UNIVERSITY OF CALIFORNIA SAN DIEGO

Tumor Necrosis Factor Receptor 1 on Schwann Cell derived Exosomes Serves as an Alternative Binding Site for Tumor Necrosis Factor α

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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The thesis of Jasmine Thien-Thu Thi Le is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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List of Abbreviations

BCA	Bicinchoninic acid
BPE	Bovine pituitary extract
BS^3	(Bis(sulfosuccinimidyl) suberate)
BSA	Bovine serum albumin
cAMP	Cyclic AMP
cIAPs	Cellular inhibitor of apoptosis proteins
CM	Conditioned medium
CNS	Central nervous system
CRD	Cysteine-rich domains
DD	Death domain
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
DRG	Dorsal root ganglia
ErbB	Epidermal growth factor
EVs	Extracellular vesicles
FADD	Fas-associated protein with death domain
FBS	Fetal bovine serum
IL	Interleukin
JNK	Jun N-terminal Kinase
LIF	Leukemia inhibitory factor
LUBAC	Linear ubiquitin chain assembly complex
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein
miR	MicroRNA
MVBs	Multivesicular Bodies
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRG1	Neuregulin 1
NTA	Nanoparticle tracking analysis
PBS	Phosphate buffered saline
PLAD	Pre-ligand binding assembly domains
PNS	Peripheral nervous system
RIP	Receptor interacting protein kinase
SC EV	Schwann cell-derived extracellular vesicles
SCp	Schwann cell precursor
SC	Schwann cell
sTNF	Soluble TNFα
TBS-T	Tris-buffered saline Tween20
tmTNF	Transmembrane TNFα
TNFα	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
TRADD	TNF receptor 1-associated protein with a death domain
TRAF	TNF receptor-associated factor
WD	Wallerian degeneration

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ABSTRACT OF THE THESIS

Tumor Necrosis Factor Receptor 1 on Schwann Cell-derived Exosomes Serves as an Alternative Binding Site for Tumor Necrosis Factor α

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Tumor necrosis factor α (TNFα) is a proinflammatory cytokine that is upregulated in Schwann cells (SC) during Wallerian degeneration (WD) and has also been implicated

in neuropathic pain. TNFα may be a connecting factor that leads the inflammatory response in WD to turn neuropathic. Understanding how TNFα is regulated during WD may provide more insight as to how elevated TNFα contributes to the occurrence of neuropathic pain after a peripheral nerve injury. Previous studies done in our lab have demonstrated that Schwann cell derived extracellular vesicles (SC EVs) block TNFα signaling on primary cultured SCs, but the biological mechanism of how SC EVs may play a role in TNFα regulation remain unclear. We observed that EVs isolated from SCs are enriched with TNFR1, the main binding receptor for soluble TNFα. To elucidate how TNFα may be regulated, we performed binding studies validating that TNFα can bind to the TNFR1 on SC EVs. We also show that SC EVs attenuate the effects of TNFα *in vitro* by analyzing the morphology of SCs treated with TNFα and SC EVs. Our studies suggest that TNFα binds to the TNFR1 on SC EVs, thus it can be argued that the presence of EVs decrease TNFα/TNFR1 signaling produced by SCs after a nerve injury. In conclusion, this mechanism is significant to understanding how elevated expression of TNFα is regulated during WD and offers some perspective on how elevated TNFα expression perpetuates neuropathic pain.

Chapter 1

Introduction

1.1 Development and anatomy of the peripheral nerve

1.1.1 Overview of the peripheral nerve

Peripheral nerves are structures that disseminate information to and from the central nervous system (CNS) through bilateral communication. Peripheral nerves contain the axons of both sensory and motor neurons, which respectively stem from either the cell bodies located in the dorsal root ganglion (DRG) or ventral horn (Purves, 2004). The sensory neurons are considered pseudounipolar neurons because their axons split into two branches from the cell body located in the DRG. One branch of axons travels and extends into PNS, which include the skin, organs, muscles and joints. Whereas the other branch from the cell body extends to the spinal cord of the CNS. Nerves form through multiple groups of fascicles, which are structures consisting of axons, glia cells, connective tissue, and blood vessels (Jessen et al., 2015).

Macrophages, fibroblasts, and Schwann cells (SCs) are the other non-neuronal cells that contribute to the structure of the peripheral nerve (Figure 1). Macrophages are phagocytic cells that arise from monocytes in the peripheral blood that migrate and then settle into tissues (van Furth et al., 1972). The two classes of macrophages are resident macrophages and infiltrating macrophages. In the peripheral nerve, resident macrophages become activated in response to injury, whereas infiltrating macrophages are recruited to the nerve by SCs secreting leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1) (Mueller et al., 2003; Sugiura et al., 2000; Tofaris et al., 2002). The main function of macrophages is to discard of both axonal and myelin debris from the nerve after injury (Hirata & Kawauchi, 2002; Lutz et al., 2017; Rosenberg et al., 2012). Both endoneurial fibroblasts and SCs are derived from SC

precursors (Joseph et al., 2004; Jessen et al., 2015). Endoneurial fibroblasts are responsible for creating and excreting the extracellular matrix protein collagen, which forms the endoneurial connective tissues in the peripheral nerve (Jessen & Mirsky, 2005; Joseph et al., 2004). SCs have two different phenotypes that are expressed in peripheral nerves, which are dependent on the axon size and cellular signals produced by the axon's state (Jessen & Mirsky, 2019). For larger diameter axons, each SC has a myelinating phenotype that will wrap around one axon multiple times to assist in propagating nerve impulses. The other non-myelinating phenotype of SCs will group together numerous smaller diameter axons to create a Remak Bundle that serves as metabolic and trophic support for the axons (Jessen & Mirsky, 2019).

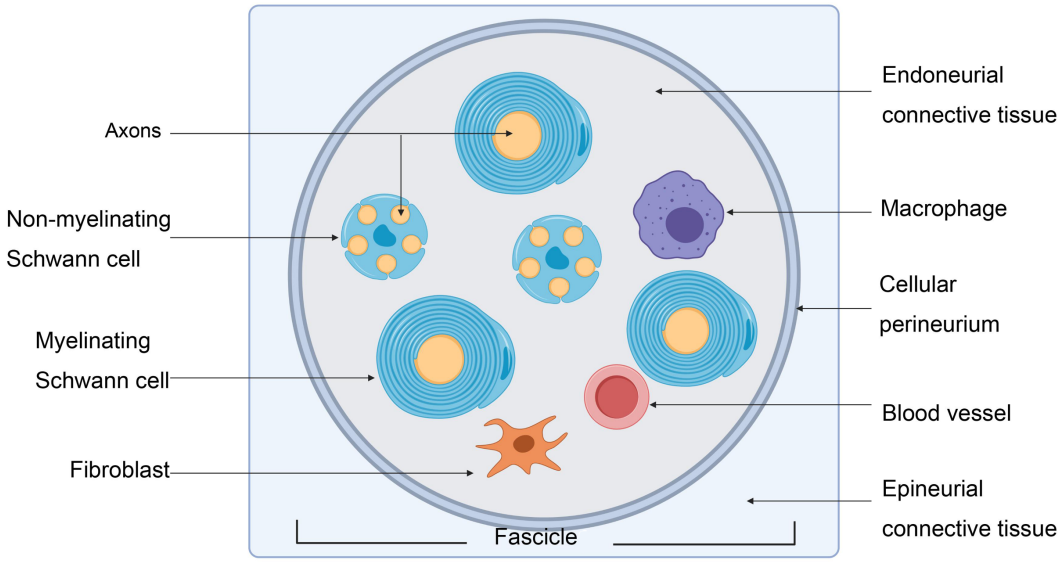


Figure 1: The diagram of a transverse section of peripheral nerve, showing the components of a fascicle. Created with Biorender.

1.1.2 Origins, development, and functions of Schwann cells

Mature SCs originate from neural crest cells, which undergo three major physiological transitions before the formation of the two types of mature SCs (Figure 2). The first transition entails neural crest cells to transforming into SC precursors. SC precursors then undergo differentiation into immature SCs, which eventually differentiate into either a myelinating SC or a Remak SC.

One of the main factors driving SC precursor proliferation and differentiation is Neuregulin-1 (NRG1) signaling, which stimulates distinctive glial features and inhibits the expression of neuronal factors (Shah et al. 1994). This process is initiated when NRG1 binds to ErbB2/3, a tyrosine kinase pair of plasma membrane proteins, that activates various signaling pathways for proliferation and migration of the SC precursors, but also is involved in the development of myelination (Newbern and Birchmeier 2010; Raphael and Talbot 2011; Garratt, et al., 2000a). Specifically, NRG1 type III is the driving molecule in determining myelination, as studies have shown that NRG1-III can induce phenotypic changes from non-myelinated to myelinated (Obremski and Bunge 1995; Taveggia et al. 2005). In previous research, inhibiting NRG1/ErbB signaling by either deleting ErbB2 or NRG1 type III in SCs caused a failure of SCs to myelinate (Riethmacher et al., 1997; Garratt, et al., 2000b), illustrating to the importance of this signaling pathway for SC development.

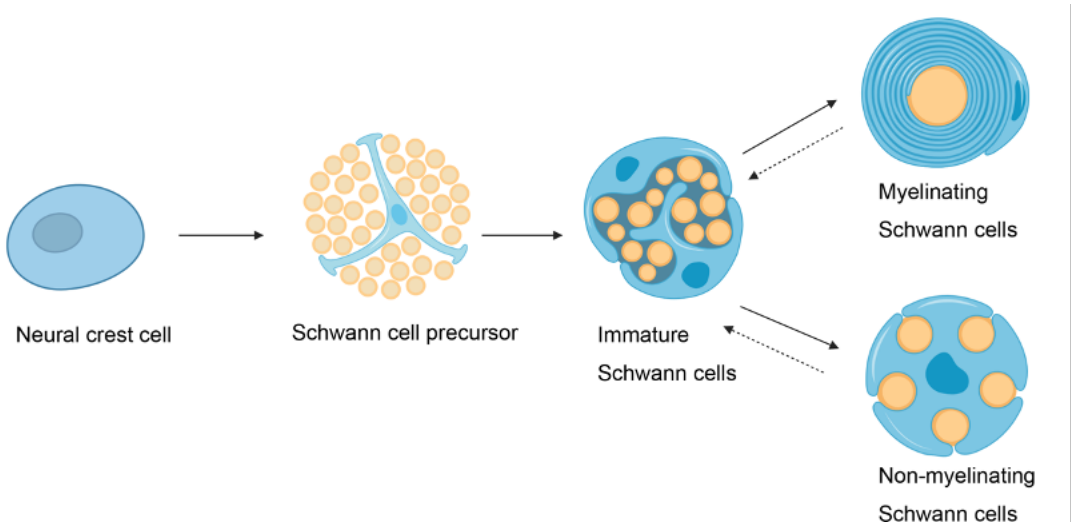


Figure 2: Schwann cell lineage. Schematic illustration of Schwann cell development. Dashed arrows indicate that the development is reversible, mature myelinating and non-myelinating Schwann cells can revert to a phenotype similar to that of the immature Schwann cells in response to injury. Created with BioRender.

SC precursors proceed to transform into immature SCs which is observed in late stages of embryonic development. Unlike SC precursors, the survival of immature SCs is not dependent on axon signals (Dong et al., 1995; Meier et al., 1999). This is because immature SCs contain autocrine survival circuits, which allow survivability without the presence of axons (Dong et al. 1999; Meier et al. 1999). In addition, it has been demonstrated that increased cAMP expression promotes and drives the proliferation of immature SCs from SC precursors (Arthur-Farraj et al., 2011; Mogha et al., 2013). Immature SCs will then differentiate into two types of mature SC types: myelinating or non-myelinating.

1.2 Wallerian degeneration after peripheral nerve injury and mechanism of regeneration

1.2.1 Wallerian degeneration and the Schwann cell response to injury

First observed by Augustus Waller, Wallerian Degeneration (WD) is an immediate response following a nerve injury, where the axons distal to the injury site will start to degenerate (Waller 1850) and the efficacy of this process is essential in optimizing axonal regeneration in peripheral nerve injuries (Chen et al., 2007). In peripheral nerve injuries (PNI), SCs are major orchestrators in initiating WD. Both myelinating and Remak SCs distal to the injury site transdifferentiate into a repair phenotype to promote regeneration and to assist in the guidance of regenerating axons (Gomez-Sanchez et al., 2017; Jopling et al., 2011). This programmed response in SCs is triggered through changes in the cellular environment of both the injured axon and SCs (Jessen & Mirsky, 2016). During transdifferentiation, myelin associate genes such as myelin transcription factor Krox20, myelin basic protein, and myelin associated glycoprotein in SCs are downregulated (Jessen & Mirsky 2008). However, the key regulator for the repair program is the transcription factor c-Jun, which is required for the activation of the repair program and works by inhibiting the expression of Krox20 (Parkinson et al., 2008; Jessen & Mirsky 2015).

1.2.2 Mechanism of myelin clearance through Schwann cells

Nerve regeneration is unique to the peripheral nervous system because in response to nerve damage, SCs rapidly degrade its own myelin during the initial stages of the repair program (Perry et al. 1995; Stoll *et al.,* 1989). This process of self-degradation in cells is known as autophagy and it is activated in SCs after peripheral nerve injuries due to an autophagy gene, Atg7 (Gomez-Sanchez et al., 2015). In a second phase, SCs

recruit macrophages to the site of injury to clear the remaining myelin debris through phagocytosis (Vargas et al. 2010; Hirata & Kawabuchi 2002; Dubový et al. 2013).

SCs initiate the clearance of myelin debris by secreting a large number of cytokines including tumor necrosis factor α (TNFα), which is a master orchestrator in recruiting other cytokines and macrophages to the injury site during WD (Shamash et al., 2002). TNFα is one the major orchestrators in early responses during PNS injuries as it plays a role in the development of neuroinflammation, changing SC phenotype and overall developing the pain process following WD (Leung & Cahill, 2010). In a previous study, it was shown that TNFα/TNFR1 signaling produces a positive feedback loop to promote increased expression of TNFα along with other proinflammatory cytokines that TNFα signaling recruits in SCs (Qin et al., 2008). This is significant because the link between how WD turns into neuropathic pain is unknown, but this suggests that the TNFα signaling feedback loop may play a role in connecting the two stages of pain.

1.2.3 Regeneration of the peripheral nerve after injury

Unlike the CNS, in the peripheral nervous system axons of neurons can repair themselves. The events following WD in the PNS promote an environment that is better for regeneration compared to the CNS, which contributes to the capability for regeneration to occur in peripheral nerves. The inability for regeneration following WD in the CNS is related to the failure of clearing myelin debris, which leads to an inhibitory microenvironment for axonal regeneration (Perry et al. 1987; George & Griffin 1994). The myelin debris contains inhibitory factors that causes an inhibitory microenvironment for axonal regeneration, thus the PNS has a better adaptable environment for

regeneration. Research has shown that if CNS axons are transferred into the PNS, the capability for regeneration is much greater (David & Aguayo, 1981).

1.2.4 Peripheral sensitization and neuropathic pain

Even though axonal regeneration is possible in the PNS, regeneration is often incomplete. Thus, neuropathic pain is often associated with peripheral nerve injuries. Neuropathic pain is defined by drastic pathological changes, such as lesions or disease in the somatosensory nervous system (Myers et al., 2006; Jensen et al., 2011). As mentioned before WD is pertinent for creating a microenvironment that is suitable for axonal regeneration to occur, however the same cellular and molecular events that contribute to the efficacy of regeneration contributes to the maintenance of neuropathic pain (Üçeyler and Sommer, 2006). Pathways initiated by TNFα that is essential in WD have been implicated in the persistence of neuropathic pain (Leung & Cahill, 2010). For example, TNFα directly activates nociceptors when injected into the nerve (Wagner and Myers, 1996). The underlying mechanisms included activation of p38MAPK and changes in sodium channels (Jin & Gereau, 2006).

1.2.5 TNFα is the major orchestrator for the responses following nerve injury

Some conditions correlated to high levels of TNFα include multiple sclerosis, psoriasis, rheumatoid arthritis, and other neurodegenerative diseases (Fischer et al., 2015). As of now, there are currently only five anti-TNF therapeutics to treat chronic inflammation in individuals: infliximab (Remicade), adalimumab (Humira), certolizumab pegol (Cimzia), golimumab (Simponi), and etanercept (Enbrel) (Monaco et al., 2015).

Some of these agents unfortunately cause variable side effects such as infections and development of autoimmune diseases (Monaco et al., 2015). Efforts to resolve neuropathic conditions have focused on inhibiting TNFα signaling by blocking the expression of the proinflammatory factor or receptor. However, what might be of interest is more clearly comprehending the mechanisms of how TNFα may is regulated during WD, which is not clearly known (Fischer et al., 2015). Understanding the process of how TNFα expression is regulated during injuries, may help to reveal novel therapeutics for pain management.

1.3 TNFα and TNFR1 signaling pathways

*1.3.1 TNF*α *and TNFR forms and functions*

TNFα is produced as a 26kDa transmembrane protein (tmTNFα) that assembles into a homotrimer and can be cleaved by TNFα converting enzyme (TACE) to release a soluble homotrimeric TNFα (sTNFα) (51kDa) (Kriegler *et al.,* 1988; Black *et al.,* 1997). Both TNFα forms bind to and activate receptors in the tumor necrosis factor receptor (TNFR) superfamily, TNFR1 and TNFR2.

TNFR1 and TNFR2 are both type I transmembrane receptors that have pre-ligand binding assembly domains (PLAD) on the extracellular domain side of their cysteine-rich domains (CRD). The PLAD on both receptors are essential for forming ligand-receptor interactions (Fischer et al., 2020). However, the two different forms of TNFα have significantly distinct binding affinities and activation of the two TNF receptors.

sTNFα can activate both TNFR1 and TNFR2, however the binding affinity is much higher for TNFR1. It has been shown that sTNFα has a higher binding affinity of K_d = 1.9 x 10^{-11}M for TNFR1, whereas the binding for TNFR2 is K_d = 4.2 x 10^{-10}M (Grell et al., 1998). The binding affinity between sTNFα and TNFR2 leads to transient signaling activation (Grell et al., 1998). It has been shown that tmTNFα can robustly bind and activate both TNFR1 and TNFR2 signaling (Grell et al., 1995). The affinity for sTNFα to bind either TNFR1 or TNFR2 has been correlated to the stabilization of TNF/TNFR complexes that is formed through the recruitment of different adaptor proteins (Krippner-Heidenreich et al., 2002). TNFR1 contains a death domain (DD) whereas TNFR2 does not have a death domain but is a TNF receptor associated factor (TRAF) (Tartaglia et al., 1993; Wajant et al., 2003).

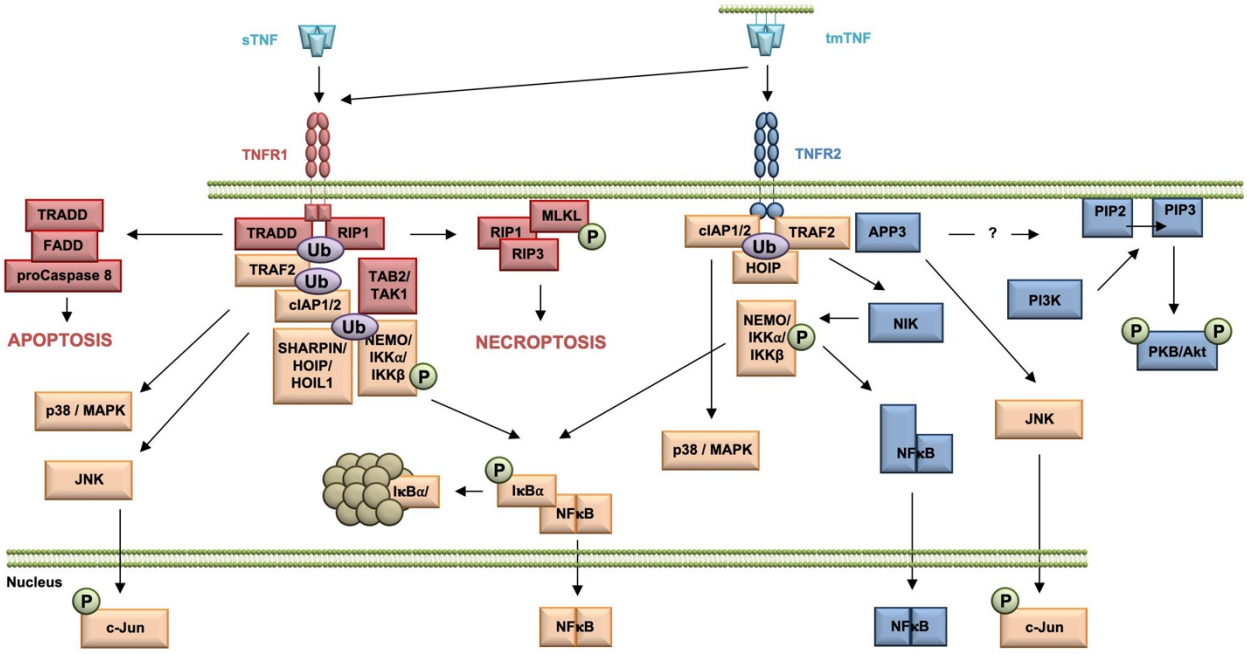


Figure 3: Diagram showing the signaling pathways activated by TNF/TNFR binding (Fischer et al., 2020).

When TNFα binds to TNFR1 the intracellular proteins that are recruited include TNF receptor 1 associated protein with a death domain (TRADD), TRAF2, receptor

interacting protein kinase 1 (RIP1), and cellular inhibitor of apoptosis proteins (cIAPs) 1 and 2 (Fischer *et al*., 2020). Common proinflammatory pathways and factors activated by TNFα/TNFR1 include NF-κB pathways, p38 MAPK, and c-jun N-terminal kinase (JNK) pathways (Guma and Firestein, 2012; Sabio and Davis, 2014) (Figure 3). NF-κB signaling pathways promote the transcription of inflammatory genes such as IL-6, IL-8, and even TNFα (Fischer et al., 2015; Kaltschmidt et al., 1993). Both p38 MAPK and c-JNK are inflammatory promoters that also can induce the expression of TNFα (Guma and Firestein, 2012; Sabio and Davis, 2014). Since both of these pathways are activated by TNFα, it can be argued that TNFα serves as its own activator for amplified expression and signaling for proinflammatory pathways (Fischer et al., 2015; Qin et al., 2008).

Whereas when TNFα binds to TNFR2, the signaling complex that forms consist of TRAF2 (Rothe et al., 1994), cIAP1/cIAP2 (Rothe et al., 1995), and HOIP, a LUBAC factor (Borghi et al., 2018). The TNFR2 signaling complex can initiate non-canonical NFκB pathways (Rauert et al., 2010) and it has also been demonstrated that the activation of TNFR2 possibly can activate c-Jun N-terminal kinase (JNK) (Jupp et al., 2001) as well as p38 MAPK pathway (He et al., 2018).

Overall, both TNFR1 and TNFR2 have been implicated in neuropathic pain (Zhang et al., 2011; Sommer et al., 1998). The activation of TNFR1 pathways is associated with inflammation and tissue degeneration, whereas TNFR2 pathways are connected to regeneration of tissue and immune homeostasis (Fischer *et al.,* 2015).

1.3.2 Pathways initiated by TNFα/TNFR1 complex in SCs

There have been previous *in vitro* studies demonstrating that TNFα signaling in SCs leads to the activation of enhanced apoptotic signaling along with decreased cell proliferation and migration (Tang et al., 2013; Skoff et al., 1998). The effects of the increasing amounts of TNFα have been shown to cause a dose dependent effect with increased expression of apoptotic related factors (Tang et al., 2013). It was also established there was an increased protein level of FADD bound to TRADD, which is associated to apoptotic signaling pathway initiated by TNFα/TNFR1 complex (Tang et al., 2013) (Figure 3). The changes of p-JNK and p- NF-κB signaling in SCs has also been shown to correlate with the amount of TNFα present (Tang et al., 2013).

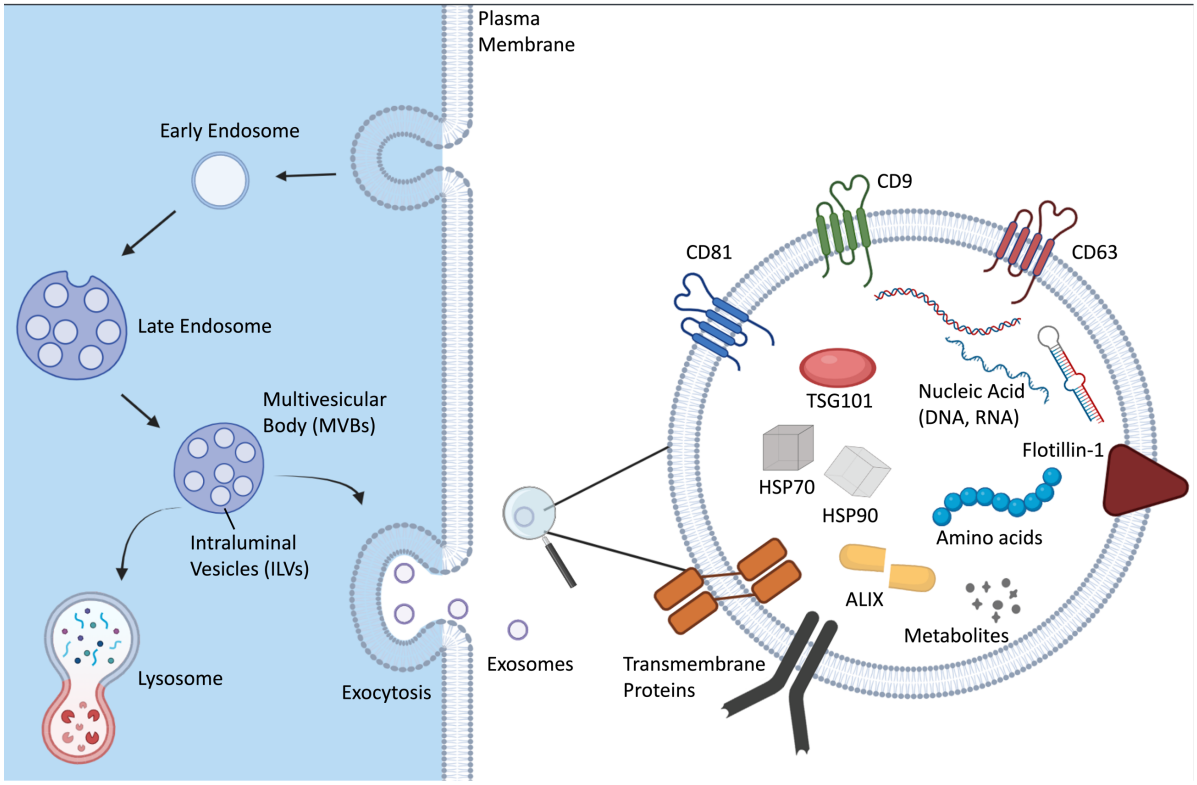


Figure 4: Schematic of exosome biogenesis and common exosome markers and cargo. Created in Biorender.

1.4 Extracellular vesicles and peripheral nervous system

1.4.1 Definition of extracellular vesicles

The release of extracellular vesicles (EVs) has recently been found to be one of the critical pathways for cell-cell communication. These nanoparticles eventually affect their destination cells by carrying different DNA, RNA, and active protein that can be transferred from the donor cell to target cells (Record *et al*., 2011; Lopez-Verrilli *et al*., 2013; Valadi *et al.,* 2007; Mathivanan *et al.*, 2010). Exosomes are the smallest type of EVs and are 30-150 nm size in diameter (Théry et al., 2006; Mathivanan et al., 2010). Exosomes are created by the inward budding of the late endosomes as intraluminal vesicles (ILVs), encapsulated with molecules within multi-vesicular bodies (MVBs) (Figure 4). These ILVs in the MVBs are destined for either (1) to fuse with lysosomes for degradation in the same cell or (2) to fuse to the plasma membrane to be released as exosomes. The release of exosomes have the potential to communicate in autocrine, paracrine and endocrine pathways both in health and disease, making them unique communicators of their original environment.

1.4.2 Importance of extracellular vesicles during peripheral nerve injury

The importance of exosomes released during the pain process is the potential for these nanoparticles to serve as a new possible biomarker of neuropathic pain. However, it is not exactly clear how they are involved in neuropathic pain and the inflammatory conditions following a nerve injury. There have been previous studies demonstrating that the cargo transfer from SCs to neurons via SC derived extracellular vesicles (SC EVs) influences sensory neurons (Chen et al., 2019; Ching et al., 2018;

Lopez-Verrilli et al., 2013). After a peripheral nerve injury, SC EVs have been shown to be selectively internalized by injured axons, which prompted axonal regeneration. (Lopez-Verrilli *et al*., 2013). The characterization of cargo within SC EVs through RNA sequencing has shown that SC EVs contain small RNAs that are associated with axon guidance and extension (Gonçalves et al., 2020). It was then identified through an *in vitro* study that the cargo transfer of miR-10b, miR-21, and miR-27b from SC EVs to DRG neurons promoted neurite growth. Another study found that SC sEVs isolated from SCs cultured in high glucose had increased miR-28, -31a, and -130a, which coincided with the acceleration of diabetic peripheral neuropathy when SC EVs were administered *in vivo* (Jia et al., 2018). Many of these studies focused on the SC EVs function to transfer cargo from donor cell to target cell for cell communication, however a less studied aspect of SC EVs is the function of receptors on the membrane surface and their potential role in cell-cell communication.

1.4.3 Schwann cell EVs with relation to neuropathic pain

The membranes of EVs consist of the same receptors as the donor cell they originate from (Record *et al*., 2011; Simons & Raposo, 2009). How the receptors on EVs play a role in cell communication or regulation is unknown. However, a previous a study showed a possible function for receptor on EVs. It was demonstrated that human vascular endothelial EVs contained TNFR1 and the release of TNFR1 on EVs have been suggested to serve as a mechanism to regulate the soluble cytokine receptor (Islam *et al.,* 2006; Hawari et al., 2004). Similarly, we have found the SC EVs contain TNFR1 and that by adding SC EVs to TNFα treated SCs the pro-inflammatory pathway

p38 MAPK can be inhibited (Sadri et al., 2021; In press). However, the molecular mechanism as to how SC EVs inhibits the effects of TNFα are not known.

1.5 Objective

We hypothesize that the TNFR1 transmembrane receptor protein on SC EVs serves as an alternative binding site for TNFα. We tested this hypothesis and found it be supported: (1) Upon measuring and calculating the copy number of TNFR1 on SC EVs, we found that SC EVs are enriched with TNFR1; (2) Using cross-linking studies, we found that TNFα binds to TNFR1 on SC EVs; (3) By analyzing the morphology of SCs treated with TNFα, plus or minus SC EVs, we found that SC EVs attenuate the effects of TNFα.

Chapter 2

Materials and Methods

2.1 Schwann cell culture and characterization through immunofluorescence

Rat SCs were isolated from the sciatic nerves of 1-day old Sprague-Dawley rats. Primary SC cultures were purified of fibroblasts with Thy1.1 antibody (M7898; Sigma-Aldrich) and rabbit complement cytolysis (Sigma-Aldrich) as previously described (Campana et al., 1998). SCs were cultured on 1 µg/µL Poly-D-Lysine (PDL)-coated plates and grown in complete medium consisting of low-glucose DMEM, 10% heat inactivated fetal bovine serum (HI-FBS), 1% Penicillin/Streptomycin, 4 µM forskolin, and 21 µg/mL BPE.

Immunofluorescence was performed on cells from a primary SC culture isolation to confirm purification of cells by staining for S100, an astrocyte marker. Cultured cells were plated on Millicell EZ SLIDE 4-well glass (Sigma PEZGS0416) at 50,000 cells per chamber. Cells were fixed with 4% PFA for 10 minutes on a shaker at 4℃ and then permeabilized with 0.1% TritonX-100 (Fisher Scientific BP151-100) in PBS. After permeabilization, cells were blocked with 5% BSA in PBS for 1hr on a shaker at 4℃. Cells were stained with a recombinant Anti-S100 beta (Abcam ab52642) overnight at 4℃ and then Alexa Fluor 488 anti-rabbit IgG (Thermofisher A-11034) was used as the secondary antibody. Nuclei of cells were then stained with mounting media containing the DNA dye DAPI (Abcam ab104139) and a cover glass was placed on the slides. The chamber slides were imaged on an Olympus CKX41 microscope using the Infinity Analyze and Capture Software.

2.2 Schwann cell EVs isolation protocol

SCs were cultured in complete medium until 80~90% confluent and then washed gently with warm Dulbecco's phosphate-buffered saline, DPBS, (Gibco-14190144) to remove exogenous and endogenous EVs. The medium was replaced with DMEM containing 10% FBS (Fisher Scientific 10-082-147) which was depleted of EVs by ultracentrifugation 100,000 × *g* for 18h (Shelke et al., 2014). The medium was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. There was no BPE or forskolin in the SC medium to avoid co-isolation of exogenous EVs and minimize cell proliferation, respectively.

SCs were allowed to condition medium for 15 h. EVs in conditioned medium (CM) were isolated by differential ultracentrifugation, as previously described (Théry et al., 2006) and modified by us. Briefly, CM was centrifuged at 2000 × *g* for 10 min to remove cellular debris and then at 11,000 × *g* for 30 min at 4 °C to remove larger EVs. The resulting supernatant was then subjected to ultracentrifugation at 108,000 x *g* for 18 h at 4 °C to pellet EVs. This pellet was washed in cold 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), re-pelleted at 130,000 x *g* for 3h at 4 °C and re-suspended in PBS. Only freshly isolated SC-derived EVs were used for analysis and in bioactivity assays.

2.3 Schwann cell EVs characterization through Nanosite

EVs were analyzed by NanoSight tracking analysis (NTA) as previously described (Almanza et al., 2018). Briefly, isolated EVs were analyzed in a NanoSight NS300 instrument equipped with a 532nm (green) laser (Malvern) that was calibrated

with polystyrene latex microbeads at 100 nm and 200 nm. EVs were re-suspended in PBS to make a 1:50-1:100 dilution and reach 50-100 objects per frame in the NanoSight. Each sample was measured in triplicate. The NTA analytical software version 2.3 was used for capturing and analyzing the data according to the manufacturer's protocol.

2.4 Transmission electron microscopy of SC EVs

Formvar-carbon-coated copper grids (100 mesh, Electron Microscopy Sciences) were placed on 20 µL drops of each EV-containing solution displayed on a parafilm sheet. After allowing adsorption of solution components for 10 min, grids were washed 3 times with 200 µL drops of Milli-Q water and then incubated for 1 min with 2% (wt/vol) uranyl acetate (Ladd Research Industries). Excess solution was removed with Whatman 3MM blotting paper. Grids were left to dry and then examined using a Tecnai G2 Spirit BioTWIN transmission electron microscope (TEM) equipped with an Eagle 4k HS digital camera (FEI).

2.5 Immunoblot analysis of Schwann Cell EVs

An equivalent amount of protein (2~5 µg) from SC and SC EV extracts, as determined by bicinchoninic acid assay (BCA), were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with Blotting grade blocker (5% nonfat dry milk) (Bio Rad-170-6404,) in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Primary antibodies diluted in 5% BSA/TBST were added and incubated overnight at 4 °C. The primary antibodies

used included: anti-CD9 (rabbit monoclonal 1:1000; Novus- NBP2-67310), anti-TSG101 (rabbit polyclonal 1:500; Abcam- ab30871), anti-Alix (mouse monoclonal 1:1000; Novus- NB100-65678), anti-GM130 (mouse monoclonal 1:1000; BD Bioscience-610822), and anti-TNFR1 (rat monoclonal 1:1000; R&D Systems- MAB425. Membranes were washed with TBS-T and incubated with secondary HRP-conjugated antibodies (1:2000; Cell Signaling Technology -7074 and Bio-Rad-1706516) in TBS-T with nonfat dry milk for 1 h at room temperature followed by enhanced chemiluminescence (GE Healthcare). Blots were scanned (Canonscan) and densitometry was performed using Image J software.

2.6 Dot blot assay

For the TNFR1 study, SC EVs were immobilized on membranes that were either blocked with 5% milk in with TBS in the absence of 0.1% (v/v) Tween-20, to avoid permeabilization or the in the presence of 0.1% (v/v) Tween-20 to induce permeabilization, for 1 h at room temperature. Subsequently, membranes were incubated with primary antibodies against TNFR1 (rat monoclonal 1:1000; R&D Systems, MAB425) and then a HRP- conjugated secondary antibody in 5% milk with either TBS or TBST followed by detection and imaging with enhanced chemiluminescence (GE Healthcare).

2.7 Identification and quantification of TNFR1 in SC EVs

First a concentration gradient of both SC EVs (0.5 to 4ug) and recombinant human soluble TNFR1 protein (2 to 20ng) was on the same blot to deduce the optimal

amount needed to produce a standard curve to calculate the amount of TNFR1 present on SC EVs. Then six independent SC EV preparations (1 µg each) were subjected to SDS-PAGE and immunoblotting with anti-TNFR1. The signal produced by the recombinant human soluble TNFR1 protein (10 to 80 ng) (R&D, 636-R1) was run on the same blot was used to calculate the amount of TNFR1 present on an individual SC EV. Total EV protein and the number of EVs in each solution, as determined by NTA, was used to determine the copy number of TNFR1 per EV.

2.8 Binding assays

For crosslinking studies, BS^3 (5mM) (Thermofisher-21580), was used as it is a water soluble crosslinker that forms stable covalent bonds. Rat SC EVs (10 µg) were incubated with TNFα (500 pM, R&D systems- 210-TA) and BS^3 at 37℃ for 10 min. TNFα (0.5 nM) alone served as a control. All samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk (Bio-Rad-170-6404) in 10 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% Tween 20 (TBS-T) for 90 min at room temperature. Blots were probed with primary TNFα-specific antibody (TNFα mouse monoclonal 1:500, Santa Cruz- sc-133192) overnight at 4° C. Subsequently, the membranes were washed with TBS-T and incubated with secondary mouse HRP-conjugated antibodies (1:2000; Cell Signaling Technology-7076S) in TBS-T for 1 h at room temperature followed by enhanced chemiluminescence (GE Healthcare).

2.9 Quantification of Schwann cell morphology

The cellular morphology of SCs was evaluated 1.5 h after treatment with TNFα (0.5 nM), TNFα plus SC EVs (10 μg), or vehicle (0.01% BSA in PBS). SCs were immunostained with anti-S100β (1:100, Abcam, ab52642), to identify SC cytoplasm, and with mounting media containing DAPI (Abcam, ab104139), to label nuclei. Stained SCs were imaged at 20X with an Olympus CKX41 inverted fluorescence microscope with a Lumenera INFINITY5-5M digital camera. Images were subjected to analysis using Image J. Nuclear area and cytoplasmic area (total cell area minus nucleus area) were determined in randomly selected cells. N/C ratios were calculated from a minimum of 30 cells per field and from 3 different experimental replicates for each treatment.

Chapter 3

Results

3.1 Isolating and characterizing Schwann cell EVs

To isolate SC EVs, we first prepared primary rat SCs from one day old neonatal rat pups using the methods previously described by (Brockes et al., 1979) (Figure 5A). The purity of SCs were validated through immunofluorescence by staining for S100, a known biomarker of SCs. The isolated primary SCs stained positive for S100, and co-localized with DAPI nucleus staining (Figure 5B).

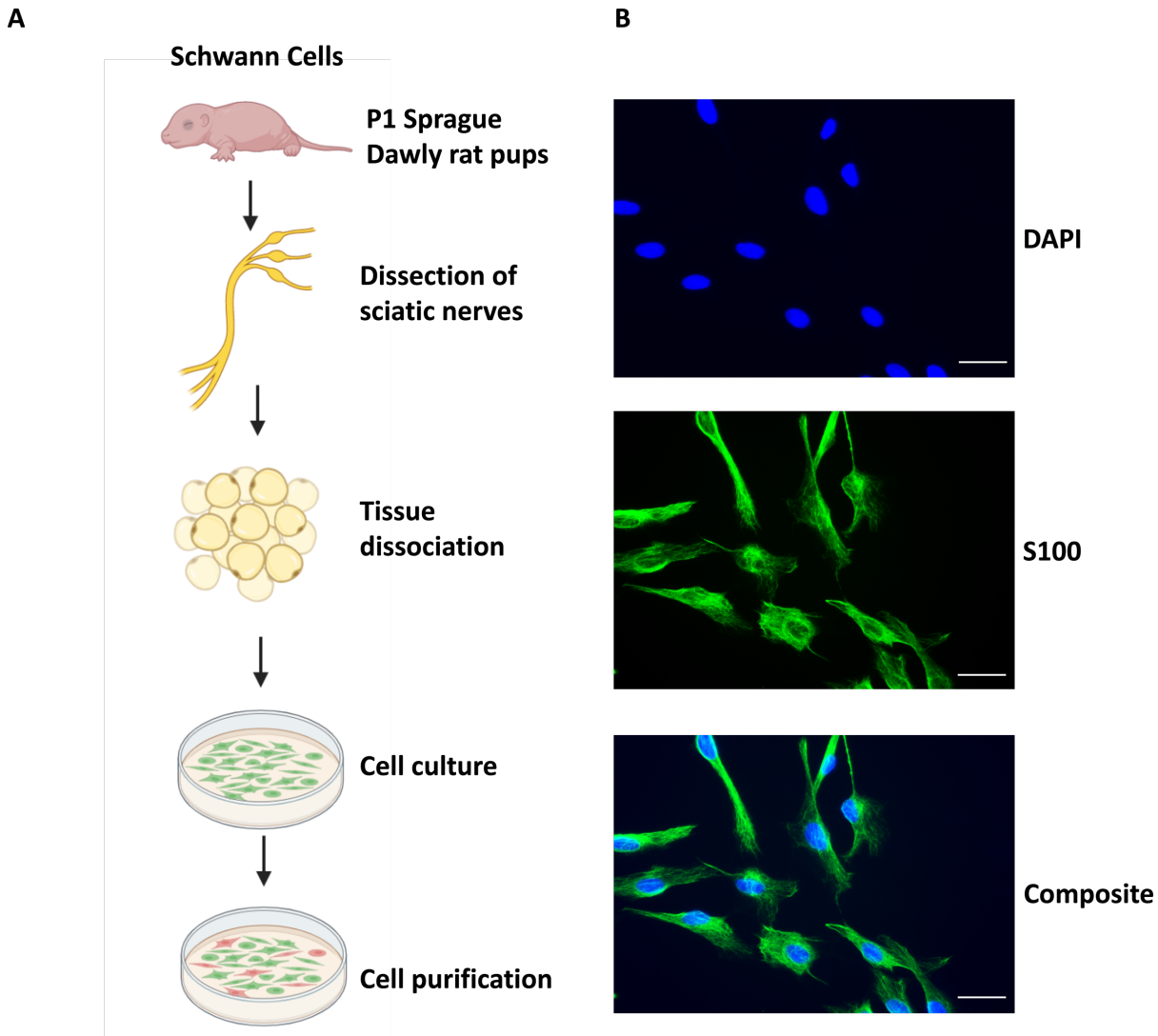


Figure 5: Primary Schwann cell characterization. (A) Schematic diagram of SC isolation strategy utilized. Created with Biorender. (B) IF microscopy of purified primary cultured SCs with anti-S100 (green) and DAPI (blue).

Next, EVs were isolated from SCs using similar methods employed by (Théry et al., 2006) (Figure 6A-B). Trypan blue analysis of originating primary SCs after 18 h treatment with EV-depleted 10% FBS containing media showed that the percentage of cell death was less than 4% (Figure 6C).

SC EVs were characterized through Nanonsight, which measures the particle size and concentration of nanoparticles that range from 10nm - 1000nm. Nanosight works by passing a laser beam through a sample chamber where the particles will scatter in a certain way that allows the program to use light scattering and Brownian motion properties to determine the concentration and size distribution of the EVs. The size distribution of three different preparations of EVs that were isolated from SCs were analyzed using the Nanosight nanoparticle tracking analysis (NTA) program. It was confirmed that EVs collected from SCs were within the appropriate range to be considered exosomes, given that they had a mean diameter of (132.1 ± 4.6nm, 152.1 ± 11.8nm, and 116.1 ± 5.0nm). Results from experimental repetition of each SC EVs preparation (n=3) revealed that the size of EVs vary between isolations (Figure 7A). SC EVs were also examined through transmission electron microscopy (TEM), which was performed by Dr. Nao Hirosawa, a visiting scholar in the Dr. Campana's lab. The EVs were found to be somewhat heterogeneous in size and frequently demonstrated cup-shaped morphology, as anticipated (Figure 7B).

To confirm the purity of SC EVs, Western Blot analysis was utilized to validate the presence of known exosome biomarkers as well as SC cell markers. Equal amounts of protein from extracts of SCs and SC EVs were loaded. GM130 is a Golgi apparatus marker that has been shown to be excluded from EVs (Merianda et al., 2009; Lötvall et

al., 2014) and we demonstrated that to be also true for our isolated SC EVs (Figure 7C). It was also found here that the isolated SC EVs contained the common exosome markers TSG101, the ESCRT-associated protein, ALIX, and the tetraspanin membrane protein CD9 (Doyle & Wang, 2019) (Figure 7C).

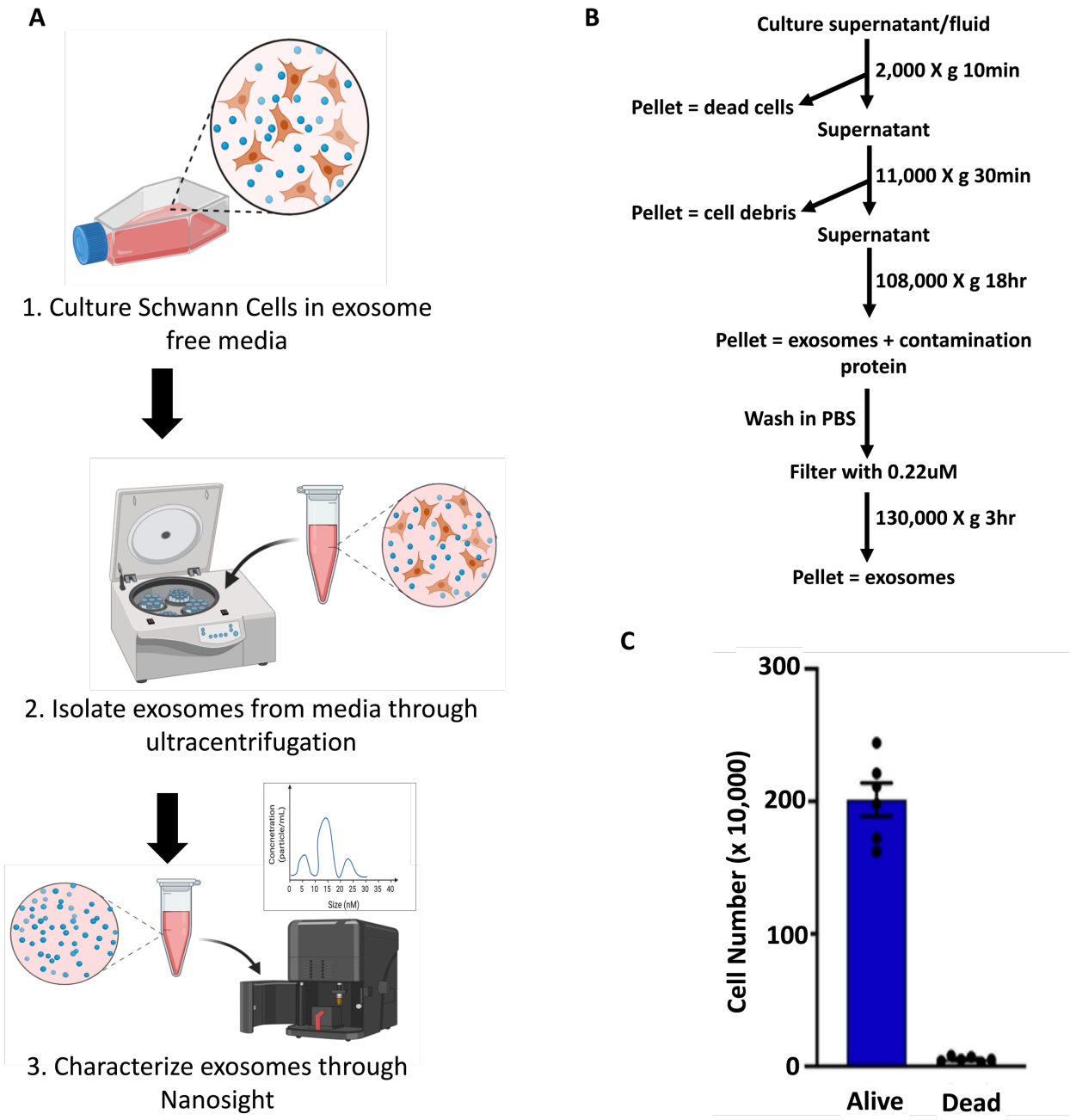


Figure 6: Schwann cell derived extracellular vesicles isolation. (A) Schematic representation of the SC EVs isolation strategy utilized. Created with Biorender. (B) Schematic of ultracentrifugation used to isolate SC EVs. (C) Trypan blue analysis of primary SCs after 12 h treatment with EV-depleted 10% FBS containing media. The percentage of cell death was less than 4%.

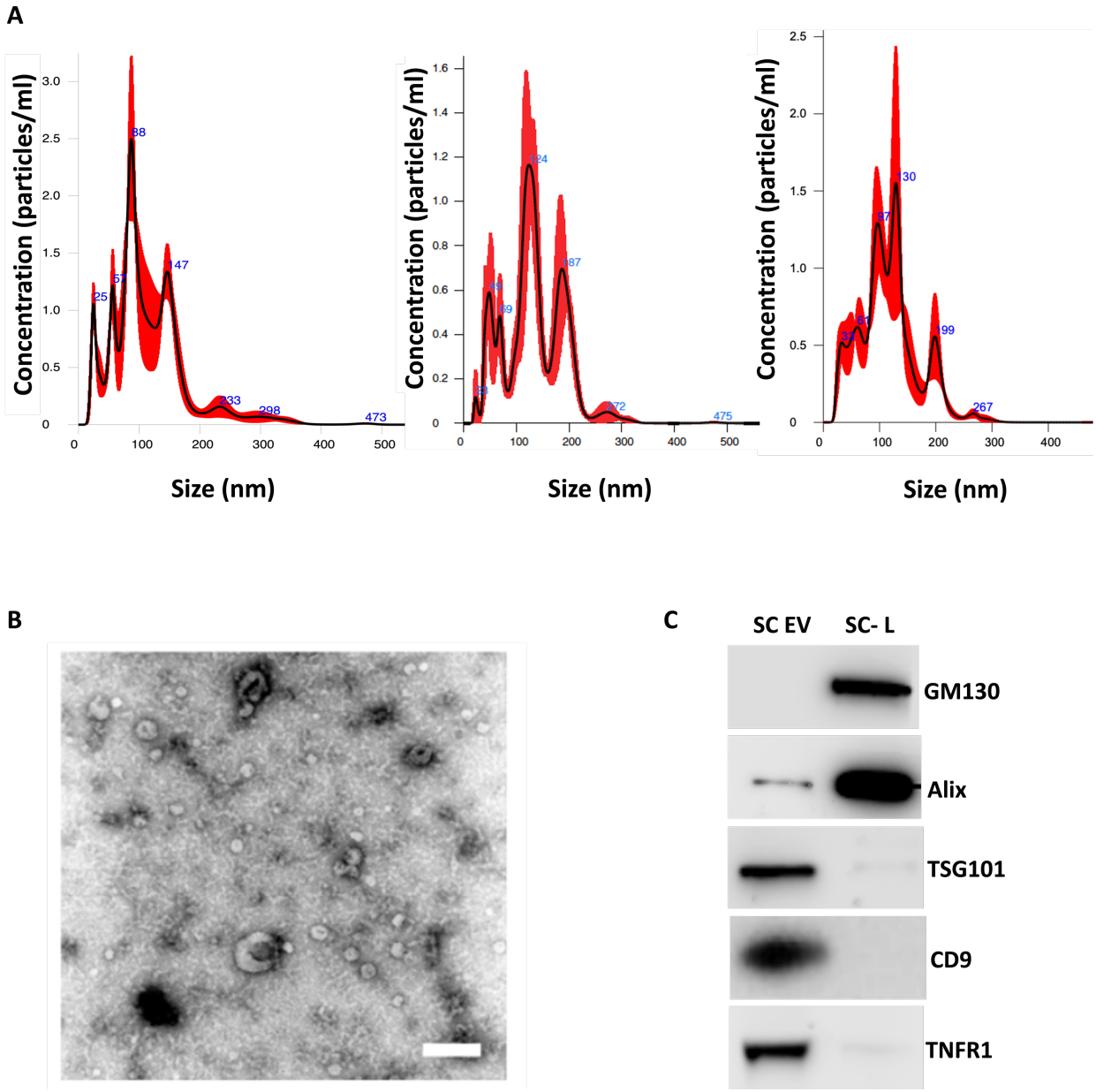


Figure 7: SC EVs Characterization. (A) Three different preparations of SC EVs isolated by ultracentrifugation from conditioned media (CM) of primary cultured SCs were analyzed by NTA. Mean diameter of SC EVs were (132.1 ± 4.6nm, 152.1 ± 11.8nm, 116.1 ± 5.0nm, respectively). (B) Transmission electron microscopy (TEM) using negative staining of EVs. Note large crescent shaped particles and smaller particles. Scale bar, 200 nm. Data are from Dr. Nao Hirosawa, Visiting Scholar in Dr. Campana's lab. (C) Immunoblot analysis of whole SC lysates (SC-L) and SC-derived EVs to detect: the exosome biomarkers, TSG101, CD9, and ALIX. GM130 is a golgi biomarker not found in exosomes. TNFR1 was found to be highly enriched in SC EVs.

3.2 SC EVs are highly enriched in TNFR1

When I was a beginning graduate student in Dr. Campana's lab, a visiting scholar, Nao Hirosawa, MD, PhD discovered that TNFR1, but not TNFR2, was highly sequestered in SC EVs. Immunoblot analysis, performed by Dr. Hirosawa, compared the expression of TNF receptor proteins in intact SCs and SC EVs (Figure 8A). Extracts of rat bone marrow-derived macrophages (BMDMs) served as a control. Both TNFR1 and TNFR2 were detected in cultured SCs. However, the level of TNFR1 was robustly increased in SC EVs compared to SCs. TNFR2 was not present in SC EVs. P0, which was used as a positive biomarker for SCs, was present in intact SCs and in SC EVs. P0 was not present in BMDMs, as anticipated.

The increased presence of TNFR1 was consistently reproduced in SC EVs that were collected by me for these experiments (Figure 7C). Next, I performed a dot blot analysis to detect TNFR1 on the surface of SC EVs. Increasing amounts of SC EVs were treated with either a detergent to permeabilize the membranes of the EVs or a non-detergent solution that would keep the membranes intact (Figure 8B). The dot blot containing the different amounts of SC EVs was then probed with a TNFR1 antibody. Permeabilized SC EVs showed an increased amount of TNFR1 compared to EVs that were not permeabilized, but it was observed that the non-permeabilized EVs also contained TNFR1 on the membrane as there is visible dot present at the lowest amount (0.3ug) of EVs. Moreover, the presence of TNFR1 increases with the higher amount of total protein (Figure 8B)

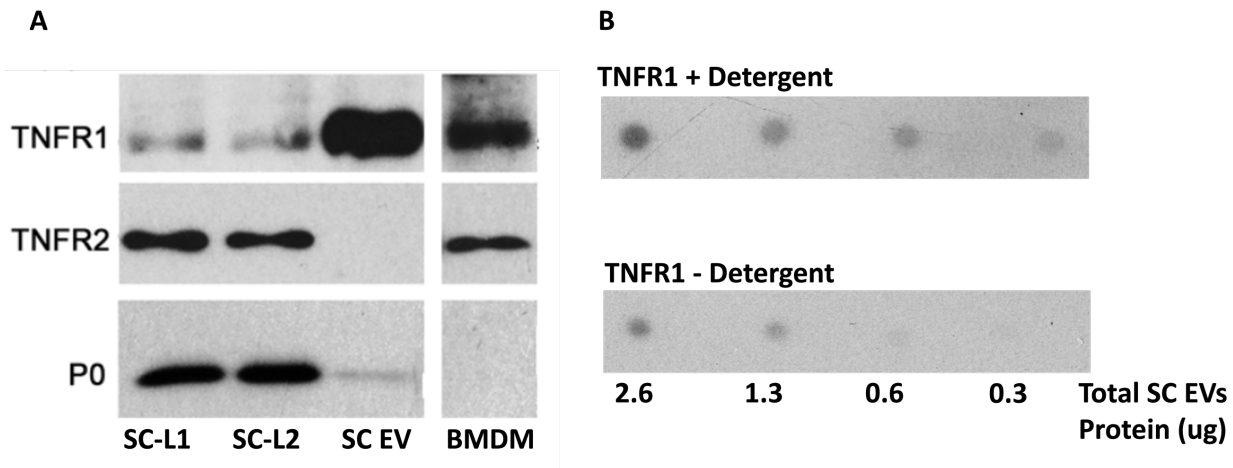
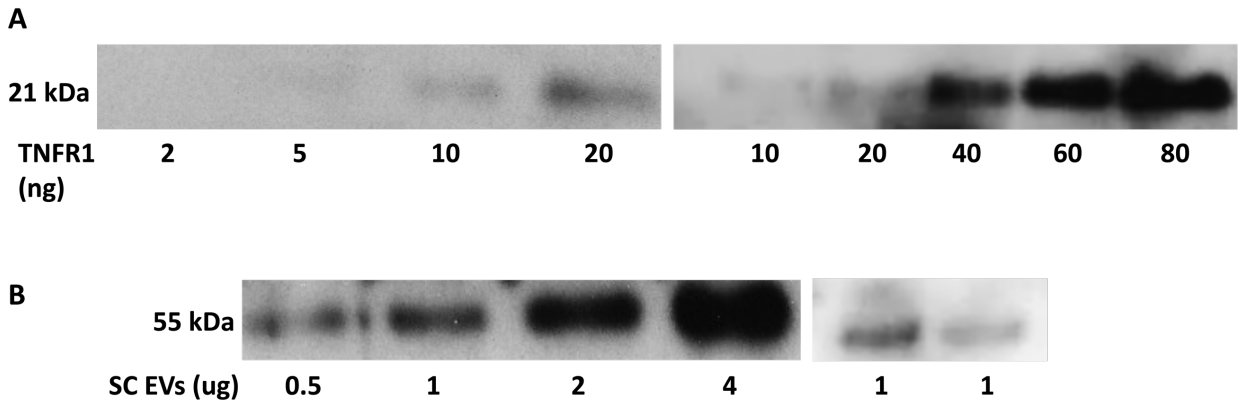


Figure 8: SC EVs are enriched with TNFR1. (A) TNFα receptors: TNFR1 (55 kDa) and TNFR2 (65 kDa) and P0 in extracts of SCs cultured in complete medium (SC-L1) or in DMEM with 10% FBS depleted of EVs (SC-L2), in SC-EVs, and in bone marrow derived macrophages (BMDMs) (2 µg/lane) were determined by immunoblot analysis. Data are from Dr. Nao Hirosawa, Visiting Scholar in Dr. Campana's lab. (B) Representative dot blot of non-permeabilized and permeabilized SC EVs (0.3-2.6 µg) probed with anti-TNFR1 antibody (n= 3 independent blots).

Next, we quantified how many copies of TNFR1 are on SC EVs. First an immunoblot containing increasing amounts SC EVs and purified soluble TNFR1 were used to determine the concentration needed to produce a standard curve. Standard curves ranging in concentration gradient of purified soluble TNFR1 from (2 to 20ug) and from (10 to 80ug) were used (Figure 9A). Based on a separate concentration gradient of SC EVs (0.5 to 4ug), it was determined that 1ug of SC EVs landed in the linear range of the standard curve (Figure 9B). Thus, six separate SC EVs isolations and a purified soluble TNFR1 standard curve were run through SDS-PAGE and immunoblotted for TNFR1. The copy number of TNFR1 for each preparation was calculated using the standard curve based on the purified TNFR1. Intensity of bands was determined through densitometry and the number of EVs/ug EV protein, which was calculated by the NTA data, were used to estimate the copies of TNFR1 from each of the six SC EVs preparations. We demonstrated that SC EVs have between 360 to 1075 copies of TNFR1, assuming an intact trimeric form of TNFR1 (Figure 9C).



C

EV ID Number	TNFR1 Trimer/EV	TNFR1 Protein (% of total EV protein)
1	1075	3.6%
2	586	2.0%
3	481	1.6%
4	395	1.3%
5	360	1.2%
6	645	1.9%
AVG ± SEM	590 ± 106	1.9 ± 0.4%

Figure 9: Calculated copy number of TNFR1 on SC EVs. (A) Concentration gradient of purified soluble TNFR1 (21 kDa) protein used to optimize a standard a curve. (B) Concentration gradient of SC EVs determined that 1ug of SC EVs was optimal for standard curve and two representative SC EV samples (1 µg/lane) in blot used for determining TNFR1 copy number. From this, the average amount of TNFR1 in SC EVs was determined to be approximately 15 ng. (C) Quantification of TNFR1 copy number based on the TNFR1 standard curve, NTA and BCA analysis of six independent EV preparations. Data are expressed as mean ± SEM.

3.3 TNFR1 on SC EVs bind TNFα

We investigated whether TNFR1 on EVs could serve as a binding site for TNFα as a possible means of regulating TNFα. To stabilize any TNFα-TNFR interaction, we used bis(sulfosuccinimidyl) suberate (BS3), a cross-linker that covalently stabilizes non-covalent bonds as previously described by Crookston and Gonias (Crookston and Gonias, 1994). The method was validated by running a time-course where purified TNFα was incubated with 5mM of BS^3 for increasing periods of time (Figure 10A). The anticipated forms of TNFα consist of a 17 kDa monomer, 34 kDa dimer, and 51 kDa trimer (Tang et al., 1996). The decreased presence of the TNFα monomer and increased presence of the dimer and trimer coincided with increasing incubation of TNFα with BS^3 as seen through SDS-PAGE. From this time course, it was determined that 10 minutes at 37°C would be the optimal parameters for measuring binding of purified TNFα to SC EVs.

Next, a binding study was performed where the treatment group consisted of SC EVs incubated at 37°C for 10 minutes with soluble TNFα and BS^3 (Figure 10B). Two different batches of EVs were used in this study. The positive control sample contained soluble TNFα and BS^3 and the negative control consisted of SC EVs and BS^3 minus TNFα. Both the control samples were incubated with the same conditions as the treatment sample. Both controls and treatment sample were subjected to a Western blot analysis, where a TNFα monoclonal antibody was used to detect TNFα expression. Predicted oligomeric forms of TNFα were found in both samples and included a monomer, dimer, and trimer, as anticipated (Tang et al., 1996). The treatment samples in both Western blots contain an additional band around the expected size of 106kDa,

as would be predicted for a TNFα trimer (51kDa) bound to TNFR1 (55kDa). However, the amount of binding reduction slightly varies between the two exosome groups. The band at 106kDa shows different expression levels of TNFα, possibly due to the different copy numbers of TNFR1 on each exosome group. A higher molecular weight band in the first blot can be seen in the treatment group that is representative of a trimer of TNFR1 (165kDa) binding to a trimer of TNFα (Figure 10B). In addition, both treatment groups of SC EVs demonstrated a decreased presence of the 34 kDa dimer and 51kDa trimer of TNFα relative to the positive control group without any EVs in both blots (Figure 10B). These results support our hypothesis that SC EVs regulate TNFα by serving as an alternative TNFR1-binding site and that the number of TNFR1 on SC EVs varies. This data provides novel insight about a possible mechanism for TNFα signaling to be downregulated in SCs during WD and pain, thus leading to a decreased activation of proinflammatory pathways that is triggered by TNFα/TNFR1 signaling.

When SC EVs were incubated with TNFα and BS^3 and re-probed for TNFR1, no band at the anticipated molecular weight of 106kDa were observed (Figure 10B). These findings suggest that binding interactions between TNFα and BS^3 may affect the immunogenicity of the TNFR1 antibody. This was confirmed by running SC EVs alone, SC EVs + BS^3, SC EVs with TNFα + BS^3, and TNFα + BS^3 (Figure 10C). When SC EVs alone is probed for TNFR1, there is clear presence of TNFR1 at the anticipated molecular weight of 55kDa (Figure 10C). However, when SC EVs + BS^3 are probed for TNFR1 the presence of TNFR1 is decreased and the bands appear shifted upwards. This is likely due to BS^3 covalently binding TNFR1 on SC EVs to one another.

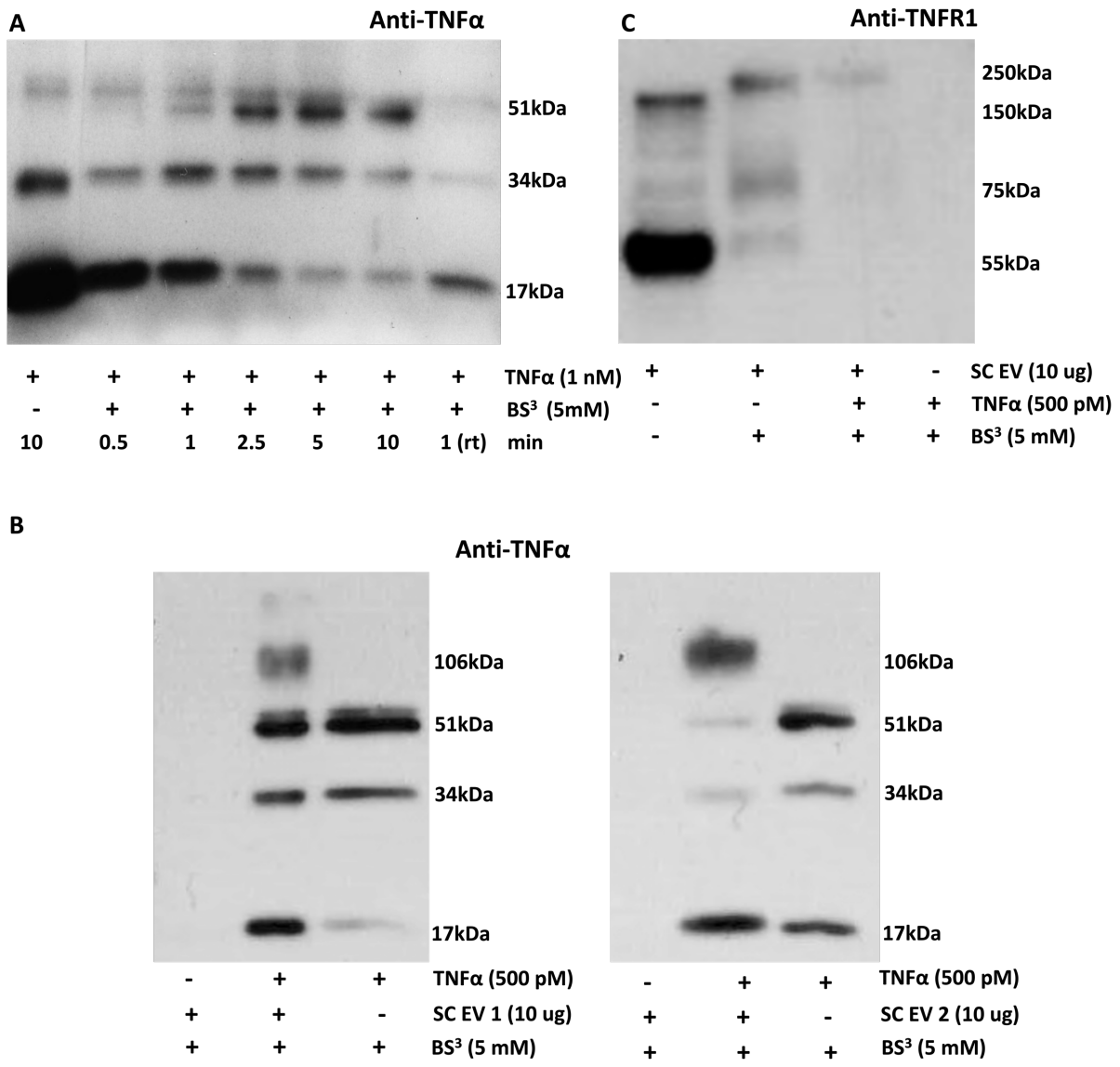


Figure 10: SC EVs bind to TNFα: crosslinking studies. (A) Representative immunoblot of purified TNFα treated with the crosslinker, BS^3 (+) or with vehicle (-) for the indicated times at 37°C. (B) Immunoblot of two different preparations of isolated SC EVs isolation, which had been incubated: SC EVs + BS^3, SC EV + TNFα treated with BS^3, or TNFα alone treated with BS^3. Note the high molecular mass band seen in the sample of SC EVs + TNFα, compared with TNFα alone. (C) Immunoblot indicating that when SC EVs are crosslinked with BS^3, TNFR1 loses immunogenicity.

3.4 SC EVs regulate the effects of TNFα on SC morphology

To validate that SC EVs can serve as an alternative binding site for TNFα, the morphology of SCs was observed because studies have shown that TNFα can affect cytoplasmic structure of cells (Silveira et al., 2018). Primary cultured SCs were treated for 1.5h with vehicle, TNFα, or TNFα + SC EVS for 1.5h. After 1.5h, the morphology of the cells was imaged by staining for the cytoplasmic SC marker S100b through immunofluorescence (Figure 11A). The TNFα treated SCs appear to have extremely thinned and narrowed cytoplasm compared to the SCs treated with the vehicle. The morphology of SCs that were treated with both TNF and SC EVs was similar to the cells treated with vehicle alone. These results demonstrate that SC EVs attenuate the effects of TNFα and essentially confirms that the TNFR1 on EVs is serving as an alternative binding site.

The morphology of SCs incubated with vehicle, TNFα, and TNFα + SC EVs was further analyzed through densitometry (90 cell/group). SCs that were treated with TNFα alone showed a significant increase in the nucleus to cytoplasm ratio (N/C). In contrast, SCs treated with the vehicle or TNFα + SC EVs showed similar N/C ratios ($p<0.0001$) (Figure 11B).

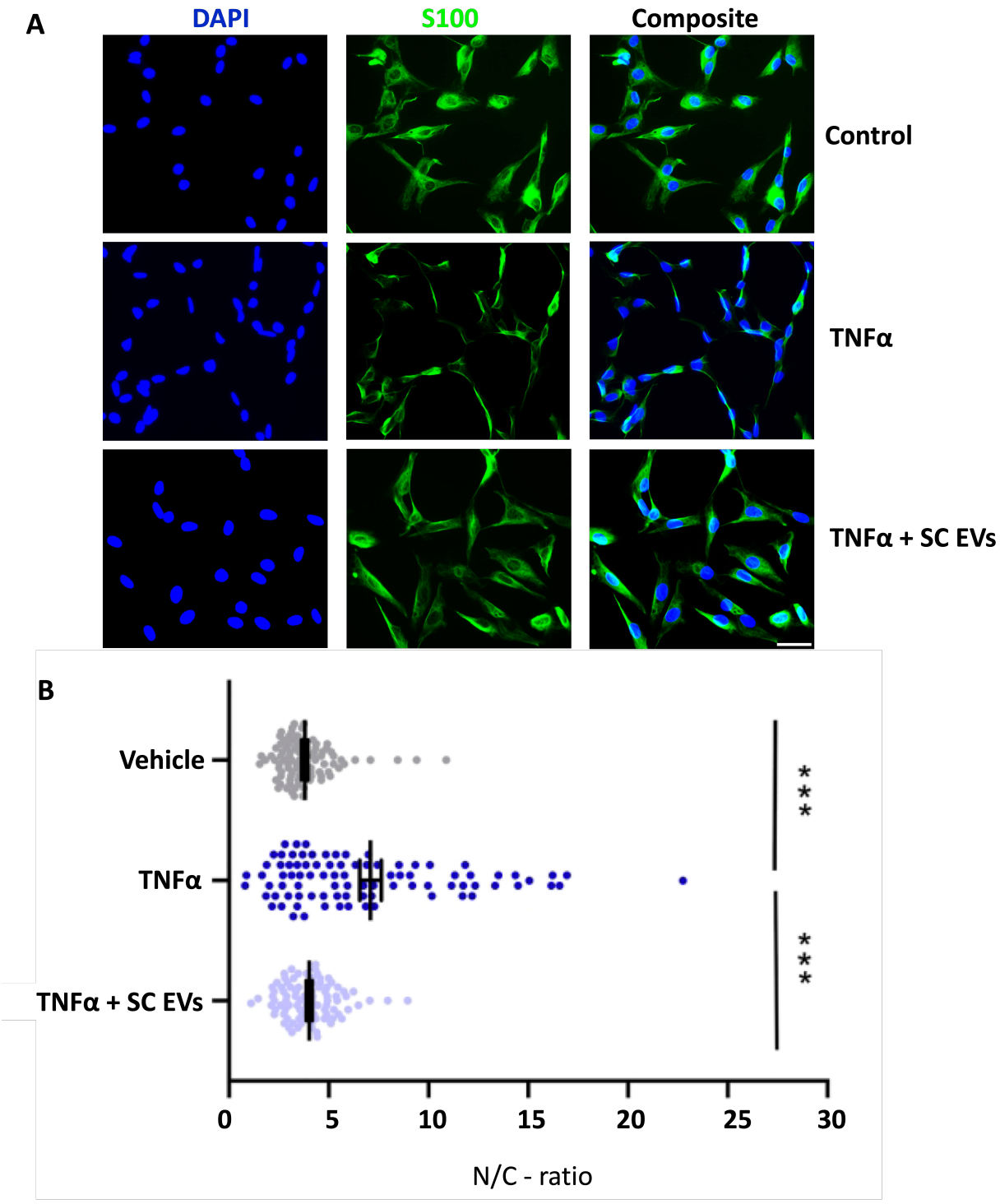


Figure 11: SC EVs attenuate the morphological response of cultured SCs to TNFα *in vitro.* (A) Immunofluorescence of S100b in primary cultured SCs treated with vehicle, TNFα (0.5 nM), or TNFα + SC EVs (10 µg) for 1.5 h. DAPI (blue) labels nuclei. Scale bar, 30 µm. (B) Quantitative analysis of S100b immunofluorescence and DAPI by densitometry. The N/C ratios of SCs treated with vehicle, TNFα or TNFα in the presence of SC EVs were calculated (mean ± SEM.; n=30-40 cells across 3 biological replicates, ****P*<0.001, one-way ANOVA and Tukey's post hoc test).

3.5 Acknowledgements

Chapter 3, in part, has been submitted for publication of the material as it may appear in Glia, 2021 Hirosawa, Nao; Sadri, Mahrou; Le, Jasmine; Romero, Haylie;Kwon, HyoJun; Pizzo, Donald; Ohtori, Seiji; Gonias, Steven; Campana, Wendy. The thesis author was the primary author of this chapter.

The transmission electron microscopy data (Figure 7B) and the immunoblot data (Figure 8A) are from Dr. Nao Hirosawa, a visiting scholar in Dr. Campana's lab.

Chapter 4

Discussion

SCs play an essential role as first responders in peripheral nerve injuries to create an environment that is optimal for nerve regeneration. During WD, SCs undergo drastic changes phenotypically that is essential for the optimization of nerve regeneration. TNFα produced by SCs is essential for signaling for the recruitment of other cytokines and macrophages in a second wave during WD (Shamash et al., 2002; Myers et al., 2006). Pathways initiated by TNFα are also responsible for the perpetuation of neuropathic pain. Therefore, it is pertinent to understand how TNFα is regulated after peripheral nerve injuries because it may contribute to the inflammatory response produced by WD turning neuropathic.

We hypothesized that the TNFR1 present on SC EVs could regulate TNFα by serving as an alternative binding site for TNFα. Expression of TNFR1 has been shown on other EV types such as from endothelial cells (Hawari et al., 2004). We have found that SC EVs isolated from primary SCs also contain TNFR1. Notably we observed that SC EVs are highly enriched with TNFR1 (Figure 9A-C). These results suggested that the TNFR1 on the membrane surface of SC EVs could serve as a binding site for TNFα and is consistent with previous findings in our lab. It is important to point out the high amount of TNFR1 on the SC EVs, as previous data in our lab has demonstrated that TNFR2, the other TNFα receptor, is present on SCs, but not SC EVs. This difference suggests that TNFR1 is actively and specifically sequestered from SCs via EVs. These results are novel and important for understanding how TNFα may be regulated during WD as well as neuropathic pain.

In addition, we found that the copy number of TNFR1 varies among the different SC EVs isolations (Figure 9C). This is consistent with the data we obtained in the cross-

linking studies when two different SC EV isolation were incubated with TNFα (Figure 10B). It demonstrated that one isolation of SC EVs had more copies of TNFR1 (55kDa), as the presence of the dimer and trimer of TNFα was more decreased and the band representing TNFα bound to the SC EV (106kDa) had a greater presence in one preparation compared to the other SC EV preparation. Even though one SC EV preparation appeared to have less copies of monomeric TNFR1, it still shows the additional band above the (106kDa) band. This band likely represents the trimer of TNFα and trimer of TNFR1, which suggests that the trimeric form of TNFR1 can be sequestered in SC EVs. The general mechanism for the biogenesis of EVs is quite defined, however how TNFR1 is processed during the biogenesis of SC EVs is yet to be elucidated.

We anticipated that TNFα binds to TNFR1 on SC EVs. However, we have yet to confirm that when incubating TNFα with SC EVs, the TNFα is bound to the TNFR1 on SC EVs. Previously, we attempted to use a TNFR1 antibody in a Western blot analysis to detect TNFR1 on SC EVs bound to TNFα (Figure 10C). However, we did not observe a band at the expected molecular weight (165kDa) after cross linking (Figure 10B). We hypothesize that immunogenicity is lost due to TNFα blocking the binding site where the TNFR1 antibody would bind on the TNFR1 of SC EVs. Based on other data, it is highly likely that TNFα is bound to the TNFR1 on the EVs since TNFR2 is not present. To verify that TNFα is binding to the TNFR1 on SC EVs other methods will need to be explored. We propose to perform co-immunoprecipitation with a TNFR1 antibody to detect that the TNFR1 on SC EVs is the receptor that is regulating TNFα. This method

uses a coupled antibody that recognizes specific proteins that can be isolated along with any other protein that may be interacting or bound to the specific target protein.

We also show that SC EVs attenuate the effects of TNFα *in vitro* indicated by the differences in SC morphology seen through S100 -immunofluorescence (Figure 11A-B). S100 is a cytoplasmic protein. This is significant to understanding how TNFα may be regulated during peripheral nerve injuries as well as the morphological changes SCs undergo in transdifferentiation after injury. The TNFα treated cells show a morphology that is indicative of how SCs initially respond after a peripheral nerve injury, when TNFα is highly upregulated and SCs start to transdifferentiate into repair SCs (Jessen & Mirsky, 2016; Gomez-Sanchez et al., 2017). Lipopolysaccharide (LPS) treated SCs can induce TNFα expression and produce a similar elongated, spindle like morphology (Cheng et al., 2007). In another study it was shown that lysophosphatidic acid (LPA) treated SCs had a flatter morphology, an indication of myelin sheath production and the cease of SC migration (Weiner et al., 2001). The flatter morphology we saw with the vehicle and SC EVs treated cells suggests that stress induced environments caused by TNFα prevents SCs from transdifferentiating back into mature SCs. Essentially the inability for SCs to reestablish myelin production after a nerve injury would contribute to neuropathic pain.

The implications of these results also suggests that SC communication and responses are regulated by EVs during peripheral pain injuries. During the initial stages of WD, SCs degrade their own myelin through the process of autophagy. It is possible that SC EVs are needed to temporarily inhibit TNFα signaling in order for SCs to have the appropriate time to perform this task. Thus, SC EVs could act as a biological buffer

between the events following injury as a way to optimize the microenvironment for regeneration. This is highly plausible as TNFα recruits other cytokines and macrophages in the second phase of WD. However, it could also be speculated that SC EVs could limit demyelination, which has been known to contribute to pain. It has been demonstrated in an *in vivo* study that p38 MAPK signaling negatively regulates the development of myelination, however signaling is needed for SCs to transdifferentiate into a repair phenotype during demyelination of WD (Roberts et al., 2017). If SC EVs inhibit demyelination by inhibiting SCs from transdifferentiating, the efficiency of WD would be slowed.

In conclusion, our results provide evidence that TNFR1 on SC EVs serve as an alternative binding site for TNFα. The implications of these novel findings indicate a potential autocrine regulation for TNFα in SCs during WD. However, it is possible that SC EVs could serve as paracrine regulator as well. In rat models, typically three to five days after peripheral nerve injuries, immune cells such as macrophages infiltrate the affected area and it is plausible that SC EVs function to communicate between SCs and macrophages (Myers et al., 2006).

In addition, because of EVs' major role in cell communication and their ability to carry various cargos, a popular emerging idea is to utilize the EVs to diagnose diseases. The contents of EVs are altered in diseased conditions, therefore there are various studies trying to utilize EVs as biomarkers for cancer and CNS degenerative conditions (Kanninen et al., 2016; Fitts et al., 2019). It is possible that SC EVs could be a potential biomarker for neuropathic conditions in the PNS. Other clinical applications that are currently being highly explored are using EVs as a drug delivery system or even

having them serve alone as a therapeutic. A previous study in human HEK293 cells engineered EVs containing TNFR1 to serve as a decoy for TNFα to attenuate the effects of inflammation (Duong et al., 2019). Because SC EVs naturally have an abundance of TNFR1, the therapeutic implications for these nanoparticle in neuropathic pain is definitely applicable and should be explored further.

Future Directions

Our studies showed that SC EVs are highly enriched with TNFR1 and that SC EVs attenuate the effects of TNFα physiologically and through cell signaling assays. Because EVs major function in cell communication, studies are under way to elucidate the potential pathways that SC EVs may activate. In addition, uptake studies to determine whether membrane bound TNFα acts as a docking and internalization site for SC EVs remains to be determined. Other studies that are being performed is treating SCs to see how the EVs will change with relation to size as well as cargo.

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