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Myelin, cPLA2, and Azithromycin: Modulation of Macrophage Activation in Spinal Cord Injury Inflammation

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MYELIN, cPLA$_2$, AND AZITHROMYCIN: MODULATION OF MACROPHAGE ACTIVATION IN SPINAL CORD INJURY INFLAMMATION

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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DISSERTATION ABSTRACT

MYELIN, $CPLA_2$, AND AZITHROMYCIN: MODULATION OF MACROPHAGE ACTIVATION IN SPINAL CORD INJURY INFLAMMATION

Spinal cord injury (SCI) produces a chronic inflammatory state primarily mediated by macrophages consisting of resident microglia and infiltrating monocytes. These chronically activated SCI macrophages adopt a pro-inflammatory, pathological state that continues to cause additional damage after the initial injury and inhibits recovery. While the roles of macrophages in SCI pathophysiology are well documented, the factors contributing to this maladaptive response are poorly understood. Here, we identify the detrimental effects of myelin debris on macrophage physiology and demonstrate a novel, activation state-dependent role for cytosolic phospholipase-A2 ($cPLA_2$) in myelin-mediated potentiation of pro-inflammatory macrophage activation. Macrophage-mediated inflammatory responses are promising therapeutic targets; however, there are very few therapeutic options to treat SCI and none that target macrophages. Here, we provide evidence that treatment with the immunomodulatory antibiotic azithromycin (AZM), initiated after SCI, improves recovery by targeting macrophage activation. There is an urgent need for the development of new therapies for the treatment of SCI. Macrophage-targeted therapies hold great promise; however, these treatment candidates require additional development before they can advance towards clinical use. Here we discuss the continued development of $cPLA_2$ as a therapeutic target, the steps necessary to advance AZM towards clinical use, and lastly, we review additional macrophage-targeted therapies currently in development. Collectively this body of work identifies key mechanisms driving macrophage pathophysiology after SCI and identifies macrophage-targeted therapies that reduce this neuroinflammation to improve recovery after SCI.

KEYWORDS: Spinal Cord Injury, Neuroinflammation, Macrophage, Microglia, Neurotrauma

Timothy Joseph Kopper

July 27th, 2021

MYELIN, $CPLA_2$, AND AZITHROMYCIN: MODULATION OF MACROPHAGE ACTIVATION IN SPINAL CORD INJURY INFLAMMATION

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Chapter 1: Introduction

1- i. Spinal Cord Injury

A spinal cord injury (SCI) is a severe condition in which damage to the spinal cord produces impairments to spinal functions. The initial damage that occurs is largely irreversible, with current medical interventions primarily seeking to stabilize the individual and prevent further damage. Unfortunately, in the initial days following SCI, the damage to the spinal cord spreads, causing additional impairments by a process called secondary injury. While the causes of secondary injury are multifaceted, a primary mechanism is the robust neuroinflammatory response mediated by infiltrating macrophages and resident microglia. Given the immense medical challenge of mending the spinal cord damage, the current body of work aims to understand the underlying mechanisms driving this detrimental cellular activity and examines therapeutics that could intervene and improve recovery after SCI.

SCI induces a robust cellular inflammatory response in which resident cells such as microglia and astrocytes become activated. In the hours and days after injury, neutrophils, monocytes, lymphocytes, and other cells infiltrate into the damaged tissue creating a unique neuroinflammatory environment not seen in typical bodily injuries (Donnelly and Popovich, 2008). There are protective or reparative events occurring in these cellular populations; however, collectively, this inflammation causes further damage. Macrophages and microglia, in particular, are capable of producing reparative or adverse responses depending

on their activation state. Indeed, macrophages can facilitate repair by increasing axon growth, stem cell differentiation, and revascularization (Gensel and Zhang, 2015; Kigerl et al., 2009); however, in SCI these, cells predominantly adopt a pro-inflammatory activation state. Pro-inflammatory macrophages and microglia contribute to pathology through secondary injury processes involving reactive oxygen species (ROS), neurotoxin, and pro-inflammatory cytokines release, as well as by causing axon retraction and dieback (Gensel and Zhang, 2015; Horn et al., 2008). Further, pro-inflammatory macrophages and microglia remain in the SCI lesion indefinitely and are thus thought to impair other repair mechanisms. Here, we seek to examine some of the underlying mechanisms causing this maladaptive macrophage response and develop therapeutics that may reduce pro-inflammatory macrophage activation.

To examine the underlying mechanisms underlying the pro-inflammatory macrophage response, we utilize an *in vitro* model using bone marrow-derived macrophages (BMDMs). As described previously (Kopper et al., 2021), BMDMs are predictive of monocyte-derived macrophage responses *in vivo* in the injured spinal cord. This has been observed at transcription (Longbrake et al., 2007) and functional levels (Gensel et al., 2009), as well as in response to therapeutic interventions (Gensel et al., 2017). *In vitro*, we reproduce the pro-inflammatory macrophage activation state with an “M1” stimulus (LPS and IFN-γ) and the anti-inflammatory reparative macrophage activation state with an “M2” stimulus (IL-4). We also utilize naïve “CTL” or “M0” macrophages that are grown to maturity but are not given an activation state stimulus. Using this system, we can add

additional stimulants, chemical inhibitors, or therapeutics to investigate cellular mechanisms and screen potential therapeutics.

1- ii. Myelin as an Inflammatory Mediator

This section is adapted from (Kopper and Gensel, 2017)

Myelin as an Inflammatory Mediator Summary:

Spinal cord injury (SCI) triggers chronic intraspinal inflammation consisting of activated resident and infiltrating immune cells (especially microglia/macrophages). The environmental factors contributing to this protracted inflammation are not well understood; however, myelin lipid debris is a hallmark of SCI. Myelin is also a potent macrophage stimuli and target of complement-mediated clearance and inflammation. The downstream effects of these neuro-immune interactions have the potential to contribute to ongoing pathology or facilitate repair. This depends in large part on whether myelin drives pathological or reparative macrophage activation states, commonly referred to as M1 (pro-inflammatory) or M2 (alternatively) macrophages, respectively. Here we review the processes by which myelin debris may be cleared through macrophage surface receptors and the complement system, how this differentially influences macrophage and microglial activation states, and how the cellular functions of these myelin macrophages and complement proteins contribute to chronic inflammation and secondary injury after SCI.

Myelin as an Inflammatory Mediator

Spinal cord injury (SCI) triggers a complex cross-talk between resident cells of the central nervous system (CNS) and infiltrating immune cells. These neuro-immune interactions can mediate recovery but also inhibit regeneration. Activated macrophages, consisting of resident microglia and recruited monocytes, contribute to this dichotomous response. Indeed, macrophages facilitate repair by increasing axon growth, stem cell differentiation, and revascularization. However, macrophages can also contribute to pathology through reactive oxygen species (ROS), neurotoxin, and pro-inflammatory cytokines release, as well as, by causing axon retraction and dieback. The extent to which macrophages are polarized toward reparative (also called M2 or alternative) or pathological (also called M1 or pro-inflammatory) phenotypes depends in large part on the stimuli present in the injured spinal cord.

While numerous studies examine how macrophage activation states affect recovery after SCI (for a review see (Gensel and Zhang, 2015)), less is understood about how the lesion environment contributes to macrophage polarization. It is well-established that myelin debris generated after SCI inhibits axonal regeneration and remyelination (McKerracher et al., 1994); however, myelin can also act as an inflammatory stimulus (Kroner et al., 2014; Wang et al., 2014; Williams et al., 1994). Lipid-laden myelin debris is taken up and processed by inflammatory cells, including neutrophils and macrophages. Myelin then becomes highly concentrated in these phagocytes, persisting in macrophages for weeks after SCI (Greenhalgh and David, 2014; Vargas and Barres, 2007; Wang

et al., 2014). In addition, myelin initiates complement-mediated inflammatory pathways with downstream effects on macrophage activation. Here we examine the myelin-macrophage and complement neuro-immune interactions after SCI. Since myelin is ubiquitously present in the acute and chronically injured spinal cord, we will explore the mechanisms of myelin debris clearance and its downstream inflammatory effects.

Spinal Cord Injury Causes Myelin Breakdown and Immune Cell Activation.

Acute spinal cord trauma ruptures vasculature leading to tissue ischemia and blood brain barrier breakdown and generates neuron and myelin debris. Myelin damage specifically occurs at the time of injury, and cellular debris is present in areas of white matter damage within 24hrs (Buss et al.; Ek et al., 2012; Imai et al., 2008). Debris increases within the first week of SCI and persists in the chronically injured spinal cord (Ek et al., 2012; Kozlowski et al., 2014). While damaged myelin is cleared within weeks of peripheral nerve injury, myelin fragments are present for the first year after SCI (Becerra et al., 1995; Vargas and Barres, 2007). Indeed, myelin debris is not cleared from the chronically injured spinal cord until years after injury (Becerra et al., 1995). This time course of acute myelin debris with protracted but eventual removal after SCI occurs across a range of mammalian models and in humans (Wang et al., 2014). The sustained presence of myelin debris inhibits axon regeneration, oligodendrocyte differentiation, and remyelination.

Immune cell activation follows a similar time course (Gensel and Zhang, 2015). Neutrophils and complement proteins (discussed below) enter the injury site within the first days after SCI. Microglia are activated at the time of injury, with peak activation within the first week of injury. Monocyte-derived macrophages infiltrate the injured spinal cord within days and peak 1–2 weeks after injury. Over time, neutrophils and complement proteins subside, but microglia and macrophages persist. Phagocytosis markers are present on these chronically activated cells (Fleming et al., 2006). It is therefore likely that myelin lipids are actively processed by macrophages and are environmental stimuli influencing chronic spinal cord inflammation. Indeed, in areas of Wallerian degeneration, macrophages disappear from the chronically injured spinal cord concomitant with myelin debris clearance (Becerra et al., 1995).

Macrophage Receptor-Mediated Myelin Removal

Receptor-mediated phagocytic myelin removal requires binding of damaged myelin to surface receptors and subsequent phagocytosis. These receptors have the potential to bind to myelin directly or to opsonized myelin particles. Both opsonized and non-opsonized myelin removal by macrophages/microglia has been reviewed extensively and will only be mentioned here briefly (Hirata and Kawabuchi, 2002; Neumann et al., 2009; Reichert and Rotshenker, 2003). Complement-mediated receptor binding, primarily through the pattern recognition receptor (PRR), complement receptor 3 (CR3/CD11b/MAC-1), is a commonly studied mechanism of opsonized myelin

clearance. Complement proteins (e.g., complement protein 3) and/or antibodies bind degenerated myelin facilitating CR3 binding and phagocytosis. This process, and the process of non-opsonized myelin clearance, is regulated in part through carbohydrate-lectin receptors, including the MAC-2/Galectin-3 receptor among others. The receptor-mediated internalization of myelin, and myelin-receptor binding itself, potentially alters the functional phenotype of the phagocyte. The downstream effects of complement and receptor-mediated myelin removal on macrophage/microglia phenotypes will be discussed below.

It is also well-documented that scavenger receptor AI/II (SRAI/II) participates in macrophage-mediated myelin clearance, for a review, see (Rotshenker, 2009). This PRR is capable of binding a variety of lipids and polyanionic ligands. SRAI/II, in combination with CR3, facilitates myelin phagocytosis by microglia and macrophages (da Costa et al., 1997; Reichert and Rotshenker, 2003). Additionally, SRAI/II is implicated as a primary mediator of oxidized lipoprotein uptake in atherosclerosis and leads to the development of foam cells (Greaves and Gordon, 2008). Emerging transcriptional evidence indicates that SCI macrophages increase lipid catabolism after injury and adopt transcription profiles closely resembling foam cells (Zhu et al., 2017). Lipoprotein receptors have been implicated in the foam cell transition, but whether SRAI/II facilities this transition in SCI has yet to be determined. This may be challenging considering that SCI results in significant free radical generation and lipid peroxidation (Hall, 2011). It is possible that these oxidative alterations to myelin lipids may alter the receptor pathways through which they are cleared, and this

caveat is important to consider when modeling myelin clearance *in-vitro* and interpreting experimental results.

Another potential mechanism of receptor-mediated myelin clearance is through the macrophage receptor with a collagenous structure (MARCO). MARCO is a scavenger receptor related to SRAI/II. Both contain collagenous and scavenger receptor cysteine-rich domains in their extracellular portions giving them similar ligand binding repertoires (Elomaa et al., 1995; Jozefowski et al., 2005). Thus, it is likely that MARCO can bind myelin lipids effectively. Although it has not been identified as a myelin receptor in SCI, we recently observed that macrophages upregulate MARCO in response to pro-inflammatory stimuli and express MARCO in the injured spinal cord (Gensel et al., 2017; Orr et al., 2017). MARCO activation, therefore, may be a potential mechanism for pro-inflammatory macrophage-mediated myelin removal in SCI.

Triggering receptor expressed on myeloid cells 2 (TREM2) facilitates microglial phagocytic activity (Takahashi et al., 2005). TREM2 is a sensor for lipid components of damaged myelin and is required for debris clearance in the cuprizone model of demyelination (Daws et al., 2003; Poliani et al., 2015). TREM2 binds polyanionic ligands, such as dextran sulphate, bacterial lipooligosaccharides, and various phospholipids (Cannon et al., 2011). In the case of myelin, the likely ligands include phosphatidylethanolamine, phosphatidylserine, and cardiolipin found in myelin membranes (Cannon et al., 2011). Individuals with TREM2 mutations or deficiencies are at higher risk for developing amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's

disease (Lill et al., 2015). Notably, individuals with mutations in TREM2 or DAP12, a key-signaling component of TREM2, develop lethal and progressive Nasu-Hakola disease characterized by early onset dementia and demyelinating brain lesions (Paloneva et al., 2002; Verloes et al., 1997). Therefore, TREM2 may be involved in removing damaged myelin following SCI, MS and other conditions. Indeed, microglial TREM2 senses lipid components of myelin debris and is important in regulating transcriptional programs essential for myelin debris clearance (Cantoni et al., 2015; Poliani et al., 2015; Siddiqui et al., 2016). Additional work is needed to examine TREM2 within the context of the myelin-macrophage interactions in SCI. Likewise, TREM2 is part of a large family of TREM and TREM-like receptors with similar ligand binding repertoires that are also unstudied in the context of myelin uptake after SCI (Cannon et al., 2011).

Myelin-Macrophage Interactions

Some of the first studies to examine macrophage-mediated myelin debris clearance in the CNS emerged from the multiple sclerosis (MS) field. Electron microscopy studies revealed myelin debris phagocytosis in active lesions and identified macrophages as facilitators of continued demyelination (Prineas, 1975; Prineas and Connell, 1978). These catalyzed further studies largely concerned with the roles of anti-myelin antibodies, complement, and other pathways in relation to the chronic demyelination observed in MS. A 1994 publication by Williams et al. in the *Journal of Neuroscience Research* identified an inflammatory role for myelin debris (Williams et al., 1994). Specifically, they

reported microglial activation with increased pro-inflammatory cytokine and ROS production with myelin phagocytosis (Williams et al., 1994). These observations indicate that myelin debris may be an inflammatory stimulus for pro-inflammatory microglia/macrophage activation.

An 1990's, work by Brück and Friede and van der Laan et al. demonstrated the importance of macrophage CR3 in mediating myelin uptake. They observed that myelin induced production of tumor necrosis factor-alpha (TNF-α) and nitric oxide (van der Laan et al., 1996a). These effects were enhanced through complement opsonization of myelin and blocked through antibody-mediated inhibition of CR3 (Brück and Friede, 1990; 1991; van der Laan et al., 1996b). This work is supported by a more recent study in an SCI model which reported myelin invoked pro-inflammatory macrophage responses *in vivo* (Sun et al., 2010). In that paper, CR3-mediated uptake of myelin and downstream activation of FAK/PI3K/Akt/NF-κβ signaling pathways increased pro-inflammatory, M1-like cytokine release and decreased M2 cytokine release (Sun et al., 2010). Collectively, these data highlight that receptor-mediated myelin removal can alter macrophage phenotypes and that removal through CR3 drives pro-inflammatory macrophage activation in SCI.

While these studies implicate myelin as a pro-inflammatory macrophage stimulus in SCI, the role of myelin and lipid processing on M1/M2 macrophage polarization *in vivo* is controversial. Specifically, myelin-laden "foamy" cells express a wide variety of anti-inflammatory molecules with some intermediate M1–M2 phenotypes in MS (Boven, 2005; Vogel et al., 2013). In atherosclerosis,

foam cell formation is associated with a downregulation of pro-inflammatory gene expression (Spann et al., 2012). In contrast, in SCI, foam cell formation and macrophage lipid accumulation is associated with decreased M2 activation (Wang et al., 2014). Further, M1 macrophage polarization predominates in SCI despite the large presence of myelin debris (Kroner et al., 2014). Interestingly, the relative expression of pro- and anti-inflammatory markers appears to depend on the lesion or tissue microenvironment. For example, in active MS lesions, anti-inflammatory marker expression predominates on foam cell macrophages in the lesion center and inner rim while pro-inflammatory marker expression is more widespread (Boven, 2005; Vogel et al., 2013). Similarly, a more recent evaluation of foam cells within the context of the M1/M2 macrophage paradigm observed a full range of M1–M2 foam cell activation states depending upon the microenvironments from which the cells were isolated (Thomas et al., 2015a; 2015b). In the case of SCI, two environmental cues that may promote M1 polarization in the presence of myelin are TNF and intracellular iron, perhaps reflective of the increased hemorrhage in SCI lesions relative to those in MS (Kroner et al., 2014).

To better elucidate the role myelin plays in macrophage polarization, researchers often stimulate macrophages with myelin *in vitro*. However, despite using fairly similar stimulation paradigms, there are contrasting reports that implicate myelin as both a pro-inflammatory and anti-inflammatory stimulus. To better understand how to reconcile these conflicting reports, we examined the myelin stimulations in detail. A few common themes emerged that may account

for the conflicting results. First, in several of these *in-vitro* paradigms, researchers load macrophages with myelin prior to applying inflammatory stimuli. In response to pro-inflammatory stimuli, these myelin-laden macrophages almost invariably express anti-inflammatory mediators and/or stop responding to the pro-inflammatory stimuli (Bogie et al., 2013; 2012; Boven, 2005). Second, myelin stimulation in isolation invokes either no phenotypic activation or causes the release of reactive oxygen species and pro-inflammatory cytokines with a few reports of subtle M2-like activation in a cell-type specific manner (Sun et al., 2010; van der Laan et al., 1996b; van Rossum et al., 1999; Wang et al., 2014; Williams et al., 1994). Third, when myelin is delivered to macrophages already stimulated to be either M1 or M2, or when myelin and other stimuli are presented at the same time, a range of responses has been reported. For example, pre-stimulation with M1 or M2 stimuli results in a myelin-induced potentiation of M1 activation (Siddiqui et al., 2016; Wang et al., 2014). Myelin co-administration with pro-inflammatory stimuli can invoke further M1 activation, albeit in a time-dependent manner with increased M2 activation over time (Liu et al., 2006). A subtlety distinct protocol added myelin to M1 polarized macrophages following the removal of the inflammatory stimuli and observed anti-inflammatory effects (Kroner et al., 2014). Notably, in that study, if pro-inflammatory stimuli are present during myelin activation, M1 polarization was observed (Kroner et al., 2014). It is also important to note that researchers utilize various cell types, including bone marrow-derived macrophages, primary microglia, cell lines, peritoneal macrophages, and blood monocytes, among others for *in vitro* models.

All of these cell types have subtle differences in their basal activation state and their ability to take up and respond to myelin (Durafourt et al., 2012; van Rossum et al., 2008). Collectively, these myelin-macrophage studies *in vitro* indicate that: 1) myelin dampens the macrophage response to subsequent stimuli; 2) in isolation, myelin may act as a pro-inflammatory stimulus that drives M1 type-activation; and 3) when combined with other stimuli, initially myelin facilitates M1 polarization but this response varies over time.

The results of these *in vitro* studies indicate that myelin is capable of producing downstream effects on macrophages that vary under different cellular and environmental conditions. This highlights the impact that stimulation type and timing, relative to myelin application, has on myelin-induced shifts in macrophage phenotype. Microglia and macrophages in the injured spinal cord would likely be exposed to inflammatory stimuli before or concurrent with the clearance of myelin debris. Macrophages also encounter distinct stimuli in the SCI vs. MS lesion environment. It is therefore possible that different macrophage activation states during myelin processing lead to varied inflammatory responses in SCI and MS. Until the molecular mechanisms through which myelin exerts its effects are better understood, it remains unclear how myelin and inflammatory stimuli synergize to produce different cellular responses.

In addition, the macrophage response to myelin uptake varies depending upon the receptors mediating phagocytosis (**Figure 1.1**). In traditional responses to infection and damage, macrophage PRRs often share redundancies in the molecular structures they target. It has been proposed that collective

engagement of different PRRs can influence the cellular response to an inflammatory insult (Jozefowski et al., 2005; Mukhopadhyay et al., 2004). Indeed, in SCI, the collaborative engagement of PRRs vs. activation in isolation yields distinct reparative or pathological functions (Gensel et al., 2015). Notably, the PRRs capable of recognizing and clearing myelin, as discussed above, appear to mediate contrasting cellular responses along the spectrum of macrophage activation states. This suggests that the specific combination of myelin clearance receptors utilized after injury may influence the inflammatory effects of myelin debris. For example, in-vitro, MARCO is important in mediating pro-inflammatory IL-12 production in response to inflammatory stimuli (Jozefowski et al., 2005). Similarly, it is well documented that macrophages increase IL-12 release with myelin stimulation (Sun et al., 2010; Wang et al., 2014). Given that MARCO is present on macrophages after SCI (Gensel et al., 2017), it is possible that MARCO-mediated myelin clearance may influence the M1-like polarization observed in SCI. Conversely, SRAI/II has been implicated in the inhibition of IL-12 production (Jozefowski, 2004; Jozefowski et al., 2005). Whether the differential function and regulation of these macrophage receptors are of similar importance in the context of myelin debris clearance after SCI is unknown (**Figure 1.1**).

Following SCI or other neurological conditions, myelin clearance is likely influenced simultaneously by multiple receptors. This receptor cross-talk could be additive, synergistic, or antagonistic in terms of intracellular signaling depending on which receptors are present and activated during clearance (Lee and Kim,

2007; Natarajan et al., 2006). Interestingly, certain bacterial strains have evolved to manipulate this PRR crosstalk by CR3, MARCO, TLRs, and C5aR (discussed below) to block the production of IL-12 and IFN-γ thereby impairing macrophage bacterial clearance (Hajishengallis and Lambris, 2011). Although, whether a similar approach could be harnessed to manipulate myelin-mediated inflammatory responses after SCI has yet to be studied. Additionally, TREM2 plays a role in myelin clearance and integrity. As discussed above, it is important in regulating the transcriptional programs essential for myelin phagocytosis (Cantoni et al., 2015; Poliani et al., 2015; Siddiqui et al., 2016). Further, TREM2 signaling facilitates the production of tropic factors important in oligodendrocyte differentiation, survival, and remyelination (Poliani et al., 2015). Interestingly, overexpression of TREM2 increases the efficiency of non-inflammatory phagocytosis (Takahashi et al., 2005; 2007). TREM2 overexpression in macrophages also induces an M2 phenotype and M2 activation is inhibited in TREM2 KO macrophages (Seno et al., 2009; Takahashi et al., 2005). In an experimental model of MS, TREM2 facilitated myelin removal while increasing anti-inflammatory cytokine production (Takahashi et al., 2007). Whether TREM2 drives M2 macrophage activation is the context of SCI is unknown but these studies suggest that it may be a pro-reparative pathway for myelin debris clearance.

CD36 (a class B scavenger receptor) is also implicated in macrophage-mediated myelin clearance after SCI. Recently, (Zhu et al., 2017), examined shifts in macrophage transcriptional profiles from 3 to 7 days post injury (dpi).

They observed a significant shift from genes controlling cytokine signaling and cellular migration at 3dpi to a profile dominated by lipid catabolism at 7dpi through the liver X and retinoid X receptor (LXR/RXR) and peroxisome proliferator-activated receptors (PPAR)/RXR canonical pathways (Zhu et al., 2017). Further, they targeted the most enriched lipid receptor identified, CD36, and found that its genetic deletion reduced lipid accumulation in macrophages and improved functional outcomes after SCI (Zhu et al., 2017). Interestingly, the RXR signaling pathways are similar to those activated by foam cells within atherosclerotic lesions.

Independent of the receptor mediating myelin clearance, a previous study of foamy macrophages in SCI showed that myelin stimulation shifted the balance of macrophage activation towards M1 activation. Specifically, myelin stimulation increased pro-inflammatory NF-κβ/STAT1 signaling and decreased M2-associated STAT3/STAT6 signaling (Wang et al., 2014). Further, while myelin increased lipid efflux and activated ATP-binding cassette transporter A1 (ABCA1) in macrophages, in foamy cells, myelin decreased the phagocytic capacity for necrotic neutrophils (Wang et al., 2014). Since foamy macrophages are present in SCI, they proposed that these spent, but non-phagocytosed, neutrophils released toxins and contribute to secondary injury after SCI. This is a novel potential mechanism through which foam-like, myelin-laden macrophages may contribute to secondary injury processes (Wang et al., 2014). Given the extensive lipid debris accumulating within SCI macrophages, additional

comparisons to foam cells and atherosclerosis may lead to new therapeutic targets.

Another important factor that likely regulates the myelin-macrophage neuro-immune interaction in SCI is the phagocytic cell origin. Microglia have the phagocytic capacity to remove damaged myelin and are the predominant phagocyte to clear debris in the acutely injured spinal cord (Greenhalgh and David, 2014). However, by three days post injury, this role is largely taken over by infiltrating macrophages (Greenhalgh and David, 2014). Specifically, in the first weeks after SCI, MAC-2 positive bone marrow-derived macrophages enter the lesion site and are positive for myelin debris (Wang et al., 2014). These infiltrating macrophages accumulate in the lesion over time and contain lipid debris for at least 42dpi (Greenhalgh and David, 2014). In contrast, CX3CR1^{high} resident microglia are primarily found along the periphery of the lesion/lipid plaque in areas of less concentrated myelin debris (Wang et al., 2014). The downstream effects of this differential distribution of macrophages and microglia relative to lipid debris has been discussed previously (Zhou et al., 2014) but it is interesting to consider how cell-specific receptor expression may mediate myelin polarization in SCI. For example, MARCO is primarily expressed by infiltrating and not resident myeloid cells (Getts et al., 2014). Similarly, MAC-2 expression is specific to monocyte derived macrophages after SCI (Wang et al., 2014). TREM2, however, is predominantly expressed by microglia (Schmid et al., 2002). As discussed above, these receptors have different propensities for pro-inflammatory or anti-inflammatory cytokine release in response to myelin

stimulation (**Figure 1.1**). It is therefore possible that varied receptor expression on microglia and monocyte-derived macrophages predisposes these cells for different inflammatory responses to SCI myelin debris. Determining the relative contribution of microglia vs. macrophages on myelin-mediated SCI inflammation will be challenging, however, based upon the observation that macrophage myelin phagocytosis varies depending upon the inflammatory cues driving recruitment to the site of injury (Slobodov et al., 2001).

Relative to the injured spinal cord, myelin may induce deleterious pro-inflammatory macrophage activation (Kroner et al., 2014; Wang et al., 2014). Myelin and myelin phagocytosis likely potentiate signaling pathways and polarization states in macrophage and function as direct inflammatory stimuli (**Figure 1.1**). Determining the mechanisms and environmental conditions through which myelin can induce these effects in immune cells could lead to novel therapies for SCI and other neuroinflammatory disorders.

Complement Mediated Myelin Clearance

In addition to macrophages, the complement system is a key inflammatory mediator of myelin debris removal. The detailed pathways and diverse roles of complement have been reviewed in the context of SCI (Peterson and Anderson, 2014). Here we discuss the contributions of complement to myelin clearance and how this may influence inflammatory pathways after SCI. Within the complement system, there are numerous proteins in the plasma that enzymatically mark pathogens for destruction. Specifically, proteins of the complement cascade

selectively recognize pathogen associated molecular patterns (PAMPs) or damaged associated molecular patterns (DAMPs) and opsonize, or tag these PAMPs or DAMPs for removal. Complement component 3 proteins, most notably C3b, act as opsonizing agents. Opsonized pathogens are targets for removal through complement receptors on phagocytes (such as CR3 discussed above) or through secondary complement pathways. Regardless of which complement pathway is initiated (classical, alternative, or lectin), a secondary complement membrane attack complex is formed, creating large holes in the target membrane and ultimately causing pathogen lysis (Parham, 2009). Complement proteins opsonize myelin debris in the CNS and thus, the complement system is a critical initiator of myelin invoked inflammatory responses to SCI in addition to its other direct functions (Peterson and Anderson, 2014; Sun et al., 2010; van der Laan et al., 1996b). Further, the activation of this immune pathway through myelin interactions, DAMPs, or other means can directly damage intact myelin, oligodendrocytes, and neurons, thereby driving inflammation by increasing myelin and cellular debris or other inflammatory mediators.

The primary source of complement is the liver. It produces substantial quantities of inactive complement. This complement is stored in the plasma until it is activated in response to infection or damage. After SCI, disruption of the blood brain barrier allows complement proteins to enter the lesion site. Indeed, complement increases in the injured spinal cord within 1 day of SCI in both rats and humans (Nguyen et al., 2008). SCI-induced inflammatory cytokines may also increase complement serum levels at these acute time points (Rebhun and

Botvin, 1980). While plasma-derived complement is likely the main initiator of early myelin clearance after SCI, complement is present in the chronically injured spinal cord (Anderson et al., 2004). Many cells of the immune and nervous systems are capable of producing complement proteins, including macrophages/monocytes, lymphocytes, and neutrophils, all of which enter the lesion after SCI, and resident astrocytes, neurons, and microglia within the CNS (Barnum, 2016; Beck et al., 2010; Peterson and Anderson, 2014). These endogenous sources of complement in the SCI microenvironment are largely unstudied but could be critical in complement-mediated reactions and myelin clearance after injury.

Many factors within the SCI lesion environment potentially activate the complement cascade with downstream effects on myelin clearance (**Figure 1.2**). For example, binding of complement protein C1q to oligodendrocyte myelin glycoprotein (OMgp) drives myelin opsonization and clearance (Johns and Bernard, 1997). OMgp contains an amino acid motif that shares homology with C1q binding sites on PAMPs/DAMPs (Johns and Bernard, 1997). OMgp and C1q levels increase after SCI (Anderson et al., 2004; Dou et al., 2009). Since C1q binding to OMgp initiates complement activation, it is likely that C1q mediates myelin debris clearance and inflammation after SCI (**Figure 1.2**). Indeed, C1q knockout mice have improved SCI recovery and tissue sparing and altered macrophage activation compared to wild-type SCI controls (Galvan et al., 2008).

Another potentially important complement protein in myelin-immune interactions after SCI is C5. Studies conducted primarily in models of MS and

Alzheimer's disease demonstrate that formation of the C5b-9 membrane attack complex on oligodendrocytes leads to extensive demyelination and oligodendrocyte cell death (Bradt et al., 1998; Liu et al., 1983; Rus et al., 2009). This lytic attack requires the proteolytic cleavage of complement into bioactive proteins including C5a among others (**Figure 1.2**). While not well understood within the CNS, in the periphery C5a binds to receptors on mast cells and basophils ultimately inducing degranulation and release of vasoactive substances. These substances, such as histamine, increase blood vessel permeability (Parham, 2009). Further, C5a is a potent chemoattractant for monocytes and neutrophils (Marder et al., 1985). C5a can therefore drive inflammation by increasing recruitment and efflux of immune cells from the blood to sites of cellular debris (Miller and Stella, 2009). In the context of myelin debris induced complement activation after SCI, increased C5a could act as an ongoing recruitment signal and mediator of macrophages entry into the injury site. A recent study demonstrated that loss of C5aR, the receptor for C5a, reduces macrophage recruitment and inflammatory cytokine production early after SCI (Brennan et al., 2015). Further, C5a has direct apoptotic effects on neurons that may contribute to secondary injury, thereby increasing cellular debris (Farkas et al., 1998; Humayun et al., 2009). It is therefore feasible that complement-mediated myelin debris clearance results in pro-inflammatory macrophage activation, increased demyelination, and further complement activation (**Figure 1.2**). Collectively, complement, myelin, and macrophage interactions may form a

positive feedback cycle maintaining a chronic inflammatory state in the injured spinal cord.

Targeting Myelin as an Inflammatory Mediator in SCI

Spinal cord injuries and other neuroinflammatory conditions inevitably result in tissue destruction and the generation of cellular and myelin debris. While debris must be cleared to begin recovery, it has direct effects on the cells that clear it, namely macrophages and microglia. The mechanisms contributing to the failed debris clearance in SCI by phagocytes remain unclear, as do the myelin-mediated pathways invoking inflammatory responses, including myelin-driven complement-mediated inflammation. Myelin is not inherently pro-inflammatory in all scenarios but is capable of producing detrimental outcomes when cleared and processed under specific cellular and environmental conditions. This indicates that the pathological effects of myelin may be receptor or activation state dependent. Targeting these pathways and receptors opens the possibility for therapeutic interventions to improve recovery after SCI. Further, given myelin's ubiquitous presence in the CNS, the development of new therapies will likely impact a variety of disorders in which complement, myelin, and macrophage interactions contribute to persistent inflammation.

1- iii. Cytosolic Phospholipase A2 as an Inflammatory Mediator

Cytosolic Phospholipase A2 as an Inflammatory Mediator Summary

$cPLA_2$ enzymatically releases arachidonic acid (AA) from cellular membranes in response to inflammatory stimuli. AA is then converted into dozens of lipid signaling molecules in the eicosanoid signaling family with diverse, albeit largely pro-inflammatory, roles in inflammation. $cPLA_2$ is implicated in the vast majority of conditions involving significant inflammatory responses, including SCI. While $cPLA_2$ is generally pro-inflammatory, it also capable of inducing protective anti-inflammatory lipids under certain cellular conditions. As a result, we seek to specifically target pro-inflammatory $cPLA_2$ activity in myelin-laden macrophages. To this aim, in this body of work, we detail the pathological roles of $cPLA_2$ in chronically pro-inflammatory myelin-laden macrophages in chapter 2 and propose alternative approaches to specifically target $cPLA_2$ in macrophages after SCI in chapters 4 and 5.

Cytosolic Phospholipase A2 as an Inflammatory Mediator

Cytosolic phospholipase A2 ($cPLA_2$) is a major mechanism initiating and regulating inflammatory responses in various cell types, including macrophages (Dennis and Norris, 2015; Gijón and Leslie, 1999). Arachidonic acid is stored at the sn-2 position of membrane phospholipids, where it is largely inactive. Enzymes from the lipid-cleaving phospholipase A2 family can release AA from the membrane, of which $cPLA_2$ is the most ubiquitous and widely studied due to

its role in the targeted release of AA in response to a variety of agonists (Gijón and Leslie, 1999). Basal $cPLA_2$ expression is increased in response to growth factors and inflammation in various cell types, notably macrophages. Enzymatic activity of $cPLA_2$ is activated by phosphorylation of its serine 505 site by mitogen-activated protein kinase (MAPK) in response to increased intracellular calcium, inflammatory stimuli, or reactive oxygen species, many of which are substantially elevated after SCI (van Rossum et al., 1999). The amount of AA released by activated $cPLA_2$ is then largely dependent on substrate availability, that is, the amount of AA present in the membranes of the endoplasmic reticulum. Additionally, the oxidation of lipids in the membrane by ROS alters its viscosity, further increasing AA availability to $cPLA_2$ (van Rossum et al., 1999).

Upon its release, AA serves as the primary precursor to the eicosanoid family of inflammatory mediators produced through three synthesis pathways. The cyclooxygenase (COX) pathways produce prostaglandins, thromboxanes, and prostacyclins, the lipoxygenase (LOX) pathways produce leukotrienes, hydroxy fatty acids, lipoxins, and hepoxilins, and finally, the cytochrome P-450 pathway produces fatty acid epoxides and hydroxy fatty acids. These eicosanoids have diverse, albeit largely pro-inflammatory functions, including the activation of the inflammatory NF-kB signaling cascade, increasing edema through vasodilation, and the chemoattraction of immune cells (Brash, 2001; Cubero and Nieto, 2012; Morcos and Ouf, 1986; Sung et al., 2007). The synthesis of eicosanoids is regulated predominately by the amount of free AA available and the activation state of the oxygenases. Together these lipid

mediators are primary drivers of the initiation and resolution of inflammation (Farooqui, 2012; Farooqui et al., 2007; 2010). In macrophages, eicosanoids have influential roles in regulating phagocytic activity and their microbial defenses (Bailie et al., 1996; Coffey et al., 1998; Knapp and Melly, 1986; Wirth and Kierszenbaum, 1985; Yong et al., 1994). The specific role of each eicosanoid on macrophage physiology is poorly understood in the context of SCI inflammation. General extrapolation from the $cPLA_2$ literature would suggest a predominantly pro-inflammatory, detrimental role for eicosanoids in macrophage activation during SCI inflammation (Dennis and Norris, 2015).

$cPLA_2$ has long been investigated as a mediator of inflammation. Early studies recognized PLA_2 enzymes as key mediators of inflammation, notably in venoms from reptiles and wasps (Fairbairn, 1948). Later work identified the role of calcium in regulating PLA_2 activity, preempting the recognition of calcium-dependent phospholipase A2 ($cPLA_2$) (Hülsmann, 1983). In the coming decades, the role of $cPLA_2$ in inflammatory responses was widely implicated in numerous disease models, including brain injury, Alzheimer's disease, ultraviolet skin damage, kidney diseases, periodontitis, arthritis, programmed cell death in response to chemotherapy in melanomas, and cervical dilation in pregnancy (Cybulsky et al., 1995; Gresham et al., 1996; Kramer et al., 1996; Liu et al., 2019; Shinohara et al., 1992; Stephenson et al., 1996; Voelkel-Johnson et al., 1996). $cPLA_2$ is a key mediator of inflammation throughout the body. As a result, $cPLA_2$ is nearly ubiquitously involved in nearly every disorder in which inflammation is a major contributor, including SCI (Liu et al., 2006).

When $cPLA_2$ is widely activated from a large stimulus, such as a major injury, the eicosanoid storm is initiated (Dennis and Norris, 2015). Within minutes of an SCI, there are rapid spikes in the production of specific eicosanoids downstream of AA. Some like 5-HETE are elevated within 5 minutes of injury and remain elevated for days; others like LTC_4 peak within 10 minutes and dissipate with the hour (Jacobs et al., 1987; Mitsuhashi et al., 1994). Similarly, other eicosanoids like TXB2 and 6-keto-PGF1 rise after injury and remain elevated long after injury (Jacobs et al., 1987). There are dozens of these AA-derived eicosanoids produced after injury; however, the cellular specific production, activity, and duration of these mediators are poorly understood in the context of SCI. While most eicosanoids are generally pro-inflammatory, some can mediate regeneration and the resolution of inflammation (Dennis and Norris, 2015). Studies that target $cPLA_2$ in SCI are thereby nonspecifically blocking all eicosanoid activities downstream of AA.

Interestingly, myelin membranes contain high concentrations of AA, stored in its inactive esterified state; however, whether $cPLA_2$ can act on these lipids remains unknown. In theory, myelin could contribute substantial quantities of AA to cellular stores in the membranes of the endoplasmic reticulum, nuclear envelope, and other potential sites (Gijón and Leslie, 1999; Schievella et al., 1995). In this body of work, we examine the activity of $cPLA_2$ across macrophage activation states and Identify $cPLA_2$ as a key mediator of myelin's effects on the macrophage inflammatory response.

$cPLA_2$ has been targeted in SCI with differential results. In a transgenic mouse study, $cPLA_2^{-/-}$ mice were found to have improved recovery after SCI highlighting $cPLA_2$ as a novel therapeutic target for SCI therapeutics and found pathological roles for related phospholipases (Lopez-Vales et al., 2008). We hypothesize that this dichotomy is a byproduct of blocking both beneficial and detrimental eicosanoids downstream of AA. Lopez et al. did not observe detrimental effects of $cPLA_2$ ablation until two weeks after injury. It is possible that blocking some of the anti-inflammatory eicosanoids typically produced later in the inflammatory response could have contributed. To this aim, in this body of work, we detail the pathological roles of $cPLA_2$ in chronically pro-inflammatory macrophages in chapter 2 and propose alternative approaches to specifically target $cPLA_2$ in macrophages after SCI in chapters 4 and 5.

1- iv. Azithromycin as an Immunomodulatory Antibiotic

Azithromycin as an Immunomodulatory Antibiotic Summary

Azithromycin is a promising therapeutic for the treatment of SCI. First, it is already FDA-approved, thereby bypassing many of the regulatory challenges of new therapeutics. Next, the concept that azithromycin drives beneficial macrophage activation states has been well established in other disease models. In SCI, our previous pre-clinical studies demonstrated AZM's ability to shift macrophage phenotype, thereby improving recovery. In this body of work, we build upon our prior AZM studies in chapter 3 by delaying the initiation of AZM

administration until minutes and hours after the injury. This subtle alteration in experimental design is a critical step in AZM's development as a therapeutic for SCI as it better reflects the clinical reality of therapeutic administration. Lastly, we discuss the remaining challenges in advancing AZM towards clinical use.

Azithromycin as an Immunomodulatory Antibiotic

While the role of the macrophage inflammatory response in SCI pathophysiology has been well characterized through basic research, there is no therapeutic available to target this pathway in humans. Further, the use of the only therapeutic available for SCI, methylprednisolone, has diminished due to potential side effects (Evaniew et al., 2015). This has created an urgent need for the development of additional therapeutics to improve SCI recovery in humans. To this aim, our prior works have investigated the FDA-approved immunomodulatory antibiotic, Azithromycin (AZM), as a therapeutic in a pre-clinical mouse model of SCI (Gensel et al., 2017; Zhang et al., 2015b).

In these proof of concept studies, we found that “AZM drives alternative macrophage activation and improves recovery and tissue sparing in contusion spinal cord injury” (Zhang et al., 2015b). These results were consistent with studies from other disease models such as stroke and lung infections with Pseudomonas aeruginosa, where AZM exerted therapeutic effects through its ability to modulate macrophage phenotype (Amantea et al., 2016b; Feola et al., 2010a). Specifically, AZM inhibits the STAT1 and NF-κB signaling pathways in

macrophages, thereby promoting the anti-inflammatory activation state (Haydar et al., 2019).

In our first proof of concept study (Zhang et al., 2015b), we administered AZM via oral gavage to three-month-old female mice daily starting three days prior to injury and then daily for up to seven days. In FACS-sorted macrophages isolated from the SCI lesion, we observed a significant increase in anti-inflammatory (M2) markers (Arginase-1 and CD206) and a significant decrease in CD86, which is associated with pro-inflammatory (M1) macrophages. Similarly, at 3dpi, AZM induced a significant increase in CD206 in microglia. Histologically at 28dpi, AZM significantly increased tissue sparing relative to vehicle. When examined in an open field during the course of 28dpi we observed significantly improved locomotion in AZM treated animals as measured by the Basso Mouse Scale (BMS). Similarly, AZM significantly improved proprioceptive locomotor function determined by the relative number of hindlimb footfalls with a Gridwalk apparatus at 28dpi. Lastly, *in vitro*, supernatant from pro-inflammatory “M1” macrophages was applied to a Neuro2A neuron culture. Supernatants from macrophages treated with AZM were significantly less toxic to neurons. Collectively, this work established the proof of concept that AZM improved SCI recovery by driving alternative macrophage activation.

In our next study developing AZM as an SCI therapeutic, we sought to better understand exactly how AZM was altering macrophage activation after SCI. To this aim, we first utilized our BMDM *in vitro* system to thoroughly examine the gene expression profiles of pro-inflammatory “M1” and anti-

inflammatory “M2” macrophages (Gensel et al., 2017). This identified a series of gene targets that are differentially expressed in M1 and M2 macrophages. Next, we administered AZM doses of 10, 40, or 160mg/kg to four-month-old female mice at the time of injury and then daily for the rest of the survival period (3 or 7 days). We then isolated macrophages from the injury site at the time of euthanasia and subjected them to the same gene panel utilized on our BMDM samples. Using this data, we were then able to detect which M1/M2 gene targets were suppressed or elevated by AZM in each dosing paradigm. Lastly, in a separate cohort of animals processed for histological analyses (160 mg/kg dosage), we examined protein expression of a series of M1 and M2 markers at 3 and 7dpi. At 3dpi, AZM significantly increased M2-associated markers CD206 and Arg-1 and decreased M1-associated markers MARCO and CD86. Similarly, at 7dpi, AZM significantly decreased M1-associated markers MARCO and CD86; however, at this timepoint CD206 and Arg-1 were unaffected. Collectively, this work demonstrated a dose-dependent immunomodulatory shift in macrophage activation following SCI (Gensel et al., 2017).

In this body of work, we build upon our prior AZM studies in chapter 3 by delaying the initiation of AZM administration until minutes and hours after the injury. This subtle alteration in experimental design is a critical step in AZM's development as a therapeutic for SCI as it better reflects the clinical reality of therapeutic administration. Lastly, we discuss the challenges in advancing AZM towards clinical use and address the required steps to continue its development.

Azithromycin Dosing Strategies

A major limitation of our early study is that we used a pre-injury and post-injury dosing paradigm because it was shown to be effective in another disease model (Feola et al., 2010b). Indeed, this approach resulted in clear therapeutic benefits by successfully shifting macrophage activation states (Zhang et al., 2015b); however, this dosing paradigm is not clinically relevant. In our second study, we administered AZM beginning 30 minutes after injury; however, we did not examine long-term functional recovery. This dosing paradigm, while improved, would still present great challenges for clinical translation. In the second and third National Acute Spinal Cord Injury Randomized Controlled Trials (NASCIS) of methylprednisolone for the treatment of SCI, many patients were unable to begin treatment until several hours after their injury (Bracken et al., 1991; 1997). Given that AZM is widely regarded as safe even when taken for extended periods (Uzun et al., 2014) and is already widely prescribed (Durkin et al., 2018), it is possible that AZM may be administered much faster than methylprednisolone; however, 30 minutes from injury to administration would remain a clinical challenge for many patients. In this body of work, we address this by examining the therapeutic effectiveness of AZM when administered starting 30-minutes, 3-hours, and 24 hours after SCI throughout a comprehensive 28 day recovery period.

1- v. Macrophage-Targeted Therapies for the Treatment of SCI and Other Neurological Conditions.

Macrophages dominate the SCI lesion environment mediating both destructive and reparative responses after injury (Kigerl et al., 2009). Generally, however, SCI macrophages predominately adopt a pro-inflammatory activation state thought to impair recovery (Gensel et al., 2017; Kigerl et al., 2009). These macrophages have thus become critical therapeutic targets to improve recovery after SCI. Specifically, macrophage-targeted therapies generally seek to reduce pro-inflammatory activation. This could reduce macrophage production of toxic mediators, including reactive oxygen species (ROS), nitric oxide, and pro-inflammatory cytokines and eicosanoids, thereby reducing excessive cellular damage and death. Conversely, macrophage-targeted therapies often aim to increase anti-inflammatory, pro-reparative macrophage activation states. This could promote the resolution of inflammation and induce an environment more supportive of healing and repair. Macrophages exist along a spectrum of activation states; macrophage-targeted therapies seek to shift the general activation characteristics of these cells to minimize damage and promote repair.

As discussed previously, AZM is a promising immunomodulatory antibiotic capable of shifting macrophage activation states to improve recovery after SCI (Gensel et al., 2017; Zhang et al., 2015b). Similarly, $cPLA_2$ is a key mediator of pro-inflammatory macrophage activation and detrimental cellular activities. As such, AZM and $cPLA_2$ inhibitors are promising macrophage-targeted therapies to improve recovery after SCI. In this body of work, we continue to build pre-clinical

data developing these macrophage-targeted therapies into therapeutics for SCI. Additionally, in chapter 4 we continue to develop additional strategies to target macrophages after SCI, including transgenic manipulation of $cPLA_2$, AZM derivative pharmaceuticals with altered immunomodulatory profiles, and the immunomodulatory cytokine leukemia inhibitory factor (LIF). Lastly, we cover the remaining macrophage-targeted therapies currently and previously in development in the SCI research field.

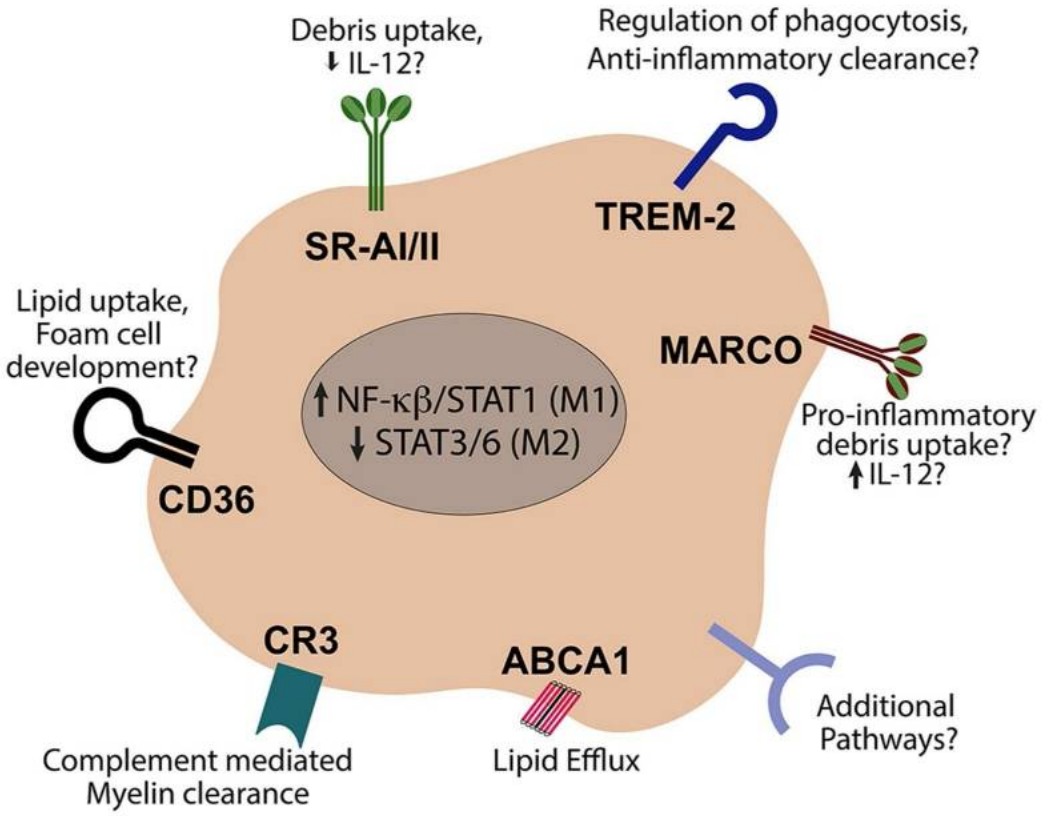


Figure 1.1. Macrophage receptors potentially mediating myelin clearance and inflammatory activity after spinal cord injury

In addition to activating a number of different receptors, in the context of SCI, myelin also drives downstream pathways (Stat1) associated with pro-inflammatory (M1) macrophage activation. References can be found in the main text.

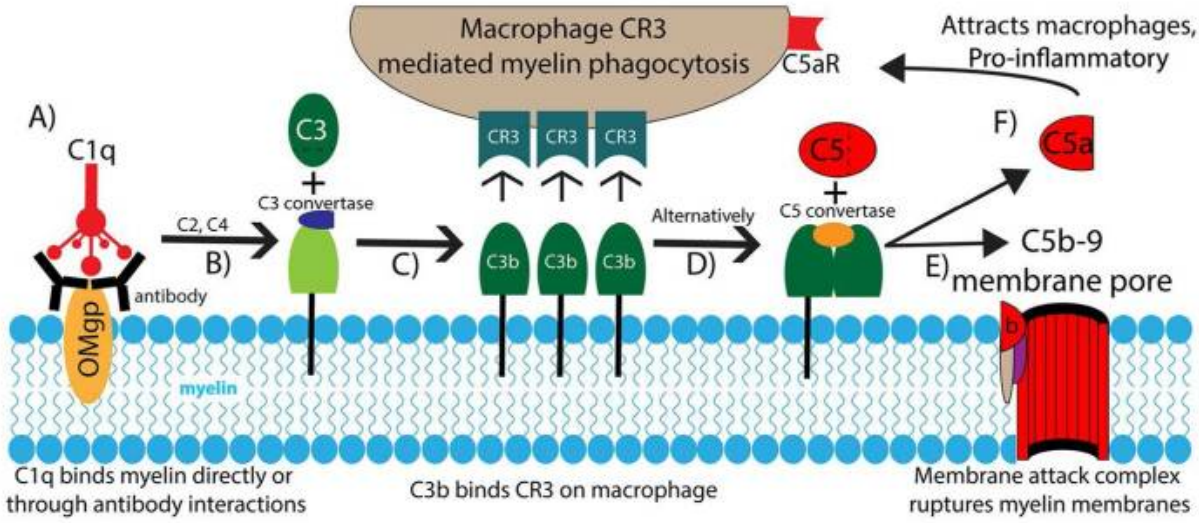


Figure 1.2. Complement system in myelin clearance and macrophage recruitment after spinal cord injury.

Numerous complement pathways are initiated by myelin debris after SCI. (A) C1q binds directly to myelin oligodendrocyte myelin glycoprotein (OMgp) or to an antibody intermediate as depicted. (B) C1q binding initiates the formation of a C3 convertase capable of cleaving C3. (C) The C3b cleavage fragment opsonizes myelin debris and binds to macrophage complement receptor 3 (CR3) initiating the phagocytosis of myelin debris. (D) C3b can also induce the formation of a C5 convertase. (E) The C5 convertase initiates the cleavage of C5 and the subsequent recruitment of C6-9 creating the C5b-9 membrane attack complex capable of rupturing myelin membrane debris or intact oligodendrocytes. (F) The C5a fragment released during this process acts as both a potent recruitment signal for macrophages to the site of complement opsonization, through the C5 receptor (C5aR) and as a pro-inflammatory, vasoactive stimulus on a variety of other cell types. References can be found in the main text.

Chapter 2: The Effects of Myelin on Macrophage Activation are Phenotypic Specific via $cPLA_2$ in the Context of Spinal Cord Injury Inflammation

This chapter is adapted from Kopper et al. 2021

2- i. Chapter Summary

Spinal cord injury (SCI) produces chronic, pro-inflammatory macrophage activation that impairs recovery. The mechanisms driving this chronic inflammation are not well understood. Here, we detail the effects of myelin debris on macrophage physiology and demonstrate a novel, activation state-dependent role for cytosolic phospholipase-A2 ($cPLA_2$) in myelin-mediated potentiation of pro-inflammatory macrophage activation. We hypothesized that $cPLA_2$ and myelin debris are key mediators of persistent pro-inflammatory macrophage responses after SCI. To test this, we examined spinal cord tissue 28-days after thoracic contusion SCI in 3-month-old female mice and observed both $cPLA_2$ activation and intracellular accumulation of lipid-rich myelin debris in macrophages. *In vitro,* we utilized bone marrow-derived macrophages to determine myelin's effects across a spectrum of activation states. We observed phenotype-specific responses with myelin potentiating only pro-inflammatory (LPS+INF-γ; M1) macrophage activation, whereas myelin did not induce pro-inflammatory responses in unstimulated or anti-inflammatory (IL-4; M2) macrophages. Specifically, myelin increased levels of pro-inflammatory cytokines, reactive oxygen species, and nitric oxide production in M1 macrophages as well as M1-mediated neurotoxicity. PACOCF3 ($cPLA_2$ inhibitor)

blocked myelin's detrimental effects. Collectively, we provide novel spatiotemporal evidence that myelin and $cPLA_2$ play an important role in the pathophysiology of SCI inflammation and the phenotype-specific response to myelin implicate diverse roles of myelin in neuroinflammatory conditions.

Keywords: Neuroinflammation, Foamy, Neurotrauma, Secondary Injury, Eicosanoid, Brain, IL-10, Foam cell, Arachidonic acid, M1/M2.

2- ii. Background:

Spinal cord injury (SCI) triggers a complex neuroinflammatory response that mediates tissue repair but also potentiates secondary injury processes. Activated macrophages, consisting of resident microglia and infiltrating monocytes, contribute to this dichotomous response. Macrophages facilitate repair by increasing axon growth, stem cell differentiation, and revascularization (Gensel and Zhang, 2015; Kigerl et al., 2009), however, macrophages can also contribute to pathology through secondary injury processes involving reactive oxygen species (ROS), neurotoxins, and pro-inflammatory cytokine release as well as by causing axon retraction and dieback (Gensel and Zhang, 2015; Horn et al., 2008). The extent to which macrophages are polarized toward reparative (also called M2 or anti-inflammatory) or pathological (also called M1 or pro-inflammatory) phenotypes largely depends on the stimuli present in the injured spinal cord.

One notable distinction between SCI and self-resolving peripheral nerve injuries is the sustained presence of myelin debris. Lipid-laden myelin debris is taken up and processed predominantly by infiltrating macrophages, rather than endogenous microglia, after SCI (Wang et al., 2014). Phagocytic markers are present on these chronically activated macrophages, which become visibly laden with debris (Fleming et al., 2006; Greenhalgh and David, 2014; Kroner et al., 2014; Vargas and Barres, 2007; Wang et al., 2014). It is therefore likely that myelin lipids are actively processed by macrophages and are environmental stimuli that drive chronic spinal cord inflammation. Indeed, in areas of Wallerian degeneration, macrophages disappear from the chronically injured spinal cord concomitant with myelin debris clearance (Becerra et al., 1995; Greenhalgh and David, 2014; Vargas and Barres, 2007; Wang et al., 2014). The accumulation of lipid debris in the days and weeks after injury also closely aligns with the temporal shift in macrophage phenotype, ending with persistent pro-inflammatory activation by 28 dpi (Kigerl et al., 2009; Wang et al., 2014). Evidence also suggests that myelin acts as an inflammatory stimulus on macrophages *in vitro*, implicating a key link between myelin debris accumulation and the grievous shift in macrophage phenotype which impairs regeneration after SCI (Kopper and Gensel, 2017; Kroner et al., 2014; Wang et al., 2014; Williams et al., 1994).

While myelin debris is implicated in macrophage activation state and recovery after SCI (Kroner et al., 2014; Wang et al., 2014; Zhu et al., 2017), the intracellular mechanisms mediating myelin's effects remain unclear. Cytosolic phospholipase A2 ($cPLA_2$) facilitates arachidonic acid (AA) release from cellular

membranes following inflammatory stimuli and is largely unstudied in SCI macrophage responses. Interestingly, myelin membranes contain high concentrations of AA, stored in its inactive esterified state; however, whether $cPLA_2$ can act on these lipids remains unknown. $cPLA_2$-mediated breakdown of AA initiates an eicosanoid storm in which a wide variety of bioactive lipids are released including prostaglandins, leukotrienes, and thromboxanes. Eicosanoids have diverse albeit largely pro-inflammatory functions including activating the inflammatory NF-kB signaling cascade and increasing edema as well as potentiating immune cell chemoattraction, fibrosis, and inflammatory responses. The role of $cPLA_2$ in macrophage physiology has been detailed in other systems; however, it is unknown if $cPLA_2$ has any differential effects in macrophages polarized along the spectrum of activation states in the presence of myelin, or if it is a contributor to prolonged pro-inflammatory activation of macrophages after SCI. $cPLA_2$ activation is induced by inflammatory stimuli such as LPS/IFN-γ, also known as the M1, pro-inflammatory paradigm *in vitro,* and is likely induced by the complex inflammatory environment observed after SCI (Dennis and Norris, 2015; Liu et al., 2006; 2014). This is the basis of our hypothesis that **$cPLA_2$ activity in myelin-laden macrophages after SCI aggravates tissue damage and contributes to chronic inflammation.** Here we establish these mechanisms *in vitro*, and provide the proof-of-concept that these pathways may play an important role *in vivo* after SCI.

2- iii. Methods:

Animals

As described previously (Kopper et al., 2019; Zhang et al., 2015b), *in vitro* experiments were performed using 2–4-month-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine). *In vivo* experiments were performed using 4-month-old female C57BL/6 mice, weighing 20.7 g ± 1.3 g (Jackson Laboratory, Bar Harbor, ME, USA). Animals were housed in IVC cages with ad libitum access to food and water. All procedures were performed in accordance with the guidelines and protocols of the Office of Research Integrity and with approval of the Institutional Animal Care and Use Committee at the University of Kentucky. All experiments were carried out in compliance with the ARRIVE guidelines (Kilkenny et al., 2010).

Cell Culture

Bone marrow-derived macrophages (BMDMs) were extracted from the femur and tibia of female C57BL/6 mice at 2–4 months old as previously reported (Gensel et al., 2009; 2015) and were plated at 0.8–1 × 10^6 cells/mL in differentiation media containing Roswell Park Memorial Institute medium (RPMI, Thermo Fisher Scientific, #21870-092) supplemented with 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific, #5140122), 1% HEPES (Sigma-Aldrich, #83264-100ML-F), 1% GlutaMAX 0.001 (Thermo Fisher Scientific, #35050061) 0.001% β-mercaptoethanol (Thermo Fisher Scientific, #21985023), 10% FBS (Life technologies, #10082147), and 20% supernatant

from sL929 cells (a generous gift from Phillip Popovich, The Ohio State University). Supernatant collected from sL929 cells contains macrophage colony-stimulating factor, which helps to promote bone marrow cells' differentiation into macrophages(Burgess et al., 1985). The BMDMs were allowed to differentiate for 7 days in culture, and cells were then replated on day 7 at a density of 1×10^6 cells/mL in 12-well plates in RPMI, containing 1% P/S, 1% GlutaMAX and 10% FBS. On day 8, cells were stimulated for 24 h to be either M1 using LPS (50 ng/mL, Invivogen, #tlrl-eblps, standard preparation) plus IFN-γ (20 ng/ml, eBioscience #14-8311-63) diluted in N2A growth medium (described below), M2 using IL-4 (20 ng/ml, R&D systems, #404-ML-010), or Control/Unstimulated (CTL) using fresh media without any stimulants. At the time of stimulation cells were immediately treated with myelin debris (50 µL/mL, preparation described below), 50 µM PACOCF3 (inhibitor of $cPLA_2$, Tocris Bioscience, CAS 141022-99-3), or PBS/DMSO vehicles to equalize volume and drug solvent concentrations across groups. 24 h after stimulation the supernatants were removed, centrifuged at 13,000 RPM (Fisher Scientific accuSpin Micro R centrifuge), and then this macrophage conditioned media (MCM) was either applied directly to N2A cells to measure cytotoxicity, or stored at − 80 °C prior to testing for IL-12p40 levels using standard ELISA kits (Thermo Fisher Scientific, Rockford, IL # EMIL12P40), Nitric Oxide with the Griess Reagent Kit (Thermo Fisher Scientific # G-7921), and phenol red-free RPMI, or a mulit-plex ELISA system measuring protein levels of TNF-alpha, IL-1Beta, IL-6, CX3CL1, and IL-10 (Meso Scale Diagnostics). BMDMs for coverslip stains were treated as above, except at a lower plating

density of 3×10^5 cells/mL. Coverslips were fixed in cold 2% PFA for 30 min, washed in PBS and stored at 4 °C until staining.

Moderate purity myelin (> 95% myelin, with small contributions from axolemma and other cellular membranes) was prepared as follows (adapted from Larocca et al. (Larocca and Norton, 2001)): brains were collected from C57BL/6 mice at the time of BMDM isolation and stored at − 80 °C prior to myelin isolation. The brains were rinsed and suspended in cold PBS with 1% P/S and placed in a Dounce homogenizer (DWK Life Science, #357544) under sterile conditions and blended with the loose and tight pestles. The solution was transferred to a 15 mL tube and pelleted at 2000 RPM (Thermo Scientific Legend XTR centrifuge) prior to discarding the soluble supernatant fraction. The pellet was resuspended in the PBS/P/S, and then 5mLs of a 30% Percoll solution (Sigma-Aldrich, #P1644-500ML) was gently underlaid below the myelin solution for density gradient centrifugation. The layers were then centrifuged at 2000 RPM for 15 min at 4 °C under gentle acceleration/deceleration, generating three distinct layers (soluble on top, myelin in middle, and Percoll/cell pellet on bottom). After removing the soluble fraction, the myelin was transferred to a fresh tube and resuspended in 10 mL distilled water with 1% P/S and incubated for 10 min (hypoosmotic shock) to separate membranes at 4 °C. The myelin was then re-pelleted at 2000 RPM, suspended in PBS/1% P/S and separated a second time by density gradient centrifugation as described above. The myelin was then suspended and pelleted twice in PBS/1% P/S to remove residual Percoll and water-soluble contaminants, and then aliquoted before storage at − 80 °C. The final protein concentration of

the myelin stock solutions produced by this protocol were 10.23 mg/mL with a standard deviation of 0.282 mg/mL as determined by a BCA Protein Assay Kit (Thermo Fisher Scientific #23225). With the application of myelin debris to BMDMs at 50 µL/mL, cells had a mean dosage of 0.51 mg/mL. Lastly, to ensure our results were not due to endotoxin contamination in our myelin preparations, we tested aliquots from each batch of myelin stimulant (Thermo Fisher Scientific #88282).

A mouse neuroblastoma cell line (Neuro-2a or N2A, a gift from Chris Richards, University of Kentucky) was maintained in N2A growth medium containing 45% DMEM, 45% OPTI-MEM reduced-serum medium, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. N2A were plated at a density of 1×10^5 cells/mL in 96-well tissue culture plates and allowed to proliferate for 48 h. The neurotoxicity of MCM was evaluated as reported previously using a MTT-based cell growth determination kit according to the manufacturer's instructions (Sigma-Aldrich CGD1-1KT) (Zhang et al., 2019). Briefly, 24 h before testing, N2A growth media was replaced with serum-free N2A media to induce differentiation. The day of testing this media was replaced by fresh MCM, and the N2A cells were incubated in MCM for 24 h before thiazolyl blue tetrazolium bromide (MTT (5 mg/ml), 20 µl per well) was added to each well and the cells further incubated for 2 h. The tetrazolium ring of MTT can be cleaved by mitochondrial dehydrogenases of viable cells, yielding purple formazan crystals, which were then dissolved in acidified isopropanol solvent. The resulting purple solution was spectro-photometrically measured at 570 nm

Epoch microplate reader (BioTek Instruments, Inc., Winooski, VT) using 690 nm as a background absorbance. This data is normalized to the non-toxic CTL values to generate proportional decrease in viability values and presented inversely as increased toxicity relative to CTL.

Macrophage reactive oxygen species (ROS) production was measured using CM-H2DCFDA (Invitrogen #C6827). In short, BMDMs were cultured and stimulated as described above except in a 96 well plate (1×10^6 cells/mL). Following the 24-h stimulation the supernatants were removed and replaced with a 5 μM solution of CM-H2DCFDA in phenol red-free RPMI with 1% GlutaMAX and penicillin/streptomycin and incubated at 37 °C for 25 min. ROS mediates the conversion of this compound to fluorescent DCF which was then detected by an Epoch microplate reader (BioTek instruments, Inc., Winooski, VT) at the compound's Excitation/Emission spectra of approx. 492–495/517–527 nm.

Macrophage $cPLA_2$ activity was measured using a Cytosolic Phospholipase A2 Assay Kit (Abcam #ab133090). In short, cells were cultured as described above except in six well culture dishes (1×10^6 cells/mL). Cells were lysed and briefly sonicated on ice in TBS-T (0.4% Triton-X) with a protease inhibitor (Sigma-Aldrich #11836170001) before proceeding directly into the manufacturer's protocol.

Spinal Cord Injury

As described previously (Kopper et al., 2019; Zhang et al., 2015b) , animals were anesthetized via intraperitoneal (i.p.) injections of ketamine

(100 mg/kg) and xylazine (10 mg/kg). Following a T9 laminectomy, a moderate-severe thoracic SCI was produced using the Infinite Horizon (IH) injury device (75-kdyn displacement; Precision Systems and Instrumentation). Any animals receiving SCI with abnormalities in the force vs. time curve generated by the IH device were excluded from analysis. These abnormalities are indicative of bone hits or instability in the spinal cord at the time of injury and occurred < 10% of the time. After injury, muscle and skin incisions were closed using monofilament suture. After surgery, animals received one subcutaneous injection of buprenorphine-SR (1 mg/kg) and antibiotic (5 mg/kg, enroloxacin 2.27%: Norbrook Inc., Lenexa, KS) in 2 mL of saline and were housed in warming cages overnight. Animals continued to receive antibiotic subcutaneously in 1 mL saline for 5 days. Food and water intake and the incision site were monitored throughout the course of the study. Bladder expression was performed on injured mice twice daily. Mice were sacrificed at 7 and 28 days post-injury (n = 8 and 10, respectively) to generate spinal cord sections for histological analyses.

Tissue processing and immunohistochemistry

As described previously (Kopper et al., 2019; Zhang et al., 2015b) , mice were anesthetized and then transcardially perfused with cold PBS (0.1 M, pH 7.4), followed by perfusion with cold 4% paraformaldehyde (PFA). Dissected spinal cords (1 cm) were post-fixed for another 2 h in 4% PFA and subsequently rinsed and stored in cold phosphate buffer (0.2 M, pH 7.4) overnight at 4 °C. On the following day, tissues were cryoprotected in 30% sucrose for 3 days at 4 °C,

followed by rapidly freezing and blocking in optimal cutting temperature (OCT) compound (SakuraFinetek USA, Inc.) on dry ice. Tissue blocks were cut in serial coronal sections (10 μm) and mounted onto Colorfrost plus slides (Fisher #12-550-17).

Spinal cord sections were stained with Eriochrome Cyanine (myelin) and anti-Neurofilament (1:1000, Aves labs, NFH) to visualize damage and thereby identify the epicenter of each lesion, as defined as the point where spared tissue constitutes the smallest proportion of spinal cord volume (Kopper et al., 2019). Immunohistochemistry on tissue sections and BMDM coverslips was performed to stain for phosphylated-cPLA$_2$ (p-cPLA$_2$; rabbit, 1:500, Cell Signaling Technology #2831S), BODIPY (2 μM 30 min, Thermo Fisher Scientific #D3922), biotinylated tomato lectin (TomL) (Sigma-Aldrich L0651-1MG, and DAPI (Sigma-Aldrich #D9542-10MG) overnight at 4 °C. Secondary antibodies were applied at 1:1000 for 1 h at RT: Alexa Fluor 546 goat anti-rabbit (Life Technologies #11010), and Streptavidin Alexa Fluor 647 conjugate (Thermo Fisher Scientific #S-21374). Antigen retrieval was performed to improve signal: 10 min in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) at 90 °C. BODIPY is specific to neutral lipids from the breakdown of myelin and cellular membranes. TomL binds to poly-N-acetyl lactosamine on macrophages and microglia. TomL also binds to large blood vessels which were excluded from analysis when possible based on their large tubular morphology (Schmid et al., 2002; Villacampa et al., 2013). Imaging was performed at or within 100 μm of lesion epicenter due to tissue loss during antigen retrieval. All images were taken

using a C2+ laser scanning confocal microscope (Nikon Instruments Inc., Melville, NY, USA). Images were quantified using using the MetaMorph analysis program (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis:

As described previously (Kopper et al., 2019; Zhang et al., 2015b) statistical analyses were completed using GraphPad Prism 6.0 (GraphPad Software). Data were analyzed using one- or two-way ANOVA followed by Dunnett's test for multiple comparisons. Results were considered statistically significant at $p \leq 0.05$. All data are presented as mean ± SEM unless otherwise noted. All *in vitro* measurements were done in triplicates, and at least three independent experiments were carried out. Imaging and quantification were performed by investigators fully blinded to all experimental conditions. *In vitro* experiments were not fully blinded during experimental procedures (due to the obvious presence of myelin in some conditions); however, all analyses were confirmed by an investigator blinded to experimental conditions. Figures were prepared using Adobe Photoshop CS6 (Adobe Systems) and Prism 6.0.

2- iv. Results:

Myelin-Laden Macrophages Contain Active $cPLA_2$ After SCI

In order to determine the extent to which $cPLA_2$ may be contributing to myelin processing by macrophages after SCI, we examined inflammation within the injured spinal cord 4 weeks after mouse contusion SCI. As reported

previously (Wang et al., 2014), we observed that SCI generated extensive infiltration of monocyte derived macrophages and activation of resident microglia (**Figure 2.1A,F,K)**. Similarly, we observed presumptive myelin debris, i.e. neutral lipid droplets detected by BODIPY staining inside TomL+ macrophages throughout the injury epicenter (**Figure 2.1B,G,L)**. Extracellular lipid droplets not clearly contained within macrophages were also present **(Figure 2.1B,G,L)**. Next, we sought to determine whether the $cPLA_2$ system is active (phosphorylation indicates activation) in these chronically activated macrophages within the injured spinal cord. As indicated in **Figure 2.1C,H,M,** $cPLA_2$ is widely activated (p-$cPLA_2$) throughout the lesion epicenter images of macrophages, and critically is observed within macrophages containing lipid debris at 28 days after injury (dpi). Similar observations were also seen at 7 dpi **(Supplemental Figure 2.1)**. Lastly**,** uninjured tissue from sham surgery animals contained TomL+ macrophage/microglia populations but no appreciable lipid debris, and minimal $cPLA_2$ activation **(Figure 2.1P-T)**. Collectively this provides spatiotemporal evidence that macrophages are chronically present in the injured spinal cord, loaded with substantial myelin-derived lipid debris, and can contain activated $cPLA_2$. Furthermore, these results provide the proof-of-concept that myelin and $cPLA_2$ may play a key role in the prolonged pro-inflammatory macrophage activation thought to impede semi-acute and chronic recovery after SCI.

Myelin Potentiates Pro-inflammatory Macrophage Activation in an Activation State Dependent Manner

To determine the role of myelin in the activation of macrophages we developed an *in vitro* model of SCI inflammation. BMDMs are predictive of monocyte-derived macrophage responses *in vivo* in the injured spinal cord. This have been observed at transcription (Longbrake et al., 2007) and functional levels (Gensel et al., 2015), as well as in response to therapeutic interventions (Gensel et al., 2017). Further, BMDMs are the primary myeloid cells phagocytizing myelin after SCI (Wang et al., 2014). Specifically, we used an M1 stimuli (LPS + IFN-γ) to model the detrimental pro-inflammatory activation state, M2 (IL-4) to model the reparative anti-inflammatory activation state, and CTL (unstimulated control or M0) to model a mature, yet naïve macrophage. Myelin stimulation was generated from mouse CNS tissue to model the myelin debris "stimulant" generated after SCI *in vivo*. Using this system, we observe both myelin up-take across activation states and increased $cPLA_2$ activation under M1 conditions ($p<0.001$) **(Supplementary Figure 2.2)**. Interestingly, we observed that many of myelin's effects on macrophage physiology were activation state dependent, with differential effects when applied in the M1, M2, or CTL activation states. In **Figure 2.2A** pro-inflammatory (M1) macrophages had increased production of the pro-inflammatory cytokine IL-12 compared to unstimulated (CTL) or anti-inflammatory (M2) macrophages, as would be expected ($p<0.001$). Upon the concurrent addition of myelin to these groups, however, the M1 production of IL-12 rises substantially ($p<0.01$), whereas CTL and M2 cells were

not significantly affected. In **Figures 2.2B and 2.2C** we performed additional stimulations and measured the production of reactive oxygen species (ROS) and nitric oxide, two toxic macrophage byproducts thought to contribute to cell death and SCI pathogenesis. With this, we observed a similar phenotype-specific effect in which only pro-inflammatory M1 cells were significantly potentiated by the addition of myelin, whereas CTL and M2 cells were not significantly affected. Specifically, M1 stimulation significantly increased levels of ROS ($p<0.001$), and nitric oxide relative to unstimulated (CTL) cells, as would be expected. Critically, each of these M1 mediated increases were significantly increased with the application of myelin alongside the M1 stimulation, indicating that myelin potentiates pro-inflammatory responses ($p<0.001$ and $p<0.001$ respectively). Interestingly, this novel phenomenon occurred despite observing similar degrees of myelin debris uptake across all treatment groups **(Supplemental Fig 2.2**), suggesting that differences in phagocytosis is not a contributing factor. The mechanisms through which the myelin was taken up under each phenotype, however, was not evaluated. To test the effects of myelin on an anti-inflammatory associated functional outcome we examined the activity of the arginase-1 enzyme in cell lysates from each of our stimulations **(Figure 2.2D)**. As would be expected M2 macrophages had higher arginase activity relative to M1 and CTL ($p<0.001$ and $p<0.0001$ respectively). The addition of myelin, however, did not alter arginase activity in any of the stimulations tested. Lastly, the myelin stimulants did not contain any endotoxin contamination that would confound our results **(Supplemental Figure 2.3)**. Collectively, this suggests that the pro-

inflammatory effects of myelin on macrophage activation states are phenotype-specific.

Next, we sought to determine the cellular implications of these shifts in macrophage polarization states by applying BMDM conditioned media from these cells to a neuronal cell line (N2A) to determine the relative neurotoxicity of each stimulation. In **Figure 2.2E**, we observed that M1 neurotoxicity was again significantly potentiated by the addition of myelin, whereas CTL and M2 cell toxicity was unaffected by the addition of myelin.

$cPLA_2$ Inhibition Blocks Myelin's Pro-inflammatory Potentiation of M1 Macrophages

The activation of $cPLA_2$ by inflammatory stimuli is thought of as the primary rate-limiting step in the release of AA and the initiation of the production of various eicosanoids during the onset of inflammation. Given that we observed significant effects of myelin in macrophages in the presence of pro-inflammatory stimuli (LPS and IFN-γ), we hypothesized that $cPLA_2$ activation in M1 macrophages may be a key mediator of myelin's cellular effects. To test this hypothesis, we targeted $cPLA_2$ in our *in vitro* model using the chemical inhibitor Palmityl trifluoromethylketone (PACOCF3) as used previously (Schaeffer et al., 2005). As in our initial studies, we found myelin induced significant increases in ROS, nitric oxide, and neurotoxicity when applied with an M1 stimulus, potentiating the M1 pro-inflammatory response (**Figure 2.3**). Critically, application of the $cPLA_2$ inhibitor, PACOCF3, significantly reduced the myelin-

mediated increases of ROS, nitric oxide, and neurotoxicity, indicating an important role for $cPLA_2$ in this system **(Figure 2.3)**. Interestingly, PACOCF3 did not influence arginase activity in any of the stimulations tested **(Figure 2.3D and Supplemental Figure 2.4D and 2.4H),** suggesting that $cPLA_2$ is not linked to cellular arginase activity. Given that myelin did not previously affect ROS, nitric oxide, neurotoxicity, or arginase in the context of CTL or M2 stimulation, we would not expect $cPLA_2$ to influence these outcomes. Indeed, PACOCF3 had no significant effects on CTL or M2 simulations groups **(Supplemental Figure 2.4)**. Collectively these data suggest that $cPLA_2$ may play a key role in mediating myelin's effects on pro-inflammatory macrophage activation.

Macrophage Cytokine Profiles Indicate a Mixed Neuroinflammatory Phenotype.

Macrophages exist along a spectrum of activation states commonly simplified to pro- vs. anti-inflammatory states *in vivo*, or M1/M2 *in vitro*. While these terms are used for practical purposes, macrophages are further characterized by many factors including morphology, surface markers, secreted cytokines, byproducts and functional outcomes. Our measures of neurotoxicity, IL-12, nitric oxide and ROS indicate a pathological phenotype; however, these are not comprehensive phenotypic indicators. To begin to address this complex phenotypic analysis we sought to measure additional inflammatory cytokines using a multiplex ELISA in our *in vitro* myelin/$cPLA_2$ system. In line with our previous observations, M1 macrophages had significantly increased production of the pro-inflammatory cytokines TNFα ($p>0.001$) and CX3CL1 ($p>0.001$) upon

the addition of myelin **(Figure 2.4A, 2.4C)**, and for TNFα, $cPLA_2$ inhibition blunted this effect ($p<0.01$) **(Figure 2.4B)**. Similarly, myelin significantly reduced the production of anti-inflammatory IL-10 in M1 cells, exacerbating their pro-inflammatory phenotype ($p<0.001$) **(Figure 2.4E)**. Conversely, the pro-inflammatory cytokine IL-1β was significantly decreased in M1 cells following the addition of myelin ($p<0.001$) **(Figure 2.4G)**. For the pro-inflammatory cytokine, IL-6, M1 cells were unaffected by the addition of myelin. Interestingly, for CX3CL1, myelin induced a small but significant increase in cytokine levels in CTL and M2 cells **(Figure 2.4C)**; IL-10 and IL-6 had similar trends in this regard, however, they were not statistically significant. Lastly, in some instances the $cPLA_2$ inhibitor (PACOCF3) alone induces significant cytokine shifts, decreasing CX3CL1 ($p<0.01$) and increasing anti-inflammatory IL-10 ($p<0.001$) **(Figures 2.4D, 2.4F)**, suggesting that $cPLA_2$ inhibition has anti-inflammatory effects outside the context of myelin stimulation. Interestingly, when examining the effects of myelin and $cPLA_2$ inhibition specifically within CTL and M2 stimulation we observed statistically significant but biologically small, relative to M1, increases in both pro (TNF-alpha, CX3CL1, IL-6) and anti-inflammatory (IL-10) cytokines suggesting myelin can induce a limited mixed phenotype in M2 and CTL groups **(Supplemental Figure 2.5)**. Collectively, these data indicate that myelin induces a mixed neuroinflammatory cytokine profile.

Myelin Increases $cPLA_2$ Activity in an Activation State Dependent Manner.

Myelin has long been proposed as an inflammatory stimulus in CNS models of neuroinflammation (Williams et al., 1994). Similarly, $cPLA_2$'s activation in response to a wide variety of chemokines and inflammatory signal has been well documented (Gijón and Leslie, 1999). Despite this, the formal hypothesis of whether myelin can directly increase macrophage $cPLA_2$ activation has not been directly tested. To this aim we performed a PLA_2 activity assay on cell lysates from CTL, M1, and M2 treated cells with or without myelin. Myelin increased PLA_2 activity in an activation state dependent manner, with M1 cells containing active PLA_2, which was further increased (* $p<0.05$) with the addition of myelin **(Figures 2.5A-C)**. Conversely, PLA_2 activity in CTL and M2 cells was below the detection limit of the assay with or without the addition of myelin. To distinguish $cPLA_2$ activity from $iPLA_2$, cell lysates were incubated in Bromoenol Lactone to inhibit $iPLA_s$. $iPLA_2$ inhibition did not significantly reduce total PLA_2 activity in any of the groups tested indicating $cPLA_2$ is likely the primary source of this enzymatic activity **(Figures 2.5A-C)**.

2- v. Discussion:

The continual inflammatory response observed after spinal cord injury (SCI) is a primary mechanism impairing recovery, however, the factors contributing to this maladaptive response are poorly understood. One unique aspect of the CNS injury environment not present in peripheral injuries is the accumulation of large volumes of myelin debris within phagocytes at the lesion

epicenter. Indeed, recent literature suggests that myelin may drive pro-inflammatory macrophage activation, supporting the notion that myelin accumulation within macrophages may be a key driver of the persistent pro-inflammatory macrophage response observed after injury (van der Laan et al., 1996c; Wang et al., 2014; Williams et al., 1994). However, there are conflicting reports regarding the role of myelin on macrophage activation (Boven, 2005; Kroner et al., 2014) and the mechanisms governing these discrepancies are not well understood. In this study we provided the proof of concept that myelin and $cPLA_2$ may play a key role in the prolonged pro-inflammatory macrophage activation thought to impede semi-acute and chronic recovery after SCI. Specifically, we found that myelin potentiates macrophage polarization in an activation state-dependent manner. The addition of myelin alongside a pro-inflammatory M1 macrophage stimulus (LPS and IFN-γ) further polarized pro-inflammatory activation as indicated by increased IL-12 production. Conversely, myelin had minimal effects on anti-inflammatory M2 (IL-4 stimulated) or control (unstimulated) macrophages. Similar patterns emerged when examining ROS, nitric oxide, arginase activity (indicative of M2 activation), and the neurotoxic potential of the macrophage supernatants. Inhibition of $cPLA_2$ significantly blunted these harmful effects while arginase activity was unaffected. *In vivo* we observed ubiquitous myelin debris in, and around, macrophages expressing active $cPLA_2$, providing the key spatiotemporal evidence that $cPLA_2$ may also mediate the detrimental effects of myelin in macrophages after SCI.

Myelin and $cPLA_2$'s pro-inflammatory activities appear to be largely exclusive to the M1 phenotype as M2 and CTL cells were unresponsive to myelin in most outcome measures. This interesting observation becomes clearer upon closer examination of $cPLA_2$ biology. AA is stored at the sn-2 position of membrane phospholipids where it is largely inactive. Enzymes from the lipid-cleaving phospholipase A2 family can release AA from the membranes, of which $cPLA_2$ is the most ubiquitous and widely studied due to its role in the targeted release of AA in response to a variety of agonists (Brash, 2001; Gijón and Leslie, 1999). Here, we demonstrate that myelin itself can induce increased $cPLA_2$ activity. Once activated, $cPLA_2$ translocates from the cytosol to the membranes of the endoplasmic reticulum and nuclear envelope. These are the primary sites of $cPLA_2$ activity under homeostatic conditions, however, any alterations in $cPLA_2$ activity within the myelin-laden macrophage is currently unknown (Schievella et al., 1995). It remains uncertain if myelin's AA rich lipids could be a direct substrate for $cPLA_2$ or if these lipids could be trafficked within the cell to the endoplasmic reticulum and other membranes and be targeted. For each AA molecule released by $cPLA_2$, lysophosphatidylcholine is also produced, which is known to cause further demyelination and thus could contribute to the prolonged myelin debris production observed after SCI (Lopez-Vales et al., 2008). Basal $cPLA_2$ expression is increased in response to growth factors and inflammation in a variety of cell types, notably macrophages. $cPLA_2$ is activated by mitogen-activated protein kinase (MAPK) phosphorylation of its serine 505 sites in response to increased intracellular calcium, inflammatory stimuli, or ROS,

many of which are substantially elevated after SCI (or in our *in vitro* model LPS and IFN-γ) (Liu et al., 2006; van Rossum et al., 1999). The amount of AA released by activated $cPLA_2$ is then largely dependent on substrate availability, of which myelin could contribute substantial quantities to cellular stores in the membranes of the endoplasmic reticulum, nuclear envelope and other potential sites. Further, the oxidation of lipids in the membrane by ROS alters its viscosity further increasing AA availability to $cPLA_2$ (van Rossum et al., 1999). Collectively, this suggests that the detrimental effects of myelin are restricted to the M1 phenotype, as only under these conditions could $cPLA_2$ become robustly activated and release AA.

Given the influential roles $cPLA_2$ activation and subsequent eicosanoid storms play in the initiation and resolution of inflammation, it is perhaps unsurprising that $cPLA_2$ has been previously studied in the context of SCI. Indeed, following SCI there is a substantial acute and sustained production of AA derived inflammatory mediators (Hanada et al., 2012; Mitsuhashi et al., 1994; Murphy et al., 1994). One such study by NK Liu et al. found that pharmacologically or genetically targeting $cPLA_2$ improved locomotor and anatomical recovery after SCI. Conversely, López-Vales et al. found $cPLA_2$ to have protective functions after SCI. Given the immensely diverse factors controlling $cPLA_2$ activation, AA release, and the downstream production of inflammatory mediators, it is quite possible that $cPLA_2$ has differential effects depending on the treatment paradigm or cell type (i.e. toxic mediators produced by M1 macrophages as in our *in vitro* model, and anti-inflammatory mediators in

other cell types with different enzymatic processing of AA released by $cPLA_2$). Indeed, in the spinal cord after injury, in addition to macrophages, we observed appreciable $cPLA_2$ activity in many non-macrophage cell types within the lesion epicenter. Given the great heterogeneity of lipid signaling molecules downstream of $cPLA_2$, and differential capacities of each cell type to produce these mediators, it is uncertain whether $cPLA_2$ is mediating detrimental or beneficial effects in these other non-macrophage cell types. Prior studies targeting $cPLA_2$ have used a global approach to target $cPLA_2$ either genetically or with chemical inhibitors with conflicting results (Liu et al., 2006; Lopez-Vales et al., 2011). Our results suggest that $cPLA_2$ plays a clear detrimental role in macrophage physiological responses to myelin, and thus future studies specifically targeting macrophage $cPLA_2$ *in vivo* may hold more therapeutic potential than the previous systemic approaches in which both detrimental and beneficial mechanisms are likely being affected. One important caveat to consider when using therapeutics targeting $cPLA_2$ is that many chemical inhibitors are cross-reactive with closely related phospholipases. In this study our $cPLA_2$ inhibitor of choice PACOCF3, for example, can also inhibit a related enzyme calcium-independent phospholipase A2 ($iPLA_2$). While our data suggests that $iPLA_2$ is not contributing significantly to total PLA_2 activity at 24-h post-stimulation, it's activities prior to cell lysis were not examined. Consequently, the influence of $iPLA_2$ cannot be ruled out in this study. Similarly, previous work has demonstrated that inhibiting $cPLA_2$ can influence the breakdown and phagocytosis of myelin during Wallerian degeneration (De et al.,

2003). Continued work is needed to determine if such a mechanism could be occurring in the SCI Myelin-macrophage and our *in vitro* model.

Our results are consistent with a number of previous observations. Specifically, a 1994 publication by Williams et al. demonstrated that the treatment of microglia with myelin debris increased microglial activation as indicated by increased pro-inflammatory cytokine and ROS production (Williams et al., 1994). Next, Van der laan et al. demonstrated that myelin increases TNF-alpha and nitric oxide when applied to peritoneal macrophages. Lastly, Wang et al. (2014), observed similar increases in pro-inflammatory macrophage activation with myelin application to bone marrow-derived macrophages, and demonstrated the key role that this infiltrating macrophage population plays in clearing myelin debris *in vivo* after SCI (Wang et al., 2014).

While these papers support a mechanism linking myelin phagocytosis to the pro-inflammatory macrophage response observed after SCI, our results conflict with other data reporting anti-inflammatory actions of myelin in *in vitro* models of various CNS disorders (Boven, 2005; Kroner et al., 2014). This suggests that the effects myelin has on macrophage activation may depend on the type of macrophage, stimulation timing, myelin source, dosage, and the CNS condition being modeled as discussed previously (Kopper and Gensel, 2017). Similarly, others have observed pro-inflammatory effects of myelin without an M1 co-stimulus (Wang et al., 2014). Our data presented here indicate that the effect of myelin is phenotype specific, which may further account for these differences. For example, Kroner et al., utilized a sequential approach: applying an M1

stimulus to the BMDMs, washed thoroughly, and then applied bovine-derived myelin (Kroner et al., 2014). While this approach is certainly appropriate for some studies, it may not capture our phenotype-specific $cPLA_2$ mediated inflammation, as $cPLA_2$ could need sustained LPS/IFN-γ to remain activated and exert its effects with myelin. Specifically, $cPLA_2$ activity is regulated by both a rapid transient calcium influx induced by inflammatory stimuli and its phosphorylation state allowing for complex regulation of either brief or sustained activation (Lee et al., 2015; Leslie, 1997). Given this, sequential or simultaneous application of myelin and LPS/IFN-γ could induce $cPLA_2$ to activate and interact with myelin under very different regulatory conditions; however, further studies are needed to better understand how phenotype, $cPLA_2$ regulation, and methodological variation such as this synergize to produce differential responses to myelin. In doing so we could better understand the immune dysregulation leading to chronic inflammation after human SCI.

Our model utilized in this work has its own strengths and limitations in modeling the complex SCI inflammatory response. First, we chose to use BMDM as our cell choice as recent literature has implicated this monocyte derived population as the primary mediator of myelin clearance, and as being more detrimental to recovery relative to microglia (Bellver-Landete et al., 2019; Wang et al., 2014). Second, we utilized a high dose of myelin which was not overtly toxic yet provided excess myelin to overwhelm the cell's phagocytic capacity over the 24-h stimulation window similar to what occurs after SCI (Becerra et al., 1995; Greenhalgh and David, 2014; Vargas and Barres, 2007; Wang et al.,

2014). Lower doses may be better suited to shorter duration stimulations or studies investigating the effects of myelin upon binding to extracellular receptors. Third, our myelin preparations are from the same species and strain as the mice from which we collected the BMDMs (i.e. mouse myelin on mouse cells, and when feasible, myelin derived from the same mouse sacrificed for BMDM isolation). This was done to minimize any unintended cross-species immune effects. A limitation here, however, is that we used myelin derived from both brain and spinal cord tissue to yield sufficient myelin for our studies, it is possible that subtle differences in myelin composition between these sites could have differential effects. Similarly, the protein and lipid composition of CNS and PNS myelin differs and was not evaluated in this study (Quarles et al.). Fourth, we utilized a prolonged 24-h stimulation paradigm to allow for complete lipid loading of the cells as occurs *in vivo.* It is certainly possible that the results could be different at earlier time points. Similarly, studies interested in true chronic effects could adapt these protocols using cell lines to overcome the short lifespan of BMDMs. Fifth, we applied the myelin and stimulants at the same time and for the entire stimulation, as this is what likely occurs after SCI. Lastly, we applied myelin with multiple types of stimulates to begin to capture how myelin may affect macrophages across the spectrum of phenotypic states. An important caveat to this, however, is that we primarily investigated pathological BMDM features associated with impaired SCI recovery. There are likely other effects of myelin on CTL and M2 macrophage physiology not captured in this study.

While in many ways our macrophage model closely replicates the cellular populations found after SCI (Gensel et al., 2017), there are certainly other factors not utilized here in our model. Notably, TNF and iron are implicated as key environmental mediators of detrimental macrophage activation (Kroner et al., 2014). Similarly, *in vivo* after SCI there are numerous other factors that can influence macrophage activation, including cross talk between macrophages and microglia, T-cell responses, and damage associated molecular patterns released by necrotic tissue (Greenhalgh et al., 2018; Wynn and Vannella, 2016). While not investigated here, these are all likely important factors contributing to pro-inflammatory macrophage activation in myelin-laden macrophages and represent an important caveat in extrapolating our results to the human SCI condition. Similarly, while the data presented here implicate $cPLA_2$ as an important regulator of myelin's activity, other cellular mechanisms are certainly involved. Myelin uptake/phagocytosis, for example, is clearly a key step prior to any intracellular mechanism. Injection of myelin directly into the spinal cord previously by Sun and colleagues was found to induce leukocyte chemoattraction, however, this effect was lost in CR3 KO animals with deficient phagocytic capacity. Next, they implicated the FAK/Akt/NF-κB signaling cascade as a mediator of myelin's activity (Sun et al., 2010). These observations are certainly compatible with our current observations as the NF-κB signaling cascade is activated by similar stimuli as the $cPLA_2$ signaling cascade including our LPS in-vitro stimulant. Interestingly, activation of NF-κB by myelin may even drive increased $cPLA_2$ expression, further demonstrating the intertwined nature of many

proposed mechanisms (Lee et al., 2011). Collectively, this demonstrates the need for continued investigation of these pathways to identify a clinically viable treatment for SCI in the human condition.

Our *in vitro* data suggest that myelin debris potentiates pro-inflammatory functions specifically within the M1 macrophage population. Intriguingly, the time course of peripheral macrophage infiltration and initial clearance of myelin debris between 3 and 7 days post-SCI correlates with the peak presence of M2 macrophage activation markers, which then progressively drop as the cells shift to a pronounced and prolonged M1 activation state (Gensel and Zhang, 2015; Kigerl et al., 2009). By chronic time points only M1 markers can be detected, indicative of the prolonged pro-inflammatory macrophage activation thought to impede recovery (Becerra et al., 1995; Vargas and Barres, 2007; Wang et al., 2014). Here we demonstrate that $cPLA_2$ is present within myelin-laden macrophages well into chronic time points. Together with our *in vitro* data, this provides the proof of concept that myelin and $cPLA_2$ may play a key role in the prolonged pro-inflammatory macrophage activation thought to impede semi-acute and chronic recovery after SCI. Collectively this highlights macrophage $cPLA_2$activity as a potential key mediator of the neuroinflammatory response after SCI and thereby warrants continued investigation as a therapeutic target.

Conclusions:

Spinal cord injury (SCI) produces chronic intraspinal inflammation consisting of resident microglia and infiltrating monocytes. These chronically

activated SCI macrophages adopt a persistent pro-inflammatory, pathological state that potentiates secondary damage and impairs SCI recovery (Gensel and Zhang, 2015; Horn et al., 2008). The mechanisms driving chronic macrophage activation in SCI are poorly understood. Here we implicate myelin debris and $cPLA_2$ as key mediators of pathological macrophage activation. *In vitro* we found that the effects of myelin on macrophage activation are phenotype-specific, with myelin potentiating only pro-inflammatory (LPS + INF-γ; M1) macrophage activation, whereas myelin had no pro-inflammatory effect on unstimulated or anti-inflammatory (IL-4; M2) macrophages. Inhibition of $cPLA_2$ significantly reduced the detrimental effects of myelin on M1 macrophages, implicating $cPLA_2$ as a key regulator of maladaptive macrophage activation. *In vivo* we observed ubiquitous myelin debris in, and around, macrophages expressing active $cPLA_2$ providing the key spatiotemporal evidence that $cPLA_2$ may also mediate the detrimental effects of myelin in macrophages after SCI. Collectively, this establishes a novel mechanism driving detrimental macrophage activation and provides key evidence identifying macrophage $cPLA_2$ activity as a therapeutic target to improve recovery after SCI.

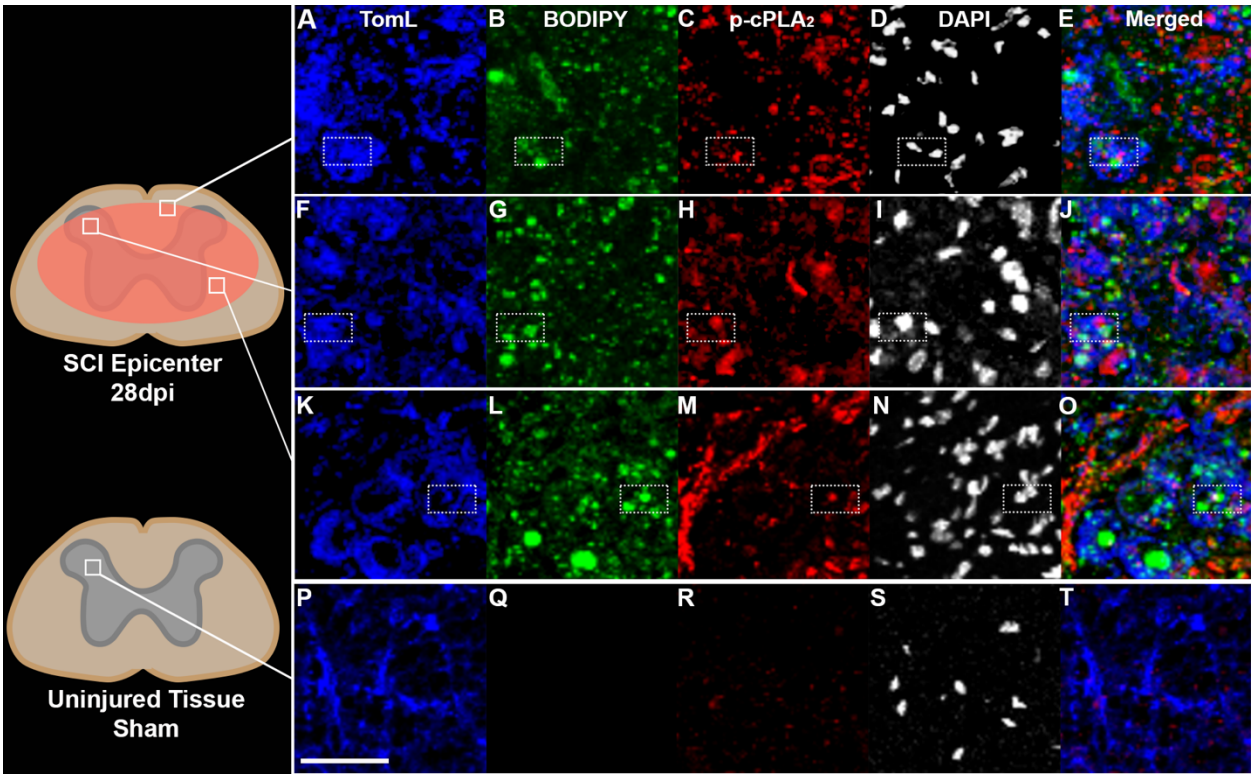


Figure 2.1. Macrophages can contain both myelin-derived lipids and active cPLA$_2$ 28 days after spinal cord injury (SCI).

Ten adult, 4-month-old C57/b female mice received a T9 75kdyn infinite horizons (IH) contusion SCI. A-E) Representative example of TomL positive macrophages (blue) in injured white matter containing both lipid debris (green, Bodipy, staining for neutral lipids) and active p-cPLA$_2$ (red) (imaged area is represented by box within spinal cord diagram). DAPI (white) was excluded from merged images. F-J) Example of TomL positive macrophages (blue) in injured grey matter containing both lipid debris (green, Bodipy, staining for neutral lipids) and active p-cPLA$_2$ (red). K-O) Example of TomL positive macrophages (blue) in injured grey matter in the core of the injury epicenter containing both lipid debris (green, Bodipy, staining for neutral lipids) and active p-cPLA$_2$ (red). P-T) Example of

TomL positive macrophages (blue) in uninjured tissue from sham animals with minimal lipid debris or active p-cPLA$_2$ (red). Boxes indicate examples of triple positive cells. Maximum intensity projection confocal images. Scale bar in image P=50μm.

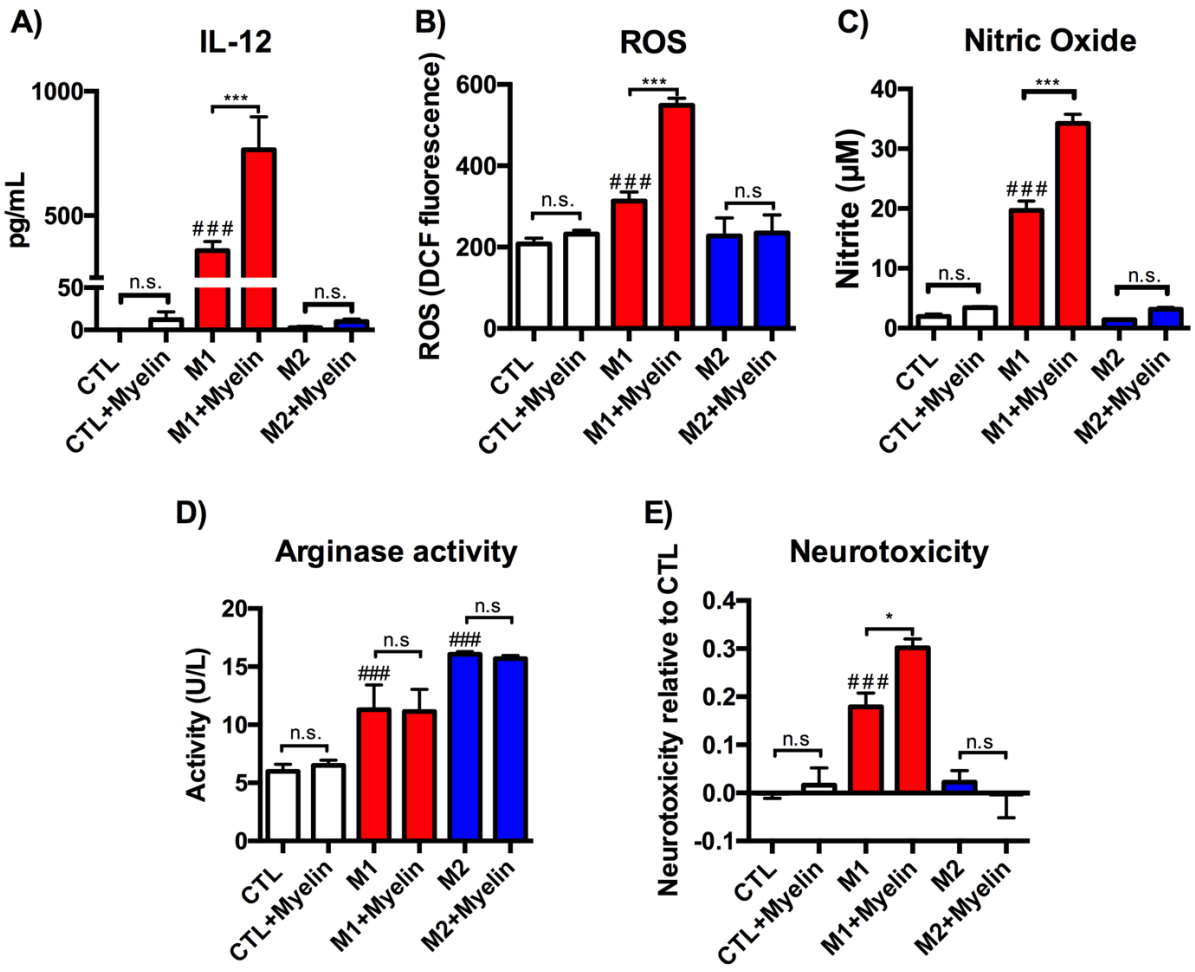


Figure 2.2. Myelin potentiates pro-inflammatory macrophage activation *in vitro*.

Bone marrow derived macrophages (BMDMs) were utilized to determine the function outcomes resulting from myelin debris application under three distinct activation states: M1 (IFN-γ and LPS), M2 (IL-4), or CTL (media, i.e. unstimulated). A) Myelin stimulation significantly increased pro-inflammatory IL-12 cytokine levels relative to M1 alone (*** $p<0.001$), however myelin did not increase IL-12 in CTL or M2 treated cells. M1 stimulations had increased IL-12 relative to CTL or M2 (### $p<0.001$). B) Following the stimulation BMDMs were washed and treated with 2 μM H2DCFDA for 25 min. Myelin stimulation

increased ROS relative to M1 alone (*** p<0.001), however myelin did not increase ROS in CTL or M2 treated cells. M1 stimulations had increased ROS relative to CTL or M2 (### p<0.001). C) Nitric oxide levels in treated supernatants were examined using the Griess assay of nitrite accumulation. Myelin stimulation increased nitric oxide relative to M1 alone (*** p<0.001), however myelin did not increase nitric oxide in CTL or M2 treated cells. M1 stimulations had increased nitric oxide relative to CTL or M2 (### p<0.001). D) Cell lysates were tested for arginase enzymatic activity. M2 macrophages had higher arginase activity relative to M1 and CTL (p<0.001 and p<0.0001 respectively). The addition of myelin however did not significantly alter arginase activity in any of stimulations tested. E) Supernatants were applied to a neuron culture (N2A cells) for 24 hours to determine supernatant toxicity. Myelin stimulation increased neurotoxicity relative to M1 alone (* p<0.05), however myelin did not increase neurotoxicity in CTL or M2 treated cells. M1 stimulations had increased ROS relative to CTL or M2 (### p<0.001). Representative of 3 independent biological replications of both BMDMs and myelin source. * p<0.05, ** p<0.01, *** p<0.001, # p<0.05, ## p<0.01, ### p<0.001 mean ± SEM.

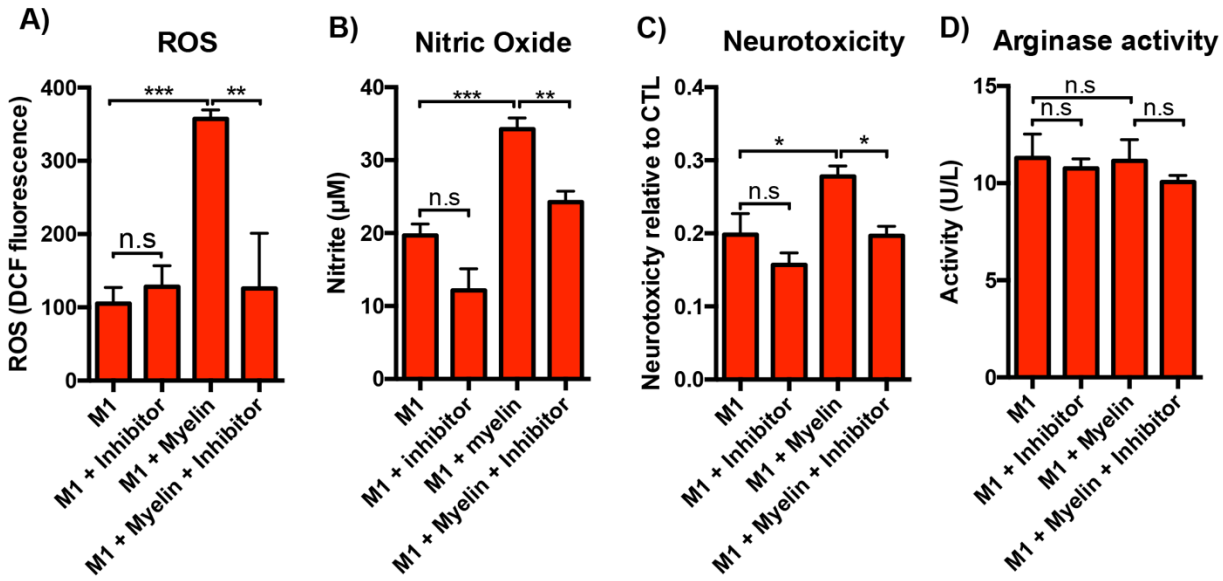


Figure 2.3. Inhibition of cPLA$_2$ with PACOCF3 reduces the pro-inflammatory effects of myelin on M1 macrophages.

BMDMs were grown and stimulated as in Figure 2, with or without the addition of cPLA$_2$ inhibitor, PACOCF3 (50 μM). A) cPLA$_2$ inhibition reduced myelin mediated ROS increases in M1 (IFN-γ and LPS) macrophages. B) cPLA$_2$ inhibition reduced myelin mediated Nitric oxide production. C) cPLA$_2$ inhibition reduced the neurotoxic potential of M1 macrophages treated with myelin as determined by an MTT assay measurement of N2a cell viability. D) cPLA$_2$ inhibition did not significantly alter arginase activity. Representative of 3 biological replications of both BMDMs and myelin source. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ mean ± SEM.

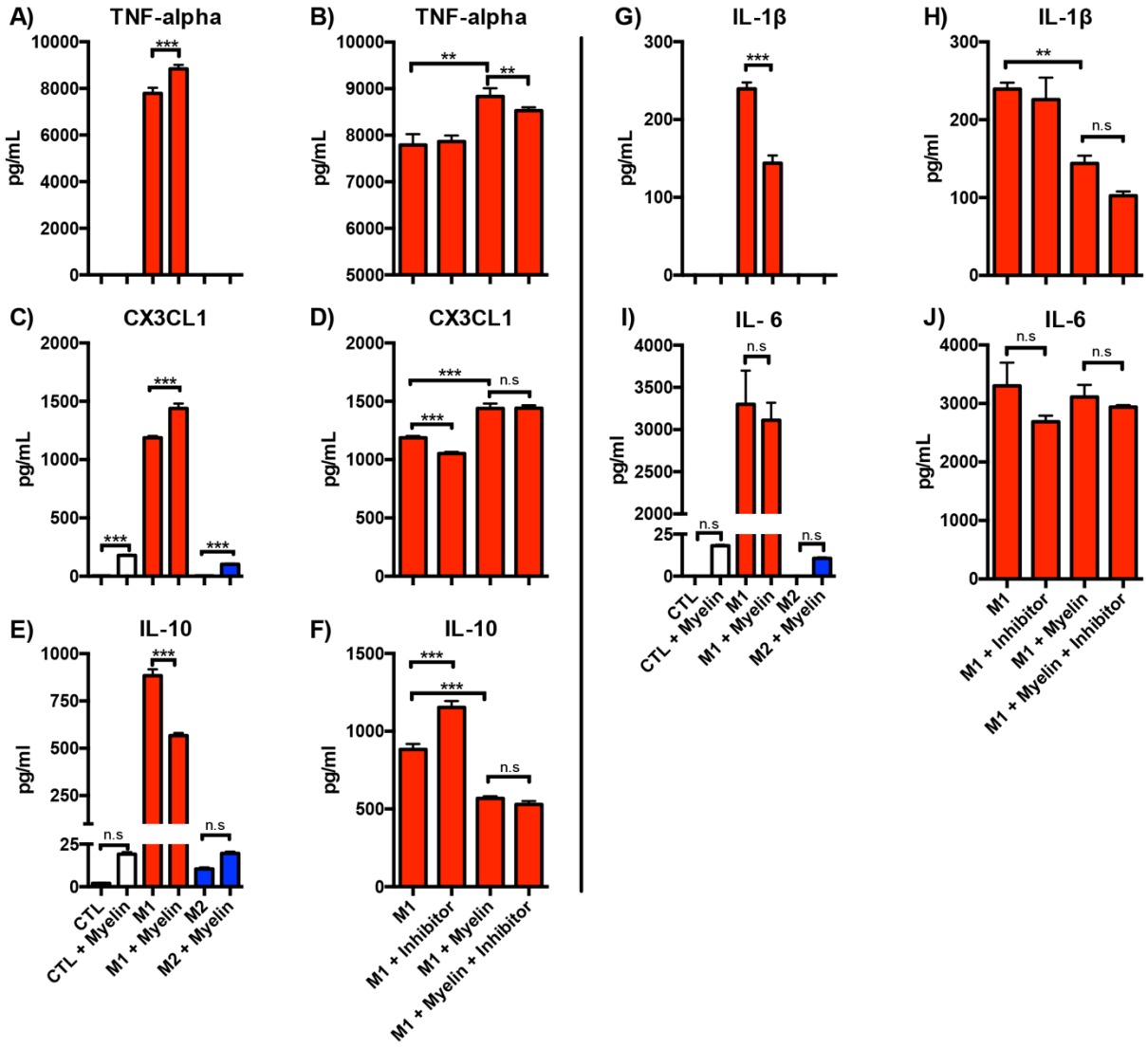


Figure 2.4. Macrophage cytokine profiles indicate a mixed neuroinflammatory phenotype.

Supernatants from treated BMDMs were collected to measure pro- and anti-inflammatory cytokine production in response to phenotype, myelin stimulation, and cPLA$_2$ inhibition with PACOCF3. A-B) Myelin significanty increases the production of TNF-alpha in M1 cells, but has no effect on CTL and M2 cells. cPLA$_2$ inhibition significantly reduced myelin potentiation of M1 TNF-alpha

production. C-D) Myelin increases CX3CL1 in CTL, M1, and M2 cells. $cPLA_2$ inhibition significantly reduced this effect in M1 cells, but not under M1 and Myelin co-stimulation. E-F) Myelin significantly decreased IL-10 production in M1 cells, but this effect was not reversed with $cPLA_2$ inhibition. $cPLA_2$ inhibition alone increased IL-10 production in M1 cells. G-H) Myelin significantly reduced pro-inflammatory IL-1beta production in M1 cells, whereas CTL and M2 cells were unaffected. $cPLA_2$ inhibition did not alter this effect. I-J) Pro-inflammatory IL-6 was not significantly altered by myelin or $cPLA_2$ inhibition. Refer to Supplementary Figure 5 for CTL and M2 $cPLA_2$ inhibition data. Representative of 3 biological replications. *$p<0.05$ ** $p<0.01$, *** $p<0.001$ mean ± SEM.

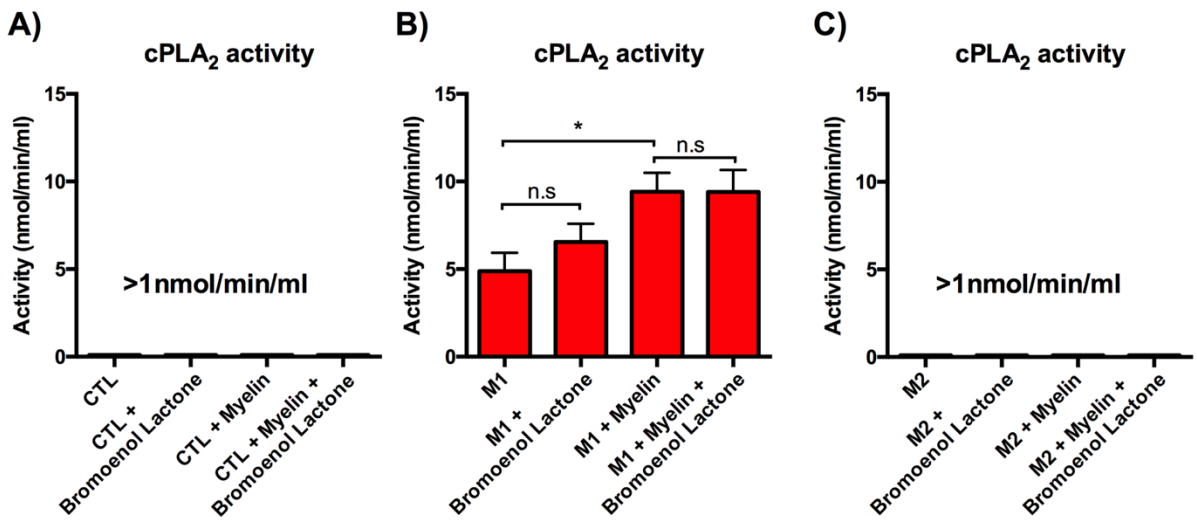
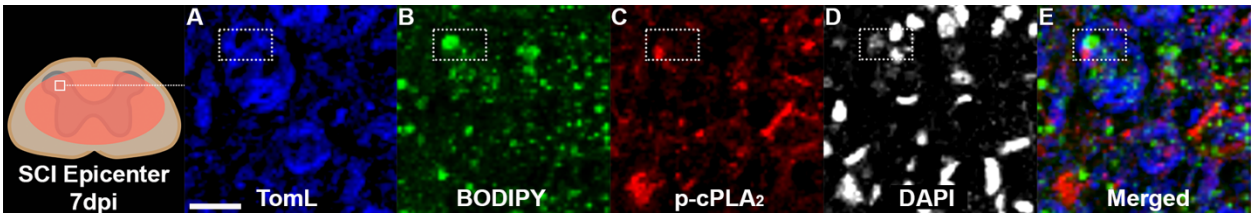


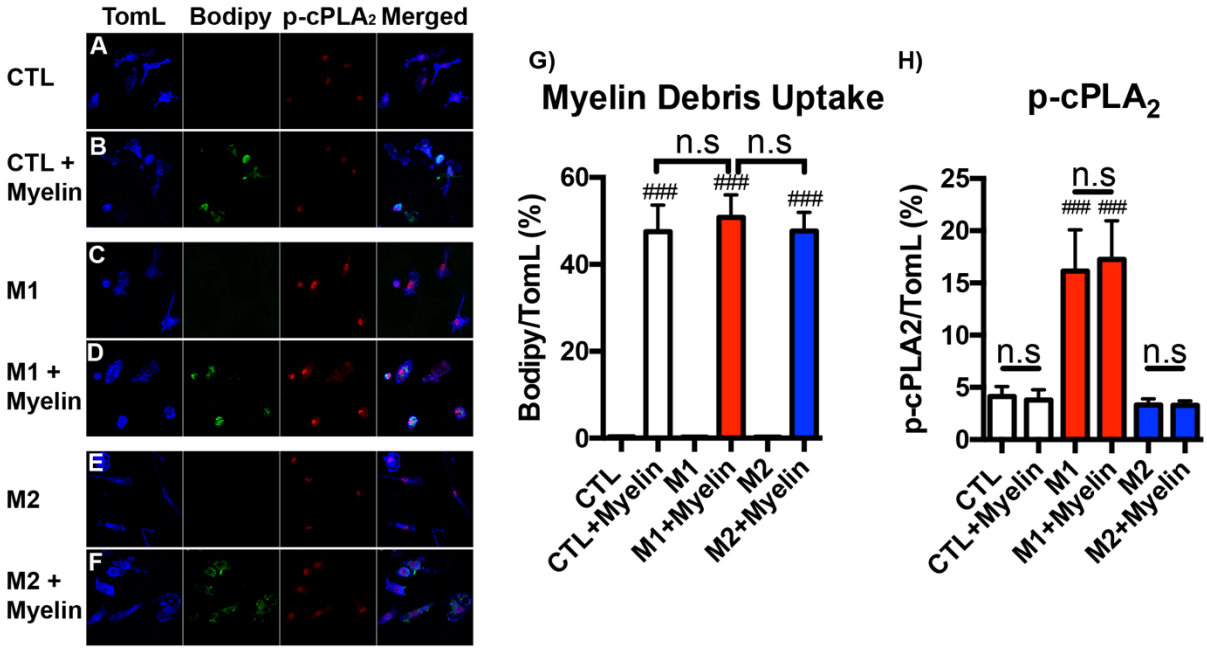
Figure 2.5. Myelin increases $cPLA_2$ activity in an activation state dependent manner.

Cell lysates were collected from BMDM cultures to measure $cPLA_2$ activity following stimulation into CTL, M1, and M2 cells with or without myelin. Samples containing Bromoenol Lactone, an inhibitor of $iPLA_2$ were used to exclude the possibility of $iPLA_2$ contributing to enzymatic activity. A) CTL treated cells had no discernable activity with or without myelin and Bromoenol Lactone. B) M1 treated cells had a mean enzymatic activity of 4.88nmol/min/ml. Addition of myelin significantly increased $cPLA_2$ enzymatic activity ($*p<0.05$). Addition of Bromoenol Lactone did not significantly alter enzyme activity. C) M2 treated cells had no discernable activity with or without myelin and Bromoenol Lactone. Representative of 3 biological replications. $*p<0.05$ $**$ $p<0.01$, $***$ $p<0.001$ mean ± SEM.



Supplemental Figure 2.1. Macrophages can contain both myelin-derived lipids and active cPLA$_2$ 7 days after spinal cord injury (SCI).

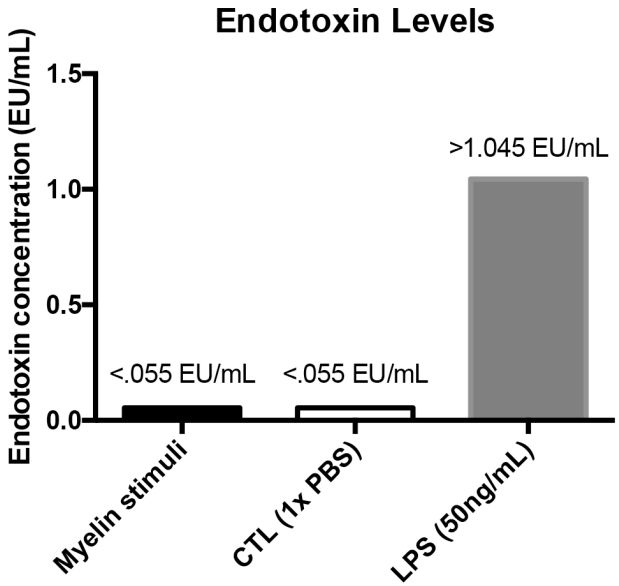
Eight adult, 4-month-old C57/b female mice received a T9 75kdyn infinite horizons (IH) contusion SCI. A-E) Representative examples of TomL positive macrophages (blue) in injured white matter containing both lipid debris (green, Bodipy, staining for neutral lipids) and active p-cPLA$_2$ (red). DAPI (white) was excluded from merged images. Imaged area is represented by box within spinal cord diagram. Boxes on image indicate examples of triple positive cells. Maximum intensity projection confocal images. Scale bar in image A=25μm



Supplemental Figure 2.2. *In Vitro* modeling of cPLA$_2$ activity in myelin loaded macrophages.

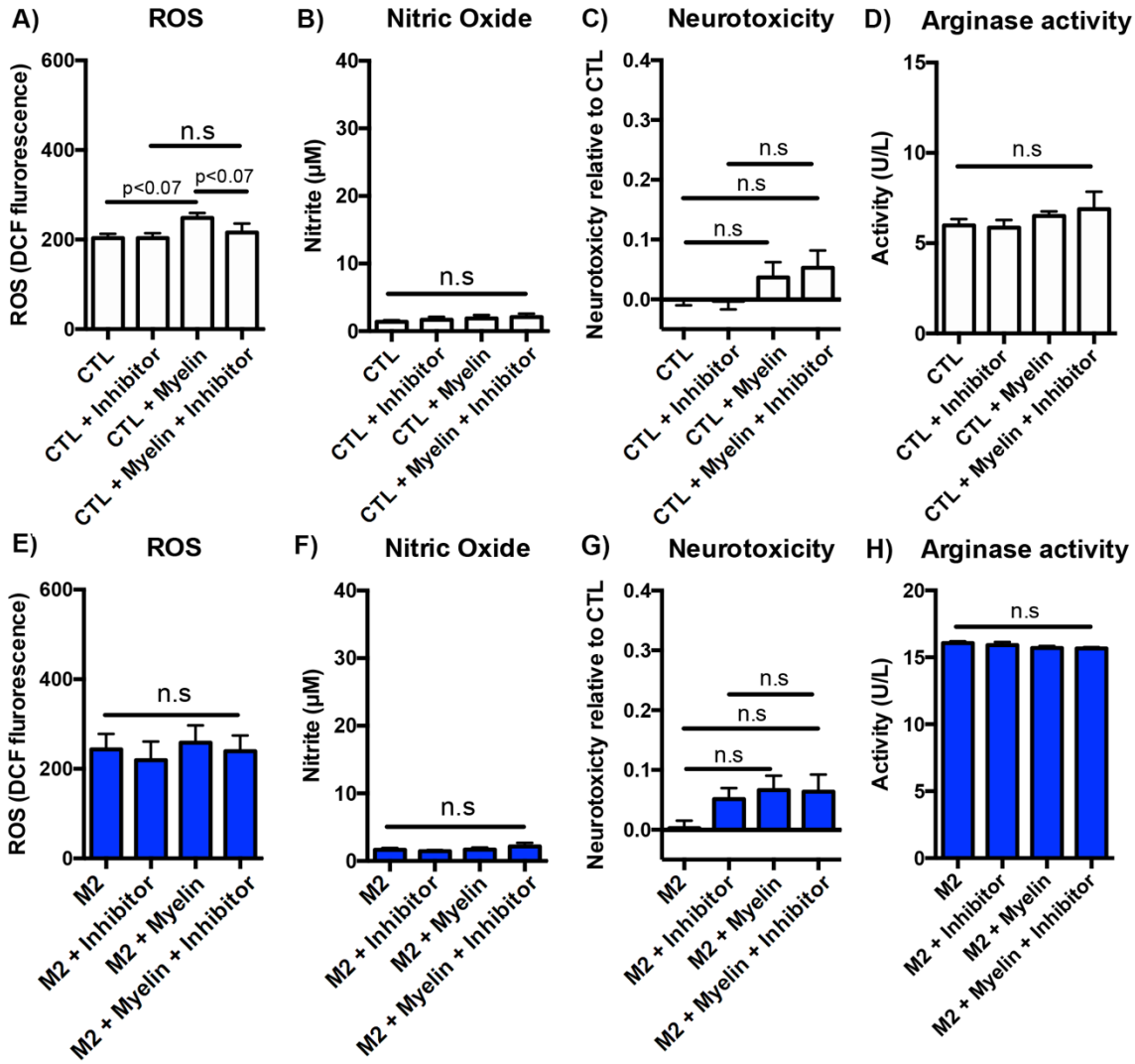
Coverslip plated BMDMs were stained for TomL (macrophage marker, blue), Bodipy (myelin derived lipids, green) and active p-cPLA$_2$ (red). Myelin uptake was quantified as the threshold area ratio of Bodipy or p-cPLA$_2$ to TomL positive stain. A-F) Representative images of CTL, M1, and M2 BMDMs treated with or without myelin debris. G) CTL, M1, and M2 BMDMs treated with myelin contained significantly more neutral lipids (Bodipy) than each of their respective untreated counterpart (### $p<0.001$); however, there were no significant differences between groups treated with myelin. H) M1 stimulated BMDMS has significantly higher p-cPLA$_2$ immunoreactivity than either M1 or M2 (###

$p<0.001$); however, myelin was not found to alter p-cPLA$_2$ immunoreactivity in any of the groups tested.



Supplemental Figure 2.3. Myelin stimulants did not contain detectable endotoxin contamination.

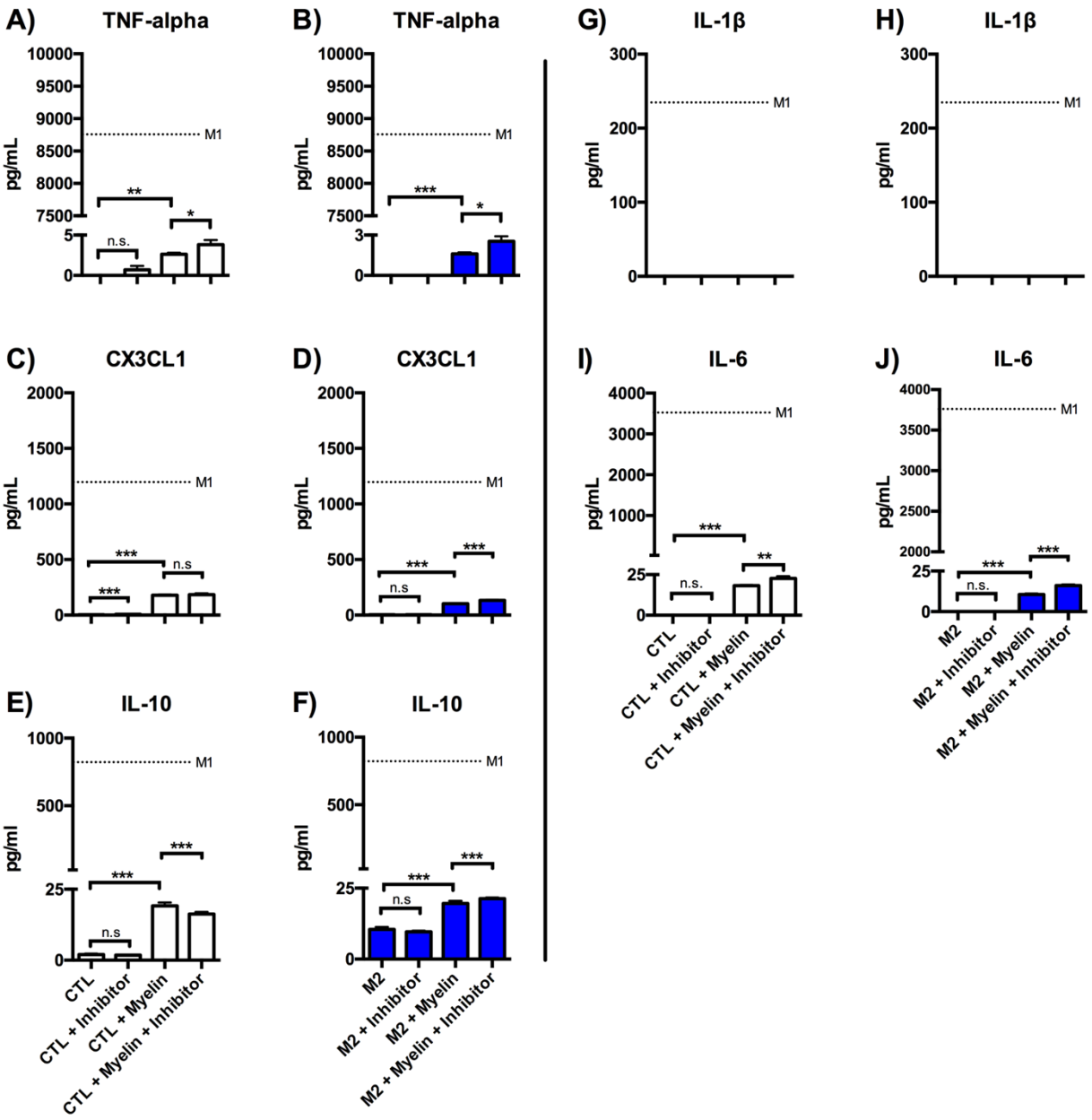
Aliquots from each myelin isolation were stored at -80 °C prior to testing. Myelin stimulants and negative control (1x sterile PBS) had endotoxin levels below the level of detection (<.055 EU/mL). The positive control (LPS 50 ng/mL) exceeded the detection limit (>1.045 EU/mL). mean ± SEM.



Supplemental Figure 2.4. cPLA$_2$ inhibition has no effects on CTL or M2 stimulated cells.

Cells were grown and stimulated as detailed in Figures 2 and 3. A-D) cPLA$_2$ inhibition did not significantly change levels of reactive oxygen species (ROS), nitric oxide, supernatant neurotoxicity, or arginase activity in CTL (unstimulated) cells with or without the addition of myelin. E-H) cPLA$_2$ inhibition did not

significantly change levels of reactive oxygen species (ROS), nitric oxide, supernatant neurotoxicity, or arginase activity in M2 (IL-4) cells with or without the addition of myelin. Representative of 3 biological replications of both BMDMs and myelin source n.s.=non-significant, $p>0.05$, mean ± SEM.



Supplemental Figure 2.5. Macrophage cytokine profiles indicate a mixed neuroinflammatory phenotype in CTL and M2 stimulated cells in response to myelin and inhibition of $cPLA_2$ with PACOCF3.

Supernatants from treated BMDMs were collected to measure pro and anti-inflammatory cytokine production in response to phenotype, myelin stimulation,

and $cPLA_2$ inhibition with PACOCF3. A-J) Myelin significantly increases the production of pro and anti-inflammatory cytokines TNF-alpha, CX3CL1, IL-10 and IL-6. IL-1Beta was unaffected. $cPLA_2$ inhibition induced small but statistically significant adjustments in cytokine levels in myelin treated CTL and M2 cells. Representative of 3 biological replications. *$p<0.05$ ** $p<0.01$, *** $p<0.001$ mean ± SEM.

Chapter 3: Delayed Azithromycin Treatment Improves Recovery After Mouse Spinal Cord Injury

This chapter is adapted from Kopper et al., 2019

3- i. Chapter Summary

After spinal cord injury (SCI), macrophages infiltrate into the lesion and can adopt a wide spectrum of activation states. However, the pro-inflammatory, pathological macrophage activation state predominates and contributes to progressive neurodegeneration. Azithromycin (AZM), an FDA approved macrolide antibiotic, has been demonstrated to have immunomodulatory properties in a variety of inflammatory conditions. Indeed, we previously observed that post-SCI AZM treatment reduces pro-inflammatory macrophage activation. Further, a combined pre- and post-injury treatment paradigm improved functional recovery from SCI. Therefore, for the current study, we hypothesize that post-injury AZM treatment will improve recovery from SCI. To test this hypothesis, we examined the therapeutic potential of delayed AZM treatment on locomotor, sensory, and anatomical recovery. We administered AZM beginning 30-min, 3-h, or 24-h following contusion SCI in female mice, and then daily for 7 days. AZM administration beginning 30-min and 3-h post-injury improved locomotor recovery with increased stepping function relative to vehicle controls. Further, delaying treatment for 30-min after SCI significantly reduced lesion pathology. Initiating AZM treatment 24-h post-injury was not therapeutically effective. Regardless of the timing of the initial treatment, AZM did not statistically

reduce the development of neuropathic pain (mechanical allodynia) nor increase neuron survival. Collectively, these results add to a growing body of evidence supporting AZM's translational potential as a therapeutic agent for SCI and other neuroinflammatory conditions in which patients currently have very few options. *This chapter was adapted from Kopper et. Al 2019*

3- ii. Background

Spinal cord injury (SCI) induces a complex heterogeneous inflammatory response largely mediated by resident microglia and infiltrating monocyte-derived macrophages. While these cells are capable of adopting a wide spectrum of beneficial and detrimental functions, the acute SCI microenvironment promotes pro-inflammatory macrophage activation (Kigerl et al., 2009). Pro-inflammatory macrophages and microglia are widely believed to be major contributors to the continued neurodegeneration and tissue loss observed following the initial mechanical SCI. Targeting macrophage activation acutely is, therefore, a promising therapeutic approach to improve recovery. However, to date there are no FDA approved drugs to target these pathways after SCI.

Azithromycin (AZM) is a widely prescribed, FDA-approved, antibiotic with a well-established safety record. AZM has significant anti-inflammatory and immunomodulatory actions across a wide array of disease states (Amantea et al., 2016a; Banjanac et al., 2012; Barks et al., 2019; Feola et al., 2010b; Gensel et al., 2017; Ivetić Tkalčević et al., 2011; Murphy et al., 2008; 2010; Nujić et al., 2012a; Osman et al., 2017; Polancec et al., 2012; Varano et al., 2017; Vrancic et

al., 2012; Zhang et al., 2015b). Specifically, AZM promotes anti-inflammatory activation by inhibiting macrophage STAT1 and NF-κB signaling pathways (Haydar et al., 2019). Emerging evidence supports the use of AZM as a treatment for neurological conditions including stroke, retinal ischemia, spinal muscular atrophy, and neonatal hypoxic–ischemic brain injury (Amantea et al., 2016a; 2019; Barks et al., 2019; Osman et al., 2017; Varano et al., 2017). Previously, we demonstrated that AZM improves tissue sparing and locomotor recovery in a mouse model of contusion SCI when dosing is initiated 3 days prior to injury (Zhang et al., 2015b). In our previous work, the neuroprotective effects of AZM were coincident with increased anti-inflammatory and decreased pro-inflammatory macrophage activation (Zhang et al., 2015b). More recently, we established that delaying treatment for 30 min post-injury substantially decreases markers associated with pro-inflammatory (M1) macrophage activation while significantly increasing anti-inflammatory (M2) macrophage markers(Gensel et al., 2017). Further, we recently found that AZM decreases neurotoxic, pro-inflammatory macrophage activation independent of its antibiotic properties (Zhang et al., 2019). Collectively, these findings highlight AZM as a promising SCI immunomodulatory therapeutic; however, the long-term effects of delayed AZM treatment are unknown. For the current study, we hypothesized that post-injury AZM treatment improves recovery from SCI. Specifically, we evaluated the therapeutic efficacy of post-SCI AZM treatment on long-term locomotor, sensory, and anatomical recovery. Initial treatment was delayed 30 min, 3-h, or 24 h post-injury and recovery evaluated for 4 weeks after injury.

3- iii. Methods

Experimental Design:

The current data includes the combined results of three independent studies.

Study One

Mice (*n* = 10/group) were treated with vehicle (initiated at 30 min post-injury) or AZM (160 mg/kg/day) by oral gavage beginning 30 min, 3 h, or 24 h after a moderate-severe (75 kdyn) T9 contusion SCI. Drug and vehicle administration was continued daily for 7 days post-injury (dpi). Locomotor function of all animals, as determined by the Basso Mouse Scale (BMS), was assessed prior to injury and again at 1, 3, 7, 14, 21 and 28 dpi. At 28 dpi, all the animals were sacrificed for the generation of spinal cord sections for histological analyses of tissue sparing, lesion length, and neuron survival. One mouse was euthanized due to surgical complications. Three mice were excluded based upon a prior exclusion criteria for abnormal impactor parameters reported by the Infinite Horizons (IH) SCI device (indicative of a bone hit or spinal cord movement during injury; *n* = 2) and abnormally high functioning locomotor behavior at 1 dpi (BMS >3) indicative of incomplete injury (*n* = 1).

Studies Two and Three

Mice (*n* = 10/group/study) were injured and treated as study one except that the 24 h delayed treatment group was discontinued due to clear therapeutic ineffectiveness in study one (**Figures 3.1A,B**; $p > 0.98$ for all outcomes). In

addition, horizontal ladder performance and measures of neuropathic pain (Von Frey, mechanical allodynia) were collected prior to injury and at 26 and 27 dpi, respectively. Group sizes for studies two and three (n = 10) were calculated based upon *a priori* power analysis of the BMS behavior data collected in study one. Specifically, we estimated that with a significance of $\alpha = 0.05$, a power of $1-\beta = 0.80$, and expected levels of animal attrition, that we would need an additional 20 animals per group. Two mice in study two and three mice in study three were excluded based upon *a priori* impact or behavioral criteria for incomplete/abnormal injuries. Rostral-caudal neuronal survival was only assessed in studies two and three because unknown temperature inconsistencies during study one tissue processing and sectioning caused tissue folding and tissue loss, making histological analysis impossible. Discrepancies between *n*'s in behavioral vs. histological analyses are due to the fact that mice without an obvious and fully intact injury epicenter (due to tissue processing complications) were not included for histological analyses. Final animal numbers are reported in the figure legends and/or results.

Animals

Experiments were performed using 4-month-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA). Animals were housed in IVC cages with *ad libitum* access to food (Teklad Irradiated Global 18% Protein Rodent Diet) and purified water. Housing is set to maintain a 14 h light/10 h dark cycle, at 70°F and 50% humidity. All experimental procedures were conducted during the light

cycle and were performed in accordance with the guidelines and protocols of the Office of Research Integrity and with approval of the Institutional Animal Care and Use Committee at the University of Kentucky.

Spinal Cord Injury

Animals were anesthetized *via* intraperitoneal (i.p.) injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). Following a T9 laminectomy, a moderate-severe thoracic SCI was produced using the IH injury device (75-kdyn; Precision Systems and Instrumentation; (Scheff et al., 2004)). Any animals receiving SCI with abnormalities in the force vs. time curve generated by the IH device were excluded from analysis. These abnormalities are indicative of bone hits or instability in the spinal cord at the time of injury and occurred <10% of the time. After injury, muscle and skin incisions were closed using monofilament suture. Post-surgery, animals received one subcutaneous injection of buprenorphine-SR (1 mg/kg) and one injection of antibiotic (5 mg/kg, enrofloxacin 2.27%: Norbrook Inc., Lenexa, KS, USA) in 2 ml of saline and were housed in warming cages overnight. Animals continued to receive antibiotic subcutaneously in 1 ml saline for 5 days. AZM (160 mg/kg) or vehicle (1% methylcellulose) was delivered in 0.1-ml volume *via* oral gavage daily beginning 30 min, 3 h or 24 h after injury and again daily for 7 days post-injury. Animal health and the incision site were monitored throughout the course of the study. Bladder expression was performed on injured mice twice daily.

Behavioral Analysis

All experimental animals were assessed using the BMS to score hindlimb function as previously described (Basso et al., 2006). Mice were tested in an open field for 4 min before surgery and at 1, 3, 7, 14, 21, and 28 days post-injury (dpi). Each hindlimb was scored separately based on movement (e.g., ankle placement and stepping) and whole-body coordination and trunk stability were also scored; the average of both hindlimb scores was used to generate a single score for each animal. A score of 0 indicated complete paralysis and a score of 9 indicated normal locomotion. Animals receiving a score of 3 or higher at 1 dpi, or less than 2 at 28 dpi were excluded from the study based on *a priori* statistical assessment of over 450 prior 75 kdyn mouse SCI surgeries. These scores are rare (greater than 2 standard deviations from mean BMS score) and are indicative of surgical/injury abnormalities (bone hit, low/high impact force, etc.).

Mechanical Allodynia Testing

Mechanical allodynia was measured using the manual up-down approach with von Frey monofilaments as described previously (Chaplan et al., 1994). Animals were first acclimated to the testing apparatus consisting of a wire mesh floor within an acrylic enclosure. A monofilament was pressed perpendicularly against the plantar surface of the hindlimb until bent, beginning with the 1.4 g monofilaments and ranging from the 0.4 g to 6.0 g monofilaments. Fifty percentage withdrawal threshold was calculated and reported as the average of both hind paws at each time point (Chaplan et al., 1994). Positive responses

include rapid paw withdrawal, paw shaking and/or paw licking. Paw movement due to normal locomotor activity and responses occurring after the removal of the filament were not considered positive responses and were excluded from analysis.

Tissue Processing and Immunohistochemistry

At 28 dpi, mice were anesthetized then transcardially perfused with cold PBS (0.1 M, pH 7.4), followed by perfusion with cold 4% paraformaldehyde (PFA). Dissected spinal cords (1 cm) were post-fixed for another 2 h in 4% PFA and subsequently rinsed and stored in cold phosphate buffer (0.2 M, pH 7.4) overnight at 4°C. On the following day, tissues were cryoprotected in 30% sucrose for 3 days at 4°C, followed by rapidly freezing and blocking in optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA, USA) on dry ice. Tissue was systematically randomized into blocks (each block contained spinal cords from four subjects) with equal group distribution to ensure uniformity of staining across groups and blocked tissue was stored at −80°C before sectioning. Tissue blocks were cut in serial coronal sections (10 μm) and mounted onto Colorfrost plus slides (Fisher #12-550-17).

Spinal cord sections were stained with Eriochrome Cyanine (myelin) and anti-Neurofilament (1:1,000, Aves labs: NF-H) to visualize damage and thereby identify the epicenter and length of each lesion as described previously (Zhang et al., 2015b). To examine the epicenter in greater detail, slides were stained with glial fibrillary acidic protein (GFAP; 1:500, Aves: GFAP) and macrophage marker

F4/80 (1:1,500, AbD serotec, MCA497) primary antibodies overnight at 4°C, followed by Alexa Fluor 488 goat anti-chicken (1:1,000, Life Technologies A11039) and goat anti-rat Alexa Fluor 633 (1:1,000, Thermo Fisher Scientific: A-21094) secondary antibodies for 1 h at room temperature. To assess neuron survival, slides were subjected to antigen retrieval for 5 min in hot citrate buffer pH 6, incubated with rabbit anti-NeuN (1:4,000, Novus Biologicals NBP1-77686) primary antibody overnight at room temperature, then biotinylated goat anti-rabbit (1:500, Vector BA-1000) secondary antibody for 2 h at room temperature, then elite-ABC (prepared according to manufacturer's instructions, Vector PK-6100) for 2 h at room temperature, and finally DAB (prepared according to manufacturer's instructions, Vector SK-4100) with nickel additives for 5 min at room temperature. Slides were cover-slipped with Permount mounting medium (Fisher Scientific, Hampton, NH, USA) or Immu-Mount (Thermo Scientific, Waltham, MA, USA) for brightfield and fluorescent stains, respectively.

Images were taken using a C2+ laser scanning confocal microscope (Nikon Instruments Inc., Melville, NY, USA) or a ZEISS Axio Scan.Z1 (Munich, Germany), then analyzed using the MetaMorph analysis program (Molecular Devices, Sunnyvale, CA, USA) or HALO Image Analysis Platform (Indica Labs, Albuquerque, NM, USA). To identify the rostral-caudal lesion length, Eriochrome Cyanine and Neurofilament (EC/NF) stained tissue sections were examined at 100 µm intervals rostral and caudal from the lesion epicenter until damage became entirely localized to the dorsal column totaling less than 2% of the total spinal cord area. To quantify tissue sparing at the lesion epicenter (i.e., the tissue

section with the least amount of intact EC/NF stained tissue) the regions of dense GFAP positive staining were outlined and measured using the MetaMorph analysis program (Molecular Devices, Sunnyvale, CA, USA) or HALO. These spared areas as defined by the GFAP+ glial scar closely align with areas of Neurofilament positive axons as determined previously (Freria et al., 2017; Zhang et al., 2015b). Lesion area, intact tissue area, and overall spinal cord size were used to calculate the percentage of spared tissue at the lesion epicenter. While not quantified directly, F4/80 positive macrophages were used as a secondary tracing guide in any areas of ambiguity as they densely accumulate within the core of the lesion as described previously (Wang et al., 2014). To quantify neurons, NeuN-stained cells within the gray matter were quantified using HALO software. Spinal cross-sections at 100 μm intervals from the epicenter were analyzed, and average values were calculated from equidistant rostral/caudal sections. Folded or torn sections were excluded from analysis.

Statistical Analysis

Investigators blinded to experimental conditions performed all data acquisition and analysis. Statistical analyses were completed using GraphPad Prism 6.0 (GraphPad Software). Data were analyzed using one- or two-way ANOVA followed by Dunnett's or Holms–Sidak *post hoc* tests for multiple comparisons to the vehicle groups. Chi square and independent-sample *t*-tests were used when appropriate. Results were considered statistically significant at $p \leq 0.05$. All data are presented as mean ± SEM unless otherwise noted.

Figures were prepared using Adobe Photoshop CC 2014 (Adobe Systems) and Prism 6.0.

3- iv. Results

Post-injury Administration of Azithromycin Improves Locomotor Recovery After SCI:

As described previously, initiating AZM treatment 30 min after SCI, followed by repeated daily doses for 7 days, mediates an immunomodulatory shift in macrophage phenotype resulting in the downregulation of markers associated with pro-inflammatory M1 macrophage activation and upregulation of anti-inflammatory M2 markers (Gensel et al., 2017). Because this type of macrophage phenotypic transition is often associated with an increase in reparative functions after SCI, we sought to determine whether post-injury administration of AZM led to long-lasting locomotor improvement up to 28 days after injury. AZM was administered beginning 30 min, 3 h, or 24 h post-injury and then daily for 7 days in three compiled studies. The 24 h delayed treatment group was discontinued due to clear therapeutic ineffectiveness in study one (**Figures 3.1A,B;** $p > 0.98$ for all outcomes). The 30 min and 3 h administration groups, however, displayed improved locomotor recovery relative to vehicle. Specifically, overall locomotor recovery out to 28 dpi, as measured by the BMS, varied as a function of treatment ($p = 0.008$ main effect of treatment; time × treatment interaction $p = 0.02$). Locomotor recovery significantly improved when AZM was administered at 30-min relative to vehicle ($p = 0.004$ main effect; **Figure 3.2A**).

This treatment effect was significant beginning at 14 dpi (p = 0.005; **Figure 3.2A**). Delaying the initial dose for 3-h after SCI improved recovery relative to vehicle (p = 0.058 vs. vehicle, main effect) with significant improvements by 28 dpi (p= 0.0004).

By 28 dpi, delaying AZM treatment by either 30 min or 3 h after SCI significantly improved locomotor function vs. vehicle with an average BMS score of ~5 for AZM groups vs. ~4 for the vehicle-treated group (**Figure 3.2A**). A transition from a score of 4–5 is largely dependent on the mouse's ability to fully support its body weight on its hind legs while stepping. Because we observed this group-dependent separation along the 4/5 score of the BMS scale, we further quantified stepping ability. As seen in **Figure 3.2B**; a significantly greater proportion of mice treated with AZM beginning at either 30 min or 3 h post-injury recovered frequent plantar stepping function vs. vehicle controls (Chi-squared, p = 0.001 and p = 0.04, respectively). We also evaluated proprioceptive hindlimb stepping function with the horizontal ladder task. There were no differences among groups prior to SCI (p = 0.99, one-way ANOVA, data not shown). However, since few animals in the vehicle group recovered stepping function and were able to perform the horizontal ladder task, results of this test were not compared among groups after SCI.

Azithromycin and Neuropathic Pain:

We recently reported that AZM is an analgesic that alleviates chronic SCI pain when dosed 30 min prior to measuring heat-induced hyperalgesia (Gensel

et al., 2019). Here, we expanded upon this by testing whether AZM limits the development of neuropathic pain (mechanical allodynia) after SCI. As seen in **Figure 3.3**, SCI induced allodynia ($p < 0.0001$), however, AZM administration beginning 30 min and 3 h following injury did not significantly reduce long-term pain responses (mechanical allodynia) relative to vehicle-treated animals at 27 dpi ($p = 0.89$).

The Effect of Post-SCI Azithromycin Treatment on Tissue Pathology:

Previously, we observed improved locomotor recovery along with increased tissue preservation using a combined pre- and post-SCI dosing paradigm for AZM (Zhang et al., 2015b). As seen in **Figures 3.4A–E**, the effect post-SCI AZM treatment on tissue sparing at the lesion epicenter did not reach statistical significance for either the 30 min or 3 h treatment groups in the current studies ($p = 0.07$ and 0.54, respectively). AZM administration significantly reduced the mean rostral-caudal lesion length when given 30 min after injury ($p = 0.03$; **Figures 3.5A,B**); the 3-h delivery timepoint was not statistically significant ($p= 0.55$; **Figure 3.5**). Given that pro-inflammatory macrophages are neurotoxic and that AZM limits this activation *in vitro* we sought to quantify whether post-SCI AZM treatment improved the number of surviving neurons at 28 dpi (Zhang et al., 2015b; 2019). However, AZM treatment did not affect neuron sparing throughout the rostral-caudal extent of the lesion (ANOVA main effect of treatment $p = 0.26$; **Figures 3.6A,B**) or total neuron sparing according to area under the neuron by distance curve (ANOVA $p = 0.98$; **Figure 3.6C**).

3- v. Discussion

The clinical use of methylprednisolone, the only FDA-approved drug to complete phase three clinical trials for SCI to date, has fallen substantially in recent years with concerns that its side effects may out-weigh its clinical benefits. As a result of this decline, many individuals with a SCI have been left with no pharmacological treatments for their injuries. Here, we provide evidence that azithromycin (AZM) treatment, initiated after SCI, improves recovery. Specifically, delaying treatment for 30 min or 3 h post-injury significantly increased locomotor recovery in mice. We observed therapeutic effects even when delayed for 3 h, although less robust than our 30-min timepoint. When delayed 24 h after injury AZM administration had no discernable therapeutic effect. Azithromycin is routinely given to SCI individuals with limited side effects (Evans et al., 2013) and our results here demonstrate that AZM may have a viable therapeutic window as a neuroprotective treatment for SCI.

Clinically, AZM is widely used for its antibiotic properties, however, increasing evidence indicates that the neuroprotective effects are a result of a distinct cellular mechanism. Specifically, AZM has a remarkable ability to accumulate within cells, in particular, within phagocytes such as macrophages (Zimmermann et al., 2018). Subsequently, AZM appears to inhibit the activation of the NF-κB signaling cascade, a potent regulator of macrophage activation states (Aghai et al., 2007; Cigana et al., 2007; Feola et al., 2010b; Haydar et al., 2019). In support of the concept that the immunomodulatory effects of AZM are independent of its antibacterial actions, we recently developed a library of AZM

derivatives with reduced antibiotic properties. Indeed, even compounds with reduced antibiotic activity retained the ability to blunt pro-inflammatory macrophage activation and macrophage-mediated toxic effects on neurons (Zhang et al., 2019). Similarly, investigators in other disease models have utilized AZM as an immunomodulatory agent and have attributed its efficacy to its anti-inflammatory effects on macrophage physiology (Amantea et al., 2016b; Feola et al., 2010b; Kitsiouli et al., 2015). We have identified a similar mechanism of action for AZM in SCI previously (Gensel et al., 2017; Zhang et al., 2015b; 2019). Specifically, we found that AZM administration 30 min post-injury at doses of 10, 40, or 160 mg/kg decreased pro-inflammatory M1 macrophage gene expression at 3 dpi while the lowest (10 mg/kg) and highest (160 mg/kg) doses increased anti-inflammatory M2 macrophage gene expression at 7 dpi (Gensel et al., 2017). One small caveat to this, and the current study, is that all of the mice receive prophylactic antibiotic (enrofloxacin) during surgical recovery. It is thereby possible that there is a drug interaction effect, although their intended antimicrobial mechanisms have distinct cellular targets. Collectively, increasing evidence demonstrates that AZM accumulates in macrophages and improves outcomes by driving a shift in macrophages from pro-inflammatory and pathological M1 phenotypes to more reparative, anti-inflammatory M2 phenotypes. These AZM mediated shifts in macrophage activation states are thereby likely to be important factors mediating the long-term therapeutic benefits detailed in the current study, however, we did not specifically evaluate the impact of treatment timing on macrophage phenotype.

Our observations are consistent with reports of AZM being neuroprotective in other neurological conditions through the promotion of anti-inflammatory macrophage polarization. For example, Amantea et al. demonstrated that administration of AZM is neuroprotective in a transient middle cerebral artery occlusion model of stroke. Importantly, they attributed AZM's protective effects to its ability to drive macrophages to an anti-inflammatory M2 phenotype (Amantea et al., 2016b). Similarly, Varano et al. utilized AZM to target CNS inflammation in a rat model of retinal ischemia/reperfusion injury (Varano et al., 2017) (pathology associated with glaucoma, diabetic retinopathy, and anterior ischemic neuropathy; (Zheng et al., 2007). In that study, a single dose of AZM (150 mg/kg, i.p.) given after 50 min of ischemia, increased retinal ganglion cells survival by reducing the excitotoxicity and propagation of the macrophage response (Varano et al., 2017). Together with our prior observation of AZM decreasing M1 activation and increasing M2 macrophage *in vivo* after SCI (Gensel et al., 2017; Zhang et al., 2015b) these studies suggest that AZM could have important therapeutic implications across many neurological conditions. In particular, CNS disorders such as traumatic brain injury, Alzheimer's disease, and multiple sclerosis are known to be heavily influenced by inflammatory pathways. However, the efficacy AZM in these models remains to be determined. In many CNS disorders there is the added complexity of having both bone marrow derived monocytes/macrophages and microglial derived macrophage populations. While emerging evidence suggests that microglia may be more neuroprotective than monocyte-derived macrophages, we do not yet know

through which population AZM exerts its therapeutic effects (Bellver-Landete et al., 2019; Greenhalgh et al., 2018). Our prior work using a combined pre- and post-injury AZM administration paradigm demonstrated that both microglia (*in vivo*) and macrophages (*in vitro*) are affected by AZM (Gensel et al., 2017; Zhang et al., 2015b). The relative contribution of microglia vs. monocyte-derived macrophages to the therapeutic effects of AZM observed in the current study, however, remains unclear. To address these uncertainties, we are currently developing small molecule labeling techniques to track AZM after administration and methods to separate and analyze microglia and monocyte populations individually after SCI.

One limitation of the current study is that only one AZM dosing paradigm (160 mg/kg/day) was tested. Using typical interspecies allometric scaling, this dose may translate to a high, but still clinically relevant dose in humans (Anroop B Nair, 2016)However, additional dose-response studies or alternative formulations may improve the neuroprotective potential or therapeutic window of AZM. Indeed, we previously observed that AZM retains its ability to modulate macrophage phenotype even at substantially lower doses (10 and 40 mg/kg) when administration begins 30 min post-SCI (Gensel et al., 2017). Similarly, using alternative dosing strategies, Amantea et al. (Amantea et al., 2016a) demonstrated that both intravenous and intraperitoneal administrations of AZM were neuroprotective in a rodent model of stroke. In the case of intraperitoneal administration, AZM remained neuroprotective after stroke at doses from 150 mg/kg down to as low as 1.5 mg/kg, suggesting that alternative administration

routes may offer greater effectiveness with reduced doses (Amantea et al., 2016b). We have also begun modifying AZM structure through medicinal chemistry to produce derivatives that lack antibiotic activity yet maintain their immunomodulatory effects on macrophages (Zhang et al., 2019). Ongoing work seeks to identify a derivative with enhanced anti-inflammatory activity, which may allow for further reductions in dosages prior to clinical translation.

The therapeutic efficacy of AZM in the current study decreased with the timing of the first oral dose after SCI with the 30-min time point being the most effective, 3-h being moderately effective, and no efficacy when the first treatment was delayed 24 h. It may be possible to extend this treatment window. For example, here we administered AZM by oral gavage, whereas faster and more direct methods such as intravenous administration may allow for more robust effects and a more flexible dosing window. In the treatment of stroke, intraperitoneal administration of AZM was effective when administered out to 4.5 h post-injury suggesting that alternative dosing approaches may be able to extend AZM's dosing window post-SCI (Amantea et al., 2016b; 2019). Collectively, while the data showed here demonstrate that early oral administration of AZM is therapeutically effective, there are ample opportunities to both improve its efficacy and minimize any associated risks.

In our current dosing paradigm, we dosed for 7 days after injury, yet our most robust behavioral improvements are seen from days 14–28 post-injury. This delayed response is consistent with observations in humans and may be due in part to AZM's ability to accumulate within macrophages. Indeed, in human

patients treated with AZM for 3 days, AZM accumulation in monocytes is still evident by 14 days with minimal depletion (Wildfeuer et al., 1996). Presumably, there is a similarly prolonged presence of AZM within macrophages after SCI contributing to the neuroprotective effects well into chronic time points. We observed a similar response in using a pre-treatment dosing strategy in that we only saw significant behavioral differences relative to vehicle starting at 14 dpi (Zhang et al., 2015b). This suggests that AZM accumulation in macrophages after SCI is sufficient to facilitate long-term improvements. Therefore, methods to enhance AZM's targeted delivery to macrophages, such as with liposome-based drug delivery systems may facilitate greater therapeutic efficacy.

In the weeks and months following SCI, animals develop neuropathic pain with both hyperalgesia (increased pain from a stimulus that normally provokes pain) and allodynia (pain due to a stimulus that does not normally provoke pain; (Deuis et al., 2017). Previously we demonstrated that AZM has analgesic properties when given to these chronically injured animals 30 min prior to pain testing (Gensel et al., 2019). This is in contrast with the current study in which AZM had no analgesic effects. The two key differences are that in the current study we dosed with AZM for 7 days, stopping 3 weeks prior to pain testing and we evaluated pain using a mechanical withdrawal (allodynia) test instead of thermal-induced pain test (allodynia and hyperalgesia). It is possible that the analgesic properties of AZM are modality-specific and AZM could block the development of heat but not mechanically induced pain responses. The results of the current study, however, suggest that AZM has no effect on the overall

development of mechanical allodynia after SCI, but maintains promise as a pain relief alternative to opioid and non-steroidal anti-inflammatory based therapeutics. Further, it has become increasingly important to include tests for the affective components of pain in animal models when identifying new drugs to treat SCI (Kramer et al., 2017). It is possible that future studies evaluating the effects of AZM treatment on affective pain may reveal additional benefits of treatment.

In the current study, we administered AZM through oral gavage after SCI and detected modest, yet significant therapeutic benefits. It is possible that alternate dosages and routes of delivery could broaden AZM's treatment window and increase its therapeutic effects. Despite these challenges, AZM holds great promise in the treatment of SCI and a broad variety of other inflammatory disorders. Fortunately, AZM's excellent safety history, and wide availability at essentially all healthcare centers would greatly reduce barriers preventing rapid use after injury (Trifirò et al., 2017; Uzun et al., 2014). The results of the current study add to a growing body of evidence supporting AZM's translational potential as a neuroprotective agent for SCI and other neuroinflammatory conditions in which patients currently have very few options.

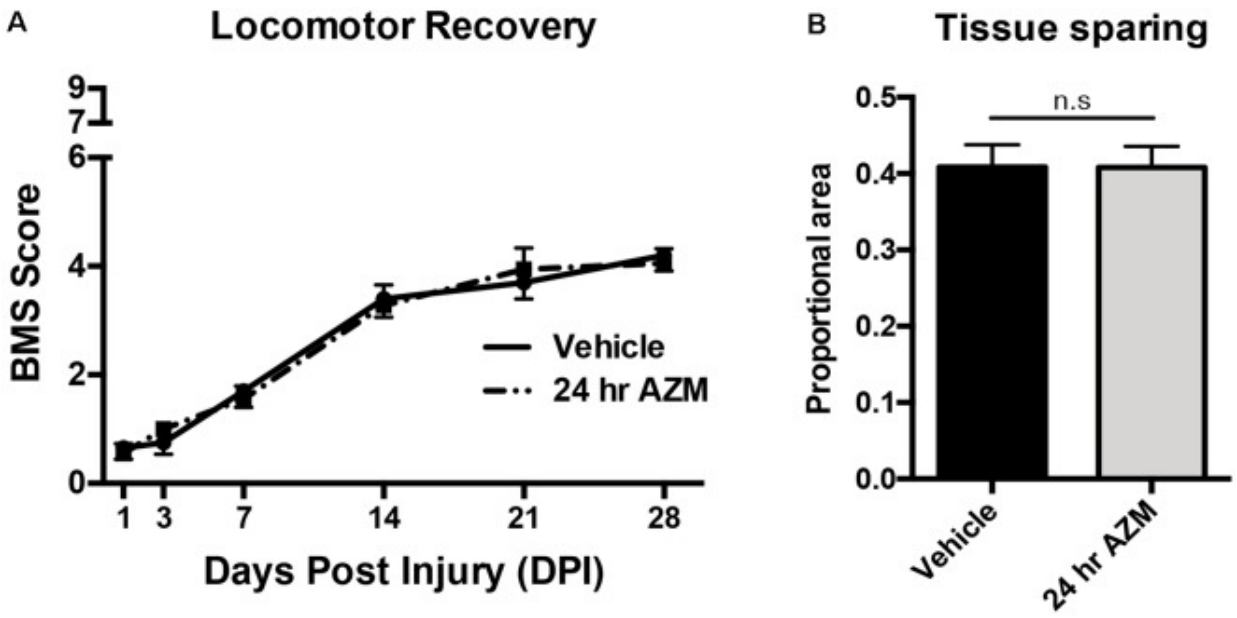


Figure 3.1. Azithromycin (AZM) administration beginning 24 h after injury is not therapeutically effective.

Adult (4-month-old) female mice received a moderate-severe thoracic T9 contusion spinal cord injury (SCI; 75-kdyn). AZM was first administered at 24-h post-injury and then daily for 7 days (160 mg/kg/day). Functional recovery was assessed before injury and at 1, 3, 7, 14, 21, and 28 dpi. **(A,B)** Initiating AZM treatment 24-h after SCI did not improve locomotor recovery relative to vehicle control ($p = 0.99$, $n = 9–10$) or improve tissue sparing [as defined by glial fibrillary acidic protein (GFAP) reactivity] at the lesion epicenter at 28 dpi relative to vehicle (n.s. = not significant, $p = 0.98$, $n = 7–8$). Mean ± SEM.

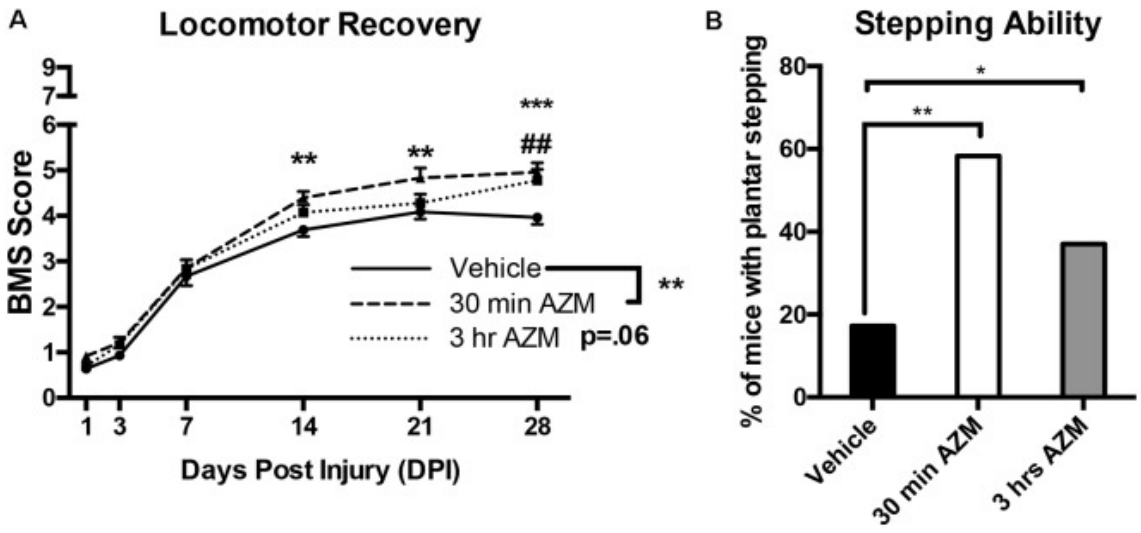


Figure 3.2. Early AZM administration improves locomotor recovery in SCI mice.

Adult (4-month-old) female mice received a moderate-severe thoracic T9 contusion SCI (75-kdyn). AZM was first administered at 30-min or 3-h post-injury and then daily for 7 days (160 mg/kg/day). Functional recovery was assessed before injury and at 1, 3, 7, 14, 21, and 28 dpi. **(A)** Mice treated with AZM beginning 30-min post-injury displayed significantly improved locomotor recovery relative to vehicle (main effect of treatment vehicle vs. 30 min, $p = 0.004$) with significant improvement from 14 to 28 dpi (**,***$p < 0.05$ 30 min vs. vehicle, Holms–Sidak's *post hoc*). Mice first treated at 3 h post-injury had increased recovery relative to vehicle ($p = 0.06$ main effect of treatment vehicle vs. 3 h) with significant improvements at 28 dpi ($^{\#\#}p < 0.05$ vs. vehicle Holms–Sidak's *post hoc*). **(B)** Mice treated at 30-min recovered significantly improved frequent plantar stepping frequency than vehicle controls (58% and 17% respectively, Chi-squared, $p = 0.001$). Similarly, mice treated at 3-h recovered significantly more

frequent plantar stepping relative to vehicle (37% and 17%, respectively, Chi-squared, $p = 0.04$). $n = 24–29$, mean ± SEM. *$p < 0.05$.

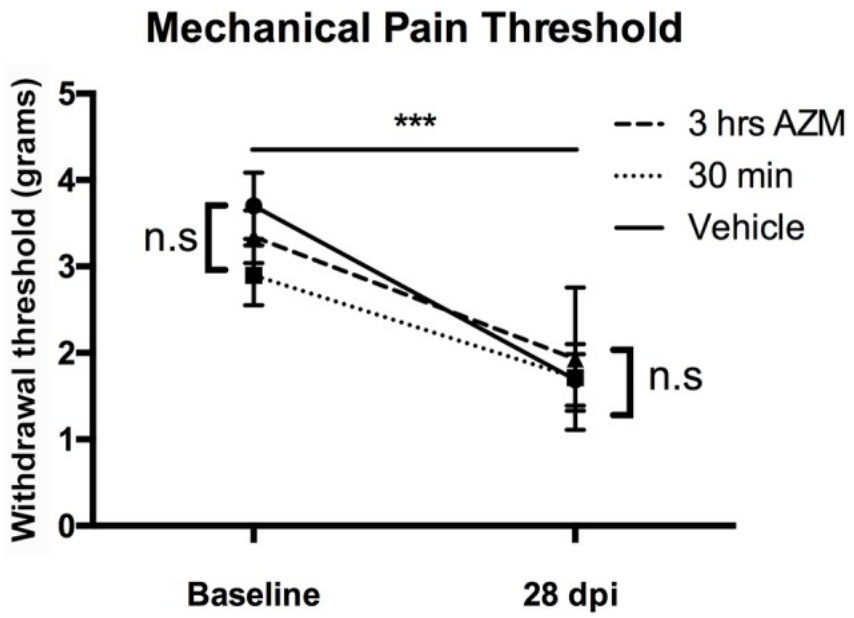


Figure 3.3. AZM administration does not alter the development of mechanical allodynia in mice after SCI.

Gram-force withdrawal threshold to Von Frey filaments stimuli decreased after SCI, relative to baseline, indicative of the development of allodynia after SCI (***$p > 0.0001$). However, AZM did not alter the development of allodynia at 27 dpi relative to vehicle (n.s. = not significant, $p = 0.89$). There were no baseline differences in withdrawal threshold prior to injury ($p = 0.29$). $n = 15–20$, mean ± SEM.

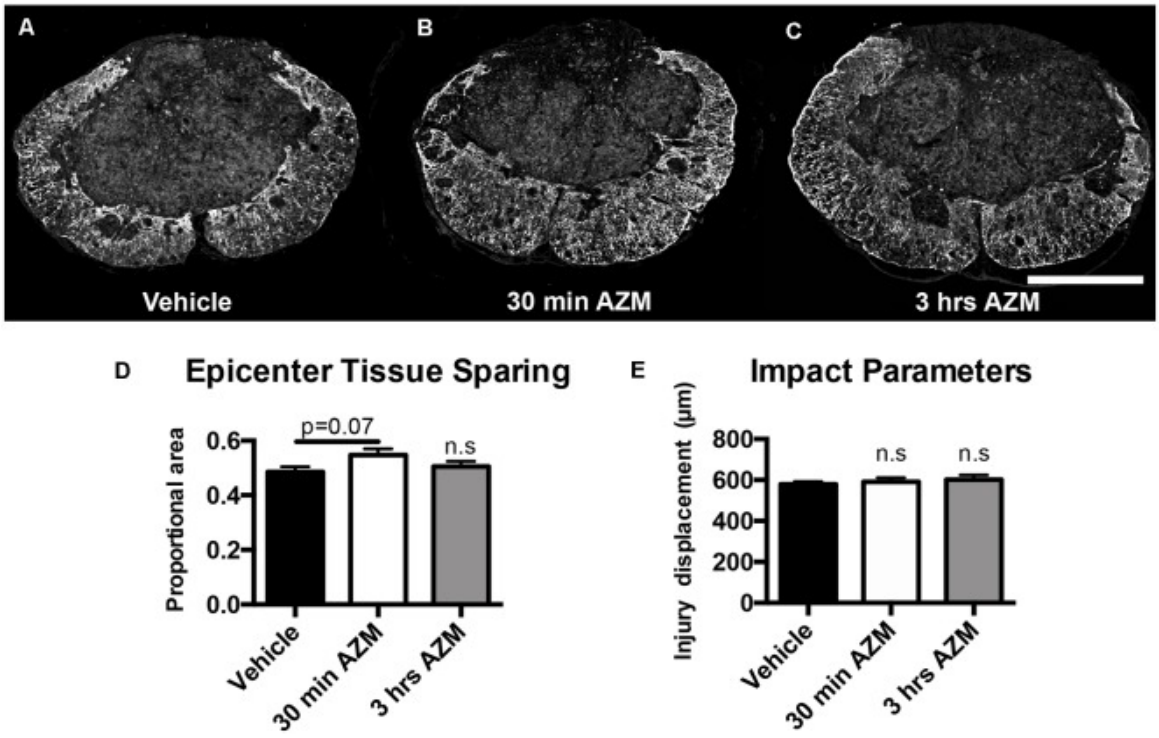


Figure 3.4. AZM does not significantly increase long-term, 28-day tissue sparing at lesion epicenter.

Tissue sections representative of the mean values for **(A)** vehicle or AZM beginning **(B)** 30 min or **(C)** 3 h post-SCI. **(D)**Quantification of tissue sparing at 28 days post-injury based on GFAP reactivity did not demonstrate any statistically significant differences across groups, however, the mean tissue sparring was higher in the 30-min group (56%) than the vehicle-treated group (49%; $p = 0.07$). **(E)** The injury displacement values were equal across groups ($p = 0.69$) indicative of comparable injury severities prior to treatment administration. $n = 19–25$, mean ± SEM. Scale bar: 500 µM. n.s = not significant.

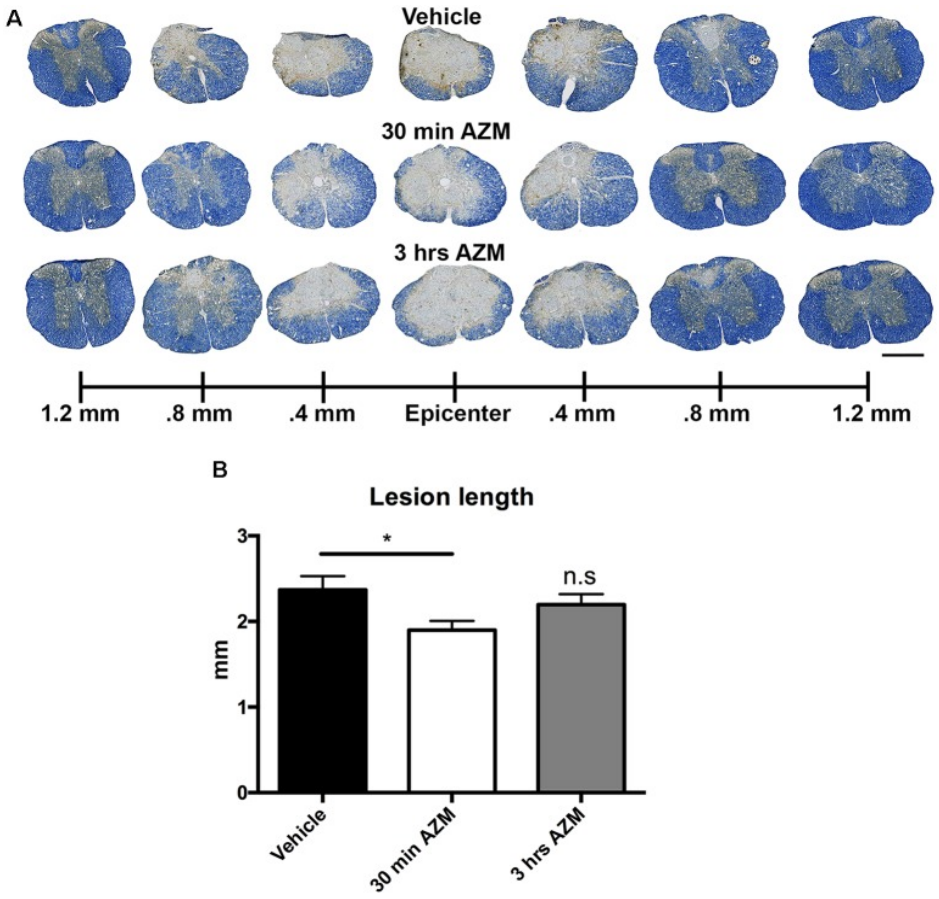


Figure 3.5. Post-SCI treatment with AZM reduces rostral-caudal lesion length.

(A) Representative images of EC/NF stained sections 28 days after SCI detailing the lesion characteristics for vehicle, 30-min AZM, and 3-h AZM administration paradigms. (B) AZM administration beginning 30 min post-injury reduced overall rostral-caudal lesion length to an average of 1.9 mm relative to the 2.4 mm average in vehicle-treated animals (p = 0.03). This is evident in (A) by the relatively intact spinal cord at 0.8 mm rostral and caudal to the epicenter in the 30 min AZM vs. vehicle. group. Mice with treatment beginning at 3-h post-injury had a slightly lower average lesion length at 2.2 mm, however this was not

statistically significant (p = 0.55). n = 23–29, mean ± SEM. Scale bar: 500 μM.

* p< 0.05; n.s = not significant.

Chapter 4: Advancing macrophage-targeted therapies into successful treatments for SCI

4- i. Summary:

Chapter Summary

There is an urgent need for the development of new therapies for the treatment of SCI. While AZM and $cPLA_2$ inhibitors are promising macrophage-targeted therapy candidates, there are numerous other approaches to target macrophages being developed. In this Chapter we examine: 1) A transgenic approach to target cPLA2 *in vitro.* 2) The immunomodulatory properties of macrolide derivatives of AZM. 3) The shift in pro-inflammatory macrophage cytokine profiles in response to leukemia inhibitory factor (LIF). Lastly, we cover a general discussion of these approaches and of additional macrophage-targeted therapies currently in development. Respective topics are indicated by section headings.

Summary: Transgenic Targeting of $cPLA_2$ *In Vitro*

We previously targeted $cPLA_2$ using a chemical inhibitor, PACOCF3. Here we build on these prior studies using a transgenic approach. We isolated BMDMs from $cPLA_2^{-/-}$ (KO) and $cPLA_2^{+/+}$ (WT) and examined the impact of $cPLA_2$ ablation on ROS, nitric oxide, neurotoxicity, and arginase enzymatic activity during myelin exposure. $cPLA_2$ reduced myelin induced ROS, nitric oxide, and neurotoxicity

relative to WT BMDMs, although arginase enzymatic activity was not affected. This is additional evidence indicating a key role of $cPLA_2$ in mediating the myelin induced potentiation of pro-inflammatory macrophage activation.

Summary: AZM Derivatives

This section is adapted, in part, from Zhang et al. 2019. All excluded content can be found in the original source material.

Azithromycin (AZM) and other macrolide antibiotics are applied as immunomodulatory treatments for CNS disorders. The immunomodulatory and antibiotic properties of AZM are purportedly independent. To improve the efficacy and reduce antibiotic resistance risk of AZM-based therapies, we evaluated the immunomodulatory and neuroprotective properties of novel AZM derivatives. We semisynthetically prepared derivatives by altering sugar moieties established as important for inhibiting bacterial protein synthesis. Bone marrow-derived macrophages (BMDMs) were stimulated *in vitro* with proinflammatory, M1, stimuli (LPS + INF-gamma) with and without derivative costimulation. Pro- and anti-inflammatory cytokine production, IL-12 and IL-10, respectively, was quantified using ELISA. Neuron culture treatment with BMDM supernatant was used to assess derivative neuroprotective potential. Azithromycin and some derivatives increased IL-10 and reduced IL-12 production of M1 macrophages. IL-10/IL-12 cytokine shifts closely correlated with the ability of AZM and derivatives to mitigate macrophage neurotoxicity. Sugar moieties that bind bacterial ribosomal complexes can be modified in a manner that retains AZM immunomodulation and

neuroprotection. Since the effects of BMDMs *in vitro* are predictive of CNS macrophage responses, our results open new therapeutic avenues for managing maladaptive CNS inflammation and support utilization of IL-10/12 cytokine profiles as indicators of macrophage polarization and neurotoxicity.

Summary: LIF

Leukemia inhibitory factor (LIF) was previously implicated in promoting oligodendrocyte survival and reducing demyelination following SCI. Here, we examine the role of LIF in our *in vitro* model of SCI inflammation previously shown to be predictive of monocyte-derived macrophage responses *in vivo* in the injured spinal cord. Application of LIF to pro-inflammatory “M1” macrophages resulted in reduced production of the pro-inflammatory cytokine IL-12, and increased production of the anti-inflammatory cytokine IL-10. This suggests that LIF exerts its protective effects at least in part by driving a shift in macrophage activation state towards an anti-inflammatory phenotype.

4- ii. Background:

There is only one therapeutic, methylprednisolone, that is available to improve neurological recovery after SCI (Hall, 2011). Unfortunately, due to the risk of side effects and a modest therapeutic benefit, many physicians no longer give methylprednisolone to their SCI patients (Evaniew et al., 2015). This means that many individuals who sustain an SCI do not receive any therapeutics to improve their recovery, highlighting the urgent need to develop new therapeutics

to treat SCI. Macrophages are key mediators of the complex neuroinflammatory response observed after SCI contributing to the secondary injury process, and thus macrophages are promising targets for therapies to treat SCI. While AZM and $cPLA_2$ inhibitors are promising macrophage-targeted therapy candidates, there are numerous other macrophage-targets therapies being developed. These include strategies to reduce macrophage infiltration into the injury site and additional methods to shift macrophage activation towards a pro-reparative state.

The macrophage inflammatory response is a key component of the pathophysiology of numerous disorders, including SCI. In addition to AZM, and $cPLA_2$ inhibition discussed previously, there have been additional approaches to modulate macrophage activation states. $cPLA_2$ inhibition, for example, is a simple approach to target $cPLA_2$; however, it comes with the major caveat of off target effects on related phospholipases. Here, we build upon this work by repeating our previous observations in transgenic mice. Complete genetic ablation of $cPLA_2$ allows for clear comparisons without the caveats of $cPLA_2$ inhibitors. Similarly, AZM is a macrolide antibiotic developed through chemical modifications to erythromycin to improve efficacy; it was not developed with immunomodulatory activities intentionally. Because of this, we hypothesized that additional chemical modifications through medicinal chemistry could allow for further enhancement of its immunomodulatory activity. Here we examine the therapeutic efficacy of these AZM derivatives in our *in vitro* model of SCI neuroinflammation. Next, we examine the ability of the cytokine leukemia inhibitory factor (LIF) to improve pro-inflammatory macrophage cytokine

production profiles. Lastly, we discuss some of the other approaches utilized to target macrophage inflammatory responses in SCI inflammation.

With continued development, macrophage-targeted therapies hold great promise in the treatment of SCI and related neuroinflammatory conditions for which patients currently have very few therapeutic options.

Transgenic Targeting of $cPLA_2$ *In Vitro*

$cPLA_2$ selectively releases arachidonic acid (AA) from cellular membranes where it is stored in its inactive state. Once released it is rapidly converted into numerous eicosanoids by enzymes such as the LOX and COX families of enzymes. These eicosanoids have diverse, albeit largely pro-inflammatory functions. Previous work has sought to target $cPLA_2$ in rodent models of SCI with conflicting results (Liu et al., 2006; Lopez-Vales et al., 2008). We hypothesized that specifically targeting macrophage $cPLA_2$ activity may be more beneficial as it would target detrimental $cPLA_2$ activity while leaving other beneficial $cPLA_2$ intact. To this aim, we previously investigated the role of $cPLA_2$ in myelin induced potentiation of pro-inflammatory macrophage activation using the chemical inhibitor of $cPLA_2$ PACOCF3 (Kopper et al., 2021). This approach includes many known and unknown caveats including off target effects on other phospholipases and the inclusion of solvents like DMSO to suspend the chemical. Here we repeat these previous studies using BMDMs derived from $cPLA_2^{-/-}$ (KO) transgenic mice and their $cPLA_2^{+/+}$ (WT) littermates. This approach allows us to confirm our previous observations without the previous caveats of PACOCF3.

AZM Derivatives

This section is adapted, in part, from Zhang et al. 2019. All excluded content can be found in the original source material.

The management of maladaptive inflammation is an emerging therapeutic target for many neuropathologies. Different macrophage phenotypes have been identified in the injured central nervous system (CNS) in conditions such as ischemic brain damage, spinal cord injury, and traumatic brain injury. After spinal cord injury, for example, there is a heterogeneous neuroinflammatory response mediated by resident microglia and infiltrating macrophages. Classically activated macrophages (M1) secrete proinflammatory cytokines and chemokines and contribute to continued cell death and a persistent inflammatory microenvironment within the injured spinal cord (Gensel and Zhang, 2015; Kigerl et al., 2009). In contrast, alternatively activated macrophages (M2) release anti-inflammatory cytokines and facilitate tissue repair (Kigerl et al., 2009). Increasingly, clinicians and researchers are testing the therapeutic potential of drugs that polarize macrophage activation toward reparative phenotypes in a variety of CNS disorders.

Macrolide antibiotics are a class of natural products consisting of a highly substituted macrocyclic 14-, 15-, or 16-membered lactone ring. Azithromycin (AZM) is a 15-membered, second generation, synthetic derivative of erythromycin with improved pharmacokinetic properties and a broad antimicrobial spectrum (Parnham et al., 2014). Azithromycin is well tolerated and commonly

prescribed. Moreover, AZM becomes highly concentrated in macrophages and other phagocytes (Miossec-Bartoli et al.; Wilms et al., 2006). Across a variety of inflammatory conditions, AZM attenuates proinflammatory cytokine production by macrophages and other immune cells (Murphy et al., 2008).

Azithromycin and other macrolide antibiotics are now being tested as immunomodulatory agents for CNS disorders. Specifically, we and others observed immunomodulatory effects and improved recovery with AZM treatment in spinal cord injury, stroke, and retinal ischemia/reperfusion injury (Amantea et al., 2016a; 2016b; Gensel et al., 2017; Petrelli et al., 2016; Varano et al., 2017; Zhang et al., 2015b).

The neuroprotective properties of AZM in these models are associated with direct effects on macrophages (Amantea et al., 2016b; Gensel and Zhang, 2015; Gensel et al., 2017). We have shown that *in vitro* application of AZM to proinflammatory M1 bone marrow-derived macrophages (BMDMs) dampens the release of proinflammatory cytokines, increases M2-associated anti-inflammatory cytokines, and reduces the neurotoxicity of M1 macrophage-conditioned medium (Gensel and Zhang, 2015).

In efforts to improve efficacy and/or reduce the risk of increasing antibiotic resistance, researchers are evaluating the immunomodulatory potential of AZM derivatives and other macrolide derivatives with the goal of separating the antibiotic from immunomodulatory properties. As a result, some macrolide derivatives have been shown to retain immunomodulatory properties in models of lung inflammation, inflammatory bowel diseases, arthritis, and skin inflammation

(Balloy et al., 2014; Bosnar et al., 2012; Hodge et al., 2017; Mencarelli et al., 2011; Rodriguez-Cerdeira et al., 2012; Sugawara et al., 2012). The ability of macrolide derivatives to reduce macrophage-mediated neurotoxicity, however, is unknown. With the increased use of AZM as an immunomodulatory agent for macrophage-mediated neurotoxicity in CNS pathologies, our goal in the present study was to determine whether macrolide derivatives retain neuroprotective properties. Using a semisynthetic approach to target modification of the sugar moieties of AZM, we generated a small library of derivatives, some of which lacked the cladinose found in the parent. We then tested the cytokine profiles and neurotoxicity of M1-stimulated BMDMs treated with derivatives and observed that unique derivatives reduce M1-macrophage activation and subsequent neuron death. Previously we determined that the effect of BMDMs *in vitro* is predictive of macrophage responses in the injured CNS (Gensel et al., 2009; 2015; 2017); therefore, the results of the current study open new therapeutic avenues for the management of maladaptive inflammation in CNS disorders.

LIF

Leukemia inhibitory factor (LIF) is an IL-6 class cytokine with numerous functions depending on cell type. In the nervous system LIF has been shown to be neuroprotective in models of stroke (Rowe et al., 2014; Suzuki et al., 2005), multiple sclerosis (Butzkueven et al., 2006; Linker et al., 2008), amyotrophic lateral sclerosis (Azari et al., 2001), and SCI (Azari et al.; Kerr and Patterson, 2005). In SCI, LIF was found to increase oligodendrocyte survival during the

secondary injury process (Kerr and Patterson, 2005). Interestingly, this was not thought to be a direct effect of LIF on oligodendrocytes, but instead through LIF's actions on an ancillary cell population (Kerr and Patterson, 2005). While not specified here, macrophages could be a possible mediator in this process. A similar work demonstrated LIF's role in arresting oligodendrocyte death and demyelination (Azari et al.). Here we examine the effects of BMDM in our pro-inflammatory *in vitro* model of neuroinflammation. As described previously (Kopper et al., 2021), BMDMs are predictive of monocyte-derived macrophage responses *in vivo* in the injured spinal cord. This has been observed at transcription (Longbrake et al., 2007) and functional levels (Gensel et al., 2009), as well as in response to therapeutic interventions (Gensel et al., 2017) as described previously (Kopper et al., 2021). These data were collected in collaboration in a study examining the role of LIF in modulating the peripheral immune response in a rat stroke model of emergent large vessel occlusion (Davis et al., 2018).

4- iii. Methods:

Transgenic Targeting of $cPLA_2$ *In Vitro:*

As described previously (Kopper et al., 2021), bone marrow-derived macrophages (BMDMs) were extracted from the femur and tibia of female C57BL/6 mice as, previously reported (Gensel et al., 2009; 2015), from both $cPLA_2^{-/-}$ (KO) and $cPLA_2^{+/+}$ (WT) mic developed previously (Bonventre, 1999). Mice were generated from breeding pairs kindly provided by Dr. Xiao-Ming Xu at

the Indiana University School of Medicine. BMDMs were plated at 0.8–1 × 10^6 cells/mL in differentiation media containing Roswell Park Memorial Institute medium (RPMI, Thermo Fisher Scientific, #21870-092) supplemented with 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific, #5140122), 1% HEPES (Sigma-Aldrich, #83264-100ML-F), 1% GlutaMAX 0.001 (Thermo Fisher Scientific, #35050061) 0.001% β-mercaptoethanol (Thermo Fisher Scientific, #21985023), 10% FBS (Life technologies, #10082147), and 20% supernatant from sL929 cells (a generous gift from Phillip Popovich, The Ohio State University). Supernatant collected from sL929 cells contains macrophage colony-stimulating factor, which helps to promote bone marrow cells' differentiation into macrophages (Burgess et al., 1985). The BMDMs were allowed to differentiate for 7 days in culture, and KO or WT cells were then replated on day 7 at a density of 1 × 10^6 cells/mL in 12-well plates in RPMI, containing 1% P/S, 1% GlutaMAX and 10% FBS. On day 8, cells were stimulated for 24 h to be "M1" cells using LPS (50 ng/mL, Invivogen, #tlrl-eblps, standard preparation) plus IFN-γ (20 ng/ml, eBioscience #14-8311-63) diluted in N2A growth medium (described below). At the time of stimulation cells were immediately treated with myelin debris (50 µL/mL, preparation described below), 24 h after stimulation the supernatants were removed, centrifuged at 13,000 RPM (Fisher Scientific accuSpin Micro R centrifuge), and then this macrophage conditioned media (MCM) was either applied directly to N2A cells to measure cytotoxicity, or stored at − 80 °C prior to testing for Nitric Oxide with the Griess Reagent Kit (Thermo Fisher Scientific # G-7921).

Moderate purity myelin (> 95% myelin, with small contributions from axolemma and other cellular membranes) was prepared as follows (adapted from Larocca et al. (Larocca and Norton, 2001)): brains were collected from C57BL/6 mice and stored at − 80 °C prior to myelin isolation. The brains were rinsed and suspended in cold PBS with 1% P/S and placed in a Dounce homogenizer (DWK Life Science, #357544) under sterile conditions and blended with the loose and tight pestles. The solution was transferred to a 15 mL tube and pelleted at 2000 RPM (Thermo Scientific Legend XTR centrifuge) prior to discarding the soluble supernatant fraction. The pellet was resuspended in the PBS/P/S, and then 5mLs of a 30% Percoll solution (Sigma-Aldrich, #P1644-500ML) was gently underlaid below the myelin solution for density gradient centrifugation. The layers were then centrifuged at 2000 RPM for 15 min at 4 °C under gentle acceleration/deceleration, generating three distinct layers (soluble on top, myelin in middle, and Percoll/cell pellet on bottom). After removing the soluble fraction, the myelin was transferred to a fresh tube and resuspended in 10 mL distilled water with 1% P/S and incubated for 10 min (hypoosmotic shock) to separate membranes at 4 °C. The myelin was then re-pelleted at 2000 RPM, suspended in PBS/1% P/S and separated a second time by density gradient centrifugation as described above. The myelin was then suspended and pelleted twice in PBS/1% P/S to remove residual Percoll and water-soluble contaminants, and then aliquoted before storage at − 80 °C. The final protein concentration of the myelin stock solutions produced by this protocol were 10.23 mg/mL with a standard deviation of 0.282 mg/mL as determined by a BCA Protein Assay Kit (Thermo

Fisher Scientific #23225). With the application of myelin debris to BMDMs at 50 µL/mL, cells had a mean dosage of 0.51 mg/mL. Lastly, to ensure our results were not due to endotoxin contamination in our myelin preparations, we tested aliquots from each batch of myelin stimulant (Thermo Fisher Scientific #88282).

A mouse neuroblastoma cell line (Neuro-2a or N2A, a gift from Chris Richards, University of Kentucky) was maintained in N2A growth medium containing 45% DMEM, 45% OPTI-MEM reduced-serum medium, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. N2A were plated at a density of 1×10^5 cells/mL in 96-well tissue culture plates and allowed to proliferate for 48 h. The neurotoxicity of MCM was evaluated as reported previously using a MTT-based cell growth determination kit according to the manufacturer's instructions (Sigma-Aldrich CGD1-1KT) (Zhang et al., 2019). Briefly, 24 h before testing, N2A growth media was replaced with serum-free N2A media to induce differentiation. The day of testing this media was replaced by fresh MCM, and the N2A cells were incubated in MCM for 24 h before thiazolyl blue tetrazolium bromide (MTT (5 mg/ml), 20 µl per well) was added to each well and the cells further incubated for 2 h. The tetrazolium ring of MTT can be cleaved by mitochondrial dehydrogenases of viable cells, yielding purple formazan crystals, which were then dissolved in acidified isopropanol solvent. The resulting purple solution was spectro-photometrically measured at 570 nm Epoch microplate reader (BioTek Instruments, Inc., Winooski, VT) using 690 nm as a background absorbance. This data is normalized to the non-toxic CTL

values to generate proportional decrease in viability values and presented inversely as increased toxicity relative to CTL.

As described previously (Kopper et al., 2021), Macrophage reactive oxygen species (ROS) production was measured using CM-H2DCFDA (Invitrogen #C6827). In short, BMDMs were cultured and stimulated as described above except in a 96 well plate (1×10^6 cells/mL). Following the 24-h stimulation the supernatants were removed and replaced with a 5 μM solution of CM-H2DCFDA in phenol red-free RPMI with 1% GlutaMAX and penicillin/streptomycin and incubated at 37 °C for 25 min. ROS mediates the conversion of this compound to fluorescent DCF which was then detected by an Epoch microplate reader (BioTek instruments, Inc., Winooski, VT) at the compound's Excitation/Emission spectra of approx. 492–495/517–527 nm.

Macrophage $cPLA_2$ activity was measured using a Cytosolic Phospholipase A2 Assay Kit (Abcam #ab133090). In short, cells were cultured as described above except in six well culture dishes (1×10^6 cells/mL). Cells were lysed and briefly sonicated on ice in TBS-T (0.4% Triton-X) with a protease inhibitor (Sigma-Aldrich #11836170001) before proceeding directly into the manufacturer's protocol.

Statistical analyses:

As described previously (Kopper et al., 2021), results are expressed as mean ± standad error od the mean (SEM) and analyzed using GraphPad Prism 6.0 (GraphPad Software). Data were compared by one-way analysis of variance

(ANOVA) among groups followed by Dunnett's multiple comparison tests. Differences were determined to be statistically significant at *P* value ≤0.05.

AZM Derivatives:

This section is adapted from Zhang et al. 2019

Semisynthesis of AZM derivatives:

The semisynthesis of AZM derivatives and detection of antibiotic activity was performed in collaboration with Xiaodong Liu, Zheng Cui, and Steven G. Van Lanen in the College of Pharmacy, University of Kentucky, Lexington, Kentucky as detailed in Zhang et al. 2019. **Table 4.1** summarizes the modifications made to each derivative of AZM.

Preparation of bone marrow-derived macrophages (BMDMs) and macrophage-conditioned medium (MCM):

BMDMs were isolated from the femurs and tibias of C57BL/6 mice at 10-16 weeks of age. In a tissue culture hood, the bones were flushed with a syringe filled with cold washing media (RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin) to extrude bone marrow into a sterile falcon tube. The bone marrow was then triturated three times using syringes fit with 18 gauge needles and then centrifuged at 1000 rpm for 5 minutes at 4°C. After removing supernatant, red blood cells were lysed in lysis buffer (0.15 mol/L NH4Cl, 10 mmol/L KHCO3, and 0.1 mmol/L Na2EDTA, pH 7.4) for 3 min. The remaining cells were washed once in washing media and then centrifuged at 212 *g* for

5 minutes at 4°C. The resulting cell pellet was resuspended in BMDM culture media (RPMI 1640 supplemented with 1% penicillin/streptomycin, 1% HEPES, 0.001% β-mercaptoethanol, 10% FBS, and 20% supernatant from sL929 cells) and then plated in T75 flasks at a density of 1×10^6 cells/mL. The sL929 cell supernatant (cells, a generous gift from Phillip Popovich at The Ohio State University) contains macrophage colony stimulating factor, which is needed to promote differentiation of bone marrow cells into macrophages (Burgess et al., 1985). Cell culture media was changed on days 2, 4, and 6, and then, cells were replated at the density of 1×10^6 cells/mL on day 7 for designated stimulation and/or azithromycin (AZM) treatment. The following day, BMDMs were stimulated to be M1 using LPS (50 ng/mL; Invivogen) plus IFN-gamma (20 ng/mL; eBioscience) diluted in N2A growth medium as previously described (Zhang et al., 2015b). AZM (Sigma PHR1088) or AZM derivatives were diluted to the concentrations of 1, 5, 25, and 125 μmol/L and then added to the BMDMs at the time of stimulation. Unstimulated BMDMs maintained in N2A growth medium were used as control. Six hours following incubation, the supernatant of the stimulated macrophages (macrophage-conditioned media (MCM)) was collected and centrifuged to remove the cell debris. The resulting media was either applied to Neuro-2a cells for the measurement of neurotoxicity or tested for IL-10 and IL-12p40 levels using standard ELISA kits (Thermo Scientific, Rockford, IL).

Assessment of macrophage viability:

BMDMs seeded in 96-well plates (1×10^6 cells/mL) were treated with a range of concentrations (1-125 μmol/L) of AZM or AZM derivatives for 24 hours. Cells were cultured in N2a growth media, which contains 45% DMEM and 45% Opti-MEM Reduced-Serum Medium (Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin (Wendy M Dlakic and Bessen, 2007).

Assessment of Neuron viability:

Assessment of neuron viability was performed by Bei Zhang. These methods can be found in full in Zhang et al. 2019 and described above

Statistical analyses:

Results are expressed as mean ± standard deviation (SD) and analyzed using GraphPad Prism 6.0 (GraphPad Software). Data were compared by one-way analysis of variance (ANOVA) among groups followed by Dunnett's or Holmes-Sidak multiple comparison tests. Differences were determined to be statistically significant at *P* value ≤0.05.

LIF

Adapted from Davis et al. 2018

Cell Culture

Bone marrow-derived macrophages (BMDMs) were isolated from C57BL/6 mice (3 months of age) as described previously and above (Gensel et

al., 2009; Zhang et al., 2015b). On the day of BMDM stimulation, a classically activated phenotype (M1) was induced using N2A medium containing LPS (50 ng/ml) and IFNγ (20 ng/ml). LIF (20 ng/ml) or PBS was co-administered with the LPS and IFNγ. Macrophage-conditioned media was collected 24 h after stimulation and centrifuged at 13,000 rpm at 4 °C for 10 min prior to measurement of IL-12 p40 and IL-10 via ELISA (Kigerl et al., 2009).

4- iv. Results:

Transgenic Targeting of $cPLA_2$ *In Vitro*

Previously we targeted $cPLA_2$ *in vitro* with the inhibitor PACOCF3 (Kopper et al., 2021), here, we repeated these studies using bone marrow derived macrophages from $cPLA_2^{-/-}$ (KO) or $cPLA_2^{+/+}$ (WT) mice **(Figure 4.1).** Under M1 conditions KO macrophages produced less reactive oxygen species (ROS), and nitric oxide in response to myelin compared to WT cells **(Figures 4.1a, and 4.1b)**. Similarly, BMDM supernatants from myelin treated KO M1 macrophages were less toxic to neurons compared to myelin treated WT M1 macrophages **(Figures 4.1c)**. Arginase enzymatic activity was unaffected by myelin treatment or genetic ablation of $cPLA_2$ **(Figure 4.1c).**

AZM Derivatives

This section is adapted, in part, from Zhang et al. 2019. All excluded content can be found in the original source material.

Assessment of Macrophage Viability

We chose to use primary bone marrow-derived macrophages (BMDMs) for our studies as BMDM responses *in vitro* are predictive of CNS macrophage response *in vivo*. When stimulated with LPS+IFN-gamma), BMDMs model proinflammatory macrophages found in neuropathologies (Gensel et al., 2009; 2017; Kigerl et al., 2009). No doses of AZM or its derivatives were toxic to BMDMs when applied directly to the cells for 24 hours **(Figure 4.2)** Interestingly, this prolonged stimulation of BMDMs with high doses of AZM and its derivatives resulted in increased readouts on the MTT assay indicative of increased BMDM proliferation or increased metabolic activity **(Figure 4.2)**. This effect was not as robust after 6 hours of stimulation (**Supplemental Figure 4.1**), and therefore, a 6-hr stimulation time point was used for subsequent assays.

Macrophage IL-12/IL-10 Levels with Derivatives

The relative expression of IL-10 and IL-12 is a defining feature of M1 and M2 macrophages (Mantovani et al., 2004) with M2 macrophages producing high levels of IL 10 and low levels of IL-12. Conversely, M1 macrophages produce substantial amounts of IL-12 and minimal IL-10. These cytokine profiles are also predictive of the neurotoxic potential of stimulated macrophages with toxic potential decreasing with increased IL-10 and reduced IL-12 production (Gensel and Zhang, 2015; Zhang et al., 2015a). Similar to previous observations (Zhang et al., 2015b), AZM reduced production of the proinflammatory cytokine, IL-12, and elevated the secretion of the anti-inflammatory cytokine IL-10 in M1

macrophages in a dose-dependent manner 6 hours after stimulation. Compared to M1-stimulated macrophages, costimulation of M1 stimulant (LPS +IFN-gamma) with either 25 or 125 μmol/L AZM significantly decreased IL-12 ($P < 0.01$ and <0.001, respectively) and increased IL-10 ($P < 0.01$ and <0.0001, respectively; (**Figure 4.3)**.

To determine the immunomodulatory properties of our azithromycin derivatives, we examined IL-10 and IL-12 production in BMDMs costimulated with derivatives and M1 stimulants (LPS + INF-gamma). We detected significantly decreased IL-12 production with 125 μmol/L cotreatment concentrations for derivatives 4 ($P < 0.001$) and 7 ($P < 0.05$) relative to M1 stimulation alone **(Figure 4.3).** Derivative 5 had significantly reduced IL-12 production with 25 μmol/L ($P < 0.01$) and 125 μmol/L ($P < 0.0001$) concentrations. There was no significant effect on IL-12 with derivative 1. We observed reciprocal significant increases in IL-10 with all derivatives at the highest dose of 125 μmol/L ($P < 0.05$, **Figure 4.3**). In addition, the 25 μmol/L stimulation with derivative 5 significantly increased production of IL-10 relative to M1 ($P < 0.001$; **Figure 4.3**). RT-PCR analyses of select genes associated with M1 or M2 macrophage phenotypes (ie, IL-6, IL-1b, TNF-a, and TGF-b) demonstrated similar immunomodulatory effects between AZM and derivatives 4 and 7 (**Supplementary Figure 4.2**).Collectively, these data demonstrate that altering the bacterial binding residues of AZM does not reduce its immunomodulatory properties with AZM5 having similar dose-response properties as the parent compound.

Macrophage-Mediated Neurotoxicity with Derivatives

Assessment of neuron viability was performed by Bei Zhang. These results can be found in full in Zhang et al. 2019 and described above.

LIF

Other strategies to target macrophage inflammatory responses typical to aim to modulate macrophage activation states including AZM and $cPLA_2$ ablation discussed previously. Another method we've utilized to reduce pro-inflammatory macrophage activation *in vitro* is with leukemia inhibitory factor (LIF), a cytokine important in cellular differentiation. When given to pro-inflammatory "M1" BMDMs (LPS + IFN-γ) LIF significantly decreased the production of pro-inflammatory IL-12, and significantly increased the production of anti-inflammatory IL-10 **(Figure 4.4, *adapted from Davis et al. 2018*)**.

4- v. Discussion:

Transgenic Targeting of $cPLA_2$ *In Vitro*

The gold standard in defining biological mechanisms in biomedical research is through the use of transgenic rodent models. In the case of $cPLA_2$, genetic ablation of this enzyme was found to improve locomotor recovery and reduce tissue damage after SCI (Liu et al., 2014) suggesting a detrimental role for $cPLA_2$ in SCI pathogenesis. Conversely, in sciatic nerve injury models, genetic ablation of $cPLA_2$ resulted in fewer regenerating axons and reduced

macrophage recruitment (Lopez-Vales et al., 2008). Similarly, chemical inhibition of $cPLA_2$ with AX059 after SCI was found to impair locomotor recovery suggesting a protective role for $cPLA_2$ (Lopez-Vales et al., 2011). Here, we demonstrate a clear pathological role of $cPLA_2$ in macrophage inflammatory responses. Specifically, we observed that macrophage inflammatory responses under pro-inflammatory "M1" conditions were significantly potentiated by the addition of myelin. When $cPLA_2$ is ablated, however, this potentiation in macrophage inflammation is significantly abated. Given that macrophages after SCI are largely pro-inflammatory and contain myelin, these results implicate $cPLA_2$ as a major contributor to detrimental macrophage activities after SCI. This highlights the need to develop safe therapeutics to target $cPLA_2$ in the human condition.

AZM Derivatives

This section is adapted, in part, from Zhang et al. 2019. All excluded content can be found in the original source material.

In this study, we demonstrate the retention of immunomodulatory activity in AZM derivatives with altered sugar moieties using our *in vitro* model of macrophage CNS inflammation. This model accurately predicts the macrophage/microglial response in the injured CNS (Gensel et al., 2009; 2015; 2017b; Zhang et al., 2015b). Specifically, we demonstrate that these derivatives, like the AZM parent compound, have no negative toxic effects on macrophage viability, retain the ability to polarize M1 macrophages toward the M2 phenotype

as determined by IL-10/12 cytokine profiles, and are equally effective in reducing the neurotoxic effects of M1 macrophage supernatants on neuronal cultures. In these studies, the ability of the derivatives to shift IL-10/12 cytokine profiles closely correlated with their ability to mitigate M1 supernatant toxicity to neurons. This supports our notion of utilizing IL-10/12 cytokine profiles as an indicator of M1/M2 macrophage polarization and neurotoxicity. Recent literature demonstrates that AZM increases reparative macrophage activation in rodent models of spinal cord injury, stroke, lung infection, skin inflammation, and in humans with cystic fibrosis (Amantea et al., 2016a; 2016b; Čulić et al., 2001; Feola et al., 2010b; Gensel et al., 2017; Murphy et al., 2010; Petrelli et al., 2016; Varano et al., 2017; Zhang et al., 2015b). This anti-inflammatory mechanism, potentially unrelated to AZM's antibacterial properties, holds great promise in the treatment of these diverse inflammatory conditions.

Further, this relatively unexplored therapeutic approach could likely be exploited more effectively with continued optimization of therapeutics such as AZM and related macrolides. In particular, one major obstacle in the clinical development of anti-inflammatory macrolide antibiotics, such as AZM, is the concern that increased use of these drugs for their secondary anti-inflammatory effects may inadvertently promote bacterial resistance to this antibiotic in the treatment of a variety of infections. In the spinal cord injury patient population, for example, AZM is the antibiotic of choice for treating recurrent respiratory infections and pneumonia (Evans et al., 2013), a leading cause of death following spinal cord injury; thus, antibiotic resistance is a major concern. Fortunately,

recent studies have indicated that macrolides modified to remove their antibacterial activity retain beneficial anti-inflammatory effects in models of inflammatory skin disorders and chronic lung diseases (Hodge et al., 2017; Rodriguez-Cerdeira et al., 2012).

Collectively, we demonstrate that AZM derivatives with altered sugar moieties retain immunomodulatory properties. We did not, however, observe uniform immunomodulatory and neuroprotective properties with all derivatives tested. AZM7, which had the most extensive chemical modifications (diacetylation and the removal of the cladinose moiety), invoked modest immunomodulatory effects exclusively at the highest concentration tested. Interestingly, however, AZM7 did not retain any antistaphylococcal activity. Similarly, AZM4, which also lacks the cladinose that is replaced by a carbonyl, had modest yet significant immunomodulatory effects with minimal antistaphylococcal activity. Although AZM1 at the concentration of 125 μmol/L significantly increases IL-10 level, it has no effect in reducing IL-12 production. Interestingly, AZM1 also induced significant but small changes, relative to AZM, in BMDM metabolic activity at this high dose. AZM5, which was the only derivative tested without the cladinose removed, closely mimicked or slightly exceeded AZM's activity at all concentrations tested including the BMDM MTT assay. Unfortunately, the acetylation of both sugars in AZM5 did not abolish the antistaphylococcal activity as desired. This may suggest, however, that chemical modifications to substitute the cladinose with functional variants may be an effective approach in developing subsequent generations of derivatives. Future

studies that systematically alter each of these components may better clarify which of these modifications are beneficial or detrimental in retaining/improving AZM's immunomodulatory activity.

We demonstrated the clear anti-inflammatory activity of these AZM derivatives in vitro*;* however, related studies utilizing derivatives of AZM and other macrolides suggest that these drug candidates likely hold great potential for treating inflammatory disorders of the CNS *in vivo.* For example, Sugawara et. al (2012) developed a series of anti-inflammatory nonantibiotic macrolide derivatives *in vitro* and then successfully used these derivatives in an *in vivo* model of inflammatory bowel disease (Sugawara et al., 2012). Together with our prior work demonstrating the predictive nature of our *in vitro* model (Gensel et al., 2009; 2015; 2017a; Zhang et al., 2015b), these results suggest that our compounds hold great promise in treating the detrimental neuroinflammatory conditions. Given that there are extremely few treatment options for most neurological disorders, our current findings clearly demonstrate the importance of these drugs and support their continued development into novel therapeutics to treat CNS inflammation.

While the current study and related publications show encouraging therapeutic outcomes following stimulations with derivatives of AZM and other macrolides, the exact mechanisms of action remain uncertain. Much of the work in this regard has converged in identifying the NF-κB signaling cascade as the core regulator of the observed shifts in cytokine profiles following macrolide treatment (Aghai et al., 2007; Cigana et al., 2007; Feola et al., 2010b; Vrancic et

al., 2012). While this is clearly important, the molecular target upstream of the NF-κB cascade on which these drugs act remains unclear (Nujić et al., 2012b). *In vitro* studies show that AZM accumulates in macrophage lysosomes, where it increases the pH, interacts with membrane lipids, induces phospholipidosis, and alters vesicular trafficking which may affect endocytosis and phagocytosis (MuniÄ et al., 2011; Nujić et al., 2012a). Other suggested mechanisms indicate that AZM may alter cellular autophagy, or alter the TLR4 signaling pathways by changing endosome trafficking (Nujić et al., 2012a). Further, it remains unknown whether these findings remain valid *in vivo* or whether derivatives of AZM retain the same mechanism of action. While complicated, continued work in these areas is essential as it could lead to new therapeutics, such as the compounds described here, or provide novel therapeutic targets for future drug development.

One interesting finding in the current study was the fairly pronounced increase in macrophage viability when treated with the highest dose of AZM or derivative for 24 hours (widely used time point for measuring drug toxicity *in vitro*). This assay measures the conversion of tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan by NAD(P)H-dependent cellular oxidoreductase enzymes, and this measure of metabolic activity is routinely used to quantify changes in cell number or vitality. How AZM and its derivatives induce this effect at high concentrations, and how this may alter inflammatory activities remains unclear, however, it is unlikely that this is directly related to our observed shifts in cytokine profiles and neurotoxicity across drug concentrations. If the observed increases in IL-10 following AZM

stimulations were simply a result of cellular proliferation, then IL-12 would also be expected to rise, instead, however, IL-12 levels fell dramatically. Similarly, measurements of cellular proliferation/metabolism at the 6-hour time point, when we measure IL-10/12 levels, displayed more modest increases in proliferation/metabolism and are thus less likely to influence our cytokine profiles. In conclusion, we have identified AZM derivatives that retain key immunomodulatory functions in our *in vitro* model of CNS inflammation. While the antiinfective properties of the derivatives were associated with neuroprotection, we also observed that some derivatives with greatly reduced antiinfective characteristics retained neuroprotective and anti-inflammatory functions. Although a limited sample size of derivatives was created and tested, this indicates that the antibiotic properties of AZM may not be required for immunomodulatory-mediated neuroprotection. With continued development, these compounds could become viable clinical neuroprotectants and immunomodulatory treatments for neuropathologies. Additionally, given the usage of AZM's anti-inflammatory properties across disciplines, these drugs hold great potential in treating a wide variety of inflammation-based human disorders.

LIF

LIF’s effects on BMDM cytokine production profiles indicate that it holds immunomodulatory properties. This and previous evidence indicating therapeutic effects in rodent models of SCI suggest that LIF is a promising therapy to improve recovery after SCI. Importantly, LIF has already been investigated in US

clinical trials to prevent chemotherapy-induced peripheral neuropathy. In the phase I study recombinant human LIF was observed to have a biological effect in hemopoietic recovery after chemotherapy without any appreciable safety concerns (Gunawardana et al., 2003). Unfortunately, in a phase II study LIF was not successful in reducing chemotherapy-induced peripheral neuropathy (Davis et al., 2005). Nonetheless, these studies provide promising evidence that LIF can be safely administers to humans. With continued work LIF thus holds significant promise in improving recovery after SCI and other neurological conditions.

Conclusions

Many individuals who sustain an SCI do not receive any pharmacological agent to improve their functional recovery. This highlights the critical need to develop new therapeutics to treat SCI. Macrophages are significant mediators of SCI pathophysiology and are thus important therapeutic targets. There are numerous approaches to target macrophages after SCI; however, there are important considerations as these therapies continue to develop.

Macrophages are known to contribute to pathological inflammation in numerous conditions including stroke, SCI, lung infections, myocardial infarction, and many others (Al-Darraji et al., 2018; Amantea et al., 2016b; Feola et al., 2010b; Zhang et al., 2015b). As a result, there have been several strategies developed to target these cellular populations. The two general approaches are those that seek utilize a therapeutic or therapeutic target to alter macrophage

activation (such as AZM and $cPLA_2$ ablation), while others target macrophages to prevent them from reaching sites of inflammation.

One frequent strategy used to target macrophages relies on their innate propensity to phagocytose debris and foreign material. A rapidly developing use of this approach is with carefully engineered liposomes. These spherical lipid structures can be engineered to be preferentially engineered to be phagocytose by macrophages and monocytes through manipulations of lipid composition, size, charge, and membrane proteins (Kelly et al., 2010). These liposomes can then be loaded with clodronate resulting in the selection depletion of macrophages. This has been used to prevent macrophages from reaching the site of injury after SCI, thereby improving recovery (Popovich et al., 1999). A similar strategy has been developed using immune-modifying microparticles, derived from microdiamonds, poly(lactic-*co*-glycolic) acid, or polystyrene. Uptake of these particles by macrophages causes these cells to sequester in the spleen instead of migrating into sites of inflammation (Getts et al., 2014). These cells are then cleared through apoptotic pathways (Getts et al., 2014)

Others have utilized the macrophage targeted liposomes strategy differently, by instead loading them with a therapeutic, allowing for a relatively specific delivery to macrophages. For example, this strategy has recently been employed to delivery AZM to macrophages in a rodent model of myocardial infarction and was shown to improve recovery (Al-Darraji et al., 2020). Similarly, another group developed vessels engineered from M1 and M2 macrophages creating nanocarriers capable of modulating macrophage phenotype (Neupane

et al., 2021). Intriguingly, another group has investigated using macrophages themselves as drug carriers across the blood brain barrier using “cellular backpacks” loaded with pharmaceuticals attached to the macrophage cell surface (Klyachko et al., 2017).

To date, there has been one macrophage-targeted therapy to reach clinical trials. This therapy was derived from pre-clinical studies in which macrophages were isolated from blood and stimulated with nerve tissue or skin tissue to stimulate the cells into a pro-reparative phenotype (Bomstein et al., 2003; Rapalino et al., 1998). These cells were then injected into transected spinal cords, resulting in partial recovery of paraplegic rats as determined by open field examination, electrophysiological recordings from hind limb muscles, or histological analysis. The phase I clinical trial indicated that the procedure was safely tolerated by the participants (Knoller et al., 2005); however, phase II trials failed to show any therapeutic benefits (Lammertse et al., 2012). While disappointing, there are numerous reasons why this macrophage-target therapy failed to improve neurological recovery. First, the time to treatment from the time of injury was a average of 12.93 days, with most patients clustered towards the maximum 14-day post-injury inclusion criteria limit (Lammertse et al., 2012). Considering many key events of SCI pathophysiology occur soon after injury, it is possible the injection delay was too late to impart significant therapeutic effects. While the procedure itself took at least 36 hours to collect cells, stimulate them, and surgically inject the cells into the injury site, any future iteration of this study should consider earlier injection criteria. Second, macrophages are highly plastic

cells that can shift between activation states depending on their environment. Given the predominantly pro-inflammatory environment observed after SCI, it is possible that the macrophages that were “trained” to be reparative with nerve or skin tissue simply reverted to a pro-inflammatory state. There is a strong precedent for this in pre-clinical research. When M2 macrophages were injected into naïve spinal cords or an SCI lesion at 7dpi, the cells in the lesion quickly lost indicators of M2 activation states (Kigerl et al., 2009). In this regard, future iterations of this study should consider this strategy for chronic injury where inflammation has subsided or investigate methods to reduce macrophage plasticity before injection. Lastly, like many therapies, differential responses to therapeutics in rodents and humans are common, so this strategy may simply be ineffective in humans. Collectively, this is an interesting macrophage-targeted therapy; however, additional work is needed before this can be successfully implemented.

The most consequential decision impacting the success of novel therapeutics is likely the route and timing of administration. First, macrophages can be targeted through a systemic administration or through macrophage-specific approaches. This selection will likely have a significant impact on dosing and potential side effects. With AZM, for example, we utilized a systemic administration and successfully improved functional outcomes (Kopper et al., 2019). Others, however, have enhanced the therapeutic benefits of AZM by loading AZM into liposomes targeting macrophages (Al-Darraji et al., 2020). Similarly, others have found that liposomal delivery can increase drug

concentrations at the site of inflammation while reducing the ED 50 of the drug, presumably from a reduction in off-target effects (Rose, 2005). While promising, whether a targeted or systemic approach results in increased efficacy is likely drug-specific. $cPLA_2$, for example, is expressed in numerous cell types with likely differential functions. While we hypothesize that a macrophage-targeted delivery may improve efficacy, others have shown significant improvement from a systemic approach (Liu et al., 2006). Whether the targeting of additional cell types improves or decreases therapeutic efficacy will likely differ for each therapy. Similarly, as discussed previously (Gensel et al., 2017; Kopper and Gensel, 2021; Kopper et al., 2019), the selection of dosage, dose timing, and route of administration are critical factors in therapeutic efficacy.

The macrophage population after SCI is predominantly composed of infiltrating myeloid-derived macrophages and resident microglia. Historically these cell types were used interchangeably; however, growing evidence has suggested they may have differential roles in inflammation, myelin clearance, and recovery (Bellver-Landete et al., 2019; Greenhalgh et al., 2018; Wang et al., 2014). Specifically, microglia may hold more protective roles than previously thought, as microglia depletion further impairs locomotor recovery and neuronal survival (Bellver-Landete et al., 2019). Conversely, infiltrating monocyte populate the core of the lesion and appear to be the primary cell type taking up myelin (Wang et al., 2014). Lastly, growing evidence suggests that microglia and peripherally derived macrophages may communicate within the lesion and differentially regulate each other's cellular activities (Greenhalgh and David,

2014). Continued work is needed to better understand the differential roles of microglia and monocyte-derived macrophages. Targeting a specific macrophage population is an important consideration when selecting routes and timing of administration, as each may have a distinct impact on each population. For example, intravenously injected liposomes may disproportionately affect circulating monocytes vs. tissue-resident microglia. Similarly, direct spinal injections may be required to target both cellular populations, particularly after the closure of the blood-brain barrier post-SCI.

While there is a critical need for therapeutics to treat acute SCI, there is also an existing population of over 250,00 individuals in the United States living with an SCI. This is an important consideration when advancing therapeutics towards clinical use. Pro-inflammatory macrophages are present in the chronically injured spinal cord and are likely a continuous barrier to recovery (Horn et al., 2008). Thus, it is especially important to evaluate macrophage-targeted therapies at chronic timepoints. While locomotor and histological recovery are common outcomes in animal research, the SCI population generally prioritize improvements to their bowel, bladder, and sexual health (Anderson, 2004). Because of this, it is essential to include these outcome measures when examining macrophage-targeted therapies in chronic injuries.

Macrophage-targeted therapies hold great promise in the treatment of SCI; however, various degrees of development are needed to advance these therapies towards clinical use. As an FDA-approved antibiotic with an excellent safety history, AZM could, in theory, be rapidly deployed into the clinic.

Realistically, however, we need additional basic research to maximize its modest effects (Kopper et al., 2019) and gain preliminary clinical before it could be widely deployed. Conversely, $cPLA_2$ displays robust effectiveness as a therapeutic target in pre-clinical studies *in vitro* (Kopper et al., 2021); however, it will require fairly extensive pre-clinical work before it can be targeted in patients. Similarly, numerous alternative therapies in development to target macrophages, which, while promising, also need significant development to advance them toward clinical use. Given the current pharmacological gap in our ability to treat individuals with SCI, it is imperative that new SCI therapies, including those discussed here, are thoroughly investigated.

Collectively, these studies highlight the critical role macrophages play in pathogenic inflammation and the urgent need to develop an immunomodulatory macrophage therapy towards FDA approval.

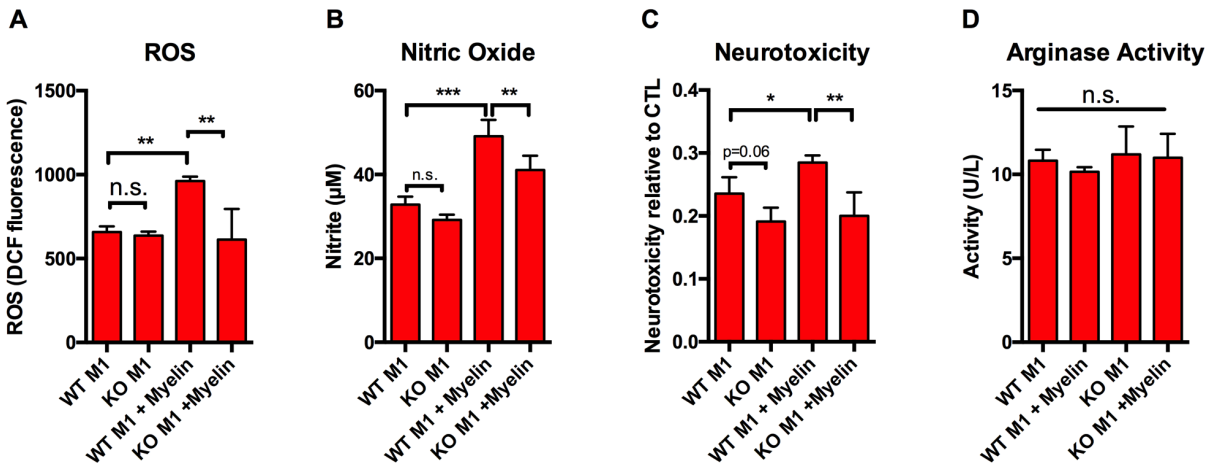


Figure 4.1. cPLA$_2$ KO macrophages exhibit reduced reactivity to myelin under pro-inflammatory conditions relative to cPLA$_2$ WT macrophages.

BMDMs were isolated from cPLA$_2^{-/-}$ (KO) or cPLA$_2^{+/+}$ (WT) mice and stimulated as in Fig 2.3. (A) Genetic ablation of cPLA$_2$ reduced myelin mediated ROS increases in M1 (IFN-γ and LPS) macrophages. (B) Genetic ablation of cPLA$_2$ reduced myelin mediated nitric oxide production. (C) Genetic ablation of cPLA$_2$ reduced the neurotoxic potential of M1 macrophages treated with myelin as determined by an MTT assay measurement of N2a cell viability. (D) Genetic ablation of cPLA$_2$ did not significantly alter arginase activity. Representative of 3 biological replications of both BMDMs and myelin source. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ mean ± SEM.

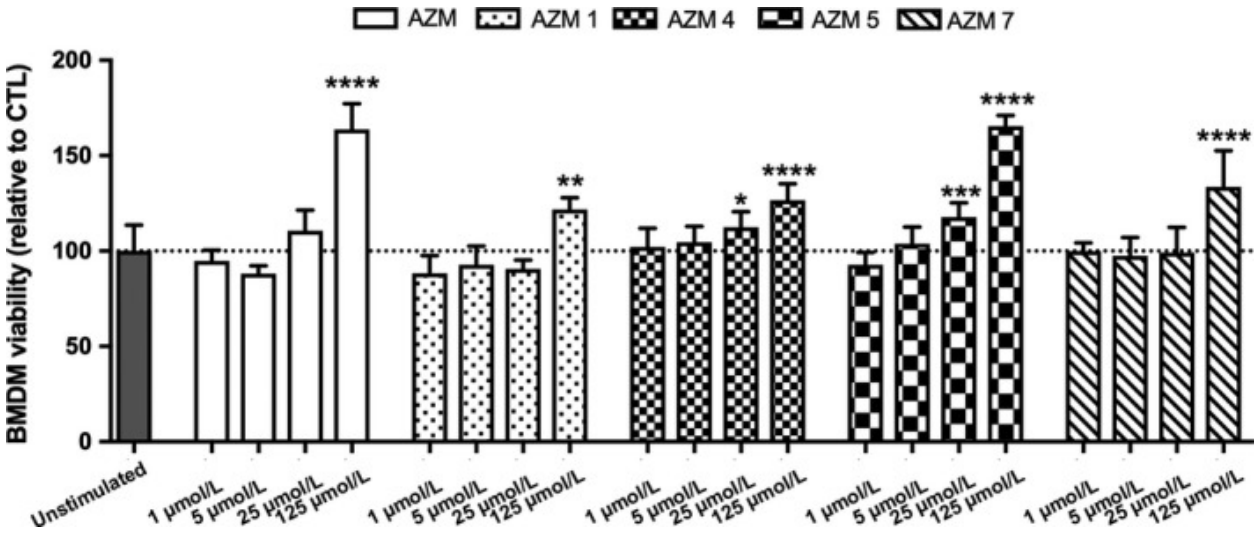


Figure 4.2. Altering the antibiotic properties of azithromycin does not decrease macrophage viability.

Bone marrow-derived macrophages (BMDMs) were isolated from adult mice and were treated with AZM, AZM1, AZM4, AZM5, and AZM7 at concentrations of 1, 5, 25, and 125 µmol/L for 24 h Cell viability was measured by using MTT assay. AZM or AZM derivatives exhibited no cytotoxicity at any tested concentration as compared to unstimulated, nontreated BMDM control (dotted line). Moreover, AZM and AZM derivatives at 25 and/or 125 µmol/L significantly increased proliferation of BMDMs as compared to unstimulated controls at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are mean ±SD and representative of three independent biological replicate experiments

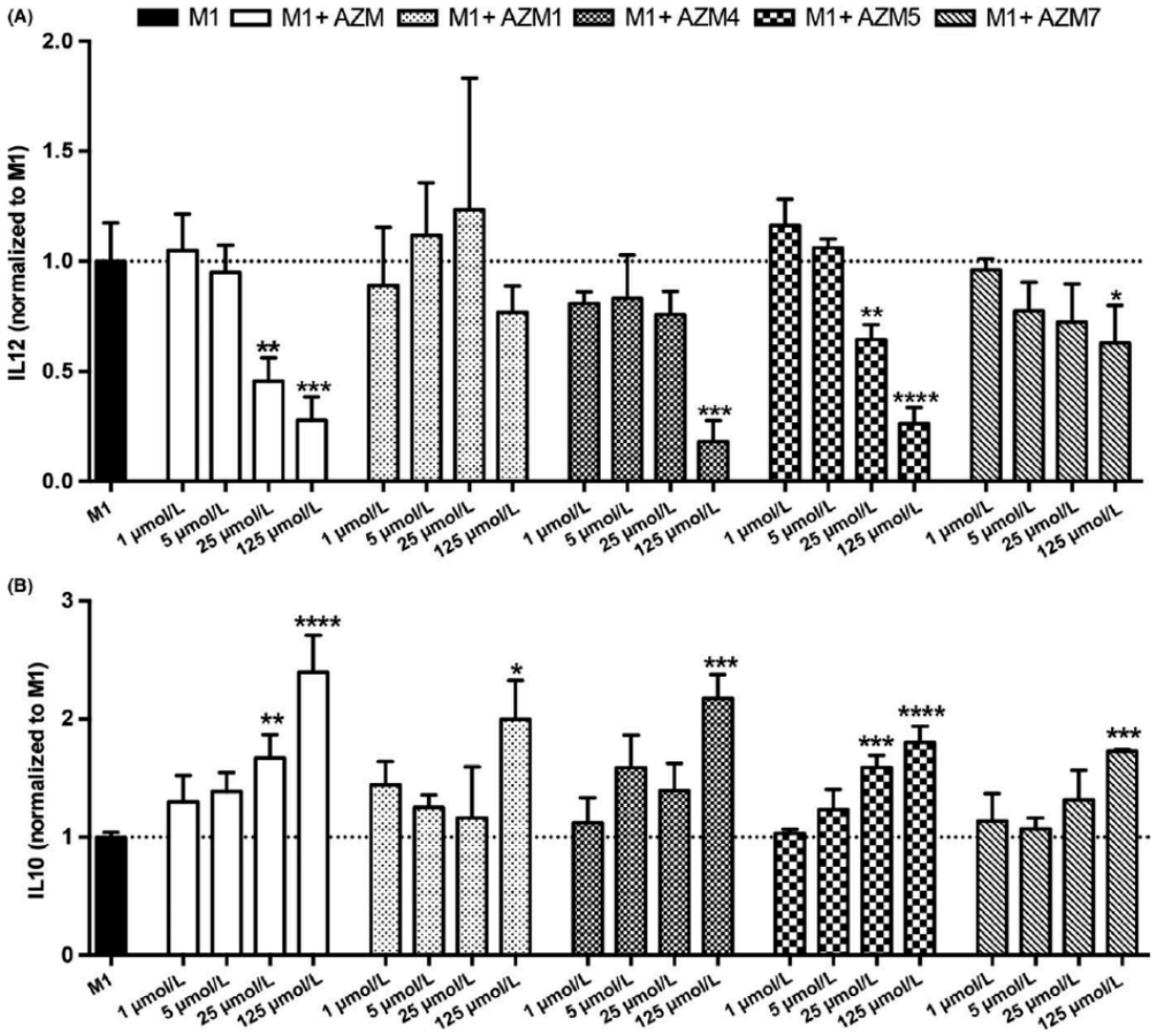
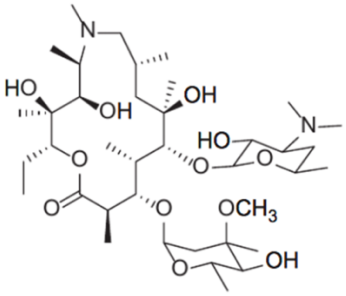
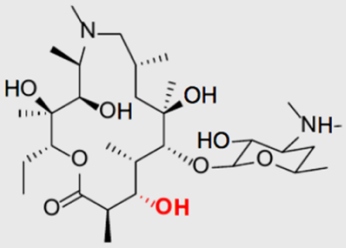
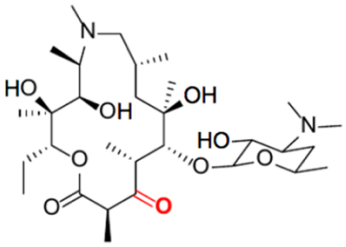
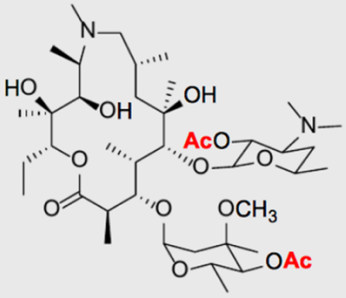
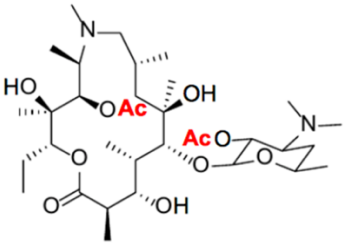


Figure 4.3. Nonantibiotic macrolides polarize proinflammatory macrophages to an anti-inflammatory phenotype.

BMDMs were polarized to be M1 macrophages by stimulating with LPS +INF-gamma. AZM, AZM1, AZM4, AZM5, and AZM7 were coapplied to M1 cells at concentrations of 1, 5, 25, and 125 μmol/L for 6 h Protein levels of IL-12 (A) and IL-10 (B) in cell culture medium were analyzed by ELISA and expressed as fold change over M1 of mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs M1. Data

are representative of three independent biological replicate experiments. Each experiment was performed in triplicates per treatment group. (A) AZM and AZM 5 at the concentrations of 25 and 125 μmol/L significantly decreased proinflammatory cytokine IL-12, while AZM4 and AZM7 significantly reduced IL-12 level only at the concentration of 125 μmol/L. AZM 1 showed no effect in changing IL-12 secretion. (B) The anti-inflammatory cytokine IL-10 level was significantly increased in M1 macrophages coincubated with AZM and AZM 5 at the concentrations of 25 and 125 μmol/L; While AZM1, AZM4, and AZM7 significantly increased IL-10 expression only at the highest tested concentration of 125 μmol/L.

Table 4.1: Structure, molecular weight, and antibiotic properties of derivatives.

Derivatives name	Structure	Molecular weight	MIC[a] (μmol/L)
AZM (parent compound)		748.51	1.0
AZM1		590.79	1000
AZM4		588.77	500
AZM5		833.06	2.0
AZM7		674.44	1000

[a]Minimum inhibitory concentration against *Staphylococcus aureus* subsp aureus Rosenbach (ATCC 6538).

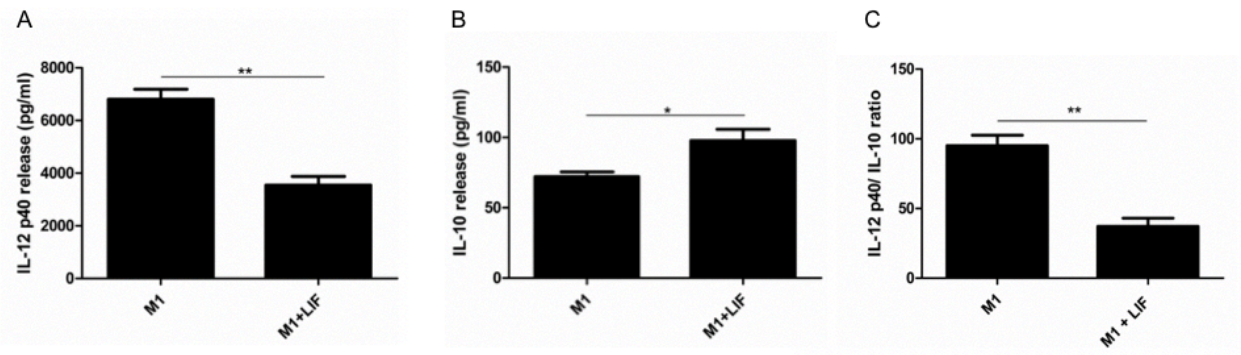
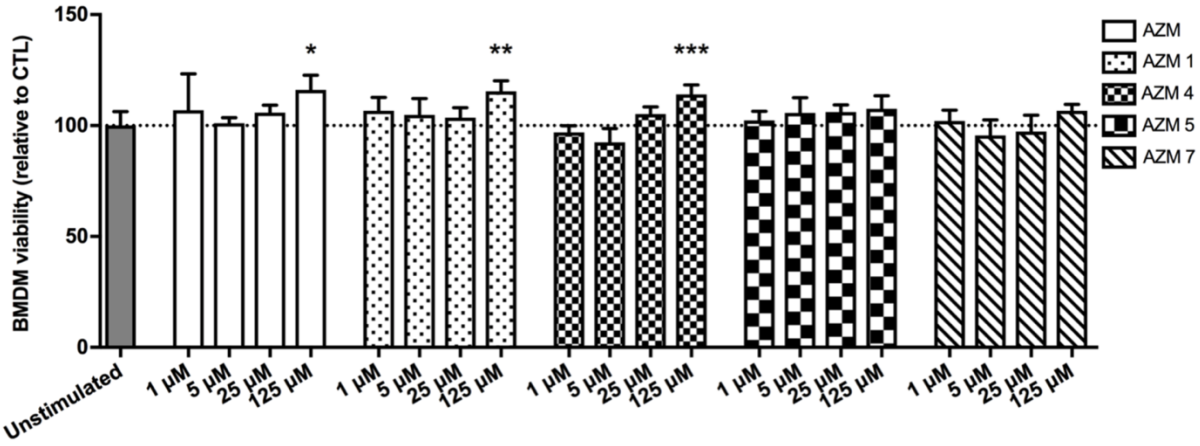


Figure 4.4. LIF treatment decreases IL-12 p40 release and increases IL-10 release in pro-inflammatory BMDMs.

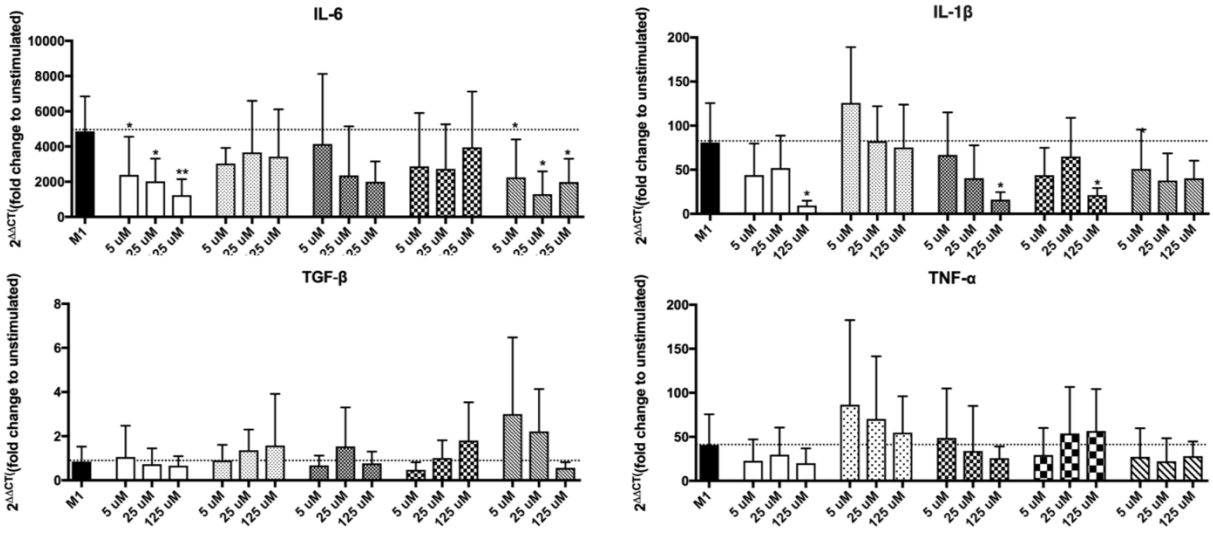
A pro-inflammatory (M1) phenotype was induced in BMDMs via stimulation with IFNγ and LPS. LIF or PBS were co-administered with stimulants. At 24 h after stimulation, macrophage-conditioned media from M1 cells treated with LIF had significantly lower IL-12 p40 release compared to media from M1 cells treated with PBS (**$p < 0.01$). b IL-10 release in macrophage-conditioned media from M1 cells treated with LIF compared to M1 cells treated with PBS (*$p < 0.05$). c The average ratio of IL-12 p40/IL-10 in the macrophage-conditioned media from LIF-treated M1 cells was significantly lower than the IL-12 p40/IL-10 ratio in the PBS-treated M1 cells (**$p < 0.01$). $n = 3$ wells per treatment group



Supplemental Figure 4.1: Altering the antibiotic properties of AZM does not decrease macrophage viability at the time of protein and RNA isolation.

Bone marrow-derived macrophages (BMDMs) were isolated from adult mice and were treated with AZM, AZM1, AZM4, AZM5, and AZM7 at concentrations of 1, 5, 25, and 125 μM for 6 hrs (timepoint at which samples are collected for protein, RNA, and neurotoxicity assays). Cell viability was measured by using MTT assay. AZM or AZM derivatives exhibited no cytotoxicity at any tested concentration as compared to unstimulated, non-treated BMDM control (dotted line). Moreover, AZM and AZM derivatives 1, and, 4 at 125 μM significantly increased proliferation of BMDMs as compared to unstimulated controls at *$p<0.05$, **$p < 0.01$, ***$p < 0.001$, however this effect was less robust than the 24hr drug toxicity timepoint in Figure 4.2. Data is mean ± SD and representative of three independent biological replicate experiments.

Methods: Performed as in Figure 4.2, utilizing a 6hr timepoint.



Supplemental Figure 4.2: AZM and AZM derivatives decrease pro-inflammatory macrophage activation. The pro- inflammatory cytokine, IL-1-beta, was down regulated by AZM, AZM 4, and AZM 7. Similarly, the pro-inflammatory cytokine, IL-6 was, down regulated by AZM and AZM 7. Shifts in the anti-inflammatory cytokine TGF-Beta and the pro-inflammatory cytokine TNF-alpha were not statistically significant. Results indicative of 2 independent biological replications. *p<0.05, **p < 0.01, ***p < 0.001

Methods: BMDMs were polarized to be M1 macrophages by stimulating with LPS + IFN-gamma. AZM, AZM1, AZM4, AZM5, and AZM7 were co-applied to M1 cells at concentrations of 1, 5, 25, and 125 μM for 6 hr. 300μL TRIzol LS reagent (Life Technologies) was added to each well (500,000 cells) to isolate RNA. Total RNA was isolated based on the manufacturer's protocol, with an additional phase separation using BCP, precipitation with isopropanol (Sigma-Aldrich, St. Louis,

MO), and wash of the isolated RNA in 70 % ethanol. Then, 1 μg RNA was reverse-transcribed using the high- capacity complementary (cDNA) reverse transcription kit (Life Technologies). Real-time PCR amplification was performed on the mixture of 100 ng cDNA sample, Taqman Universal PCR Master Mix, and Taqman Probes (Life Technologies) using the Applied Biosystems Step One Plus Real-Time PCR System. Expression of genes was normalized to 18S mRNA for each sample, and reported values were calculated as $2^{-\Delta\Delta CT}$.

Chapter 5: Conclusions and Future Directions

5- i. Chapter Summary:

Macrophages are influential mediators of the inflammatory environment thought to propagate secondary injury processes and impair recovery after SCI. Macrophage phenotype is a critical determinant on whether the macrophage response supports reparative vs. destructive functions after SCI (Kigerl et al., 2009). The pro-inflammatory macrophage activation state predominates after SCI; however, the underlying mechanisms of this prolonged destructive process are poorly understood. Here we examine the role of myelin debris in propagating macrophage inflammatory responses under the spectrum of macrophage activation states. We then seek to target these macrophages and shift their activation state as a strategy to improve recovery in mouse models of SCI. Specifically, we identify $cPLA_2$ as a key mediator of myelin's pathological effect on macrophage and utilize the immunomodulatory antibiotic AZM as a means to shift macrophage activation state and improve SCI recovery. Lastly, we aim to develop $cPLA_2$ inhibitors, AZM, AZM derivatives, and related therapeutics as macrophage-targeted therapeutics for use in human SCIs for which there is an urgent clinical need.

5- ii. Review of Major Findings:

As detailed in chapter 2, myelin debris is an important environmental factor contributing to the pathological macrophage response after SCI. *In vitro,*

myelin robustly potentiates pro-inflammatory macrophages. Pro-inflammatory "M1" treated with myelin increased production of reactive oxygen species, nitric oxide, IL-12, and had increased neurotoxicity toxicity. Anti-inflammatory "M2" macrophages and unstimulated macrophages were largely unaffected by myelin, indicating that the effects of myelin are specific to the "M1" phenotype. *In vivo* after SCI, macrophages contain extensive myelin debris and are predominantly in a pro-inflammatory activation state, implicating myelin as a contributing factor to the prolonged destructive macrophage response after SCI.

We then found that inhibition of the enzyme $cPLA_2$ significantly reduced myelin's ability to potentiate pro-inflammatory macrophage responses. This reduction implicates $cPLA_2$ as a key mechanism in the macrophage myelin response and thus as an important therapeutic target. *In vivo* after SCI, myelin-loaded macrophages express active $cPLA_2$ providing the key spatiotemporal evidence for $cPLA_2$'s role in pathological SCI inflammation.

We previously targeted macrophage-mediated inflammatory responses with the immunomodulatory antibiotic AZM (Gensel et al., 2017; Zhang et al., 2015b). Here in chapter 3, we expand on these prior works by administering AZM beginning 30-minutes, 3-hours, or 24-hours following SCI, and then daily for seven days. The 30-minute and 3-hour timepoints improved locomotor recovery (BMS scale) and increased stepping frequency. The 30-minute timepoint also exhibited histological improvements with a reduced lesion length. The 24-hour timepoint was therapeutically ineffective in this model. Lastly, in chapter 4 we continue to develop additional strategies to target macrophages after SCI,

including transgenic manipulation of $cPLA_2$, AZM derivative pharmaceuticals with altered immunomodulatory profiles, and the immunomodulatory cytokine leukemia inhibitory factor (LIF).

There are few treatment options to improve SCI recovery in humans. Here we review the steps to advance macrophage-targeted therapies towards use in humans. While $cPLA_2$ is a promising therapeutic target, there are no established therapies to target it in humans. Because of this, there are substantial stages of basic research required to prove efficacy and safety in animal models of SCI. Conversely, AZM is an FDA-approved antibiotic with widespread clinical use; however, its effects are modest. Because of this, we need to optimize routes of delivery and develop non-invasive methods to collect data on patients prescribed AZM near the time of their injury. Lastly, given the urgent clinical need for SCI therapeutics, all potential therapeutics need to be investigated. These include macrophage-targeted therapies, including AZM derivatives, LIF, and liposome-based therapies.

Collectively we identify an underlying mechanism for maladaptive macrophage activation and investigate macrophage-targeted therapies to improve recovery after SCI.

5- iii. Continued Development of Macrophage $cPLA_2$ as a Therapeutic Target for the Treatment of Spinal Cord Injury and Other Neurological Conditions

Macrophage $cPLA_2$ activity has been identified as a novel therapeutic target to improve recovery after SCI; however, additional preclinical research is required to develop these concepts. First, we need definitive *In vivo* data showing therapeutic benefits from targeting macrophage $cPLA_2$ activity after SCI. Next, we need to identify a chemical $cPLA_2$ inhibitor most likely to be safely tolerated in humans from the numerous compounds used in the literature. Lastly, we need to continue to develop methods to deliver therapeutics specifically to macrophages.

Arachidonic acid, released by $cPLA_2$ activity, can be processed into a vast collection of lipid mediators of inflammation with diverse functions. Further, this process is differentially regulated in numerous cell types. Given this, it is possible that $cPLA_2$ can possess both protective and detrimental roles depending on cell type and environmental conditions. The next step in developing macrophage $cPLA_2$ activity as a therapeutic target is to specifically target only macrophage $cPLA_2$ activity. Unfortunately, no macrophage-specific transgenic mouse line has been developed. Instead, to determine the role of $cPLA_2$ specifically in infiltrating macrophages following SCI, we will use a bone marrow chimera KO approach as detailed in **Figure 5.1.** Specifically, wild-type (WT) C57BL/6 will be irradiated to deplete bone marrow and peripheral macrophages and then injected with bone marrow cells isolated from WT or $cPLA_2^{-/-}$ mice. These mice will then be housed for approximately two months to allow for complete bone marrow reconstitution

(Fenn et al., 2014). This approach will create a controlled scenario in which $cPLA_2$ can be studied exclusively in myeloid-derived cells, notably infiltrating macrophages following SCI, leaving $cPLA_2$'s other diverse physiological functions intact. Functional recovery will be regularly monitored throughout using the Basso mouse scale (BMS); a gridwalk, and an activity box monitoring generativity and rearing (Basso et al., 2006; Zhang et al., 2015b). Lastly, we will assess measures of neuropathic pain (Von Frey, mechanical allodynia) prior to injury and at 27 dpi. Including pain outcomes is important as many eicosanoids downstream of $cPLA_2$ are known mediators of pain (Dennis and Norris, 2015; Deuis et al., 2017). The animals should be sacrificed at 3, 7, and 28 days to capture acute, peak, and chronic $cPLA_2$ activity (Liu et al., 2006; 2014). The effect of $cPLA_2$ depletion from infiltrating macrophage on white matter tissue sparing, total and macrophage-specific ROS production, and acute and chronic macrophage polarization states will be determined using histology as in (Zhang et al., 2015a; 2016). Collectively, these studies will provide key evidence on the impact of $cPLA_2$ on SCI pathogenesis. This data is essential to justify the continued effort to develop macrophage $cPLA_2$ as a therapeutic target for treating spinal cord injury and other neurological conditions.

$cPLA_2$ (and related phospholipases) has previously been targeted in rodent and *in vitro* models of SCI using a variety of chemical inhibitors, including PACOCF3, AACOCF3, AX059, AX115, and FKGK2 (Kopper et al., 2021; Liu et al., 2014; Lopez-Vales et al., 2011). Further, there are additional $cPLA_2$ inhibitor candidates, such as AVX001, which treats psoriasis (Ashcroft et al., 2020). In this

case, AVX001 even demonstrated efficacy against plaque psoriasis in a phase I/IIa clinical trial when used topically (Omland et al., 2017); however, to date, no known $cPLA_2$ inhibitor is available for clinical use in humans. Fingolimod (FTY720), an FDA-approved immunomodulatory sphingosine-1-phosphate agonist, which sequesters lymphocytes to treat multiple sclerosis (Chun and Hartung, 2010), has also been suggested to inhibit $cPLA_2$ (Payne et al., 2007); however, whether it could be utilized to inhibit macrophage $cPLA_2$ activity remains unknown. Interestingly, it has been separately investigated as an SCI therapeutic in mice with observed improvements in locomotor function (Norimatsu et al., 2012). Lastly, there are numerous other proposed $cPLA_2$ inhibitors that are commercially available that have not been widely tested. In order to reach the ultimate goal of targeting $cPLA_2$ in human SCI, a drug will need to be selected/developed that can be safely administered and pass all FDA requirements. Current drug candidates to inhibit the PLA2 family of enzymes are summarized in **Table 5.1**. Continued work is necessary to screen these pharmaceutical candidates for $cPLA_2$ inhibition efficacy, relative PLA_2 specificity, and cellular toxicity. Additional collaboration with medicinal chemists could help screen out candidates unfit for further investigation into human use.

Before any macrophage $cPLA_2$ inhibitor could be developed for human use, we must first optimize existing methods to target drug delivery specifically to macrophages. This would likely be achieved by methods designed to target phagocytes through the use of liposomes or related engineered vesicles (Al-Darraji et al., 2020; Kelly et al., 2010; Neupane et al., 2021). The ability to

package drugs into lipid vessels is highly dependent on the drug's chemical structure, so this would be an additional consideration when selecting $cPLA_2$ inhibitor candidates (Sercombe et al., 2015). Once these technicalities are addressed, these inhibitor-vesicles would need to be tested for efficacy in rodent models of SCI at multiple doses or dosing paradigms. If successful, these experiments would then need to be replicated in a larger species (rat and/or pig). Lastly, in preparation for any potential use in human patients, investigations to screen for possible side effects and general toxicity (LD50s) would need to be performed (Mosedale, 2018).

Macrophage $cPLA_2$ activity is a potent regulator of inflammatory reactions and thus is a promising therapeutic target. While the observed data is encouraging, there are still significant challenges and continued work needed to turn a $cPLA_2$ inhibitor into a successful macrophage-targeted therapy for the treatment of SCI **(Figure 5.2).**

5- iv. Continued Development of Azithromycin as a Neuroprotective Therapeutic for the Treatment of Spinal Cord Injury and other Neurological Conditions

This section was adapted from Kopper and Gensel 2021

Spinal cord injury (SCI) induces a robust inflammatory response largely mediated by resident microglia and infiltrating macrophages across the blood-brain barrier. While these cell populations are capable of promoting repair and regenerative responses, in the days and weeks after SCI they predominately

adopt pro-inflammatory profiles known to inhibit recovery and potentiate secondary injury pathways. Continued work is needed to develop clinically viable immunomodulatory therapeutics and promote pro-reparative macrophage responses. Recently we published on the therapeutic benefits of the macrolide antibiotic azithromycin (AZM), which improves locomotor and histological recovery after SCI in 3-month-old female mice (Kopper et al., 2019). Specifically, we initiated AZM beginning 30 minutes, 3, or 24 hours after injury and then daily for 7 days. AZM administration initiated at 30 minutes and 3 hours post-injury improved locomotor function as detected by an open field locomotor scale and significantly improved stepping frequency. The 24- hour time point, however, was ineffective suggesting the importance of early administration. Histologically we observed modest improvements with the 30-minute treatment time point with significantly reduced lesion length and evidence of slight increases in tissue sparing at the lesion epicenter. Previously, we observed that the same AZM dosing strategy after SCI reduces pro-inflammatory microglia and macrophage activation as determined by a diverse panel of inflammatory markers (Gensel et al., 2017). These neuroprotective findings are consistent with recent studies finding AZM to be therapeutically effective in multiple stroke models (Amantea et al., 2016b, 2019) a rat model of retinal ischemia/reperfusion injury (Zheng et al., 2007), and in a rat neonatal hypoxic-ischemic brain injury model (Barks et al., 2019). AZM is the most commonly prescribed antibiotic due in part to its safety profile and large therapeutic index (Durkin et al., 2018). Collectively, these studies highlight the potential for AZM to be developed into a safe,

neuroprotective treatment for SCI and other neurological conditions. Here, we highlight additional areas of study that will facilitate the translation of AZM as a neuroprotective agent.

Extending the therapeutic window of AZM treatment would maximize its therapeutic development. Based upon studies in animal models of stroke and SCI, AZM remains effective if the initial dose is delayed up to 3 (oral) or 4.5 (intraperitoneal) hours after SCI and stroke, respectively, with earlier administration time points being most effective (Amantea et al., 2016a; Kopper et al., 2019). This may present a challenge for implementation in SCI. Indeed, the average time of acute methylprednisolone administration in a previous SCI clinical trial was between 8–9 hours after injury (Bracken et al., 1990). An extended therapeutic window may be achieved by investigating alternative routes of administration (e.g., intravenous or intrathecal-we used oral administration in our studies); improving dosing paradigms (initiation time point, concentrations, frequency, and duration) or developing more targeted delivery approaches (i.e., liposomal formations for targeted macrophage delivery). Similarly, continued work in medicinal chemistry holds the potential to improve AZM's therapeutic benefits and/or pharmacokinetics. In our previous work, we were able to introduce a series of changes to the molecular structure of AZM in which its antibiotic activity was reduced but the newly generated derivatives retained their immunomodulatory and neuroprotective effects (Zhang et al., 2019). Continued work in this area could identify a closely related drug with improved therapeutic efficacy. Collectively, given AZM's large therapeutic index and safety profile is it

likely that optimization of AZM administration in animal models can be achieved to fully develop AZM as an effective therapeutic in SCI and other neurological conditions.

Insight into AZM's therapeutic mechanisms of action will further facilitate translation. For example, in our recent work we found that AZM did not prevent the overall development of neuropathic pain over the course of 28 days (Kopper et al., 2019), however, previously we found that AZM has analgesic properties when administered 30 minutes prior to pain testing at chronic time points when neuropathic pain (heat hypersensitivity) is already established (Gensel et al., 2019). Microglial activation in the lumbar spinal cord is implicated in chronic pain after SCI yet acute monocyte- derived macrophage infiltration after injury is postulated to contribute to neurodegeneration. The current body of *in vivo* literature has utilized systemic administrations of AZM targeting both microglia, infiltrating macrophages, and likely other cell types yet to be examined. Currently, the relative contribution of each of these cell populations to the therapeutic effects of AZM is unknown, however, it is possible that AZM's effects are localized to one population. Further investigations into the cell-specific effects of AZM may improve therapeutic strategies and the relatively recent introduction of microglia- specific antibodies provides new tools to probe these questions.

As we continue to optimize immunomodulatory therapies for SCI, it is important that we prepare for the logistical challenges of clinical implementation. AZM has a unique benefit among the many promising drug candidates for SCI in that AZM is already Food and Drug Administration approved and heavily utilized

by the general population (Durkin et al., 2018). While these properties ease the barrier for human treatment, discontinuities in outcome measures of efficacy in animals models and humans may confound our ability to determine the therapeutic properties of AZM treatment in a clinical setting. In a clinical application there are uncertainties as to what would be the best marker of efficacy in humans. Analyses targeting blood or cerebrospinal fluid would presumably be the first choices given their clinical availability and non-invasive nature, however, the best analyte or cellular outcome to detect efficacy is unknown. Similarly, imaging techniques such as MRI are frequently utilized after SCI, and thus could be a useful tool in quantifying any therapeutic effects. As such, ongoing animal studies involving AZM should begin to test and incorporate blood, cerebrospinal fluid, and/or live animal imaging in order to determine the best approaches to detect AZM's therapeutic efficacy in humans. Fortunately, new SCIs are relatively rare in the United States; however, this may produce a logistical problem in the optimization of biomarkers of efficacy. In this scenario, the SCI research community could benefit from collaborating with the larger stroke field when determining the most useful therapeutic indicators. Once collected, any data indicating therapeutic and anti-inflammatory activities in the patient population would then serve as the final push likely needed to initiate full-scale clinical trials in humans.

Initial clinical studies utilizing AZM should certainly investigate potential improvements in the American Spinal Injury Association Impairment Scale impairment scale, however, this outcome would likely prove to be a difficult

threshold to detect efficacy. Given the modest effects of AZM treatment, patient and injury variability during this early stage of investigation would likely make a statistically significant shift in American Spinal Injury Association Impairment Scale grades unlikely. Even in a larger-scale clinical trial, this may still prove to be too demanding of a therapeutic threshold to detect efficacy. In contrast, rodent models of SCI research regularly utilize locomotor recovery as a primary outcome using highly consistent injuries and optimized testing paradigms capable of detecting treatments with smaller effect sizes. The discontinuities between animal and clinical research were recently highlighted at “SCI 2020: Launching a Decade for Disruption in Spinal Cord Injury Research”, a meeting hosted by the National Institute of Neurological Disorders and Stroke. Clinicians, individuals with SCI, and researchers at the meeting emphasized the importance of incorporating clinically relevant outcomes such as bladder, bowel, and sexual function, as well as, neuropathic pain and autonomic dysreflexia both into basic research and when determining therapeutic efficacy in the clinic. In our recent works, we investigated the ability of AZM to either prevent or suppress neuropathic pain (Gensel et al., 2019; Kopper et al., 2019), however, AZM’s impacts on these other important clinical outcomes are currently unknown. Therefore, continued research into AZM’s impact on other key outcomes in animal models could highlight specific measures to consider when designing clinical studies.

As a Food and Drug Administration (FDA) approved drug with an excellent safety history, AZM holds great promise as a therapeutic to treat SCI and other

neuroinflammatory conditions. Although the current research in animal models of neurological conditions is promising, the clinical variability in the human population and AZM's relatively modest effect size will likely become a challenge in detecting efficacy. To address this, as summarized in **Figure 5.3**, we need to better understand AZM's underlying mechanisms, improve its efficacy by optimizing dosing paradigms, and begin developing approaches to detect therapeutic effects non-invasively in humans. Once these challenges are overcome AZM will have greatly improved chances of moving towards successful clinical implementation as a neuroprotective treatment.

5- v. Limitations and Alternative Approaches:

Macrophage $cPLA_2$ is a promising therapeutic *in vitro;* however, it has never been specifically manipulated *in vivo.* The genetic $cPLA_2$ chimeric approach described here, while promising, has several limitations. First, all hematopoietic cells will be affected. While this is significantly more specific than a systemic drug injection, it could certainly induce unknown effects in other cell types such as leukocytes. Similarly, microglia, a significant macrophage population in the SCI lesion environment, would be unaffected. While infiltrating macrophages are the primary cell type mediating long-term myelin clearance, the importance of macrophage vs. microglial $cPLA_2$ activity is unknown. Alternative approaches to improve specificity could include direct injections of a $cPLA_2$

inhibitor into the CNS or incorporated them into macrophage-targeted liposomes. While interesting, each of these approached have unique limitations.

$cPLA_2$ is expressed in many cell types, and eicosanoids downstream of AA release are commonly exchanged between cells (Dennis and Norris, 2015). Because of this, despite complete genetic ablation of $cPLA_2$, macrophages could still produce eicosanoids with AA or downstream products released from other cell types. While this would presumably be a significant reduction, the extent of this process is unknown. Use of exogenously released AA in macrophages could be reduced by using combinatorial approaches with multiple inhibitors of both $cPLA_2$ and the other major LOX/COX enzymes downstream of $cPLA_2$. This limitation is less of a concern in less specific approaches to target $cPLA_2$.

The genetic $cPLA_2$ chimeric approach described here is not fully specific to macrophages, but it is the closest approach currently available. While technically challenging, macrophage-specific conditional KO mice have been developed in the past for other gene targets using the Cre-LoxP recombination system (Haydar et al., 2021; Shi et al., 2018). With continued work, a conditional KO could be developed to target $cPLA_2$ in macrophages; however, a $cPLA_2$-LoxP mouse line would first need to be created. While more macrophage-specific than myeloid genetic chimeras, additional myeloid cells, notably neutrophils, are also affected under the LysM gene. Similarly, conditional KOs often allow for residual gene expression compared to a global KO. Each approach has its own set of strengths and caveats to consider in future studies targeting $cPLA_2$.

A significant limitation in macrophage-targeted therapies is that they are specific. The immune response is clearly more complicated than solely macrophage-induced inflammation. Because of this, it is possible that strategies that solely target macrophages may not be capable of producing robust improvements in the complex post-SCI inflammatory environment. Because of this, it is important to design outcomes that are sensitive to modest improvements. Given the severe gap in therapeutic options for patients, any drug with any proven benefits would be a significant improvement. With time, combinatorial therapies could be developed to improve therapeutic efficacy further.

While $cPLA_2$ is often considered the most important PLA_2 isoform, as it is critical in the initiation and modulation of inflammatory responses and specifically targets phospholipids containing AA acyl groups, other PLA_2 enzymes, notably $iPLA_2$, can also release AA non-specifically. These isoforms have even been shown play pathological roles following SCI (Lopez-Vales et al., 2011). Therefore, it is possible that our $cPLA_2$ deficient macrophages isolated from the SCI lesion may still release free AA capable of being synthesized into various eicosanoids. Although unlikely, if AA levels in KO's were substantial relative to the WT chimeric control, we could perform preliminary studies utilizing other commercially available PLA_2 deficient mice such as $iPLA_2^{-/-}$ mice to define the novel role of additional PLA_2s in myelin macrophages *in-vitro.* This would then be followed by a repetition of our chimeric mice approach targeting an alternative PLA2 isoform.

We performed these studies predominantly on young female mice. Given that the human SCI population is predominantly male, with an increasing average age of injury occurrence (National Spinal Cord InjuryStatistical Center, 2014), the use of young female mice is a major limitation. Sex and age are important biological variables where therapeutics frequently have differential effects. Macrophage and microglial inflammatory gene profiles examining, activation, redox, and debris metabolism/clearance, differ in response to sex and age (Stewart et al., 2021). It is possible that $cPLA_2$, AZM, and the other therapeutics discussed here have differential roles across these variables not captured in the current data. In Stewart et al. 2021, $cPLA_2$ did not have notable differences across age and sex; however, there was higher $cPLA_2$ in microglia relative to monocyte-derived macrophages. Examining sex and age as biological variable is an important consideration as we continue to develop macrophage-targeted therapies. Similarly, going forward studies need to incorporate outcome measures important to the SCI community, including sexual, bowel, and bladder health (Anderson, 2004). This is important as therapies without notable benefits to locomotor recovery could induce sensory recovery which could be missed when only observing the traditional set of outcome measures.

5. vi. Interactions of Azithromycin and $cPLA_2$

Initial evidence suggests that intracellular membranes, the site of $cPLA_2$ activity, may be a target of macrolide antibiotics, including AZM (Banjanac et al., 2012; Tyteca et al., 2003). Interestingly, AZM can reduce AA release from the

J774A.1 macrophage cell line, with similar reductions in eicosanoid profiles as a $cPLA_2$ inhibitor (Banjanac et al., 2012). Similarly, in human leukocytes, AZM was found to reduce expression of $cPLA_2$ as well as cyclooxygenase (COX)-1 and COX-2 (enzymes downstream of $cPLA_2$ in AA processing) (Miyazaki et al., 2003). This resulted in reduced LPS-induced prostaglandin E2 synthesis (Miyazaki et al., 2003). These data suggest that AZM may reduce the release of AA by $cPLA_2$ either through direct inhibition or indirectly through downregulation of $cPLA_2$. Given that substrate availability is a major regulator of LOX/COX activity, reducing AA release would reduce the total level of eicosanoids produced. Recent work has shown that AZM has significant antihyperalgesic properties in treating neuropathic pain after SCI in mice (Gensel et al., 2019). Because eicosanoids have an established role in pain mechanisms (Dennis and Norris, 2015), it is possible that AZM exerts these effects through its iterations with the $cPLA_2$, COX-1, and COX-2 (Miyazaki et al., 2003)

While the majority of these lipid metabolites of AA have pro-inflammatory functions largely detrimental after SCI, under certain conditions, AA can be synthesized into the potent anti-inflammatory eicosanoid Lipoxin A4. The synthesis of lipoxins requires the concerted transcellular activity of 12/15 Lipoxygenase (LOX) in macrophages and 5-LOX in other cell types. In macrophages, these enzymes are expressed in response to the M2 macrophage cytokine IL-13 stimulation (Mabalirajan et al., 2013; Nassar et al., 1994). Given our published data showing Azithromycin's (AZM) ability to polarize toward the M2 phenotype after SCI (Gensel et al., 2017; Zhang et al., 2015b), I hypothesize

that AZM treatment will allow for increased Lipoxin A4 production from myelin derived free AA. This would almost certainly be beneficial after SCI as Lipoxin A4, and its direct precursors have been found to be neuroprotective and beneficial when added exogenously after SCI (Liu et al., 2015; Martini et al., 2014; 2016). Further Lipoxin A4 has been implicating in activating the PPAR-γ pathway which is strongly associated with polarization to the M2 phenotype (Bouhlel et al., 2007; Sobrado et al., 2009).

Together after SCI, AZM would decrease the total amount of myelin derived bioactive AA released from the membrane and promote the conversion of any free AA present into the anti-inflammatory eicosanoid Lipoxin A4 thereby promoting the resolution of inflammation and reducing secondary damage after SCI. Importantly, myelin's prolonged persistence in these lipid-laden macrophages drives the hypothesis that the chronic pro-inflammatory macrophage activation observed months after SCI is a product of persistent AA release. AZM's mechanisms of action could interrupt this pathway, specifically within macrophages, likely even at chronic injury timepoints for which there is a significant lack of treatment options. While intriguing, additional work is needed to validate these observations and determine if AZM can be used to interfere with $cPLA_2$ mediated pathophysiology after SCI.

5- vii. Significance

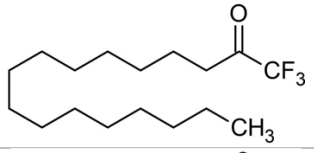
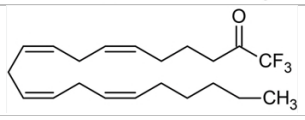
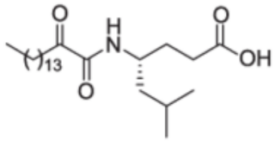
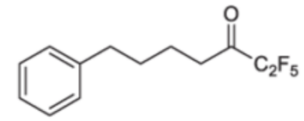
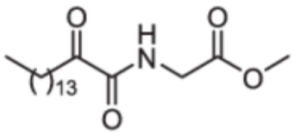
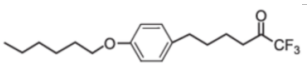
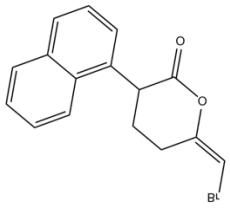
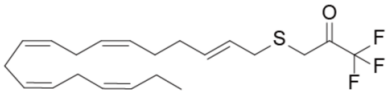
Myelin is an integral part of the CNS, carefully maintained by oligodendrocytes around many types of neurons. In SCI, however, the

homeostatic balance in the spinal cord is severely disrupted through physical damage and the massive infiltration of immune cells, blood, and inflammation. Myelin sheaths soon constitute the bulk of cellular debris as the neurons they encapsulate begin to die at the site of injury. Macrophages attempt to clear the extensive debris during which they become loaded with myelin-derived lipids nearly indefinitely, adopt pro-inflammatory activities, and begin to cause further damage (Gensel et al., 2009; Horn et al., 2008; Kigerl et al., 2009; Wang et al., 2014). This process, while critically important, is poorly understood. Here we examine in detail how macrophage activation affects myelin uptake, how myelin uptake affects macrophage polarization, and the mechanisms through which myelin impacts macrophage physiology. This evidence provides meaningful improvements in our understanding of how macrophages develop from a well-intended immune cell into a destructive, pro-inflammatory, and long-lasting presence in the core of the SCI lesion.

An SCI is a life-changing, often permanent, event impacting an individual's physical and mental health. Unfortunately, despite great advances in our understanding of SCI pathophysiology, most individuals who sustain an SCI have few or no therapeutic options to help their recovery. Here we identify $cPLA_2$ as an important therapeutic target mediating detrimental macrophage activation. Next, we provide continued evidence that the immunomodulatory antibiotic AZM has neuroprotective effects in a mouse model of SCI. Lastly, we examine the steps needed to continue to develop macrophage-targeted therapies towards clinical use. Given the clinical lack of a therapeutic standard of care for SCI, continued

work towards new therapeutics and combinatorial therapies is critical (Emerich et al., 2012; Hall and Springer, 2004; Hurlbert, 2000). With continued development, a macrophage-targeted therapy could eventually promote recovery in patients who currently have very few treatment options available (Emerich et al., 2012; Hurlbert, 2000).

Table 5.1. Chemical inhibitors of the PLA_2 family

PLA_2 Inhibitor Structure	Name	PLA_2 Specificity
	PACOCF3	$cPLA_2$ $iPLA_2$
	AACOCF3	$cPLA_2$ $iPLA_2$
	AX059	$cPLA_2$
	FKGK11	$iPLA_2$ $cPLA_2$ (partial) $sPLA_2$ (partial)
	AK115	$cPLA_2$ $iPLA_2$ $sPLA_2$
	FKGK22	$cPLA_2$ $iPLA_2$ $sPLA_2$
	Bromoenol lactone	$iPLA_2$
	AVX001	$cPLA_2$

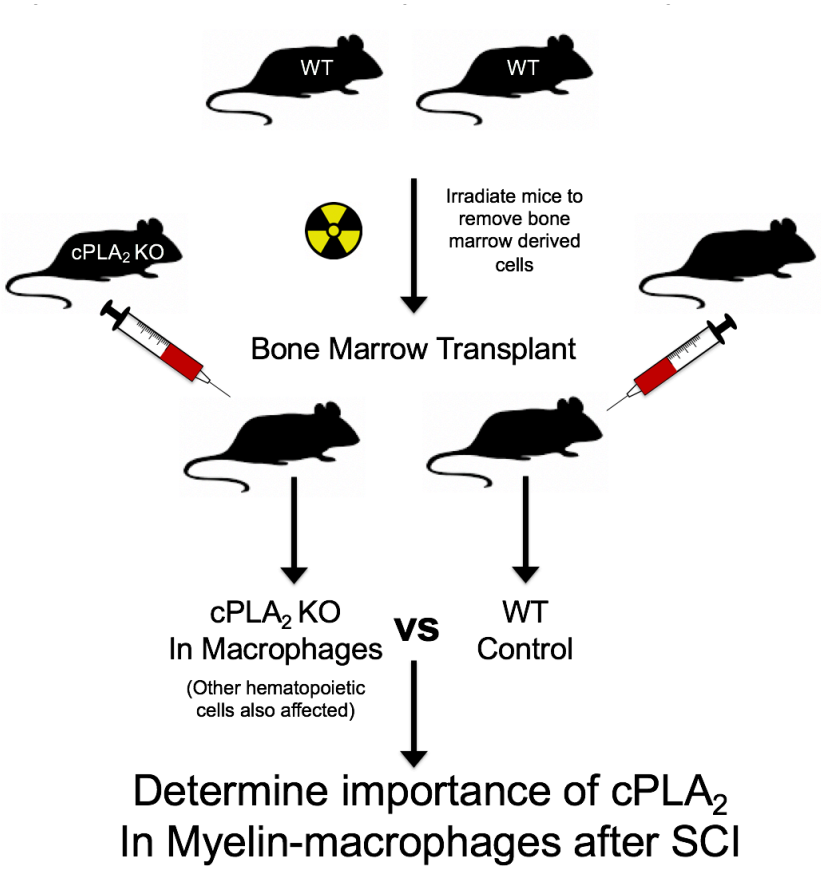


Figure 5.1. Targeting macrophage $cPLA_2$ with transgenic mouse chimeras.

Wild-type $cPLA_2^{+/+}$ (WT) C57BL/6 will be irradiated to deplete bone marrow and peripheral macrophages and then injected with bone marrow cells isolated from WT or $cPLA_2^{-/-}$ (KO) mice. These mice will then be housed for approximately two months to allow for complete bone marrow reconstitution (Fenn et al., 2014). This approach will create a controlled scenario in which $cPLA_2$ can be studied exclusively in myeloid-derived cells, notably infiltrating macrophages following SCI. Following SCI, the animals will be evaluated for locomotor, sensory, and histological recovery.

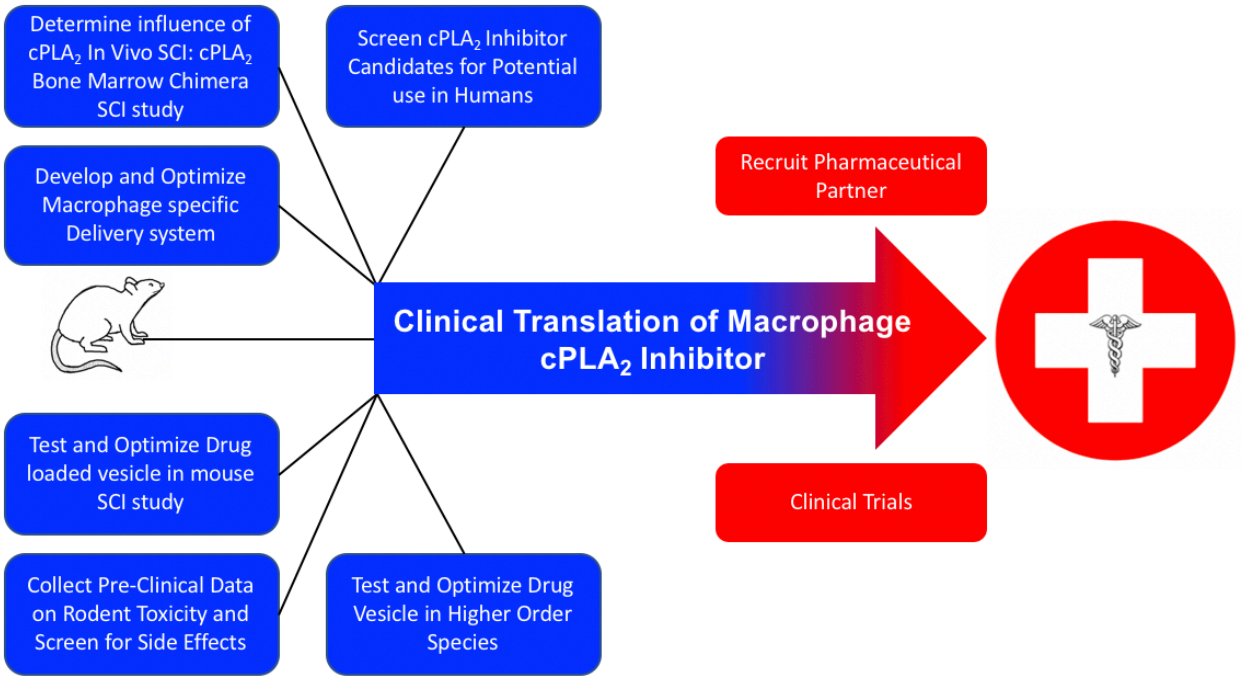


Figure 5.2. Important steps in developing macrophage $cPLA_2$ as a therapeutic target for spinal cord Injury.

To advance $cPLA_2$ as a therapeutic target for the treatment of SCI we need to complete several stages of pre-clinical research. First, we must establish Macrophage $cPLA_2$'s pathological role in SCI through mechanistic studies using $cPLA_2$ transgenic studies in mice. Next, a suitable $cPLA_2$ inhibitor candidate needs to be selected and screened for suitability for human use. Lastly, this final drug candidate would likely require replicate studies in higher order species before it could progress towards clinical trials and human use.

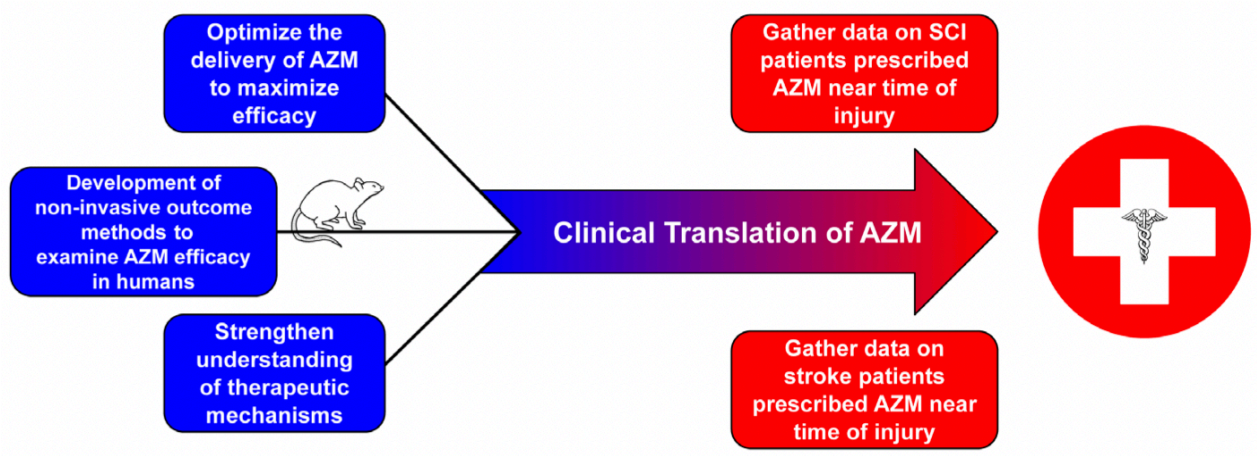


Figure 5.3. Important steps in repurposing of AZM as a neurotherapeutic agent.

Additional work in animal models is needed to both improve AZM's efficacy and develop outcome measures feasible for use in non-invasive human studies. Application of these outcome measures in the patient population already taking AZM near time of injury could provide key evidence for the transition of AZM into clinical trials and use as a therapeutic for SCI. AZM: Azithromycin; SCI: spinal cord injury.

Appendix: List of abbreviations

AA: Arachidonic acid

ABCA1: ATP-binding cassette transporter A1

ANOVA: Analysis of variance

Arg-1: Arginase-1

AZM: Azithromycin

BODIPY: Dipyrrometheneboron difluoride (Neutral lipid stain)

BMDM: Bone marrow-derived macrophage

BMS: Basso Mouse Scale

CNS: Central nervous system

COX: Cyclooxygenase

$cPLA_2$: cytosolic phospholipase A2

CR3/CD11b/MAC-1: complement receptor 3

CTL: Control

DAMP: Damage associate molecular pattern

DAPI: 4′,6-diamidino-2-phenylindole (DNA stain)

DCF: 2', 7' –dichlorofluorescein (Fluorescent ROS indicator)

DMEM: Dulbecco's Modified Eagle Medium

Dpi: Days post injury

EC/NF Eriochrome cyanine and neurofilament

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

FDA: Food and drug administration

GFAP: Glial fibrillary acidic protein

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)

IFN: Interferon

IL: Interleukin

IH: Infinite Horizons impactor device

$iPLA_2$: Calcium-independent phospholipase A2

Kdyn: Kilodyne force

KO: Knock out

LD50: median lethal dose

LIF: Leukemia inhibitory factor

LOX: Lipoxygenase

LPS: Lipopolysaccharide

LXR/RXR: Liver X and retinoid X receptor

MAC-2: Galectin-3 (activated macrophage)

MAPK: Mitogen-activated protein kinase

MARCO: Macrophage receptor with a collagenous structure

MCM: Macrophage conditioned media

MIC: Minimum Inhibitory Concentration

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NASCIS: National Acute Spinal Cord Injury Study

NF-κB: Nuclear factor kappa B

NIH: National Institutes of Health

NINDS: National Institute of Neurological Disorders and Stroke

n.s: Not significant

N2a: Neuro-2a

OCT: Optimal cutting temperature compound

OMgp: oligodendrocyte myelin glycoprotein

Opti-MEM: Reduced-Serum Medium is an improved Minimal Essential Medium

PBS: Phosphate-buffered saline

p-cPLA$_2$: phosphorylated cytosolic phospholipase A2 (activated)

PFA: Paraformaldehyde

PNS: Peripheral nervous system

ROS: Reactive oxygen species

RPM: Rotations per minute

PPAR/RXR: peroxisome proliferator-activated receptors

RPMI: Roswell Park Memorial Institute medium

PRR: Pattern recognition receptor

P/S: penicillin/ streptomycin

SCI: spinal cord injury

SD: Standard deviation

SEM: standard error of the mean

sPLA2: Secretory phospholipase A2

SRAI/II: Scavenger receptor AI/II

TLR: Toll like receptor

TNF: Tumor Necrosis Factor

TomL: Tomato lectin

TREM2: Triggering receptor expressed on myeloid cells 2

VCP: Valosin-containing protein

WT: Wild type

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Education

2014 - Present	University of Kentucky Doctor of Philosophy- Physiology
2016 (2 weeks)	Ohio State University: Spinal Cord Injury Research Training Program
2010-2014 Bachelor of Arts	University of Colorado Boulder Majors- Molecular, Cellular, and Developmental Biology Integrative Physiology Minor- Biochemistry

Research Experience:

Graduate Research Fellow
Dr. John Gensel, Physiology Department, University of Kentucky
2014-Present

Undergraduate Research Assistant
Dr. Xuedong Liu, Biochemistry department, University of Colorado Boulder
August 2013 to May 2014

Bioengineering Student Internship
Dr. Daewon Park, Bioengineering department
University of Colorado Denver- Anschutz Medical Campus
May 2013- September 2013

Phage Genomics Laboratory
Dr. Nancy Guild, Molecular, Cellular and Developmental Biology department
University of Colorado Boulder
August 2010-May 2011

Selected Skills:

Fluorescent immunohistochemistry, confocal microscopy, isolation and primary cell culture of bone marrow derived macrophages, culture of N2a, PC12, and

F11 neural cell lines, culture of L929 fibroblast cells, mouse colony management, Basso Mouse Scale and other behavioral methods of monitoring locomotion, generation of mouse genetic chimeras through cesium irradiation, tissue RNA isolation, PCR, qPCR, ELISA, cell death assays, sliding microtome, cryostat sectioning, mouse T9 spinal cord injuries, culture of bacterial clones, His-tag affinity purification, fast protein liquid chromatography, Graph Pad, Prism, Excel, Photoshop, Illustrator, Metamorph and HALO image analyses.

Professional Membership:

2015-Present	Bluegrass Chapter of the Society for Neuroscience
2016-Present	National Neurotrauma Society
2017-Present	Society for Neuroscience

Academic and Professional Honors

2016	Travel award to attend Neurotrauma (2016) from the Kentucky Spinal Cord and Head Injury Research Trust
2017	Trainee Professional Development Travel Award, from the Society for Neuroscience to attend annual conference
2018	Top graduate student poster award at the Kentucky Center for Clinical and Translational Spring Research Day.
2018	Travel award to attend the International Symposium on Neural Regeneration.

Grant Funding:

NIH NINDS T32 NS077889: Neurobiology of CNS Injury and Repair. PI: Edward Hall, PhD. (2016-2018)

NIH NINDS F31 NS105443: Contributions of Myelin Derived Arachidonic Acid to Macrophage Polarization and Secondary Damage after Spinal Cord Injury. PI: Timothy Kopper. (2018-2021)

Research Publications:

Kopper, T.J., Zhang, B., Bailey, W.M., Bethel, K.E., and Gensel, J.C. (2021). The effects of myelin on macrophage activation are phenotypic specific via cPLA 2 in the context of spinal cord injury inflammation. Sci. Rep. *11*, 1–13.

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Pope, W.H., Bowman, C.A., Russell, D.A., Jacobs-Sera, D. **et. al (100+ authors)** (2015). Whole genome comparison of a large collection of mycobacteriophages reveals a continuum of phage genetic diversity. Elife. doi: 10.7554/eLife.06416.

Other Publications:

Kopper, T.J., and Gensel, J.C. (2021). Continued development of azithromycin as a neuroprotective therapeutic for the treatment of spinal cord injury and other neurological conditions. Neural Regeneration Research *16*, 508–509.

Venditto, V.J., Haydar, D., Abdel-Latif, A., Gensel, J.C., Anstead, M.I., Pitts M.G., Creameans J., **Kopper, T.J.,** Peng, C., Feola, D.J.in, J.G.F., 2021 Immunomodulatory Effects of Azithromycin Revisited: Potential Applications to COVID-19. Frontiersin.org. doi: 10.3389/fimmu.2021.574425

Kopper, T.J., and Gensel, J.C. (2017). Myelin as an inflammatory mediator: Myelin interactions with complement, macrophages, and microglia in spinal cord injury. Journal of Neuroscience Research *21*, 1831–1839.

Select Abstracts

Kopper TJ, Zhang B, Bethel KE, Bailey WM, Gensel JC. The Effects of Myelin on Macrophage Activation are Phenotype-Specific: A Novel Role for $cPLA_2$ in SCI Inflammation. International Symposium on Neural Regeneration. Pacific Grove, CA 2020.

Kopper TJ, Zhang B, Bethel KE, Bailey WM, Gensel JC. The Effects of Myelin on Macrophage Activation are Phenotype-Specific: A Novel Role for $cPLA_2$ in SCI Inflammation. Neuroscience 2019, Chicago, IL 2019.

Kopper TJ, Zhang B, Bethel KE, Bailey WM, Gensel JC. The Effects of Myelin on Macrophage Activation are Phenotype-Specific: A Novel Role for $cPLA_2$ in SCI Inflammation. Colorado Immunology Conference, Steamboat Springs, CO 2019

Kopper TJ, Zhang B, Bethel KE, Bailey WM, Gensel JC. The Effects of Myelin on Macrophage Activation are Phenotype-Specific: A Novel Role for $cPLA_2$ in SCI Inflammation. Neuroscience 2018, San Diego, CA 2018

Kopper TJ, Zhang B, Gensel JC. Myelin modulates macrophage inflammatory responses after spinal cord injury. Neuroscience 2017, Washington, D.C 2017

Kopper TJ, Zhang B, Gensel JC. Myelin modulates macrophage inflammatory responses after spinal cord injury. International symposium on neural regeneration, Pacific Grove, CA 2017

Kopper TJ, Liu X, Zhang B., Veldhorst A., Bailey W.M, S.G. Van Lanen, Gensel J.C. Developing Azithromycin derivatives for altering macrophage phenotype. National Neurotrauma Symposium. Lexington, KY 2016

Kopper TJ, Liu X, Zhang B., Veldhorst A., Bailey W.M, S.G. Van Lanen, Gensel. Developing Azithromycin derivatives for altering macrophage phenotype. J.C. Bluegrass Society for Neuroscience Spring Research Day. Lexington, KY 2016

Zhang B, Bailey WM, **Kopper TJ**, Orr MB, Feola DJ, Gensel JC. Pharmacological manipulation of macrophage phenotype with azithromycin improves recovery and tissue sparing in spinal cord injury. 2015 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2015. Online.

Weekman E.M, **Kopper TJ**, Sudduth T.L, Wilcock DM. Reduced efficacy of anti-A immunotherapy in a mouse model of amyloid deposition and vascular cognitive impairment co-morbidity. Alzheimer's Association International Conference 2015.

Weekman EM, Caverly C.N, **Kopper TJ**, Sudduth T.L, Wilcock DM. Reduced efficacy of anti-A immunotherapy in a mouse model of amyloid deposition and vascular cognitive impairment co-morbidity. 2015 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2015. Online.

Oral Presentations

Kopper TJ, Liu X, Zhang B., Veldhorst A., Bailey W.M, S.G. Van Lanen, Gensel J.C . Developing Azithromycin derivatives for altering macrophage phenotype. Department of Physiology Research Retreat. Lexington, KY 2016.

Kopper TJ, Zhang B, Bethel KE, Bailey WM, Gensel JC. Contribution of Myelin Derived Arachidonic Acid to Macrophage Polarization and Secondary Damage after Spinal Cord Injury. Kentucky Spinal Cord & Head Injury Research Trust Symposium. Louisville, KY 2019.

Related Work Experience:

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