve regeneration and those that believe inflammation of the sciatic nerve predominantly drives regeneration. A classic paper by Richardson and Lu showed that heightened inflammation in DRGs through local injection of *Corynebacteerium parvum* or isogenous macrophages prompted increased sciatic nerve regeneration after crush injury (X. Lu & Richardson, 1991). Niemi et al., suggested that loss of global CCR2 expression—the major chemoattractant for circulating monocytes—impaired macrophage accumulation in DRGs and impaired conditioning-injury-induced regeneration (Niemi et al., 2013). Similarly, the overexpression of the CCR2 ligand, CCL2, in DRGs produced a conditioning-injury-like effect through increased STAT3 signaling (Niemi et al., 2016). Contrastingly inflammation and signaling from immune cells in the nerve have also shown to promote regeneration. Enhancing GM-CSF signaling, endogenously produced by macrophages, potentiated early axonal growth after sciatic nerve crush (Bombeiro et al., 2018). Polarization of nerve macrophages toward an anti-inflammatory phenotype with thrombomodulin altered their protein production and improved regeneration (Huang et al., 2020). Interestingly, increased levels of NP-1, normally released by neutrophils, increased pro-growth signals in the nerve (Yu et al., 2020). Further, macrophage-derived vascular endothelial growth factor is required for axonal reinnervation of the NMJ following injury (C. Y. Lu et al., 2020). In terms of placement within this field, our work primarily supports the thought that signals originating from the sciatic nerve drive its post-injury regeneration.

As stated earlier, our experiments show a significant influx of immune cells including granulocytes, monocyte/macrophages, and dendritic cells into the sciatic nerve following crush injury. However, immune infiltration into the DRG was shown to be quite minimal. Via flow cytometry, there are very modest increases in monocyte/macrophages and dendritic cells in the DRG. There is an increase in CD11b and Iba1 protein following sciatic nerve crush, though this could be from up-regulation of DRG-resident immune cells which are known to upregulate the protein following activation (Donninelli et al., 2020; Pei et al., 2019). Increases observed in Iba1 and F4/80 immunoreactivity could also be due to local proliferation of DRG-resident macrophages. Further, with our parabiont paradigm composed of a $tdTom^-$ mouse and $tdTom^+$ mouse, we found very modest numbers of $tdTom^+$ cells in the DRGs of $tdTom^-$ mice after nerve crush, suggesting minimal amounts of immune infiltration from circulation. Contrastingly within this same paradigm, we show the injury site and distal stump of the sciatic nerve harbor significant immune infiltrates. We did however find significant morphological changes of DRG macrophages following injury, which by immunofluorescence alone can appear to indicate increased numbers of infiltrating macrophages. An additional intriguing difference observed between DRG and sciatic nerve is that of Ly6C expression. It is known that circulating monocytes express high levels of Ly6C while differentiated macrophages are often $Ly6C^-$ (Epelman et al., 2014; Kimball et al., 2018). The naïve nerve is composed of predominately $Ly6C^-$ nerve-resident macrophages, but following crush injury we found the sciatic nerve has an influx of $Ly6C^{hi}$ monocytes 1dpi that differentiate into $Ly6C^-$ macrophages over the course of several days. Contrastingly in the DRG following crush injury, there is an increase in $Ly6C^-$ macrophages while numbers of potential infiltrating $Ly6C^{hi}$ monocytes remain relatively low. Overall, our evidence suggests that infiltration of immune cells in the sciatic nerve rather than that of the DRG play a larger role in the degenerative and regenerative response to injury.

While we show there is only a small contribution of circulating immune cells to DRG inflammation, we also demonstrate the presence of diverse transcriptional changes. Through DRG bulk RNA-seq we found upregulation of a previously identified module of gene expression networks following injury (Chandran et al., 2016) composed of many regeneration-associated genes like *Jun*, *Stat3*, and *Atf3*. Our analysis also identified a turquoise module that progressively increased each day after injury. Gene ontology analysis showed a role for immune cell activation and regulation with inflammatory factors like IFNγ and TNFα as well as pro-resolution factors like

IL-10 and IL-4. Importantly increased expression of these modules may originate from DRG-resident immune cells or non-immune cells including the DRGs themselves or surrounding support cells. Our transcriptional analysis also showed low expression levels of DRG GM-CSF and CCL2 receptors *Csf2r*, and *Ccr2*. Signaling through these receptors promotes monocyte migration and macrophage polarization, further suggesting a less involved role for infiltrating monocytes in the DRG. Finally, data collected from *Csf2* (GM-CSF) knock-out mice in our conditioning-lesion paradigm showed that signaling through CSF2R is important for the transition of inflammatory $Ly6C^{hi}$ monocytes to pro-resolution $Ly6C^{-}$ macrophages in the sciatic nerve. Impedance of this transition negated the effects of conditioning-lesion-induced dorsal column axon regeneration. Our *Csf2* knock-out *in vitro* neurite outgrowth experiment showed that the intrinsic growth programs of the DRGs was unaffected, suggesting the extrinsic pro-regenerative signaling from pro-resolving $Ly6C^{-}$ macrophages is imperative for promoting repair.

4.4 Evidence of Efferocytosis in the Injured Sciatic Nerve

As mentioned previously, the role of efferocytosis has been shown in several systems including atherosclerosis in blood vessels, immune cell development in the thymus and spleen, and across the whole body in systemic lupus erythematosus (**Chapter I**). Immune cell development is an example where efferocytosis maintains a constant state of balance and homeostasis, with defective immune cells (like B cells) becoming apoptotic to be cleared by patrolling efferocytes. Though in cases like atherosclerosis and lupus, efferocytosis has gone awry either due to efferocytes becoming overwhelmed by too many lipids or the overall efferocytic machinery breaking down, impeding efferocytes' ability to maintain systemic tolerance. Very recently a handful of studies have investigated the contribution of this process in the central nervous system, primarily following stroke and ischemia (Damisah et al., 2020; Mazaheri et al., 2014; Mike & Ferriero, 2021). However, evidence for the involvement of efferocytosis following peripheral nerve injury has never been published—until now.

We showed, as one would expect, there is an increase in the number of dying cells in the nerve following sciatic nerve crush. We also found that there is a significant increase in transcription of efferocytic machinery including specific bridging molecules and cognate engulfment receptors in the injury condition compared to the naïve. Many of these receptors are predominately expressed by the Mac4 population and significantly less so on others, suggesting

this is the macrophage subset that carries out efferocytosis in the injured nerve. Interestingly, we found immune cells tended to express lower transcript levels compared to other cells of classic efferocytic receptors *Axl* and *MerTK*, and rather preferred to employ LRP1, CD300, scavenger receptors, and opsonic receptors. This finding points to a possible preference for specific cells for certain receptors. As discussed earlier, efferocytic macrophages must also intake and digest a massive quantity of apoptotic cell components including carbohydrates, lipids, and cholesterol (**Chapter I**). Macrophages upregulate many export molecules like ABCA1 and ApoE to effectively process and export these molecules (Linton et al., 2019; Yvan-Charvet et al., 2010). Intriguingly, we found that our Mac4 population was the highest expresser for many of these transcripts. Further, some of the highest gene sets designating this population include general lysosome and phagosome machinery and promotion of an anti-inflammatory environment—a known role for efferocytic macrophage as discussed previously—solidifying the population's role in carrying out efferocytosis.

While the transcriptional data collected from scRNA-seq strongly suggests the presence of this efferocytic macrophage population in the nerve, we wanted to demonstrate *in vivo* the activity of these cells. To accomplish this, we employed the surgical paradigm of parabiosis, a novel technique in this area of research. This allowed us to establish a shared circulatory system between two mice of different genotypes while tissue-resident immune cells remain unaffected. Here we found a significant population of CD45.1^{+} tdTom^{+} monocytes/macrophages in the CD45.1^{+} tdTom^{-} mouse partner, suggesting circulating immune cells from the tdTom animal entered the injured sciatic nerve of this mouse and were efferocytosed by CD45.1^{+} tdTom^{-} cells. While we cannot yet verify that these are the Mac4 population we see in the wild-type scRNA-seq data, this is likely the case. Most studies of efferocytosis have focused on the actions of macrophages, though we also found that dendritic cells participate in this process, though to a much lesser extent than monocytes/macrophages. Overall we believe the evidence presented here strongly supports the presence of a specific macrophage population following nerve crush that actively engages in efferocytosis and promotes and environment of immune resolution and neuroregeneration.

4.5 Future Directions

While our description of the DRG and nerve transcriptional and inflammatory response to crush injury is extensive, there remain a wealth of additional questions to be answered in this

paradigm. One intriguing insight is the differential expression of various efferocytic receptors across the differenct cell types of the injured nerve. As mentioned previously, many efferocytic receptors that bind to apoptotic cell phosphatidylserine are also used in myelin debris clearance after injury (**Chapter I**). Our data show Schwann cell clusters 1 and 3 express different transcript levels of *Axl* and *MerTK*, two of the most classic efferocytic receptors. While most likely utilized in myelin phagocytosis, different Schwann cell populations may employ these receptors to contribute to efferocytosis, an area of interest lacking sufficient study. Surprisingly the Mac4 population expressed relatively low levels of these receptors in favor of higher expression of transcripts for the receptors LRP1, CD300, CD36, and Fcgr1/3/4. Macrophages in other tissues strongly employ MER and Axl (Nagata, 2018), positing potential tissue-to-tissue variation in receptor expression. Further study of these differences is warranted to assess if this results in differential downstream signaling or efferocytosis efficacy.

An additional area to follow-up upon is deeper analysis of the immune response in our *Csf2* knock-out mice. $Ly6C^{hi}$ monocytes in these mice have a delayed or impaired transition toward pro-resolution $Ly6C^{-}$ macrophages and reduced conditioning-lesion-induced regeneration. I suspect the nerves and DRGs of these mice would also have prolonged inflammatory and reduced pro-regenerative signaling following crush. This could be assessed by bulk nerve RNA-seq and/or analysis of released cytokines through ELISA or western blotting. Further studies should also assess which of the five macrophage populations identified by scRNA-seq in wild-type mice are reduced or lacking in $Csf2^{-/-}$ mice. As the nerve likely remains in a prolonged inflammatory state, I would posit a reduction in the efferocytic Mac4 population that promotes inflammation resolution through efferocytosis. An additional future direction would be to embrace recent advances in sequencing technology to perform special transcriptomics on the injured nerve. While our separation of the nerve segments proved effective, this technique would allow for a unique combination of visual, special, and transcriptomic information. Further, if this technique could be combined with expansion microscopy to expand the nerve within a polymer matrix, it could offer an unparalleled and extremely high-resolution look at the cellular and molecular events following peripheral nerve injury.

One of the most exciting avenues for future inquiry is the manipulation of efferocytosis following injury. To further verify the role of efferocytosis in promoting immune resolution and nerve regeneration one could impair efferocytic machinery following crush injury and assess the

resulting inflammatory milieu and regeneration. As mentioned earlier, while it may seem there is significant redundancy in efferocytic ligands and receptors, the deletion of a single receptor in many cases is sufficient to cause break-down in the efferocytic process (Doran et al., 2020). In the case of the sciatic nerve, as the Mac4 population preferentially expresses LRP1, Trem2, and CD300, one could conditionally ablate one or multiple of these receptors in macrophages and assess accumulation of apoptotic cells, prolonging of injury-induced inflammation, and axon regeneration. Interestingly, thinking about common methods used for macrophage depletion like clodronate liposomes, these techniques generally ablate nearly all macrophage populations. With our data showing the existence of five different macrophage populations with such diverse transcriptomic profiles, one wonders the many different functions of each of these macrophages that are missing upon their depletion. One is not only ablating pro-inflammatory macrophages but also those that specifically aid in Wallerian degeneration (Mac2/3) as well as efferocytic populations (Mac4). While indeed some macrophage populations can be harmful to regeneration, it would be ideal to have the capability to alter each of these populations individually in order to reduce unwanted damaging signals while reinforcing those that are pro-regenerative. To our knowledge this technology does not exist, but with techniques of the future this may become a reality. On the opposing side of ablation, and of even more interest for therapeutic potential, would be to drive efferocytosis to promote resolution and regeneration more effectively. Several groups have utilized this technique, primarily to promote resolution in chronic inflammatory states like diabetes, arthritis, and GvHD, some in human clinical trisl (Bonnefoy et al., 2016; Mevorach et al., 2014; Xia, Chang-Qing, 2007). In the context of the sciatic nerve, apoptotic cells could be injected either locally or systemically to kick-start resolution and moderate harmful inflammation following injury.

4.6 Concluding Remarks

Billions of cells die in the body every day, hundreds of millions per second. Though the astounding frequency of this process progresses with us remaining unawares, thanks to the remarkable efficiency of the efferocytic process. Great strides in understanding this phenomenon have been made over the last several decades, though the role of efferocytosis in the nervous system is only recently coming to light and many unanswered questions remain. The work herein contributes significantly to the field by being the first to show evidence of this process in an injured

peripheral nerve. We also provide one of the most comprehensive and in-depth analyses of the multicellular complexity of the injury response by employing multiple sequencing techniques, flow cytometry, protein analysis, immunofluorescent imaging, *in vitro* cultures, and novel surgical paradigms. Additional studies are necessary to ascertain the specific molecular machinery at play and therapeutic potential of peripheral nerve efferocytosis. Improved understanding of these mechanisms will help identify new therapies for the many patients afflicted with nervous system disorders including chronic neuropathic pain, diabetic and chemotherapy-induced neuropathy, Guillain-Barré syndrome, and others.

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