Elucidating the Role of Neuregulin-1 in Glial and Immune Response Following Traumatic Spinal Cord Injury

By

Arsalan Alizadeh

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology and Pathophysiology

Rady Faculty of Health Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

Abstract

Spinal cord injury (SCI) elicits a robust glial and neuroinflammatory response that governs secondary injury processes and causes progressive neural degeneration and loss of neurological function. Activated glial and immune cells produce a plethora of inhibitory and toxic mediators that lay the foundation for a non-permissive microenvironment for neural repair and regeneration. Resident astrocytes and microglia together with blood-borne monocytes, T cells and B cells are the main orchestrators of secondary injury processes following SCI. Despite detrimental effects, these cells possess the ability to play pro-regenerative roles in response to proper modulatory signals from their microenvironment. Hence, identifying new therapeutic targets to promote the supportive aspect of glial and immune response is a viable approach for the treatment of SCI. To develop such targeted therapies, it is necessary to understand the endogenous mechanisms that regulate astrogliosis and neuroinflammation following SCI.

Our team has previously discovered an acute and sustained depletion in spinal cord tissue levels of the neuronally derived Neuregulin-1 (Nrg-1) following SCI. Further studies by our group established a correlation between the injury-induced depletion of Nrg-1 and the inadequate oligodendrocyte replacement after SCI. Similar to oligodendrocytes, glial and immune cells also express Nrg-1 receptors, ErbB2, 3, 4, suggesting potential ramifications of Nrg-1 dysregulation on glial activity and neuroinflammation. Recent studies have also identified an immunomodulatory role for Nrg-1 in ischemic brain injuries. Based on this body of evidence, we hypothesized that diminished tissue levels of Nrg-1 in the injured spinal cord contribute to the imbalanced glial and immune response following SCI.

Using an *in vitro* model of activated astrocytes and microglia and a clinically relevant *in vivo* model of rat compressive-contusive SCI, we unravel the role of Nrg-1 in regulating

astrogliosis and immune response following SCI. We show for the first time that Nrg-1 treatment moderates several detrimental characteristic aspects of activated glia such as chondroitin sulfate proteoglycans (CSPGs), nitric oxide (NO) and pro-inflammatory cytokine production. Mechanistically, we demonstrate that Nrg-1 effects on activated glia are mediated through an ErbB2/3 heterodimer complex and activation of Erk1/2 and STAT3 pathways. In SCI, our comprehensive analysis of immune and glial response using Western blotting, qPCR, immunohistochemistry and flow cytometry shows that Nrg-1 treatment reduces glial scar formation and induces a pro-regenerative regulatory phenotype in T and B cells and macrophages in the spinal cord and peripheral blood during the acute and chronic stages of SCI. Nrg-1 fosters a more balanced post-SCI microenvironment by attenuating antibody deposition and expression of pro-inflammatory cytokines and chemokines such as IL-6, IL-1β and TNF-α while upregulating pro-regenerative mediators such as arginase-1, CCL11 and IL-10. Moreover, Nrg-1 bio-availability significantly improves neural tissue preservation and recovery of neurological function following SCI.

Our work provides novel insights to into the role and mechanisms of Nrg-1 modulation of glial activation and neuroinflammation in SCI. It also establishes the promise of Nrg-1 treatment as a candidate immunotherapy for traumatic SCI and other CNS neuroinflammatory conditions.

Acknowledgements

I would like to thank a number of people whose support and guidance made this work possible:

First and Foremost, I would like to express my sincerest gratitude to my mentor and advisor, Dr. Soheila Karimi. I owe a great debt of gratitude to Soheila for her unparalleled support, patience and guidance. She taught me how to think critically and write scientifically, perform sophisticated surgical and experimental procedures and most importantly, how to be a great team player. Soheila's exceptional mentoring and guidance guarantee her students' success in every step along their academic journey. I was fortunate to be mentored by her which enabled me not only to flourish as a graduate student but also to become a better human being.

My special thanks go to members of my advisory committee: Dr. Christopher Anderson, Dr. Paul Fernyhough and Dr. Phillip Gardiner. Thank you for your support and encouragement throughout these years. I am extremely grateful for your time and dedication in writing reference letters which greatly aided me to win awards and scholarships. I am also grateful for your time and insightful feedbacks during my thesis review.

This work could not be completed without the help and support of a team of great colleagues at Dr. Karimi's lab. I would like to thank Marie-Krystel Gauthier who taught me laboratory techniques during my first year in the training program. I would also like to thank Dr. Scott Dyck who patiently helped me in behavioural analysis of our rodent model of spinal cord injury. His critical feedbacks helped me to find possible flaws and improve the design of my projects. Special thanks to Dr. Hardeep Kataria and Dr. Santhosh Thomas Kallivalappil for their continuous support, critical feedback and guidance. They greatly helped me in both of my PhD projects and were always there during the time of need. Lastly, I would like to thank Ghazaleh

Shahriary, Christopher Hart, Hoang Nguyen, Evan Proulx and Ryan Henrie for either helping or working in different projects alongside me during past 5 years.

I would also like to thank Ms. Gail McIndless and Ms. Judith Olfert for their patience and availability to guide and support during these years.

I would also like to extend my thanks to Dr. Parvin Babaei and Dr. Bahram Soltani my research advisors at Guilan University of Medical Sciences in Iran. They provided me the opportunity to be exposed to basic science research and learn to conduct scientific experiments. Thank you so much for all the support and great opportunities.

Lastly, I would like to thank the funding agencies that supported me and my research project; Research Manitoba and the Children's Hospital Research Institute, the University of Manitoba and the Manitoba Health Research Council. I am also thankful for the travel awards provided by Faculty of Graduate Studies, University of Manitoba Graduate Student Association, Manitoba Neuroscience Network, the Nick Shepel Travel award, Canadian Association for Neuroscience, Manitoba Medical Services Foundation, Drewry Travel Award, Gairdner Foundation, Children's Hospital Research Institute of Manitoba. Thank you for supporting my research and providing me with opportunities to attend great scientific meetings.

This thesis is dedicated to my loving family

My grandmother

Zahra Asadinia

An angel who thought me the art of living, unconditional love and forgiveness.

My grandfather

Ahmad Naghash Souratgar

An example of broad-heartedness and wisdom, from whom I learned dedication and invaluable lessons of life.

My mother

Dr. Narges Naghash Souratgar

Whose heavenly love and endless support paved the way for my success.

This journey was impossible without you.

My father

Alireza Alizadeh

Whose empathy and advise enlightened my path throughout this journey.

My beloved wife

Saba Sedaghat

Although miles away from each other, our hearts were close.

Her love and support was the incentive to continue this challenging journey.

I was blessed to have these wonderful people in my life that cared and supported me.

Thank you all for your endless love and support.

Table of contents

Contribution of the Authors

Components of two manuscripts published in peer-reviewed scientific journals (as listed below are presented in Chapters 2 and 3 of this dissertation. The content of introduction section will be prepared as two review articles: one on the pathophysiology of SCI and the other one on Neurogulin-1 in the central nervous system. These two articles are expected to be submitted in summer 2018. Published papers are listed below.

i) Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315. © John Wiley & Sons, Inc.

My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. I contributed to over 80% of the total experimental procedures, data and analysis. Manuscript preparation was done in collaboration with my supervisor. Scott Dyck contributed to functional assessments and astrocyte activation in vitro. Pure microglia culture and data related to microglia presented in Figure 3 were provided by Hardeep Kataria and Ghazaleh Shahriary. Immunoprecipitation data presented in Figure 4 was provided by Dr. Hardeep Kataria. PCR data in figure 2 were provided by Dr. Santhosh Thomas Kallivalappil.

ii) Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9. © The Author (s).

My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. I contributed to over 80% of the total experimental procedures, data and analysis. Manuscript preparation was performed in collaboration with my supervisor. The collection of PCR data shown in Figure 5 and 6 were collected by Dr. Kallivalappil Thomas Santhosh. Analysis was performed by myself. Dr. Hardeep Kataria assisted with flow cytometry sample preparation. Dr. A.S. Gounni provided guidance and essential feedback in flow cytometry studies.

List of Tables

List of Figures

List of abbreviations

AChR: acetylcholine receptor

AIF: apoptosis inducing factor

AIS: ASIA impairment score

ALS: amyotrophic lateral sclerosis

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APC: antigen presenting cells

APRIL: a proliferation-inducing ligand

ARIA: acetylcholine receptor-inducing activity

ASIA: American spinal cord injury association

BACE: β-site of amyloid precursor protein cleaving enzyme

BAFF: B-cell activating factor

BBB: blood-brain barrier

BCMA: B-cell maturation antigen

BMP: bone morphogenetic proteins

B_{reg}: regulatory B cell

BSB: blood-spinal cord barrier

CCL: C-C motif chemokine ligand

CHL1: neural cell adhesion molecule L1-like protein

CNS: central nervous system

CSF: cerebrospinal fluid

CXCL: C-X-C motif ligand

DC: dendritic cells

EAE: experimental autoimmune encephalomyelitis

ECD: extracellular domain

ECM: extracellular matrix

EGF: epidermal growth factor

EMG: electromyography

ETC: electron transport chain

Fc: fragment crystallisable

FGF: fibroblast growth facor

GABA: γ-aminobutyric acid

GFAP: glial fibrillary acidic protein

I-CLiPs: intramembrane-cleaving proteases

ICAM: intercellular adhesion molecule

ICD: intracellular domain

IgG: immunoglobulin G

IgM: immunoglobulin M

IFN-Ɣ: interferon gamma

IL-1β: interleukin-1 beta

IL-6: interleukin 6

IL-12: interleukin 12

ISP: intracellular sigma peptide

LPS: lipopolysaccharide

MBP: myelin basic protein

MCP-1: monocyte chemoattractant protein

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

NFkBIZ: NF-kappa-B inhibitor zeta

NGF: nerve growth factor

NLI: neurological level of injury

NMDA: N-methyl-D-aspartate

nNOS: neuronal nitric oxide synthase

NOD SCID: non-obese diabetic severe combined immunodeficient

NPC: neural precursor cell

Nrg-1: neuregulin-1

NT-3: neurotrophin-3

OPC: oligodendrocyte precursor cell

PARP: poly (ADP ribose) polymerase

PI3K: Phosphoinositide 3-kinase

PNS: peripheral nervous system

PSD-95: postsynaptic density protein of 95 kDa

PTPσ: protein tyrosine phosphate sigma

RAG2: recombinant activating gene 2

rhNrg-1β1: recombinant human Nrg-1β1

SCI: spinal cord injury

SNP: single nucleotide polymorphism

TACE: tumor necrosis factor-α converting enzyme

TCR: T-cell receptor

T_h: helper T cell

TIA: trauma induced autoimmunity

TNF: tumor necrosis factor

T_{reg}: regulatory T cell

VCAM: vascular cell adhesion molecule

VEGF: vascular endothelial growth factors

Chapter 1: Introduction

The content of this section will be prepared as two review articles: one on the pathophysiology of SCI and the other one on Neurogulin-1 in the central nervous system. These two articles are expected to be submitted in summer 2018.

1.1 Traumatic Spinal Cord Injury: Overview of Clinical Impact and Epidemiology

Spinal cord injury (SCI) is a devastating event with tremendous psychological and financial burdens on the patients and society [1]. The annual global incidence of SCI reaches 250-500 thousand new cases per year, with over 4000 new cases in Canada [1, 2]. Currently, over 85,000 SCI patients live in Canada [1, 2]. Annual medical costs of SCI in Canada are estimated at $2.7 billion with the total life-time medical cost of one SCI patient ranging from $1.5 million for paraplegia to $3 million for tetraplegia [3]. Etiologically, more than 90% of SCI cases are traumatic and the rest are caused by diseases or degenerative conditions. The majority of traumatic cases are due to preventable causes such as traffic accidents, road rage, violence or falls [1]. Studies have reported a male-to-female ratio of 2:1 for SCI, which happens more frequently in adults compared to children [1]. Demographically, men are mostly affected during their early and late adulthood (3^{rd} and 8^{th} decades of life) [1], while women are at higher risk during their adolescence (15-19 years) and 7^{th} decade of their lives [1]. The outcomes of SCI depend on the severity and location of the lesion, and may include partial or complete loss of sensory and/or motor function below the level of injury. Lower thoracic lesions can cause paraplegia while lesions at cervical level are associated with quadriplegia [4]. Given the socioeconomic impact of SCI, it is critical to unravel SCI mechanisms and develop new effective treatments for this devastating condition.

1.2 Pathophysiology of Spinal Cord Injury

1.2.1 Primary Spinal Cord Injury

Primary SCI is referred to the tissue damage caused by the mechanical forces delivered to the spinal cord at the time of injury [5]. The most common form of traumatic SCI in clinical settings is compressive-contusive caused by displaced vertebrae [5]. Other mechanisms of primary injury include but are not limited to distraction, acceleration-deceleration and laceration [6]. These forces directly damage the axons and spinal pathways and disrupt blood vessels and cell membranes [6, 7]. The extent of the primary injury determines the severity and outcome of SCI [8, 9].

1.2.1.1 An Overview on SCI Clinical Classification Systems

Functional classification of SCI has been developed to establish reproducible scoring systems by which the severity of SCI could be measured, compared and correlated with the clinical outcomes [10]. Several scoring systems have been used for clinical classification of neurologic deficits following SCI. The first classification system was developed by Frankel and colleagues in 1969. They assessed the severity and prognosis of SCI using numerical sensory and motor scales [4]. It was a 5-grade system in which Grade A was the most severe SCI with complete loss of sensory and motor function below the level of injury [4]. Grade B represented complete motor loss with preserved sensory function and sacral sparing [4]. Patients in Grade C and D had different degrees of motor function preservation and Grade E represented normal sensory and motor function [4]. The Frankel scale was widely used after publication due to its ease of use. However, lack of clear distinction between Grades C and D and inaccurate categorization of motor improvements in patients over time, led to its replacement by other scoring systems [4].

Other classification methods followed Frankel's system. In 1987, Bracken et al. at Yale University School of Medicine classified motor and sensory functions separately in a 5 and 7-scale systems, respectively [11]. This scoring system failed to account for sacral function [4]. Moreover, integration of motor and sensory classifications was impossible in this system [4] and it was abandoned due to complexity and impracticality in clinical settings [4]. Several other scoring systems were developed in 1970's and 1980's by different groups such as Lucas and Ducker at the Maryland Institute for Emergency Medical Services in late 1970's [12], by Klose and colleagues at the University of Miami Neuro-spinal Index (UMNI) in early 1980s [13] and by Chehrazi and colleagues (Yale Scale) in 1981 [14]. All of these scoring systems also became obsolete due to failure in evaluation of sacral functions, difficulty of use or discrepancies between their motor and sensory scoring sub-systems [4].

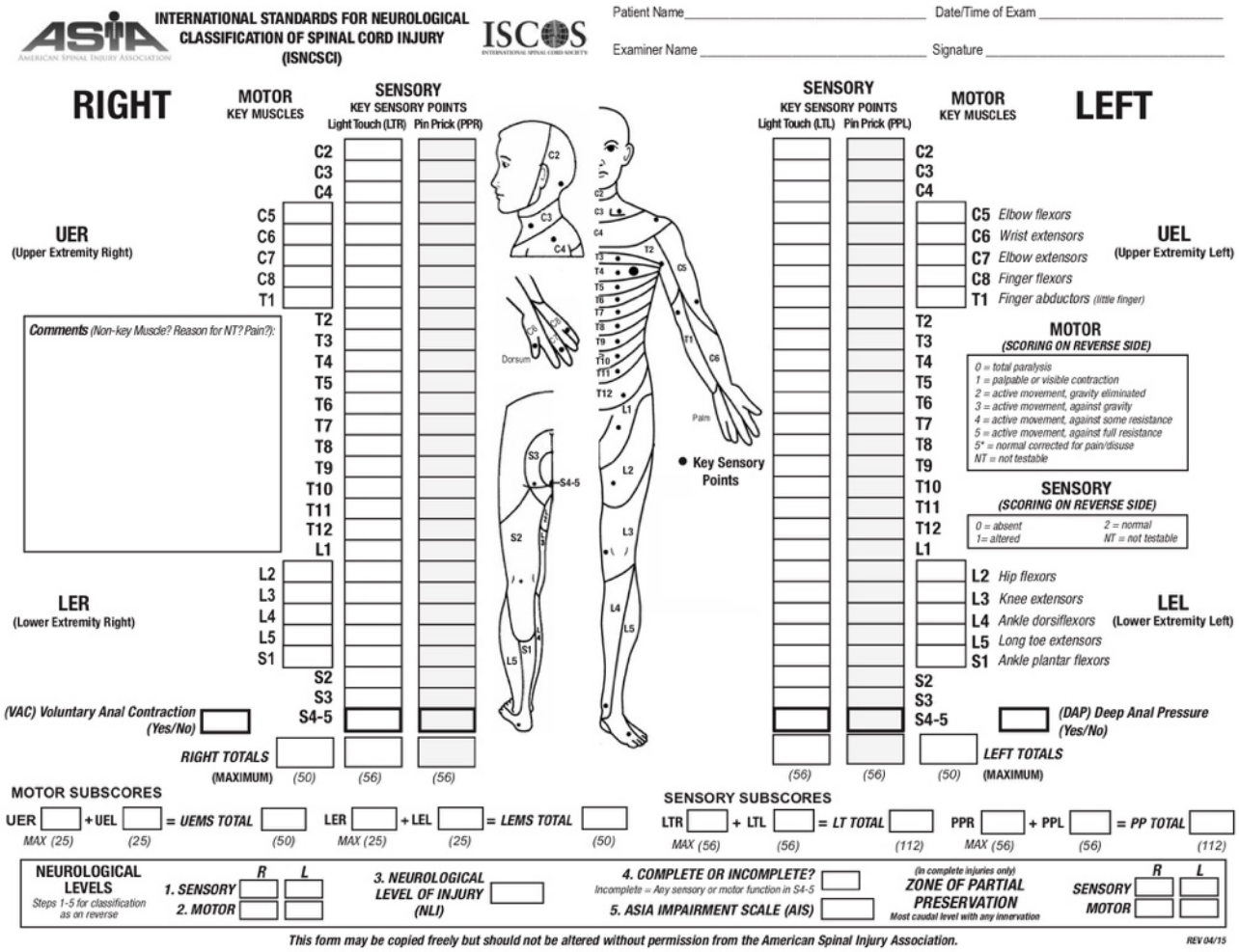
1.2.1.2 American Spinal Injury Association (ASIA) Scoring System

The ASIA scoring system is currently the most widely accepted and employed clinical scoring system for SCI. ASIA was developed in 1984 by the American Spinal Cord Injury Association [4] and has been updated over time to improve its reliability [4]. In this system sensory function is scored from 0-2 and motor function from 0-5 [4]. The aim is to classify the SCI outcomes based on ASIA impairment score (AIS) which ranges from complete loss of sensation and movement (AIS=A) to normal neurological function (AIS=E) [4]. Details of the AIS and motor scoring components of the ASIA scoring system are provided in Fig. 1.1. The first step is to identify the neurological level of injury (NLI) [4]. To determine NLI properly, it is critical to distinguish it from the skeletal level of injury. Except upper cervical spinal column that closely overlay the underlying spinal cord level, the anatomical relationship between the spinal cord segments and

their corresponding vertebrae is not reciprocally aligned [4]. At thoracic and lumbar levels, each spinal column overlay an spinal cord segment one or two levels below [4] and as the result, a T11 vertebral burst fracture results in neurological deficit at and below L1 spinal cord segment [4]. Hence, the neurological level of injury (NLI) is defined as the most caudal neurological level at which all sensory and motor functions are normal [4].

Upon identifying the NLI, if the injury is complete (AIS=A), zone of partial preservation (ZPP) should be determined [4]. ZPP is defined as all of the segments below the NLI that have some preserved sensory or motor function [4]. A precise record of ZPP enables the examiners to distinguish spontaneous from treatment-induced functional recovery, thus, essential for evaluating the therapeutic efficacy of treatments [4]. Complete loss of motor and preservation of some sensory functions below the neurological level of the injury is categorized as AIS B [4]. If motor function is also partially preserved below the level of the injury, AIS score can be C or D [4]. The AIS is scored D when the majority of the muscle groups below the level of the injury exhibit strength level of 3 or higher (for more details see Fig. 1.1) [4]. ASIA classification is the most current scoring system which combines the assessments of motor, sensory and sacral functions thus eliminating the weaknesses of previous scoring systems [4]. Its validity and reproducibility combined with its accuracy in prediction of patients' outcome, have made it the most accepted and reliable SCI scoring system utilized in clinical settings [4].

Fig. 1.1: ASIA scoring for the assessment of the SCI. a sample scoring sheet used for ASIA scoring in clinical setting is shown above (adopted from: http://asia-spinalinjury.org)



1.2.1.3 Spontaneous Recovery and Prognosis

In clinical management of SCI, physicians classify the severity of SCI using ASIA scoring system at 72 hours after injury to choose an appropriate management strategy for the patient [4]. As time passes, SCI patients experience some spontaneous recovery of motor and sensory functions [4]. Most of the functional recovery occurs during the first 3 months and in most cases reaches a plateau by 9 months after injury [4]. However, additional recovery may occur up to 12 to 18 months post-injury [4]. Long term outcomes of SCI are closely related to the level of the injury and the severity of the primary injury that will be described below [4].

Paraplegia is defined as an impairment of sensory or motor function in lower extremities [4]. Patients with incomplete paraplegia usually have a good prognosis in regaining full locomotor ability (~76% of patients) within a year [15]. Complete paraplegics however, experience no recovery of lower limb function if their NLI is above T9 [16]. An NLI below T9 is associated with 38% chance of regaining some lower extremity functions [16]. In patients with complete paraplegia, the chance of recovery to an incomplete status is only 4% with only half of them regaining bladder and bowel control [16].

Tetraplegia is defined as partial or total loss of sensory or motor function in all four limbs [4]. Patients with incomplete tetraplegia will gain better recovery than complete tetra- and paraplegia [17]. Unlike complete plegia, recovery from incomplete tetraplegia usually happens at multiple levels below the NLI [4]. Patients reach a plateau of recovery within 9 to 12 months after injury [4]. Regaining some motor function within the first month after the injury is associated with a better functional outcome [4]. Moreover, appearance of muscle flicker in the lower extremities is highly associated with recovery of function [18]. Patients with complete tetraplegia, mostly (66-90%) regain function at one level below the injury [17]. Initial muscle strength is an important

predictor of functional recovery in these patients [4]. Complete tetraplegic patients with cervical SCI can regain antigravity muscle function in 27% of the cases when their initial muscle strength is 0 on a 5-point scale [19]. However, the rate of regaining antigravity muscle strength at one caudal level below the injury increases to 97% when the patients have initial muscle strength of 1 to 2 out of 5 [20].

Studies have demonstrated an association between sensory and motor recovery [21]. Spontaneous sensory recovery usually follows the pattern of motor recovery [4] and maintenance of pinprick sensation at the zone of partial preservation or in sacral segments is a reliable predictor of motor recovery [22]. The reason for this association is that pinprick fibers in lateral spinothalamic tract travel in proximity of motor fibers in the lateral corticospinal tract, thus preservation of sensory fibers can be a good indicator of the motor fiber integrity [4]. Diagnosis of an incomplete injury is of great importance and failure to detect sensory preservation at sacral segments results in an inaccurate assessment of prognosis [4].

1.2.1.4 Animal Models of SCI

1.2.1.4.1 An Overview of Available Experimental Models

Animal models of SCI have been developed to allow understanding the biomedical mechanisms of SCI, and developing therapeutic strategies for this condition. [23]. Treatment of SCI is challenged by a multitude of factors including a complex inflammatory and glial response, vascular disruption, substantial loss of neurons and oligodendrocytes and consequently degeneration or demyelination of axons [23]. Therefore, attempts have been made to develop animal models that can reproduce the complex pathophysiology of SCI. An ideal animal model should have several characteristics including its relevance to the pathophysiology of human SCI,

reproducibility, availability, and its potential to produce various severities of injury [23]. Among the currently available animal models of SCI, rodent models are the most frequently used due to their availability, ease of use and cost-effectiveness compared to primates and larger non-primate models of SCI [23]. Among rodents, rats are preferred to mice as they more closely mimic pathophysiological, electrophysiological, functional and morphological features of human SCI [24]. However, mouse models have been used extensively in genetic studies to identify injury mechanisms [24]. In recent years, primates and other larger animals have been used in pre-clinical evaluation [25-27]. The following sections will overview available SCI models based on the mechanism of injury, their specifications and relevance to human SCI.

1.2.1.4.2 Transection Models

A complete transection model is relatively easy to reproduce [25]. However, this model is less relevant to human SCI as a complete transection of the spinal cord rarely happens in clinical cases [25]. Transection models are specifically suitable for studying axonal regeneration or developing biomaterials to bridge the gap between proximal and distal stamps of the severed spinal cord [25]. Due to complete disconnection from higher motor centers, this model is also suitable for studying the role of propriospinal motor and sensory circuits in partial recovery of locomotion following SCI [25, 28]. Partial transection, hemi-section and unilateral transection of the spinal cord are other variants of transection models that are more likely to happen in clinical settings [25]. Partial transection can be used to compare the histopathological outcome between healthy and injured tissue in an injured animal [25]. The hemi-section method leads to a less severe injury and higher magnitude of spontaneous recovery rendering this method unsuitable for studying therapeutic effects of new therapies [25]. Partial transection methods are more suitable for investigating nerve

grafting, plasticity and wherever a comparison between injured and non-injured pathways in the same animal is needed [25]. Inconsistency of severity is the major limitation of the partial transection methods [25].

1.2.1.4.3 Contusive Models

Contusion is caused by a transient acute impact to the spinal cord [25]. There are currently three types of devices that can inflict contusion injury in animal models: weight-drop apparatus, electromagnetic impactor and a recently introduced air gun device [25]. The impactor model was first introduced in New York University (NYU) by Gruner in 1992 [29]. The original NYU impactor included a metal rod of specific weight (10 g) that could be dropped on the exposed spinal cord from a specific height to induce SCI [25]. The severity of SCI could be defined by adjusting the height from which the rod fell [29]. Parameters such as time, velocity at impact and biomechanical response of the tissue could be recorded for analysis and verification [25]. The NYU impactor was later renamed to Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor, and conditions surrounding the study and use of the MASCIS impactor were standardized [25]. The MASCIS impactor was revised twice. The most recent version, MACIS III, was introduced in 2012 and included both electromagnetic control and digital recording of the impact parameters [25]. However, inability to control duration of impact and "weight bounce", that could cause multiple impacts, were of known limitations of MASCIS impactors [25].

The Infinite Horizon (IH) impactor is another type of impactor that utilizes a stepping motor to generate force controlled impact in contrast to free fall in the MASICS impactor [25]. This allows for better control over the force of impact and prevents "weight bounce" as the computer controlled metal impounder could be immediately retracted upon transmitting a desired

force to the spinal cord [25]. Three different force levels (100, 150, 200 kdyn) have been available in IH impactors to generate mild, moderate and severe SCI in rats [25]. A limitation with IH impactors is unreliability of their clamps in holding the spinal column firmly during the impact that can cause inconsistent parenchymal injury and neurological deficits [25].

Ohio State University (OSU) impactor is a computer controlled electromagnetic impactor that was developed in 1987 and refined in 1992 to improve reliability [30]. As the impactor is electromagnetically controlled, multiple strikes are avoided [25]. A modified version of OSU impactor was developed in 2000 for use in mice [31]. However, OSU impactor is limited by its inability to determine the precise initial contact point with the spinal cord due to displacement of CSF upon loading the device [25]. MASCIS, IH and OSU impactor devices have been employed successfully to induce SCI and are available for small and large animals such as mice, rats, marmosets, cats and pigs [25, 32].

1.2.1.4.4 Compressive Models

Compressive models of SCI have been also developed and employed for several decades [33]. While contusion injury is achieved by applying a force for a very brief period (milliseconds), the compression injury consists of an initial contusion for milliseconds followed by a prolonged compression through force application for a longer duration (seconds to minutes) [25]. Thus, compression injury can be categorized as contusive-compressive models [25]. Various models of compressive SCI are available.

1.2.1.4.4.1 Clip Compression

Clip compression is the most commonly used compression model of SCI [25, 33-35]. It was first introduced by Rivlin and Tator in 1978 [33]. In this model, following laminectomy at desired spinal segment, a modified aneurism clip with a specific closing force is applied to the spinal cord for a specific duration of time (usually 1 minute) to induce a contusive-compressive- injury [25]. The severity of injury can be modified by adjusting the force of the clip and duration of compression [25]. Applying a 50g clip for 1 minute typically produces a severe SCI while a 35g clip creates a moderate to severe injury with the same duration [35]. Aneurysm clips were originally designed for use in rat SCI, however, recently smaller and larger clips have been developed for use in mice [34] and pig models [26]. The clip compression model has many advantages compared to other SCI models. This method is less expensive and easier to perform [25]. Importantly, in contrast to the impactor injury that contusion is only applied dorsally to the spinal cord, the clip compression model provides contusion and compression simultaneously both dorsally and ventrally. Hence, clip compression model more closely mimics the most common form of human SCI which is caused by dislocation and burst compression fractures [35]. However, despite injury severity is calibrated in clip compression model, variables such as the velocity of closing and actual delivered force could not be measured precisely at the time of application [25].

1.2.1.4.4.2 Calibrated Force Compression

Forceps technique is a simple and inexpensive compressive model of SCI which was first used in 1991 for SCI induction in guinea pigs [36]. In this method, a calibrated forceps with a spacer is used to compress the spinal cord bilaterally [25]. This model does not mimic human SCI as it lacks the first impact and contusive injury which happens in most human SCI cases. Accordingly, the

forceps model of compressive SCI is not a relevant model for reproducing human SCI pathology and therapeutic development [25].

1.2.1.4.4.3 Balloon Compression

In the balloon compression model, a catheter with an inflatable balloon is inserted in the epidural or subdural space [25]. The inflation of the balloon with air or saline for a specific duration of time provides the force for SCI induction [25]. This model has been extensively used in primates, dogs and cats [37-39]. It has the same flaw as forceps compression model as it lacks primary contusive impact [25]. Moreover, the velocity and amount of force are unmeasurable [25].

Between compressive and contusive models described above, none can cover all aspects of human SCI [25]. However when compared, impactor and clip compression models more closely mimic the pathophysiology of human SCI and are the most commonly used methods to induce compressive and contusive injuries [25]. Between these two, clip compression models have the advantage of accessibility and lower cost together with closer simulation of the most frequent mechanism of SCI in clinical settings, which includes an initial impact and secondary compressive effect of the fractured vertebrae [35]. In this thesis, we have employed a clip compression model of SCI in rats.

1.2.2 Secondary Mechanisms of Spinal Cord Injury

The concept of secondary SCI was first introduced by Allen in 1911[40]. Studying SCI in dogs, he observed that removal of the post traumatic hematomyelia improved their neurological outcome [41]. He hypothesized that presence of some detrimental factors in the necrotic hemorrhagic lesion causes further damage to the spinal cord [41]. He termed this “biochemical factor” [40]. The term

of secondary injury is now referred to as a series of cellular, molecular and biochemical phenomena that continue to damage spinal cord tissue after the primary impact in traumatic SCI (Fig. 1.2) [41]. These events begin shortly after the initial mechanical injury and continues chronically to further self-destruct spinal cord tissue and impede neurological recovery [41]. In the following sections, I will describe the major components of the secondary mechanisms of SCI.

1.2.2.1 Vascular Damage, Ischemia and Hypoxia

Disruption of spinal cord vascular supply and hypo-perfusion is one of the early consequences of primary injury [42]. Hypovolemia and hemodynamic shock in SCI patients due to excessive bleeding and neurogenic shock result in compromised spinal cord perfusion and ischemia [42]. Larger vessels such as anterior spinal artery usually remain intact [43, 44], while rupture of smaller intramedullary vessels and capillaries which are susceptible to traumatic damage leads to extravasation of leukocytes and red blood cells [42]. Moreover, increased tissue pressure in edematous injured spinal cord and hemorrhage-induced vasospasm in intact vessels further disrupts blood flow to the spinal cord [42, 44]. Grey matter is more prone to ischemic damage as it is highly vascularized and contains neurons with high metabolic demand [44]. Cellular necrosis and release of cytoplasmic content increase extracellular glutamate load causing glutamate excitotoxicity [42]. Ischemia also leads to energy depletion, and reversal of the Na^+ dependent glutamate transport which exacerbates glutamate excitotoxicity [45]. Moreover, re-establishment of blood flow in ischemic tissue leads to further damage due to generation of free radicals [42]. Importantly, ischemia-induced tissue damage elicits an inflammatory response that includes activation of resident astrocyte and microglia, recruitment of immune cells and production of pro-inflammatory cytokines such as IL-1β and TNF-α that will be discussed [42].

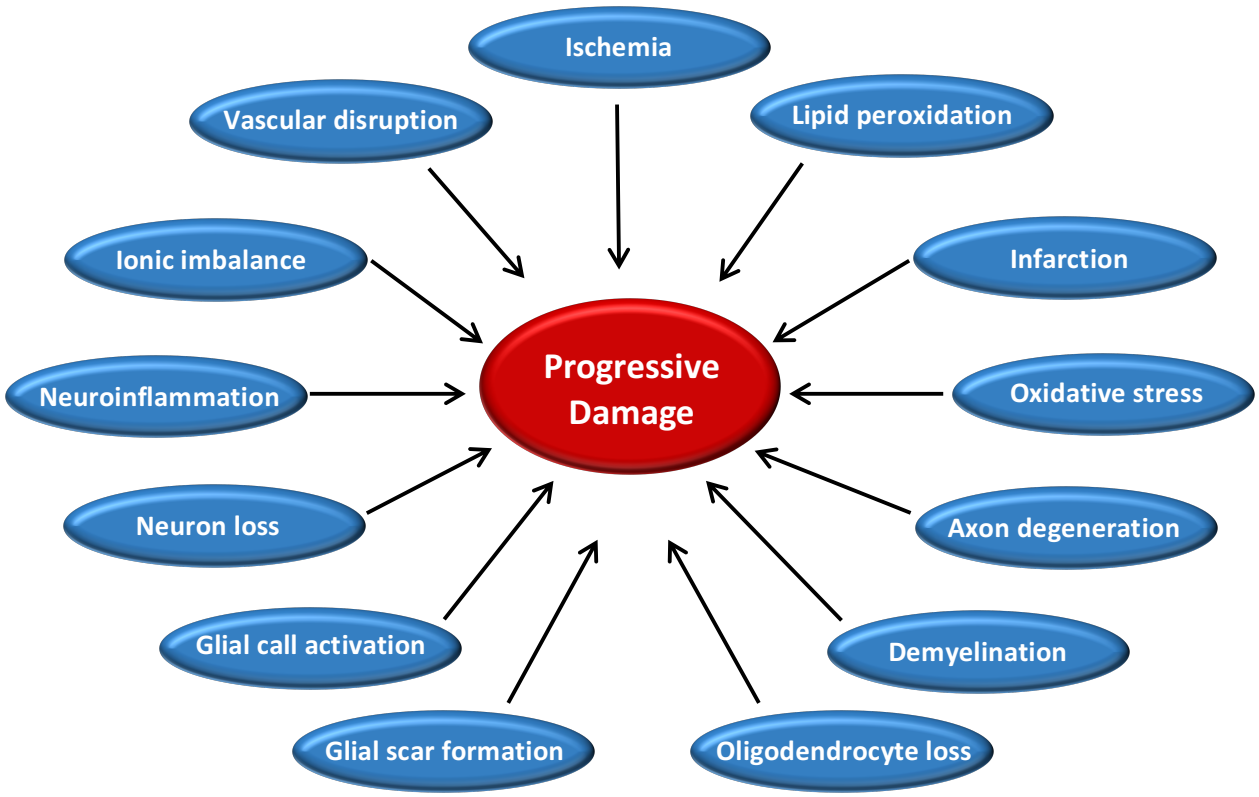


Fig. 1.2: Secondary injury processes following SCI.

Diagram shows the secondary pathophysiological events following SCI that lead to progressive neural tissue damage. Glial cell activation, scar formation and neuroinflammation, the focus of this thesis, are three major pathophysiological events that greatly contribute to neurodegeneration and failure of repair following SCI.

1.2.2.2 Ionic Imbalance and Excitotoxicity

Within few minutes after primary injury, the combination of direct cellular damage and ischemia/hypoxia triggers a significant rise of extracellular level of glutamate, the main excitatory neurotransmitter in the CNS [46]. Glutamate binds to ionotropic (NMDA, AMPA and Kainate receptors) as well as metabotropic receptors [47-49] that results in calcium influx inside the cells [42]. The effect of glutamate is not restricted to neurons as its receptors are vastly expressed on the surface of glial cells such as astrocytes, microglia, endothelial cells and oligodendrocytes [47-50]. Astrocytes can also release excess glutamate extracellularly upon elevation of their intracellular Ca^{++} levels [42]. Reduced ability of astrocytes for glutamate re-uptake from the interstitial space due to lipid peroxidation leads to further accumulation of glutamate in the SCI milieu [42].

In normal condition, concentration of free Ca^{2+} can considerably vary in different parts of the cell [51]. In the cytosol, Ca^{2+} ranges from 50 to 100 nM while it approaches 0.5-1.0 mM in the lumen of endoplasmic reticulum [52-54]. A long lasting abnormal increase in Ca^{2+} concentration in cytosol, mitochondria or endoplasmic reticulum is called calcium overload, which can have detrimental consequences for the cell [51-55]. Mitochondria play the central role in calcium dependent neuronal death [55]. In neurons, during glutamate induced excitotoxicity, NMDA receptor over-activity leads to calcium overload, which can cause apoptotic or necrotic cell death [55]. While the amount of mitochondrial calcium is limited during resting state, they possess the ability to store enormous amount of Ca^{2+} following stimulation [55]. It is known that NMDA receptor over-activity targets mitochondria causing a mitochondrial calcium overload [56, 57]. Calcium overload impedes mitochondrial respiration and results in ATP depletion [58]. Lack of ATP disables Na^+/K^+ ATPase and increases intracellular Na^+ [59]. This reverses the function of

the Na^+ dependent glutamate transporter which normally utilizes Na^+ gradient to transfer glutamate into the cells [59-61]. Moreover, the excess intracellular Na^+ reverses the activity of Na^+-Ca^{2+} exchanger allowing more Ca^+ enter the cells [62]. Cellular depolarization activates voltage gated Na^+ channels that results in entry of Cl^- and water into the cells along with Na^+ causing swelling and edema [63]. Increased Na^+ concentration over-activates Na^+/H^+ exchanger causing a rise in intracellular H^+ [64, 65]. Resultant intracellular acidosis leads to increased membrane permeability to Ca^{2+} that exacerbates the ionic imbalance [64, 65]. Axons are more susceptible to the damage caused by ionic imbalance due to their high concentration of voltage gated sodium channels in the nodes of Ranvier [46].

Evidence shows that administration of Na^+ channel blockers such as Riluzole reduces lesion size and improves functional recovery in compressive SCI in rats underlining sodium as a key player in secondary injury mechanisms [66]. While in the white matter glutamate excitotoxicity is triggered by ionic imbalance, in the grey matter, it is mostly associated with activity of neuronal NMDA receptors [67, 68]. Activation of NMDA receptors and consequent Ca^{2+} overload induces intrinsic apoptotic pathways in neurons and oligodendrocytes and causes cell death during the first week of SCI in the rat [59, 69]. Administration of NMDA receptor antagonist (MK-801) shortly following SCI has been associated with improved functional recovery and reduced edema [70]. Calcium overload also activates a host of protein kinases and phospholipases that results in calpain mediated protein degradation and oxidative damage due to mitochondrial respiratory failure [42].

1.2.2.3 Free Radicals and Oxidative Damage

Following injury, NMDA receptors and mitochondria are shown to contribute to free radical production. NMDA receptors are coupled with Ca^{2+} dependent neuronal nitric oxide synthases

(nNOS) which generate NO upon calcium influx [71]. Shortly after SCI, Ca^{2+} enters mitochondria through the mitochondrial calcium uniporter (MCU) [71]. Mitochondrial Ca^{2+} overload activates NADPH oxidase (Nox) and induce generation of superoxide by electron transport chain (ETC) [71]. Reactive oxygen and nitrogen species (ROS and RNS) produced by the activity of Nox and ETC activates poly (ADP ribose) polymerase (PARP) which resides in the cytosol [71]. PARP consumes and depletes NAD^+ causing failure of glycolysis, ATP depletion and cell death [71]. Moreover, PAR polymers produced by PARP activity, cause release of apoptosis inducing factor (AIF) from mitochondria and induce cell death [71]. On the other hand, acidosis caused by SCI results in the release of intracellular iron stores from ferritin and transferrin [42]. Spontaneous oxidation of Fe^{2+} to Fe^{3+} gives rise to more superoxide radicals [42]. Subsequently, the Fenton reaction between Fe^{3+} and hydrogen peroxide produces highly reactive hydroxyl radicals [72]. The resultant ROS and RNS react with numerous targets including lipids in the cell membrane with the most deleterious effects [42]. ROSs react with polyunsaturated fatty acids in the cell membrane and generate reactive lipids that will then form lipid peroxyl radicals upon interacting with free superoxide radicals in the SCI microenvironment [73, 74]. Each lipid peroxyl radical can react with a neighboring fatty acid, turn it into an active lipid and start a chain reaction that continues until no more unsaturated lipids are available or terminates when the reactive lipid quenches with another radical [42]. The final products of this "termination" step of the lipid peroxidation is 4-hydroxynonenal (HNE) and 2-propenal which are highly toxic to the cells [73-75]. Lipid peroxidation causes ionic imbalance through destabilizing cellular membranes such as cytoplasmic membrane and endoplasmic reticulum [42]. Moreover, lipid peroxidation leads to Na^+/K^+ ATPase dysfunction which exacerbates the intracellular Na^+ overload [76]. In addition to ROS associated lipid peroxidation, amino acids are subject to significant RNS associated oxidative damage

following SCI [42]. RNSs (containing $ONOO^-$) can nitrate the tyrosine residues of amino acids to form 3-nitrotyrosine (3-NT), which is a marker for peroxynitrite ($ONOO^-$) mediated damage to cellular proteins [74]. Oxidation of lipids and proteins is one of the key mechanisms of secondary injury following SCI [42]. Lipid and protein oxidation following SCI has many detrimental consequences at cellular level. These consequences include mitochondrial respiratory failure, DNA alteration and metabolic collapse leading to necrotic and apoptotic cell death [76].

1.2.2.4 Necrotic and Apoptotic Cell Death in SCI

During the acute phase of SCI, neurons and glial cells at the site of impact die due to the mechanical damage [46]. Moreover, excitotoxicity and ionic imbalance cause cell swelling and ruptures the cell membrane [46]. This instantaneous non-programmed cell death is called necrosis [46]. There is no energy requirement for this process in which the damaged cell releases its contents into the extracellular matrix (ECM) which triggers an inflammatory response [77]. On the other hand, within hours of primary injury, a programmed, energy dependent mechanism of cell death called apoptosis will conquer the stage and begin neural tissue destruction in a controlled manner [46]. This process takes place in cells that survive the primary injury but suffer enough insult to activate their apoptotic pathways [77]. In rat SCI, apoptosis occurs in the injured spinal cord as early as 4 hours after the injury reach a peak at 7 day and can be observed at a diminished rate at sub-acute phase (3 weeks post-SCI) [78, 79]. More than 90% of oligodendrocytes at the site of injury are lost within 7 days after SCI [78]. Apoptotic cell death has also been observed in microglia and astrocytes [80, 81]. Even seemingly unaffected neurons and oligodendrocytes distant from the injury epicenter continue to undergo apoptosis at chronic stages of SCI leading to progressive

neurological deficits in SCI patients [82]. Evidence suggests that chronic oligodendrocyte apoptosis is caused by lack of trophic support from degenerating neurons [83, 84].

Apoptosis can occur through extrinsic and intrinsic pathways based on the triggering mechanisms [85]. The extrinsic pathway is triggered by activation of death receptors such as FAS and TNFR1, which eventually activates caspase 8 [82]. The intrinsic pathway, however, is regulated through a balance between intracellular pro- and anti-apoptotic proteins, and is triggered by the release of cytochrome C from mitochondria activating caspase 9 [82]. In SCI lesion, apoptosis primarily happens due to injury induced Ca^{2+} influx, which activates caspases and calpain; enzymes involved in breakdown of cellular proteins [46]. Moreover, it is believed that the death of neurons and oligodendrocytes in remote areas from the lesion epicenter can be mediated through cytokines such as TNF-α, free radical damage and excitotoxicity since calcium from damaged cells within the lesion barely reaches these remote areas [86, 87]. Studies have shown Fas mediated cell death as a key mechanism of apoptosis following SCI [88-90]. Post-mortem studies on acute and chronic human SCI and animal models revealed that Fas mediated apoptosis plays a role in oligodendrocyte apoptosis and inflammatory response at acute and subacute stages of SCI [91]. Fas deficient mice exhibited a significant reduction in apoptosis and inflammatory response evidenced by reduced macrophage infiltration and inflammatory cytokine expression following SCI [91]. Interestingly, Fas deficient SCI mice showed a significantly improved functional recovery [91].

One of the protective mechanisms of cells that can prevent cell death following SCI is autophagy. In autophagy, cells degrade harmful, defective or unnecessary cytoplasmic proteins and organelles by a lysosomal dependent mechanism [92, 93]. The process of autophagy begins with the formation of a phagosome around the proteins and organelles that are tagged for

autophagy [93]. The phagosome will then fuse with a lysosome to form an autolysosome that begins a recycling process [93]. The whole dynamic process of autophagy including formation of autophagosome, fusion with lysosome and degradation in autolysosome is called "autophagic flux" [93]. Studies have suggested a neuroprotective role for autophagy following SCI [92]. Autophagy promotes cell survival through elimination of toxic proteins and damaged mitochondria [94, 95]. Following SCI, dysregulation of "autophagic flux" contributes to neuronal loss [96]. Accumulation of autophagosomes in ventral horn motor neurons have been detected acutely following SCI [97]. Neurons with dysregulated "autophagic flux" exhibit higher expression of caspase 12 and become more prone to apoptosis [97]. Recent studies have also unraveled a crucial role for autophagy in neuronal survival and axon growth after SCI [98]. Autophagy is shown to be crucial in cytoskeletal remodeling in neurons [98]. Autophagy seems to stabilize neuronal microtubules by degrading SCG10, a protein involved in microtubule disassembly in neurons, hence promoting axon growth [98]. Pharmacological induction of autophagy in a hemi-section model of SCI in mice was associated with improved neurite outgrowth and axon regeneration, indicating the critical role of "autophagic flux" in overcoming neuronal stress following SCI [98].

1.2.2.5 Neuroinflammation

1.2.2.5.1 An Overview of Adaptive and Innate Immune Response in SCI

Neuroinflammation is a key component of the SCI secondary injury mechanisms with local and systemic consequences. Neuroinflammation has been implicated in both tissue damage and repair [99, 100]. Following SCI, resident astrocytes and microglia initiate an inflammatory response by producing inflammatory chemokines and cytokines and facilitating the recruitment of blood-borne leukocytes [101, 102]. Neutrophils and macrophages, the main players of the innate immune

response, infiltrate the spinal cord during acute stages of SCI and play essential roles in shaping the adaptive immune response by T and B lymphocytes at later stages [103, 104]. T lymphocytes become activated in response to antigen presentation by macrophages, microglia and other antigen presenting cells (APCs) [105]. $CD4^+$ helper T cells produce cytokines that stimulate B cell antibody production and activate phagocytes [106]. In SCI, B cells produce autoantibodies against spinal cord tissue, which exacerbate neuroinflammation and cause tissue destruction [107]. Interestingly, based on the signals received from their microenvironment, all immune cells are capable of differentiating into pro-regenerative phenotypes and contribute to spinal cord repair and regeneration [108-111]. The role of each immune cell in the pathophysiology of SCI will be discussed in detail in upcoming sections.

1.2.2.5.2 Astrocytes

Astrocytes are the most abundant glial cells in the CNS [112]. While they are not considered an immune cell, they play pivotal roles in the neuroinflammatory processes in CNS injury and disease. Their histo-anatomical localization in the CNS has placed them in a strategic position for participating in physiological and pathophysiological processes in the CNS [113]. In normal CNS, astrocytes play major roles in maintaining CNS homeostasis. They contribute to the structure and function of blood-brain-barrier (BBB), provide nutrients and growth factors to neurons [113], and remove excess fluid, ions, and neurotransmitters such as glutamate from synaptic spaces and extracellular microenvironment [113]. Astrocytes also play key roles in the pathologic CNS by regulating BBB permeability and reconstruction as well as immune cell activity and trafficking [112]. Importantly, astrocytes undergo astrogliosis and form a glial scar around the lesion that aids in limiting the extent of immune mediated tissue destruction [114, 115]. In addition to scar

formation, astrocytes contribute to both innate and adaptive immune responses following SCI [112] by differential activation of their intracellular signaling pathways in response to microenvironmental signals [112].

Astrocytes react acutely to CNS injury by increasing cytokine and chemokine production [116]. It is shown that astrocytes mediate chemokine production and recruitment of neutrophils through an IL-1R1-Myd88 pathway [116]. Activation of the nuclear factor kappa b (NF-κB) pathway, one of the key downstream targets of IL1R-Myd88 axis, increases expression of intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) both of which are necessary for adhesion and extravasation of leukocytes in inflammatory conditions such as SCI [112, 116]. Within minutes of injury, the levels of IL-1β becomes significantly elevated in astrocytes and microglia [117]. Moreover, chemokines such as monocyte chemoattractant protein (MCP)-1, chemokine C-C motif ligand 2 (CCL2), C-X-C motif ligand 1 (CXCL1) and CXCL2 are produced by astrocytes, and enhance the recruitment of neutrophils and pro-inflammatory macrophages following injury [112, 116]. Astrocytes also promote pro-inflammatory M1 polarization in macrophages and microglia in the injured spinal cord through their production of TNF-α, IL-12 and IFN-ɣ [118-120]. Interestingly, astrocytes have shown to produce anti-inflammatory cytokines, such as TGF-β and IL-10, which can promote a pro-regenerative M2 phenotype in macrophages and microglia [112, 121, 122]. This evidence collectively demonstrates the ability of astrocytes to regulate the outcomes of the inflammatory response in the acute phase of CNS injury [112].

In addition to the acute regulation of innate immune response, astrocytes modulate adaptive immunity through regulation of T and B lymphocytes [112]. Astrocytes produce a host of chemokines such as CCL5, CXCL10 and CXCL12 in CNS infection models and in patients with

multiple sclerosis [123-127]. These chemokines are known to act as chemoattractant for T and B lymphocytes [112]. Moreover, studies on animal experimental autoimmune encephalomyelitis (EAE) models revealed that astrocytes can direct T cell phenotype by producing pro- or anti-inflammatory cytokines such as TNF-α, IL17, IFN-ɣ and IL-10 [112, 122, 128]. Interestingly, astrocytes can also activate T lymphocytes by presenting antigens through an MHCII dependent antigen presenting system [129].

The role of astrocytes as pro- or anti-inflammatory mediators is defined by the signaling pathways activated through a wide variety of surface receptors [113]. For example, activation of gp130, a member of IL-6 cytokine family, activates SHP2/Ras/Erk signaling cascade in astrocytes and limits neuroinflammation in animal models of EAE [128]. TGF-β signaling in astrocytes has been implicated in modulation of neuroinflammation through inhibition of NF-κB activity and nuclear translocation [112, 130]. STAT3 is another signaling pathway in astrocytes with beneficial properties in neuroinflammation. Increases in STAT3 phosphorylation promote rapid scar formation and limit spreading of inflammatory cells in mouse SCI, which is associated with improved functional recovery [131]. Detrimental signaling pathways in astrocytes are known to be induced by cytokines, sphingolipids and neurotrophins [113]. As an example, IL-17 is an important pro-inflammatory cytokine released by effector T cells that can bind to IL-17R on the astrocyte surface [113]. Activation of IL-17R results in the activation of NF-κB, which promotes transcription of pro-inflammatory cytokines, activation of oxidative pathways and exacerbation of neuroinflammation [113, 132]. This evidence shows the significance of astrocytes in the inflammatory processes following SCI and autoimmune diseases of the CNS.

1.2.2.5.3 Neutrophils

Neutrophils infiltrate the spinal cord from the bloodstream within the first few hours after injury [133]. Their population increases acutely in the injured spinal cord tissue and reaches a peak within 24 hours post-injury [103]. The presence of neutrophils is mostly limited to the acute phase of SCI as they are rarely found sub-acutely in the injured spinal cord [103]. The role of neutrophils in SCI pathophysiology is controversial. Evidence shows that neutrophils contribute to phagocytosis and clearance of tissue debris[134]. They release inflammatory cytokines, proteases and free radicals that degrade extracellular matrix (ECM), activate astrocytes and microglia and initiate neuroinflammation [134]. Although neutrophils have been conventionally associated with tissue damage [134, 135], their elimination compromises healing process and impedes functional recovery [136].

To elucidate the role of neutrophils in SCI, Stirling and colleagues used a specific antibody to reduce circulating LyG6/Gr1^{+} neutrophils in a mouse model of thoracic contusive SCI [136]. This approach significantly reduced neutrophil infiltration in the injured spinal cord by 90% at 24 and 48 hours after SCI [136]. Surprisingly, neutrophil depletion aggravated the neurological and structural outcomes in the injured animals suggesting a beneficial role for neutrophils in the acute phase of injury [136]. Evidence shows that stimulated neutrophils release IL-1 receptor antagonist that can exert neuroprotective effects following SCI [137]. Moreover, studies have suggested a critical role for neutrophils in growth factor regulation following SCI [136]. Ablation of neutrophils results in altered expression of cytokines and chemokines and downregulation of growth factors such as fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and bone morphogenetic proteins (BMPs) in the injured spinal cord that seemingly disrupt the normal healing process [136]. Interestingly, this downregulation is followed by a significant upregulation of the above-mentioned growth factors at later time-points following SCI.

This could represent a detrimental dysregulation of growth factor expression in the absence of neutrophils as extra growth factor is not always favorable [136]. As an example, increase in FGFs can upregulate the production of an adhesion molecule named CHL1 (neural cell adhesion molecule L1-like protein), which can potentially hinder SCI repair by inhibiting neurite outgrowth [138]. Moreover, studies have shown that inhibition of BMPs, growth factors involved in CNS development, can improve SCI outcome [139]. This evidence emphasizes that extra growth factors can exert detrimental effects and indicates the critical role of neutrophils in balancing growth factor milieu during acute phase of SCI.

In conclusion, neutrophils play key roles in regulating neuroinflammation at the early stage of SCI that shapes the immune response and repair processes at later stages. While neutrophils were originally viewed as being detrimental in SCI, emerging evidence shows their critical role in the repair process. More research is necessary to further elucidate the role of neutrophils in SCI pathophysiology.

1.2.2.5.4 Microglia and Macrophages

Following neutrophil invasion, macrophages and microglia begin to populate injured spinal cord within 2-3 days post-SCI. The population of macrophages and microglia reaches its peak at 7 to 10 post-injury in mouse SCI, followed by a decline in the subacute and chronic phases [4, 140]. While macrophages and microglia share many functions and immunological markers, they are believed to have different origins. Microglia are resident immune cells of the CNS that originate from yolk sac during the embryonic period [141]. Macrophages are derived from blood monocytes, which originate from myeloid progeny in the bone marrow [99, 142]. Under normal circumstances, blood-spinal cord barrier (BSB) prevents immune cells from entering the spinal cord [99]. Upon

injury, acute disruption of BSB enables monocyte to infiltrate the spinal cord tissue and transform into macrophages [99]. Monocyte derived macrophages populate the injury epicenter while resident microglia are mainly located in the perilesional area [99]. Once activated, macrophages and microglia are morphologically and immunohistologically indistinguishable [4]. Macrophages and microglia play a beneficial role in CNS regeneration. They promote the repair process by expression of growth promoting factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3) and thrombospondin [143, 144]. Macrophages and microglia are important for wound healing process following SCI due to their ability for phagocytosis and scavenging damaged cells and myelin debris following SCI [99, 145].

Based on microenvironmental signals, both macrophages and microglia can be polarized to either pro-inflammatory (M1) or pro-regenerative (M2) phenotype and accordingly contribute to injury or repair processes following SCI [146]. Classically activated M1 macrophages and microglia can be induced by exposure to T_h1 specific cytokine, interferon (IFN)-γ [146]. Moreover, studies have revealed that the SCI microenvironment drives M1 polarization of activated macrophages [146]. M1 macrophages and microglia produce pro-inflammatory cytokines such as IL-1β, TNF-α and free radicals which contribute to secondary injury process in SCI [146-148]. Following SCI, activated M1 macrophages and microglia highly express MHCII and present antigens to T cells and contribute to the activation and regulation of innate and adaptive immune response (Fig. 1.3) [146]. Studies on acute and subacute SCI and EAE models have shown that M1 macrophages are associated with higher expression of chondroitin sulfate proteoglycans (CSPGs) and increased EAE severity and tissue damage [149-152]. *In vitro,* addition of activated M1 macrophages to dorsal root ganglion (DRG) neuron cultures leads to axonal retraction and failure of regeneration as the expression of CSPGs is 17 times higher in M1 compared to M2

macrophages [152, 153]. M1 macrophages also produce other repulsive factors such as repulsive guidance molecule A (RGMA) that is shown to induce axonal retraction following SCI [154, 155].

Alternatively activated (M2) macrophages and microglia, are polarized by T_h2 cytokines, IL-4 and IL-13 and exhibit a high level of IL-10, TGF-β and arginase-1 with reduced NF-κB pathway activity [146]. IL-10 is a potent immune-regulatory cytokine with positive roles in repair and regeneration following CNS injury [156-158]. Studies on IL-10 knock-out mice showed that absence of IL-10 after SCI was associated with increased pro-inflammatory cytokines such as IL-1β, TNF-α and oxidative substances [159]. Lack of IL-10 was also associated with increased expression of pro-apoptotic factors such as Bax and decreased expression of anti-apoptotic factors such Bcl-2 [159]. Local IL-10 administration was shown to attenuate astrocyte activation in a focal brain injury model in the mouse [160]. SCI mice that lacked IL-10 exhibited worsened recovery of function compared to wild-type mice [159]. Low numbers of M2 macrophages following SCI contributes to robust pro-inflammatory response with detrimental effects on neural tissue integrity seen following SCI [146].

In addition to immune modulation, M2 macrophages promote axonal regeneration [146]. However, similar to prolonged M1 macrophage response that is detrimental to spinal cord repair, excessive M2 activity promotes fibrotic scar formation through the release of factors such as TGF-β, PDGF, VEGF, IGF-1, and Galactin-3 [146, 161-163]. Hence, a moderated balance between M1 and M2 macrophage response is essential for the repair of SCI [146].

1.2.2.5.5 T and B Lymphocytes

T and B lymphocytes are the key regulators of the adaptive immune response after SCI [105]. Lymphocytes infiltrate the injured spinal cord acutely during the first week of injury and remain

chronically in mouse and rat SCI [105, 107, 164, 165]. In contrast to the innate immune response which can be activated directly by foreign antigens, the adaptive immune response requires a complex signaling process in T cells that is elicited by antigen presenting cells [166]. Similar to other immune cells, T and B lymphocytes adopt different phenotypes and contribute to both injury and repair processes in response to microenvironmental signals [105, 167]. SCI elicits a CNS-specific autoimmune response in T and B cells, which remains active chronically [107]. Autoreactive T cells can exert direct toxic effects on neurons and glial cells [105, 168]. Moreover, T cells can indirectly affect neural cell function and survival through pro-inflammatory cytokine and chemokine production (e.g. IL-1β, TNF-α, IL-12, CCL2, CCL5 and CXCL10) [105, 168]. Genetic elimination of T cells (in athymic nude rats) or pharmacologic T cell inhibition (using cyclosporine A and tacrolimus) leads to improved tissue preservation and functional recovery after SCI [105, 169] showing the impact of T cells in SCI pathophysiology and repair.

Under normal circumstances, systemic autoreactive effector $CD4^+$ helper T cells (T_{eff}) are suppressed by $CD4^+FoxP3^+$ regulatory T cells (T_{reg}) (Fig. 1.3) [105, 170]. This inhibition is regulated through various mechanisms such as production of anti-inflammatory cytokines IL-10 and TGF-β by the T_{reg} cells (Fig. 1.3) [105]. Moreover, it is known that T_{reg} mediated inhibition of antigen presentation by dendritic cells (DCs) prevent T_{eff} cell activation [105]. Following SCI, this T_{reg} -T_{eff} regulation is disrupted, and increase in the activity of autoreactive T_{eff} cells contributes to tissue damage through production of pro-inflammatory cytokines and chemokines, promoting M1 macrophage phenotype and induction of Fas mediated neuronal and oligodendroglial apoptosis (Fig. 1.3) [91]. Moreover, autoreactive T_{eff} cells promote proliferation and differentiation of antigen specific B cells to plasma cells in which produce autoantibodies and contribute to tissue damage after SCI [171]. In SCI and MS patients, myelin specific proteins such as myelin basic

protein (MBP) significantly increase the population of circulating T cells [172, 173]. Moreover, serological assessment of SCI patients has shown high levels of CNS reactive IgM and IgG isotypes confirming SCI-induced autoimmune activity of T and B cells (Fig. 1.3) [107, 174, 175]. In animal models of SCI, serum IgM level increases acutely followed by an elevation in the levels of IgG1 and IgG2a at later time-points [107]. In addition to autoantibody production, autoreactive B cells contribute to CNS injury through pro-inflammatory cytokines that stimulate and maintain the activation states of T_{eff} cells [105, 176]. SCI studies on B cell knockout mice (BCKO) that have no mature B cell but with normal T cells, showed a reduction in lesion volume, lower antibody levels in the cerebrospinal fluid and improved functional recovery compared to wild-type mice [171]. Of note, antibody mediated injury is regulated through complement activation as well as macrophages/microglia that express immunoglobulin receptors [164, 171].

The effect of SCI on systemic B cell response is controversial. Evidence shows that SCI can suppress B cell activation and antibody production [177]. Studies in murine SCI have shown that B cell function seems to be influenced by the level of injury [178]. While injury to upper thoracic spinal cord (T3) suppressess the antibody production, a mid-thoracic (T9) injury has no effect on B cell antibody production [178]. An increase in the level of corticosterone in serum together with elevation of splenic norepinephrine found to be responsible for the suppression of B cell function acutely following SCI [177]. Elevated corticosterone and norepinephrine leads to upregulation of lymphocyte beta-2 adrenergic receptors eliciting lymphocyte apoptosis [105]. This suggests a critical role of sympathetic innervation of peripheral lymphoid tissues in regulating B cell response following CNS injury [177]. Despite their negative roles, B cells also contribute to spinal cord repair following injury through their immune-modulatory B_{reg} phenotype (Fig. 1.3)

[179]. B_{reg} cells control antigen-specific T cell autoimmune response through IL-10 production (Fig. 1.3) [180].

Detrimental effects of SCI-induced autoimmunity are not limited to the spinal cord. Autoreactive immune cells contribute to the exacerbation of post-SCI sequelae such as cardiovascular, renal and reproductive dysfunctions [105]. For example, presence of an autoantibody against platelet prostacyclin receptor has been associated with a higher incidence of coronary artery disease in SCI patients [181]. Collectively, evidence shows the critical role of adaptive immune system in SCI pathophysiology and repair. Thus, treatments that harness the pro-regenerative properties of the adaptive immune system can be utilized to reduce immune mediated tissue damage and improve neural tissue preservation and repair following SCI.

Fig. 1.3: SCI induced neuroinflammation.

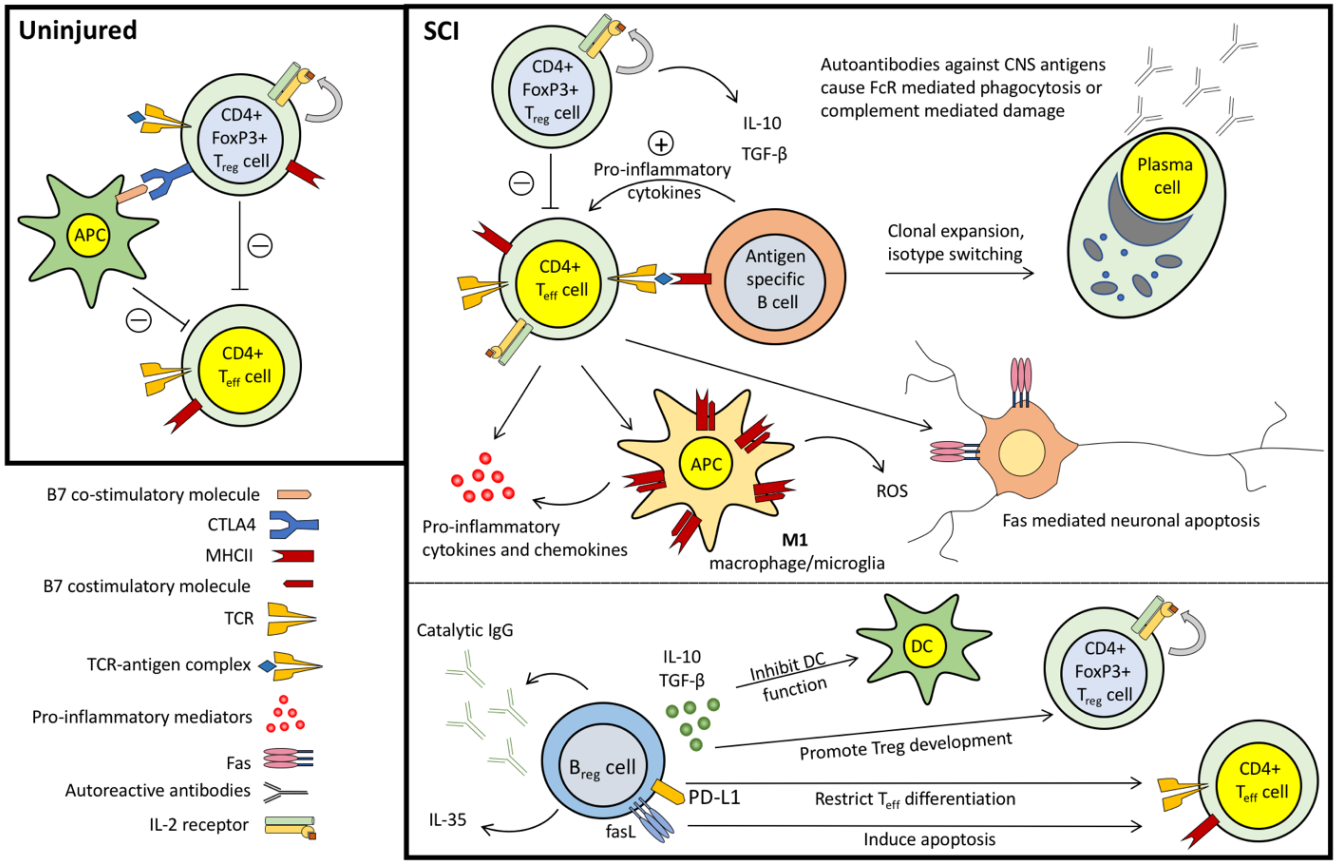


Fig. 1.3: SCI induced neuroinflammation. Under normal circumstances, there is a balance between pro-inflammatory effects of $CD4^+$ T_{eff} and anti-inflammatory effects of T_{reg} and B_{reg} cells where IL-10 and TGF-β produced by regulatory T and B cells suppress the activation of antigen specific $CD4^+$ T_{eff} cells. Injury disrupts this balance. Activated microglia and macrophages release pro-inflammatory cytokines and chemokines and present antigens to $CD4^+$ T cells causing activation of antigen specific effector T cells. T_{eff} cells activate antigen specific B cells to undergo clonal expansion and produce autoantibodies against spinal cord tissue antigens. These autoantibodies cause neurodegeneration through FcR mediated phagocytosis or complement mediated cytotoxicity. M1 macrophages/microglia produce pro-inflammatory cytokines and reactive oxygen species (ROS) that are detrimental to neurons and oligodendrocytes. B_{reg} cells possess the ability to promote T_{reg} development and restrict T_{eff} cell differentiation. B_{reg} cells could also induce apoptosis in T_{eff} cells through Fas mediate mechanisms [Parts of this figure is modified and reproduced with permission from [105]].

1.2.2.6 Glial Scar Formation

Traumatic SCI triggers the formation of a glial scar tissue around the injury epicenter [114]. Activated astrocytes, $NG2^+$ oligodendrocyte precursor cells (OPCs) and microglia contribute to the glial scar by forming a cellular barrier and secreting extracellular matrix (ECM) components such as CSPGs (Fig. 1.4) [182]. Among these cells, activated astrocytes play a significant role in the formation of the glial scar [183]. Following injury, astrocytes increase their expression of intermediate filaments, GFAP, nestin and vimentin, and become hypertrophied [184, 185]. Activated astrocytes proliferate and migrate to the site of injury and form a mesh like structure of intermingled filamentous processes around the injury epicenter [186-188]. The glial scar serves as a barrier that prevents the spread of inflammation into the adjacent segments [186-188]. Reactive astrocytes also upregulate their CSPG production within 24h after injury which persists in the chronically injured spinal cord tissue [189-191].

Infiltrating immune cells also contribute to the process of glial activation and scar formation by producing cytokines (e.g. IL-1β and IL-6) chemokines and enzymes that activate glial cells or disrupt BSB [183]. Activated microglia and macrophages produce proteolytic enzymes such as matrix metalloproteinases (MMPs) that increase vascular permeability by further disruption of the BSB [192]. Inhibition of MMPs has been shown to improve neural preservation and functional recovery in animal models of SCI [192-194].

In addition to glial and immune cells, fibroblasts, pericytes and ependymal cells also contribute to the structure of the glial scar [183]. In penetrating injuries where meninges are compromised, meningeal fibroblasts infiltrate the lesion epicenter and contribute to scar formation [195]. Fibroblasts secrete ECM components such as collagen, laminin and fibronectin [183]. They are also a source of axon-repulsing molecules such as semaphorins that impede axonal

regeneration following SCI [196]. Fibroblasts have also been found in contusive injuries where meninges are intact [197, 198]. Studies using genetic fate mapping in these injuries have revealed that perivascular pericytes and fibroblasts migrate to the injury site and form a fibrotic core in the scar which matures within 2 weeks post-injury [197, 198]. SCI also triggers proliferation and migration of the stem/progenitor cell pool of the spinal cord parenchyma and ependyma. These cells give rise to new astrocytes and $NG2^+$ oligodendrocyte precursor cells that contribute to the formation of glial scar [186, 199, 200]. In a mature glial scar, activated microglia/macrophages occupy the innermost portion closer to the injury epicenter surrounded by $NG2^+$ OPCs (Fig. 1.4) [183], while reactive astrocytes reside in the injury penumbra and form a cellular barrier which limits the spread of injury-related toxins and inflammatory mediators [183].

Although glial scar might initially be beneficial due to establishment of the BBB and limiting the extension of inflammatory cells, it ultimately hinders CNS repair and regeneration in sub-acute and chronic stages of injury when most of the repair and regeneration takes place [for review see 201, 202]. Many studies have demonstrated CSPGs as the main inhibitory component of the glial scar [203-205] that limit axonal regeneration [204, 206-210], sprouting [211-214], conduction [215-217] and remyelination [218-222]. In normal condition, low levels of CSPGs are expressed in the CNS that play critical roles in neuronal guidance and synapse stabilization [223, 224]. Following injury, neurocan, versican, brevican and NG2 are four main CSPGs that are upregulated reach their peak of expression at 2 weeks post-SCI and remain upregulated chronically [225, 226]. Mechanistically, disruption of BSB and hemorrhage following traumatic SCI triggers upregulation of CSPGs in the glial scar by exposing the scar forming cells to factors in plasma such as fibrinogen [227]. Studies in cortical injury have shown that fibrinogen induces CSPG expression in astrocytes through TGFβ/Smad2 signaling pathway [227]. Intracellular Smad2

translocation is essential for Smad2 signal transduction process and its inhibition reduces scar formation [228].

Extensive research in the past decade has demonstrated the inhibitory effect of CSPGs on axon regeneration [229, 230]. The first successful attempt on improving long axon tract regeneration by enzymatic degradation of CSPGs using chondroitinase ABC (ChABC) in a rat SCI model was published in 2002 by Bradbury and colleagues [207]. In that study, significant improvement in recovery of locomotor and proprioceptive functions was observed following intrathecal delivery of ChABC in a rat model of dorsal column injury [207]. This observation was followed by several other studies demonstrating the promise of CSPGs degradation in improvement of regeneration and sprouting of the serotonergic [210, 212, 214, 218], sensory [213, 231, 232], corticospinal [207, 212, 218, 233] and rubrospinal fibers [234] in animal models of CNS injury. In addition to promoting regeneration and sprouting of injured axons, ChABC treatment is shown to be neuroprotective by preventing CSPG induced axonal dieback and degeneration [218, 235, 236]. A study by Karimi-Abdolrezaee and colleagues in 2010 showed that degradation of CSPGs using ChABC attenuates axonal dieback in corticospinal fibers in chronic SCI model in the rat [218]. ChABC administration has also been shown to moderate macrophage-mediated axonal degeneration in neural cultures and after SCI [153]. In this studies, ChABC induced improvement of axonal regeneration and prevention of axonal dieback led to significant recovery of function following SCI [218].

In addition to inhibition of axon regeneration, CSPGs are also known to inhibit oligodendrocyte differentiation and remyelination [182]. Myelin repair is a critical process for repair and regeneration in SCI and other demyelinating conditions such as MS [224]. Activation of endogenous OPCs or transplantation of exogenous NPCs and OPCs have been shown to be

feasible treatment strategies for the replacement of lost oligodendrocytes [218, 219, 237]. SCI triggers activation of endogenous OPCs which migrate to the injury site [238, 239]. However, an inhibitory SCI microenvironment does not support oligodendrocyte survival, differentiation and formation of compact myelin sheath [237-241]. Moreover, studies by our group identified components of the glial scar such as CSPGs as an underlying cause of the failure of survival and oligodendrocyte differentiation of transplanted NPCs following chronic SCI [218]. The inhibitory role of CSPGs in regulating endogenous precursor cell response to SCI was confirmed when studies by our group and others showed that perturbing CSPGs by ChABC administration enhances endogenous NPC and OPC proliferation, oligodendrocyte differentiation and remyelination following SCI [219].

Mechanistically, the inhibitory effects of CSPGs on NPCs and axon growth is governed by signaling through receptor protein tyrosine phosphatase sigma (RPTPσ) and leukocyte common antigen-related phosphatase receptor (LAR) [242]. RPTPσ is the main receptor mediating the inhibition of axon growth by CSPGs [242, 243]. Improved neuronal regeneration was shown in RPTPσ-/- mice model of SCI and peripheral nerve injury [243, 244]. Using a recently developed inhibitory sigma peptide (ISP), Lang and colleagues showed improvement in axonal growth following inhibition of RPTPσ signaling in DRG neurons cultured on a CSPG-reach substrate [242]. Moreover, this study showed a significant improvement in locomotor and bladder function associated with serotonergic re-innervation below the level of injury following ISP treatment in a rat SCI model [242]. In addition to the RPTPσ role in axonal growth inhibition, other studies have shown that downregulation of RPTPσ or inhibition of RPTPσ/ROCK signaling pathway reduces CSPG-mediated inhibition of oligodendrocyte maturation and myelination [220].

Overall, recent evidence has clearly demonstrated the inhibitory role of glial scar on axon survival and growth, OPC integration, migration, maturation and myelination which limit the regenerative capacity of the injured spinal cord [245]. Of note, in human, the glial scar begins to form within the first hours after the SCI and remains chronically in the spinal cord tissue [246]. The glial scar has been found within the injured human spinal cord up to 42 years after the injury [183]. These findings indicate the clinical implications of treatment strategies that aim to inhibit the production and function of CSPGs in the injured CNS.

In conclusion, compelling evidence indicates the contribution of dysregulated glial and immune response in developing a hostile and inhibitory post-SCI microenvironment that result in failure of repair and regeneration. Considering the determining role of microenvironmental signals on regulation of secondary injury and regenerative processes following SCI, it is imperative to unravel the mechanisms that contribute to this dysregulation. A recent discovery by our group has correlated an acute and sustained downregulation of neuronally derived Nrg-1 with dysregulated glial and immune response following SCI [247]. Nrg-1 replacement could restore the ability of endogenous NPCs for oligodendrocyte differentiation and remyelination [247, 248]. Moreover studies by other groups have established a positive immune modulatory role for Nrg-1 therapy in brain injury [249, 250]. Building on available evidence from our group and others, this thesis focuses on elucidating the impact of Nrg-1 on glial and immune response following SCI. The following sections provide an introduction to neuregulin-1 structure, signaling and role in CNS physiology and disease.

Fig. 1.4: Neuroinflammation and glial scar formation in SCI.

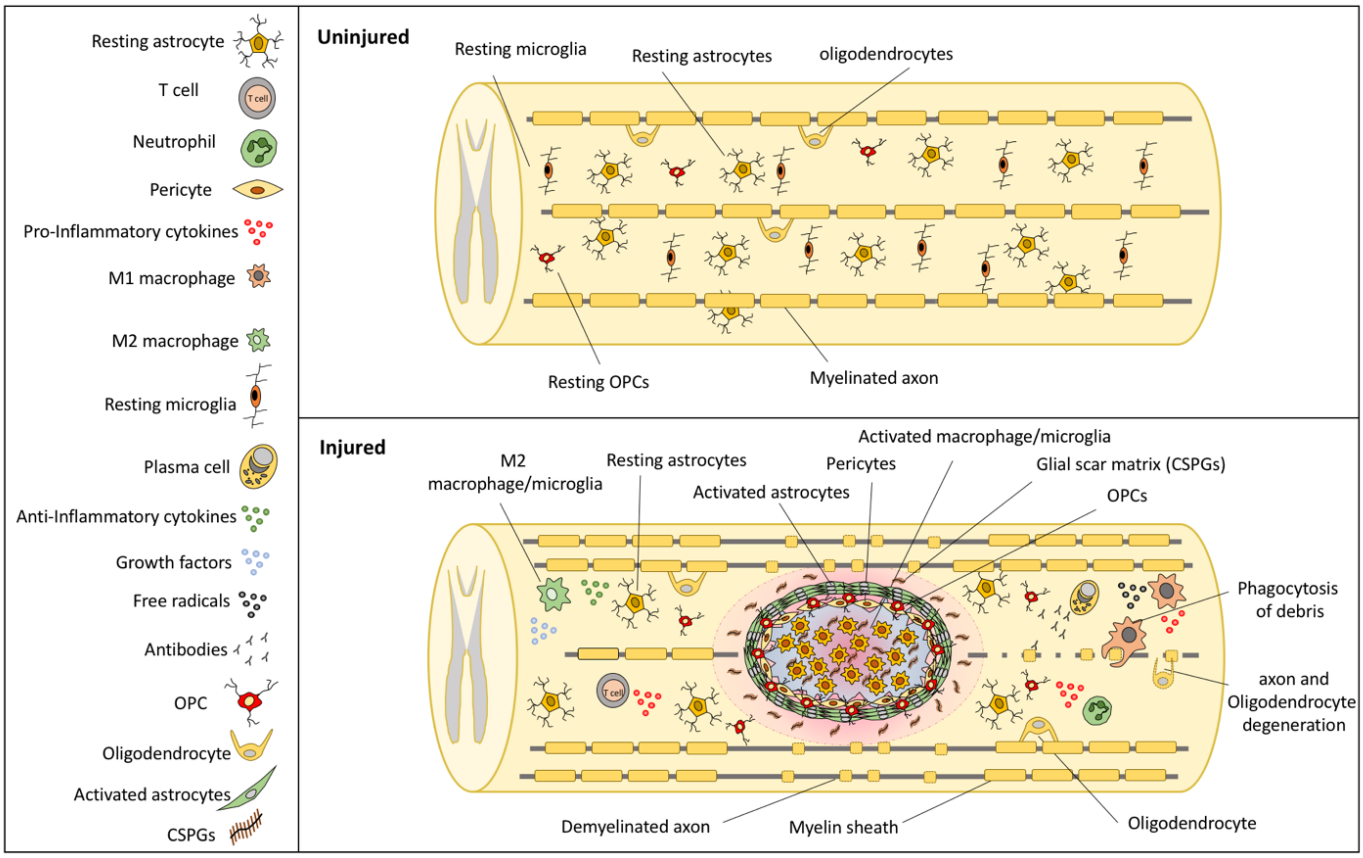


Fig. 1.4: Neuroinflammation and glial scar formation in SCI. Following SCI, activation of astrocytes and microglia and infiltration of blood-borne immune cells orchestrate secondary injury mechanisms that lead to glial scar formation, neural tissue degeneration and axonal demyelination. Astrocytes, OPCs and pericytes, which normally reside in the spinal cord parenchyma, proliferate and migrate to the site of injury and contribute to the formation of the glial scar. Glial scar surrounds the injury epicenter and acts as a barrier that plays both beneficial and detrimental roles. Glial scar limits the expansion of neuroinflammation to normal tissue while impeding axonal regeneration. Macrophages occupy the epicenter of the injury to scavenge tissue debris. Pericytes, OPCs and astrocytes surround the injury epicenter. These cells produce CSPGs as a major component of the glial scar with inhibitory effects on axonal regeneration and myelin repair. Most of the infiltrated immune cells such as T cells, B cells and M1 macrophages produce pro-inflammatory cytokines, chemokines, autoantibodies reactive oxygen and nitrogen species that foster neuronal and oligodendroglial degeneration. Loss of oligodendrocytes leaves the demyelinated axons dysfunctional and susceptible to damage due to the hostile SCI microenvironment. M2 macrophages and regulatory T and B cells produce growth factors and pro-regenerative cytokines such as IL-10 that promote tissue remyelination, moderate glial scar formation and support axonal regeneration.

1.3 Neuregulin-1

1.3.1 Neuregulin Family and Neuregulin-1 Structure

Neuregulins (also known as heregulins in humans) belong to a family of growth factors that are encoded by four genes (Nrg-1–4) and possess similar sequences with epidermal growth factor (EGF) [251]. Nrg-1 is the most well-known member of this family [252]. Nrg-1 plays an important role in developing nervous system including neural differentiation, neuronal guidance, synapse formation, myelination, and formation of neuromuscular junction [253-257]. Nrg-1 is encoded by a gene spanning 2.4 mbp in mice and 2.6mbp in humans and rats [252]. Due to its specific 5′ flanking regulatory elements and alternative splicing, Nrg-1 gene can generate six types of proteins (type I–VI) and approximately 31 different isoforms in humans [251, 258-260]. Each type of Nrg-1 has a distinct amino-terminal region, however, all Nrg-1 isoforms contain an EGF-like domain that is located in the extracellular domain of the protein [260, 261]. The EGF-like domain is necessary and sufficient for the activation of ErbB receptor tyrosine kinase [260, 261]. Since Nrg-1 consists of more than 30 isoforms, various nomenclatures have been proposed for their identification. The most accepted nomenclature of the identified Nrg-1 isoforms is derived from different exons located downstream of the EGF-like domain.

Nrg-1 type I, type II and type III are encoded in a wide variety of vertebrates, while type IV appears to be restricted to mammals [262-268] and Neuregulin type V and VI to primates [252]. There are distinct similarities and differences between various Nrg-1 isoforms. An immunoglobulin (Ig)-like domain located between the N-terminal sequence and the EGF-like domain distinguishes Nrg-1 types I, II, IV, and V (with the presence or absence of a spacer region), whereas Nrg-1 types III and VI are characterized by an N-terminal region connected directly to the EGF-like domain (Fig. 1.5) [269]. This IgG like domain contributes to specific interactions

with extracellular matrix components (e.g. heparan-sulfate proteoglycans; HSPGs) and define the distance and concentration over which these growth factors act [270]. It has been proposed that HSPGs situated on the cell surface concentrate Nrg-1 ligands in a micro-environment neighboring their ErbB receptors, thereby increasing the local ligand concentration. Furthermore, HSPGs may present Nrg-1 molecules to their higher affinity ErbB receptors [270].

The N-termini of the type I isoforms (and likely types IV–VI) lack a signal sequence or alternative mechanism to release from the cell membrane. Their expression on the cell surface and proteolytic release of their growth factor domain is determined by their central transmembrane and intracellular domains [271-273]. In contrast, type II isoforms readily enter the secretory pathway as they possess the classic signal peptide. Nrg-1 type III isoforms are unique compared to all other types due to several reasons that include the presence of an N-terminal sequence containing a cysteine-rich domain (CRD), an additional transmembrane domain (TM) [272] and their limited expression by neurons [274, 275]. Consequently, Nrg-1 type III isoforms have cytosolic N-termini and an EGF-like domain which is attached to the cell membrane. This topology limits type III signaling to cell–cell interactions, it enables bidirectional signaling by acting as a ligand as well as a receptor that modulates the behavior of neurons (Fig. 1.5) [276].

Fig. 1.5: Structural diversity of Nrg-1.

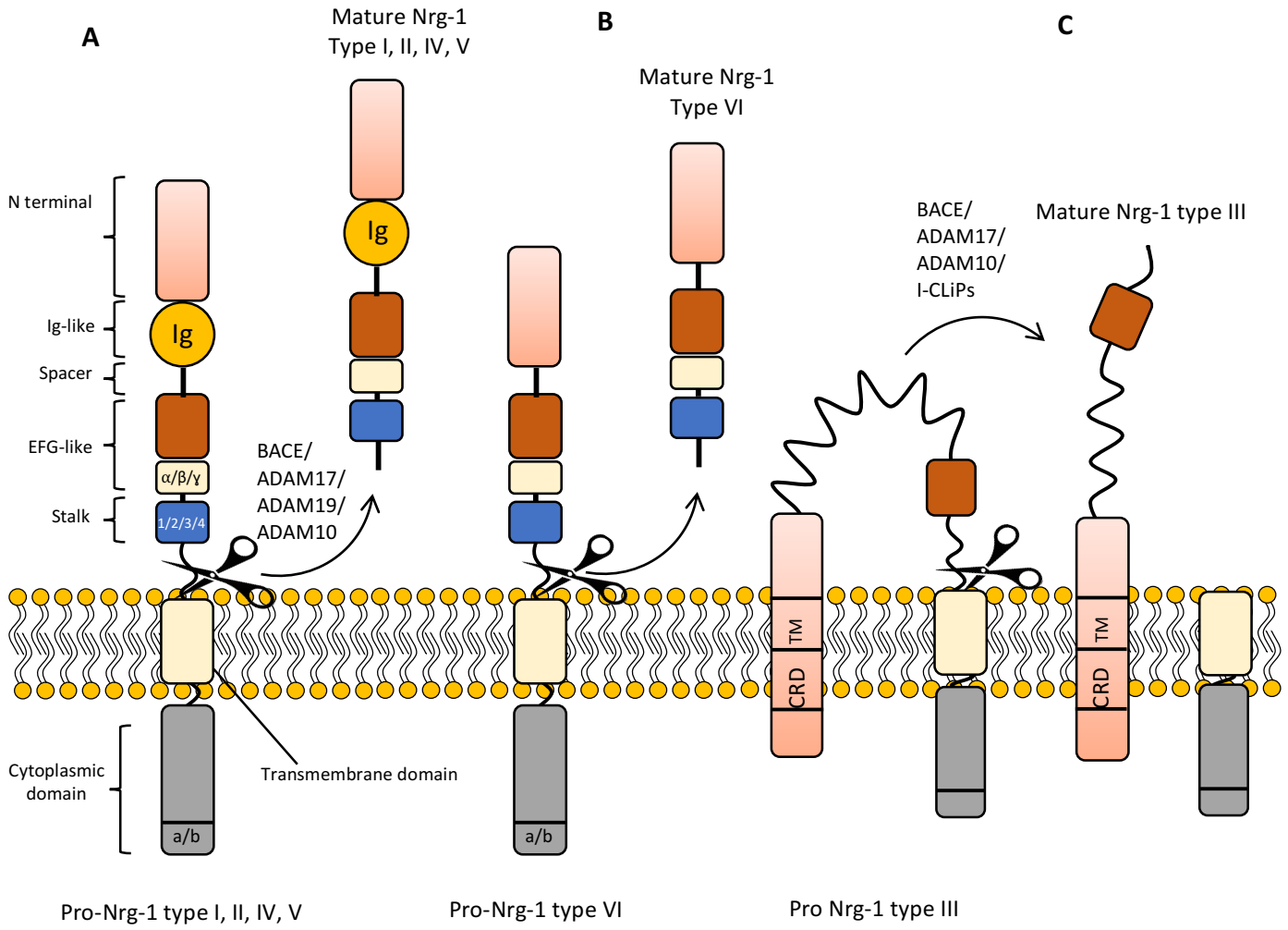


Fig. 1.5: Structural diversity of Nrg-1. Six different isoforms of Nrg-1 are distinguished based on their unique N-terminal sequences. Most Nrg-1 isoforms are synthetized as a membrane bound pro-Nrg-1 that undergos enzymatic cleavage to release mature Nrg-1 as a soluble ligand except for Nrg-1 type III. (A) Nrg-1 type I, II, IV, V are consist of an N terminal, Ig-like domain with or without the spacer region, EGF-like domain, stalk and cytoplasmic domain. (B, C) Nrg-1 type III and VI lack Ig domain. (C) Nrg-1 type III isoforms are unique compared to all other types due to the presence of an N-terminal sequence containing a cysteine-rich domain (CRD) and an additional transmembrane domain (TM). Nrg-1 type III isoforms have cytosolic N-termini and an EGF-like domain, which is attached to the cell membrane. This topology limits type III signaling to cell–cell interactions. Nrg-1 type III is cleaved by multiple enzymes including BACE1, ADAM10 and ADAM17, and also processed by three different intramembrane-cleaving proteases (I-CLiPs).

1.3.2 Neuregulin-1 Signaling

Most Nrg-1 isoforms are synthesized as pro-Nrg-1 which is bound to the cell membrane and possess an extracellular EGF domain [272, 277]. Mature Nrg-1 is released following proteolytic cleavage of the pro-Nrg-1 at the juxta-membrane region close to the carboxy-terminal of the EGF-like domain. Three types of transmembrane proteases catalyze this cleavage: TNF-α converting enzyme (TACE, also known as ADAM17) [277, 278], β-site of amyloid precursor protein cleaving enzyme (BACE, also known as memapsin 2) [279-281] and meltrin beta (also known as ADAM19) [282]. Following cleavage, Nrg-1 isoforms that lack transmembrane domain are directly released into the extracellular space [251]. Genetic studies have shown distinct roles of Nrg-1 isoforms in neural development [283-285]. Moreover, patterns and levels of Nrg-1 expression and processing are strictly regulated throughout the CNS [276]. Differential processing of Nrg-1 by proteolytic enzymes leads to distinct functional consequences. For instance, BACE-1 cleavage of axonal Nrg-1 type III positively regulates myelination [279, 281] and remyelination [286], while TACE cleavage has a negative effect on myelination [287]. Recent evidence shows that Nrg-1 type III is the first protein substrate that is not only cleaved by multiple sheddases (BACE1, ADAM10 and ADAM17) but is also processed by three different intramembrane-cleaving proteases (I-CLiPs) (Fig. 1.5) [288].

The EGF-like domain of Nrg-1 isoforms is necessary for interaction with ErbB receptors [289]. The EGF-like domain structure contains three sections: 1) N-terminal, which is shared among all Nrg-1 isoforms, 2) α or β type exon, and 3) a variable 'stalk' region, which located close to the membrane on the extracellular surface and lies in between the EGF-like and transmembrane domains. Variations in the stalk region directly affect the affinity of the Nrg-1 ligands for receptors

[271]. Due to such differences, β isoforms exhibit 100-folds higher affinity and activity compared to α isoforms [290].

Nrg-1 signals by activating a family of ErbB tyrosine kinases receptor (in humans called HER) proteins [291]. The ErbB family consists of ErbB1, 2, 3 and 4 receptors which share significant structural homology [292-296]. In canonical forward signaling, upon binding to its ligand, an ErbB receptor undergoes structural modifications in the juxta-membrane region. This leads to an increase in its affinity for another ErbB receptor, resulting in homo- or heterodimerization (Fig. 1.6) [297]. The heterodimers of ErbB2–ErbB3, ErbB2–ErbB4, ErbB3–ErbB4 and the homodimer ErbB4–ErbB4 are induced upon ligand binding [297]. ErbB4 can also form a heterodimer with EGFR (also known as ErbB1) [298]. However, the functional significance of this heterodimer in the CNS is not well understood [269]. Among the ErbB receptors, ErbB3 lacks is unable to phosphorylate other ErbB receptors in the dimer complex due to lack of the active kinase domain [299]. However, in presence of a ligand, ErbB3 participates in a heterodimer complex with another ErbB leading to phosphorylation by the partner and signal transduction [300]. ErbB2, on the other hand, has an active kinase domain, but no high-affinity ligand has been yet discovered for this receptor [301, 302]. ErbB2 functions as a co-receptor by forming heterodimers with other ErbB receptors but its homodimers are catalytically inactive (Fig. 1.6) [303]. Among the ErbB receptors, the function of ErbB4 in the CNS has been widely investigated and characterized. ErbB-4 is unique as it can form functional homo- or heterodimers upon ligand binding.

Intracellularly, activation of ErbB receptors through homo- or heterodimerization allows for trans-phosphorylation of the ErbB partners and recruits adaptor/effecter molecules such as phosphatidylinositol 3-kinase (PI3K) subunit p85, Src, and Shc leading to signal transmission to

their transducers [304]. The Raf–MEK–ERK and PI3K–Akt–S6K pathways are commonly triggered by the Nrg-1 induced activation of ErbB receptor homo- or heterodimers [269, 305]. Other downstream kinases known to be activated by Nrg-1 include c-Abl, JNK, CDK5, Fyn and Pyk2 [306-308]. Of note, Nrg-1 is not an ErbB1 (EGFR) ligand, however, it possess the ability to form heterodimers with ErbB-4 [309]. Thus, Nrg-1 stimulation can activate signaling pathways that are typically associated with the EGFR, such as the JNK, Src family kinases and PLCγ [269].

In non-canonical forward signaling, first, TACE cleaves the juxta-membrane region of ErbB4 releasing a soluble peptide (ecto-ErbB4) that contains the Nrg-1 binding site. Second, γ-secretase cleaves the residual 80kDa membrane-bound fragment within its transmembrane domain and releases the ErbB4 intracellular domain (ErbB4-ICD) [310, 311]. ErbB4-ICD can translocate into the nucleus and regulate transcription [312].

The transmembrane nature of pro-Nrg-1 provides the likelihood of these molecules to act as a receptor and mediate backward signaling [276]. Either the transmembrane form or soluble ecto-ErbB-4 may act as a ligand for the putative receptor pro-Nrg-1 or transmembrane Nrg-1 type III resulting in activation of intracellular signaling [276, 313]. Following the proteolytic processing of the extracellular juxta-membrane region of Nrg-1, the stump can be further cleaved by γ-secretase to release intracellular domain (Nrg-1-ICD). Depolarization of neurons or interaction with ecto-ErbBs can prompt the translocation of Nrg-1-ICD into the nucleus and regulate the transcriptional activity [276, 314]. Thus, the release of Nrg-1-ICD depends on the proteolytic activity of the γ-secretase complex, while its nuclear signaling depends on unknown enzymatic cleavage and is enhanced by synaptic activity [314]. This evidence indicates that not only Nrg-1 functions as a ligand for ErbB receptors but also acts as a receptor for the soluble ecto-ErbB4.

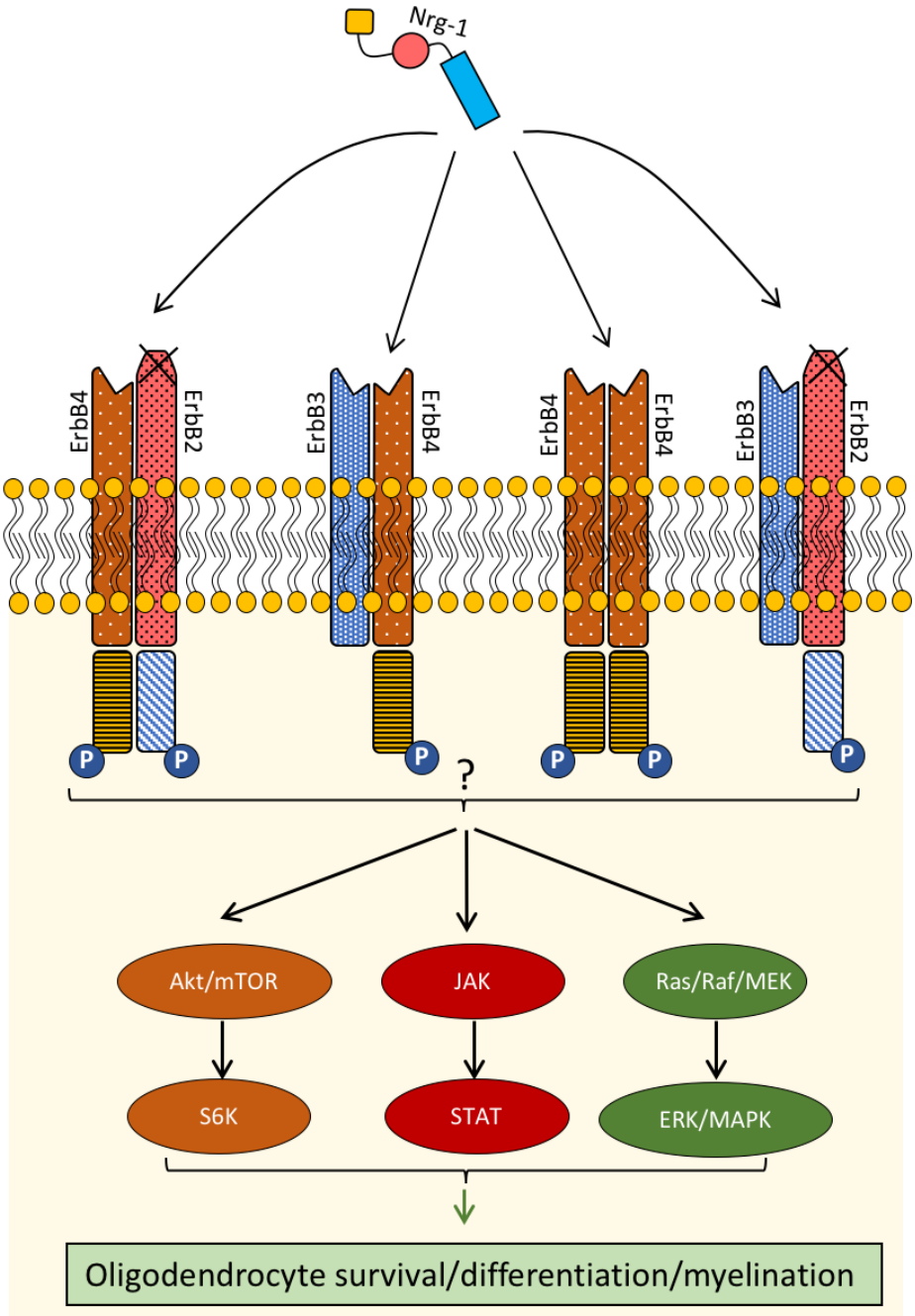

Nrg-1
ErbB4
ErbB2
ErbB3
ErbB4
ErbB4
ErbB4
ErbB3
ErbB2
P
?
Akt/mTOR
JAK
Ras/Raf/MEK
S6K
STAT
ERK/MAPK
Oligodendrocyte survival/differentiation/myelination

Fig. 1.6: Regulation of myelination by Nrg-1 signaling. Nrg-1 and its signaling receptors are a major regulatory network of oligodendrocyte development and maturation. Nrg-1 ligand can bind to ErbB3 and ErbB4 with high affinity. Upon binding to Nrg-1, ErbB receptors can form homo- or heterodimers and activate multiple pathways through their intracellular tyrosine kinase domain. Nrg-1 signaling has shown to activate Jak/STAT, Erk/MAPK and PI3/Akt that are associated with cell survival, proliferation and differentiation. However, the impact of each specific ErbB receptor in mediating Nrg-1 effects on each stage of oligodendrocyte lineage development and myelination remains to be elucidated.

Modified with permission from: Arsalan Alizadeh, Soheila Karimi-Abdolrezaee. **Microenvironmental regulation of oligodendrocyte replacement and remyelination in spinal cord injury.** The Journal of Physiology. 2016 Jul; 3539-3552. DOI: 10.1113/JP270895.

1.3.3 Neuregulin-1 and Nervous System Development

Although the complexity of the Nrg-1 gene and its receptors has been acknowledged for decades, little is known about the functional diversity of the Nrg-1 isoforms in the nervous system. Developmental studies have shown that Nrg-1 signaling is indispensable for heart development as homozygous-null embryos for Nrg-1 or ErbBs die at 10.5–13.5 days of gestation [260, 274, 283, 285, 315-317]. Nrg-1 also plays important role in the formation and regulation of neuromuscular junctions [278, 279]. Inhibition of ErbB tyrosine kinase activity has been associated with the attenuation of acetylcholine receptor (AChR) expression in myocytes [306, 307, 318, 319]. Postsynaptic density of AChR is reduced in heterozygous *Nrg1* mutant mice leading to dysfunctional neuromuscular transmission [320]. Moreover, Nrg-1 is presumed to signal to myocytes indirectly through Schwann cells in neuromuscular junctions [274].

Nrg-1 and ErbB receptors are expressed in the developing and adult nervous system and play important role in neural differentiation [321, 322]. Since lacking Nrg-1 or its receptors, ErbB2, ErbB3 and ErbB4, is embryonically lethal due to its critical role in cardiac development and function, direct assessment of Nrg-1 signaling in the developing and adult nervous system remains a limitation. Therefore, most of the experimental studies of Nrg-1 and its signaling in the CNS and PNS have relied on heterozygous mutant mice, conditional knockout, transgenic overexpression of Nrg-1, transplantation of mutant oligodendrocyte precursors or dominant-negative ErbB receptor expression in oligodendrocytes [323-326]. These studies have shown that Nrg-1 is necessary for neural pathfinding and migration [327]. In the hindbrain, Nrg-1 signaling is involved in patterning and proper migration of neural crest cells [328]. Nrg-1 is also essential for the migration of cerebral cortical neurons and cerebellar granule cells by regulating the formation and extension of radial glial cells [329]. In the developing cortex, Nrg-1 signaling has

been implicated in the radial migration of glutamatergic neurons as well as the tangential migration of GABA (γ-aminobutyric acid)-ergic neurons [253, 257, 330, 331]. Furthermore, Nrg-1 promotes neurite outgrowth in hippocampal neurons, retinal neurons, cerebellar granule cells and thalamic neurons [332-335]. Nrg-1-ErbB-4 signaling has been implicated in the regulation of thalamocortical axon projections which convey sensory and motor inputs to the cerebral cortex [334]. Different isoforms of Nrg-1 have been shown to influence the expression of different excitatory and inhibitory receptors of the CNS. Nrg-1 type I and type II influence the expression of GABA-A receptors whereas type III regulates the expression of neuronal AChRs [275, 333, 336, 337]. Moreover, Postsynaptic ErbB4 signalling regulates the activity-dependent maturation of excitatory synapses [338]. In addition to neurogenesis, neurite outgrowth, neuronal migration and activity, Nrg-1 signaling plays an important role in myelination [339, 340].

1.3.4 Neuregulin-1 and Peripheral Myelination

Nrg-1 plays a principal role in the development of Schwann cells and axon myelination in the PNS [339, 341]. It also directs the commitment of neural crest cells to a gliogenic fate [342], and promotes their proliferation, migration and differentiation to mature myelinating Schwann cells along the axons [283, 339, 343, 344]. PNS axons mainly express transmembrane Nrg-1 type III, while Schwann cells express soluble type I/II Nrg-1 [345]. Evidence shows that the release of soluble Nrg-1 by axons enhances Schwann cell survival [346]. Ligand binding to ErbB3 leads to ErbB2-ErbB3 heterodimerization and activation of ErbB2 tyrosine kinase domain, which activates intracellular signaling proteins and eventually regulates the genes responsible for Schwann cell survival, migration, differentiation, proliferation, and myelination [299, 347, 348]. Ablation of Nrg-1 isoforms or ErbB receptors (ErbB2 and ErbB3) leads to a nearly complete loss of Schwann

cells as well as the sensory and motor neurons supported by them in the PNS [260, 283, 316]. In injured peripheral nerves, Nrg-1/ErbB signaling reprograms Schwann cells by downregulating myelin associated genes such as Krox20, Oct6, MBP, MPZ, Sox10 and upregulating nerve repair signals such as cJun, Sox2, p75NTR, BDNF and GDNF [349]. Transcription of these genes is regulated by a vast array of signaling molecules downstream of ErbB receptors [349]. These downstream signaling molecules include but not limited to MAPK family of signaling molecules such as Ras/Raf/Erk, Rak/JNK, P38 MAPK and PI3K/Akt/mTOR pathways [349]. Reprogramming and trans-differentiation of Schwann cells allow them to contribute to and orchestrate peripheral nerve repair [349]. Studies have demonstrated that high level of Erk activity confers Schwann cell dedifferentiation by activating proliferation and repair genes such as p75NTR and GDNF [349]. Meanwhile low or basal activity of Erk would activate Schwann cell differentiation and myelination [349]. This evidence suggests the context dependency of the effect of these signaling pathways. Thus, further studies are needed to decipher the role of these complex signaling pathways in nervous system injury and repair.

During PNS development, Nrg-1 type III promotes Schwann cell myelination through an ErbB2/3 dependent mechanism and its tissue level determines myelin sheath thickness [341, 350]. In recent years, invaluable information has been gathered on the role of Nrg-1 in myelination using DRG neurons in culture. DRG neurons provide an excellent system to study CNS and PNS myelination as they possess both central and peripheral processes that can be myelinated by both Schwann cells and oligodendrocytes [341]. DRG axons express multiple forms of membrane bound Nrg-1 in addition to releasing a soluble form that can activate ErbB receptor signaling on myelinating cells [341]. Moreover, it has been shown that Nrg-1 type III is uniformly expressed along the internode in sciatic nerve axons in adult mice [350]. Nrg-1 type III has emerged as a key

regulator at essentially every stage of Schwann cell development [350-354]. Absence of Nrg-1 type III leads to a lack of Schwann cell precursors [274, 353]. Moreover, a recent study has established a role for Nrg-1 type III in Schwann cell migration as type III isoform specific knockout mice exhibited a delayed Schwann cell precursor migration [355].

Nrg-1 is also implicated in organization of myelin. Large myelinated axons express higher levels of Nrg-1 type III, which is associated with increased myelin thickness and internodal length [282, 326, 354, 356]. Downregulation of Nrg-1/ErbB signaling in the PNS results in hypomyelination and reduces axonal conduction velocity while Nrg-1 overexpression leads to hypermyelination [350]. Paradoxically, type II isoform of Nrg-1, induces demyelination in neuron/Schwann cell co-cultures [357], and transgenic mice with Nrg-1 type II over-expression exhibit demyelination and development of other abnormalities by approximately 7 months of age [358]. These contradictory outcomes suggest a delicate balance and differential role for Nrg-1 signaling in PNS myelination during development and adulthood.

Nrg1 regulates myelination by modulating several pathways including Ras/Raf/MAPK/Erk, PI3K/Akt, PLCγ, focal adhesion kinase (FAK), Rho-GTPases, and JNK, [348, 359-364]. Erbin is another important factor for myelination, which binds to ErbB2 and promotes Nrg-1 signaling [365]. Another signaling molecule, Shp2 mediates Nrg-1 effects downstream of ErbB2/3. Shp2 is required for sustained activation of MAPK/Erk1/2 and its ablation impairs Nrg-1 mediated cellular responses such as proliferation, migration, or myelination [366]. Ablation of Erk1/2 in Schwann cells causes deficits that are remarkably similar to those observed in Shp2 or Nrg1/ErbB2/ErbB3 mutants [367]. Instead, mild activation of MAPK signaling was sufficient to mimic Nrg-1/ErbB3 without ErbB3 activation and reactivate myelination in mature Schwann cells [368].

1.3.5 Neuregulin-1 and CNS Myelination

The role of Nrg-1 in CNS myelination remains unclear. To date, the most substantiated role of Nrg-1/ErbB signaling in CNS myelination is its impact on the establishment of the oligodendroglial lineage [340]. Early studies showed that in neural tube explants prepared from Nrg-1 knockout mice, oligodendroglial cells were not produced, and exogenous Nrg-1 was able to rescue oligodendrogenesis [340]. It is known that OPCs and oligodendrocytes express all Nrg-1 receptors (ErbB2, ErbB3, ErbB4) [341]. Nrg-1 has been shown to promote proliferation of O2A progenitor cells, $O4^+$ oligodendrocyte precursor cells, and $O1^+$ cells [369] as well as the survival of oligodendrocytes *in vitro* [369-371]. In the rat subventricular zone, Nrg-1 expression coincides with oligodendrocyte differentiation, and leads to the differentiation of oligodendrocytes from bipotential O2A glial progenitor cells [372]. Role of Nrg-1 in oligodendrogenesis was further substantiated when disruption of ErbB signaling suppressed oligodendrocyte differentiation from progenitor cells [373]. High ErbB3 expression is found in oligodendrocytes and their precursors in the brain and is involved in their propagation and differentiation [374]. ErbB3 expression is also observed in neural precursor cells in the adult hippocampus and contributes to their proliferation, although ErbB3 expression is modest [375]. However, ErbB3 seems to be dispensable for the oligodendrocyte development, probably because of the expression and overlapping functions of autonomous ErbB4 receptor [324]. Interestingly, studies of ErbB2-null mice showed that oligodendrocyte development ceases at the OPC stage indicating a critical role for ErbB2 in oligodendrocyte differentiation [376]. Further studies, revealed that Nrg-1 dependent oligodendrocyte differentiation in the CNS seems to be mediated through an ErbB2/4 heterodimer complex [341].

Interestingly, despite an essential role for Nrg-1 signaling in oligodendrocyte development, genetic manipulation of Nrg-1 and ErbB receptors revealed no disruption in CNS myelination during the development [377]. Conditional knockout of ErbB3/ErbB4 receptors in mice suggests that axonal control of myelination is markedly different between the CNS and PNS [378]. Nonetheless, albeit a proposed dispensable role for Nrg-1 in the initiation of myelination, activity of Nrg-1 signaling seems to play an important role in the quality of myelination in the CNS [378]. Nrg-1 overexpression was associated with CNS hypermyelination [378], while dominant negative ErbB4 reduces myelin thickness and conduction velocity in CNS axons [323]. In conclusion, while recent studies have increased our understanding on the role of Nrg-1 and its signaling in the CNS, further work is required to unveil the significance of Nrg-1 on the processes of myelination and remyelination in the normal and pathologic CNS.

1.3.6 Role of Neuregulin-1 in Nervous System Injury and Disease

1.3.6.1 Neuregulin-1 and Myelin Repair

As previously discussed, Nrg-1 plays important roles in the development and maintenance of oligodendrocytes and myelin [251]. Interestingly, despite its role in the developing CNS, Nrg-1 is has been under-studied in the context of CNS injury. In recent years, emerging evidence from our group and others has signified a role for Nrg-1 in SCI. Our laboratory provided the original evidence that Nrg-1 transcript and protein levels are significantly downregulated in a compressive model of SCI in the rats as early as one day post-injury [247]. Importantly, this study identified that SCI-induced downregulation of Nrg-1 evolved over time as injury progressed, and persisted chronically without restoration of its levels in the chronic phase of SCI. Interestingly, partial restoration of Nrg-1 levels through intrathecal administration of human recombinant Nrg-1

treatment promoted endogenous oligodendrogenesis in the injured spinal cord, and fostered preservation of mature oligodendrocytes and axons following SCI [247]. These studies also found a strong correlation between Nrg-1 downregulation and increased astrocyte proliferation following SCI in adult rats [247]. Furthermore, in a rat model of focal demyelination, our group has unraveled a significant role for Nrg-1 in enhancing oligodendrogenesis and remyelination process in demyelinating spinal cord [248]. Interestingly, Nrg-1 showed the ability to modulate both populations of neural precursor cells (NPCs) and OPCs. Nrg-1 enhanced proliferation and fate specification of adult derived NPCs towards an oligodendrocyte lineage, promoted the maturation of adult OPCs into MBP expressing oligodendrocytes, and enhanced their the ability to ensheath and myelinate axons [248]. Importantly, Nrg-1 also augmented morphological complexity of oligodendrocytes during the maturation process signifying its impact on myelination. In demyelinating lesions of the spinal cord, increasing the bioavailability of Nrg-1 enhances oligodendrocyte and Schwann cell mediated remyelination and augments myelin thickness in newly remyelinated axons [248]. Mechanistically, *in vitro* studies by our groups have shown that Nrg-1 appears to modulate oligodendrocyte myelination through ErbB2 and ErbB4 receptor activation [248] which is in agreement with previous studies [341]. A recent study by Bartus and colleagues has demonstrated that Schwann cells contribute to remyelination of the spinal cord dorsal column axons in mouse SCI [379]. Interestingly, in this study, conditional knockout mice lacking Nrg-1 failed to demonstrate remyelination by endogenous Schwann cells following injury [379]. This evidence suggests that Nrg-1 promotes Schwann cell differentiation of endogenous precursor cells and remyelination of demyelinated axons following SCI [379]. Collectively, existing evidence from our groups and others indicates a positive modulatory role for Nrg-1 in the central and peripheral myelination, myelin maintenance and quality of myelin. Hence, Nrg-1

represents a potential candidate as a treatment for myelin repair in demyelinating conditions such as SCI and multiple sclerosis [380].

1.3.6.2 Neuroprotective Effects of Neuregulin-1

Emerging evidence has implicated Nrg-1 as a neuroprotective treatment in cerebral ischemic lesions [381, 382]. Minimizing neuronal death and axonal degeneration acute phase of injury are the main goals in neuroprotective strategies for ischemic and traumatic CNS injuries [383]. In humans, three hours post cerebral ischemia is shown to be an optimal time-window for neuroprotective treatments [383]. A number of ischemic and chemical injury models have been utilized to develop new neuroprotective pharmacological approaches and determine their effectiveness at various time-points after the insult [381, 382, 384, 385]. In an animal model of cerebral ischemia, Nrg-1 administration up to 13.5 hours after the ischemic insult was able to improve neuronal preservation through inhibition of apoptosis and neuroinflammation [381]. In a similar study, Nrg-1 treatment showed a 7-day therapeutic window for improvement of neurological function in cerebral ischemia [383]. Studies on chemical models of CNS injury have suggested a narrower time-window for the effectiveness of Nrg-1 treatment [384]. In a study using diisopropylfluorophosphate (DFP), an organophosphate-like neurotoxin, Nrg-1 administration prior or up to 1 hour after the injury significantly reduced apoptosis and oxidative damage in neurons, while Nrg-1 treatment 4 hours after the injury was unable to confer any significant neuroprotection [384]. In cerebral ischemia and reperfusion models, Nrg-1 treatment has been associated with reduced caspase-3 mediated apoptosis in neurons and a decline in the expression of pro-inflammatory cytokines such as IL-1β and TNF-α [382, 386]. ErbB4 receptor and activation of CDK5/Akt/PI3 kinase pathway has been identified as a mechanism underlying the

neuroprotective effects of Nrg-1 [384, 385]. Significant increase in Akt phosphorylation was observed in both neurons and glial cells after Nrg-1 treatment in animal models of chemically induced cerebral injury [384]. Altogether, availability of Nrg-1 appears to augment the ability of neurons to survive in ischemic injuries of the brain. Further investigations are needed to identify Nrg-1 mechanisms of neuronal protection.

Dysregulation of Nrg-1 and its receptors ErbB3 and ErbB4 has been recently reported in motor neurons during the onset of Amyotrophic Lateral Sclerosis (ALS), a neurodegenerative disease affecting spinal motor neurons [387]. Nrg-1 is normally expressed at C-boutons of cholinergic synapses [388]. In ALS, the size and quantity of these boutons are altered [389]. In superoxide dismutase (SOD)1-ALS mice model, delivery of Nrg-1 type III increased the number of synaptic c-boutons and improved motor neuron survival suggesting therapeutic potential of Nrg-1 signaling in ALS [387]. Interestingly, studies by our group also demonstrated a neuroprotective role for Nrg-1 in rats with SCI [247]. Restoration of dysregulated levels of Nrg-1 in the injured spinal cord by intrathecal delivery of Nrg-1 treatment was associated with improved overall neural tissue preservation and oligodendrocyte survival [247].

In conclusion, initial evidence suggests a neuroprotective and neuroregenerative role for Nrg-1 in CNS injury and disease. Further studies are required to elucidate the ramification of Nrg-1 dysregulation in SCI, and unravel the cellular and molecular mechanisms by which Nrg-l regulates the secondary injury mechanisms following SCI.

1.4 Thesis Overview

1.4.1 Study Rationale

As discussed in previous sections, SCI leads to a complex cascade of cellular and molecular events

referred as “secondary injury” [41, 45, 237, 241, 390-397]. This secondary injury process is mainly characterized by glial activation, neuroinflammation, cell death, scar formation, progressive axonal degeneration, demyelination, and consequent sensorimotor deficits [41, 45, 237, 241, 390-397]. Shortly after injury, astrocytes and resident microglia respond to the injury and aid in recruitment of blood borne macrophages, neutrophils and lymphocytes. These populations together orchestrate complex glial and immune responses within the injured spinal cord that have a determining role in both injury and repair processes [398]. These cells produce a host of pro-inflammatory cytokines and chemokines, free oxygen radicals, autoantibodies and inhibitory extracellular matrix components that cause progressive neural tissue degeneration [171, 398, 399]. Additionally, activated astrocytes undergo phenotypical changes, secrete inhibitory ECM components such as inhibitory CSPGs and form a glial scar which poses a barrier to axon regeneration and cell replacement [218, 219, 224]. These changes lay the foundation for a non-permissive microenvironment for neural repair and regeneration. Despite these detrimental effects, astrocytes and immune cells also play pro-regenerative roles [110, 111, 146, 202, 400-405]. Studies have shown that increased number of anti-inflammatory and immune-regulatory microglia and leukocytes has been associated with improved tissue preservation and recovery of function following SCI [109, 179, 403]. Development of repair strategies to promote a pro-regenerative response in astrocytes and immune cells is essential for the preservation and restoration of neurological functions after SCI. Hence, understanding the endogenous mechanisms that regulate astrogliosis and neuroinflammation is imperative to allow development of such targeted therapies for SCI.

Previous studies in our laboratory revealed, for the first time, that SCI results in a rapid and long-lasting dysregulation of Nrg-1 [247]. Further studies by our group established a correlation

between the injury-induced depletion of Nrg-1 and inadequate oligodendrocyte replacement and potentially suboptimal remyelination that occur after SCI [406]. Similar to oligodendrocytes, glial and immune cells also express Nrg-1 receptors, ErbB2, 3, 4, suggesting potential ramifications of Nrg-1 dysregulation on glial activity and neuroinflammation. Recent studies have also identified an immunomodulatory role for Nrg-1 in ischemic brain injuries [249, 250]. This evidence collectively suggests that disruption of Nrg-1 signaling may cause an imbalanced immune and glial response after SCI, and create an impermissible microenvironment for repair processes following SCI. Currently, the impact of Nrg-1 on secondary injury mechanisms of traumatic SCI is largely unknown.

1.4.2 General Hypothesis and Research Objectives

The overall goal of my PhD thesis was to unravel the role and mechanisms of Nrg-1 in regulating glial and immune responses in traumatic SCI.

1.4.3 Hypotheses:

1. Diminished tissue levels of Nrg-1 in the injured spinal cord contribute to the imbalanced astrocyte activity and inflammatory processes following SCI.
2. Nrg-1 positively modulates the response of resident microglia and peripherally recruited leukocytes, and fosters a pro-regenerative immune response in SCI that is associated with improved functional recovery.

1.4.4 Specific Research Objectives:

1. To investigate the role of Nrg-1 on glial response using *in vitro* models of reactive astrocytes and microglia (Chapter 2).

2. To investigate the effect of Nrg-1 treatment on scar formation, neuroinflammation and functional recovery in a model of compressive/contusive SCI in the rat (Chapter 2).
3. To investigate the effect of Nrg-1 treatment on recruitment and phenotype of infiltrating macrophages and resident microglia following SCI (Chapter 3).
4. To investigate the effect of Nrg-1 treatment on T and B cell infiltration and phenotype in the spinal cord and blood following SCI (Chapter 3).

These questions have been addressed in two published articles that are reported in Chapters 2 and 3 of this thesis.

1.5 References

1. WHO, *WHO | Spinal cord injury.* WHO, 2013. **Fact sheet N°384**.
2. Noonan, V.K., et al., *Incidence and prevalence of spinal cord injury in Canada: a national perspective.* Neuroepidemiology, 2012. **38**(4): p. 219-26.
3. Singh, A., et al., *Global prevalence and incidence of traumatic spinal cord injury.* Clin Epidemiol, 2014. **6**: p. 309-31.
4. Michael G. Fehlings, A.R.V., Maxwell Boakye, Srge Rossignol, John F. Ditunno Jr., Anthony S. Burns *Essentials of Spinal Cord Injury Basic Research to Clinical Practice*. 2013: Thieme Medical Publishers, Inc. 658.
5. Sekhon, L. and M. Fehlings, *Epidemiology, Demographics, and Pathophysiology of Acute Spinal Cord Injury.* Spine, 2001. **26**(24S): p. S2-S12.

6. Rowland, J.W., et al., *Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon.* Neurosurg Focus, 2008. **25**(5): p. E2.

7. Figley, S.A., et al., *Characterization of vascular disruption and blood-spinal cord barrier permeability following traumatic spinal cord injury.* J Neurotrauma, 2014. **31**(6): p. 541-52.

8. Wilson, J.R., et al., *Clinical prediction model for acute inpatient complications after traumatic cervical spinal cord injury: a subanalysis from the Surgical Timing in Acute Spinal Cord Injury Study.* J Neurosurg Spine, 2012. **17**(1 Suppl): p. 46-51.

9. Wilson, J.R., D.W. Cadotte, and M.G. Fehlings, *Clinical predictors of neurological outcome, functional status, and survival after traumatic spinal cord injury: a systematic review.* J Neurosurg Spine, 2012. **17**(1 Suppl): p. 11-26.

10. Furlan, J.C., et al., *Assessment of disability in patients with acute traumatic spinal cord injury: a systematic review of the literature.* J Neurotrauma, 2011. **28**(8): p. 1413-30.

11. Bracken, M.B., S.B. Webb, Jr., and F.C. Wagner, *Classification of the severity of acute spinal cord injury: implications for management.* Paraplegia, 1978. **15**(4): p. 319-26.

12. Lucas, J.T. and T.B. Ducker, *Motor classification of spinal cord injuries with mobility, morbidity and recovery indices.* Am Surg, 1979. **45**(3): p. 151-8.

13. Klose, K.J., et al., *University of Miami Neuro-Spinal Index (UMNI): a quantitative method for determining spinal cord function.* Paraplegia, 1980. **18**(5): p. 331-6.

14. Chehrazi, B., et al., *A scale for evaluation of spinal cord injury.* J Neurosurg, 1981. **54**(3): p. 310-5.

15. Waters, R.L., et al., *Motor and sensory recovery following incomplete tetraplegia.* Arch Phys Med Rehabil, 1994. **75**(3): p. 306-11.

16. Waters, R.L., et al., *Recovery following complete paraplegia.* Arch Phys Med Rehabil, 1992. **73**(9): p. 784-9.

17. Ditunno, J.F., Jr., et al., *Wrist extensor recovery in traumatic quadriplegia.* Arch Phys Med Rehabil, 1987. **68**(5 Pt 1): p. 287-90.

18. Folman, Y. and W. el Masri, *Spinal cord injury: prognostic indicators.* Injury, 1989. **20**(2): p. 92-3.

19. Fawcett, J.W., et al., *Guidelines for the conduct of clinical trials for spinal cord injury as developed by the ICCP panel: spontaneous recovery after spinal cord injury and statistical power needed for therapeutic clinical trials.* Spinal Cord, 2007. **45**(3): p. 190-205.

20. Kirshblum, S.C. and K.C. O'Connor, *Predicting neurologic recovery in traumatic cervical spinal cord injury.* Arch Phys Med Rehabil, 1998. **79**(11): p. 1456-66.

21. Stauffer, E.S., *Neurologic recovery following injuries to the cervical spinal cord and nerve roots.* Spine (Phila Pa 1976), 1984. **9**(5): p. 532-4.

22. Oleson, C.V., et al., *Prognostic value of pinprick preservation in motor complete, sensory incomplete spinal cord injury.* Arch Phys Med Rehabil, 2005. **86**(5): p. 988-92.

23. Nardone, R., et al., *Rodent, large animal and non-human primate models of spinal cord injury.* Zoology (Jena), 2017. **123**: p. 101-114.

24. Kjell, J. and L. Olson, *Rat models of spinal cord injury: from pathology to potential therapies.* Dis Model Mech, 2016. **9**(10): p. 1125-1137.

25. Cheriyan, T., et al., *Spinal cord injury models: a review.* Spinal Cord, 2014. **52**(8): p. 588-95.

26. Lee, J.H., et al., *A novel porcine model of traumatic thoracic spinal cord injury.* J Neurotrauma, 2013. **30**(3): p. 142-59.

27. Nout, Y.S., et al., *Animal models of neurologic disorders: a nonhuman primate model of spinal cord injury.* Neurotherapeutics, 2012. **9**(2): p. 380-92.

28. Barbeau, H., et al., *Tapping into spinal circuits to restore motor function.* Brain Res Brain Res Rev, 1999. **30**(1): p. 27-51.

29. Gruner, J.A., *A monitored contusion model of spinal cord injury in the rat.* J Neurotrauma, 1992. **9**(2): p. 123-6; discussion 126-8.

30. Stokes, B.T., *Experimental spinal cord injury: a dynamic and verifiable injury device.* J Neurotrauma, 1992. **9**(2): p. 129-31; discussion 131-4.

31. Jakeman, L.B., et al., *Traumatic spinal cord injury produced by controlled contusion in mouse.* J Neurotrauma, 2000. **17**(4): p. 299-319.

32. Petteys, R.J., et al., *Design and testing of a controlled electromagnetic spinal cord impactor for use in large animal models of acute traumatic spinal cord injury.* J Clin Neurosci, 2017. **43**: p. 229-234.

33. Rivlin, A.S. and C.H. Tator, *Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat.* Surg Neurol, 1978. **10**(1): p. 38-43.

34. Joshi, M. and M.G. Fehlings, *Development and characterization of a novel, graded model of clip compressive spinal cord injury in the mouse: Part 1. Clip design, behavioral outcomes, and histopathology.* J Neurotrauma, 2002. **19**(2): p. 175-90.

35. Poon, P.C., et al., *Clip compression model is useful for thoracic spinal cord injuries: histologic and functional correlates.* Spine (Phila Pa 1976), 2007. **32**(25): p. 2853-9.

36. Blight, A.R., *Morphometric analysis of a model of spinal cord injury in guinea pigs, with behavioral evidence of delayed secondary pathology.* J Neurol Sci, 1991. **103**(2): p. 156-71.

37. Aslan, A., et al., *Does dexmedetomidine reduce secondary damage after spinal cord injury? An experimental study.* Eur Spine J, 2009. **18**(3): p. 336-44.

38. Nesathurai, S., et al., *Model of traumatic spinal cord injury in Macaca fascicularis: similarity of experimental lesions created by epidural catheter to human spinal cord injury.* J Med Primatol, 2006. **35**(6): p. 401-4.

39. Fukuda, S., et al., *New canine spinal cord injury model free from laminectomy.* Brain Res Brain Res Protoc, 2005. **14**(3): p. 171-80.

40. AR, A., *Surgery of experimental lesion of spinal cord equivalent to crush injury of fracture dislocation of spinal column.* JAMA, 1911. **57**: p. 878-880.

41. Tator, C.H. and M.G. Fehlings, *Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms.* J Neurosurg, 1991. **75**(1): p. 15-26.

42. Couillard-Despres S., B.L., Vogl M., *Pathophysiology of Traumatic Spinal Cord Injury*, in *Neurological Aspects of Spinal Cord Injury*, R.R. Weidner N., Tansey K., Editor. 2017, Springer International Publishing: Switzerland.

43. Koyanagi, I., C.H. Tator, and E. Theriault, *Silicone rubber microangiography of acute spinal cord injury in the rat.* Neurosurgery, 1993. **32**(2): p. 260-8; discussion 268.

44. Tator, C.H. and I. Koyanagi, *Vascular mechanisms in the pathophysiology of human spinal cord injury.* J Neurosurg, 1997. **86**(3): p. 483-92.

45. Agrawal, S.K. and M.G. Fehlings, *Mechanisms of secondary injury to spinal cord axons in vitro: role of Na+, Na(+)-K(+)-ATPase, the Na(+)-H+ exchanger, and the Na(+)-Ca2+ exchanger.* J Neurosci, 1996. **16**(2): p. 545-52.

46. Oyinbo, C.A., *Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade.* Acta Neurobiol Exp (Wars), 2011. **71**(2): p. 281-99.

47. Karadottir, R. and D. Attwell, *Neurotransmitter receptors in the life and death of oligodendrocytes.* Neuroscience, 2007. **145**(4): p. 1426-38.

48. Verkhratsky, A. and C. Steinhauser, *Ion channels in glial cells.* Brain Res Brain Res Rev, 2000. **32**(2-3): p. 380-412.

49. Gottlieb, M. and C. Matute, *Expression of ionotropic glutamate receptor subunits in glial cells of the hippocampal CA1 area following transient forebrain ischemia.* J Cereb Blood Flow Metab, 1997. **17**(3): p. 290-300.

50. Vanzulli, I. and A.M. Butt, *mGluR5 protect astrocytes from ischemic damage in postnatal CNS white matter.* Cell Calcium, 2015. **58**(5): p. 423-30.

51. Fernyhough, P. and N.A. Calcutt, *Abnormal calcium homeostasis in peripheral neuropathies.* Cell Calcium, 2010. **47**(2): p. 130-9.

52. Leist, M. and P. Nicotera, *Calcium and neuronal death.* Rev Physiol Biochem Pharmacol, 1998. **132**: p. 79-125.

53. Verkhratsky, A. and O.H. Petersen, *The endoplasmic reticulum as an integrating signalling organelle: from neuronal signalling to neuronal death.* Eur J Pharmacol, 2002. **447**(2-3): p. 141-54.

54. Verkhratsky, A. and E.C. Toescu, *Endoplasmic reticulum Ca(2+) homeostasis and neuronal death.* J Cell Mol Med, 2003. **7**(4): p. 351-61.

55. Pivovarova, N.B. and S.B. Andrews, *Calcium-dependent mitochondrial function and dysfunction in neurons.* FEBS J, 2010. **277**(18): p. 3622-36.

56. Duchen, M.R., *Mitochondria in health and disease: perspectives on a new mitochondrial biology.* Mol Aspects Med, 2004. **25**(4): p. 365-451.

57. Starkov, A.A., C. Chinopoulos, and G. Fiskum, *Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury.* Cell Calcium, 2004. **36**(3-4): p. 257-64.

58. Pandya, J.D., V.N. Nukala, and P.G. Sullivan, *Concentration dependent effect of calcium on brain mitochondrial bioenergetics and oxidative stress parameters.* Front Neuroenergetics, 2013. **5**: p. 10.

59. Li, S. and P.K. Stys, *Na(+)-K(+)-ATPase inhibition and depolarization induce glutamate release via reverse Na(+)-dependent transport in spinal cord white matter.* Neuroscience, 2001. **107**(4): p. 675-83.

60. LoPachin, R.M., et al., *Experimental spinal cord injury: spatiotemporal characterization of elemental concentrations and water contents in axons and neuroglia.* J Neurophysiol, 1999. **82**(5): p. 2143-53.

61. McAdoo, D.J., et al., *The effect of glutamate receptor blockers on glutamate release following spinal cord injury. Lack of evidence for an ongoing feedback cascade of damage --> glutamate release --> damage --> glutamate release --> etc.* Brain Res, 2005. **1038**(1): p. 92-9.

62. Stys, P.K., S.G. Waxman, and B.R. Ransom, *Ionic mechanisms of anoxic injury in mammalian CNS white matter: role of Na+ channels and Na(+)-Ca2+ exchanger.* J. Neurosci, 1992. **12**(2): p. 430-439.

63. Regan, R.F. and D.W. Choi, *Glutamate neurotoxicity in spinal cord cell culture.* Neuroscience, 1991. **43**(2-3): p. 585-91.

64. Agrawal, S. and M. Fehlings, *Mechanisms of secondary injury to spinal cord axons in virto: role of Na+, Na(+)-K(+)-ATPase, the Na(+)-H+ exchanger, and the Na(+)-Ca2+ exchanger.* Journal of Neuroscience, 1996. **16**(2): p. 545-52.

65. Reithmeier, R.A., *Mammalian exchangers and co-transporters.* Curr Opin Cell Biol, 1994. **6**(4): p. 583-94.

66. Schwartz, G. and M.G. Fehlings, *Evaluation of the neuroprotective effects of sodium channel blockers after spinal cord injury: improved behavioral and neuroanatomical recovery with riluzole.* J Neurosurg, 2001. **94**(2 Suppl): p. 245-56.

67. Dingledine, R., et al., *The glutamate receptor ion channels.* Pharmacol Rev, 1999. **51**(1): p. 7-61.

68. MacDermott, A.B., et al., *NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones.* Nature, 1986. **321**(6069): p. 519-22.

69. Faden, A.I. and R.P. Simon, *A potential role for excitotoxins in the pathophysiology of spinal cord injury.* Ann Neurol, 1988. **23**(6): p. 623-6.

70. Wada, S., et al., *Apoptosis following spinal cord injury in rats and preventative effect of N-methyl-D-aspartate receptor antagonist.* J Neurosurg, 1999. **91**(1 Suppl): p. 98-104.

71. Duchen, M.R., *Mitochondria, calcium-dependent neuronal death and neurodegenerative disease.* Pflugers Arch, 2012. **464**(1): p. 111-21.

72. Pruss, H., et al., *Non-resolving aspects of acute inflammation after spinal cord injury (SCI): indices and resolution plateau.* Brain Pathol, 2011. **21**(6): p. 652-60.

73. Hall, E.D., *Antioxidant therapies for acute spinal cord injury.* Neurotherapeutics, 2011. **8**(2): p. 152-67.

74. Hall, E.D., *The Contributing Role of Lipid Peroxidation and Protein Oxidation in the Course of CNS Injury Neurodegeneration and Neuroprotection: An Overview*, in *Brain Neurotrauma: Molecular, Neuropsychological, and Rehabilitation Aspects*, F.H. Kobeissy, Editor. 2015: Boca Raton (FL).

75. Springer, J.E., et al., *4-hydroxynonenal, a lipid peroxidation product, rapidly accumulates following traumatic spinal cord injury and inhibits glutamate uptake.* J Neurochem, 1997. **68**(6): p. 2469-76.

76. Cuzzocrea, S., et al., *Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury.* Pharmacol Rev, 2001. **53**(1): p. 135-59.

77. Beattie, M.S., et al., *Cell death in models of spinal cord injury.* Prog Brain Res, 2002. **137**: p. 37-47.

78. McTigue, D.M., P. Wei, and B.T. Stokes, *Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord.* J Neurosci, 2001. **21**(10): p. 3392-400.

79. Mizuno, Y., et al., *Apoptosis in neurodegenerative disorders.* Intern Med, 1998. **37**(2): p. 192-3.

80. Liu, X.Z., et al., *Neuronal and glial apoptosis after traumatic spinal cord injury.* J Neurosci, 1997. **17**(14): p. 5395-406.

81. Beattie, M.S., A.A. Farooqui, and J.C. Bresnahan, *Review of current evidence for apoptosis after spinal cord injury.* Journal of neurotrauma, 2000. **17**(10): p. 915-25.

82. Zhang, N., et al., *Inflammation & apoptosis in spinal cord injury.* Indian J Med Res, 2012. **135**: p. 287-96.

83. Crowe, M.J., et al., *Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys.* Nat Med, 1997. **3**(1): p. 73-6.

84. Guest, J.D., E.D. Hiester, and R.P. Bunge, *Demyelination and Schwann cell responses adjacent to injury epicenter cavities following chronic human spinal cord injury.* Exp Neurol, 2005. **192**(2): p. 384-93.

85. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.

86. Dumont, R.J., et al., *Acute spinal cord injury, part I: pathophysiologic mechanisms.* Clin Neuropharmacol, 2001. **24**(5): p. 254-64.

87. Amemiya, S., et al., *Anti-apoptotic and neuroprotective effects of edaravone following transient focal ischemia in rats.* Eur J Pharmacol, 2005. **516**(2): p. 125-30.

88. Davis, A.R., et al., *FasL, Fas, and death-inducing signaling complex (DISC) proteins are recruited to membrane rafts after spinal cord injury.* J Neurotrauma, 2007. **24**(5): p. 823-34.

89. Casha, S., W.R. Yu, and M.G. Fehlings, *FAS deficiency reduces apoptosis, spares axons and improves function after spinal cord injury.* Exp Neurol, 2005. **196**(2): p. 390-400.

90. Casha, S., W.R. Yu, and M.G. Fehlings, *Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat.* Neuroscience, 2001. **103**(1): p. 203-18.

91. Yu, W.R. and M.G. Fehlings, *Fas/FasL-mediated apoptosis and inflammation are key features of acute human spinal cord injury: implications for translational, clinical application.* Acta Neuropathol, 2011. **122**(6): p. 747-61.

92. Zhou, K., et al., *The Temporal Pattern, Flux, and Function of Autophagy in Spinal Cord Injury.* Int J Mol Sci, 2017. **18**(2).

93. Mizushima, N., *Autophagy: process and function.* Genes Dev, 2007. **21**(22): p. 2861-73.

94. Zhang, H.Y., et al., *Regulation of autophagy and ubiquitinated protein accumulation by bFGF promotes functional recovery and neural protection in a rat model of spinal cord injury.* Mol Neurobiol, 2013. **48**(3): p. 452-64.

95. Yu, D., et al., *Induction of neuronal mitophagy in acute spinal cord injury in rats.* Neurotox Res, 2013. **24**(4): p. 512-22.

96. Chen, J., et al., *Increased oligodendrogenesis by humanin promotes axonal remyelination and neurological recovery in hypoxic/ischemic brains.* Hippocampus, 2015. **25**(1): p. 62-71.

97. Liu, J., et al., *Nitric Oxide Interacts with Caveolin-1 to Facilitate Autophagy-Lysosome-Mediated Claudin-5 Degradation in Oxygen-Glucose Deprivation-Treated Endothelial Cells.* Mol Neurobiol, 2016. **53**(9): p. 5935-5947.

98. He, M., et al., *Autophagy induction stabilizes microtubules and promotes axon regeneration after spinal cord injury.* Proc Natl Acad Sci U S A, 2016. **113**(40): p. 11324-11329.

99. Zhou, X., X. He, and Y. Ren, *Function of microglia and macrophages in secondary damage after spinal cord injury.* Neural Regen Res, 2014. **9**(20): p. 1787-95.

100. Alexander, J.K. and P.G. Popovich, *Neuroinflammation in spinal cord injury: therapeutic targets for neuroprotection and regeneration.* 2009. **175**: p. 125-137.

101. Rice, T., et al., *Characterization of the early neuroinflammation after spinal cord injury in mice.* J Neuropathol Exp Neurol, 2007. **66**(3): p. 184-95.

102. Bartholdi, D. and M.E. Schwab, *Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an in situ hybridization study.* Eur J Neurosci, 1997. **9**(7): p. 1422-38.

103. Neirinckx, V., et al., *Neutrophil contribution to spinal cord injury and repair.* J Neuroinflammation, 2014. **11**: p. 150.

104. Shin, T., et al., *Alternatively activated macrophages in spinal cord injury and remission: another mechanism for repair?* Mol Neurobiol, 2013. **47**(3): p. 1011-9.

105. Jones, T.B., *Lymphocytes and autoimmunity after spinal cord injury.* Exp Neurol, 2014. **258**: p. 78-90.

106. Abul K. Abbas, A.H.L., Shiv Pillai, *Cellualar and Molecular Immunology*. 9th ed. 2018, USA: Elsevier.

107. Ankeny, D.P., et al., *Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis.* J Neurochem, 2006. **99**(4): p. 1073-87.

108. Vazquez, M.I., J. Catalan-Dibene, and A. Zlotnik, *B cells responses and cytokine production are regulated by their immune microenvironment.* Cytokine, 2015. **74**(2): p. 318-26.

109. Walsh, J.T. and J. Kipnis, *Regulatory T cells in CNS injury: the simple, the complex and the confused.* Trends Mol Med, 2011. **17**(10): p. 541-7.

110. Kigerl, K.A., et al., *Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord.* J Neurosci, 2009. **29**(43): p. 13435-44.

111. Wattananit, S., et al., *Monocyte-Derived Macrophages Contribute to Spontaneous Long-Term Functional Recovery after Stroke in Mice.* Journal of Neuroscience, 2016. **36**(15): p. 4182-4195.

112. Cekanaviciute, E. and M.S. Buckwalter, *Astrocytes: Integrative Regulators of Neuroinflammation in Stroke and Other Neurological Diseases.* Neurotherapeutics, 2016. **13**(4): p. 685-701.

113. Colombo, E. and C. Farina, *Astrocytes: Key Regulators of Neuroinflammation.* Trends Immunol, 2016. **37**(9): p. 608-20.

114. Fitch, M.T. and J. Silver, *CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure.* Exp Neurol, 2007.

115. Rolls, A., R. Shechter, and M. Schwartz, *The bright side of the glial scar in CNS repair.* Nat Rev Neurosci, 2009. **10**(3): p. 235-41.

116. Pineau, I., et al., *Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion.* Brain Behav Immun, 2010. **24**(4): p. 540-53.

117. Pineau, I. and S. Lacroix, *Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved.* J Comp Neurol, 2007. **500**(2): p. 267-85.

118. Sun, N., et al., *Activation of astrocytes in the spinal cord of mice chronically infected with a neurotropic coronavirus.* Virology, 1995. **213**(2): p. 482-93.

119. Constantinescu, C.S., et al., *Astrocytes as antigen-presenting cells: expression of IL-12/IL-23.* J Neurochem, 2005. **95**(2): p. 331-40.

120. Park, C., et al., *TLR3-mediated signal induces proinflammatory cytokine and chemokine gene expression in astrocytes: differential signaling mechanisms of TLR3-induced IP-10 and IL-8 gene expression.* Glia, 2006. **53**(3): p. 248-56.

121. Zamanian, J.L., et al., *Genomic analysis of reactive astrogliosis.* J Neurosci, 2012. **32**(18): p. 6391-410.

122. Brambilla, R., et al., *Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury.* J Exp Med, 2005. **202**(1): p. 145-56.

123. Krumbholz, M., et al., *BAFF is produced by astrocytes and up-regulated in multiple sclerosis lesions and primary central nervous system lymphoma.* J Exp Med, 2005. **201**(2): p. 195-200.

124. Babcock, A.A., et al., *Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS.* J Neurosci, 2003. **23**(21): p. 7922-30.

125. Sauder, C., et al., *Chemokine gene expression in astrocytes of Borna disease virus-infected rats and mice in the absence of inflammation.* J Virol, 2000. **74**(19): p. 9267-80.

126. Krumbholz, M., et al., *Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment.* Brain, 2006. **129**(Pt 1): p. 200-11.

127. Kim, B.O., et al., *Induction of C chemokine XCL1 (lymphotactin/single C motif-1 alpha/activation-induced, T cell-derived and chemokine-related cytokine) expression by HIV-1 Tat protein.* J Immunol, 2004. **172**(3): p. 1888-95.

128. Haroon, F., et al., *Gp130-dependent astrocytic survival is critical for the control of autoimmune central nervous system inflammation.* J Immunol, 2011. **186**(11): p. 6521-31.

129. Stuve, O., et al., *The role of the MHC class II transactivator in class II expression and antigen presentation by astrocytes and in susceptibility to central nervous system autoimmune disease.* J Immunol, 2002. **169**(12): p. 6720-32.

130. Cho, M.L., et al., *Transforming growth factor beta 1(TGF-beta1) down-regulates TNFalpha-induced RANTES production in rheumatoid synovial fibroblasts through NF-kappaB-mediated transcriptional repression.* Immunol Lett, 2006. **105**(2): p. 159-66.

131. Okada, S., et al., *Conditional ablation of Stat3 or Socs3 discloses a dual role of reactive astrocytes after spinal cord injury.* Nat Med, 2006. **12**(7): p. 829-834.

132. Colombo, E., et al., *Fingolimod may support neuroprotection via blockade of astrocyte nitric oxide.* Ann Neurol, 2014. **76**(3): p. 325-37.

133. Dusart, I. and M.E. Schwab, *Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord.* Eur J Neurosci, 1994. **6**(5): p. 712-24.

134. Taoka, Y., et al., *Role of neutrophils in spinal cord injury in the rat.* Neuroscience, 1997. **79**(4): p. 1177-82.

135. Farooque, M., J. Isaksson, and Y. Olsson, *Improved recovery after spinal cord trauma in ICAM-1 and P-selectin knockout mice.* Neuroreport, 1999. **10**(1): p. 131-4.

136. Stirling, D.P., et al., *Depletion of Ly6G/Gr-1 leukocytes after spinal cord injury in mice alters wound healing and worsens neurological outcome.* J Neurosci, 2009. **29**(3): p. 753-64.

137. Schroder, A.K., et al., *Polymorphonuclear leucocytes selectively produce anti-inflammatory interleukin-1 receptor antagonist and chemokines, but fail to produce pro-inflammatory mediators.* Immunology, 2006. **119**(3): p. 317-27.

138. Jakovcevski, I., et al., *Glial scar expression of CHL1, the close homolog of the adhesion molecule L1, limits recovery after spinal cord injury.* J Neurosci, 2007. **27**(27): p. 7222-33.

139. Matsuura, I., et al., *BMP inhibition enhances axonal growth and functional recovery after spinal cord injury.* J Neurochem, 2008. **105**(4): p. 1471-9.

140. Greenhalgh, A.D. and S. David, *Differences in the phagocytic response of microglia and peripheral macrophages after spinal cord injury and its effects on cell death.* J Neurosci, 2014. **34**(18): p. 6316-22.

141. Ginhoux, F., et al., *Origin and differentiation of microglia.* Front Cell Neurosci, 2013. **7**: p. 45.

142. Epelman, S., K.J. Lavine, and G.J. Randolph, *Origin and functions of tissue macrophages.* Immunity, 2014. **41**(1): p. 21-35.

143. Elkabes, S., E.M. DiCicco-Bloom, and I.B. Black, *Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function.* J Neurosci, 1996. **16**(8): p. 2508-21.

144. Chamak, B., V. Morandi, and M. Mallat, *Brain macrophages stimulate neurite growth and regeneration by secreting thrombospondin.* J Neurosci Res, 1994. **38**(2): p. 221-33.

145. Davalos, D., et al., *ATP mediates rapid microglial response to local brain injury in vivo.* Nat Neurosci, 2005. **8**(6): p. 752-8.

146. David, S. and A. Kroner, *Repertoire of microglial and macrophage responses after spinal cord injury.* Nat Rev Neurosci, 2011. **12**(7): p. 388-99.

147. Kroner, A., et al., *TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord.* Neuron, 2014. **83**(5): p. 1098-116.

148. Boato, F., et al., *Absence of IL-1beta positively affects neurological outcome, lesion development and axonal plasticity after spinal cord injury.* J Neuroinflammation, 2013. **10**: p. 6.

149. Mikita, J., et al., *Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration.* Mult Scler, 2011. **17**(1): p. 2-15.

150. Rathore, K.I., et al., *Ceruloplasmin protects injured spinal cord from iron-mediated oxidative damage.* J Neurosci, 2008. **28**(48): p. 12736-47.

151. Bao, F., et al., *Early anti-inflammatory treatment reduces lipid peroxidation and protein nitration after spinal cord injury in rats.* J Neurochem, 2004. **88**(6): p. 1335-44.

152. Martinez, F.O., et al., *Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression.* J Immunol, 2006. **177**(10): p. 7303-11.

153. Busch, S.A., et al., *Overcoming Macrophage-Mediated Axonal Dieback Following CNS Injury.* J. Neurosci, 2009. **29**(32): p. 9967-9976.

154. Hata, K., et al., *RGMa inhibition promotes axonal growth and recovery after spinal cord injury.* J Cell Biol, 2006. **173**(1): p. 47-58.

155. Schwab, J.M., et al., *Spinal cord injury-induced lesional expression of the repulsive guidance molecule (RGM).* Eur J Neurosci, 2005. **21**(6): p. 1569-76.

156. Knoblach, S.M. and A.I. Faden, *Interleukin-10 improves outcome and alters proinflammatory cytokine expression after experimental traumatic brain injury.* Exp Neurol, 1998. **153**(1): p. 143-51.

157. Thompson, C.D., et al., *The therapeutic role of interleukin-10 after spinal cord injury.* J Neurotrauma, 2013. **30**(15): p. 1311-24.

158. Zhou, Z., et al., *IL-10 promotes neuronal survival following spinal cord injury.* Exp Neurol, 2009. **220**(1): p. 183-90.

159. Genovese, T., et al., *Absence of endogenous interleukin-10 enhances secondary inflammatory process after spinal cord compression injury in mice.* J Neurochem, 2009. **108**(6): p. 1360-72.

160. Balasingam, V. and V.W. Yong, *Attenuation of astroglial reactivity by interleukin-10.* J Neurosci, 1996. **16**(9): p. 2945-55.

161. Braga, T.T., J.S. Agudelo, and N.O. Camara, *Macrophages During the Fibrotic Process: M2 as Friend and Foe.* Front Immunol, 2015. **6**: p. 602.

162. Lech, M. and H.J. Anders, *Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair.* Biochim Biophys Acta, 2013. **1832**(7): p. 989-97.

163. Lin, Y.H., et al., *Aldosterone induced galectin-3 secretion in vitro and in vivo: from cells to humans.* PLoS One, 2014. **9**(9): p. e95254.

164. Beck, K.D., et al., *Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment.* Brain, 2010. **133**(Pt 2): p. 433-47.

165. Sroga, J.M., et al., *Rats and mice exhibit distinct inflammatory reactions after spinal cord injury.* J Comp Neurol, 2003. **462**(2): p. 223-40.

166. Chen, L. and D.B. Flies, *Molecular mechanisms of T cell co-stimulation and co-inhibition.* Nat Rev Immunol, 2013. **13**(4): p. 227-42.

167. Popovich, P.G. and E.E. Longbrake, *Can the immune system be harnessed to repair the CNS?* Nat Rev Neurosci, 2008. **9**(6): p. 481-93.

168. Jones, T.B., et al., *Pathological CNS autoimmune disease triggered by traumatic spinal cord injury: implications for autoimmune vaccine therapy.* J Neurosci, 2002. **22**(7): p. 2690-700.

169. Potas, J.R., et al., *Augmented locomotor recovery after spinal cord injury in the athymic nude rat.* J Neurotrauma, 2006. **23**(5): p. 660-73.

170. Walsh, J.T., et al., *Regulatory T cells in central nervous system injury: a double-edged sword.* J Immunol, 2014. **193**(10): p. 5013-22.

171. Ankeny, D.P., Z. Guan, and P.G. Popovich, *B cells produce pathogenic antibodies and impair recovery after spinal cord injury in mice.* J Clin Invest, 2009. **119**(10): p. 2990-9.

172. Kil, K., et al., *T cell responses to myelin basic protein in patients with spinal cord injury and multiple sclerosis.* J Neuroimmunol, 1999. **98**(2): p. 201-7.

173. Zajarias-Fainsod, D., et al., *Autoreactivity against myelin basic protein in patients with chronic paraplegia.* Eur Spine J, 2012. **21**(5): p. 964-70.

174. Hayes, K.C., et al., *Elevated serum titers of proinflammatory cytokines and CNS autoantibodies in patients with chronic spinal cord injury.* J Neurotrauma, 2002. **19**(6): p. 753-61.

175. Popovich, P.G., B.T. Stokes, and C.C. Whitacre, *Concept of autoimmunity following spinal cord injury: possible roles for T lymphocytes in the traumatized central nervous system.* J Neurosci Res, 1996. **45**(4): p. 349-63.

176. Lund, F.E. and T.D. Randall, *Effector and regulatory B cells: modulators of CD4+ T cell immunity.* Nat Rev Immunol, 2010. **10**(4): p. 236-47.

177. Ankeny, D.P. and P.G. Popovich, *B cells and autoantibodies: complex roles in CNS injury.* Trends Immunol, 2010. **31**(9): p. 332-8.

178. Lucin, K.M., et al., *Impaired antibody synthesis after spinal cord injury is level dependent and is due to sympathetic nervous system dysregulation.* Exp Neurol, 2007. **207**(1): p. 75-84.

179. Ren, X., et al., *Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke.* J Neurosci, 2011. **31**(23): p. 8556-63.

180. Yoshizaki, A., et al., *Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions.* Nature, 2012. **491**(7423): p. 264-8.

181. Kahn, N.N., W.A. Bauman, and A.K. Sinha, *Appearance of a novel prostacyclin receptor antibody and duration of spinal cord injury.* J Spinal Cord Med, 2005. **28**(2): p. 97-102.

182. Dyck, S.M., et al., *Chondroitin Sulfate Proteoglycans Negatively Modulate Spinal Cord Neural Precursor Cells by Signaling through LAR and RPTPsigma and modulation of the Rho/ROCK Pathway.* Stem Cells, 2015.

183. Cregg, J.M., et al., *Functional regeneration beyond the glial scar.* Exp Neurol, 2014. **253**: p. 197-207.

184. Barrett, C.P., et al., *Astroglial reaction in the gray matter lumbar segments after midthoracic transection of the adult rat spinal cord.* Exp Neurol, 1981. **73**(2): p. 365-77.

185. Bignami, A. and D. Dahl, *The astroglial response to stabbing. immunofluorescence studies with antibodies to astrocyte-specific protein (GFA) in mammalian and submammalian vertebrates.* Neuropathology and Applied Neurobiology, 1976. **2**(2): p. 99-110.

186. Barnabe-Heider, F., et al., *Origin of new glial cells in intact and injured adult spinal cord.* Cell Stem Cell, 2010. **7**(4): p. 470-82.

187. Wanner, I.B., et al., *Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury.* J Neurosci, 2013. **33**(31): p. 12870-86.

188. Faulkner, J.R., et al., *Reactive Astrocytes Protect Tissue and Preserve Function after Spinal Cord Injury.* J Neurosci, 2004. **24**(9): p. 2143-2155.

189. Herrmann, J.E., et al., *STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury.* J Neurosci, 2008. **28**(28): p. 7231-43.

190. Rabchevsky, A.G., et al., *A role for transforming growth factor alpha as an inducer of astrogliosis.* J. Neurosci, 1998. **18**: p. 10541-10552.

191. McKeon, R.J., M.J. Jurynec, and C.R. Buck, *The chondroitin sulfate proteoglycans neurocan and phosphacan are expressed by reactive astrocytes in the chronic CNS glial scar.* J Neurosci, 1999. **19**(24): p. 10778-88.

192. Noble, L.J., et al., *Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events.* J Neurosci, 2002. **22**(17): p. 7526-35.

193. Agrawal, S.M., L. Lau, and V.W. Yong, *MMPs in the central nervous system: where the good guys go bad.* Semin Cell Dev Biol, 2008. **19**(1): p. 42-51.

194. Wells, J.E., et al., *An adverse role for matrix metalloproteinase 12 after spinal cord injury in mice.* J Neurosci, 2003. **23**(31): p. 10107-15.

195. Bundesen, L.Q., et al., *Ephrin-B2 and EphB2 regulation of astrocyte-meningeal fibroblast interactions in response to spinal cord lesions in adult rats.* J Neurosci, 2003. **23**(21): p. 7789-800.

196. Pasterkamp, R.J., et al., *Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal CNS.* Mol Cell Neurosci, 1999. **13**(2): p. 143-66.

197. Goritz, C., et al., *A pericyte origin of spinal cord scar tissue.* Science, 2011. **333**(6039): p. 238-42.

198. Soderblom, C., et al., *Perivascular fibroblasts form the fibrotic scar after contusive spinal cord injury.* J Neurosci, 2013. **33**(34): p. 13882-7.

199. Meletis, K., et al., *Spinal cord injury reveals multilineage differentiation of ependymal cells.* PloS Biol, 2008. **6**(e182).

200. Hughes, E.G., et al., *Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain.* Nat Neurosci, 2013. **16**(6): p. 668-76.

201. Sofroniew, M.V., *Molecular dissection of reactive astrogliosis and glial scar formation.* Trends Neurosci, 2009. **32**(12): p. 638-647.

202. Karimi-Abdolrezaee, S. and R. Billakanti, *Reactive Astrogliosis after Spinal Cord Injury-Beneficial and Detrimental Effects.* Mol. Neurobiol, 2012.

203. Grimpe, B. and J. Silver, *A Novel DNA Enzyme Reduces Glycosaminoglycan Chains in the Glial Scar and Allows Microtransplanted Dorsal Root Ganglia Axons to Regenerate beyond Lesions in the Spinal Cord* J Neurosci, 2004. **24**(6): p. 1393-1397.

204. Cafferty, W.B.J., et al., *Functional Axonal Regeneration through Astrocytic Scar Genetically Modified to Digest Chondroitin Sulfate Proteoglycans.* J. Neurosci, 2007. **27**(9): p. 2176-2185.

205. Buss, A., et al., *NG2 and phosphacan are present in the astroglial scar after human traumatic spinal cord injury.* BMC Neurol, 2009. **9**(32).

206. Galtrey, C.M. and J.W. Fawcett, *The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system.* Brain Research Reviews, 2007. **54**(1-18): p. 1.

207. Bradbury, E.J., et al., *Chondroitinase ABC promotes functional recovery after spinal cord injury.* Nature, 2002. **416**(6881): p. 636-40.

208. Fournier, A.E., B.T. Takizawa, and S.M. Strittmatter, *Rho Kinase Inhibition Enhances Axonal Regeneration in the Injury CNS.* J. Neurosci, 2003. **24**(44): p. 1416-1423.

209. Massey, J.M., et al., *Increased chondroitin sulfate proteoglycan expression in denervated brainstem targets following spinal cord injury creates a barrier to axonal regeneration overcome by chondroitinase ABC and neurotrophin-3.* Exp. Neurol, 2008. **209**: p. 426-445.

210. Tom, V.J., et al., *Combining Peripheral Nerve Grafts and Chondroitinase Promotes Functional Axonal Regeneration in the Chronically Injured Spinal Cord.* J. Neurosci, 2009. **29**(47): p. 14881-14890.

211. Chen, Z., et al., *Oligodendrocyte precursor cells: reactive cells that inhibit axon growth and regeneration.* J. Neurocytol, 2002. **6**(481=495): p. 481.

212. Barritt, A.W., et al., *Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury.* J Neurosci, 2006. **26**(42): p. 10856-67.

213. Massey, J.M., et al., *Chondroitinase ABC Digestion of the Perineuronal Net Promotes Functional Collateral Sprouting in the Cuneate Nucleus after Cervical Spinal Cord Injury.* J. Neurosci, 2006. **26**(16): p. 4406-4414.

214. Alilain, W.J., et al., *Functional regeneration of respiratory pathways after spinal cord injury.* Nature, 2011. **475**(7355): p. 196-200.

215. Petrosyan, H.A., et al., *Neutralization of Inhibitory Molecule NG2 Improves Synaptic Transmission, Retrograde Transport, and Locomotor Function after Spinal Cord Injury in Adult Rats.* J Neurosci, 2013. **33**(9): p. 4032-4043.

216. Arvantan, V.I., et al., *Chronic spinal hemisection in rats induces a progressive decline in transmission in uninjured fiber to motorneurons.* Exp. Neurol, 2009. **216**: p. 471-480.

217. Hunanyan, A.S., et al., *Role of chondroitin sulfate proteoglycans in axonal conduction in Mammalian spinal cord*, in *J. Neurosci*. 2010: 30. p. 7761.

218. Karimi-Abdolrezaee, S., et al., *Synergistic Effects of Transplanted Adult Neural Stem/Progenitor Cells, Chondroitinase, and Growth Factors Promote Functional Repair and Plasticity of the Chronically Injured Spinal Cord.* J. Neurosci, 2010. **30**(5): p. 1657-1676.

219. Karimi-Abdolrezaee, S., et al., *Chondroitinase and Growth Factors Enhance Activation and Oligodendrocyte Differentiation of Endogenous Neural Precursor Cells after Spinal Cord Injury.* Plos One, 2012. **7**(5): p. 1-16.

220. Pendleton, J.C., et al., *Chondroitin Sulfate Proteoglycans Inhibit Oligodendrocyte Myelination through PTPσ.* Exp. Neurol, 2013.

221. Lau, L.W., et al., *Chondroitin sulfate proteoglycans in demyelinated lesions impair remyelination.* Ann Neurol, 2012. **72**(3): p. 419-32.

222. Larsen, P.H., et al., *Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan.* J Neurosci, 2003. **23**(35): p. 11127-35.

223. Kurazono, S., et al., *Expression of brain specific chondroitin sulfate proteoglycans, neurocan and phosphacan, in the developing and adult hippocampus of Ihara's epileptic rats.* Brain Res, 2001. **898**: p. 36-48.

224. Dyck, S.M. and S. Karimi-Abdolrezaee, *Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system.* Exp Neurol, 2015. **269**: p. 169-87.

225. Asher, R.A., et al., *Versican Is Upregulated in CNS Injury and Is a Product of Oligodendrocyte Lineage Cells.* J. Neurosci, 2002. **22**(6): p. 2225-2236.

226. Buss, A., et al., *NG2 and phosphacan are present in the astroglial scar after human traumatic spinal cord injury.* BMC Neurol, 2009. **9**: p. 32.

227. Schachtrup, C., et al., *Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-B after vascular damage.* J. Neurosci, 2010. **30**(17): p. 5843-5854.

228. Hellal, F., et al., *Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury.* Science, 2011. **331**(6019): p. 928-31.

229. Levine, J.M., *Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury.* J Neurosci, 1994. **14**(8): p. 4716-30.

230. Asher, R.A., et al., *Neurocan is upregulated in injured brain and in cytokine-treated astrocytes.* J Neurosci, 2000. **20**(7): p. 2427-38.

231. Cafferty, W.B., et al., *Functional axonal regeneration through astrocytic scar genetically modified to digest chondroitin sulfate proteoglycans.* J Neurosci, 2007. **27**(9): p. 2176-85.

232. Cafferty, W.B.J., et al., *Chondroitinase ABC-Mediated Plasticity of Spinal Sensory Function.* J. Neurosci, 2008. **28**(46): p. 11998-12009.

233. Wang, D., et al., *Chondroitinase Combined with Rehabilitation Promotes Recovery of Forelimb Function in Rats with Chronic Spinal Cord Injury.* J. Neurosci, 2011. **31**(25): p. 9332-9344.

234. Yick, L., et al., *Lithium chloride reinforces the regeneration-promoting effect of chondroitinase ABC on rubrospinal neurons after spinal cord injury.* J. Neurotrauma, 2007. **21**(7): p. 932-943.

235. Carter, L.M., S.B. McMahon, and E.J. Bradbury, *Delayed treatment with chondroitinase ABC reverses chronic atrophy of rubrospinal neurons following spinal cord injury.* Exp Neurol, 2011. **228**(1): p. 149-56.

236. Carter, L.M., et al., *The Yellow Fluorescent Protein (YFP-H) Mouse Reveals Neuroprotection as a Novel mechanism Underlying Chondroitinase ABC-Mediated Repair after Spinal Cord Injury.* J. Neurosci, 2008. **28**(52): p. 14107-14120.

237. Karimi-Abdolrezaee, S., et al., *Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury.* J Neurosci, 2006. **26**(13): p. 3377-89.

238. Wang, Y., et al., *Astrocytes from the Contused Spinal Cord Inhibit Oligodendrocyte Differentiation of Adult Oligodendrocyte Precursor Cells by Increasing the Expression of Bone Morphogenetic Proteins.* J. Neurosci, 2011. **31**(16): p. 6053-6058.

239. Pohl, H.B.F., et al., *Genetically Induced Adult Oligodendrocyte Cell Death Is Associate with Poor Myelin Clearance, Reduced Remyelination, and Axonal Damage.* J. Neurosci, 2011. **31**(3): p. 1069-1080.

240. Alizadeh, A. and S. Karimi-Abdolrezaee, *Microenvironmental Regulation of Oligodendrocyte Replacement and Remyelination in Spinal Cord Injury.* J Physiol, 2016.

241. Keirstead, H.S., et al., *Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury.* J Neurosci, 2005. **25**(19): p. 4694-705.

242. Lang, B.T., et al., *Modulation of the proteoglycan receptor PTPsigma promotes recovery after spinal cord injury.* Nature, 2015. **518**(7539): p. 404-8.

243. Fry, E.J., et al., *Corticospinal tract regeneration after spinal cord injury in receptor protein tyrosine phosphatase sigma deficient mice.* Glia, 2010. **58**(4): p. 423-33.

244. McLean, J., et al., *Enhanced Rate of Nerve Regeneration and Directional Errors After Sciatic Nerve Injury in Receptor Protein Tyrosine Phosphatase σ Knock-Out Mice.* J Neurosci, 2002. **22**(13): p. 5481-5491.

245. Lau, L.W., et al., *Pathophysiology of the brain extracellular matrix: a new target for remyelination.* Nat Rev Neurosci, 2013. **14**(10): p. 722-9.

246. Huang, L., et al., *Glial scar formation occurs in the human brain after ischemic stroke.* Int J Med Sci, 2014. **11**(4): p. 344-8.

247. Gauthier, M.-K., et al., *Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury.* Eur. J. Neurosci, 2013. **38**(5): p. 2693-2715.

248. Kataria, H., et al., *Neuregulin-1 promotes remyelination and fosters a pro-regenerative inflammatory response in focal demyelinating lesions of the spinal cord.* Glia, 2017.

249. Ryzhov, S., et al., *Activation of ERBB3 Receptor Prevents TNFα Production from Human Non-Classical Monocytes.* The FASEB Journal, 2015. **29**(1 Supplement).

250. Li, Y., et al., *Neuregulin-1 inhibits neuroinflammatory responses in a rat model of organophosphate-nerve agent-induced delayed neuronal injury.* J Neuroinflammation, 2015. **12**(1): p. 64.

251. Falls, D.L., *Neuregulins: functions, forms, and signaling strategies.* Exp Cell Res, 2003. **284**(1): p. 14-30.

252. Chou, C.F. and M. Ozaki, *In silico analysis of neuregulin 1 evolution in vertebrates.* Biosci Rep, 2010. **30**(4): p. 267-75.

253. Anton, E.S., et al., *Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex.* Development, 1997. **124**(18): p. 3501-10.

254. Calaora, V., et al., *Neuregulin signaling regulates neural precursor growth and the generation of oligodendrocytes in vitro.* J Neurosci, 2001. **21**(13): p. 4740-51.

255. Zhang, Z., et al., *Neuregulin isoforms in dorsal root ganglion neurons: effects of the cytoplasmic domain on localization and membrane shedding of Nrg-1 type I.* J Neurosci Res, 2006. **84**(1): p. 1-12.

256. Zhong, C., et al., *Presynaptic type III neuregulin 1 is required for sustained enhancement of hippocampal transmission by nicotine and for axonal targeting of alpha7 nicotinic acetylcholine receptors.* J Neurosci, 2008. **28**(37): p. 9111-6.

257. Yau, H.J., et al., *Neural development of the neuregulin receptor ErbB4 in the cerebral cortex and the hippocampus: preferential expression by interneurons tangentially migrating from the ganglionic eminences.* Cereb Cortex, 2003. **13**(3): p. 252-64.

258. Tan, W., et al., *Molecular cloning of a brain-specific, developmentally regulated neuregulin 1 (NRG1) isoform and identification of a functional promoter variant associated with schizophrenia.* J Biol Chem, 2007. **282**(33): p. 24343-51.

259. Steinthorsdottir, V., et al., *Multiple novel transcription initiation sites for NRG1.* Gene, 2004. **342**(1): p. 97-105.

260. Meyer, D., et al., *Isoform-specific expression and function of neuregulin.* Development, 1997. **124**(18): p. 3575-86.

261. Carraway, K.L., 3rd, et al., *Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases.* Nature, 1997. **387**(6632): p. 512-6.

262. Peles, E., et al., *Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells.* Cell, 1992. **69**(1): p. 205-16.

263. Holmes, W.E., et al., *Identification of heregulin, a specific activator of p185erbB2.* Science, 1992. **256**(5060): p. 1205-10.

264. Jessell, T.M., R.E. Siegel, and G.D. Fischbach, *Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord.* Proc Natl Acad Sci U S A, 1979. **76**(10): p. 5397-401.

265. Falls, D.L., et al., *ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family.* Cell, 1993. **72**(5): p. 801-15.

266. Raff, M.C., et al., *Schwann cell growth factors.* Cell, 1978. **15**(3): p. 813-22.

267. Lemke, G.E. and J.P. Brockes, *Identification and purification of glial growth factor.* J Neurosci, 1984. **4**(1): p. 75-83.

268. Marchionni, M.A., et al., *Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system.* Nature, 1993. **362**(6418): p. 312-8.

269. Mei, L. and W.C. Xiong, *Neuregulin 1 in neural development, synaptic plasticity and schizophrenia.* Nat Rev Neurosci, 2008. **9**(6): p. 437-52.

270. Li, Q. and J.A. Loeb, *Neuregulin-heparan-sulfate proteoglycan interactions produce sustained erbB receptor activation required for the induction of acetylcholine receptors in muscle.* J Biol Chem, 2001. **276**(41): p. 38068-75.

271. Montero, J.C., et al., *Differential shedding of transmembrane neuregulin isoforms by the tumor necrosis factor-alpha-converting enzyme.* Mol Cell Neurosci, 2000. **16**(5): p. 631-48.

272. Wang, J.Y., S.J. Miller, and D.L. Falls, *The N-terminal region of neuregulin isoforms determines the accumulation of cell surface and released neuregulin ectodomain.* J Biol Chem, 2001. **276**(4): p. 2841-51.

273. Golding, M., et al., *Mapping nucleolar and spliceosome localization sequences of neuregulin1-beta3.* Exp Cell Res, 2004. **299**(1): p. 110-8.

274. Wolpowitz, D., et al., *Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses.* Neuron, 2000. **25**(1): p. 79-91.

275. Yang, X., et al., *A cysteine-rich isoform of neuregulin controls the level of expression of neuronal nicotinic receptor channels during synaptogenesis.* Neuron, 1998. **20**(2): p. 255-70.

276. Bao, J., et al., *Back signaling by the Nrg-1 intracellular domain.* J Cell Biol, 2003. **161**(6): p. 1133-41.

277. Loeb, J.A., E.T. Susanto, and G.D. Fischbach, *The neuregulin precursor proARIA is processed to ARIA after expression on the cell surface by a protein kinase C-enhanced mechanism.* Mol Cell Neurosci, 1998. **11**(1-2): p. 77-91.

278. Montero, J.C., et al., *The extracellular linker of pro-neuregulin-alpha2c is required for efficient sorting and juxtacrine function.* Mol Biol Cell, 2007. **18**(2): p. 380-93.

279. Hu, X., et al., *Bace1 modulates myelination in the central and peripheral nervous system.* Nat Neurosci, 2006. **9**(12): p. 1520-5.

280. Hu, X., et al., *BACE1 regulates the proliferation and cellular functions of Schwann cells.* Glia, 2017. **65**(5): p. 712-726.

281. Willem, M., et al., *Control of peripheral nerve myelination by the beta-secretase BACE1.* Science, 2006. **314**(5799): p. 664-6.

282. Yokozeki, T., et al., *Meltrin beta (ADAM19) mediates ectodomain shedding of Neuregulin beta1 in the Golgi apparatus: fluorescence correlation spectroscopic observation of the dynamics of ectodomain shedding in living cells.* Genes Cells, 2007. **12**(3): p. 329-43.

283. Meyer, D. and C. Birchmeier, *Multiple essential functions of neuregulin in development.* Nature, 1995. **378**(6555): p. 386-90.

284. Fischbach, G.D. and K.M. Rosen, *ARIA: a neuromuscular junction neuregulin.* Annu Rev Neurosci, 1997. **20**: p. 429-58.

285. Kramer, R., et al., *Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development.* Proc Natl Acad Sci U S A, 1996. **93**(10): p. 4833-8.

286. Hu, X., et al., *Genetic deletion of BACE1 in mice affects remyelination of sciatic nerves.* FASEB J, 2008. **22**(8): p. 2970-80.

287. La Marca, R., et al., *TACE (ADAM17) inhibits Schwann cell myelination.* Nat Neurosci, 2011. **14**(7): p. 857-65.

288. Fleck, D., et al., *Proteolytic Processing of Neuregulin 1 Type III by Three Intramembrane-cleaving Proteases.* J Biol Chem, 2016. **291**(1): p. 318-33.

289. Talmage, D.A., *Mechanisms of neuregulin action.* Novartis Found Symp, 2008. **289**: p. 74-84; discussion 84-93.

290. Tzahar, E., et al., *ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms.* J Biol Chem, 1994. **269**(40): p. 25226-33.

291. Bublil, E.M. and Y. Yarden, *The EGF receptor family: spearheading a merger of signaling and therapeutics.* Curr Opin Cell Biol, 2007. **19**(2): p. 124-34.

292. Downward, J., et al., *Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences.* Nature, 1984. **307**(5951): p. 521-7.

293. Schechter, A.L., et al., *The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen.* Nature, 1984. **312**(5994): p. 513-6.

294. Semba, K., et al., *A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma.* Proc Natl Acad Sci U S A, 1985. **82**(19): p. 6497-501.

295. Kraus, M.H., et al., *Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors.* Proc Natl Acad Sci U S A, 1989. **86**(23): p. 9193-7.

296. Plowman, G.D., et al., *Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family.* Proc Natl Acad Sci U S A, 1993. **90**(5): p. 1746-50.

297. Olayioye, M.A., et al., *The ErbB signaling network: receptor heterodimerization in development and cancer.* EMBO J, 2000. **19**(13): p. 3159-67.

298. Olayioye, M.A., et al., *ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner.* Mol Cell Biol, 1998. **18**(9): p. 5042-51.

299. Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.

300. Sierke, S.L., et al., *Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein.* Biochem J, 1997. **322 (Pt 3)**: p. 757-63.

301. Cho, H.S., et al., *Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab.* Nature, 2003. **421**(6924): p. 756-60.

302. Garrett, T.P., et al., *The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors.* Mol Cell, 2003. **11**(2): p. 495-505.

303. Guy, P.M., et al., *Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity.* Proc Natl Acad Sci U S A, 1994. **91**(17): p. 8132-6.

304. Iwakura, Y. and H. Nawa, *ErbB1-4-dependent EGF/neuregulin signals and their cross talk in the central nervous system: pathological implications in schizophrenia and Parkinson's disease.* Front Cell Neurosci, 2013. **7**: p. 4.

305. Li, K.X., et al., *Neuregulin 1 regulates excitability of fast-spiking neurons through Kv1.1 and acts in epilepsy.* Nat Neurosci, 2011. **15**(2): p. 267-73.

306. Si, J., Q. Wang, and L. Mei, *Essential roles of c-JUN and c-JUN N-terminal kinase (JNK) in neuregulin-increased expression of the acetylcholine receptor epsilon-subunit.* J Neurosci, 1999. **19**(19): p. 8498-508.

307. Fu, A.K., et al., *Cdk5 is involved in neuregulin-induced AChR expression at the neuromuscular junction.* Nat Neurosci, 2001. **4**(4): p. 374-81.

308. Bjarnadottir, M., et al., *Neuregulin1 (NRG1) signaling through Fyn modulates NMDA receptor phosphorylation: differential synaptic function in NRG1+/- knock-outs compared with wild-type mice.* J Neurosci, 2007. **27**(17): p. 4519-29.

309. Kim, J.H., K. Saito, and S. Yokoyama, *Chimeric receptor analyses of the interactions of the ectodomains of ErbB-1 with epidermal growth factor and of those of ErbB-4 with neuregulin.* Eur J Biochem, 2002. **269**(9): p. 2323-9.

310. Lee, H.J., et al., *Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4.* J Biol Chem, 2002. **277**(8): p. 6318-23.

311. Ni, C.Y., et al., *gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase.* Science, 2001. **294**(5549): p. 2179-81.

312. Sardi, S.P., et al., *Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain.* Cell, 2006. **127**(1): p. 185-97.

313. Leimeroth, R., et al., *Membrane-bound neuregulin1 type III actively promotes Schwann cell differentiation of multipotent Progenitor cells.* Dev Biol, 2002. **246**(2): p. 245-58.

314. Bao, J., et al., *Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos.* Nat Neurosci, 2004. **7**(11): p. 1250-8.

315. Gassmann, M., et al., *Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor.* Nature, 1995. **378**(6555): p. 390-4.

316. Lee, K.F., et al., *Requirement for neuregulin receptor erbB2 in neural and cardiac development.* Nature, 1995. **378**(6555): p. 394-8.

317. Erickson, S.L., et al., *ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice.* Development, 1997. **124**(24): p. 4999-5011.

318. Si, J., D.S. Miller, and L. Mei, *Identification of an element required for acetylcholine receptor-inducing activity (ARIA)-induced expression of the acetylcholine receptor epsilon subunit gene.* J Biol Chem, 1997. **272**(16): p. 10367-71.

319. Si, J., Z. Luo, and L. Mei, *Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase.* J Biol Chem, 1996. **271**(33): p. 19752-9.

320. Sandrock, A.W., Jr., et al., *Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo.* Science, 1997. **276**(5312): p. 599-603.

321. Corfas, G., et al., *Differential expression of ARIA isoforms in the rat brain.* Neuron, 1995. **14**(1): p. 103-15.

322. Steiner, H., et al., *Differential expression of ErbB3 and ErbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat.* Exp Neurol, 1999. **159**(2): p. 494-503.

323. Roy, K., et al., *Loss of erbB signaling in oligodendrocytes alters myelin and dopaminergic function, a potential mechanism for neuropsychiatric disorders.* Proc Natl Acad Sci U S A, 2007. **104**(19): p. 8131-6.

324. Schmucker, J., et al., *erbB3 is dispensable for oligodendrocyte development in vitro and in vivo.* Glia, 2003. **44**(1): p. 67-75.

325. Sussman, C.R., T. Vartanian, and R.H. Miller, *The ErbB4 neuregulin receptor mediates suppression of oligodendrocyte maturation.* J Neurosci, 2005. **25**(24): p. 5757-62.

326. Taveggia, C., et al., *Type III neuregulin-1 promotes oligodendrocyte myelination.* Glia, 2008. **56**(3): p. 284-93.

327. Liu, Y., et al., *Neuregulin-1 increases the proliferation of neuronal progenitors from embryonic neural stem cells.* Dev Biol, 2005. **283**(2): p. 437-45.

328. Golding, J.P., et al., *Defects in pathfinding by cranial neural crest cells in mice lacking the neuregulin receptor ErbB4.* Nat Cell Biol, 2000. **2**(2): p. 103-9.

329. Schmid, R.S., et al., *Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4251-6.

330. Rio, C., et al., *Neuregulin and erbB receptors play a critical role in neuronal migration.* Neuron, 1997. **19**(1): p. 39-50.

331. Flames, N., et al., *Short- and long-range attraction of cortical GABAergic interneurons by neuregulin-1.* Neuron, 2004. **44**(2): p. 251-61.

332. Gerecke, K.M., J.M. Wyss, and S.L. Carroll, *Neuregulin-1beta induces neurite extension and arborization in cultured hippocampal neurons.* Mol Cell Neurosci, 2004. **27**(4): p. 379-93.

333. Bermingham-McDonogh, O., K.L. McCabe, and T.A. Reh, *Effects of GGF/neuregulins on neuronal survival and neurite outgrowth correlate with erbB2/neu expression in developing rat retina.* Development, 1996. **122**(5): p. 1427-38.

334. Lopez-Bendito, G., et al., *Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation.* Cell, 2006. **125**(1): p. 127-42.

335. Rieff, H.I., et al., *Neuregulin induces GABA(A) receptor subunit expression and neurite outgrowth in cerebellar granule cells.* J Neurosci, 1999. **19**(24): p. 10757-66.

336. Liu, Y., et al., *Neuregulins increase alpha7 nicotinic acetylcholine receptors and enhance excitatory synaptic transmission in GABAergic interneurons of the hippocampus.* J Neurosci, 2001. **21**(15): p. 5660-9.

337. Okada, M. and G. Corfas, *Neuregulin1 downregulates postsynaptic GABAA receptors at the hippocampal inhibitory synapse.* Hippocampus, 2004. **14**(3): p. 337-44.

338. Li, B., et al., *The neuregulin-1 receptor erbB4 controls glutamatergic synapse maturation and plasticity.* Neuron, 2007. **54**(4): p. 583-97.

339. Dong, Z., et al., *Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors.* Neuron, 1995. **15**(3): p. 585-96.

340. Vartanian, T., G. Fischbach, and R. Miller, *Failure of spinal cord oligodendrocyte development in mice lacking neuregulin.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 731-5.

341. Vartanian, T., et al., *Axonal neuregulin signals cells of the oligodendrocyte lineage through activation of HER4 and Schwann cells through HER2 and HER3.* J Cell Biol, 1997. **137**(1): p. 211-20.

342. Shah, N.M., et al., *Glial growth factor restricts mammalian neural crest stem cells to a glial fate.* Cell, 1994. **77**(3): p. 349-60.

343. Winseck, A.K., et al., *In vivo analysis of Schwann cell programmed cell death in the embryonic chick: regulation by axons and glial growth factor.* J Neurosci, 2002. **22**(11): p. 4509-21.

344. Mahanthappa, N.K., E.S. Anton, and W.D. Matthew, *Glial growth factor 2, a soluble neuregulin, directly increases Schwann cell motility and indirectly promotes neurite outgrowth.* J Neurosci, 1996. **16**(15): p. 4673-83.

345. Syed, N., et al., *Soluble neuregulin-1 has bifunctional, concentration-dependent effects on Schwann cell myelination.* J Neurosci, 2010. **30**(17): p. 6122-31.

346. Ma, Z., et al., *Critical period of axoglial signaling between neuregulin-1 and brain-derived neurotrophic factor required for early Schwann cell survival and differentiation.* J Neurosci, 2011. **31**(26): p. 9630-40.

347. Fricker, F.R. and D.L. Bennett, *The role of neuregulin-1 in the response to nerve injury.* Future Neurol, 2011. **6**(6): p. 809-822.

348. Newbern, J. and C. Birchmeier, *Nrg1/ErbB signaling networks in Schwann cell development and myelination.* Semin Cell Dev Biol, 2010. **21**(9): p. 922-8.

349. Boerboom, A., et al., *Molecular Mechanisms Involved in Schwann Cell Plasticity.* Front Mol Neurosci, 2017. **10**: p. 38.

350. Michailov, G.V., et al., *Axonal neuregulin-1 regulates myelin sheath thickness.* Science, 2004. **304**(5671): p. 700-3.

351. Ho, W.H., et al., *Sensory and motor neuron-derived factor. A novel heregulin variant highly expressed in sensory and motor neurons.* J Biol Chem, 1995. **270**(24): p. 14523-32.

352. Garratt, A.N., S. Britsch, and C. Birchmeier, *Neuregulin, a factor with many functions in the life of a schwann cell.* Bioessays, 2000. **22**(11): p. 987-96.

353. Nave, K.A. and J.L. Salzer, *Axonal regulation of myelination by neuregulin 1.* Curr Opin Neurobiol, 2006. **16**(5): p. 492-500.

354. Taveggia, C., et al., *Neuregulin-1 type III determines the ensheathment fate of axons.* Neuron, 2005. **47**(5): p. 681-94.

355. Miyamoto, Y., et al., *Neuregulin-1 type III knockout mice exhibit delayed migration of Schwann cell precursors.* Biochem Biophys Res Commun, 2017. **486**(2): p. 506-513.

356. Chen, S., et al., *Neuregulin 1-erbB signaling is necessary for normal myelination and sensory function.* J Neurosci, 2006. **26**(12): p. 3079-86.

357. Zanazzi, G., et al., *Glial growth factor/neuregulin inhibits Schwann cell myelination and induces demyelination.* J Cell Biol, 2001. **152**(6): p. 1289-99.

358. Huijbregts, R.P., et al., *Hypertrophic neuropathies and malignant peripheral nerve sheath tumors in transgenic mice overexpressing glial growth factor beta3 in myelinating Schwann cells.* J Neurosci, 2003. **23**(19): p. 7269-80.

359. Kim, H.A., J.E. DeClue, and N. Ratner, *cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the cAMP and neuregulin/tyrosine kinase pathways.* J Neurosci Res, 1997. **49**(2): p. 236-47.

360. Li, Y., et al., *Neuregulin signaling through a PI3K/Akt/Bad pathway in Schwann cell survival.* Mol Cell Neurosci, 2001. **17**(4): p. 761-7.

361. Ogata, T., et al., *Opposing extracellular signal-regulated kinase and Akt pathways control Schwann cell myelination.* J Neurosci, 2004. **24**(30): p. 6724-32.

362. Maurel, P. and J.L. Salzer, *Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI 3-kinase activity.* J Neurosci, 2000. **20**(12): p. 4635-45.

363. Vartanian, T., et al., *Neuregulin induces the rapid association of focal adhesion kinase with the erbB2-erbB3 receptor complex in schwann cells.* Biochem Biophys Res Commun, 2000. **271**(2): p. 414-7.

364. Grove, M., et al., *FAK is required for axonal sorting by Schwann cells.* J Cell Biol, 2007. **176**(3): p. 277-82.

365. Tao, Y., et al., *Erbin regulates NRG1 signaling and myelination.* Proc Natl Acad Sci U S A, 2009. **106**(23): p. 9477-82.

366. Grossmann, K.S., et al., *The tyrosine phosphatase Shp2 (PTPN11) directs Neuregulin-1/ErbB signaling throughout Schwann cell development.* Proc Natl Acad Sci U S A, 2009. **106**(39): p. 16704-9.

367. Newbern, J., et al., *Mouse and human phenotypes indicate a critical conserved role for ERK2 signaling in neural crest development.* Proc Natl Acad Sci U S A, 2008. **105**(44): p. 17115-20.

368. Sheean, M.E., et al., *Activation of MAPK overrides the termination of myelin growth and replaces Nrg1/ErbB3 signals during Schwann cell development and myelination.* Genes Dev, 2014. **28**(3): p. 290-303.

369. Canoll, P.D., et al., *GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors.* Neuron, 1996. **17**(2): p. 229-43.

370. Fernandez, P.A., et al., *Evidence that axon-derived neuregulin promotes oligodendrocyte survival in the developing rat optic nerve.* Neuron, 2000. **28**(1): p. 81-90.

371. Flores, A.I., et al., *Akt-mediated survival of oligodendrocytes induced by neuregulins.* J Neurosci, 2000. **20**(20): p. 7622-30.

372. Vartanian, T., et al., *A role for the acetylcholine receptor-inducing protein ARIA in oligodendrocyte development.* Proc Natl Acad Sci U S A, 1994. **91**(24): p. 11626-30.

373. Kim, J.Y., et al., *The role of ErbB2 signaling in the onset of terminal differentiation of oligodendrocytes in vivo.* J Neurosci, 2003. **23**(13): p. 5561-71.

374. Makinodan, M., et al., *A critical period for social experience-dependent oligodendrocyte maturation and myelination.* Science, 2012. **337**(6100): p. 1357-60.

375. Mahar, I., et al., *Subchronic peripheral neuregulin-1 increases ventral hippocampal neurogenesis and induces antidepressant-like effects.* PLoS One, 2011. **6**(10): p. e26610.

376. Park, S.K., D. Solomon, and T. Vartanian, *Growth factor control of CNS myelination.* Dev Neurosci, 2001. **23**(4-5): p. 327-37.

377. Zhang, J., et al., *Promoting myelin repair and return of function in multiple sclerosis.* FEBS Lett, 2011. **585**(23): p. 3813-20.

378. Brinkmann, B.G., et al., *Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system.* Neuron, 2008. **59**(4): p. 581-95.

379. Bartus, K., et al., *Neuregulin-1 controls an endogenous repair mechanism after spinal cord injury.* Brain, 2016. **139**(Pt 5): p. 1394-416.

380. Birchmeier, C. and D.L. Bennett, *Neuregulin/ErbB Signaling in Developmental Myelin Formation and Nerve Repair.* Curr Top Dev Biol, 2016. **116**: p. 45-64.

381. Xu, Z., et al., *Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke.* Biochem Biophys Res Commun, 2004. **322**(2): p. 440-6.

382. Guo, W.P., et al., *Neuroprotective effects of neuregulin-1 in rat models of focal cerebral ischemia.* Brain Res, 2006. **1087**(1): p. 180-5.

383. Iaci, J.F., et al., *Glial growth factor 2 promotes functional recovery with treatment initiated up to 7 days after permanent focal ischemic stroke.* Neuropharmacology, 2010. **59**(7-8): p. 640-9.

384. Li, Y., et al., *Neuregulin-1 is neuroprotective in a rat model of organophosphate-induced delayed neuronal injury.* Toxicol Appl Pharmacol, 2012. **262**(2): p. 194-204.

385. Croslan, D.R., et al., *Neuroprotective effects of neuregulin-1 on B35 neuronal cells following ischemia.* Brain Res, 2008. **1210**: p. 39-47.

386. Shyu, W.C., et al., *Neuregulin-1 reduces ischemia-induced brain damage in rats.* Neurobiol Aging, 2004. **25**(7): p. 935-44.

387. Lasiene, J., et al., *Neuregulin 1 confers neuroprotection in SOD1-linked amyotrophic lateral sclerosis mice via restoration of C-boutons of spinal motor neurons.* Acta Neuropathol Commun, 2016. **4**: p. 15.

388. Issa, A.N., et al., *Neuregulin-1 at synapses on phrenic motoneurons.* J Comp Neurol, 2010. **518**(20): p. 4213-25.

389. Schutz, B., *Imbalanced excitatory to inhibitory synaptic input precedes motor neuron degeneration in an animal model of amyotrophic lateral sclerosis.* Neurobiol Dis, 2005. **20**(1): p. 131-40.

390. Karimi-Abdolrezaee, S., et al., *Synergistic effects of transplanted adult neural stem/progenitor cells, chondroitinase, and growth factors promote functional repair and plasticity of the chronically injured spinal cord.* J Neurosci, 2010. **30**(5): p. 1657-76.

391. Eftekharpour, E., S. Karimi-Abdolrezaee, and M.G. Fehlings, *Current status of experimental cell replacement approaches to spinal cord injury.* Neurosurg Focus, 2008. **24**(3-4): p. E19.

392. Thuret, S., L.D. Moon, and F.H. Gage, *Therapeutic interventions after spinal cord injury.* Nat Rev Neurosci, 2006. **7**(8): p. 628-43.

393. Sinha, K., et al., *Functional changes in genetically dysmyelinated spinal cord axons of shiverer mice: role of juxtaparanodal Kv1 family K+ channels.* J Neurophysiol, 2006. **95**(3): p. 1683-95.

394. Eftekharpour, E., et al., *Structural and functional alterations of spinal cord axons in adult Long Evans Shaker (LES) dysmyelinated rats.* Exp Neurol, 2005. **193**(2): p. 334-49.

395. Park, E., A.A. Velumian, and M.G. Fehlings, *The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration.* J Neurotrauma, 2004. **21**(6): p. 754-74.

396. Nashmi, R. and M.G. Fehlings, *Mechanisms of axonal dysfunction after spinal cord injury: with an emphasis on the role of voltage-gated potassium channels.* Brain Res Brain Res Rev, 2001. **38**(1-2): p. 165-91.

397. Povlishock, J.T. and C.W. Christman, *The pathobiology of traumatically induced axonal injury in animals and humans: a review of current thoughts.* J Neurotrauma, 1995. **12**(4): p. 555-64.

398. Anwar, M.A., T.S. Al Shehabi, and A.H. Eid, *Inflammogenesis of Secondary Spinal Cord Injury.* Front Cell Neurosci, 2016. **10**: p. 98.

399. Hausmann, O.N., *Post-traumatic inflammation following spinal cord injury.* Spinal Cord, 2003. **41**(7): p. 369-78.

400. Donnelly, D.J. and P.G. Popovich, *Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury.* Exp Neurol, 2008. **209**(2): p. 378-88.

401. Gensel, J.C., et al., *Macrophages promote axon regeneration with concurrent neurotoxicity.* J Neurosci, 2009. **29**(12): p. 3956-68.

402. Hamby, M.E. and M.V. Sofroniew, *Reactive astrocytes as therapeutic targets for CNS disorders.* Neurotherapeutics, 2010. **7**(4): p. 494-506.

403. Shechter, R., et al., *Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice.* PLoS Med, 2009. **6**(7): p. e1000113.

404. Pennati, A., et al., *Regulatory B cells induce formation of IL-10 expressing T cells in mice with autoimmune neuroinflammation.* J Neurosci, 2016.

405. Ulndreaj, A., et al., *Characterization of the antibody response after cervical spinal cord injury.* J Neurotrauma, 2016.

406. Gauthier, M.K., et al., *Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury.* Eur J Neurosci, 2013. **38**(5): p. 2693-715.

Chapter 2: Neuregulin-1 Positively Modulates Glial Response and Improves Neurological Recovery following Traumatic Spinal Cord Injury

This collaborative work was published as an original article in *Glia* (PMID: 28456012, Glia, 2017. **65**(7): p. 1152-1175. https://doi.org/10.1002/glia.23150)

Arsalan Alizadeh, Scott M. Dyck, Hardeep Kataria, Ghazaleh M. Shahriary, Dung H. Nguyen, Kallivalappil T. Santhosh and *Soheila Karimi-Abdolrezaee

Authors' affiliation: Regenerative Medicine Program, Department of Physiology and Pathophysiology, Spinal Cord Research Centre, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0J9

Author Contributions: My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. I contributed to over 80% of the total experimental procedures, data and analysis. Manuscript preparation was done in collaboration with my supervisor. Scott Dyck contributed to functional assessments and astrocyte activation *in vitro*. Pure microglia culture and data related to microglia presented in Figure 3 were provided by Hardeep Kataria and Ghazaleh Shahriary. Immunoprecipitation data presented in Figure 4 was provided by Dr. Hardeep Kataria. PCR data in figure 2 were provided by Dr. Santhosh Thomas Kallivalappil.

Acknowledgement: This work was supported by operating grants awarded to S.K-A from the Canadian Institutes of Health Research (CIHR, Grant No: MOP 133721), the Canadian Paraplegic Association of Manitoba, and the Manitoba Paraplegic Foundation. Salary support for A.A, S.M.D, H.K was provided by CHRIM, Research Manitoba, the Rick Hansen Institute and the U of M. D.H.N was jointly supported by studentships from the CIHR, the American Association of Neurology (AAN) and the U of M.

Conflict of Interest: The authors declare no competing financial interests

---

Chapter 2 is reprinted with permission (Glia) as it appears in: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.1 Abstract

Spinal cord injury (SCI) results in glial activation and neuroinflammation, which play pivotal roles in the secondary injury mechanisms with both pro- and anti-regeneration effects. Presently, little is known about the endogenous molecular mechanisms that regulate glial functions in the injured spinal cord. We previously reported that the expression of neuregulin-1 (Nrg-1) is acutely and chronically declined following traumatic SCI. Here, we investigated the potential ramifications of Nrg-1 dysregulation on glial and immune cell reactivity following SCI. Using complementary in vitro approaches and a clinically-relevant model of severe compressive SCI in rats, we demonstrate that immediate delivery of Nrg-1 (500ng/day) after injury enhances a neuroprotective phenotype in inflammatory cells associated with increased interleukin-10 and arginase-1 expression. We also found a decrease in pro-inflammatory factors including IL-1β, TNF-α, matrix metalloproteinases (MMP-2 and 9) and nitric oxide after injury. Additionally, Nrg-1 modulates astrogliosis and scar formation by reducing inhibitory chondroitin sulfate proteoglycans after SCI. Mechanistically, Nrg-1 effects on activated glia are mediated through ErbB2 tyrosine phosphorylation in an ErbB2/3 heterodimer complex. Furthermore, Nrg-1 exerts its effects through downregulation of MyD88, a downstream adaptor of Toll-like receptors, and increased phosphorylation of Erk1/2 and STAT3. Nrg-1 treatment with the therapeutic dosage of 1.5μg/day significantly improves tissue preservation and functional recovery following SCI. Our findings for the first time provide novel insights into the role and mechanisms of Nrg-1 in acute SCI and suggest a positive immunomodulatory role for Nrg-1 that can harness the beneficial properties of activated glia and inflammatory cells in recovery following SCI.

2.2 Introduction

Spinal cord injury (SCI) results in profound changes in the activity of resident glial cells and infiltrating immune cells. These cells dramatically modify the post-SCI microenvironment by releasing a plethora of inflammatory cytokines, nitric oxide (NO), free radicals, excitotoxic neurotransmitters, and matrix metalloproteinases (MMPs) [1-8]. Additionally, astrocytes cause astrogliosis leading to the formation of the glial scar and inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) [9, 10]. Glial activation has traditionally been viewed as a detrimental phenomenon associated with poor regenerative outcome following SCI [11-13]. However, emerging evidence points to the neuroprotective potential of activated glia following injury through their secretion of anti-inflammatory cytokines and pro-regenerative factors that can be exploited to foster tissue repair [14-18]. Currently, it is not clearly understood how the inhibitory and protective phenotypes of glia is regulated in acute SCI. Given the impact of glial cells in secondary injury mechanisms, it is imperative to unravel the endogenous processes involved in their modulation.

Our group has previously identified that the tissue levels of neuregulin-1 (Nrg-1) protein decline acutely and chronically in the injured spinal cord [19]. Nrg-1 is a neuronally-derived factor that is best known for its essential roles in oligodendrocyte and Schwann cell differentiation and myelin maintenance in developing and adult nervous system, and following peripheral nerve injury [20-22]. We previously demonstrated that restoring the deficient level of Nrg-1 through local delivery to the injured spinal cord remarkably decreases astrocyte differentiation of endogenous neural precursor cells (NPCs), promotes oligodendrocyte survival and replacement, and attenuates axonal degeneration [19]. Moreover, evidence from other groups points to a positive role for Nrg-1 in attenuating neurotoxicity and improving neuronal survival and recovery following ischemic

brain injuries [23-25]. Positive effect of Nrg-1 in reducing pro-inflammatory cytokines has been shown in cultures of activated microglial cell lines [25, 26]. In SCI, astrocytes and microglia express all Nrg-1 receptors, ErbB-2, ErbB-3 and ErbB-4, suggesting that acute reduction in Nrg-1 levels after SCI may have important ramifications on their response following injury [19]. To date, the impact and mechanisms of Nrg-1 in regulating astrogliosis and inflammatory response have not been explored in SCI.

In the present study, by utilizing a recombinant human Nrg-1 (rhNrg-1β1) peptide containing the bioactive EGF-like domain of all Nrg-1 isoforms, we investigated the impact of Nrg-1 bioavailability on glial functions using complementary in vitro and in vivo SCI models. Our work introduces, for the first time, a positive role for Nrg-1 in moderating astrogliosis and promoting a pro-regenerative immune response in the injured spinal cord, and its promise as a new therapeutic target for traumatic SCI.

2.3 Materials and Methods

2.3.1 Animals, Rat Model of Compressive Spinal Cord Injury and Animal Care

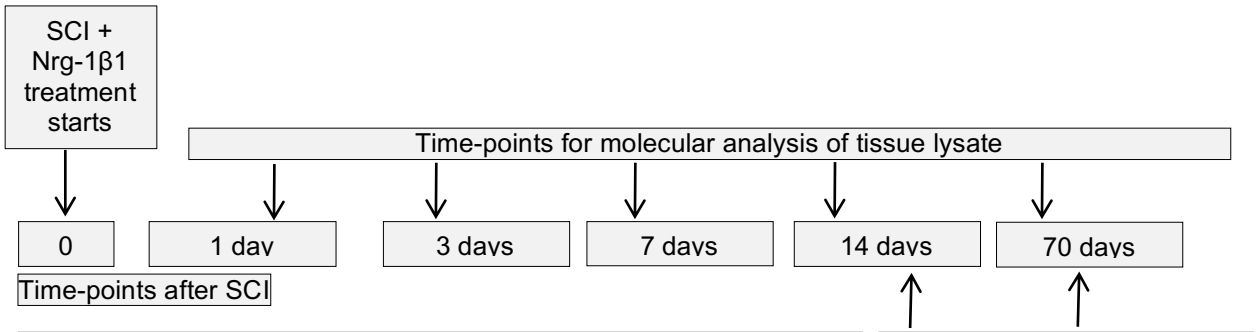
All experimental protocols in this study were approved by the University of Manitoba in accordance with the guidelines and policies established by the Canadian Council on Animal Care. For in vivo SCI studies, a total of 106 age and weight matched adult female Sprague Dawley (SD) rats (8-10 weeks, 250 g) and for in vitro experiments, 30 SD rat pups (1-3 days) and 2 C57BL/6 mice (8 weeks) were used (Local Facility at the University of Manitoba, Canada). Under deep Isoflurane anesthesia, rats received laminectomy at thoracic levels T6–T8 and then, a 35g aneurysm clip (University Health Network, Toronto, Ontario, Canada) was applied for 1 min at T7 of the spinal cord extradurally inducing a compression injury. Rats were housed in standard

plastic cages at 22° C before SCI and at 26°C afterwards in a 12:12 h light/dark cycle with soft paper bedding to prevent skin erosions and urine scalding. Pelleted food and drinking water were available ad libitum. Immediately after surgery, each animal received a combination of buprenorphine (Temgesic®, 0.05 mg/kg) and meloxicam (Metacam® Boehringer Ingelheim GmbH, 2 mg/kg) followed by three additional doses of buprenorphine with 8-hour intervals for pain management. SCI rats were examined daily and their bladder was expressed manually three times a day until the return of reflexive bladder control.

2.3.2 *In vivo* Experimental Groups and Treatments

Before surgical procedure, animals were randomly assigned to two experimental groups: 1) SCI/vehicle control, receiving rhNrg-1β1 vehicle solution (0.1% bovine serum albumin, BSA, in 0.9% saline); 2) SCI/Nrg-1, receiving rhNrg-1β1 500ng/day intrathecally. In vivo rhNrg-1β1 dosing was determined in our previous study where we found positive effects of rhNrg-1β1on inducing oligodendrocyte replacement after SCI [19]. Each experimental group included five time-points: 1d, 3d, 7d, 14d and 70d post-SCI. Nrg-1 or vehicle treatment was delivered at the time of SCI using an osmotic mini-pump and an indwelling intrathecal catheter (Alzet® –model #2001D for 1-day, model #1003D for 3d, model #2001 for 7d, model #2002 for 14d). For 10-week assessment of Nrg-1 effects, we used Alzet® pump model #2006 with 6-week delivery of Nrg-1. For long-term functional assessments, we tested three different daily doses of Nrg-1 that included 500ng, 1µg, and 1.5µg per day. A summary of our experimental approaches and groups is provided in Table 1.

Table 2.1: Summary of experimental procedures, treatment groups, time-points, dosing and sample size for *in vivo* experiments.



Experimental Groups:

1) Uninjured

2) SCI/ vehicle (saline + BSA)

3) SCI/ rhNrg-1β1

Nrg-1 dosing:

1) 0.5μg/day for all time-point molecular and histological analysis

2) 0.5μg/day, 1μg/day, and 1.5μg/day for 10wk neurological evaluations

Sample size:

N= 4 rats/group/time-point for acute and subacute molecular and histological analysis

N= 8 rats/group for 10 weeks chronic neurological testing

Total number of animal used in our *in vivo SCI* assessments:

106 female SD rats

2.3.3 Tissue Processing

At the end of each time-point, SCI rats were deeply anesthetized. Five millimeters of the spinal cord tissue centered at the injury site was dissected and processed for Western or slot blotting. For histological analyses, rats were perfused transcardially with 2.5% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). A 1.5-2 cm length of the spinal cord tissue centered at the injury site was excised and embedded (Tissue-Tek®-O.C.T., Electron Microscopy Sciences). Cross sections (35μm) were cut serially by a cryostat (Leica Biosystems GmbH) and mounted onto Superfrost® Plus Micro Slides (Fisher Scientific) and stored at -80°C until immunostaining procedure.

2.3.4 Isolation, Culture and Activation of Primary Culture of Astrocytes and Microglia

Primary glial cells were isolated from the cortex of postnatal rats (P1-P3) [27, 28]. Briefly, postnatal (P1-P3) rat pup cortices were mechanically dissociated and cells were seeded into 75 cm^2 flasks in DMEM/F12 (1:1) supplemented with 10% FBS and 1% Penicillin-Streptomycin-Neomycin (PSN). Mixed glial cultures were maintained with media change every three days until confluency (2-3 weeks). Our immunostaining showed that the culture was comprised of astrocytes (~80%) and microglia (~20%). We refer to this mixed culture of astrocytes and microglia as "astro-microglia culture" throughout the study. To induce global reactivity in the glia in vitro, LPS (10μg/ml) was added to the cultures for various time-points. LPS is widely used as an inducer of pro-inflammatory response in glial cells resulting in the release of NO, TNF-α and IL-1β as well as increased expression of astrogliosis markers including CSPGs and nestin [1]. In all experimental conditions, serum free media (SFM) was used to specifically determine the effects of Nrg-1 and rule out the influence of other factors in serum. Four conditions were examined in vitro: 1) Non-

activated glia as “control”, 2) Non-activated glia + rhNrg-1β1 termed as “Nrg-1”, 3) LPS-activated glia termed as “LPS”, 4) LPS-activated glia + rhNrg-1β1 as “LPS+Nrg-1”. We added rhNrg-1β1 (150ng/ml) to the cultures at the time of LPS activation. In vitro, Nrg-1 concentration was chosen based on our dose-response studies on astro-microglia cultures (Supp. Fig. 2.1). This concentration induced significant decrease in nitrite and CSPGs production in LPS activated astro-microglia culture (Sup. Fig. 2.1A, B). Conditioned media (CM) and cell lysates were analyzed at different time points after activation and treatments.

Pure Primary Microglia were isolated from mixed glial cultures as described above. Once mixed cultures became confluent, the cultures were then shaken at 200 rpm for 6 h at 37 °C. Floating microglia were collected and seeded over poly-D-lysine coated dishes in 1:1 mixture of fresh media (DMEM/F12) and glial CM (collected and filter sterilized after shaking). Remaining astrocytes devoid of microglia were used as pure astrocyte cultures after 48h of resting in refreshed media. Optimal dosing for LPS and Nrg-1 in pure microglial cultures were determined based on our dosing assay.

2.3.5 Immunostaining Procedures *In vitro* and *In vivo*

For all immunostaining procedures, the blocking solution contained 5% non-fat milk, 1% BSA, and 0.3% Triton X-100 in 0.1 M PBS unless otherwise noted. A list of the primary antibodies is provided in Table 2. Specificity of all antibodies was confirmed using both a negative control, omitting the primary antibody in our immunostaining protocol, and a positive control, testing the antibody on tissues or cell preparations known to express the target antigen.

2.3.6 Immunocytochemistry in Mixed Glial Culture

Following PFA fixation, cells were incubated with primary antibodies Ki67, GFAP and Iba-1 for 2 hours followed by goat-anti-mouse or goat-anti rabbit Alexa Fluor 488 or 568 secondary antibodies for 1 hour at room temperature (Table 2). To stain the nucleus, cells were incubated with 4, 6-diamidino-2-phenylindole (DAPI, Sigma D8417). Fluorescence images were taken at 20x magnification, and 10 random fields were acquired containing an average of 400 cells for each condition. The percentage of proliferating astrocytes and microglia was determined by counting the total number of GFAP+/Ki67+/DAPI+ cells for astrocytes and Iba-1+/Ki67+/DAPI+ cells for microglia in each treatment condition normalized to the total number of GFAP+/DAPI+ and Iba-1+/DAPI+ cells. The fold changes in proliferating cells were reported by normalizing the percentage of Ki67+/DAPI+ cells in each treatment condition to that of non-activated control group.

2.3.7 Histological Assessment of Chronic SCI Lesion

Spinal cord transections were stained using Luxol Fast Blue (LFB) and Hematoxylin and Eosin (H&E). Tissue section showing the least amount of preserved tissue was chosen as the epicenter. For assessment of tissue preservation, a total of 7 sections were chosen centering the injury epicenter (thickness, 35μm). Sections were evenly spaced being 1mm apart from each other. Images were taken using Zeiss Axio Imager M2® and tiling function of ZEN® software. Quantification was done using NIH ImageJ software calculating the percentage of the preserved spinal cord tissue by following formula: [(total spinal cord area - cavity area)/total area] x 100. Statistical analysis was done using Two-way ANOVA followed by Holm-Sidak post-hoc test.

Table 2.2: List of primary antibodies and sequence of qPCR primers used in this study. All antibodies, as well as their sources, usage and dilution factors are displayed. Sequence of the primers for real-time PCR is depicted. **ICC:** Immunocytochemistry, **IHC:** Immunohistochemistry, **WB:** Western blotting

Antibody	Source	Usage	Dilution Factor
IL-1β	Serotec (Rabbit, AAR15G)	WB	1:1000
TNF-α	Serotec (Rabbit, AAR33)	WB	1:1000
CS56	Sigma (Mouse, C8035)	IHC/WB	1:150/1:500
Nestin	Chemicon (Mouse, MAB 353)	WB	1:400
GFAP	DAKO (Rabbit, Z0334)	ICC	1:800
GFAP	Cell Signaling (Mouse, #3670)	WB	1:6000
Iba-1	Wako (Rabbit, 016-20001)	WB	1:1000
Iba-1	Wako (Rabbit, 019-19741)	IHC/ICC	1:500
Olig2	Chemicon (Rabbit, AB9610)	ICC	1:2000
Ki67	BD (Mouse, 550609)	ICC	1:50
MyD88	Cell Signaling (Rabbit, D80F5)	WB	1:1000
p-STAT3	Cell Signalling (Rabbit, #9145)	WB	1:1000
STAT3	Cell Signalling (Mouse, #9139)	WB	1:1000
p-ERK	Cell Signalling (Rabbit, #4370)	WB	1:500
ERK	Cell Signalling (Rabbit, #4695)	WB	1:1000
Beta actin	Sigma (Rabbit, A2066)	WB	1:300
Beta actin	Chemicon (Mouse, MAB1501R)	WB	1:300
DAPI	Vector (H-1200)	ICC/IHC	1:10000/1:5000
ErbB2	Santa Cruz (Rabbit, SC-284)	IP, WB	1:200
ErbB3	Santa Cruz (Rabbit, SC-285)	IP, WB	1:200
ErbB4	Santa Cruz (Rabbit, SC-283)	IP, WB	1:200
pTyr	Santa Cruz (Mouse, SC-7020)	WB	1:1000
OX42	AbD Serotec (Mouse, MCA275G)	ICC	1:100
Arg1 Primers	Forward- ACA AGA CAG GGC TAC TTT CAG G Reversed- ACA AGA CAA GGT CAA CGC CA		
Beta-actin primers	Forward- CGTGCGTGACATCAAAGAGAA Reversed- GGCCATCTCCTGCTCGAA		

2.3.8 Immunohistochemical Assessment of CSPGs, GFAP and Iba-1 Expression following SCI

Frozen spinal cord cross-sections (35 μm) from the injury epicenter as well as the 1mm rostral and caudal to the lesion center underwent immunostaining using CS56 (Sigma C8035), GFAP (DAKO Z0334) and Iba-1 (Wako, 019-19741) overnight at 4^{o}C followed by goat anti-mouse or goat anti-rabbit Alexa Fluor 568 and 488 secondary antibodies. The slides were washed three times in PBS, stained with DAPI and cover-slipped using Mowiol mounting media. All samples were processed in parallel under the same condition and imaged using Zeiss AxioImager M2 fluorescence microscope (Zeiss) under consistent exposure time as we described previously [19, 29, 30]. Using NIH ImageJ analysis software (imagej.nih.gov), entire spinal cord was traced in each section. After setting the threshold automatically, the immunodensity above the threshold was calculated. The following formula was used to calculate the percentage of CSPG, GFAP and Iba-1 area: normalized immunointensity of tissue section X = total immunointensity of tissue section X / total area of the spinal cord section X.

2.3.9 Immunoblotting Procedures *In vitro* And *In vivo*

For all Western and slot blot analyses, proteins were extracted from cell lysate or tissues using RIPA buffer containing proteinase and phosphatase inhibitor (Sigma, Cat #S8820). Conditioned media (CM) was concentrated 30 times using centrifugal column with molecular weight cut-off at 3 kDa (VWR). The protein concentration of each sample was measured using the modified Lowry assay. The blocking and antibody solution contained 5% non-fat milk in Tris-buffered saline with 0.5% Tween-20 (TBST) unless otherwise mentioned. A list of the antibodies used in

immunoblotting is provided in Table 2. Specificity of all antibodies in detecting the protein of interest was confirmed by specified molecular weight for each protein.

2.3.10 Western Blotting on Glial Cell Lysate, Conditioned Media and SCI Tissues

10 to 50 μg of protein of glial cell lysate, CM or spinal cord tissue per each sample was loaded into SDS-PAGE gels and transferred onto nitrocellulose membranes. The membrane was blocked for 1 hour at room temperature and then incubated with various primary antibodies overnight at 4^{o}C (Table 2). For MyD88 Western blotting, blocking solution contained 5% BSA in 1X TBS+0.1% Tween-20. The membranes were then incubated with secondary antibodies and developed as described previously [19]. Densitometry analysis of the immunoreactive bands was performed using NIH ImageJ software. To control for equal protein values were normalized to β-actin on the same membrane. For CM, membranes were stained with Ponceau S that stains all protein bands on a membrane. The densitometry value of each protein was then normalized to the total amount of protein in that lane identified by Ponceau S. All samples were processed in parallel on the same gel for comparison and bands were then quantified and normalized to the control condition from the same membrane.

2.3.11 Slot Blot Analysis of CSPGs Expression in Glial Conditioned Media and SCI Tissues

Slot blotting was done with 3 to 10μg of protein of glial CM or SCI tissue per sample, respectively, on a nitrocellulose membrane using Bio-Dot® slot blot system. The membrane was then blocked and incubated with mouse-anti CSPGs for 2 hours at room temperature. The membranes were developed in the same manner as described for Western blotting.

2.3.12 Griess Assay for Detection Of Nitrite

Griess assay (Promega Corp. USA) was used to measure nitrite level as a representative of iNOS activity in glial CM collected at 72hr after LPS activation according to the manufacturer instructions. To eliminate any possible interference in Griess assay readings, phenol red free media was used for these experiments.

2.3.13 Dose-Response Assay for Nrg-1 in Activated Astro-microglia and Pure Microglia Cultures

To determine in vitro dosage of Nrg-1 for astro-microglia cultures, activated cultures (using LPS, 10μg/ml) were treated with 25, 75, 150, 300 ng/ml of Nrg-1 representing low, mid and high doses for this type of culture. Nrg-1 effects on glial activation were then assessed by Griess nitrite assay and slot blot for CSPGs (Supp. Fig. 2.1). Our assessments identified 150ng/ml as the most effective Nrg-1 dose for treating activated astro-microglia cultures (Supp. Fig. 2.1A, B). For pure microglia, our dosing assay (10, 25, 50, 150 ng/ml) identified that 25 ng/ml of Nrg-1 can most effectively decrease nitrite production in activated pure microglia cultures (using 1μg/ml LPS).

2.3.14 Gelatin Zymography on SCI Tissues

To assess MMP-2 and MMP-9 enzymatic activity in the injured spinal cord tissue, 30μg of protein from the SCI tissue was loaded on 10% SDS-polyacrylamide gel, copolymerized with 1 mg/ml gelatin as substrate and were separated by electrophoresis. Proteins were renatured by 2.5% Triton X-100 to restore gelatinase activity. Gels then incubated with developing buffer overnight at 37°C to allow the MMPs to cleave the gelatin. Gels were stained with Coomassie blue for 30 minutes and de-stained in 30% Ethanol/10% acetic acid until appropriate color contrast was achieved.

Areas of gelatinase activity appeared as clear bands against a dark blue background. MMPs were identified based on their molecular weight and their density was measured as described in our Western blot procedures.

2.3.15 RNA Extraction and Quantitative Real-time PCR

Expression of Arginase 1 (Arg1) mRNA in vitro was studied using quantitative PCR (ABI, Perkin-Elmer PE Biosystems, USA) and the double-stranded DNA-specific fluorophore SYBR Green I (Invitrogen, Canada) as we described before [19]. RNA was extracted in Trizol using an RNeasy plus mini kit (Qiagen). First-strand cDNA was synthesized with Superscript III Reverse Transcriptase (Invitrogen). Primer information is depicted in Table 2.

2.3.16 Co-immunoprecipitation (co-IP) and Recapture Assay

For analysis of Nrg-1 induced activation of ErbB receptors, glial cultures were treated with Nrg-1 (150ng/ml) for 15, 30 and 60 minutes and then washed with cold PBS and solubilized in NP-40 lysis buffer with protease and phosphatase inhibitor cocktail (Thermo Fisher) on ice. Cell lysate samples containing 500ug of protein were incubated with protein A/G agarose beads (Santa Cruz) at 4 °C for 1 h on an orbital rotor before centrifugation at 12,000 rpm for 10 min. For immunoprecipitation, samples were incubated with specific antibodies [ErbB2, ErbB3 or ErbB4 (Santa Cruz)] for 1h followed by protein A/G overnight on an orbital rotor at 4°C. The immune-complexes were spun at 1000g for 3 min and washed four times with ice-cold lysis buffer. The immunoprecipitated proteins were solubilized by boiling in SDS-PAGE sample buffer for 5 min and were subject to SDS-PAGE and Western blotting. For immunoprecipitation–recapture the protocol in Current Protocols in Molecular Biology (1999) was followed.

2.3.17 Neurological Assessments: BBB Open Field Locomotor Score and Tail Flick Thermal Reactivity

Longitudinal hind-limb locomotion testing was performed weekly for 10 weeks following SCI using the Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale as was done previously [29, 31]. The test was performed by two examiners, who were blinded to the animal treatments. The duration of each session was 4 minutes per rat.

Thermal reactivity was examined at 4, 7 and 10 weeks after SCI by tail flick as we and others described previously [29, 32, 33]. In brief, thermal reactivity was assessed in response to immersion of 5 cm of the end of the tail in a 46^{o}C water bath. Three attempts were made per animal in each session and each attempt was separated by a 20-scond interval period. The latency of the rat to remove its tail from the heat was recorded.

2.3.18 Statistical Analysis and Randomization

Using SigmaStat Software, we performed two-way analysis of variance (ANOVA) repeated measures followed by Holm-Sidak post-hoc test for distance analysis in immunohistological assessments and for time-points analysis in functional testing. One-way ANOVA followed by Holm-Sidak post-hoc was used in all slot blot and Western blot analyses. Student t-test was used when two groups were compared. The data was reported as means ± standard error of the mean (SEM). $P \leq 0.05$ was considered statistically significant. Proper randomization and blinding was employed in all immunohistological and neurological assessments.

2.4 Results

2.4.1 Nrg-1 Positively Regulates The Activity of Astrocytes and Microglia in Co-culture

The acute response of microglia and astrocytes to injury is an essential process in the onset of neuroinflammation, astrogliosis and eventually scar formation [1, 14]. In our astro-microglia

culture, we found that Nrg-1 (150ng/ml) significantly reduces proliferation of activated astrocytes with no effects on microglial proliferation. Our quantitative immunocytochemistry against GFAP, Iba-1 and Ki67 that mark astrocytes, microglia and proliferating nuclei, respectively, showed a 2-fold increase in the number of proliferating astrocytes (GFAP+/Ki67+ cells) at 72h following LPS activation compared to the control condition (Fig. 2.1A-J). Treating LPS-activated astro-microglia with Nrg-1 resulted in a significant 1.6-fold decrease in astrocyte proliferation (one-way ANOVA, $P<0.05$, N=4) (Fig. 2.1A-D, I). Nrg-1 had no significant effects on the basal proliferative activity of astrocytes in non-activated state. Analysis Iba-1+/Ki67+ cells showed that microglial proliferation in both LPS and LPS+NRG-1 conditions was significantly reduced compared to the normal and Nrg-1 control counterparts (Fig. 2.1 E-H, J). This observation possibly reflects the reduced attachment of scavenging activated microglia in the LPS treated conditions and further detachment during staining procedures. Nonetheless, Nrg-1 treatment did not alter the proliferation rate of LPS-activated microglia suggesting Nrg-1 neither under normal nor activated state affects microglial proliferation (Fig. 2.1J).

Our slot blot analysis of conditioned media (CM) obtained from activated astro-microglia culture showed a significant 2-fold increase in CSPG release at 72h post-LPS activation compared to non-activated control cultures (Fig. 2.1K). Nrg-1 treatment significantly attenuated the rise in CSPG release by 1.47-fold compared to LPS activated glial cultures (one-way ANOVA, $P<0.001$, N= 4) (Fig. 2.1K). We found no significant effect on the constitutive expression of CSPGs under normal non-activated condition (Fig. 2.1K). Similar to CSPGs, Nrg-1 treatment significantly reduced nestin expression in LPS treated astro-microglia (1.7-fold decrease) with no effect on the basal expression of nestin under normal state (one-way ANOVA, $**P<0.01$, $*P<0.05$, N=3) (Fig. 2.1L).

Figure 2.1. Nrg-1 significantly mitigates astrogliosis and CSPGs production with no effect on microglial proliferation *in vitro*.

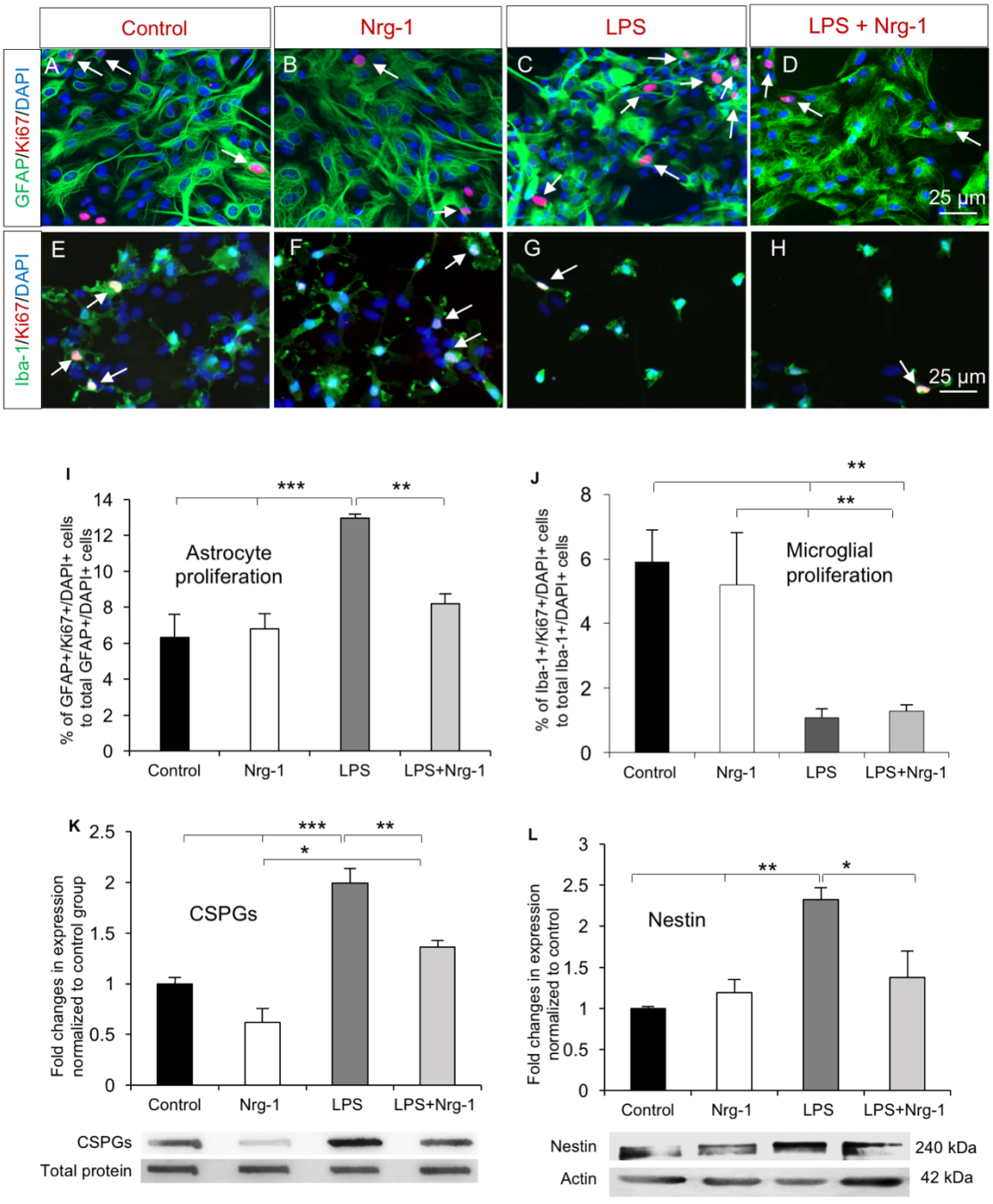


Figure 2.1. Nrg-1 significantly mitigates astrogliosis and CSPGs production with no effect on microglial proliferation *in vitro*. (A-D, I) Immunocytochemical analysis of astrocytes and microglia using the cell proliferation marker Ki67 revealed that 72h exposure to LPS significantly increases astrocyte proliferation rate evident by increased number of GFAP+/Ki67+/DAPI+ cells normalized to the total number of GFAP+/DAPI+ astrocytes. Astrocytes in LPS+Nrg-1 treated cultures showed a significant 1.5-fold reduction in the number of GFAP+/Ki67+/DAPI+ proliferating astrocytes ($P<0.01$, N=4). Nrg-1 itself had no effect on astrocytes proliferation in non-activated state. **(E-H, J)** Quantitative analysis of Iba-1+/Ki67+ microglia indicated no apparent difference in proliferation between normal and Nrg-1 treated control conditions. However, following LPS activation, in contrast to activated astrocytes, we found less proliferating microglia as the number of Iba-1+ cells were significantly reduced in both LPS and LPS+Nrg-1 conditions compared to the normal and Nrg-1 control counterparts. Regardless, Nrg-1 treatment did not change the proliferative activity of LPS-treated microglia as we found a comparable number of Iba-1+/Ki67+ in LPS treated cultures with or without Nrg-1 treatment. **(K)** Slot blot analysis of glial conditioned media (CM) with CS56 antibody showed a significant 2-fold increase in CSPGs at 72h following LPS activation. Nrg-1 treatment significantly reduced CSPGs production by 1.4 folds compared to non-treated activated glial cultures. Representative slot blot with corresponding Ponceau S protein loading control are depicted. **(L)** Western blot analysis of the glial cell lysate 72h after activation revealed a significant (1.6-fold) decrease in nestin expression in Nrg-1 treated group. No significant difference in nestin and CSPGs expression was observed between control and Nrg-1 treated groups. Actin was used as protein loading control. The data represent mean ± SEM, N=4/condition, * $P<0.05$, ** $P<0.01$, ***$P<0.001$, One-Way ANOVA, followed by Holm-Sidak post-hoc test.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.4.2 Nrg-1 Mitigates Pro-inflammatory Cytokine and Nitrite Production while Increases Arginase-1 Expression in Activated Astro-microglia Co-culture

Activated astrocytes and microglia release NO following traumatic and ischemic injuries to the CNS that initiates oxidative stress and neurotoxic cascades in neurons [8, 34]. At 24 and 72 hours following LPS activation, there was a significant 3 to 3.25-fold increase in nitrite content of activated astro-microglia CM compared to non-activated control condition, respectively ($P<0.001$, one-way ANOVA, N=4) (Fig. 2.2A, B). This elevation was reduced significantly by the availability of Nrg-1 in LPS+Nrg-1 treated cultures compared to LPS condition at all time-points (1.4-fold at 24h and 1.9-fold at 72h) ($P<0.01$, one-way ANOVA, N=4/time-point) (Fig. 2.2A, B). Given these results, we next assessed the expression level of Arginase-1 (Arg1) in LPS-activated astro-microglia under Nrg-1 treatment. Arg1 is known to reduce NO production in glia through metabolizing L-arginine that is required by iNOS for NO production [35]. Quantitative real-time PCR showed a higher expression of Arg1 transcript in LPS+Nrg-1 cultures compared to LPS condition (Fig. 2.2C). While there was no detectable constitutive expression of Arg1 in normal and Nrg-1 control conditions, LPS activation elicited a rise in Arg1 expression. Interestingly, treatment with Nrg-1 promoted a significant 2.2-fold increase in Arg1 expression in activated glia compared to LPS condition suggesting that increased expression of Arg1 may underlie the reduced NO production in Nrg-1 treated culture ($P<0.01$, one-way ANOVA, N=4).

In our mixed astro-microglia culture, we sought to determine whether Nrg-1 plays a role in modulating the release of IL-1β and TNF-α, two major pro-inflammatory cytokines associated with excitotoxicity and cell death after SCI [36, 37]. At 24h and 72h post-LPS activation, our Western blot analysis of glial CM revealed a significant elevation in the levels of pro and active forms of IL-1β in LPS activated glial cultures compared to the non-activated astro-microglia with

undetectable levels of pro and active IL-1β ($P<0.001$, one-way ANOVA, N=4) (Fig. 2.2D-G). While addition of Nrg-1 to LPS-treated glia had no significant effect on IL-1β release at 24h after activation ($P>0.05$, N=4), we found a 50% reduction in production of both pro and active forms of IL-1β in the CM of LPS+Nrg-1 treated glia at 72h compared to LPS condition ($P<0.001$, one-way ANOVA, N=4) (Fig. 2.2D-G). Assessment of TNF-α also revealed similar results. Nrg-1 treatment significantly reduced the elevated levels of TNF-α at both 24h and 72h after activation (Fig. 2.2H-K) ($P<0.001$, one-way ANOVA, N=3). Moreover, when normal glial cells were exposed to Nrg-1, there was no change in the basal levels of TNF-α or IL-1β.

Figure 2.2. Nrg-1 significantly ameliorates pro-inflammatory cytokines and nitrite production while increases Arg1 expression in LPS-activated astro-microglial cells.

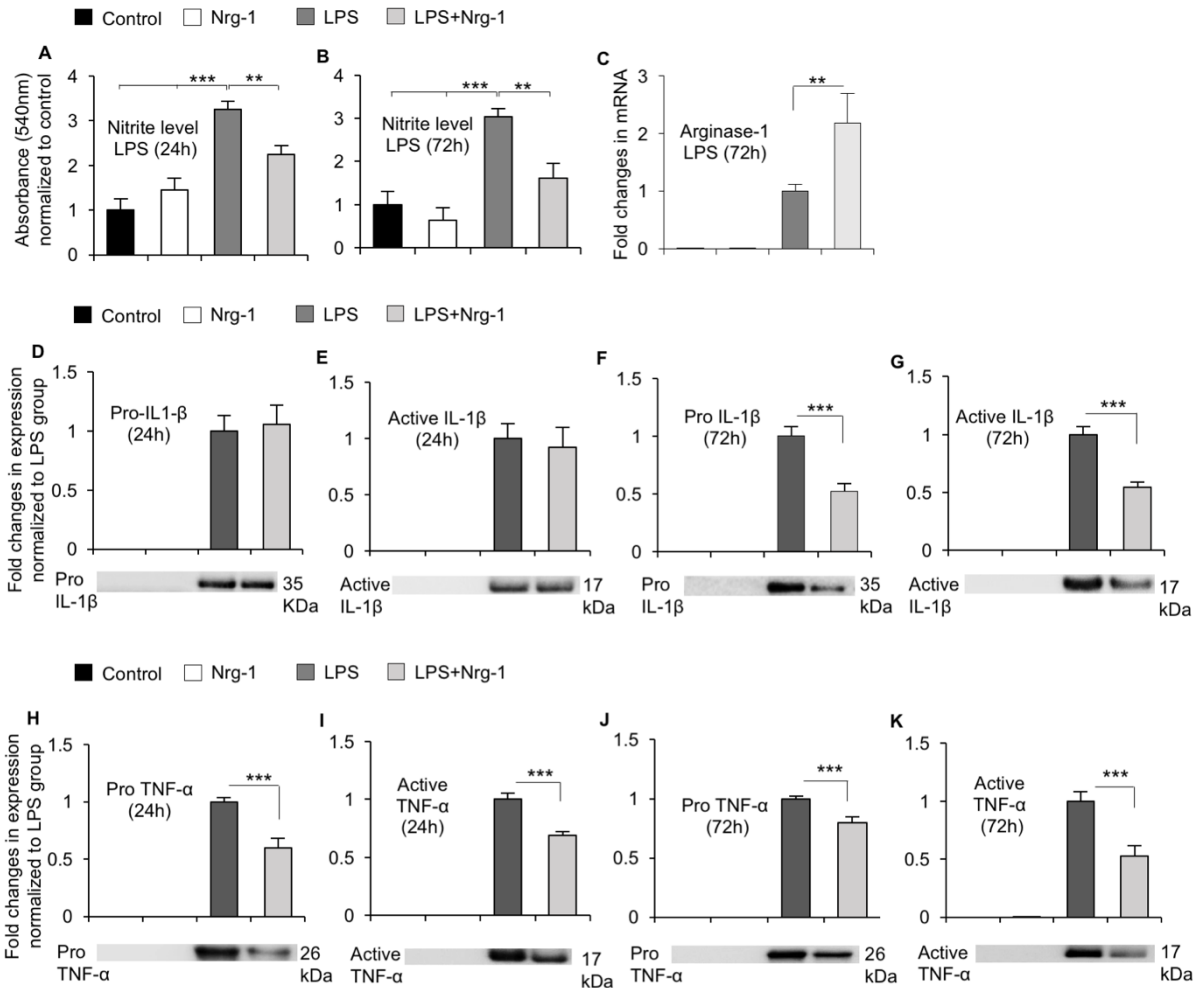


Figure 2.2. Nrg-1 significantly ameliorates pro-inflammatory cytokines and nitrite production while increases Arg1 expression in LPS-activated astro-microglial cells. (A, B) Analysis of conditioned media (CM) at 24h and 72h post LPS activation showed over 3-fold increase in nitrite production as compared to non-activated glia. Nrg-1 treatment significantly attenuated nitrite levels in activated glia. **(C)** Quantitative PCR at 72h after LPS exposure showing upregulation of Arg1 transcript in glial cells. Treatment with rhNrg-1β1 resulted in a significant 2.2-fold increase in Arg1 expression (**P<0.01, one-way ANOVA, N=4). We found no detectable Arg1 expression in non-activated normal and Nrg-1 control conditions (N=5 independent experiments for nitrite and N=3 for Arg1 experiments). **(D- G)** Western blot analysis shows the relative levels of pro and active interleukin-1β (IL-1β) in CM under different treatments. **(D, E**) At 24h after LPS activation, there were significantly higher levels of pro and active IL1-β compared to non-activated glia; however, Nrg-1 had no effects on LPS induced release of IL-1β at this time-point. **(F, G)** At 72h after LPS treatment, Nrg-1 treatment could significantly reduce the production of both forms. Of note, normal and Nrg-1 treated glia showed undetectable levels of IL-1β in their CM indicating that Nrg-1 did not induce active IL-1β under normal state of glia. Representative blots are depicted for each experimental condition. (**H, I**) Our quantitative Western blot analysis shows induced expression of pro and active TNF-α in CM of LPS activated glia at 24h and 72h after activation. There was a significant reduction in pro and active TNF-α in CM of LPS+Nrg-1 treated glia compared to LPS activated cultures at both time-points. Normal and Nrg-1 treated glia showed no detectable levels of TNF-α. For loading control of CM, normalization to total protein visualized by Ponceau S was used and data were normalized to the values obtained from LPS group. The data represent mean ± SEM, *P<0.05, ***P<0.001, One-Way ANOVA, followed by Holm-Sidak post hoc test (N= 3-5 independent experiments).

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.4.3 Effects of Nrg-1 on pure activated microglia and astrocytes *in vitro*

To unravel the specific effects of Nrg-1 on pure microglia, a culture of microglia with over 95% purity was used. LPS and Nrg-1 dosing assays were conducted using nitrite and cytokine production as markers of activity. We identified 1μg/ml of LPS and 25ng/ml of Nrg-1 as optimal concentrations of LPS and Nrg-1 in pure microglia cultures. LPS activation caused a significant increase in nitrite, IL-1β and TNF-α production (Fig. 2.3A-E). Nrg-1 treatment (25ng/ml) significantly attenuated production of nitrite and active IL-1β in LPS-activated pure microglia and caused a non-significant reduction in the TNF-α levels (N=4, $P<0.05$, one-way ANOVA) (Fig. 2.3A- E).

We additionally assessed the response of pure astrocytes to LPS activation. In our purified astrocyte culture (over 95% purity), we treated astrocytes with 1, 10 and 20 μg/ml LPS as representatives of low, medium and high concentrations. None of these concentrations could activate pure astrocytes indicated by no changes in nitrite and CSPGs levels in astrocyte CM after 72h of activation(N=4) (Fig. 2.3F, H). Interestingly, addition of CM from LPS-activated microglia could robustly activate these pure astrocytes and significantly increase their nitrite (5.3 folds) and CSPGs (4 folds) production (N=3, $P<0.001$, one-way ANOVA) (Fig. 2.3G, H). We confirmed that CSPGs upregulation in astro-microglia cultures was attributed to astrocyte activation and not microglia as we found that pure microglia produce a rather low level of CSPGs after LPS activation (Fig. 2.3I). These observations indicate that the presence of microglia and their paracrine modulation was essential to induce astrocyte activation following LPS treatment in our astro-microglia cultures. Moreover, these findings show that the beneficial effect of Nrg-1 on attenuating CSPGs in mixed astro-microglia studies reflects changes in astrocytes and not microglia.

Figure 2.3. Effects of LPS and Nrg-1 on pure microglia and astrocytes.

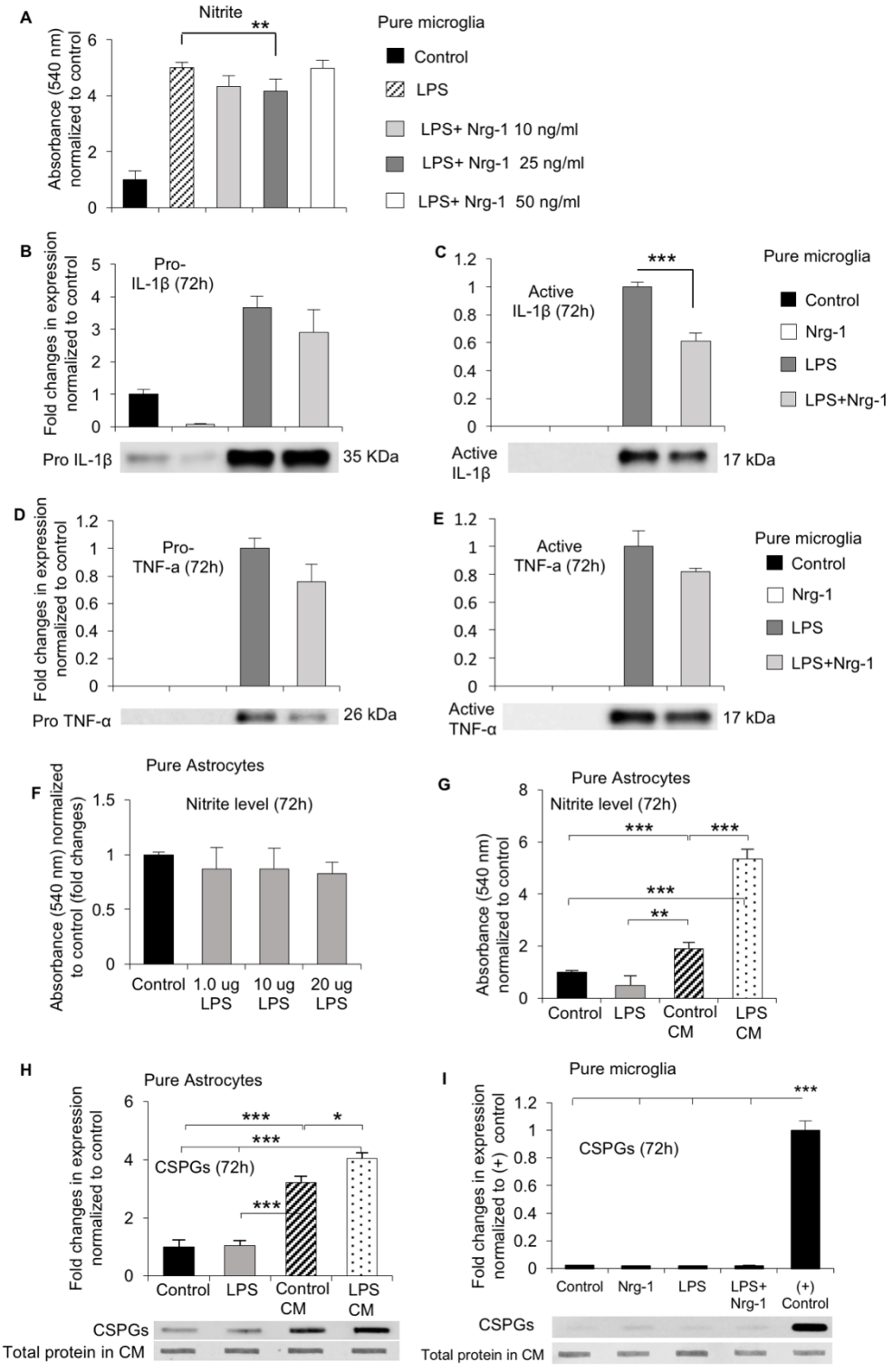


Figure 2.3. Effects of LPS and Nrg-1 on pure microglia and astrocytes. (A) Three doses of Nrg-1 (10, 25, 50 ng/ml) were assessed. Nrg-1 at 25ng/ml concentration significantly reduced nitrite production by LPS-activated pure microglia. **(B-E)** Western blot analysis of cytokine release in CM of LPS-activated pure microglia showed no significant reduction in pro form of IL-1β following Nrg-1 treatment (25 ng/ml), while there was a significant reduction in active IL-1β. **(D, E)** There was also a non-significant reduction in active TNF-α under Nrg-1 treatment (N=4, P>0.05). **(F-I)** Pure astrocytes were treated with LPS at different concentrations (1, 10 and 20μg/ml). They did not respond to LPS as there was no change in their nitrite and CSPGs production (N=4, P>0.05). When CM from LPS activated microglia was added to pure astrocyte, it elicited a significant increase in both **(G)** nitrite and **(H)** CSPGs production by pure astrocytes. (I) Normal and LPS-activated pure microglia are not a source of CSPGs. Representative Slot blots is shown under respective figures. The data represent mean ± SEM, ***P<0.001, **P<0.01, *P<0.05, N=4, One-Way ANOVA, followed by Holm-Sidak post hoc test.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.4.4 Nrg-1 Regulates Activated Glial Cells through ErbB2/3 Hetero-dimerization and Activation of Erk1/2 Signaling.

We next investigated the mechanisms underlying the effects of Nrg-1 on glial cells. We first confirmed that astrocytes and microglia express all ErbB receptors using co-immunostaining of GFAP (astrocytes) and OX42 (microglia) with ErbB-2, 3 and 4 receptors (Fig. 2.4 A-F). For studying ErbB activity, astro-microglia cultures were treated with Nrg-1 for 15, 30 and 60 min. Using co-immunoprecipitation (co-IP), each individual ErbB receptor was immunoprecipitated with a specific antibody and analyzed for tyrosine phosphorylation using anti-phosphotyrosine (pTyr) antibody (Fig. 2.4 G-I). Nrg-1 treatment resulted in a significant increase in the tyrosine phosphorylation of ErbB2 receptor at 15 min (1.65 folds) and 30 min (1.4 folds) time-points. ErbB2 tyrosine phosphorylation remained elevated 60 min after Nrg-1 treatment, however it was not statistically significant compared to its basal level (Fig. 2.4G, J). While ErbB4 expression was confirmed in glial cells (Fig. 2.4C, F, I), there was no detectable activation of ErbB4 in Nrg-1 treated astro-microglial cultures (Fig. 2.4I). Interestingly, we also detected pTyr activity in ErbB3 immunoprecipitates at 180 kDa as opposed to the expected 240 kDa for ErbB3 (Fig. 2.4 H). These data suggested a potential heterodimerization of ErbB2/ErbB3 in astro-microglial cells following Nrg-1 treatment since ErbB3 lacks the tyrosine kinase domain as reported in the literature [38]. This observation prompted us to perform co-IP of ErbB3 and confirm the presence of ErbB2 and ErbB3 receptors in the co-immunoprecipitate complex. We therefore performed co-IP for ErbB3, followed by immune-recapture of ErbB2 and ErbB3 sequentially (Fig. 2.4K). Interestingly, ErbB2 was detected in ErbB3 immunoprecipitates from Nrg-1 treated cells confirming that Nrg-1 treatment leads to heterodimerization of ErbB2/3 and the pTyr activity in ErbB3 immunoprecipitate likely reflects the presence of ErbB2 (Fig. 2.4H, K).

Intracellularly, we focused on Erk1/2 pathway that is a known downstream effector in Nrg-1 signaling cascade [39, 40]. Western blot analysis of the ratio of phosphorylated Erk1/2 (pErk1/2) to total Erk1/2 (tErk1/2) showed a significant increase (2.6-fold) in Erk1/2 phosphorylation in astro-microglia culture upon activation ($P<0.05$, $N=3$). Treating LPS activated astro-microglia with Nrg-1 further increased Erk1/2 phosphorylation (1.45-fold) ($P<0.05$, $N=3$) (Fig. 2.4L). Interestingly, Nrg-1 also enhanced Erk1/2 phosphorylation when added to normal non-activated glial cells to a comparable level as LPS activated glia but significantly lower than LPS+Nrg-1 treated group. Altogether, these results suggest that Nrg-1 signals through ErbB2/3 heterodimers and activates ErbB2 tyrosine phosphatase domain resulting in an increase in Erk1/2 phosphorylation in our primary astro-microglial cultures.

Figure 2.4. Nrg-1 signals through ErbB-2/3 heterodimer complex and activates Erk1/2 signaling pathway in mixed glial cultures.

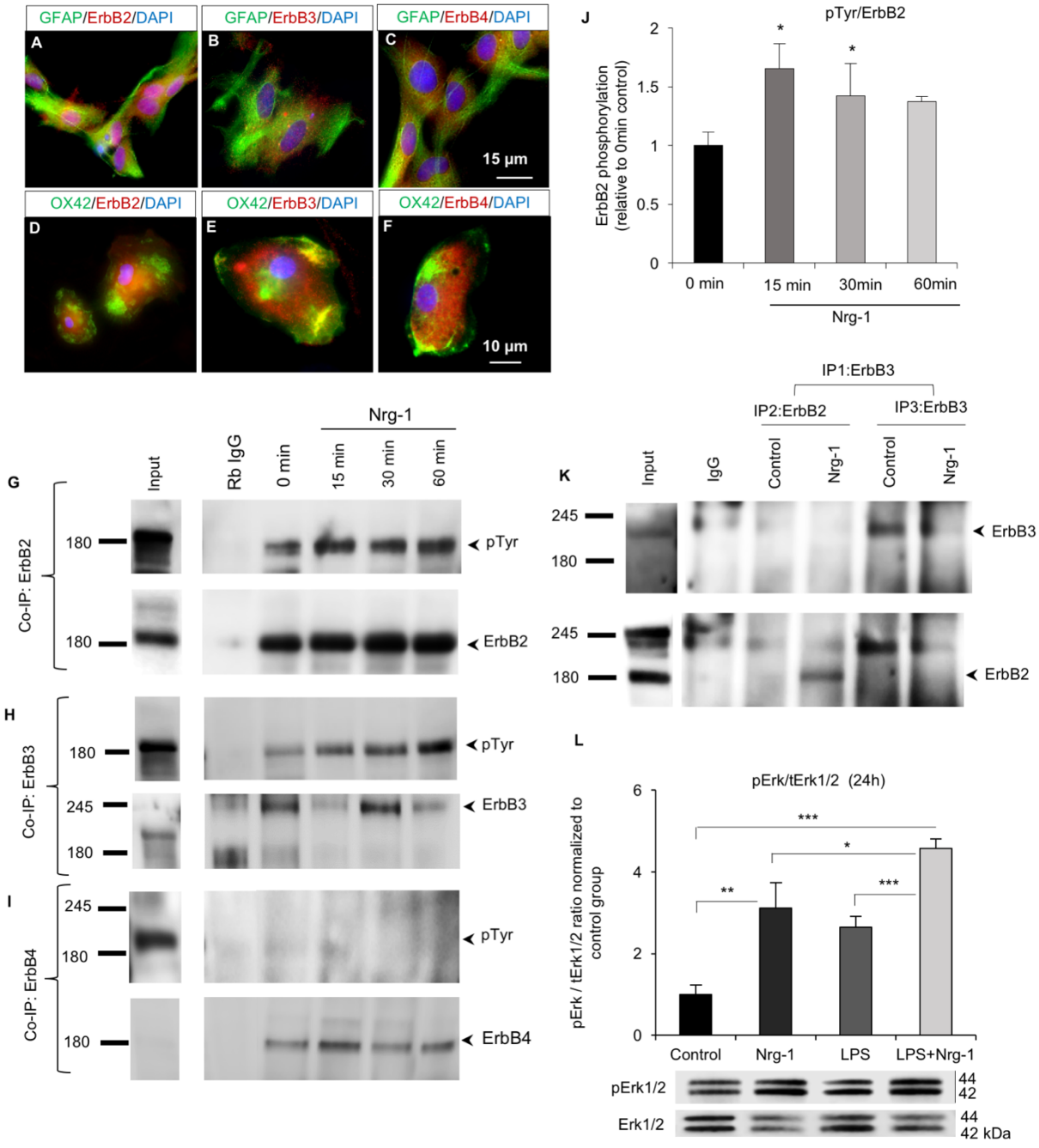


Figure 2.4. Nrg-1 Regulates Activated Glial Cells through ErbB2/3 Hetero-dimerization and Activation of Erk1/2 Signaling. (A-F) Co-immunostaining of astrocytes (GFAP) and microglia (Ox42) with anti-ErbB 2, 3, 4 antibodies in glial culture shows expression of all three ErbB receptors in both astrocytes and microglia. **(G-I)** In serum free media, glial cultures were treated without (0 min) or with Nrg-1 (150 ng/ml) for 15, 30 and 60 min. Co-IP was performed on cell lysate of Nrg-1 treated glia using anti-ErbB2, anti-ErbB3 or anti-ErbB4 antibodies, followed by Western blotting for anti-phosphotyrosine (pTyr), anti-ErbB2, anti-ErbB3 or anti-ErbB4 on immunoprecipitates. Our data show that Nrg-1 induced phosphorylation of ErbB2 at all the treatment time-points. In ErbB3 immunoprecipitates, a lower (180 kDa) pTyr band was observed despite the expected molecular weight of 240 kDa for ErbB3 possibly due to co-IP of ErbB2/ErbB3 heterodimers. No pTyr activity was observed in ErbB4 co-immunoprecipitates. **(J)** Tyrosine phosphorylation was significantly increased in ErbB2 Co-IP following Nrg-1treatment at 15min (1.65-fold) and 30min (1.4-fold) as compared to control untreated glial cultures. ErbB2 phosphorylation data was normalized to protein content detected by ErbB2 antibodies. **(K)** Presence of ErbB2 was confirmed in the ErbB3 co-immunoprecipitate complexes with sequential recapture immunoprecipitation for ErbB2 (IP2) and ErbB3(IP3) suggesting that pTyr detection in ErbB3 Co-IP may reflect heterodimerization of the two receptors. (L) Our Western blot analysis for total and phosphorylated forms of Erk1/2 (tErk1/2 and pErk1/2) on glial cell lysate showed an increase in the activity of Erk1/2 in LPS-activated glia compared to non-activated conditions. Treatment with Nrg-1 significantly increased the ratio of pErk1/2 to tErk1/2 in both normal and LPS-activated glia compare to normal non-treated cells. LPS+Nrg-1 treated glia demonstrated significantly higher Erk1/2 phosphorylation compared to both LPS treated and Nrg-1 treated

conditions at 24 hr in culture. The data show mean ± SEM. *P<0.05 N=3/group/time-point, one-way ANOVA followed by Holm-Sidak post-hoc test.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.4.5 Nrg-1 Treatment Moderates CSPGs Expression and Glial Scarring following SCI

We next evaluated the effects of acute Nrg-1 treatment on astrogliosis at various time-points after SCI representing acute (1d and 3d), subacute (7d and 14d) and chronic (70d) stages of injury. Of note, we have confirmed the thermal stability and efficacy of rhNrg-1β1 peptide at 37°C in our previous studies (data not shown). Assessment of CSPGs in the SCI lesion showed a significant 2-fold upregulation as early as 1d after SCI, which remained elevated (1.7 to 3 fold) compared to the uninjured group at all examined time-points (Fig. 2.5 A-D) ($P<0.05$, one-way ANOVA, N=4/group/time-points). In agreement with our in vitro data, Nrg-1 treatment significantly attenuated CSPGs production within the glial scar at 1d (2.7-fold), 7d (2.3 fold) and 14d (3-fold) time-points compared to vehicle treated SCI groups ($P<0.01$, one-way ANOVA, N=4/group/time-point) (Fig. 2.5 A-D). We further studied the effects of Nrg-1 treatment on GFAP expression as an indicative of astrocytic scar following SCI. Western blot analysis confirmed a significant 1.4 to 5-fold increase in GFAP expression at all examined time-points after SCI ($P<0.05$, N=4, one-way ANOVA) (Fig. 2.5 E-H). Nrg-1 treatment could attenuate GFAP expression at all time-points with a statistically significant effect at 1d compared to the vehicle treated group ($P<0.05$, N=4/group/time-point, one-way ANOVA).

We performed complementary immunohistological assessments of CSPGs and GFAP to specifically study the lesion and perilesional areas at subacute (14d) and chronic (70d) stages of SCI. Nrg-1 treatment resulted in a reduction in CSPGs immunoreactivity within the epicenter of injury at both 14d (30%) and 70d (41%) post-SCI which was significantly lower than the vehicle treated group ($P<0.05$, N=5, two-way repeated measure ANOVA) (Fig. 2.5 I, K, M, O). Analysis of GFAP immunointensity also showed a 47% significant reduction at the epicenter in Nrg-1 treated group compared to the vehicle group at 14d post-SCI with no significant difference in

GFAP expression at 70d between the two groups ($P<0.05$, N=6, two-way ANOVA) (Fig. 2.5 J, L, N, P). Collectively, our in vitro and in vivo SCI data indicate a positive role for Nrg-1 in moderating the response of activated astrocytes and scar formation particularly deposition of the inhibitory CSPGs in the extracellular matrix following injury.

Figure 2.5. Nrg-1 treatment reduces CSPGs and GFAP expression following SCI and moderates scar formation in the SCI lesion.

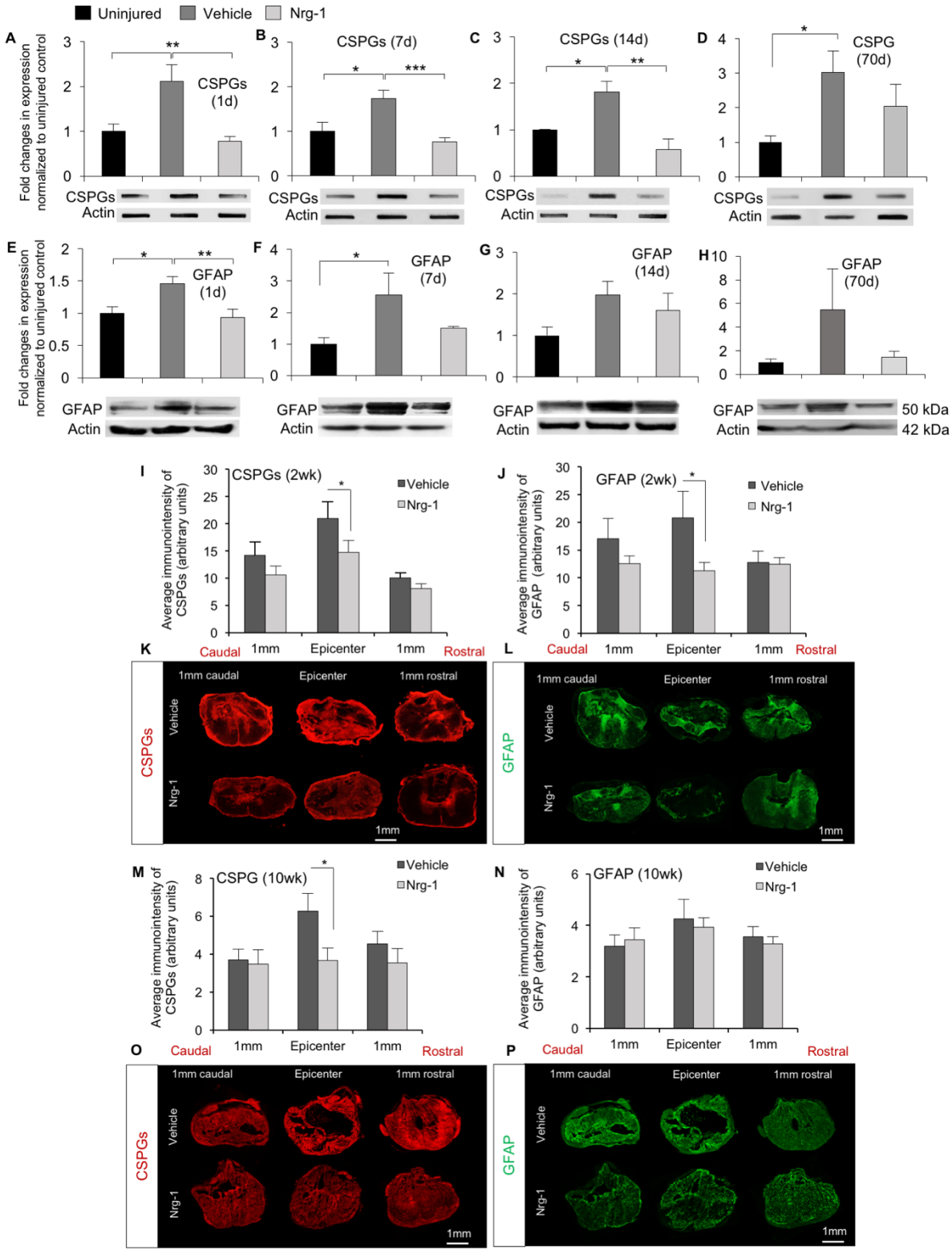


Figure 2.5. Nrg-1 treatment reduces CSPGs and GFAP expression following SCI and moderates scar formation in the SCI lesion. (A-D) Our slot blot tissue analysis of CSPGs is depicted at different time-points (1d, 7d, 14d and 70d) following injury representing acute, subacute and chronic stages of SCI. Nrg-1 treatment significantly decreased CSPGs level in relation to vehicle-treated group at 1d, 7d and 14d time-points following SCI. Actin was used for normalization as a loading control. **(E-H)** Western blot analysis of GFAP expression was also performed for the same time-points following SCI. Nrg-1 treatment resulted in a significantly reduced expression of GFAP at 1d time-point compared to the vehicle treated counterparts. The data show mean ± SEM. *P<0.05, **P<0.01, ***P<0.0001, N=4/group/time-point, one-way ANOVA followed by Holm-Sidak post-hoc test. Actin was used for normalization as a loading control. Representative Western and slot blot images are provided under each graph. **(I- P)** Our Immunohistological assessment of CSPGs and GFAP immunointensity was performed on the epicenter and perilesional areas at 14d and 70d post-SCI representing subacute and chronic stages of injury. **(I, J)** comparison between vehicle and Nrg-1 treated SCI rats at 14d time-point showed a significant reduction in CSPGs and GFAP immunointensity in Nrg-1 treated group at the injury epicenter. **(M, N)** At 70d post-injury, there was a significant reduction in CSPGs deposition in Nrg-1 treated group at the injury epicenter with no significant difference in GFAP immunointensity between the Nrg-1 and vehicle treated groups. **(K, L, O, P)** Representative images are depicted at the epicenter and 1mm rostral and caudal to the lesion center. The data show mean ± SEM. *P<0.05, Two-way ANOVA followed by Holm-Sidak post-hoc test, N=5/group/time-point.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315.

2.4.6 Nrg-1 Treatment Promotes a Shift from a Pro-inflammatory Immune Response to an Anti-inflammatory Profile Following SCI

Early recruitment of microglia/macrophages in SCI is characterized by the release of pro-inflammatory cytokines, which cause cell death and tissue degeneration following injury [41, 42]. Using Western blot analysis, uninjured spinal cord tissue did not show a detectable basal level of IL-1β as expected (Fig. 2.6A-C). At 1d post-injury, the tissue level of IL-1β increased pronouncedly in the vehicle group compared to the uninjured rats followed by a decremental pattern at later time-points after SCI. Interestingly, Nrg-1 treatment significantly mitigated the release of IL-1β at 1d and 7d following injury in comparison to the vehicle treated SCI rats ($P<0.05$, one-way ANOVA, N=5/group/group/time-points). The same trend was also observed in 14d time-point but the decrease was not statistically significant (Fig. 2.6C). Similarly, TNF-α level was upregulated in the injured spinal cord as early as 1d following injury and remained significantly elevated until 7d (approximately 4-fold increase at 1d and 7d time-points, $P<0.001$, one-way ANOVA, N=4/group/time-point) (Fig. 2.6 D-F). Nrg-1 treatment resulted in a significant reduction in TNF-α at 1d and 7d post-injury compared to the vehicle treated group (Fig. 2.6 D-F).

Given the decrease in IL-1β and TNF-α release in response to Nrg-1, we asked whether this decline is secondary to a reduction in the recruitment of microglia/macrophages to the lesion site after Nrg-1 treatment. Western blot analysis for Iba-1 showed a sizeable increase in the presence of microglia/macrophages following SCI compared to the uninjured group as anticipated (Fig. 2.6 G-I). Iba-1 expression reached its peak at 7d by approximately 29-fold increase compared to the uninjured level followed by a decline at 14d with approximately 10 fold higher than Iba-1 expression in the uninjured group ($P<0.001$, N=5/group/timeline, one-way ANOVA). We found no statistically significant difference in Iba-1 levels between Nrg-1 and vehicle treated groups at

any examined time-point in our study (Fig. 2.6 G-I). Our complementary immunohistological examination of Iba-1 expression at the injury epicenter and perilesional areas also confirmed that Nrg-1 treatment had no apparent effects on the recruitment of microglia/macrophages to the lesion (Suppl. Fig 2.2A, B).

We additionally assessed the expression of interleukin-10 (IL-10) and Arg1, two well-studied factors associated with M2 microglia/macrophages with beneficial role in repair and regeneration following CNS injury [43-45]. Western blot analysis at 3d, 7d and 14d post-SCI showed upregulation of IL-10 in the injured spinal cord compared to its undetectable level in the uninjured tissue (Fig. 2.6 J-L). Interestingly, Nrg-1 treated animals showed significantly higher levels of IL-10 at all examined time-points (Fig. 2.6J-L) ($P<0.05$, $N=4$, one-way ANOVA, followed by Holm-Sidak post-hoc test). Nrg-1 treatment also resulted in a 7-fold significant increase in Arg1 expression at 3d post-injury, representative of acute infiltration of inflammatory cells into the SCI lesion, compared to the vehicle counterparts (Fig. 2.6M, N). Arg1 was downregulated at 7d post-injury to a non-significant level compared to the uninjured control group although it was still higher than the basal uninjured level. However, Nrg-1 treated SCI rats showed a significantly higher (2.3-fold) expression of Arg1 at 7d post-injury ($P<0.01$, $N=4$, One-way ANOVA) (Fig. 2.6M, N). Taken together, our in vitro and SCI cytokine analyses indicate a beneficial role for Nrg-1 treatment in shifting the phenotype of microglia/macrophages from an M1 proinflammatory to a pro-regenerative M2 phenotype without altering the recruitment of microglia/macrophages to the SCI lesion.

Figure 2.6. Nrg-1 treatment modulates inflammatory response without altering the recruitment of macrophages/microglia following SCI.

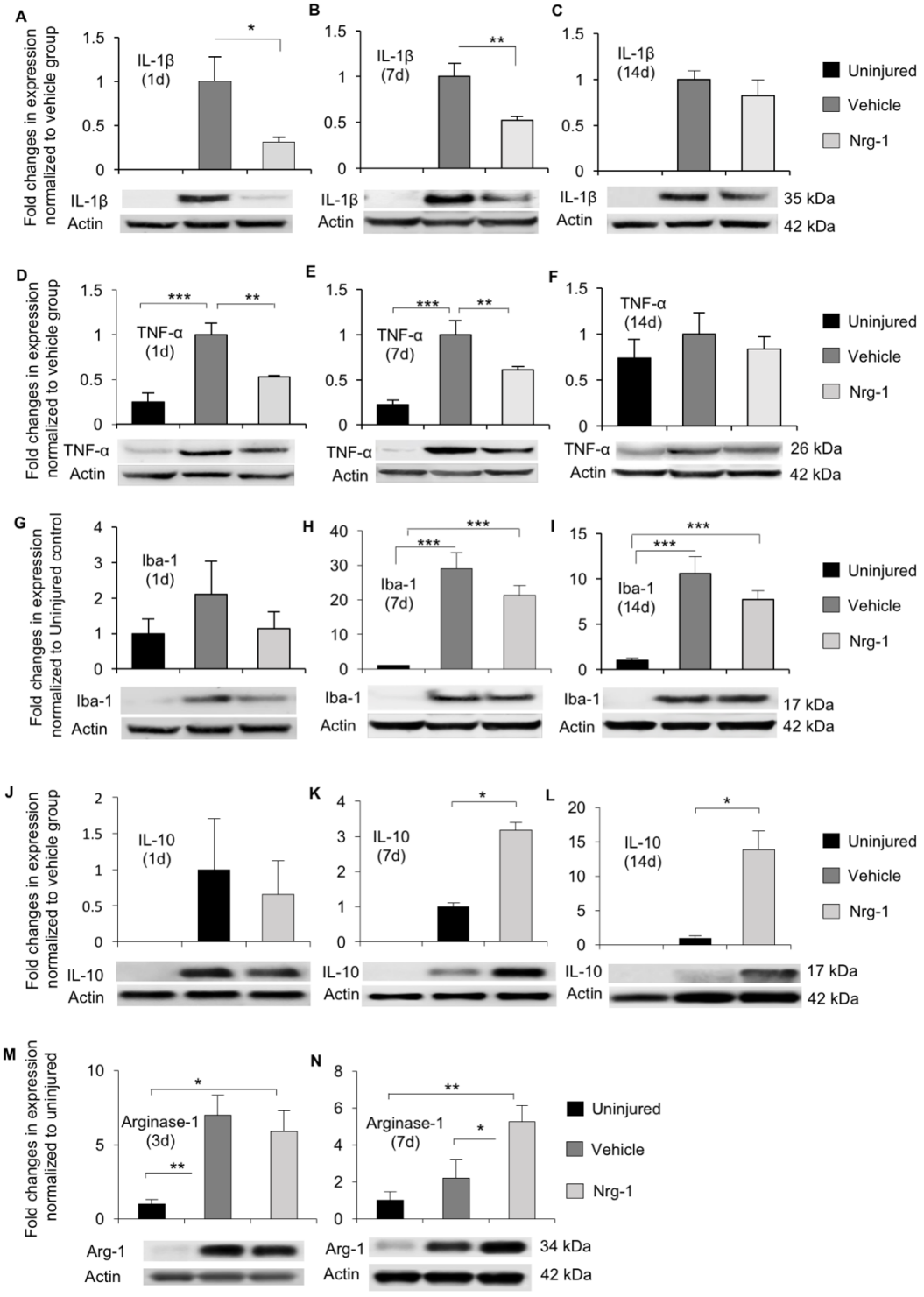


Figure 2.6. Nrg-1 treatment modulates inflammatory response without altering the recruitment of macrophages/microglia following SCI. (A-C) Time-point analysis of the active form of IL-1β by Western blotting showed an anticipated upregulation of IL-1 β at 1d following SCI. While expression of IL-1β was decreased at 7d and 14d time-points after injury, it was still detectable. Nrg-1 treatment significantly reduced IL-1β at 1d and 7d post-SCI. **(D- F)** TNF-α level was significantly elevated at 1day and 7d time-points after injury and Nrg-1 could reduce TNF-α at all time-points compared to the vehicle treated group. However, this reduction was statistically significant at 1d and 7d time-points post-SCI. **(G- I)** Western blot analysis on spinal cord tissue depicts the changes in the expression of Iba-1. At 1d after SCI, Iba-1 expression was increased in the spinal cord tissue; however, this increase was not significantly different from uninjured rats. Increase in Iba-1 reached its peak (approximately 30 fold) by 7d post-injury and then declined at 14d while still being significantly higher (10 fold) than the uninjured control rats. Nrg-1 treatment had no significant effect on Iba-1 expression compared to the vehicle treated animals at all time-points. **(J- L)** Western blot analysis of the IL-10 showed a significant increase at 7d and 2w post-SCI. Nrg-1 treated animals demonstrated significantly higher IL-10 production at these time-points. No significant change in IL-10 level was observed at 1d post SCI. **(M, N)** Western blot analysis of the spinal cord tissue at 3d and 7d following injury shows increase in Arg1 expression following SCI. This increase was significant at 3d time-point ($P<0.01$, $N=4$). While there was no significant difference between vehicle and Nrg-1 treated groups at 3d post-SCI, Nrg-1 treated rats showed significant increase in Arg1 level at 7d post-injury compared to vehicle treated group ($P<0.05$, $N=4$). Representative Western blot images together with actin as the loading control are shown below each graph. Actin was used for normalization as a loading control. Results have been normalized to the actin loading control prior to subsequent normalization to the uninjured control

values. The data represent mean ± SEM, *P<0.05, **P<0.01, ***P<0.001, One-Way ANOVA, followed by Holm-Sidak post hoc test, N=4/group/time-point.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.4.7 Nrg-1 Treatment Attenuates MyD88 Expression while Enhances the Activity of Erk1/2 and STAT3 Pathways Following SCI

To identify the potential cellular mechanisms of Nrg-1 in attenuating pro-inflammatory cytokines, we first focused on MyD88, which is an adaptor protein essential for activation of TLR and IL-1 receptors signalling pathways [46, 47]. Increased MyD88 level in the injured spinal cord has been associated with the release of pro-inflammatory mediators and secondary tissue damage following SCI [11, 48]. Western blot analysis of SCI tissue showed an incremental rise in Myd88 expression as early as 1d after SCI that peaked at 7 days (Fig. 2.7A-C). We found a significant 1.82-, 9- and 3.65- fold increase in MyD88 protein expression at 1d, 7d and 14d post-SCI, respectively, compared to the uninjured basal level that was significantly attenuated by Nrg-1 treatment at all studied time-points (Fig. 2.7A-C) ($p<0.05$, one-way ANOVA, N=4/time-point/group). These results suggest that Nrg-1 may reduce TNF-α and IL-1β at least in part by modulating the expression of MyD88 following SCI.

To further elucidate the mechanisms through which Nrg-1 modulates immune response following SCI, we focused on Erk1/2 and STAT3 pathways; two well-established pathways involved in regulation of IL-10 expression and signaling [49, 50]. Our Western blot analysis revealed a significant 2.5-fold increase in Erk1/2 phosphorylation in Nrg-1 treated rats compared to vehicle treated group confirming our in vitro observation in astro-microglial cultures ($P<0.05$, N=4) (Fig. 2.7D). Our analysis of pSTAT/tSTAT3 ratio by Western blotting at 3d and 14d time-points also showed that Nrg-1 treated SCI rats had a significantly higher STAT3 phosphorylation compared to the vehicle treated SCI group (3.7- and 1.5-fold increase at 3d and 14d respectively, $P<0.05$, N=4) (Fig. 2.7 E, F).

Figure 2.7. Nrg-1 down-regulates MyD88 expression and increases phosphorylation of Erk1/2 and STAT3 following SCI.

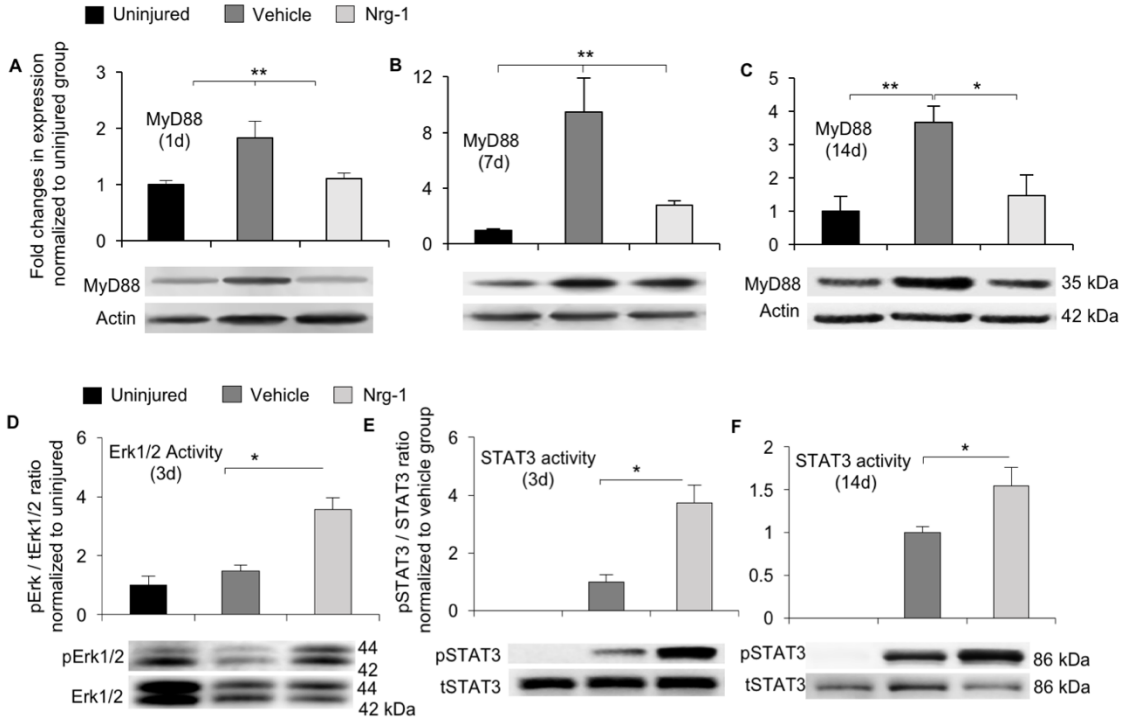


Figure 2.7. Nrg-1 down-regulates MyD88 expression and increases phosphorylation of Erk1/2 and STAT3 following SCI. (A-C) Expression of MyD88 protein in the injured spinal cord tissue was significantly upregulated at 7d and 14d post-injury compared to the uninjured basal level. Nrg-1 treatment significantly mitigated MyD88 elevation at all examined time-points compared to the vehicle treated injured group. **(D)** Western blot analysis for total and phosphorylated forms of Erk1/2 (tErk1/2 and pErk1/2) shows significant increase in the activity of Erk1/2 (pErk/tErk) in Nrg-1 treated group at 3d post-SCI. There was no significant difference in Erk1/2 activity between injured vehicle treated and non-injured control group at this time-point. **(E, F)** Western blot analysis also demonstrated a significant increase in STAT3 activation (pSTAT3/STAT3) at 3d and 14d following SCI. Nrg-1 treated SCI group showed a significantly higher STAT3 phosphorylation compared to the vehicle treated group at both examined time-points. Representative Western blots are shown under each graph. Results have been normalized to the actin loading control prior to subsequent normalization to the uninjured control values. The data represent mean ± SEM, *P<0.05, **P<0.01, One-Way ANOVA, followed by Holm-Sidak post hoc test, N=4/group/time-point.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.4.8 Nrg-1 Treatment Attenuates Activity of MMP-2 and MMP-9 and Promotes Tissue Preservation Following SCI

Since we identified a role for Nrg-1 in attenuating pro-inflammatory mediators, we next examined whether Nrg-1 treatment would moderate the activity of matrix metalloproteinases (MMPs) following SCI. MMP-2 and MMP-9 play major roles in tissue degeneration and re-modeling following injury [51, 52]. Using gelatin zymography, we found an anticipated robust increase in the activity of both MMP-2 and MMP-9 in the injured spinal cord tissue at 1d and 7d post-injury (Fig. 2.8 A-F) ($P<0.01$, one-way ANOVA, N=5/time-point/group). MMP-2 activity remained elevated at 14d post-injury in the injured spinal cord, while MMP-9 activity was reduced to the undetectable basal levels in the uninjured tissue (data not shown). Analysis of zymograms identified 33% decline in the activity of MMP-9 in Nrg-1 treated group at 1d and 7d post-injury. Moreover, Nrg-1 treatment significantly reduced MMP-2 activity 30%-50% compared to vehicle SCI rats at all time-points except 14d ($P<0.01$, one-way ANOVA, N=5/time-point/group, data not shown for 14d SCI) (Fig. 2.8 A-F).

We additionally assessed the degree of tissue preservation at chronic stage of SCI. We performed morphometric analysis on spinal cord tissue stained with LFB/HE at 10 weeks. Preserved tissue was quantified in the cross section of the injured spinal cord at various distances including the epicenter, 1, 2 and 3 mm rostral and caudal points to the injury center. While groups treated with 500 ng/day and 1 µg/day Nrg-1 showed no significant improvement in tissue preservation, treatment with 1.5 µg/day Nrg-1 significantly attenuated tissue degeneration at the epicenter, 1mm rostral and 1, 2 and 3mm caudal to the injury center as compared to the vehicle treated group (Fig. 2.8 G).

Figure 2.8. Nrg-1 treatment mitigates injury-induced activity of MMP-2 and MMP-9 and promotes tissue preservations following SCI.

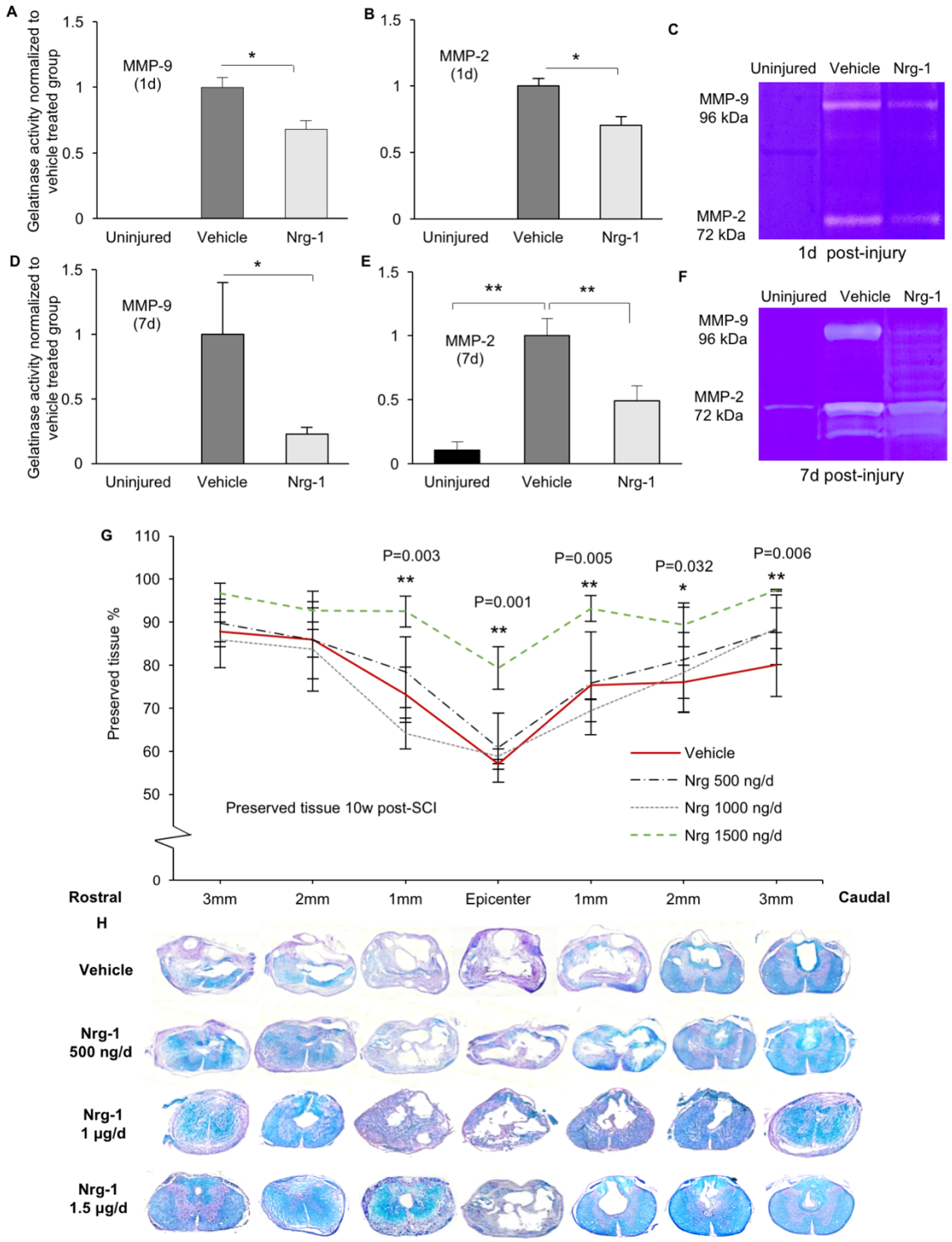


Figure 2.8. Nrg-1 treatment mitigates injury-induced activity of MMP-2 and MMP-9 and promotes tissue preservations following SCI. (A-F) Using gelatin zymography, we investigated the changes in MMP-2 and MMP-9 enzymatic activity in the spinal cord at different time-points following SCI. **(A-F)** SCI rats showed a robust increase in the activity of both MMP-2 and MMP-9 at 1d and 7d post-injury. Nrg-1 treatment significantly attenuated the injury-induced activity of MMP-2 and MMP-9 compared to the vehicle SCI group at these timepoints. **(C, F)** representative zymograms are shown. **(G)** Graph shows the percentage of preserved tissue assessed by LFB-H&E counterstaining on serial cross sections of injured spinal cord spanning 3 mm rostrally and caudally from the epicenter. The area of preserved tissue was quantified and normalized to the total area of each spinal cord section. Results were compared between all treatment groups at 10 weeks after injury. Comparing to vehicle treated group (N=4), SCI rats treated with Nrg-1 1.5 μg/day (N= 3) showed significantly improved tissue preservation at the epicenter, 1mm rostral and all assessed caudal sections. There was no significant improvement in SCI rats treated with 0.5 and 1 μg/day Nrg-1. **(H)** Representative LFB-H&E images are sown under the graph. The data represent mean ± SEM, *P<0.05, **P<0.01, One-Way ANOVA for zymography and Two-Way ANOVA for tissue preservation data, followed by Holm-Sidak post hoc test, N=4/group/time-point unless otherwise stated.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315

2.4.9 Nrg-1 Treatment Improves Locomotor Recovery after Chronic SCI with No Effects on Pain Reactivity.

We sought to determine whether beneficial effects of Nrg-1 treatment would translate to improved recovery of function following SCI. We conducted longitudinal assessments of locomotion and pain over a 10-week period after SCI. For functional analysis, we evaluated three daily doses of Nrg-1, at 0.5µg, 1µg and 1.5µg per day. Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale was assessed hind-limb functions of rats weekly for 10 weeks after SCI conducted by two examiners blinded to treatment groups (Fig. 2.9A, B). The vehicle SCI group that represents the injury baseline reached a plateau in BBB score at 8 weeks post-SCI, with an average score of 8.7±0.42. Rats treated with 0.5µg/day Nrg-1 showed similar trend in their hind-limb motor recovery comparable to the vehicle treated rats. However, we found a dose dependent improvement in BBB score of rats that received 1.0 µg and 1.5µg of Nrg-1 daily. At 10wk post-SCI, the 1.0µg Nrg-1 group scored 9.4±0.8 compared to the vehicle and 0.5 µg/day rhNrg-1β groups with a BBB score of 8.6±0.37 and 8.9±0.77, respectively. The 1.5µg Nrg-1 group showed the highest BBB score starting at 5 weeks. There was a significantly higher BBB score in 1.5 µg Nrg-1 SCI rats at 8wk (10.2±0.3), 9wk (10.6±0.2) and 10w (10.7±0.3) time-points compared to the vehicle baseline SCI group with BBB score of 8.7±0.42 (8wk), 8.8±0.39 (9wk) and 8.64±0.37 (10wk) (two-way repeated measures ANOVA followed by the pairwise multiple-comparison Holm-Sidak method, N=8 rats per group) (Fig. 2.9 A). Rats treated with 1.5 µg Nrg-1 also showed higher BBB scores compared to the 0.5 and 1 µg per day Nrg-1 group although this difference was not significant. Of note, BBB score of 10 and higher indicates the recovery of weight supported plantar stepping in SCI rats and is considered a milestone in locomotor recovery. Interestingly, we found a greater frequency of BBB score of ≥10-11 in both 1.0 and 1.5 µg Nrg-1 groups than in the

0.5µg/day Nrg-1 and vehicle groups from weeks 5 to 10 post-SCI. At the 10wk time-point, 80% of the rats in 1.5µg Nrg-1 group had a mean BBB score of 10.5 and higher compared to 1.0µg/day, 0.5µg/day and vehicle groups with 50%, 20% and 15% BBB score of ≥10, respectively (Fig. 2.9 B).

We additionally examined nociception in our SCI rats using tail flick analysis at week 4, 7 and 10 post-injury. At their pre-injury basal state, rats showed a mean latency of 20 seconds to withdraw their tail from heat. Following SCI, all injured rats showed hypersensitivity to thermal stimuli at all examined time-points compared to their normal condition (Fig. 2.9 C). At 10wk post SCI, the injury baseline withdrawal latency in vehicle group was reduced to 15.23s±1.24, which was not significantly different from the latency recorded from 0.5µg/day Nrg-1 (11.8±1.2), 1µg/day Nrg-1 (13.76±1.3) and 1.5µg Nrg-1 (14.2±1.56) (Fig. 2.9 C). These findings suggest that Nrg-1 treatment improves recovery of locomotion without affecting the response to painful stimuli following SCI.

Figure 2.9. Nrg-1 therapy improves locomotor recovery.

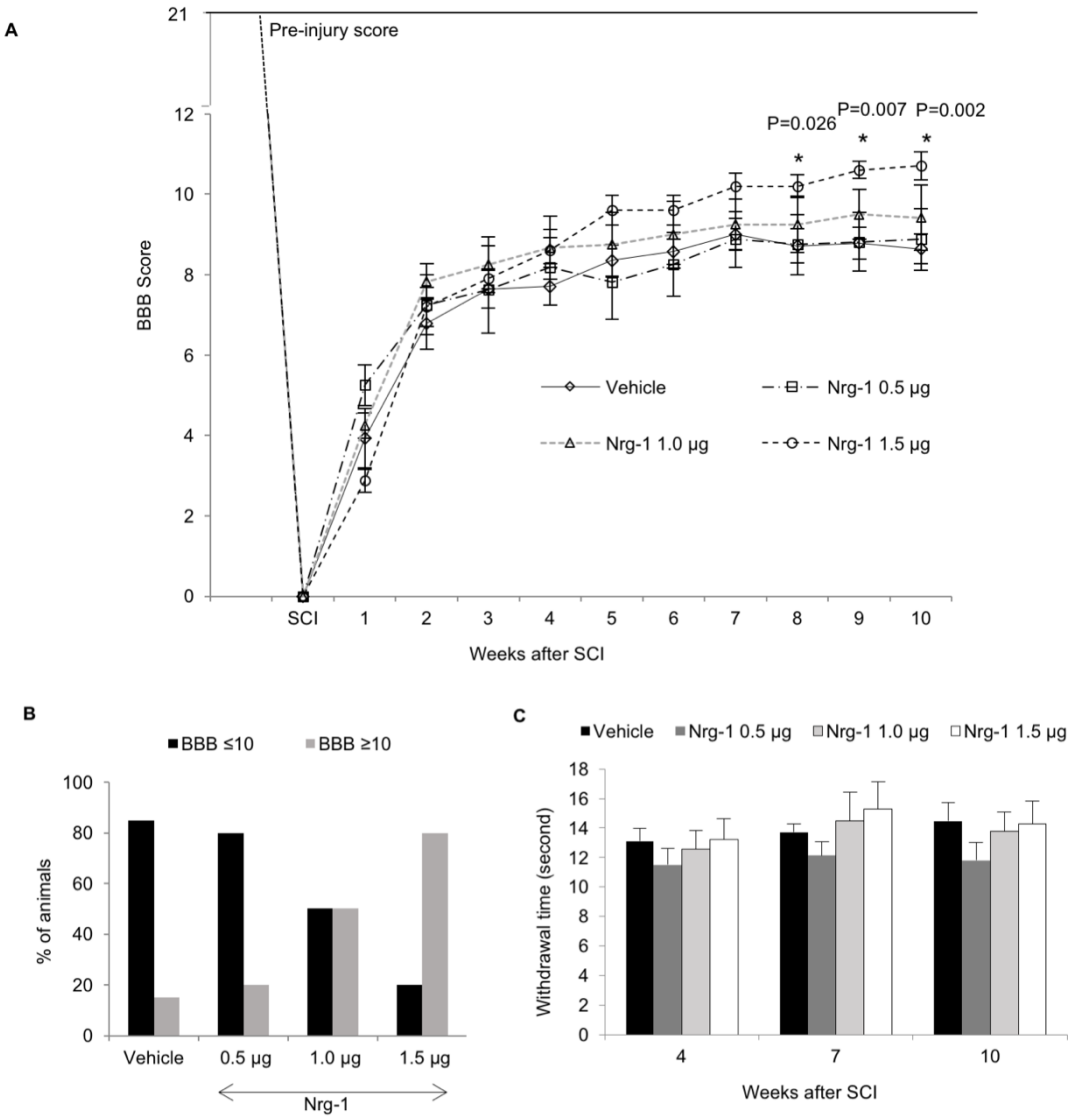


Figure 2.9. Nrg-1 therapy improves locomotor recovery. (A) Hind-limb locomotor function was assessed weekly using BBB open field scale over a 10-week period in vehicle and Nrg-1 treated (0.5µg, 1.0µg or 1.5µg per day) SCI rats (N=8 rats/group). Weekly BBB scoring showed that the vehicle SCI group reached a plateau at 8th week with an average BBB score of 8.7±0.42 with no further significant changes beyond that point. Experimental group treated with 1.5µg/day Nrg-1 showed the highest BBB score (10.7±0.35) compared to the vehicle SCI group with BBB score of 8.64±0.37. Rats treated with 1.5µg Nrg-1 also showed higher BBB score compared to the 0.5µg and 1µg Nrg-1 group although this difference was not significant. **(B)** We found a greater frequency of occasional to consistent weight-supported plantar stepping (BBB score of ≥10-11) in both 1.0 and 1.5µg Nrg-1 groups than in the 0.5µg/day Nrg-1 and vehicle groups from week 5 to 10 post-SCI. Notably, at the 10-week time-point, 80% of the rats in 1.5µg Nrg-1 group had a mean BBB score of 10.5 and higher compared to 1.0µg/day, 0.5µg/day and vehicle groups with 50%, 20%, and 15% BBB score of ≥10. **(C)** Thermal allodynia was examined in rats by immersion of 5 cm of the end of the tail in a 46^{o}C water bath and measuring the latency (seconds) of tail retraction from the heat. Following SCI, all injured rats developed hypersensitivity to thermal stimuli at all examined time-points compared to their normal condition. At 10-week post SCI, the injury baseline withdrawal latency in vehicle group was reduced to 15.23s±1.24 which was comparable to the latency recorded from 0.5µg/day Nrg-1 (11.8±1.2), 1µg/day Nrg-1 (13.76±1.3) and 1.5µg Nrg-1 (14.2±1.56) with no statistically significant difference among the groups. The data represent mean ± SEM. *P<0.05, Two-way repeated measures ANOVA followed by the pairwise multiple-comparison Holm-Sidak method, N=8 rats/group.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315.

2.5 Discussion

In this study, we provide novel insights into the impact and mechanisms of Nrg-1 in pathophysiology of SCI. We demonstrate, for the first time, a multifaceted role for Nrg-1 in maintaining a balanced microenvironment in the injured spinal cord. Our findings indicate that increasing the deficient levels of Nrg-1 after SCI favorably moderates astrogliosis and fosters an immune response that promotes a shift from pro-inflammatory to a protective anti-inflammatory profile. We demonstrate that Nrg-1 effects are mediated through ErbB2/3 dependent mechanisms and modulation of multiple regulatory cascades involved in glial and leukocyte activation including Myd88, Erk1/2 and STAT3, Arg1 and IL-10. Importantly, the positive cellular effects of Nrg-1 treatment culminate in significant improvement of function following SCI.

Nrg-1 and its signalling receptors, ErbB2, ErbB3 and ErbB4, are known for their critical role in Schwann cell and oligodendrocyte differentiation and myelination [53-56]. Despite its important roles in in the adult and developing nervous system, Nrg-1 has a poorly defined role in SCI. Our recent work in compressive SCI revealed that Nrg-1 protein expression declines robustly within hours after SCI due to neuronal and axonal degeneration and does not recover chronically [19]. We previously reported that Nrg-1 treatment confers neuroprotection to oligodendrocytes and axons and promotes oligodendrogenesis following SCI [19]. Other studies have also established a supportive role for Nrg-1 in neuronal survival following cerebral ischemia [24, 57] and identified a role for Nrg-1 in Schwann cell mediated remyelination in the injured spinal cord [58]. To date, the role of Nrg-1 in regulating astrogliosis and immune response has not been addressed in SCI and the present work is the first demonstration of such effects.

Resident glial cells and infiltrating immune cells express all Nrg-1 receptors [19, 59, 60] suggesting that inflammation can be influenced by Nrg-1 availability in acute SCI. Here, we

demonstrate that Nrg-1 treatment remarkably attenuates the production and activity of IL-1β, TNF-α, MMP-2, MMP-9 and NO following injury. These factors are key mediators of secondary injury mechanisms in SCI including oxidative stress, glutamate excitotoxicity, necrosis, apoptosis, matrix degradation and impairment of axonal conduction [36, 52, 61, 62]. TNF-α induces a pro-inflammatory environment by switching a protective M2 to an inhibitory M1 phenotype in microglia/macrophages through an incremental rise in the iron level in these cells [63]. Moreover, increased levels of TNF-α and IL-1β is associated with oligodendrocyte apoptosis after SCI [62, 64]. Here, Nrg-1 attenuated the increased expression of TNF-α and IL-1β within the first week of SCI, which coincides with domination of M1 microglia/macrophages and the peak of oligodendrocyte apoptosis [65, 66]. Interestingly, increase in IL-1β and TNF-α causally induces MMP-2 and MMP-9 production by astrocytes and microglia in acute and subacute phases of brain and spinal cord injuries [52, 67]. Increased MMP-9 activity is particularly associated with several pathologies in SCI including enhanced astrocyte migration and glial scar formation [68] as well as disruption of blood-spinal barrier [52]. MMP-9-null mice show less susceptibility to the destruction of blood-spinal barrier (BSB) and reduced neutrophil infiltration after SCI that is accompanied by improved functional recovery [52]. We found that Nrg-1 attenuates the increased activity of MMP-2 and MMP-9 following SCI, which could be secondary to a decrease in IL-1β and TNF-α release and an underlying mechanism of improved functional recovery.

A switch from M1 to M2 phenotype in microglia/macrophages after Nrg-1 treatment was accompanied by a remarkable increase in Arg1 and IL-10 expression. Arg1 and IL-10 are known for their roles in moderating immune response and secondary tissue damage after CNS injury [1, 69-71]. IL-10 is shown to attenuate astrocyte reactivity [72] and pro-inflammatory cytokine production by activated macrophages following CNS injury [73]. Moreover, IL-10 fosters

regeneration by promoting wound healing and phagocytosis of myelin debris by macrophages/microglia after CNS injury [74]. Of note, microglia polarized by IL-10 promote oligodendrocytes survival in demyelinating conditions suggesting a neuroprotective role for IL-10 [75]. IL-10 itself can increase Arg1 expression which is known to attenuate NO production in M1 microglia/macrophages by metabolizing L-arginine, a required substrate by iNOS for NO synthesis [35, 76, 77]. Thereby, augmented levels of Arg1 following Nrg-1 treatment can be an underlying cause of NO reduction in our in vitro studies. Taken together, our finding suggests a positive impact for Nrg-1 on modulation of immune response and secondary tissue damage following SCI, and provide mechanisms for the therapeutic benefits of Nrg-1 on oligodendroglial, neuronal and axonal preservation observed in our previous SCI studies [19] and those of others in cerebral ischemia [78].

Another novel finding drawn from our studies is the regulatory role of Nrg-1 in astrogliosis following injury. We show for the first time a remarkable capacity for Nrg-1 to attenuate the upregulation of CSPGs in activated astrocytes in vitro and in SCI. CSPGs play multifaceted inhibitory roles in neural regeneration [10, 79]. In addition to posing an obstacle to axonal regeneration, CSPGs impede proliferation and oligodendrocyte differentiation of neural precursor cells (NPCs) and oligodendrocyte progenitor cells (OPCs) in SCI and demyelinating lesions [29, 30, 80]. Our recent in vitro and SCI studies have shown that CSPGs directly limit oligodendrocytes differentiation through receptor mediated mechanisms [29, 30, 80], and targeting CSPGs in SCI with chondroitinase ABC (ChABC) improves oligodendrocyte replacement by endogenous and transplanted precursor cells in SCI [29, 30]. In MS-like demyelinating lesions, CSPGs also hinder the migration and recruitment of OPCs into the lesion, and thereby inhibit their capacity for replacement of mature oligodendrocytes and axon remyelination [81]. Our new findings showing

the benefits of Nrg-1 in reducing CSPGs in SCI lesion provide another mechanism for our previous results that identified a positive role for Nrg-1 in enhancing oligodendrocyte differentiation of NPCs and OPCs after SCI [19].

Mechanistically, we demonstrate that Nrg-1 modulates multiple intracellular pathways involved in inflammatory response. We show that Nrg-1 effects seems to be mediated through ErbB2/ErbB3 receptor complex and an increase in the phosphorylation of Erk1/2 pathway, a known downstream pathway for Nrg-1 signaling [82, 83]. Activation of Erk1/2 signaling is an established mechanism of glial activation following CNS ischemic and traumatic injury which seems to be highly dependent on the type of ligand and receptors that initiate Erk1/2 activity as well as the timing of the activation [84-87]. We found a correlation between increased Erk1/2 phosphorylation and a remarkable increase in IL-10 release under Nrg-1 treatment in subacute phase of SCI. This is in agreement with previous studies that established a link between Erk1/2 activation and IL-10 release by macrophages/microglia and lymphocytes [50, 88, 89]. Nrg-1 treatment also activated STAT3 in SCI that is implicated as a signaling mechanism in IL-10 mediated anti-inflammatory response (AIR) in microglia/macrophages that results in a decrease in the expression of pro-inflammatory cytokines such as TNF-α [49, 72, 73, 90, 91]. With regards to pro-inflammatory cascades, we show that Nrg-1 attenuates the expression of MyD88 following injury. MyD88 is an intracellular adaptor protein downstream of all Toll-like receptors (TLRs) except TLR3 [92, 93]. TLRs/MyD88 pathways are acutely activated in resident microglia after SCI and contribute to the onset of inflammation by activating NF-κB pathway and ultimately the release of proinflammatory IL-1β and TNF-α [48, 94]. MyD88 down-regulation has been associated with microglial M2 phenotype change and reduced production of pro-inflammatory cytokines [47]. Astrocytes also play major roles in initiating the neuroinflammation following SCI

through an IL-1 receptor/MyD88 dependent signaling pathway [11]. Interestingly, our in vitro data identified that pure astrocytes are not responsive to LPS, and their activation depend on their interactions with microglia for activation. Evidence for such dependency has been shown in other studies in the context of LPS activation [95, 96]. Our findings have collectively uncovered multiple mechanisms by which Nrg-1 plays its immunomodulatory roles.

Multifaceted benefits of Nrg-1 treatment culminated in enhanced locomotor recovery in following SCI without affecting pain response. We found a significant improvement in BBB hind-limb motor score of rats that received daily intrathecal infusion of 1.5 μg Nrg-1. Nrg-1 treatment resulted in regaining frequent to consistent weight supported plantar stepping in Nrg-1 treated rats compared to the vehicle treated group that their recovery plateaued without regaining their weight support. Our analysis of thermal sensitivity showed no significant effects of Nrg-1 on the onset and development of pain reactivity in our SCI rats. An earlier study in SCI showed that transplanted OPCs can diminish pain response by producing Nrg-1 and promoting remyelination [97]. This was concluded when inhibition of Nrg-1 by siRNA approaches abolished the beneficial effects of OPCs on pain reduction after injury. Of note, in these studies exogenous Nrg-1 treatment was not employed to assess its role in pain modulation. In the same context, there are other contradicting studies that propose a role for Nrg-1/ErbB signalling in inducing neuropathic pain through its effects on microgliosis following peripheral nerve injury [98]. Overall, our results showed no significant effects of Nrg-1 treatment on microgliosis in vitro and following SCI. We also found no changes in pain response in chronic SCI as the result of sustained Nrg-1 treatment. While it needs further investigations, the discrepancy between these findings on the role of Nrg-1 in pain could reflect the difference in the mechanisms of peripheral nerve injury and SCI.

In conclusion, our present work provides novel findings uncovering that bioavailability of Nrg-1 improves the microenvironment of SCI by modulating the response of astrocytes and immune cells to injury resulting in an improved recovery from SCI. We have identified several mechanisms by which Nrg-1 may have promoted functional recovery following SCI that include promoting an anti-inflammatory and protective phenotype in activated glia and immune cells as well as attenuating CSPGs accumulation in the glial scar. We also show that Nrg-1 exerts its effects via activation of ErbB2 in glial cells and upregulation of Erk and STAT3 pathways. Given the pronounced depletion of Nrg-1 following SCI and its multifaceted role as a neuroprotective and pro-regenerative factor, we propose that Nrg-1 therapy may represent a promising treatment strategy for SCI and other CNS conditions characterized by neuroinflammation and astrogliosis.

2.6 References

1. David, S. and A. Kroner, *Repertoire of microglial and macrophage responses after spinal cord injury.* Nat Rev Neurosci, 2011. **12**(7): p. 388-99.
2. Alizadeh, A., S.M. Dyck, and S. Karimi-Abdolrezaee, *Myelin damage and repair in pathologic CNS: challenges and prospects.* Front Mol Neurosci, 2015. **8**: p. 35.
3. Conti, A., et al., *Nitric oxide in the injured spinal cord: synthases cross-talk, oxidative stress and inflammation.* Brain Res Rev, 2007. **54**(1): p. 205-18.
4. Sato, A., et al., *Interleukin-1 participates in the classical and alternative activation of microglia/macrophages after spinal cord injury.* J Neuroinflammation, 2012. **9**: p. 65.
5. Vecil, G.G., et al., *Interleukin-1 is a key regulator of matrix metalloproteinase-9 expression in human neurons in culture and following mouse brain trauma in vivo.* J Neurosci Res, 2000. **61**(2): p. 212-24.
6. Su, Z., et al., *Reactive astrocytes inhibit the survival and differentiation of oligodendrocyte precursor cells by secreted TNF-alpha.* J Neurotrauma, 2011. **28**(6): p. 1089-100.
7. Li, S. and P.K. Stys, *Mechanisms of ionotropic glutamate receptor-mediated excitotoxicity in isolated spinal cord white matter.* Journal of Neuroscience, 2000. **20**(3): p. 1190-8.
8. Zhao W, X.W., Le W, Beers DR, He Y, Henkel JS, Simpson EP, Yen AA, Xiao Q, Appel SH, *Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism.* J Neuropathol Exp Neurol, 2004. **63**(9): p. 964-977.
9. Sofroniew, M.V., *Molecular dissection of reactive astrogliosis and glial scar formation.* Trends Neurosci, 2009. **32**(12): p. 638-647.

10. Dyck, S.M. and S. Karimi-Abdolrezaee, *Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system.* Exp Neurol, 2015. **269**: p. 169-87.

11. Pineau, I., et al., *Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion.* Brain Behav Immun, 2010. **24**(4): p. 540-53.

12. Wang, Y., et al., *Astrocytes from the Contused Spinal Cord Inhibit Oligodendrocyte Differentiation of Adult Oligodendrocyte Precursor Cells by Increasing the Expression of Bone Morphogenetic Proteins.* J. Neurosci, 2011. **31**(16): p. 6053-6058.

13. Sofroniew, M.V., *Reactive astrocytes in neural repair and protection.* Neuroscientist, 2005. **11**(5): p. 400-7.

14. Karimi-Abdolrezaee, S. and R. Billakanti, *Reactive Astrogliosis after Spinal Cord Injury-Beneficial and Detrimental Effects.* Mol. Neurobiol, 2012.

15. Donnelly, D.J. and P.G. Popovich, *Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury.* Exp Neurol, 2008. **209**(2): p. 378-88.

16. Kigerl, K.A., et al., *Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord.* J Neurosci, 2009. **29**(43): p. 13435-44.

17. Hamby, M.E. and M.V. Sofroniew, *Reactive astrocytes as therapeutic targets for CNS disorders.* Neurotherapeutics, 2010. **7**(4): p. 494-506.

18. Wattananit, S., et al., *Monocyte-Derived Macrophages Contribute to Spontaneous Long-Term Functional Recovery after Stroke in Mice.* Journal of Neuroscience, 2016. **36**(15): p. 4182-4195.

19. Gauthier, M.-K., et al., *Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury.* Eur. J. Neurosci, 2013. **38**(5): p. 2693-2715.

20. Taveggia, C., M.L. Feltri, and L. Wrabetz, *Signals to promote myelin formation and repair.* Nat Rev Neurol, 2010. **6**(5): p. 276-87.

21. Talmage, D.A., *Mechanisms of neuregulin action.* Novartis Found Symp, 2008. **289**: p. 74-84; discussion 84-93.

22. Vartanian, T., G. Fischbach, and R. Miller, *Failure of spinal cord oligodendrocyte development in mice lacking neuregulin.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 731-5.

23. Li, Y., et al., *Neuregulin-1 inhibits neuroinflammatory responses in a rat model of organophosphate-nerve agent-induced delayed neuronal injury.* J Neuroinflammation, 2015. **12**(1): p. 64.

24. Xu, Z., et al., *Extended therapeutic window and functional recovery after intraarterial administration of neuregulin-1 after focal ischemic stroke.* J Cereb Blood Flow Metab, 2006. **26**(4): p. 527-35.

25. Mencel, M., M. Nash, and C. Jacobson, *Neuregulin upregulates microglial alpha7 nicotinic acetylcholine receptor expression in immortalized cell lines: implications for regulating neuroinflammation.* PLoS One, 2013. **8**(7): p. e70338.

26. Simmons, L.J., et al., *Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial cells in vitro involves the NF-kappa B pathway.* J Neuroinflammation, 2016. **13**(1): p. 237.

27. Allen, J.W., L.A. Mutkus, and M. Aschner, *Isolation of neonatal rat cortical astrocytes for primary cultures.* Curr Protoc Toxicol, 2001. **Chapter 12**: p. Unit12 4.

28. Black, P., et al., *Models of spinal cord injury: Part 1. Static load technique.* Neurosurgery, 1986. **19**(5): p. 752-62.

29. Karimi-Abdolrezaee, S., et al., *Synergistic Effects of Transplanted Adult Neural Stem/Progenitor Cells, Chondroitinase, and Growth Factors Promote Functional Repair and Plasticity of the Chronically Injured Spinal Cord.* J. Neurosci, 2010. **30**(5): p. 1657-1676.

30. Karimi-Abdolrezaee, S., et al., *Chondroitinase and Growth Factors Enhance Activation and Oligodendrocyte Differentiation of Endogenous Neural Precursor Cells after Spinal Cord Injury.* Plos One, 2012. **7**(5): p. 1-16.

31. Karimi-Abdolrezaee, S., et al., *Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury.* J Neurosci, 2006. **26**(13): p. 3377-89.

32. Bruce, J.C., M.A. Oatway, and L.C. Weaver, *Chronic pain after clip-compression injury of the rat spinal cord.* Exp Neurol, 2002. **178**(1): p. 33-48.

33. Fleming, J.C., et al., *Alpha4beta1 integrin blockade after spinal cord injury decreases damage and improves neurological function.* Exp Neurol, 2008. **214**(2): p. 147-59.

34. Marsala, J., et al., *Traumatic injury of the spinal cord and nitric oxide.* Prog Brain Res, 2007. **161**: p. 171-83.

35. Chang, C.I., J.C. Liao, and L. Kuo, *Arginase modulates nitric oxide production in activated macrophages.* Am J Physiol, 1998. **274**(1 Pt 2): p. H342-8.

36. Ferguson, A.R., et al., *Cell death after spinal cord injury is exacerbated by rapid TNF alpha-induced trafficking of GluR2-lacking AMPARs to the plasma membrane.* J Neurosci, 2008. **28**(44): p. 11391-400.

37. Nesic, O., et al., *IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury.* J Neurotrauma, 2001. **18**(9): p. 947-56.

38. Wandinger, S.K., et al., *Quantitative Phosphoproteomics Analysis of ERBB3/ERBB4 Signaling.* PLoS One, 2016. **11**(1): p. e0146100.

39. Iwakura, Y. and H. Nawa, *ErbB1-4-dependent EGF/neuregulin signals and their cross talk in the central nervous system: pathological implications in schizophrenia and Parkinson's disease.* Front Cell Neurosci, 2013. **7**: p. 4.

40. Kovacs, T., et al., *Neuregulin 1-induced AKT and ERK phosphorylation in patients with fragile X syndrome (FXS) and intellectual disability associated with obstetric complications.* J Mol Neurosci, 2014. **54**(1): p. 119-24.

41. Hausmann, O.N., *Post-traumatic inflammation following spinal cord injury.* Spinal Cord, 2003. **41**(7): p. 369-78.

42. Klusman, I. and M.E. Schwab, *Effects of pro-inflammatory cytokines in experimental spinal cord injury.* Brain Res, 1997. **762**(1-2): p. 173-84.

43. Ledeboer, A., et al., *Interleukin-10, interleukin-4, and transforming growth factor-beta differentially regulate lipopolysaccharide-induced production of pro-inflammatory cytokines and nitric oxide in co-cultures of rat astroglial and microglial cells.* Glia, 2000. **30**(2): p. 134-42.

44. Norden, D.M., et al., *TGFbeta produced by IL-10 redirected astrocytes attenuates microglial activation.* Glia, 2014. **62**(6): p. 881-95.

45. Thompson, C.D., et al., *The therapeutic role of interleukin-10 after spinal cord injury.* J Neurotrauma, 2013. **30**(15): p. 1311-24.

46. Muzio, M., et al., *IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling.* Science, 1997. **278**(5343): p. 1612-5.

47. Yao, A.H., et al., *Early Blockade of TLRs MyD88-Dependent Pathway May Reduce Secondary Spinal Cord Injury in the Rats.* Evid Based Complement Alternat Med, 2012. **2012**: p. 591298.

48. Kigerl, K.A., et al., *Toll-like receptor (TLR)-2 and TLR-4 regulate inflammation, gliosis, and myelin sparing after spinal cord injury.* J Neurochem, 2007. **102**(1): p. 37-50.

49. Hutchins, A.P., D. Diez, and D. Miranda-Saavedra, *The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges.* Brief Funct Genomics, 2013. **12**(6): p. 489-98.

50. Liu, B.S., et al., *TLR-mediated STAT3 and ERK activation controls IL-10 secretion by human B cells.* Eur J Immunol, 2014. **44**(7): p. 2121-9.

51. Yong, V.W., et al., *Metalloproteinases in biology and pathology of the nervous system.* Nat Rev Neurosci, 2001. **2**(7): p. 502-11.

52. Noble, L.J., et al., *Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events.* J Neurosci, 2002. **22**(17): p. 7526-35.

53. Syed, N., et al., *Soluble neuregulin-1 has bifunctional, concentration-dependent effects on Schwann cell myelination.* J Neurosci, 2010. **30**(17): p. 6122-31.

54. Mei, L. and W.C. Xiong, *Neuregulin 1 in neural development, synaptic plasticity and schizophrenia.* Nat Rev Neurosci, 2008. **9**(6): p. 437-52.

55. Falls, D.L., *Neuregulins: functions, forms, and signaling strategies.* Exp Cell Res, 2003. **284**(1): p. 14-30.

56. Chen, Y.J., et al., *Type III neuregulin-1 is required for normal sensorimotor gating, memory-related behaviors, and corticostriatal circuit components.* J Neurosci, 2008. **28**(27): p. 6872-83.

57. Li, Y., et al., *Neuroprotection by neuregulin-1 in a rat model of permanent focal cerebral ischemia.* Brain Res, 2007. **1184**: p. 277-83.

58. Bartus, K., et al., *Neuregulin-1 controls an endogenous repair mechanism after spinal cord injury.* Brain, 2016.

59. Ryzhov, S., et al., *Activation of ERBB3 Receptor Prevents TNFα Production from Human Non-Classical Monocytes.* The FASEB Journal, 2015. **29**(1 Supplement).

60. Tynyakov-Samra, E., et al., *Reduced ErbB4 Expression in Immune Cells of Patients with Relapsing Remitting Multiple Sclerosis.* Mult Scler Int, 2011. **2011**: p. 561262.

61. Sharma, H.S., et al., *Involvement of nitric oxide in acute spinal cord injury: an immunocytochemical study using light and electron microscopy in the rat.* Neurosci Res, 1996. **24**(4): p. 373-84.

62. Takahashi, J.L., et al., *Interleukin-1beta promotes oligodendrocyte death through glutamate excitotoxicity.* Ann Neurol, 2003. **53**(5): p. 588-95.

63. Kroner, A., et al., *TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord.* Neuron, 2014. **83**(5): p. 1098-116.

64. Yune, T.Y., et al., *Increased production of tumor necrosis factor-alpha induces apoptosis after traumatic spinal cord injury in rats.* J Neurotrauma, 2003. **20**(2): p. 207-19.

65. Casha, S., W. Yu, and M. Fehlings, *Oligodendrocyte apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat.* Neuroscience, 2001. **103**(1): p. 203-218.

66. Beattie, M.S., et al., *Cell death in models of spinal cord injury.* Prog Brain Res, 2002. **137**: p. 37-47.

67. Hansen, C.N., et al., *Elevated MMP-9 in the lumbar cord early after thoracic spinal cord injury impedes motor relearning in mice.* J Neurosci, 2013. **33**(32): p. 13101-11.

68. Hsu, J.Y., et al., *Matrix metalloproteinase-9 facilitates glial scar formation in the injured spinal cord.* J Neurosci, 2008. **28**(50): p. 13467-77.

69. Brewer, K.L., J.R. Bethea, and R.P. Yezierski, *Neuroprotective effects of interleukin-10 following excitotoxic spinal cord injury.* Exp Neurol, 1999. **159**(2): p. 484-93.

70. Zhou, Z., et al., *IL-10 promotes neuronal survival following spinal cord injury.* Exp Neurol, 2009. **220**(1): p. 183-90.

71. Zhang, B., et al., *Age decreases macrophage IL-10 expression: Implications for functional recovery and tissue repair in spinal cord injury.* Exp Neurol, 2015. **273**: p. 83-91.

72. Balasingam, V. and V.W. Yong, *Attenuation of astroglial reactivity by interleukin-10.* J Neurosci, 1996. **16**(9): p. 2945-55.

73. Fiorentino, D.F., et al., *IL-10 inhibits cytokine production by activated macrophages.* J Immunol, 1991. **147**(11): p. 3815-22.

74. Puntambekar, S.S., et al., *Interleukin-10 is a critical regulator of white matter lesion containment following viral induced demyelination.* Glia, 2015. **63**(11): p. 2160-2120.

75. Miron, V.E., et al., *M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination.* Nat Neurosci, 2013. **16**(9): p. 1211-8.

76. Corraliza, I.M., et al., *Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages.* Biochem Biophys Res Commun, 1995. **206**(2): p. 667-73.

77. Lang, R., et al., *Shaping gene expression in activated and resting primary macrophages by IL-10.* J Immunol, 2002. **169**(5): p. 2253-63.

78. Li, Q., et al., *Effect of neuregulin on apoptosis and expressions of STAT3 and GFAP in rats following cerebral ischemic reperfusion.* J Mol Neurosci, 2009. **37**(1): p. 67-73.

79. Bradbury, E.J., et al., *Chondroitinase ABC promotes functional recovery after spinal cord injury.* Nature, 2002. **416**: p. 636-640.

80. Dyck, S.M., et al., *Chondroitin Sulfate Proteoglycans Negatively Modulate Spinal Cord Neural Precursor Cells by Signaling through LAR and RPTPsigma and modulation of the Rho/ROCK Pathway.* Stem Cells, 2015.

81. Lau, L.W., et al., *Chondroitin sulfate proteoglycans in demyelinated lesions impair remyelination.* Ann Neurol, 2012. **72**(3): p. 419-32.

82. Chen, M., et al., *Time-dependent regulation of neuregulin-1beta/ErbB/ERK pathways in cardiac differentiation of mouse embryonic stem cells.* Mol Cell Biochem, 2013. **380**(1-2): p. 67-72.

83. Monje, P.V., M. Bartlett Bunge, and P.M. Wood, *Cyclic AMP synergistically enhances neuregulin-dependent ERK and Akt activation and cell cycle progression in Schwann cells.* Glia, 2006. **53**(6): p. 649-59.

84. Maddahi, A. and L. Edvinsson, *Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the MEK/ERK pathway.* J Neuroinflammation, 2010. **7**: p. 14.

85. Mori, T., et al., *Mitogen-activated protein kinase inhibition in traumatic brain injury: in vitro and in vivo effects.* J Cereb Blood Flow Metab, 2002. **22**(4): p. 444-52.

86. Saud, K., R. Herrera-Molina, and R. Von Bernhardi, *Pro- and anti-inflammatory cytokines regulate the ERK pathway: implication of the timing for the activation of microglial cells.* Neurotox Res, 2005. **8**(3-4): p. 277-87.

87. Szydlowska, K., et al., *Prolonged activation of ERK triggers glutamate-induced apoptosis of astrocytes: neuroprotective effect of FK506.* J Neurochem, 2010. **113**(4): p. 904-18.

88. Chanteux, H., et al., *LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms.* Respir Res, 2007. **8**: p. 71.

89. Saraiva, M., et al., *Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose.* Immunity, 2009. **31**(2): p. 209-19.

90. Hedrich, C.M. and J.H. Bream, *Cell type-specific regulation of IL-10 expression in inflammation and disease.* Immunol Res, 2010. **47**(1-3): p. 185-206.

91. Marchant, A., et al., *Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia.* Eur J Immunol, 1994. **24**(5): p. 1167-71.

92. Kielian, T., *Toll-like receptors in central nervous system glial inflammation and homeostasis.* J Neurosci Res, 2006. **83**(5): p. 711-30.

93. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

94. Babcock, A.A., H. Toft-Hansen, and T. Owens, *Signaling through MyD88 regulates leukocyte recruitment after brain injury.* J Immunol, 2008. **181**(9): p. 6481-90.

95. Facci, L., et al., *Toll-like receptors 2, -3 and -4 prime microglia but not astrocytes across central nervous system regions for ATP-dependent interleukin-1beta release.* Sci Rep, 2014. **4**: p. 6824.

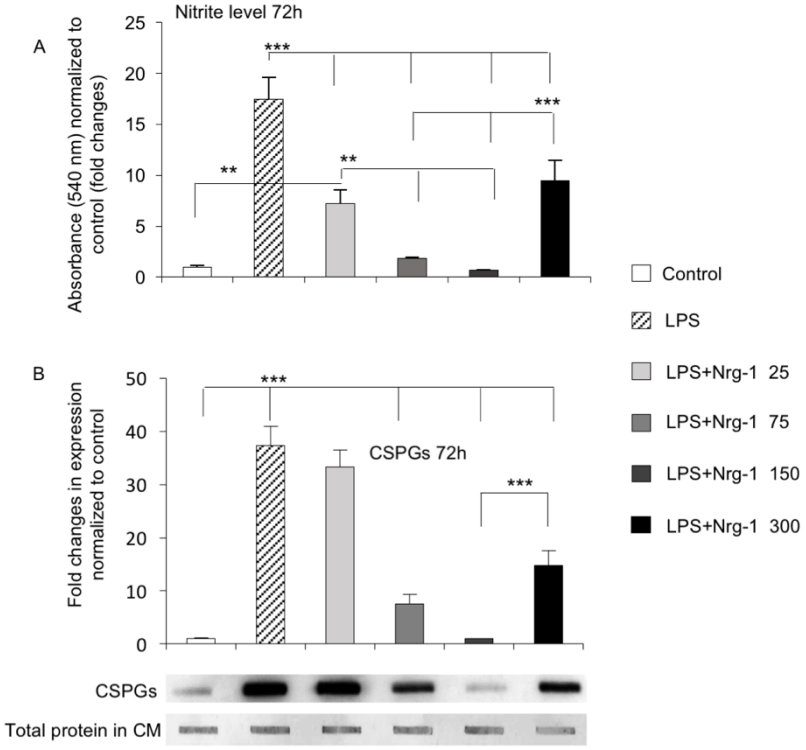
96. Lehnardt, S., et al., *The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS.* J Neurosci, 2002. **22**(7): p. 2478-86.

97. Tao, F., et al., *Role of Neuregulin-1/ErbB Signaling in Stem Cell Therapy for Spinal Cord Injury-induced Chronic Neuropathic Pain.* Stem Cells, 2012.

98. Calvo, M., et al., *Following nerve injury neuregulin-1 drives microglial proliferation and neuropathic pain via the MEK/ERK pathway.* Glia, 2011. **59**(4): p. 554-68.

2.7 Supplementary figures

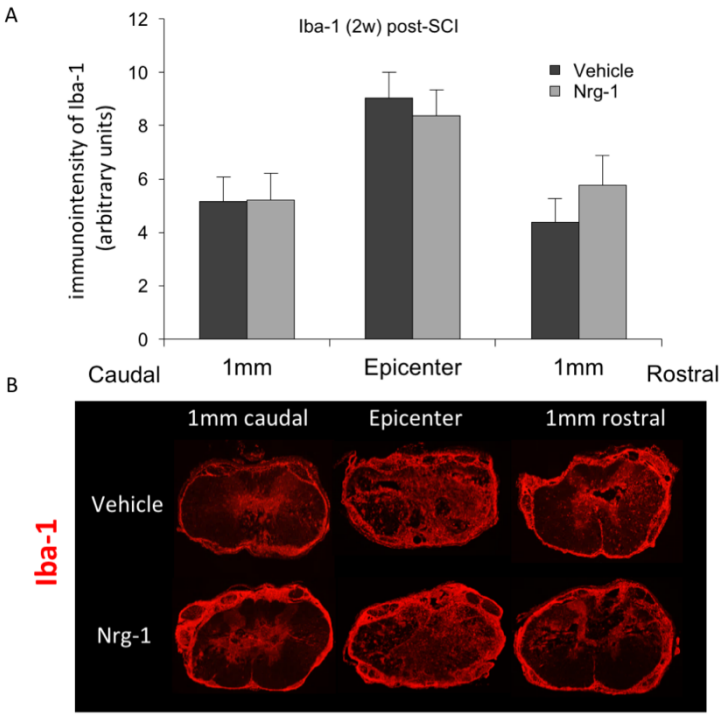
Supplementary figure 2.1. Dosing study for Nrg-1 treatment in mixed astro-microglia culture.



Supp. figure 2.1. Dosing study for Nrg-1 treatment in mixed astro-microglia culture. (A) Mixed astro-microglia activated by LPS (10μg/ml) were treated with 25, 75, 150 and 300 ng/ml of Nrg-1. State of activation was assessed using **(A)** Griess assay for nitrite and **(B)** slot blot for CSPGs. Results show that 75 and 150 ng/ml of Nrg-1 could significantly reduce nitrite and CSPGs production in activated astro-microglial cells. However, 150ng/ml was more effective in reducing glial activation. While 25 ng/ml of Nrg-1 was sufficient to significantly reduce nitrite production, it was not enough to reduce CSPGs in our model. Based on these data, 150ng/ml was used as an optimal dose for our *in vitro* studies. The data represent mean ± SEM, ***P<0.001, **P<0.01, *P<0.05, N=4 independent culture experiments, One-Way ANOVA, followed by Holm-Sidak post hoc test.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315.

Supplementary figure 2.2. Nrg-1 does not affect the presence of microglia/macrophages in the SCI lesion.



Supp. Fig. 2.2: Nrg-1 does not affect the presence of microglia/macrophages in the SCI lesion. Immunohistological assessment of Iba-1 immunointensity was performed on the epicenter and perilesional areas at 14d post-SCI. **(A)** Comparison between vehicle and Nrg-1 treated SCI rats showed no significant change in Iba-1 immunointensity between Nrg-1 and vehicle treated groups. **(B)** Representative images of spinal cord cross-sections immunostained for Iba-1 are depicted at the epicenter and 1mm rostral and caudal to the lesion center. The data show mean ± SEM. *P<0.05, Two-way ANOVA followed by Holm-Sidak post-hoc test, N=4 rats/group.

Reprinted figure: Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, Karimi-Abdolrezaee S, (2017). **Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.** Glia, 2017. **65**(7): p. 1152-1175. DOI: 10.1002/glia.2315.

Chapter 3: Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury

This collaborative work was published as an original article in *Journal of Neuroinflammation* (PMID: 25703008, Stem Cells. 2015 Aug;33(8):2550-63. doi 10.1002/stem.1979)

Arsalan Alizadeh[1], Kallivalappil T. Santhosh[1], Hardeep Kataria[1], Abdelilah S. Gounni[2], *Soheila Karimi-Abdolrezaee[1]

[1]Regenerative Medicine Program, Department of Physiology and Pathophysiology, Spinal Cord Research Centre, University of Manitoba, Winnipeg, Manitoba, Canada
[2]Department of Immunology, University of Manitoba, Winnipeg, Manitoba, Canada

Author Contributions: My contribution to this work includes concept and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript. I contributed to over 80% of the total experimental procedures, data and analysis. Manuscript preparation was performed in collaboration with my supervisor. The collection of PCR data shown in Figure 5 and 6 were collected by Dr. Kallivalappil Thomas Santhosh. Analysis was performed by myself. Dr. Hardeep Kataria assisted with flow cytometry sample preparation. Dr. A.S. Gounni provided guidance and essential feedback in flow cytometry studies.

Acknowledgement: This work was supported by operating grants awarded to S.K-A from the Canadian Institutes of Health Research (CIHR, Grant No: MOP 133721), the Canadian Paraplegic Association of Manitoba, and the Manitoba Paraplegic Foundation. Salary support for A.A was provided by CHRIM, Research Manitoba and GETS program of the U of M. H.K was supported by a post-doctoral fellowship from the Research Manitoba and the Rick Hansen Institute.
The authors would like to thank Dr. Hesam Movassagh for providing technical training.

Conflict of Interest: The authors declare no competing financial interests

Chapter 3 is reprinted as it appears in: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

3.1 Abstract

3.1.1 Background: Spinal cord injury (SCI) triggers a robust neuroinflammatory response that governs secondary injury mechanisms with both degenerative and pro-regenerative effects. Identifying new immunomodulatory therapies to promote the supportive aspect of immune response is critically needed for the treatment of SCI. We previously demonstrated that SCI results in acute and permanent depletion of the neuronally derived Neuregulin-1 (Nrg-1) in the spinal cord. Increasing the dysregulated level of Nrg-1 through acute intrathecal Nrg-1 treatment enhanced endogenous cell replacement and promoted white matter preservation and functional recovery in rat SCI. Moreover, we identified a neuroprotective role for Nrg-1 in moderating the activity of resident astrocytes and microglia following injury. To date, the impact of Nrg-1 on immune response in SCI has not yet been investigated. In this study, we elucidated the effect of systemic Nrg-1 therapy on the recruitment and function of macrophages, T cells and B cells, three major leukocyte populations involved in neuroinflammatory processes following SCI.

3.1.2 Methods: We utilized a clinically-relevant model of moderately severe compressive SCI in female Sprague Dawley rats. Nrg-1 (2 μg/day) or saline was delivered subcutaneously through osmotic mini-pumps starting 30 min after SCI. We conducted flow cytometry, quantitative real time PCR and immunohistochemistry at acute, subacute and chronic stages of SCI to investigate the effects of Nrg-1 treatment on systemic and spinal cord immune response as well as cytokine, chemokine and antibody production.

3.1.3 Results: We provide novel evidence that Nrg-1 promotes a pro-regenerative immune response after SCI. Bioavailability of Nrg-1 stimulated a regulatory phenotype in T and B cells and augmented the population of M2 macrophages in the spinal cord and blood during the acute and chronic stages of SCI. Importantly, Nrg-1 fostered a more balanced microenvironment in the

injured spinal cord by attenuating antibody deposition and expression of pro-inflammatory cytokines and chemokines while upregulating pro-regenerative mediators.

3.1.4 Conclusion: We provide the first evidence of a significant regulatory role for Nrg-1 in neuroinflammation after SCI. Importantly, the present study establishes the promise of systemic Nrg-1 treatment as a candidate immunotherapy for traumatic SCI and other CNS neuroinflammatory conditions.

3.2 Background

Pathophysiology of spinal cord injury (SCI) is characterized by a robust humoral and cellular neuroinflammatory response that is driven by the interplay between the peripherally recruited leukocytes and resident glial cells [1, 2]. Microglia/macrophages, T cells and B cells play central roles in orchestrating the innate and adaptive immune responses following SCI through a plethora of inflammatory cytokines, chemokines, antibodies and proteolytic enzymes [2-4]. Activated microglia/macrophages mediate the initial neuronal injury through pro-inflammatory mediators and oxidative damage [5]. They also activate T cells through their antigen presenting function [1]. Activated T cells stimulate B cells, through a host of cytokines and signaling molecules, to produce autoreactive antibodies against spinal cord tissue [6] causing tissue damage through antibody mediated cytotoxicity [7, 8]. These processes collectively lead to an imbalanced and dysregulated milieu that impedes repair and regeneration after SCI [1]. Despite their undisputed role in degenerative processes following SCI, emerging evidence indicates that immune cells can be modulated in their microenvironment to adopt regulatory and pro-regenerative phenotypes [9, 10]. Given the profound impact of neuroinflammation on SCI pathophysiology, it is critical to unravel endogenous mechanisms that regulate immune cells after injury. This knowledge is vital for

developing immunotherapies that can harness the potential of immune cells in fostering a supportive microenvironment for spinal cord repair and regeneration.

We previously identified that SCI results in a rapid and long-lasting decline in the tissue levels of the neuronally derived growth factor, Neuregulin-1 (Nrg-1) [11]. Nrg-1 is primarily known for its essential role in Schwann cell and oligodendrocyte differentiation, maintenance and myelination in the central and peripheral nervous systems [12]. In a preclinical model of compressive/contusive SCI in rats, we demonstrated that increasing the deficient bio-availability of Nrg-1 in the injured spinal cord improves neurological recovery following injury[13]. In our efforts to elucidate the mechanisms underpinning the recovery of function after Nrg-1 treatment [13], we identified that Nrg-1 promotes oligodendrogenesis and protects oligodendrocytes and axons resulting in white matter preservation after SCI [11]. Importantly, we found a remarkable positive role for Nrg-1 in regulating astrogliosis and glial scar formation in the injured spinal cord [13]. Moreover, Nrg-1 treatment through intrathecal infusion attenuated the release of pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) in acute SCI, while increasing the tissue levels of the anti-inflammatory cytokine, IL-10 at subacute SCI [13]. Other studies have also shown a neuroprotective role for Nrg-1 in attenuating neuronal injury *in vitro* and in ischemic brain injury models by reducing neurotoxicity and pro-inflammatory mediators [14, 15]. These initial findings suggest a potential immunomodulatory mechanism of Nrg-1 in fostering a pro-regenerative microenvironment that improves tissue preservation and neurological recovery following SCI [13].

In the present study, we dissected the impact of Nrg-1 on the peripheral and spinal cord immune responses at various stages of SCI. Using a clinically relevant model of severe compressive SCI in rat, we investigated whether systemic delivery of recombinant human Nrg-1

(rhNrg-1) could regulate the recruitment, phenotype and secretory properties of SCI relevant leukocytes in the blood and injured spinal cord. We demonstrate, for the first time, that Nrg-1 promotes a comprehensive immune regulatory response by macrophages and T and B lymphocytes at acute and chronic stages of SCI through modulation of a repertoire of cytokines and chemokines in the spinal cord tissue. Our new findings establish that bioavailability of Nrg-1 activates a neuroinflammatory process that provides a supportive environment for endogenous repair and recovery of function following SCI.

3.3 Methods

3.3.1 Rat model of Compressive Spinal Cord Injury and Animal Care

All animal experiments were approved by the University of Manitoba Animal Care Committee in agreement with the Canadian Council on Animal Care guidelines and policies. A total of 120 age and weight matched (8-10 weeks, 250g) adult female Sprague Dawley (SD) rats (Central Animal Care Facility at the University of Manitoba) were used in this study. For SCI surgeries, animals underwent laminectomy at thoracic levels T6-T8 under deep isoflurane anesthesia. To induce compressive SCI, a 35g aneurysm clip (University Health Network, Toronto, Ontario) was applied for 1 minute at mid-thoracic (T7) level. Each animal received a mixture of buprenorphine (Temgesic®, 0.05 mg/kg) and meloxicam (Metacam®, Boehringer Ingelheim GmbH, 2 mg/kg) supplemented by three additional doses of buprenorphine every 8 hours for pain management. Rats were housed in a 12:12 h light/dark cycle in standard plastic cages before SCI and afterwards in cages covered with soft paper bedding to prevent skin erosions and urine scalding. Pelleted food and drinking water were available *ad libitum*. Animals received daily examination with their bladders expressed three time a day until regaining full reflexive bladder control.

3.3.2 Experimental Groups and Treatments

Prior to surgeries, animals were randomly divided into three experimental groups: (1) un-injured control (2) SCI/vehicle control, which received vehicle solution used for rhNrg-1β1 delivery (0.1% bovine serum albumin, BSA, in 0.9% saline); (3) SCI/Nrg-1, which received rhNrg-1β1, 2μg/day systemically using subcutaneously implanted osmotic mini-pumps (Alzet® model #1003D for 3 days, model #2001 for 7 days, model #2002 for 14 days and model #2006 for 42 days). The dosage of rhNrg-1β1 (Shenandoah Biotechnology Inc., Cat # 100-46-50) was determined based on our previous study [13] where we found significant structural and functional recovery with a dose range of 0.5 μg to 1.5 μg per day of rhNrg-1β1 delivered intrathecally into the subarachnoid space. In this study, we delivered Nrg-1 systemically, thus the dose was increased to 2 μg/day to ensure adequate delivery of Nrg-1 to the spinal cord. Treatment was started approximately 30 minutes after SCI.

3.2.3 Flow Cytometric Assessment of Spinal Cord Immune Cells

At each end-point, animals were deeply anesthetized using a mixture of 40% isoflurane + 60% propylene glycol. Then, animals were sacrificed and spinal columns were excised and placed on dry ice for 5 minutes. The spinal cords were then exposed using laminectomy and 1.5 centimeter of tissue centering the epicenter of the injury was excised. Mechanical dissociation was performed in Hank's Balanced Salt Solution (HBSS) using fine scissors and tissue was retrieved by centrifugation at 1000 rpm for 1 minute at room temperature. Tissue particles were then enzymatically dissociated by incubating with 2.5 mg trypsin + 5 mg collagenase in 5 ml Dulbecco's Modified Eagle's Media (DMEM, 20 min, at 37℃). Following trituration, enzymatic reaction was stopped using 10 ml DMEM + 10% fetal bovine serum (FBS) and tissue mixture

was filtered through a 40 μm cell strainer. Cells were pelleted and reconstituted in 6 ml of HBSS and overlaid on OptiPrep® (Sigma-Aldrich, D1556) gradient and centrifuged at 1900 rpm for 15 minutes at 20℃ for separation of myelin debris from immune cells [16]. Supernatant, containing myelin and tissue debris was carefully discarded and cells were washed and re-suspended in 2.5 ml of HBSS. Cells were then incubated with RBC lysis buffer (Biolegend, 420301), washed and counted. A total of 7.5 million cells were harvested from each animal. For each antibody panel, 2 million cells per animal were used. Non-specific binding sites were blocked using 10% normal mouse serum for 30 min (Invitrogen, 10410). Cells were then incubated with antibody cocktail containing surface antibodies for each panel (listed in Table 1) for 30 min in dark at 4℃. Cells were then fixed using BD ^{TM} Cytofix Fixation Buffer for 15 min at 4^{o}C (BD, 554655).

For intracellular (cytokine) staining, following fixation, cells were incubated with permeabilizing buffer (0.1% Saponin + 10% FBS in HBSS) for 30 min and incubated with a cocktail of intracellular antibodies for 30 min in dark. For FoxP3 staining, after surface antibody staining and before fixation, cells were incubated with FoxP3 fix/perm buffer for 20 min and washed in FoxP3 perm buffer (FoxP3 fix/perm buffer set, Biolegend, 421403). Cells were then incubated with perm buffer for 15 min and incubated with cocktail of FoxP3 and IL-10 antibodies for 30 min. Finally, cells were washed with flow cytometry staining buffer (10% FBS in PBS) and reconstituted with 500 μl of this buffer and analyzed using BD FACS Canto II flow cytometer counting 200,000 events per sample. Compensation was done using single stained beads (OneComp eBeads, 501129031, eBioscience). For each antibody panel, specific isotype controls were used to account for non-specific antibody binding (flow cytometry antibodies and their isotype controls are listed in Table 1. Gating strategies for macrophages, T cells and B cells are

shown in Figures 3.1-3.4. Verification of antibody specificity is shown in supplementary figures 3.1-3.3).

3.2.4 Flow Cytometric Assessment of Blood Leukocytes

Upon anesthesia (described above) and before excising spinal cords, 1 ml of blood was collected through cardiac puncture in syringes coated with and containing 0.5 ml of 100 mM EDTA. Each 200 µl of whole blood was mixed with 2 ml of RBC lysis buffer and incubated for 5 min at room temperature (420301, Biolegend). Cells were centrifuged at 600 g for 10 min at 4℃, and the supernatant containing lysed RBCs was discarded. This procedure was repeated one or two more times until all RBCs were lysed and a clean pellet was obtained. Cells from each animal were then pooled together, washed twice in PBS and re-suspended in MACS buffer (PBS + 2 mM EDTA+ 0.5% BSA, PH=7.2-7.4). Leukocytes were then counted and underwent staining and flow cytometry procedures as described above.

3.2.5 Tissue Processing for Histological Studies

At each time-point, animals were deeply anesthetized (as described earlier) and were perfused transcardially with 2.5% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). Then the spinal tissues were post-fixed in 2.5% PFA and 10% sucrose in PBS overnight. Cryoprotection continued by incubation of tissues in 20% sucrose in PBS for 2 days. A 2-cm length of the spinal cord centered at the injury epicenter was excised and embedded (Tissue-Tek® O.C.T., Electron Microscopy Sciences). Serial cross sections (35 µm thickness) were cut by a cryostat (Leica Biosystems GmbH), mounted and cover-slipped on Superfrost® Plus Micro Slides (Fischer Scientific) and stored at -80℃ until immunostaining procedure.

Table 3.1: List of antibodies used for flow-cytometric and immunohistochemical assessment of immune cells.

Antibody	Color	Application	Cat number	Concentration
CD3	PerCP	Flow	eBioscience, 46-00-30-82	1:20
CD4	BV510	Flow, IHC	BD, 740138	1:20, (1:100 for IHC)
Ms IgG2a, k	BV510	Flow	BD, 563027	1:20
CD45	APC-Cy7	Flow	BD, 561586	1:20
Ms IgG1, k	APC-Cy7	Flow	BD, 557873	1:20
IFN-γ	FITC	Flow	BD, 559498	1:20
Ms IgG1, k	FITC	Flow	BD, 554679	1:20
IL-10	PE	Flow	BD, 555088	1:20
Ms IgG2b, k	PE	Flow	BD, 555058	1:20
FoxP3	APC	Flow, IHC	eBioscience, 17-5773-80	1:20, (1:100 for IHC)
Ms IgG2a, k	APC	Flow	eBioscience, 17-4724-42	1:20
CD68	FITC	Flow, IHC	Bio-Rad, MCA341F	1:20, (1:100 for IHC)
Ms IgG1	FITC	Flow	Bio-Rad, MCA1209F	1:20
CD163	PE	Flow, IHC	Bio-Rad, MCA342R	1:20, (1:100 for IHC)
Ms IgG1	PE	Flow	Bio-Rad, MCA1209PE	1:20
CD86	BV-421	Flow, IHC	BD, 743211	1:20, (1:100 for IHC)
Ms IgG1, k	BV-421	Flow	BD, 562438	1:20
IL-10	Alexa 647	Flow	BD, 562156	1:20
Ms IgG2b, k	Alexa 647	Flow	BD, 557903	1:20
CD45RA	APC-Cy7	Flow	BD, 561624	1:20
Armenian Hamster IgG isotype control	PE-Cy7	Flow	eBioscience, 25-4888-82	1:20
Anti Iba-1, Rabbit	Unconjugated	IHC	Wako, 019-19741	1:500
Anti CD3, Rabbit	Unconjugated	IHC	Abcam, GR295232-1	1:300
Anti IL-10, Mouse	Unconjugated	IHC	R&D, MAB519	1:300
Goat anti-Rabbit secondary Ab	Alexa Fluor® 647	IHC	Invitrogen, A21245	1:400
Goat anti-mouse secondary Ab	Alexa Fluor® 568	IHC	Invitrogen, A11031	1:400

3.2.6 Immunohistochemical Detection of Macrophages and Lymphocytes in The Injured Spinal Cord

We utilized the same flow cytometry antibodies for immunohistochemical staining of T cells, B cells and macrophages. Three tissue sections around 2 mm rostral or caudal to the epicenter were selected. Sections were washed twice with TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, PH 7.6) for 15 minutes at room temperature. Sections were blocked using 5% normal mouse serum in TBST for 1hr at room temperature and incubated with antibodies against cell surface markers for B cells (CD45RA), T cells (CD3) and macrophages (Iba-1, CD68, CD86, CD163),1:500 for Iba-1 and 1:100 for the rest of the antibodies over-night at 4℃. Sections were then washed twice with TBST and incubated with their corresponding intracellular (IL-10 for B cells, IL-10 and FoxP3 for T cells) antibodies. FoxP3 antibody was diluted in an antibody solution containing 1% BSA, 0.1% cold water fish skin gelatin, 0.5% Triton X-100 and 0.05% sodium azide in TBS. Images were taken at 20x and 63x using Axio Imager M2 fluorescent microscope (Zeiss) and were processed using ZEN software (Zeiss).

3.2.7 Immunohistochemical Assessment of IgG and IgM in the Injured Spinal Cord

Seven serial cross sections spanning 7 mm length of the injured spinal cord centered at the injury epicenter were selected with 1 mm interval. Sections were blocked for 1h with 5% mouse serum + 1% BSA + 0.3% Triton X-100 in PBS at room temperature, and then incubated with a mixture of anti-IgG (Invitrogen, #31470, 1:100) and anti-IgM (eBioscience, 14-0990-82, 1:100) antibodies overnight at 4℃. Sections were then washed 3 times with PBS and incubated with a mixture of Alexa Fluor® 568 donkey anti-goat (A1105, Invitrogen, 1:400) and Alexa Fluor® 647 goat anti-mouse (A21236, Invitrogen, 1:400) secondary antibodies for 1h at room temperature. Slides were

then washed with PBS three times and cover-slipped with Mowiol (Sigma) mounting medium. To avoid variability in our immunohistochemical studies, all samples were processed at the same time under the same conditions. Images were taken at 10X magnification from whole spinal cord section using AxioImager M2 fluorescence microscope (Zeiss) under consistent exposure time as described previously [11, 17]. Fluorescence intensity was measured using ImageJ analysis software (imagej.nih.gov). After subtracting the background automatically, the immunointensity above threshold was measured for entire spinal cord section excluding the cavities and dura matter. To eliminate the differences in the total size of the sections, the following formula was used: normalized immunointensity of tissue section = total immunointensity of tissue section x /total area of tissue section x.

3.2.8 RNA Extraction and Quantitative Real-Time PCR

At endpoints, spinal cords were dissected in ice-cold artificial cerebrospinal fluid (aCSF, containing 124 mM NaCl, 3 mM KCl, 1 mM NaHPO4, 26 mM NaHCO3, 1.5 mM MgSO4, 1.5 mM CaCl2, and 10 mM glucose). Five millimeters of the spinal cord centering the injury epicenter was excised and homogenized in TRIzol reagent (Invitrogen®). RNA was extracted using an RNeasy plus mini kit (Qiagen®) and first-strand cDNA was synthesized with 5X All-In-One RT MasterMix (ABM®). Real time qPCR reactions were conducted with the PowerUp™ CYBR® Green Master Mix (Applied Biosystems®) in an ABI7500 fast thermocycler (Applied Biosystems) as we described before [11]. Primer information is provided in Table 2.

3.2.9 Statistical Analysis

All statistical analyses were performed using SigmaStat Software. For distance analysis in

immunohistological assessments we used two-way analysis of variance (ANOVA) followed by Holm–Sidak post hoc test. One-way ANOVA followed by Holm–Sidak post hoc was used in all flow cytometry and real time qPCR analyses. The data was reported as mean ± standard error of the mean (SEM) and $p \leq 0.05$ was considered as statistically significant. Proper randomization and blinding was employed in all assessments.

Table 3.2. List of primers used in this study.

Rat-IFN-g-F: ATT CAT GAG CAT CGC CAA GTT C **Rat-IFN-g-R:** TGA CAG CTG GTG AAT CAC TCT GAT
Rat-IL6-F: TAG TCC TTC CTA CCC CAA CTT CC **Rat-IL6 R:** TTG GTC CTT AGC CAC TCC TTC
Rat-CCL11-F: TGC TGC TTG AAC ACC TTG GA **Rat-CCL11-R:** AGC CTG AAT ATT ACA GCT GGG T
Rat-CCL5-F: GCA GTC GTC TTT GTC ACT CG **Rat-CCL5-R:** ATC CCC AGC TGG TTA GGA CT
Rat-IL10-F: TGC GAC GCT GTC ATC GAT TT **Rat-IL10-R:** GTA GAT GCC GGG TGG TTC AA
Rat-CXCL1-F: CAA TGA GCT GCG CTG TCA GT **Rat-CXCL1-R:** TTG AAG TGA ATC CCT GCC ACT
Rat-CXCL2-F: AGG GTA CAG GGG TTG TTG TG **Rat-CXCL2-R:** CGA TCC TCT GAA CCA AGG GG
Rat-CXCL3-F: ACA TCC AGA GCT TGA CGG TG **Rat-CXCL3-R:** TTG GAT GGA TCG CTG CTC TG
Rat-CXCL10-F: CCG CAT GTT GAG ATC ATT GCC **Rat-CXCL10-R:** CTA GCC GCA CAC TGG GTA AA
Rat-NFKBIZ-F: GTG GAG GCG AAG GAT CGT AA **Rat-NFKBIZ-R:** CAT CCA ACT GTG TCA CCC GA

3.3 Results

3.3.1 Systemic Administration of Neuregulin-1 Alters the Population of M1 And M2 Macrophages Following SCI

Monocyte derived macrophages play a central role in the inflammatory response following SCI and contribute to both secondary injury mechanisms and repair processes owing to their

phenotypic diversity [18]. We previously demonstrated that intrathecal infusion of Nrg-1 reduces the tissue level of pro-inflammatory cytokines in rat compressive SCI while increases IL-10 levels [13]. Here, we delivered Nrg-1 systemically and investigated the recruitment and phenotype of monocyte derived macrophages in the injured spinal cord. Our flow cytometric assessment of un-injured spinal cord tissue showed minimal or undetectable number of macrophages ($CD45^+CD68^+$). Our SCI temporal analysis at 3, 7, 14, and 42 days post-injury, representing acute, subacute and chronic stages of injury, showed infiltration of $CD45^+CD68^+$ macrophages to the lesion as expected. We found a significant 13.6 and 184-fold increase in $CD45^+CD68^+$ cell population at 3 and 7 days post-injury, respectively (Fig. 3.1C). While remained significantly higher than un-injured control animals, the number of macrophages was decreased to a lower level at 14 days post-injury and onwards up to 42 days, the latest examined time-point (Fig 3.1C-F). Nrg-1 treatment significantly increased the population of infiltrated macrophages at 3 days post-injury (1.9 folds) with no significant effect on their population at 7 and 14-day time-points. However, chronically at 42 days post-SCI, Nrg-1 treatment led to a significantly higher number of $CD45^+CD68^+$ macrophages in the spinal cord compared to vehicle treatment (1.34 times).

We next performed immunophenotyping to determine whether Nrg-1 alters subpopulations of macrophages in the injured spinal cord. Our analysis of pro-inflammatory (M1) macrophages ($CD45^+CD68^+CD86^+$) comparing un-injured and vehicle treated injured rats showed an overall increase in M1 population at all examined time-points following SCI. This increase was statistically significant at 7, 14 and 42 days post-SCI ($P<0.01$) (Fig 3.1C-F). Compared to the baseline of SCI in vehicle treated groups, Nrg-1 treatment had no apparent effect on the population of M1 macrophages at 3, 7 and 14-day time-points. However, the population of M1 macrophages were significantly increased (1.6-fold) in Nrg-1 treated animals at the chronic 42-day time-point

compared to vehicle group ($P<0.01$) (Fig 3.1F). Our phenotypic analysis revealed that Nrg-1 significantly increased the number of immunomodulatory ($CD45^+CD68^+CD163^+$) M2 macrophages (3-fold) as well as their IL-10 expressing sub-population ($CD45^+CD68^+CD163^+IL\text{-}10^+$, 4.1-fold) at 3-day time-point. Moreover, we observed an increasing trend in the number of M2 macrophages at 7, 14 and 42-day time-points following Nrg-1 treatment, although these changes were not statistically significant. Using immunohistochemistry, we verified and confirmed the presence of M1 ($CD68^+CD86^+$) and M2 ($CD68^+CD163^+$) macrophages in the perilesional area in the injured spinal cord (Fig. 3.1G). Altogether, our results indicate that systemic Nrg-1 treatment following SCI, augments the recruitment of blood-borne macrophages to the spinal cord tissue at acute and chronic stages with an increase in M2 phenotype at acute (3-day) and M1 phenotype during chronic (42-day) stage of SCI (Fig. 3.1C, F, verification of antibody specificity and gating strategy are shown in spp. Fig. 3.1).

Figure 3.1. Nrg-1 treatment alters M1 and M2 macrophage populations after SCI.

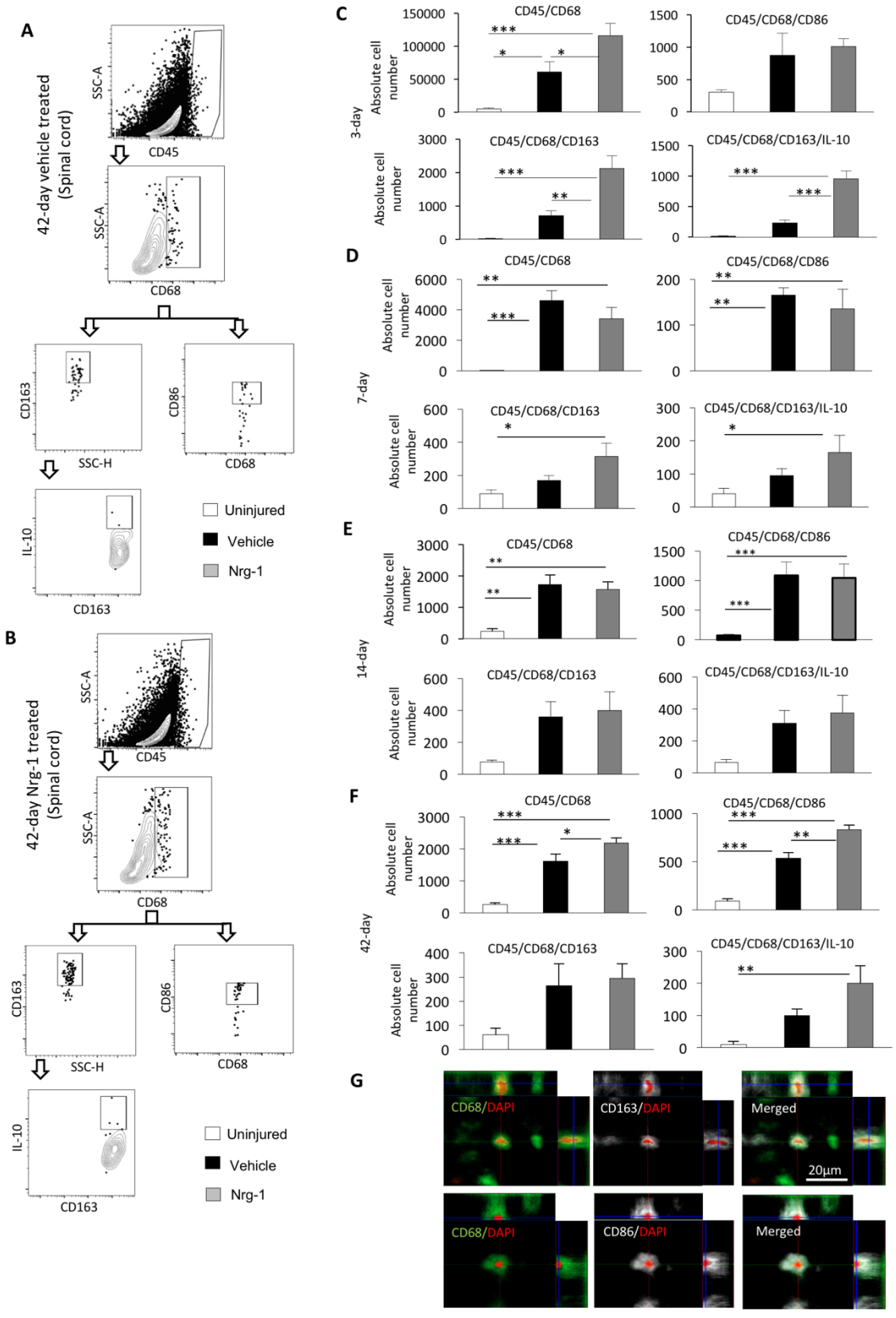


Figure 3.1. Nrg-1 treatment alters M1 and M2 macrophage populations after SCI.

(A, B) Representative images of the gating strategy for flow cytometry are provided for infiltrated spinal cord macrophages at 42-day post-injury under each treatment group. **(C)** Flow cytometric analysis 3 days post-SCI showed a robust increase in the number of macrophages ($CD45^+CD68^+$) in the injured spinal cord. Nrg-1 treated animals demonstrated significantly higher number of macrophages compared to the vehicle treated group. Phenotypical analysis of macrophages at 3-day time-point showed no significant difference in the number of infiltrated M1 macrophages ($CD45^+CD68^+CD86^+$) between vehicle and Nrg-1 treated groups. However, Nrg-1 treated SCI animals had a significantly higher population of M2 macrophages ($CD45^+CD68^+CD163^+$ and $CD45^+CD68^+CD163^+IL\text{-}10^+$) in the spinal cord. **(D, E)** At 7 and 14 days post-SCI, the total number of tissue macrophages and their M1 subpopulation was significantly higher in the injured animals compared to un-injured group, while M2 macrophage population remained unaltered. There was also no significant difference in the total number of macrophages and their M1 or M2 phenotype between the vehicle and Nrg-1 treated groups. **(F)** At 42 days post-SCI, the number of infiltrated macrophages was 30 times less than the acute 3-day time-point in SCI baseline condition but still significantly higher than un-injured animals. Compared to both vehicle and un-injured rats Nrg-1 treated animals showed a significantly higher number of M1 macrophages. There was no significant difference in the number of M2 macrophages between the vehicle and Nrg-1 treatment groups at this time-point, although Nrg-1 treated rats had a significantly higher number of M2 macrophages as compared to un-injured animals. **(G)** Immunohistochemical analysis verified the presence of M1 ($CD68^+CD86^+$) and M2 ($CD68^+CD163^+$) macrophages in the perilesional area (N=5/group/time-point, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Holm-Sidak post-hoc test).

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

3.3.2 Neuregulin-1 Promotes a Regulatory T cell Response in the Blood and Spinal Cord following SCI

We next studied how systemic Nrg-1 infusion affects T cell response in peripheral blood and within the injured spinal cord. T cells orchestrate the adaptive immune response following SCI [19] with differential roles through their diverse production of pro- and anti-inflammatory cytokines and chemokines [20]. Using the same time-points described above, our flow cytometric analysis of spinal cord tissue in baseline vehicle SCI rats showed an overall significant increase in the population of infiltrated helper T cells ($CD3^+CD4^+$) in the injured spinal cord at 3, 7, 14 and 42 days post injury by 7.7, 16.3, 4.5 and 13.5 fold, respectively ($P<0.01$ at 3-day and $P<0.001$ at all later time-points). Interestingly, while $CD3^+CD4^+$ helper T cells were significantly present at all stages of SCI, we found a decline in their number subacutely on day 14 post-injury. However, this decline was followed by an increase in their population reaching a maximum at the chronic stage of SCI on day 42 (13.5 times increase) ($P<0.001$) (Fig 3.2 C-F). The biphasic baseline pattern of T cell recruitment after SCI is in agreement with previous studies [1]. Nrg-1 treatment had no apparent effect on the population of helper T cells in acute and subacute SCI, whereas it significantly reduced their recruitment in the chronic SCI at the 42-day time-point.

We next performed immunophenotypic analysis within the $CD3^+CD4^+$ helper T cell population to determine whether Nrg-1 treatment can alter T cell subsets in the injured spinal cord. Our analysis showed that SCI promotes T regulatory (T_{reg}) ($CD3^+CD4^+FoxP3^+$ and $CD3^+CD4^+IL\text{-}10^+$) subpopulation after SCI. Nrg-1 treated SCI animals had a significantly higher population of T_{reg} cells at 3 days (3.5 folds, $P<0.01$), 14 days (5.6 folds, $p<0.01$) and 42 days (1.8 folds, $P<0.05$) time-points compared to vehicle treated (Fig. 3.2C-F). Analysis of T effector (T_{eff}) cell subpopulation ($CD3^+CD4^+IFN\text{-}\gamma^+$) also showed a remarkable positive effect for Nrg-1 in

modulating T cell response in subacute SCI. At 7 days post injury, compared to un-injured group, SCI induced a significant increase in T_{eff} cells within the injured spinal cord of vehicle treated rats (100-fold, $P<0.001$) which was significantly reduced by Nrg-1 treatment to nearly 50% (Fig. 3.2D). At other time-points, no significant change in T_{eff} cell subpopulation was detected under Nrg-1 treatment (data not shown). Our immunohistochemical assessments verified the presence of $CD3^+FoxP3^+IL10^+$ T_{reg} cells in the perilesional area of the injured spinal cord (Fig. 3.2G).

We further investigated the systemic effects of Nrg-1 on T cell recruitment and phenotype in the blood following SCI by flow cytometric analysis (Fig. 3.3). We observed an increasing trend in the population of helper T cells ($CD3^+CD4^+$) in the blood of SCI animals. However, this increase was not statistically significant at any of the examined time-points and Nrg-1 treatment had no apparent effect on the population of helper T cells after SCI (Fig. 3.3B-D). Our analysis of $CD3^+CD4^+FoxP3^+IL10^+$ cells also showed no change in the population of Treg cells in the blood under the baseline of SCI. While Nrg-1 treatment had no noticeable effect on T_{reg} cell population in the blood at 3 and 14 days post-SCI, it stimulated a significant (3.7-fold) increase in the number of IL-10 expressing T_{reg} cells ($CD3^+CD4^+FoxP3^+IL\text{-}10^+$) chronically at 42 days post-injury compared to their vehicle treated counterparts (Fig. 3.3D, verification of antibody specificity and gating strategy are shown in spp. Fig. 3.2). In conclusions, our analysis of T lymphocyte response demonstrates the ability of Nrg-1 to promote the population of immune-modulatory T_{reg} cells in the blood and spinal cord after SCI.

Figure 3.2. Nrg-1 promotes T_{reg} cell response in the injured spinal cord.

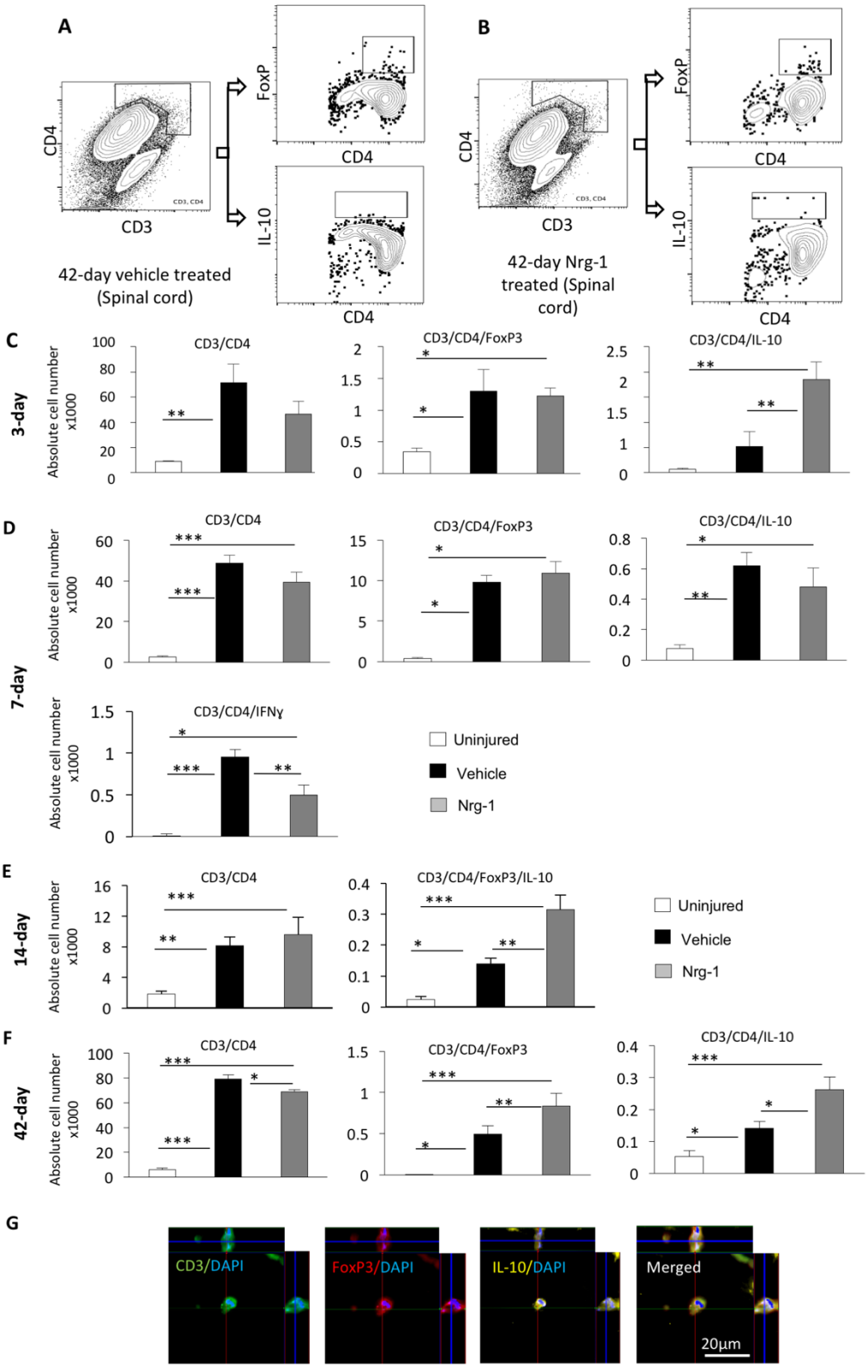


Figure 3.2. Nrg-1 promotes T_{reg} cell response in the injured spinal cord.

(A, B) Representative images of the gating strategy for flow cytometry after singlet selection are provided for vehicle and Nrg-1 treated groups. **(C)** At 3-day post-injury, the number of $CD3^+CD4^+$ T cells in the spinal cord was significantly higher in vehicle treated animals compared to uninjured control group. There was no significant difference in the population of infiltrated helper T cells and the number of $FoxP3^+$ T_{reg} cells ($CD3^+CD4^+FoxP3^+$) between vehicle and Nrg-1 treated groups. However, population of IL-10 producing $CD4^+$ T cells ($CD3^+CD4^+IL\text{-}10^+$) was significantly increased in Nrg-1 treated animals in comparison to vehicle treated group. **(D)** At 7 days post-SCI, despite no significant difference in the total helper and regulatory T cell populations between vehicle and Nrg-1 treated groups, a significant reduction (1.9-fold) was observed in IFNγ producing effector T cell population in Nrg-1 treated SCI rats compared to their vehicle treated counterparts. **(E)** At 14 days post-SCI, infiltrated helper T cells reached their lowest level among all examined time-points, and Nrg-1 treatment had no significant effect on total helper T cell population. IL-10 expressing T_{reg} cells were significantly higher in both vehcile and Nrg-1 injured rats compared to un-injured animals. Nrg-1 treated animals showed a significantly higher number of T_{reg} cells in their spinal cord at 14 day time-point compared to vehcile treated rats. **(F)** However, At chronic (42-day) time-point, the number of T helper cells reached a maximum, with Nrg-1 treated animals harboring a significantly decreased population of $CD4^+$ T-cells in their spinal cord compared to vehicle treated group. Most importantly, $CD3^+CD4^+FoxP3^+$ and $CD3^+CD4^+IL\text{-}10^+$ regulatory T cell populations were significantly increased in Nrg-1 treated groups. **(G)** Immunohistochemical images shows the presence of T_{reg} cells in the perilesional area (N=5/group/time-point, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Holm-Sidak post-hoc test).

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

Figure 3.3. Nrg-1 treatment increases the number of circulating T_{reg} cells in the blood following chronic SCI.

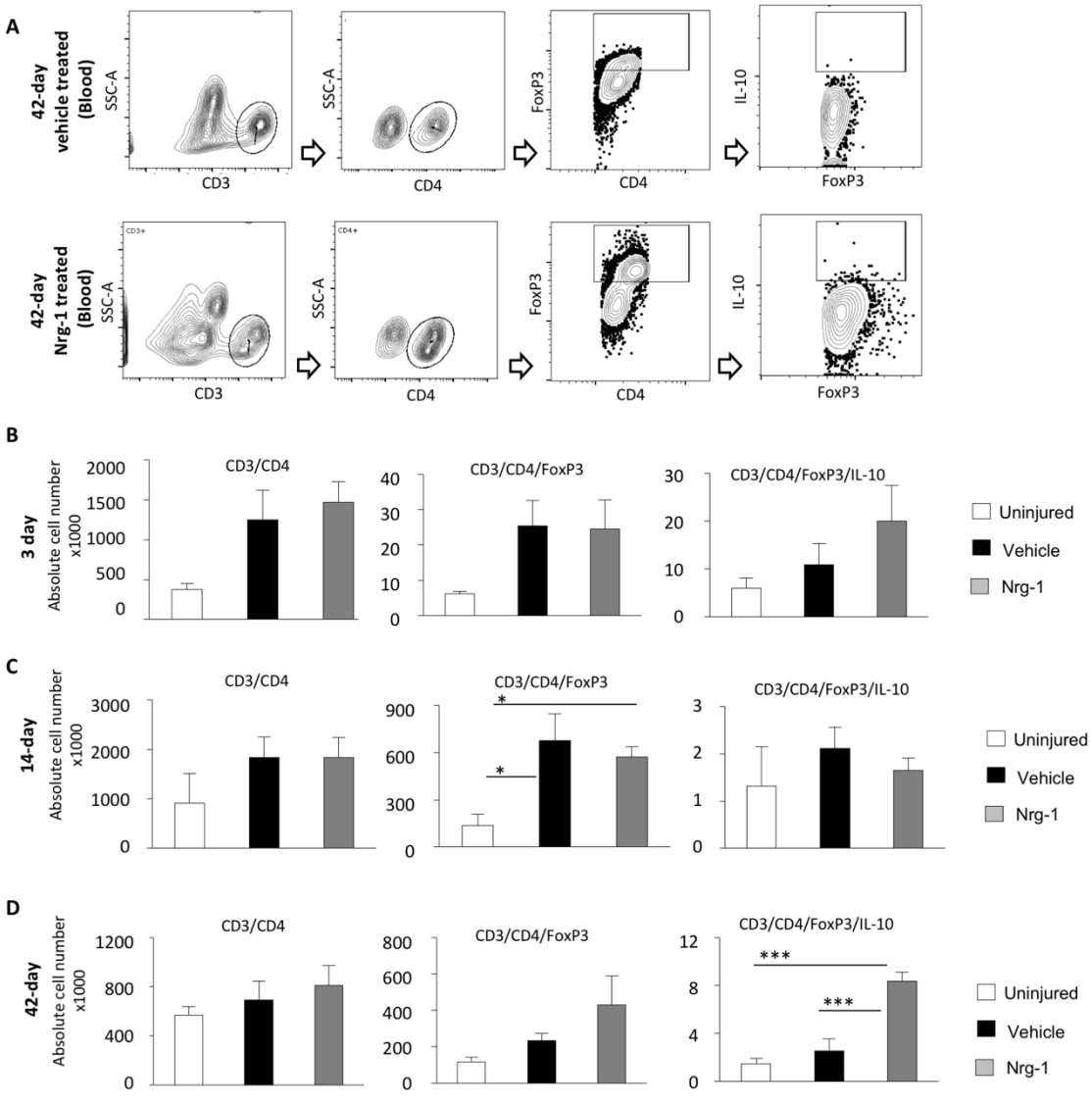


Figure 3.3. Nrg-1 treatment increases the number of circulating T_{reg} cells in the blood following chronic SCI. (A) Representative images are provided for the gating strategy for flow cytometry detection of T cells and their regulatory phenotype in the blood. **(B- D)** Flow cytometric analysis showed a slight increase in total CD3+CD4+ helper T cell population upon injury. However, at all examined time-points, the total population of helper T cells was not significantly different among any of the studied groups (P>0.05). Population of $FoxP3^+$ helper T cells was significantly increased at 14 days post-injury in the vehicle and Nrg-1 treated rats as compared to the un-injured levels while there was no difference between the two injured groups. **(D)** At 42-day time-point, Nrg-1 treatment significantly increased the number of IL-10 expressing ($CD3^+CD4^+FoxP3^+IL\text{-}10^+$) T_{reg} cells compared to vehicle and un-injured groups while there was no significant difference in the total population of helper and Fox3+ T cells between the vehicle and un-injured groups, (N=5/group/time-point, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Holm-Sidak post-hoc test).

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

3.3.3 Nrg-1 Treatment Is Associated with an Increase in Regulatory B cell Phenotype following SCI

B cells contribute to SCI secondary tissue damage after SCI by producing cytokines and autoantibodies [10, 21]. Despite their detrimental roles in tissue degeneration, B cells have the ability to positively modulate immune response through adopting an IL-10 producing immune regulatory (B_{reg}) phenotype [22]. Our flow cytometric analysis showed that SCI triggers the recruitment of B cells to the spinal cord. We found a significant increase in the total number of $CD45RA^+$ B cells in the vehicle treated injured spinal cord at all examined time-points compared to un-injured group (5.4-fold at 7-day, 2.4-fold at 14-day and 61-fold at 42-day) ($P<0.01$) (Fig 3.4 B-D). While Nrg-1 treatment did not alter B cell infiltration at the subacute 7-day and 14-day time-points, it significantly induced B cell recruitment to the injured spinal cord chronically at the 42-day time-point (2.1-fold, $P<0.001$) compared to vehicle treated group (Fig 3.4. D). Analysis of B_{reg} cells subpopulation ($CD45RA^+IL\text{-}10^+$) in spinal cord tissue showed the ability of Nrg-1 treatment to significantly promote IL-10 producing B_{reg} population at 14 days (2 folds) and 42 days (1.6 folds) post-SCI ($P<0.05$) compared to vehicle treatment (Fig. 3.4C, D). Presence of $CD45RA^+IL10^+$ cells in perilesional area of the injured spinal cord was verified in our immunohistochemical analysis (Fig. 3.4E). Interestingly, our flow cytometric analysis showed a significant 3-fold reduction in $CD45RA^+$ B cell population in the blood at 7-day post-SCI in both vehicle and Nrg-1 treated SCI rats compared to un-injured control group ($P<0.001$), which was recovered to the normal baseline by the 14-day time-point (Fig 3.4G, H). This observation is in line with other studies that reported a transient cessation of B lymphopoiesis in the bone marrow following SCI [23]. Nonetheless, there was no significant difference in the number of circulating B_{reg} cells in the blood between vehicle and Nrg-1 treated animals at any examined time-point (Fig

3.4G-I, verification of antibody specificity and gating strategy are shown in spp. Fig. 3.3). Altogether, our data show that systemic administration of Nrg-1 induces a B_{reg} cell response following SCI only at the level of spinal cord tissue without affecting the phenotype of circulating B cells.

Figure 3.4. Nrg-1 treatment promotes B_{reg} cell population following SCI.

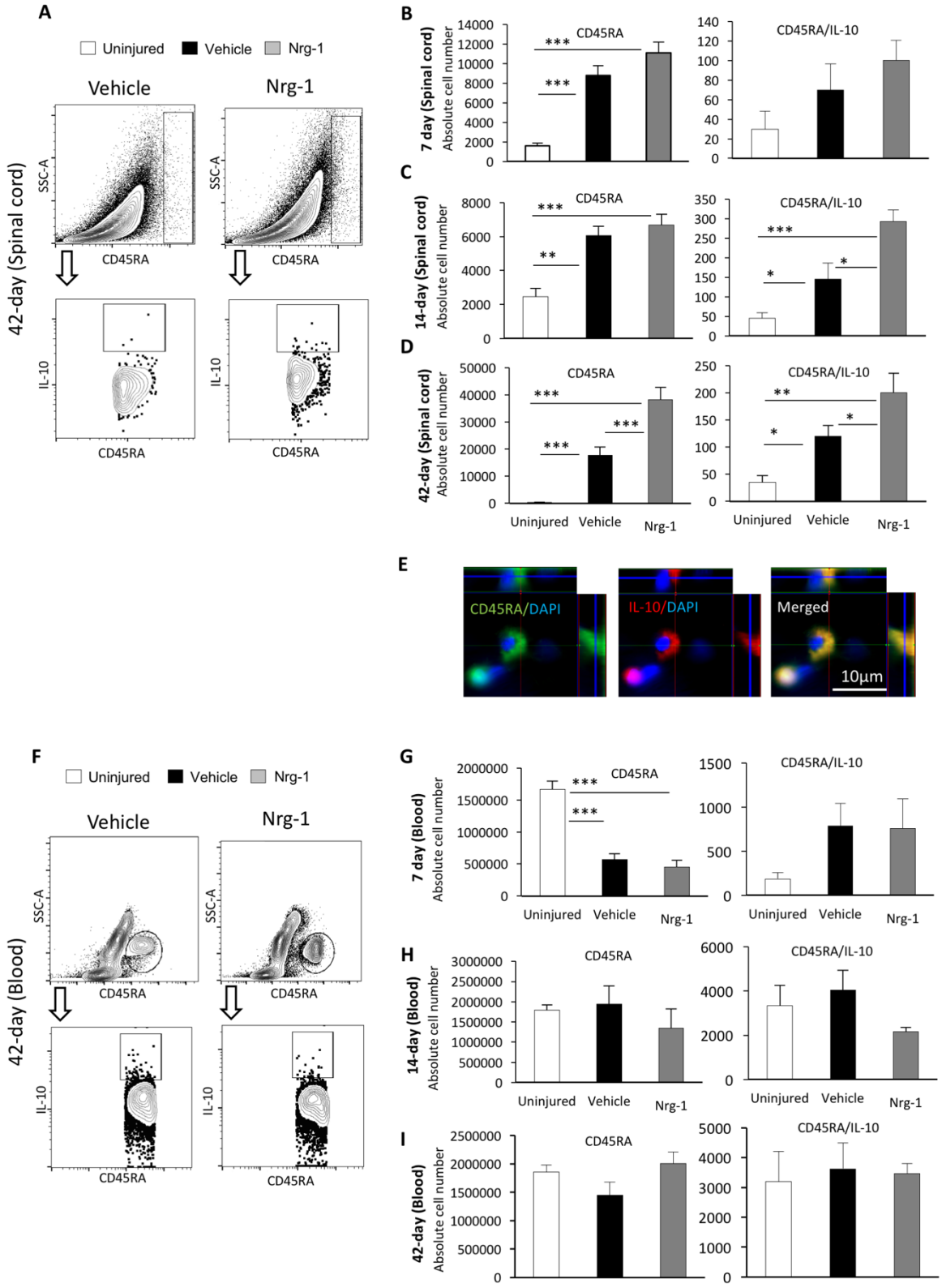


Figure 3.4. Nrg-1 treatment promotes B_{reg} cell population following SCI.

(A) Representative images of the gating strategy for flow cytometry of spinal cord are provided. **(B)** At 7 days post-injury, the number of $CD45RA^+$ B cells was significantly increased in the spinal cord without any significant difference in the total and regulatory B cell populations between vehicle and Nrg-1 treated groups. **(C)** At 14-day post-SCI, a significant increase in the number of B_{reg} cells was observed in Nrg-1 treated animals compared to vehicle treated group. **(D)** Chronically at 42 days post-SCI, the number of B cells in the spinal cord reached the highest level compared to all earlier time-points. Nrg-1 treatment resulted in a significant increase in the number of infiltrated B cells in the spinal cord and promoted IL-10 expressing B_{reg} cells compared to vehicle treatment. **(E)** Immunohistochemical analysis verified the presence of B_{reg} cells in in the perilesional area of the injured spinal cord. **(F)** Representative images of the gating strategy for flow cytometry of blood are provided. **(G)** Analysis of blood revealed a significant decline in B cell population at 7 days post-SCI without any significant change in the B_{reg} cell population at this time-point. **(H, I)** No significant change in total and regulatory B cell populations were observed in the blood at 14-day and 42-day time-points. (N=5/group/time-point, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA followed by Holm- Sidak post-hoc test)

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

3.3.4 Nrg-1 Treatment Positively Modulates Inflammatory Cytokine Expression following SCI

We previously showed that intrathecal infusion of Nrg-1 reduces the level TNF-α and IL-1β in the injured spinal cord [13]. Here, we extended these initial findings to investigate the effect of systemic Nrg-1 treatment on the expression of several key inflammatory cytokines involved in the regulation of immune response following SCI including IFN-γ, IL-6, IL-12 and IL-10 [24-27]. Our real time qPCR analysis of IFN-γ expression, a pro-inflammatory cytokine involved in M1 macrophage activation and cytotoxic T cell proliferation [28, 29], showed a significant 6.5 and 12 folds increase at 7-day and 42-day time-points, respectively, in vehicle treated rats as compared to un-injured group (Fig. 3.5A). Interestingly, Nrg-1 treatment significantly attenuated IFN-γ expression subacutely at 7 days post-SCI by 47% compared to vehicle treatment with no apparent effect at the 42-day chronic time-point ($P<0.01$) (Fig. 3.5A). Next, we assessed the expression of the pro-inflammatory cytokine IL-6 which is known to induce cellular injury and tissue degeneration following SCI [30]. IL-6 expression was transiently and significantly increased (5.1-fold) at 3 days after injury in vehicle treated animals compared to un-injured control group and returned to its normal baseline levels at 7 days post-SCI and onwards (Fig. 3.5B). Nrg-1 treated animals showed a significant 2.4-fold reduction in IL-6 expression in their injured spinal cord at acute 3-day time-point ($P<0.05$) (Fig. 3.5B). There was no significant difference in IL-6 expression between vehicle and Nrg-1 treated animals at any later time-point ($P>0.05$) (Fig. 3.5 B). Expression of IL-12A, a pro-inflammatory cytokine involved in microglia/macrophage activation with both beneficial and detrimental roles [26, 31], was also significantly elevated at subacute 7-day post-injury (2.4 fold) which remained upregulated up to the chronic 42-day time-point (2.7

fold) ($P<0.05$) (Fig. 3.5C). Nrg-1 treatment was not associated with any significant change in IL-12A expression at any stage after SCI (Fig. 3.5C).

Given Nrg-1 promoted an IL-10 regulatory phenotype in macrophages and lymphocytes, we also assessed IL-10 transcript expression. Seven days after injury, the expression of IL-10 increased significantly (8.3-fold) in the injured spinal cord (Fig. 3.5D). In agreement with our flow cytometry data, Nrg-1 treatment resulted in a significantly higher (2.6-fold) expression of IL-10 compared to vehicle treatment (Fig. 3.5E). Change in IL-10 expression was more pronounced in subacute stage of SCI (Fig. 3.5 D).

Finally, we investigated the expression of NFκB inhibitor zeta (NFkBIZ or IκBζ), an atypical IκB which is known to be an essential activator of IL-6 expression and acts as a transcription co-activator of pro-inflammatory cytokines such as IL-12 [32, 33]. The expression of NFκBIZ was significantly increased at 3 and 7 days post-injury (9 and 6.6-fold respectively) and reached its maximum at 42-day time-point (14.9-fold) (Fig. 3.5E). This elevation was reduced significantly (45%) by Nrg-1 treatment chronically at 42 days post injury (Fig 3.5E). NFκBIZ expression was also reduced by Nrg-1 treatment acutely, however, the effect was not statistically significant. Altogether, these results show a positive role for Nrg-1 in regulating inflammatory cytokine profile of the injured spinal cord tissue which seemed to be modulated, at least in part, by NFκB pathway following SCI.

Figure 3.5. Nrg-1 treatment regulates inflammatory cytokines in the injured spinal cord.

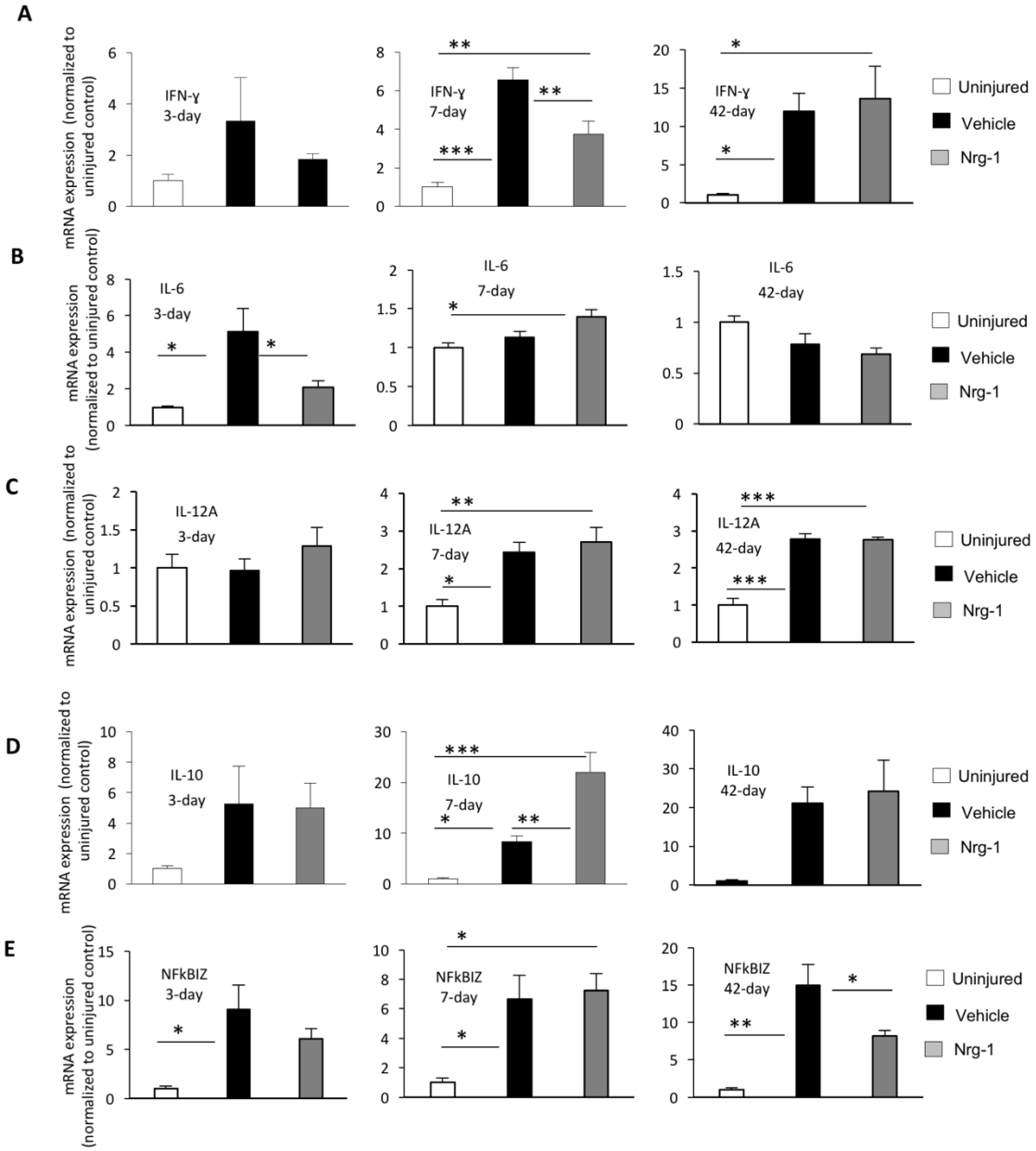


Figure 3.5. Nrg-1 treatment regulates inflammatory cytokines in the injured spinal cord.

(A) Transcript analysis of spinal cord tissue using real time qPCR released a significant increase in expression of IFN-γ at 7-day post-injury ($P<0.001$). Nrg-1 treatment significantly decreased the SCI-induced expression of IFN-γ in the injured spinal cord tissue. **(B)** Expression of IL-6 was significantly increased at 3 days post-injury, which was significantly attenuated by Nrg-1 treatment ($P<0.05$). No significant difference in IL-6 expression was observed between vehicle and Nrg-1 treated groups at 7-day and 42-day time-points. **(C)** IL-12A was significantly increased at 7- days post-injury and remained significantly elevated until 42 days. Nrg-1 had no significant effect on IL-12A expression at any examined time-point. **(D)** Transcript levels of IL-10 was significantly increased at 7 days post-injury. Interestingly, Nrg-1 treated animals showed a significantly higher expression of IL-10 compared to vehicle group at is time-point. No significant change in IL-10 expression was observed at any other examined time-points. **(E)** NFkBIZ transcript level was significantly elevated at 3 days and reached its maximum at 42 days post-injury. Nrg-1 treatment significantly attenuated NFkBIZ expression chronically at 42 days post-SCI (N=4/group/time-point, *$P<0.05$, **$P<0.01$, ***$P<0.001$, One-way ANOVA followed by Holm- Sidak post-hoc test).

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

3.3.5 Pro-regenerative Modulation of Chemokine Expression by Nrg-1 Treatment following SCI

In addition to cytokine assessment, we sought to determine the effect of systemic Nrg-1 treatment on chemokine profile in the spinal cord tissue. We focused on C-C motif ligands (CCL) 5, 11 and C-X-C motif ligands (CXCL) 1, 2, 3, 10 that are involved in the pathophysiology of SCI [34-39].

3.3.5.1 Effects of Nrg-1 on the Expression of CCL 5 and 11

C-C motif chemokine 5 (CCL5 or RANTES) is produced by resident glial and peripherally recruited immune cells including T cells, macrophages and astrocytes and is known to induce neuroinflammation by attracting leukocytes to the site of injury [40, 41]. Increased CCL5 level has been associated with microvascular dysfunction and tissue damage following CNS injury [42]. At 3-day post-SCI, the expression of CCL5 was increased (Fig. 3.6A). However, this increase was not statistically significant and Nrg-1 treatment had no apparent effect on CCL5 expression acutely. At 7 and 42 days post-SCI, CCL5 expression in the spinal cord was remarkably increased by 12 and 16 folds, respectively, compared to un-injured animals ($P<0.01$). Nrg-1 treated animals demonstrated a significant 47% reduction in CCL5 expression at 7 days post-SCI ($P<0.05$) with an insignificant 24% decline at 42-day chronic time-point (Fig. 3.6A).

CCL11 or eotaxin-1 is a small chemokine that is mainly produced by macrophages and neurons with positive effects in CNS injury including tightening the blood brain barrier and fostering a Th2 immunomodulatory response [39]. CCL11 is also considered a pro-regenerative chemokine by promoting proliferation and migration of neural progenitor cells [43]. Our qPCR analysis of spinal cord tissue at 3 and 7-day time-points in both vehicle and Nrg-1 treated animals showed no significant change in CCL11 expression (Fig. 3.6B). However, at the chronic 42-day time-point,

SCI baseline expression of CCL11 reached a significant 6.8-fold increase compared to the un-injured control group (Fig. 3.6B). Interestingly, Nrg-1 treatment induced a significant 37% increase in CCL11 expression compared to vehicle treatment (Fig. 3.6B).

3.3.5.2 Effects of Systemic Nrg-1 Treatment on the Expression of CXC Family of Chemokines

CXCL1, 2, 3, 10 play a substantial role in SCI neuroinflammation and induction of neuropathic pain [36, 44]. CXCL1 and CXCL2 are produced by mast cells and macrophages and control neutrophil recruitment during tissue inflammation [45]. Using qPCR, we observed a significant increase in the expression of CXCL1 and CXCL2 at 3 days post-injury (10 and 16 folds respectively). Nrg-1 treatment significantly reduced the expression of both chemokines acutely (74% and 64% decrease respectively) (Fig. 3.6C, D). There was no significant change in the expression of CXCL1 at subacute and chronic time-points. However, expression level of CXCL2 was significantly increased at the 42-day chronic time-point compared to un-injured control group (8.5-fold), although Nrg-1 treated animals showed no significant change in the expression of CXCL2 compared to vehicle treated group at this time-point (Fig. 3.6D). Next, we assessed the expression of CXCL3, which is a ligand for CXC chemokine receptor (CXCR) 2 [34]. It has been shown that inhibition of CXCR2 attenuates neuroinflammation and improves tissue preservation following SCI [34]. CXCL3 expression was increased significantly at the acute 3-day (3.9-fold) and chronic 42-day (15.2-fold) post-injury (Fig. 3.6E). Nrg-1 treatment remarkably decreased (96%) the expression of CXCL3 at the 42-day time-point.

CXCL10 is another member of CXC family of chemokines which is known for its distinct role in T cell recruitment and exacerbation of secondary tissue degeneration following SCI [35]. Neutralization of CXCL10 has been associated with better structural and functional outcomes

following SCI [35]. Following injury, expression of CXCL10 was increased as early as 3 days post-SCI. However, this increase was only statistically significant at the chronic 42-day post-injury (34-fold increase) compared to un-injured group (Fig. 3.6F). Similar to the other examined CXCL chemokines, Nrg-1 treated animals showed a significant 36% decrease in CXCL10 expression chronically (Fig. 3.6F). Taken together, these results demonstrate that Nrg-1 treatment can positively modulate the repertoire of cytokines and chemokines in the spinal cord tissue following SCI. Importantly, our expression data, in agreement with our flow cytometry data, indicate a more prominent role for Nrg-1 in modulating neuroinflammation at the chronic stage of SCI.

Figure 3.6. Nrg-1 treatment positively modulates chemokine expression following SCI.

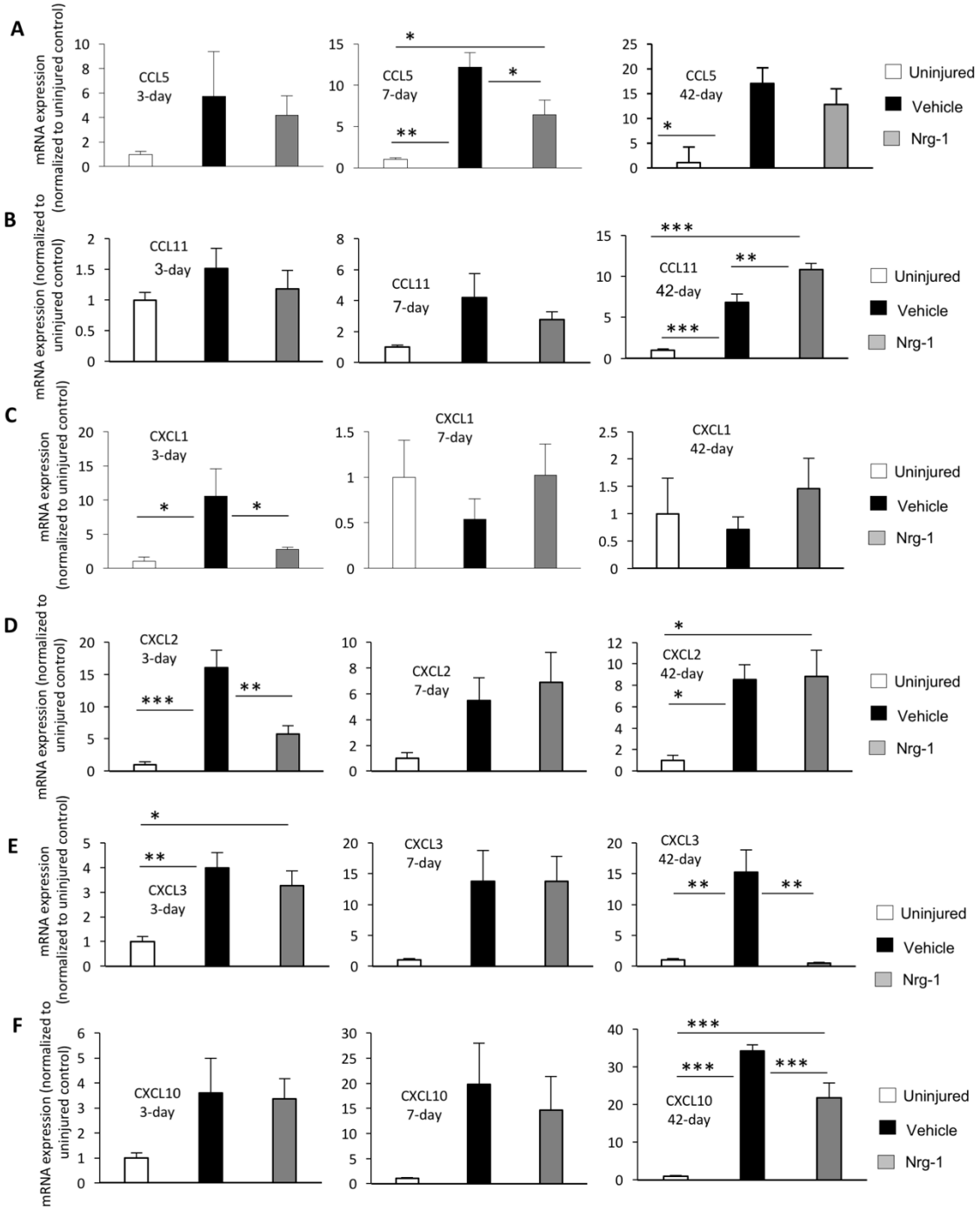


Figure 3.6. Nrg-1 treatment positively modulates chemokine expression following SCI.

(A) Analysis of chemokine expression in spinal cord tissue was performed using real time qPCR. There was a significant increase in the expression of pro-inflammatory chemokine CCL5, at 7-day post-injury, which was significantly reduced by Nrg-1 treatment. CCL5 mRNA level reached its maximum level at 42 days post-injury. However, no significant difference was found between vehicle and Nrg-1 treated animals at this time-point. **(B)** Although the expression of CCL11, an immune-modulatory chemokine, was not significantly changed at 3 and 7 days post-injury, it underwent a significant increase chronically at 42-day time-point. Nrg-1 treatment was able to significantly increase CCL11 expression compared to vehicle treated group. **(C, D)** Expression of pro-inflammatory chemokines CXCL1 and CXCL2 was significantly elevated at 3-day post-injury. Nrg-1 treatment significantly reduced CXCL1 and CXCL2 expressions at this time-point. The overall tissue level of CXCL1 mRNA reached baseline levels at 7-day post-injury and remained stable until 42 days post-injury. CXCL2 expression however, remained elevated at 7 and 42 days post-injury compared to uninjured group (significant 8.5-fold increase at 42-day). Nrg-1 treatment had no significant effect on CXCL2 expression at 7 and 42-day time-points. **(E)** Expression of the pro-inflammatory chemokine CXCL3was significantly upregulated 3 days after injury and reached its maximum levels chronically at 42 days post-SCI ($P<0.01$). Nrg-1 treated animals showed a significant reduction in CXCL3 expression at 42-day time-point (31-times). **(F)** CXCL10 expression was not significantly changed following injury at 3 and 7 days post-SCI, while it was significantly increased in vehicle treated group compared to un-injured animals at 42 days. Nrg-1 significantly decreased CXCL10 expression at this time-point ($P<0.001$) (N=4/group/time-point, *$P<0.05$, **$P<0.01$, ***$P<0.001$, One-way ANOVA followed by Holm- Sidak post-hoc test).

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

3.3.6 Systemic Nrg-1 Treatment Decreases IgM and IgG Deposition in the Injured Spinal Cord Acutely

Following SCI, autoantibodies are produced by B cells against spinal cord neoepitopes and contribute to neuroinflammation and secondary tissue damage [21]. Since we identified a role for Nrg-1 in regulating B cell response in SCI, we sought to study antibody deposition in the injured spinal cord. We performed immunohistochemical analysis of IgM and IgG in the injured spinal cord, spanning 6 mm of injured tissue around the injury epicenter rostrally and caudally. At 7 days post-injury, we found a significant reduction in IgM deposition in Nrg-1 treated group as compared to vehicle treated rats. This reduction was significant at the epicenter (163-fold), 1 and 2 mm rostral (1.8 and 2.2-fold respectively) as well as 1 mm caudal (1.8-fold) points to the injury epicenter ($P<0.05$, two-way ANOVA, N=5/group) (Fig. 3.7A). In a similar immunointensity analysis, we also found a reduction in IgG deposition in the spinal cord of Nrg-1 treated animals compared to vehicle treated group. This reduction was statistically significant at 1mm rostral to the injury epicenter (Fig. 3.7B). Analysis of IgG and IgM deposition at the chronic 42-day time-point showed no significant difference between vehicle and Nrg-1 treated groups (Fig. 3.7C, D). These results show the ability of Nrg-1 to reduce antibody deposition at subacute stage of SCI and a plausible mechanism through which Nrg-1 improved neural tissue preservation in our previous studies.

Figure 3.7. Nrg-1 therapy significantly attenuates antibody deposition in injured spinal cord tissue at subacute stage of SCI.

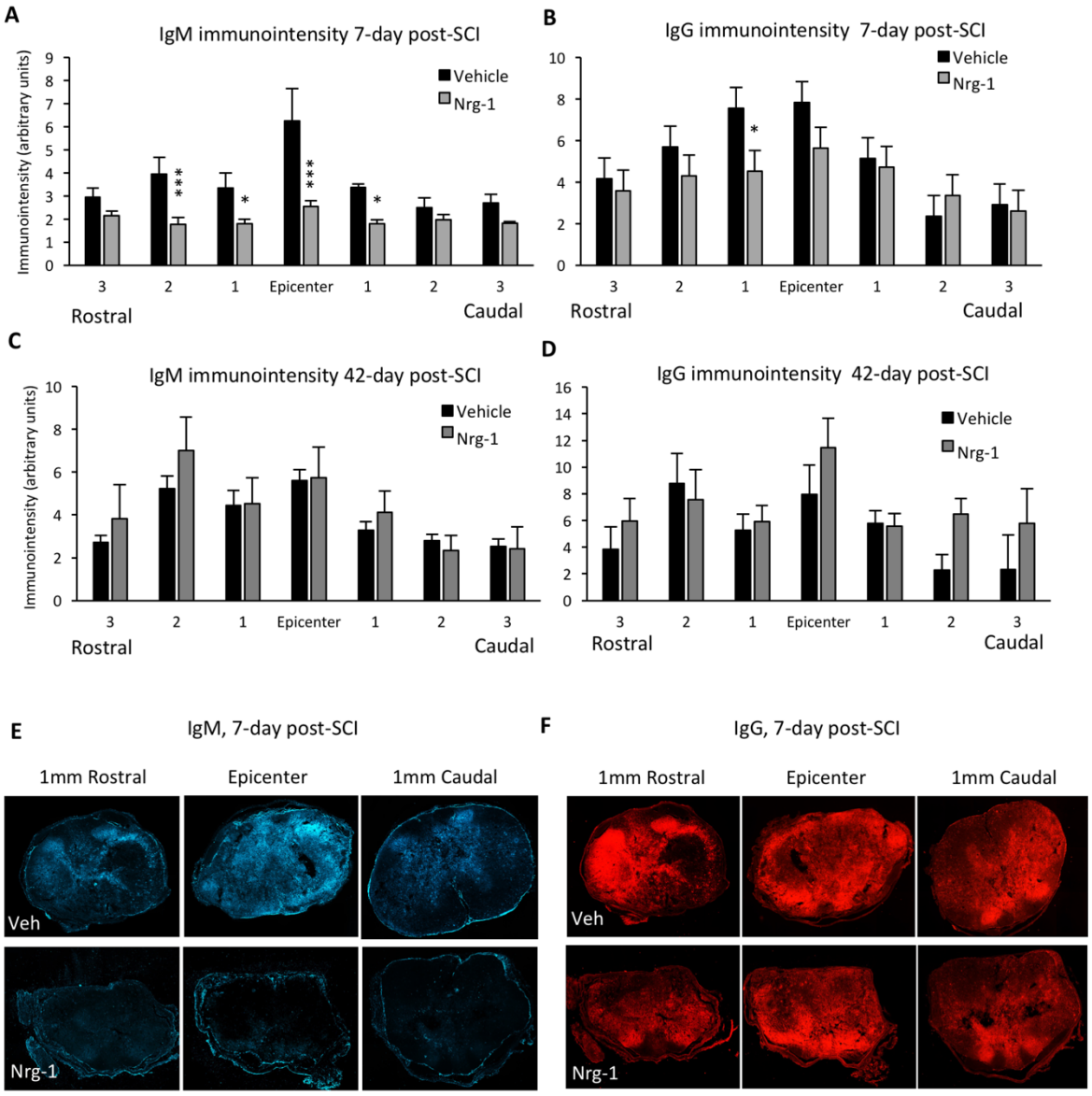


Figure 3.7. Nrg-1 therapy significantly attenuates antibody deposition in injured spinal cord tissue at subacute stage of SCI. (A-D) Immunohistochemical assessment of injured spinal cord tissue for IgM and IgG deposition was performed at the epicenter and perilesional areas at subacute (7 days) and chronic (42 days) stages of SCI. (A, C) Analysis of IgM Immunointensity showed a significantly lower IgM deposition in Nrg-1 treated animals compared to vehicle treated group at 7-day post-SCI. This decrease was significant at the epicenter, 1mm caudal and 1 and 2mm rostral to the injury site. No significant difference in IgM deposition was detected at 42-day time-point. (B, D) Comparison of IgG immunointensity between vehicle and Nrg-1 treated animals revealed a significant decrease in IgG deposition only at 1mm rostral to the epicenter at 7-day post-injury. No significant difference was detected in IgG deposition between vehicle and Nrg-1 treated groups at 42-day time-point. (E, F) Representative images are provided at the epicenter and 1 mm rostral and caudal to the lesion center (N=4/group/time-point, *P<0.05, **P<0.01, ***P<0.001, two-way ANOVA followed by Holm- Sidak post-hoc test).

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

3.4 Discussion

In the current study, using a clinically relevant model of compressive/contusive SCI in rats, we have identified a novel positive immunomodulatory role for Nrg-1 in SCI. Systemic Nrg-1 therapy augments regulatory populations of macrophages, T cells and B cells both peripherally and in the injured spinal cord tissue during the acute and chronic phases of SCI. Moreover, Nrg-1 treatment promotes pro-regenerative immune mediators such as IL-10 and CCL11 while attenuating pro-inflammatory cytokines and chemokines, IL-6, IFN-γ, CXCL1, CXCL2 and CXCL3. Importantly, Nrg-1 positively regulates B cell activity and moderates SCI-induced deposition of IgG and IgM in the spinal cord. To our knowledge, this is the first study that investigates the impact of Nrg-1 on neuroinflammation in traumatic SCI. Importantly, we have demonstrated the potential of systemic Nrg-1 as a new promising immunomodulatory strategy for traumatic SCI in a clinically-relevant model. Of note, Nrg-1 treatment used in our study is shown to pass the blood brain and spinal cord barrier and enter the central nervous system (CNS) readily [46], and importantly has shown therapeutic efficacy in previous studies by our group and others [13, 47].

Nrg-1 and ErbB network is known for its critical role in the developing central and peripheral nervous systems [48]. In the spinal cord, while the implication of Nrg-1 in neural differentiation and myelination is established during development [49, 50], our knowledge on the role of Nrg-1 in SCI pathophysiology and neuroinflammation is still in its infancy. In recent years, work by our group and others has begun unraveling the importance of Nrg-1 in the pathologic CNS [11, 13-15]. We originally identified that Nrg-1 protein expression is severely depleted in acute traumatic SCI and remains down-regulated chronically [11]. We established a close correlation between Nrg-1 dysregulation and impaired endogenous precursor response after SCI [11]. Restoration of Nrg-1 was sufficient to activate an endogenous repair program that promoted oligodendrocyte

replacement and white matter repair following SCI [11, 13]. A recent study has also shown an essential role for Nrg-1 in Schwann cell driven remyelination after SCI that is known to be instrumental for endogenous myelin repair in the injured spinal cord [12]. Interestingly, we found that Nrg-1 treatment exerts a prominent neuroprotective effect on oligodendrocytes and axons after SCI [11], which complements the recent reports showing increased neuronal survival following ischemic brain insult [15]. In addition to the anticipated impact of Nrg-1 on oligodendrocytes and myelination, our recent *in vitro* and *in vivo* studies uncovered a novel role for Nrg-1 in regulating astrocyte response to injury and evolution of the glial scar in the injured spinal cord [13]. More specifically, Nrg-1 treatment had a remarkable effect on matrix remodeling in the glial scar by moderating the SCI induced upregulation of chondroitin sulfate proteoglycans (CSPGs), a key regulator of SCI pathophysiology and a master inhibitor of repair processes [51]. We unraveled that Nrg-1 mediates its effects through activation of ErbB2/3 complex in glial cells and an increase in phosphorylation of Erk1/2 and STAT3 pathways following SCI [13]. Most importantly, we showed that Nrg-1 treatment improves neurobehavioral recovery in a dose dependent manner after SCI [13]. Interestingly, these studies also provided the initial evidence for immunomodulatory effects of Nrg-1, which seems to be an underpinning mechanism for its beneficial effects in SCI.

We and others have demonstrated that Nrg-1 positively influences microglia *in vitro* and attenuates their response to stressful conditions as evidenced by reduced production of pro-inflammatory mediators such as nitric oxide (NO), IL-1β and TNF-α [13, 52]. In rat SCI, we found that intrathecal Nrg-1 treatment can remarkably enhance an IL-10 dominant cytokine balance [13] that is shown to be beneficial for SCI repair processes such as remyelination [53]. Several immune regulatory populations such as T_{reg} cells, B_{reg} cells and M2 microglia/macrophages produce IL-10 after SCI suggestive of a broader role for Nrg-1 in modulating neuroinflammation [54-56].

Notably, T and B lymphocytes, macrophages and microglia express Nrg-1 receptors [13, 57-59] and therefore all these populations can potentially be affected by the dysregulation of Nrg-1 signaling following SCI. To date, the role of Nrg-1 in modulating the innate and adaptive immune response in SCI has remained elusive. In this study, we employed systemic Nrg-1 delivery to understand how Nrg-1 influences the recruitment and function of immune cells not only within the injured spinal cord tissue but also in peripheral blood. Additionally, systemic delivery provides a more clinically relevant therapeutic strategy for SCI.

Our immunophenotypic investigation of the injured spinal cord tissue revealed that systemic Nrg-1 increases macrophage infiltration into the injured spinal cord, which seems to be temporally regulated. We found that Nrg-1 promotes the recruitment of macrophages acutely and chronically with no apparent effect at the subacute stage. Interestingly, previous studies on acute peripheral nerve injury have also shown that intrathecal infusion of Nrg-1 induces recruitment and proliferation of resident microglia in the dorsal horn where injured sensory afferents enter the spinal cord [60, 61]. Increased microgliosis and macrophage proliferation in response to over-activation of Nrg-1 and ErbB receptors have been also shown in direct *in vitro* studies [60, 61]. Although Nrg-1 induced microgliosis has been associated with neuropathic pain in peripheral nerve injury [60, 61], our previous studies in rat SCI showed no significant change in pain sensation following Nrg-1 treatment [13]. It is noteworthy that Nrg-1 treatment in our SCI studies augments the downregulated levels of Nrg-1 in the injured spinal cord as opposed to over-activating its signaling. Interestingly, although Nrg-1 increased the population of macrophages in acute SCI, this increase mainly reflected an elevation in the subpopulation of alternatively activated M2 macrophages ($CD45^+CD68^+CD163^+IL10^+$) with no change in the population of classically activated pro-inflammatory M1 ($CD45^+CD68^+CD86^+$) macrophages. Notably, the

increase in M2 macrophage population at acute stage of SCI is in agreement with our previous cytokine study in SCI where we observed elevated levels of M2 markers such as arginase-1 and IL-10 under Nrg-1 therapy [13]. M2 macrophages are shown to promote oligodendrocyte differentiation, survival and remyelination [62] and are associated with overall tissue preservation and improved recovery of function following SCI [63]. In the chronically injured spinal cord tissue, however, it was intriguing that Nrg-1 treatment more prominently promoted the population of M1 macrophages with a concurrent increase in IL-10 expressing M2 subpopulation. Further studies are required to fully elucidate the underlying mechanisms of Nrg-1 in macrophage regulation.

We show that Nrg-1 treatment provided a more balanced chemokine profile after SCI. Nrg-1 elevated the expression level of CCL11 in chronic SCI. CCL11 is produced by microphages/microglia, astrocytes and pericytes [64-66]. Similar to IL-10, CCL11 is known to have anti-inflammatory function and protects neural tissue during inflammatory process [39]. CCL11 also increases migration and proliferation of neural precursor cells following cerebral ischemic injury [43], which is a prerequisite for endogenous cell replacement. We additionally showed that Nrg-1 attenuated the expression of CXCL1 in acute SCI. This chemokine is produced by mast cells, macrophages and astrocytes, and contributes to neutrophil recruitment and exacerbate neuroinflammation following CNS injury [45]. Interestingly, CXCL1 is known to be regulated by IL-6 [67], an inflammatory cytokine produced by macrophages, microglia and astrocytes in SCI [68, 69]. Thus, these data suggest a correlation between downregulation of CXCL1 and reduction in IL-6 expression by Nrg-1 in our acute SCI studies. Notably, inhibition of IL-6 signaling is associated with reduced glial scarring and improves functional recovery [70] and neuropathic pain following SCI [25]. Our chemokine profiling also showed the potential of Nrg-1

in attenuating the expression of CXCL2 and CXCL10. CXCL2 is known to induce neuronal injury in motoneuron cultures [71] supportive of a neuroprotective role for Nrg-1 in CNS injury. CXCL10, also known as interferon-gamma-induced protein 10, is a pro-inflammatory chemokine produced by macrophages, fibroblasts, astrocytes and endothelial cells upon IFN-ɣ stimulation [72]. Neutralization of CXCL10 has been associated with decreased secondary tissue degeneration and improvement of functional recovery in murine SCI [35]. Taken together, our chemokine analysis indicates that while Nrg-1 promotes the overall recruitment of macrophages into the injured spinal cord, it activates a phenotype in macrophages that can facilitate repair processes following SCI.

A novel finding in our study is the modulatory effect of Nrg-1 on T cells after SCI. We have provided the first evidence that Nrg-1 treatment remarkably enhances a T_{reg} response in acute, subacute and chronic SCI. Most interestingly, Nrg-1 exerts its beneficial effects systemically by increasing the population of circulating T_{reg} cells in the blood stream in chronic SCI. T_{reg} cells play pivotal roles in regulating an adaptive immune response and preventing autoimmune reactions [73]. Ablation of T_{reg} cells elicits an intensive T_{eff} response that induces neuronal death following CNS injury [54]. In our study, we showed the ability of systemic Nrg-1 treatment to suppress the population of pro-inflammatory $CD3^{+}CD4^{+}IFN\text{-}\gamma^{+}$ effector T cells and reduce $IFN\text{-}\gamma^{+}$ within the injured spinal cord tissue at subacute SCI. Of note, IFN-γ is primarily produced by lymphocytes including effector Th1 cells and promotes classic macrophage activation [29] and neuropathic pain [24]. Importantly, IFN-γ is implicated in inducing white matter degeneration and necrosis following cerebral ischemia/reperfusion injury and increasing the susceptibility of neurons to apoptosis [74, 75]. SCI studies have shown that IFN-γ directs its degenerative effects by promoting proliferation of cytotoxic T cells [8, 76]. Indeed, reduction in IFN-γ expression in subacute SCI

under Nrg-1 treatment in this study provides an underlying mechanism by which Nrg-1 ameliorated white matter degeneration in the injured spinal cord in our previous studies [11, 13]. Nrg-1 also induced a reduction in the expression of CCL5 (RANTES), a pro-inflammatory chemokine produced by astrocytes as well as T cells upon macrophage stimulation [68, 77]. This observation correlates well with the increased M2 macrophages and decreased population of effector T cells that we observed in our immunophenotypic studies. Increase in in CCL5 is implicated in microvascular dysfunction following CNS injury [42]. Altogether, our observations indicate that Nrg-1 is a positive modulator of T cell response after SCI. Therefore, increasing the deficient levels of Nrg-1 after injury has therapeutic value to augment a T regulatory immune response during the repair processes following SCI.

SCI also elicits a B cell response in the spleen and bone marrow characterized by increased B cell number and elevated serum immunoglobulin levels [4]. Our findings provide new evidence that Nrg-1 promotes recruitment and regulatory phenotype of B cells in the injured spinal cord with a more pronounced effect at the chronic stage of injury. Similar to T cells, the increase in B cell response mainly represented a rise in the B_{reg} population in response to Nrg-1 treatment. Interestingly, in contrast to the spinal cord, number of B cells was initially dropped in the blood after SCI which was independent of Nrg-1 treatment. This observation is consistent with previous reports that showed SCI induces an initial cessation of B cell production in the bone marrow [23]. There is also evidence that B cells undergo extensive apoptosis following SCI. B cells are the integral component of the adaptive humoral immune response and capable of producing a wide variety of cytokines [10]. Following SCI, B cells produce autoantibodies against spinal cord tissue that can aggravate the secondary injury processes through complement and Fc Receptor (FcR)-dependent mechanisms [21]. Similar to other immune cells, phenotype and function of B cells can

be regulated by the signaling molecules and cytokines available in their microenvironment [10]. B_{reg} cells support repair processes as they can suppress activation of helper T cell and their production of TNF-α and IFN-ɣ as well as TNF-α production by monocytes [78-80]. Importantly, B_{reg} cells play essential roles in the formation and maintenance of T_{reg} cell population [81]. Moreover, B_{reg} cell-mediated IL-10 production has been shown to limit tissue damage and improve recovery of function in a murine model of brain ischemia [22]. For the first time, we uncovered that Nrg-1 attenuates antibody deposition in the injured spinal cord. Our analysis of IgG and IgM showed that Nrg-1 was mainly effective subacutely. It is plausible that Nrg-1 mediated reduction in the spinal cord levels of IgM and IgG in subacute SCI reflects an indirect role for Nrg-1in modulation of vascular permeability. We have previously shown that Nrg-1 treatment attenuates the activity of matrix metalloproteinase (MMP)-9 in the injured spinal cord tissue [13]. MMP-9 is involved in disruption of blood-spinal barrier [3]. Moreover, IL-6 can increase antibody production by B cells [82]. Thus, the Nrg-1 induced decline in IL-6 expression in our acute SCI studies can be another underlying cause for the reduced antibody deposition in the injured spinal cord at the subacute stage without alteration in B cell number. Our immunohistochemical analysis showed an increasing trend in IgG deposition in the spinal cord tissue with Nrg-1 treatment at chronic (42-day) time-point. This observation may reflect the modulatory effect of Nrg-1 on B cells that reside chronically in the spinal cord tissue. Our analysis of spinal cord B cells showed a close correlation between increased B_{reg} population in Nrg-1 treated animals and a trend towards higher production of IgG within the chronically injured spinal cord. This is in line with other studies that have attributed IgG production to B_{reg} cells [83]. Interestingly, IgG deposition by B_{reg} cells has been associated with beneficial immunomodulatory roles that include neutralizing harmful antigens from the microenvironment, inhibiting macrophages and dendritic cell activation, and enhancing

the clearance of apoptotic bodies that contain self-antigens [83]. Moreover, studies by Nguyen and colleagues have also shown a positive role for IgG in recovery from SCI [84]. These studies revealed that systemic IgG administration increased IgG deposition in the injured spinal cord, which was associated with improved neural tissue preservation and functional recovery in a rat model of cervical SCI [84]. This evidence suggests that increased level of IgG may exert beneficial effects in SCI. Of note, our previous studies identified that Nrg-1 treatment improves tissue preservation in chronic SCI [13] that could be attributed, at least in part, to the increase in B_{reg} cells and IgG production.

3.5 Conclusions

The present study, for the first time, implicates Nrg-1 as a positive regulator of neuroinflammation after SCI. We demonstrate that systemic bio-availability of Nrg-1 induces a pro-regenerative immune response in leukocytes that fosters a supportive environment for repair and regeneration in the injured spinal cord. Identification of a multifaceted immunoregulatory role for Nrg-1 establishes a novel therapeutic target for treating traumatic SCI and other CNS neuroinflammatory conditions.

3.6 References

1. Trivedi, A., A.D. Olivas, and L.J. Noble-Haeusslein, *Inflammation and Spinal Cord Injury: Infiltrating Leukocytes as Determinants of Injury and Repair Processes.* Clin Neurosci Res, 2006. **6**(5): p. 283-292.
2. Fitch, M.T., et al., *Cellular and molecular mechanisms of glial scarring and progressive cavitation: in vivo and in vitro analysis of inflammation-induced secondary injury after CNS trauma.* J Neurosci, 1999. **19**(19): p. 8182-98.
3. Noble, L.J., et al., *Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events.* J Neurosci, 2002. **22**(17): p. 7526-35.
4. Ankeny, D.P., et al., *Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis.* J Neurochem, 2006. **99**(4): p. 1073-87.
5. Zhao W, X.W., Le W, Beers DR, He Y, Henkel JS, Simpson EP, Yen AA, Xiao Q, Appel SH, *Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism.* J Neuropathol Exp Neurol, 2004. **63**(9): p. 964-977.
6. Hoffman, W., F.G. Lakkis, and G. Chalasani, *B Cells, Antibodies, and More.* Clin J Am Soc Nephrol, 2016. **11**(1): p. 137-54.
7. Kil, K., et al., *T cell responses to myelin basic protein in patients with spinal cord injury and multiple sclerosis.* J Neuroimmunol, 1999. **98**(2): p. 201-7.
8. Sun, D., et al., *Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice.* J Immunol, 2001. **166**(12): p. 7579-87.
9. Lodge PA., S.S., *Regulation of microglial activation by TGF-beta, IL-10, and CSF-1.* Journal of Leukocyte Biology, 1996. **60**(4): p. 502-508.

10. Vazquez, M.I., J. Catalan-Dibene, and A. Zlotnik, *B cells responses and cytokine production are regulated by their immune microenvironment.* Cytokine, 2015. **74**(2): p. 318-26.

11. Gauthier, M.-K., et al., *Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury.* Eur. J. Neurosci, 2013. **38**(5): p. 2693-2715.

12. Bartus, K., et al., *Neuregulin-1 controls an endogenous repair mechanism after spinal cord injury.* Brain, 2016.

13. Alizadeh, A., et al., *Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.* Glia, 2017. **65**(7): p. 1152-1175.

14. Simmons, L.J., et al., *Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial cells in vitro involves the NF-kappa B pathway.* J Neuroinflammation, 2016. **13**(1): p. 237.

15. Guo, W.P., et al., *Neuroprotective effects of neuregulin-1 in rat models of focal cerebral ischemia.* Brain Res, 2006. **1087**(1): p. 180-5.

16. Nguyen, H.X., K.D. Beck, and A.J. Anderson, *Quantitative assessment of immune cells in the injured spinal cord tissue by flow cytometry: a novel use for a cell purification method.* J Vis Exp, 2011(50).

17. Karimi-Abdolrezaee, S., et al., *Synergistic Effects of Transplanted Adult Neural Stem/Progenitor Cells, Chondroitinase, and Growth Factors Promote Functional Repair and Plasticity of the Chronically Injured Spinal Cord.* J. Neurosci, 2010. **30**(5): p. 1657-1676.

18. Shechter, R., et al., *Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice.* PLoS Med, 2009. **6**(7): p. e1000113.

19. Zhang, N., et al., *Inflammation & apoptosis in spinal cord injury.* Indian J Med Res, 2012. **135**: p. 287-96.

20. Jones, T.B., *Lymphocytes and autoimmunity after spinal cord injury.* Exp Neurol, 2014. **258**: p. 78-90.

21. Ankeny, D.P., Z. Guan, and P.G. Popovich, *B cells produce pathogenic antibodies and impair recovery after spinal cord injury in mice.* J Clin Invest, 2009. **119**(10): p. 2990-9.

22. Ren, X., et al., *Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke.* J Neurosci, 2011. **31**(23): p. 8556-63.

23. Oropallo, M.A., R. Goenka, and M.P. Cancro, *Spinal cord injury impacts B cell production, homeostasis, and activation.* Semin Immunol, 2014. **26**(5): p. 421-7.

24. Tsuda, M., et al., *IFN-gamma receptor signaling mediates spinal microglia activation driving neuropathic pain.* Proc Natl Acad Sci U S A, 2009. **106**(19): p. 8032-7.

25. Guptarak, J., et al., *Inhibition of IL-6 signaling: A novel therapeutic approach to treating spinal cord injury pain.* Pain, 2013. **154**(7): p. 1115-28.

26. Yaguchi, M., et al., *Functional recovery after spinal cord injury in mice through activation of microglia and dendritic cells after IL-12 administration.* J Neurosci Res, 2008. **86**(9): p. 1972-80.

27. Brewer, K.L., J.R. Bethea, and R.P. Yezierski, *Neuroprotective effects of interleukin-10 following excitotoxic spinal cord injury.* Exp Neurol, 1999. **159**(2): p. 484-93.

28. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.

29. Su, X., et al., *Interferon-gamma regulates cellular metabolism and mRNA translation to potentiate macrophage activation.* Nat Immunol, 2015. **16**(8): p. 838-849.

30. Kaplin, A.I., et al., *IL-6 induces regionally selective spinal cord injury in patients with the neuroinflammatory disorder transverse myelitis.* J Clin Invest, 2005. **115**(10): p. 2731-41.

31. Pagenstecher, A., et al., *Astrocyte-targeted expression of IL-12 induces active cellular immune responses in the central nervous system and modulates experimental allergic encephalomyelitis.* J Immunol, 2000. **164**(9): p. 4481-92.

32. Gunther, J., et al., *Comparative kinetics of Escherichia coli- and Staphylococcus aureus-specific activation of key immune pathways in mammary epithelial cells demonstrates that S. aureus elicits a delayed response dominated by interleukin-6 (IL-6) but not by IL-1A or tumor necrosis factor alpha.* Infect Immun, 2011. **79**(2): p. 695-707.

33. Seshadri, S., et al., *MAIL regulates human monocyte IL-6 production.* J Immunol, 2009. **183**(8): p. 5358-68.

34. Marsh, D.R. and J.M. Flemming, *Inhibition of CXCR1 and CXCR2 chemokine receptors attenuates acute inflammation, preserves gray matter and diminishes autonomic dysreflexia after spinal cord injury.* Spinal Cord, 2011. **49**(3): p. 337-44.

35. Gonzalez, R., et al., *Therapeutic neutralization of CXCL10 decreases secondary degeneration and functional deficit after spinal cord injury in mice.* Regen Med, 2007. **2**(5): p. 771-83.

36. Zhang, Z.J., et al., *Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons.* Pain, 2013. **154**(10): p. 2185-97.

37. Hassanshahi, G., et al., *Temporal expression profile of CXC chemokines in serum of patients with spinal cord injury.* Neurochem Int, 2013. **63**(5): p. 363-7.

38. Lin, M.S., et al., *Curcumin attenuates the expression and secretion of RANTES after spinal cord injury in vivo and lipopolysaccharide-induced astrocyte reactivation in vitro.* J Neurotrauma, 2011. **28**(7): p. 1259-69.

39. Adzemovic, M.Z., et al., *Expression of Ccl11 associates with immune response modulation and protection against neuroinflammation in rats.* PLoS One, 2012. **7**(7): p. e39794.

40. Hiura, T.S., S.J. Kempiak, and A.E. Nel, *Activation of the human RANTES gene promoter in a macrophage cell line by lipopolysaccharide is dependent on stress-activated protein kinases and the IkappaB kinase cascade: implications for exacerbation of allergic inflammation by environmental pollutants.* Clin Immunol, 1999. **90**(3): p. 287-301.

41. Kim, M.O., et al., *Regulation of RANTES/CCL5 expression in human astrocytes by interleukin-1 and interferon-beta.* J Neurochem, 2004. **90**(2): p. 297-308.

42. Terao, S., et al., *Blood cell-derived RANTES mediates cerebral microvascular dysfunction, inflammation, and tissue injury after focal ischemia-reperfusion.* Stroke, 2008. **39**(9): p. 2560-70.

43. Wang, F., et al., *CCL11 promotes migration and proliferation of mouse neural progenitor cells.* Stem Cell Res Ther, 2017. **8**(1): p. 26.

44. Tyagi, P., et al., *Spontaneous Recovery of Reflex Voiding Following Spinal Cord Injury Mediated by Anti-inflammatory and Neuroprotective Factors.* Urology, 2016. **88**: p. 57-65.

45. De Filippo, K., et al., *Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation.* Blood, 2013. **121**(24): p. 4930-7.

46. Kastin, A.J., V. Akerstrom, and W. Pan, *Neuregulin-1-beta1 enters brain and spinal cord by receptor-mediated transport.* J Neurochem, 2004. **88**(4): p. 965-70.

47. Xu, Z., et al., *Extended therapeutic window and functional recovery after intraarterial administration of neuregulin-1 after focal ischemic stroke.* J Cereb Blood Flow Metab, 2006. **26**(4): p. 527-35.

48. Brinkmann, B.G., et al., *Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system.* Neuron, 2008. **59**(4): p. 581-95.

49. Hancock, M.L., et al., *Type III neuregulin 1 regulates pathfinding of sensory axons in the developing spinal cord and periphery.* Development, 2011. **138**(22): p. 4887-98.

50. Vartanian, T., G. Fischbach, and R. Miller, *Failure of spinal cord oligodendrocyte development in mice lacking neuregulin.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 731-5.

51. Dyck, S.M. and S. Karimi-Abdolrezaee, *Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system.* Exp Neurol, 2015. **269**: p. 169-87.

52. Mencel, M., M. Nash, and C. Jacobson, *Neuregulin upregulates microglial alpha7 nicotinic acetylcholine receptor expression in immortalized cell lines: implications for regulating neuroinflammation.* PLoS One, 2013. **8**(7): p. e70338.

53. Puntambekar, S.S., et al., *Interleukin-10 is a critical regulator of white matter lesion containment following viral induced demyelination.* Glia, 2015. **63**(11): p. 2160-2120.

54. Walsh, J.T., et al., *Regulatory T cells in central nervous system injury: a double-edged sword.* J Immunol, 2014. **193**(10): p. 5013-22.

55. Das, A., et al., *IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection.* J Immunol, 2012. **189**(8): p. 3925-35.

56. Chung, E.Y., et al., *Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1.* Immunity, 2007. **27**(6): p. 952-64.

57. Cannella, B., et al., *Neuregulin and erbB receptor expression in normal and diseased human white matter.* J Neuroimmunol, 1999. **100**(1-2): p. 233-42.

58. Ryzhov, S., et al., *ERBB signaling attenuates proinflammatory activation of nonclassical monocytes.* Am J Physiol Heart Circ Physiol, 2017. **312**(5): p. H907-H918.

59. Tynyakov-Samra, E., et al., *Reduced ErbB4 Expression in Immune Cells of Patients with Relapsing Remitting Multiple Sclerosis.* Mult Scler Int, 2011. **2011**: p. 561262.

60. Calvo, M., et al., *Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury.* J Neurosci, 2010. **30**(15): p. 5437-50.

61. Calvo, M., et al., *Following nerve injury neuregulin-1 drives microglial proliferation and neuropathic pain via the MEK/ERK pathway.* Glia, 2011. **59**(4): p. 554-68.

62. Miron, V.E., et al., *M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination.* Nat Neurosci, 2013. **16**(9): p. 1211-8.

63. Shin, T., et al., *Alternatively activated macrophages in spinal cord injury and remission: another mechanism for repair?* Mol Neurobiol, 2013. **47**(3): p. 1011-9.

64. Kovac, A., M.A. Erickson, and W.A. Banks, *Brain microvascular pericytes are immunoactive in culture: cytokine, chemokine, nitric oxide, and LRP-1 expression in response to lipopolysaccharide.* J Neuroinflammation, 2011. **8**: p. 139.

65. Parajuli, B., et al., *CCL11 enhances excitotoxic neuronal death by producing reactive oxygen species in microglia.* Glia, 2015. **63**(12): p. 2274-84.

66. Yang, L.P., X.A. Zhu, and M.O. Tso, *Minocycline and sulforaphane inhibited lipopolysaccharide-mediated retinal microglial activation.* Mol Vis, 2007. **13**: p. 1083-93.

67. Roy, M., et al., *CXCL1 can be regulated by IL-6 and promotes granulocyte adhesion to brain capillaries during bacterial toxin exposure and encephalomyelitis.* J Neuroinflammation, 2012. **9**: p. 18.

68. Choi, S.S., et al., *Human astrocytes: secretome profiles of cytokines and chemokines.* PLoS One, 2014. **9**(4): p. e92325.

69. Hunter, C.A. and S.A. Jones, *IL-6 as a keystone cytokine in health and disease.* Nat Immunol, 2015. **16**(5): p. 448-57.

70. Nakamura, M., et al., *Role of IL-6 in spinal cord injury in a mouse model.* Clin Rev Allergy Immunol, 2005. **28**(3): p. 197-204.

71. De Paola, M., et al., *Chemokine MIP-2/CXCL2, acting on CXCR2, induces motor neuron death in primary cultures.* Neuroimmunomodulation, 2007. **14**(6): p. 310-6.

72. Luster, A.D., J.C. Unkeless, and J.V. Ravetch, *Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins.* Nature, 1985. **315**(6021): p. 672-6.

73. Zhou, R., et al., *The living eye "disarms" uncommitted autoreactive T cells by converting them to Foxp3(+) regulatory cells following local antigen recognition.* J Immunol, 2012. **188**(4): p. 1742-50.

74. Bate, C., et al., *Interferon-gamma increases neuronal death in response to amyloid-beta1-42.* J Neuroinflammation, 2006. **3**: p. 7.

75. Yilmaz, G., et al., *Role of T lymphocytes and interferon-gamma in ischemic stroke.* Circulation, 2006. **113**(17): p. 2105-12.

76. Deb, C., et al., *CD8+ T cells cause disability and axon loss in a mouse model of multiple sclerosis.* PLoS One, 2010. **5**(8): p. e12478.

77. Swanson, B.J., et al., *RANTES production by memory phenotype T cells is controlled by a posttranscriptional, TCR-dependent process.* Immunity, 2002. **17**(5): p. 605-15.

78. Bouaziz, J.D., et al., *IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro.* Eur J Immunol, 2010. **40**(10): p. 2686-91.

79. Blair, P.A., et al., *CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients.* Immunity, 2010. **32**(1): p. 129-40.

80. Iwata, Y., et al., *Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.* Blood, 2011. **117**(2): p. 530-41.

81. Pennati, A., et al., *Regulatory B cells induce formation of IL-10 expressing T cells in mice with autoimmune neuroinflammation.* J Neurosci, 2016.

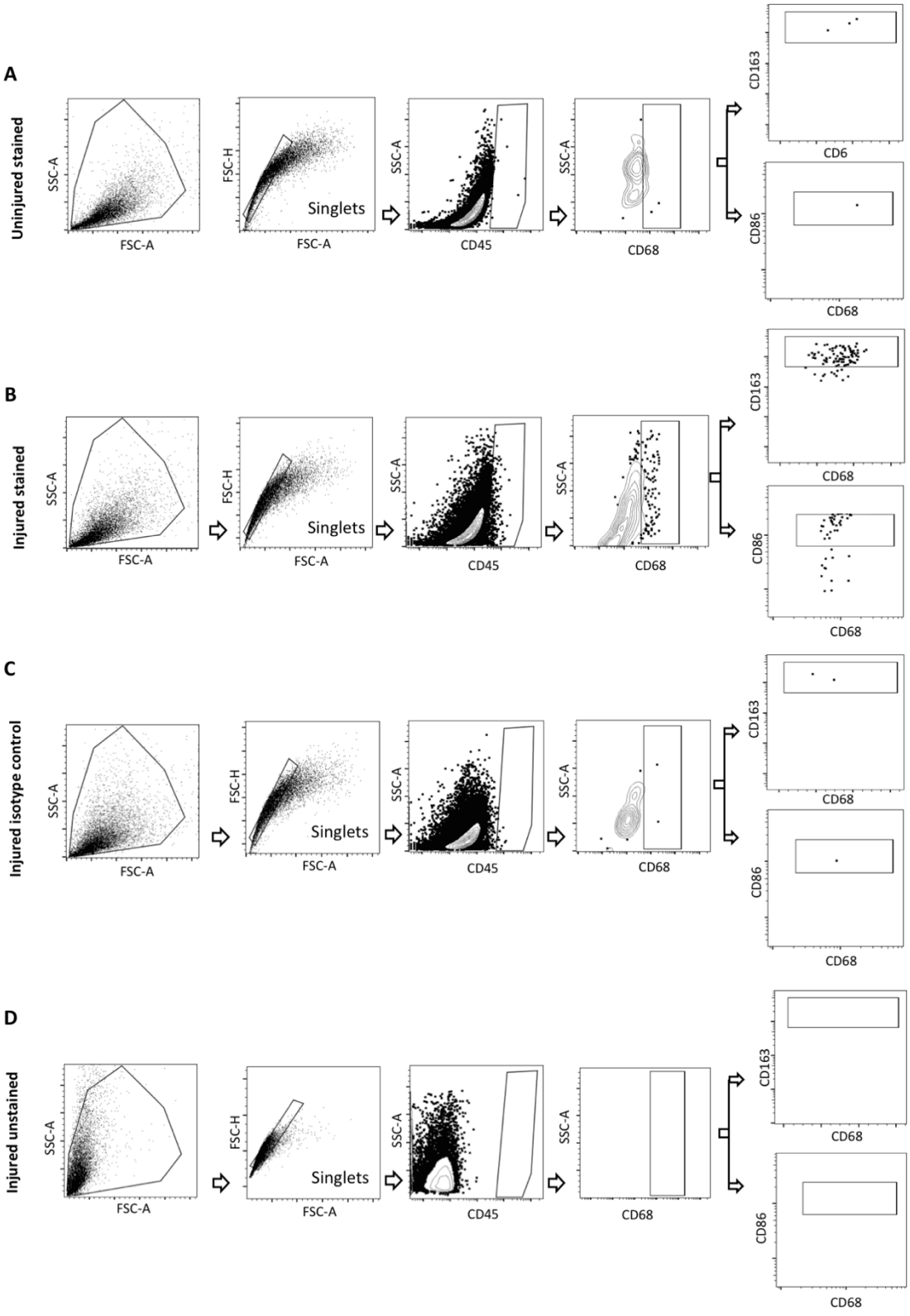
82. Maeda, K., et al., *IL-6 increases B-cell IgG production in a feed-forward proinflammatory mechanism to skew hematopoiesis and elevate myeloid production.* Blood, 2010. **115**(23): p. 4699-706.

83. Mizoguchi, A. and A.K. Bhan, *A case for regulatory B cells.* J Immunol, 2006. **176**(2): p. 705-10.

84. Nguyen, D.H., et al., *Immunoglobulin G (IgG) attenuates neuroinflammation and improves neurobehavioral recovery after cervical spinal cord injury.* J Neuroinflammation, 2012. **9**: p. 224.

3.7 Supplementary materials

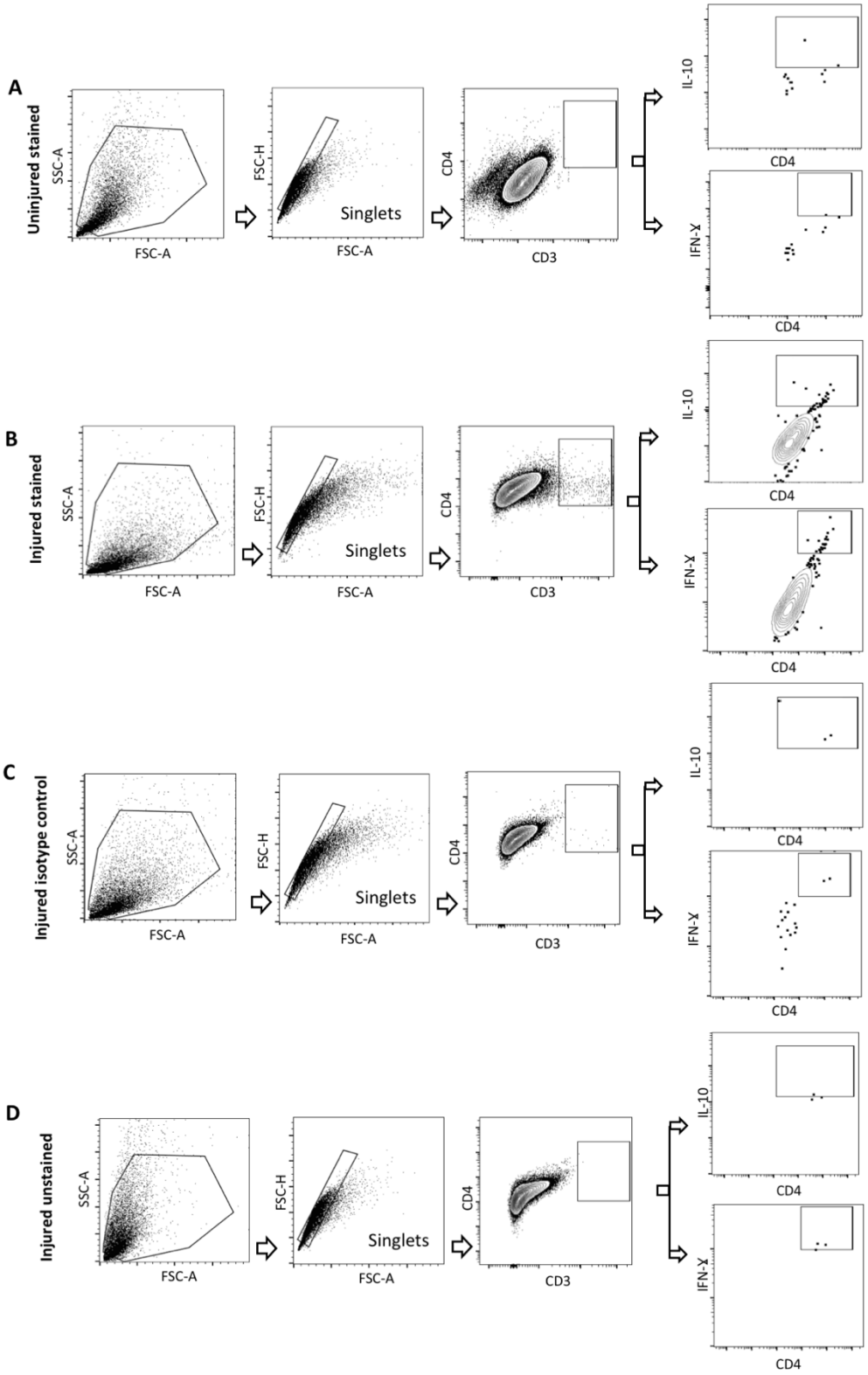
Supplementary figure 3.1. Flow cytometric verification of antibody specificity for macrophage detection.



Supplementary figure 3.1. (A-C) Flow cytometric verification of antibody specificity was performed on injured and uninjured spinal cord tissue. Stained and unstained spinal cord immune cells were gated for the detection of macrophages and their pro-inflammatory (M1, $CD45^{+}CD68^{+}CD86^{+}$) and pro-regenerative (M2, $CD45^{+}CD68^{+}CD163^{+}$) sub-populations. **(A, B)** While macrophages and their sub-populations were clearly detectable in the stained injured group, there were a minimal number of macrophages in the uninjured spinal cord tissue. **(C)** Injured tissues were stained with specific isotype controls for each antibody and analyzed with the same gating strategy. **(D)** Unstained immune cells isolated from injured spinal cord tissue were analyzed with the same gating strategy as above. Our validation studies showed a negligible number of macrophages in the injured isotype control and no positively stained cells in the unstained control compared to the stained injured group confirming the specificity of antibodies used in our macrophage panel.

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

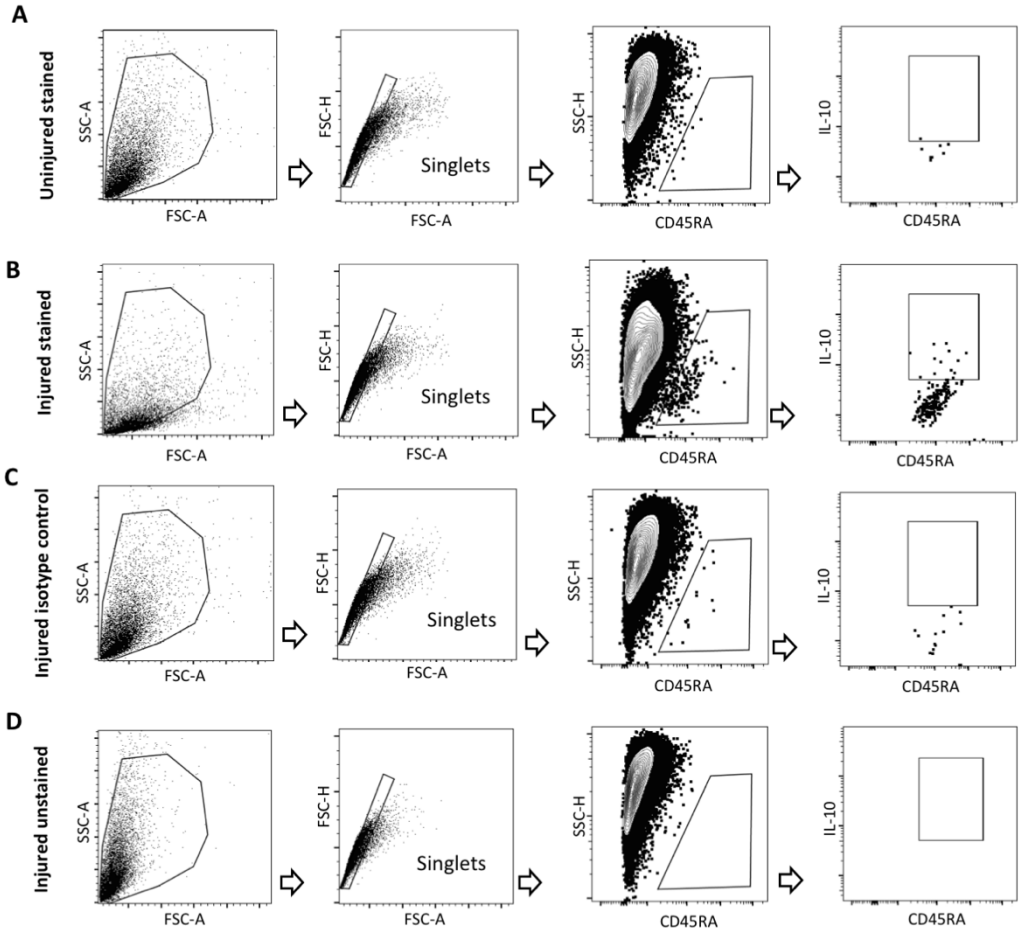
Supplementary figure 3.2. Flow cytometric verification of antibody specificity for T cell detection.



Supplementary figure 3.2. (A-C) Flow cytometric verification of antibody specificity for T cell detection. Isolated spinal cord immune cells were stained and gated for the detection of helper T cells and their effector (T_{eff}, $CD3^+CD4^+IFN\gamma^+$) and regulatory (T_{reg}, $CD3^+CD4^+IL\text{-}10^+$) sub-populations. **(A, B)** While T cells and their sub-populations were clearly detectable in the injured stained group, there were a minimal number of helper T cells in uninjured spinal cords. **(C)** Injured spinal cord tissues were stained with specific isotype controls for each antibody and analyzed with the same gating strategy. **(D)** Unstained injured spinal cord immune cells were analyzed with the same gating strategy as above. A negligible number of T cells were detected in the injured isotype and the unstained control compared to the stained injured group confirming the specificity of antibodies used in our T cell panel.

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

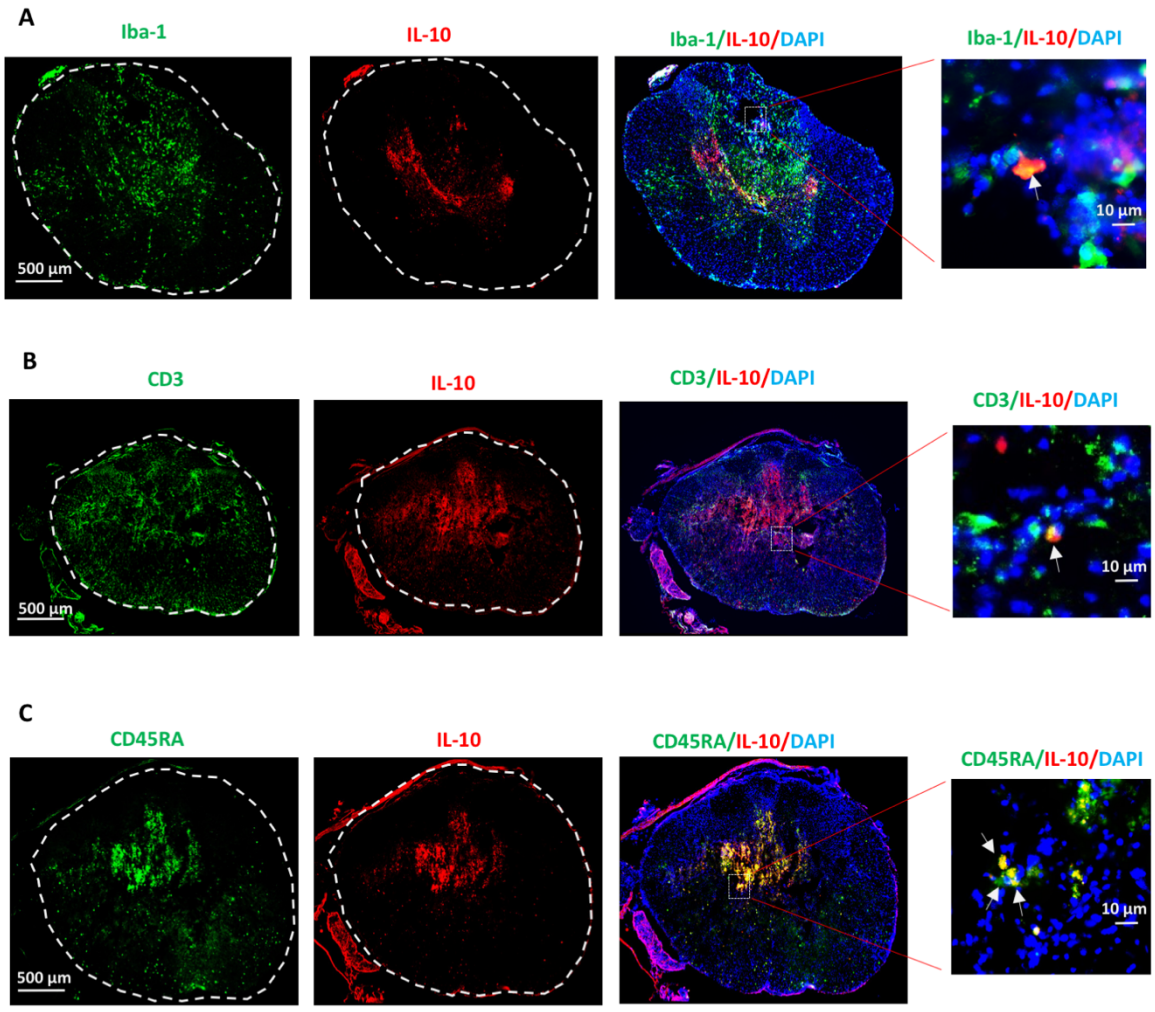
Supplementary figure 3.3. Flow cytometric verification of antibody specificity for B cell detection.



Supplementary figure 3.3. (A-C) Specificity of the antibodies used for B cell detection was verified as shown above. Isolated spinal cord immune cells were stained and gated for the detection of B cells and their (B_{reg}, $CD45RA^{+}IL\text{-}10^{+}$) sub-population. **(A, B)** B cells were clearly detectable in the injured stained group. However, B cell number was ignorable in uninjured spinal cords. **(C)** Injured spinal cord tissues were stained with specific isotype controls for each antibody and analyzed with the same gating strategy. **(D)** Unstained spinal cord immune cells obtained from injured animals were analyzed with the same gating strategy as above. Our analysis showed a negligible number of B cells in the injured isotype control and no B cells in the unstained control group compared to stained injured group confirming the specificity of our B cell antibody panel.

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

Supplementary figure 3.4.



Supplementary figure 3.4. Immunohistochemical staining of the spinal cord sections at 1mm caudal to the injury epicenter was performed to verify the tissue distribution of **(A)** macrophages/microglia (Iba-1^+), **(B)** T cells ($CD3^+$) and **(C)** B cells ($CD45RA^+$) at 2 weeks after the injury. Dashed lines show the contour of the spinal cord section. Immune cells were mostly found within the SCI lesion. Magnified pictures and white arrows show the presence of **(A)** Iba-1^+/IL-10^+ macrophages/microglia, **(B)** $CD3^+$/IL-10^+ T cells and **(C)** $CD45RA^+$/L-10^+ B cells. These data validate our flow cytometry data confirming the presence of the M2 macrophages, T_{reg} and B_{reg} cells in the injured spinal cord tissue.

Reprinted from: Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S, (2018). **Neuregulin-1 Elicits a Regulatory Immune Response Following Traumatic Spinal Cord Injury.** Journal of Neuroinflammation, 2018. 15:53, DOI: s12974-018-1093-9.

Chapter 4: General Discussion

4.1 General Overview of Findings

The current thesis focused on unraveling the role of Nrg-1 on glial activation and neuroinflammation following traumatic SCI. Previously, our team discovered an acute and long lasting depletion of Nrg-1 in the injured spinal cord tissue following injury [1]. In the current study, using *in vitro* models of primary astrocytes and microglia, and a clinically relevant *in vivo* model of compressive/contusive SCI in the rat, we have identified a multifaceted role for Nrg-1 in regulating astrogliosis, glial scar formation and neuroinflammation [2]. Bio-availability of Nrg-1 positively modulates astrocytes response and moderates their expression of inhibitory CSPGs *in vitro* and in the injured spinal cord. Mechanistically, our findings revealed that Nrg-1 effects on activated glia is mediated through an ErbB2/3 heterodimer complex and correlates with activation of Erk1/2 and STAT3 pathways [2].

We demonstrate that availability of Nrg-1fosters a neuroprotective phenotype in resident microglia and infiltrating leukocytes. Nrg-1 ameliorates inflammatory response and results in reduced production of pro-inflammatory cytokines and mediators such as IL-1β and TNF-α and matrix metalloproteinases (MMP-2 and 9). Importantly, Nrg-1 promoted a pro-regenerative inflammatory response by increasing the expression of regulatory mediators such as arginase-1 and IL-10 [2]. Our in-depth analysis of the immune response following systemic Nrg-1 administration in rat SCI unraveled an immunoregulatory role for Nrg-1. We found that Nrg-1 promotes populations of M2 macrophages, as well as regulatory T and B lymphocytes in the injured spinal cord. Increase in these regulatory populations fostered a more permissive milieu by providing pro-regenerative cytokines and chemokines such as IL-10, arginase-1 and CCL11 [3]. Interestingly, systemic Nrg-1 treatment was able to induce a regulatory phenotype in circulating T

cells in chronic SCI. Nrg-1 seemed to modulate the inflammatory response, at least in part, by attenuating the expression of MyD88 and NFκBIZ, adaptor proteins involved in NFκB pathway activation and inflammatory cytokine production [2, 3]. In B cells, Nrg-1 induced a significant reduction in autoantibody production evident by reduced IgG and IgM deposition in the injured spinal cord tissue [3]. Importantly, beneficial effects of Nrg-1 treatment in modulation of immune response and astrogliosis was translated into improved tissue preservation and motor recovery after SCI. This thesis, for the first time, uncovers a significant role for Nrg-1 in modulating secondary injury mechanisms following SCI, and identifies Nrg-1 treatment as a promising candidate therapy for SCI and other CNS pathologies characterized by glial scar formation and neuroinflammation.

4.2 Nrg-1, a New Modulator of Astrocyte Reactivity and Scar Formation

In the current study, we have discovered a novel positive role for Nrg-1 in regulating astrogliosis and formation of the glial scar and CSPGs following SCI. Astrocytes play a principal role in the secondary injury mechanisms of SCI [4]. Phenotypic changes that occur in activated astrocytes have a profound impact on the microenvironment of the injured spinal cord and in particular the composition of ECM. Upregulation of CSPGs in the ECM by scar forming astrocytes is one of the hallmarks of SCI and a key determinant of the repair processes after injury [5-8]. CSPGs are known for their deleterious inhibitory effects on axonal plasticity and regeneration in SCI [9, 10]. In the past two decades, extensive attempts have been made to target CSPGs and their signaling in the injured spinal cord in order to promote axonal regeneration and sprouting after SCI [5, 11-14]. In these studies, attenuation of CSPGs by chondroitinase ABC (ChABC), inhibition of their synthesis or modulation of their signaling receptors have resulted in improved axonal regeneration and functional recovery. Moreover, work from our group and others has also established an inhibitory

role for CSPGs in cell replacement activities in the injured spinal cord [5, 15]. CSPGs limit the survival, proliferation and oligodendrocyte differentiation of the two endogenous populations of precursor cells in the spinal cord; NPCs and OPCs [4, 15, 16]. Reduction in CSPGs promotes their regenerative response and facilitates oligodendrocyte replacement and remyelination [16]. CSPGs are also upregulated in demyelinating lesions of MS-like conditions such as EAE where they inhibit the recruitment of OPCs to the lesion and hinder their maturation to myelinating oligodendrocytes [17]. Thus, our findings that show a potential of Nrg-1 to moderate the expression of CSPGs is of great importance for several repair processes in SCI including axon regeneration, oligodendrocyte replacement and remyelination. Of note, parallel studies by our group have shown a supportive role for Nrg-1 in facilitating oligodendrocyte replacement and remyelination in SCI and focal demyelinating lesions [1, 18]. Therefore, Nrg-1-mediated reduction in CSPG expression provides a plausible mechanism by which Nrg-1 promotes oligodendrocyte differentiation and remyelination in injury conditions.

Mechanistically, in our SCI studies, we were not able to determine whether Nrg-1 directly reduces reactive astrogliosis and CSPGs production in the injured spinal cord, as this could be a secondary outcome of its immune-modulatory effects. It is known that microglia and other inflammatory cells induces astrocyte reactivity through pro-inflammatory cytokines such as IL-1β and TNF-α [19]. However, our *in vitro* studies, with pure astrocyte cultures, we found that Nrg-1 was able to directly attenuate astrocyte reactivity and CSPGs release. It is noteworthy to mention that the anti-CSPGs antibody utilized in this study (CS-56) detects the glycosaminoglycans (GAG) side chains of CSPG molecules [20] thus it is unable to specify which type of CSPGs are downregulated by Nrg-1. To address this shortcoming, further studies using specific antibodies against various lectican core protein are required to determine whether Nrg-1 can additionally modulate the proteoglycan

component of CSPGs. Ultimately, further work and genetic systems are required to elucidate Nrg-1 mediated mechanisms of astrocyte regulation. Interestingly, Nrg-1 treatment only moderated astrocyte reactivity and CSPG deposition in the injured spinal cord without preventing scar formation. This is a favorable outcome because astrocyte response and formation of the glial scar is also beneficial in SCI [21]. Glial scar stabilizes damaged spinal cord tissue following injury, enhances the re-establishment of BBB and BSB and limits the spread of inflammation to the intact tissue [22, 23]. Thus, while Nrg-1 treatment reduces detrimental inhibitory effects of the glial scar without compromising its beneficial effects for the injured spinal cord.

4.3 Nrg-1 and Regulation of Neuroinflammation

Previous research in our laboratory and others has established that inflammatory cells including macrophages, microglia and lymphocytes express Nrg-1 receptors [2, 24-26], indicating that dysregulation of Nrg-1 signaling can potentially affect these populations and the immune response after SCI. In the current study, we demonstrate that Nrg-1 positively influences the inflammatory processes in the post-SCI environment. Nrg-1 treatment promotes the populations of regulatory lymphocytes and M2 macrophages/microglia in the spinal cord and peripheral blood during acute and chronic phases of SCI [3]. Increase in these populations has been generally associated with better outcomes after SCI, and may underpin, at least in part, the beneficial role of Nrg-1 in improving tissue preservation and recovery of function in our studies [27-29].

4.3.1 Macrophages and Microglia

We demonstrate, for the first time, that systemic Nrg-1 treatment induced a biphasic increase in the infiltration of macrophages in the injured spinal cord. This Nrg-1 mediated increase in

macrophage population occurs at acute and chronic stages of SCI with no significant change at subacute stage. Previous studies in rodent models of peripheral nerve injury have also been associated Nrg-1 treatment with increased microglial proliferation in the dorsal horn of the spinal cord [30, 31]. Moreover, direct *in vitro* evidence has established a relationship between the activation of Nrg1/ErbB signaling and increased macrophages and microglial proliferation [30, 31]. Our phenotypical studies showed that the increase in the number of macrophages in the injured spinal cord mainly reflected as an elevation of alternatively activated M2 macrophages ($CD45^+CD68^+CD163^+IL10^+$) without any significant change in the population of classically activated M1 ($CD45^+CD68^+CD86^+$) macrophages. Increase in M2 macrophage population resulted in a remarkable increase in arginase-1 and IL-10, two M2 mediators with established positive roles in moderating neuroinflammation and secondary tissue damage following CNS injury [32-35]. IL-10 is known to enhance the process of wound healing and myelin clearance by macrophages and microglia following CNS injury [36], and attenuate glial activation and production of pro-inflammatory cytokines [37, 38]. Importantly, IL-10 promotes oligodendrocyte survival, differentiation and myelination in demyelinating conditions indicating its neuroprotective and neurodegenerative properties [39]. Our new findings in this study also provide a putative mechanism by which Nrg-1 treatment enhanced oligodendrogenesis and remyelination in our previous studies in SCI and focal demyelinating lesions [1, 18].

In our study, Nrg-1 increased arginase-1 expression in activated glial cultures and in the injured spinal cord. Up-regulation of arginase-1 in macrophages has been previously reported following IL-10 treatment [40]. Arginase-1 is involved in L-arginine metabolization, which is necessary for iNOS enzymatic activity and NO production [40-42]. Interestingly, we found a reduction in NO production in astro-microglia cultures under Nrg-1 treatment that can be explained

by the increase in arginase-1. In agreement with our findings, a recent study on peripheral blood monocytes of patients with heart failure has shown attenuation of monocyte pro-inflammatory activation following Nrg-1 treatment [25]. Overall, our results implicate a positive role for Nrg-1 in regulating macrophage response following traumatic SCI. Further studies are required to elucidate the cellular and molecular mechanisms of Nrg-1 in modulating macrophage and microglial response in SCI.

4.3.2 T cells

We found a prominent role for Nrg-1 in regulating T cells after SCI. Nrg-1 promoted a T_{reg} ($CD3^+CD4^+IL\text{-}10^+$) response in the spinal cord during the acute, subacute and chronic stages of SCI. Most interestingly, regulatory effects of Nrg-1 were not limited to spinal cord tissue as an increase in the T_{reg} population was also observed systemically during chronic stages of SCI. T_{reg} cells are essential for preventing autoimmune reaction by inhibiting the activity of autoreactive T_{eff} cells [43]. SCI induces an imbalance in the T_{eff}-T_{reg} relationship that is associated with increased pro-inflammatory cytokine production, M1 macrophage polarization and Fas mediated neuronal and oligodendroglial apoptosis [44, 45]. Interestingly, at subacute stage of SCI, Nrg-1 treatment suppressed the population of pro-inflammatory $CD3^+CD4^+IFN\text{-}\gamma^+$ effector T cells and reduced the expression of IFN-γ within the injured spinal cord. IFN-γ is implicated in inducing neural tissue degeneration, inducing neuropathic pain and increasing neuronal susceptibility to apoptosis [46-48]. Moreover, IFN-γ is a potent activator of macrophages/microglia, and has been shown to activate adult human astrocytes and induce gliosis in adult mouse brain [49]. In the absence of T_{reg} cells, CNS injury elicits an intensive T_{eff} response that results in extensive neuronal death and loss of neurologic function [50]. The cytokines produced by T_{reg} cells (IL-10 and TGF-

β) are essential for T_{eff} inhibition [45]. In addition to these cytokines, T_{reg} cells prevent T_{eff} cell activation by inhibiting antigen presentation by APCs [45]. Mechanistically, inflammatory response is regulated through a cross-talk between monocytes and T cells. It has been established that M2 macrophages can induce regulatory phenotype in T cells through production of TGF-β and IL-10 [51, 52]. Reciprocally, T_{reg} cells possess the ability to promote M2 phenotype in activated macrophages and microglia [45, 53]. Thus, increase in T_{reg} cell population by Nrg-1 treatment may represent an underlying mechanism for the positive astrocytes and microglial/macrophages response we observed in this study. The increase in M2 macrophages/microglia population with Nrg-1 treatment could be in part secondary to an increase in T_{reg} cell population [54]. Positive immunomodulatory and neuroprotective role of T_{reg} cells could be translated into an improvement of neural tissue preservation and recovery of function seen in our study [50, 55]. In conclusion, while our studies have unraveled a novel impact for Nrg-1 in regulating T cell response in SCI with potential immunotherapeutic benefits, future studies are required to elucidate the mechanism of Nrg-1/ErbB signaling on T cell phenotype and function.

4.3.3 Nrg-1 Regulation of B cells and Antibody Deposition

B cells are key regulators of adaptive immune response in SCI that work closely with T cells [45, 56]. We have provided new evidence that Nrg-1 can promote B cell recruitment to the injured spinal cord in particular at the chronic stage of injury. Interestingly, similar to T cells, Nrg-1 treatment significantly promoted B_{reg} cell population. Increase in B_{reg} cell population has several therapeutic implications. B_{reg} cells prevent tissue damage by suppressing the activation of autoreactive T cells and macrophages and their production of TNF-α and IFN-γ, two pro-inflammatory cytokines known to be involved in the secondary injury processes of SCI [57-59].

Interestingly, our study has demonstrated a reduction in these pro-inflammatory cytokines in Nrg-1 treated SCI rats. B_{reg} cells contribute to repair by promoting T_{reg} differentiation of T cells and maintaining T_{reg} cell population [60]. B_{reg} cells also contribute to the production of IL-10 that can limit neural tissue degeneration and improve functional recovery following CNS injury as it was discussed earlier [27].

In contrast to the increase in the recruitment of B cells in the injured spinal cord, we found an initial reduction in the total circulating B cells in acute phase of SCI. This systemic reduction, which was not affected by Nrg-1 treatment in our study, has been attributed to B cell apoptosis and cessation of B cell production in the bone marrow [61, 62]. At later stages, SCI causes a significant increase in B cell population in the bone marrow and spleen followed by an increase in serum immunoglobulin levels [63]. This is in line with our study where we observed an increase in circulating B cells at later stages after the initial acute reduction.

Nrg-1 treatment attenuated post-SCI antibody deposition in the spinal cord tissue. Auto-antibodies produced by autoreactive B cells promote neuroinflammation and aggravate neural tissue damage through complement or FcR dependent mechanisms [64]. In our study, Nrg-1 reduced IgG and IgM deposition during subacute stage of SCI. Hypothetically, the reduction of immunoglobulin deposition at this time-point could be reflective of an indirect role for Nrg-1 in modulating vascular permeability or enhancing BSB preservation or repair. Supporting this hypothesis, our study showed that Nrg-1 treatment attenuates the activity of MMP-9 in the injured spinal cord [2]. MMP-9 is known for its involvement in BSB disruption [65]. Downregulation of MMP-9 leads to improved BBB preservation and repair [65]. Another mechanism through which Nrg-1 could reduce antibody deposition in the injured spinal cord is the reduction of IL-6 expression which we observed in our study. IL-6 is known to promote antibody production by B

cells [66]. Reduction of IL-6 by Nrg-1 at acute stage of SCI may underlie the reduction that we detected in antibodies at later time-points without altering the B cell number. In contrast to our acute and subacute findings, Nrg-1 caused an increasing trend in IgG deposits in chronically injured spinal cord. Our flow cytometric analysis of spinal cord B cells showed a close temporal correlation between Nrg-1 induced increase in B_{reg} cell population and the elevated levels of IgG in chronic SCI. This is in line with other studies that have attributed IgG production to B_{reg} cells [67]. IgG produced by B_{reg} cells appears to be beneficial in SCI [67]. B_{reg} antibodies are shown to neutralize harmful antigens in SCI microenvironment, reduce the activation of APCs and enhance the clearance of apoptotic bodies that contain self-antigens [67]. Our observation is also in agreement with a study by Nguyen and colleagues where they showed systemic IgG administration promotes tissue preservation and improves recovery of neurological function in a rat model of compressive/contusive cervical SCI [68]. Collectively, our new evidence unravels another positive modulatory role of Nrg-1 treatment on B cells with significant neuroprotective implications. Further studies are required to elucidate whether Nrg-1 exerts a direct effect on B cells or its effects are governed indirectly thorough modulation of T cells and macrophages.

4.3.4 Nrg-1 Effects on Inflammatory Cytokines, Chemokines and Mediators

Our *in vitro* and SCI studies showed that Nrg-1 moderates the expression of TNF-α, IL-1β and IL-6 [2]. These key pro-inflammatory cytokines are produced downstream of NF-κB pathway activation, and contribute to secondary injury processes including astrocyte activation [69-71]. Following SCI, danger associated molecular patterns (DAMPs) such as heat shock protein (HSPs) , hyaluronic acid, necrotic cell particles and mRNA that are released following tissue damage, bind to TLR2 and TLR4 receptors and activate NF-κB pathway [72]. We have shown that Nrg-1

treatment reduces the expression of MyD88, an intracellular adaptor protein downstream of TLR receptors (except TLR3), which is involved in NF-κB pathway activation [73, 74]. TLRs/MyD88 pathways are activated in microglia after SCI and promote pro-inflammatory cytokine production through NF-κB pathway activation [75, 76]. Studies have also associated MyD88 downregulation with M2 phenotype induction in macrophages/microglia and decreased pro-inflammatory cytokine production [72]. Moreover, MyD88 downregulation can moderate astrocyte activation and pro-inflammatory cytokine production by reducing the activity of IL-1R/MyD88 dependent signaling pathways [77]. Therefore, Nrg-1 mediated reduction in astrogliosis in our studies may reflect the reduction in pro-inflammatory cytokine production after Nrg-1 treatment.

Nrg-1 treatment results in an acute reduction in the expression of CXCL1 following SCI. CXCL1 is a chemokine produced by mast cells, macrophages and astrocytes, which exacerbates neuroinflammation by promoting neutrophil recruitment to the injured CNS [78]. Interestingly CXCL1 expression is regulated by IL-6 [79]. In our study, Nrg-1 treatment reduced the expression of IL-6 in acute SCI suggesting a correlation between IL-6 downregulation and reduction of CXCL1 expression in the injured spinal cord tissue. Of note, IL-6 inhibition has been associated with reduced glial scarring, enhanced functional improvement and reduction of neuropathic pain in SCI studies [80, 81]. As discussed earlier, Nrg-1 also reduced the expression of IFN-γ by T_{eff} cells. Elevation in IFN-γ is associated with neuronal apoptosis and white matter degeneration following CNS injury [47, 48]. Nrg-1 treatment also attenuated the expression of CXCL2, CXCL10 and CCL5 (RANTES) that are known to promote neuronal injury, microvascular dysfunction and secondary tissue damage following SCI [82-84]. As an example, CXCL10 is produced by fibroblasts, astrocytes, macrophages and endothelial cells upon IFN-γ stimulation. Its elimination is associated with decreased neural tissue degeneration and improved functional

recovery in a mouse model of SCI [85]. CCL5 is produced by activated astrocytes and effector T cells [86, 87]. Nrg-1 induced increase in M2 macrophage population and reduction in T_{eff} cell population explains the reduction in CCL5 we observed in this study.

We show that intrathecal and systemic Nrg-1 treatment was able to elicit a remarkable increase in the spinal cord levels of IL-10. IL-10 is commonly produced by M2 macrophages and microglia, T_{reg} and B_{reg} cells and play key roles in moderating neuroinflammation following CNS injury [45, 88, 89]. Our phenotyping assessments revealed that Nrg-1 induces an IL-10 phenotype in M2 macrophages as well as regulatory B and T cell populations. As discussed earlier, IL-10 is an emerging beneficial factor that promotes regeneration process including remyelination after SCI [33, 34, 90-92]. Additionally, Nrg-1 treatment enhanced the expression of CCL11, a chemokine known for its positive immunomodulatory roles in CNS injury [93]. CCL11 is produced by macrophages, astrocytes and pericytes [94-96], and shows anti-inflammatory properties [93]. Moreover, CCL1 promotes endogenous cell replacement by stimulating proliferation and migration of neural precursor cells [97].

Mechanistically, we found a correlation between Erk1/2 activation by Nrg-1 treatment and IL-10 upregulation in our SCI studies. This observation is in agreement with other studies that established a relationship between IL-10 upregulation and Erk1/2 phosphorylation in macrophages and lymphocytes [98-100]. These studies suggest an essential role for Erk activation in IL-10 production by macrophages, T and B cells [98, 99]. We additionally observed an increase in STAT3 phosphorylation in Nrg-1 treated SCI rats. STAT3 is also implicated in IL-10 upregulation and down-regulation of TNF-α in macrophages/microglia [37, 38, 101-103]. Moreover, studies on human T cells have shown that IL-10 potentiates FoxP3 expression of human T cells through activation of STAT3 and Foxo1 transcription factors [51]. IL-10 binding to its receptor, IL-10R,

activates IL-10R/Jak1/STAT3 pathway [101]. Phosphorylated STAT3 translocates into the nucleus and activates anti-inflammatory response genes [101]. IL-10/STAT3 signaling is known to oppose the pro-inflammatory effects of IL-6 in macrophages and dendritic cells [104]. Of note, STAT3 function is complex as it can play both pro- and anti-inflammatory roles in a context-dependent manner [104]. For example, STAT3 can be anti-inflammatory when dendritic cells are stimulated by IL-10, while it is pro-inflammatory when the same cell type is stimulated by IL-6 [104]. Further work is needed to understand the mechanisms by which Nrg-1 and its signaling receptors regulate Erk and STAT3 activation in immune cells.

Altogether, with our new evidence, it is logical to postulate that Nrg-1 treatment is neuroprotective and fosters tissue preservation by altering the pro-inflammatory landscape of SCI to a pro-regenerative and protective environment.

4.4 Nrg-1 and Neuroprotection

Previous studies by our group have established a correlation between dysregulated Nrg-1 signaling and impaired endogenous precursor response after traumatic SCI [1]. Importantly, Nrg-1 treatment facilitated oligodendrogenesis and protected mature oligodendrocytes and axons in the injured spinal cord. Other studies have also demonstrated neuroprotective ability of Nrg-1 in animal models of cerebral ischemic and chemical injuries [105-108]. Nrg-1 bioavailability is known to improve neuronal preservation through inhibition of apoptosis, neuroinflammation and oxidative damage [105, 106, 109]. In cerebral ischemic models, reduced expression of caspase-3 and apoptosis was observed in Nrg-1 treated animals following reperfusion [107, 110]. Interestingly, our findings confirm these studies by showing a significant improvement in spinal cord tissue preservation in chronic SCI [2]. Evidence suggests that Nrg-1 mediates its neuroprotective effects

through ErbB4 receptor signaling and activation of CDK5/Akt/PI3 kinase pathway [106, 108]. In animal models of chemically induced cerebral injury, Akt phosphorylation was observed in both neurons and glial cells following Nrg-1 treatment [106]. MAPK/ERK is another survival pathway that is activated by Nrg-1 signaling network [111]. The role of Erk signaling pathway in the pathophysiology of SCI is not well understood due to the complexity and controversial findings [112, 113]. Activation of ERK signaling pathway has been generally associated with improved neuronal survival [114]. Studies also have implicated MAPK/ERK signaling in initiation of myelination and regulation of myelin sheath thickness [115]. However, recent evidence has implicated Erk pathway as a pro-apoptotic pathway following SCI [112, 116, 117]. Our SCI study showed a significant increase in the phosphorylation of Erk1/2 following Nrg-1 treatment compared to non-treated SCI rats [2]. However, this increase was associated with neuroprotective effects. Thus, our findings are in agreement with the studies that suggest a positive neuroprotective role for Erk1/2 activity [114, 115].

In conclusion, our study demonstrates a neuroprotective role for Nrg-1 and suggests that Nrg-1/ErbB signaling is a potential therapeutic candidate for CNS injuries and neurodegenerative diseases. However, due to the complexity of Nrg-1associated pathways, further studies are needed to unveil the underlying mechanisms of Nrg-1 effects following SCI.

4.5 Functional Outcomes of Nrg-1 Treatment in SCI

In the field of SCI research, effectiveness of an experimental treatment is determined by its functional outcomes. Here, we demonstrate that the multifaceted beneficial roles of Nrg-1 in regulating glial and immune responses can be translated into a meaningful functional recovery. Our longitudinal locomotion assessment showed that Nrg-1 promotes locomotor recovery without

affecting nociception. While vehicle treated SCI rats never regained weight support in their hind-limbs, Nrg-1 treatment resulted in regaining frequent to consistent weight supported plantar stepping in SCI rats. This is the first study that has shown the promise of Nrg-1 treatment in functional recovery from SCI.

Nrg-1 effect on pain sensation is controversial. A study by Tao and colleagues showed that transplanted OPCs can reduce pain sensation in SCI rats by producing Nrg-1 and promoting remyelination [118]. This beneficial effect was abolished after Nrg-1 downregulation using siRNA approaches [118]. However, this study did not test the effect of exogenous Nrg-1 on nociception. Our analysis of pain sensation showed no significant change in nociception in Nrg-1 treated compared to vehicle treated SCI rats. Other studies have shown contradicting results in the same context. A study by Calvo et al. in 2011 showed that Nrg-1/ErbB signaling induces neuropathic pain and promotes microgliosis following peripheral nerve injury [31]. In our *in vitro* assessments, we found no increase in microglial proliferation following Nrg-1 treatment. In contrast, our flow cytometric assessment of injured spinal cord tissue showed a slight but significant increase in macrophage recruitment following Nrg-1 treatment at acute and chronic stages of SCI. However, Nrg-1 treatment was not associated with any change in pain sensation in chronic SCI rats. Overall, our study did not find any association between Nrg-1 treatment and hyperalgesia following SCI. Discrepancies among these studies might reflect differential mechanisms of Nrg-1 in peripheral nerve injury and SCI.

4.6 Clinical Implications

SCI is a life changing event with devastating socioeconomic burdens to the patients, their families and the health care system [119]. In Canada, more than 85,000 patients live with SCI and 4000

new cases are added each year [119, 120]. As SCI mainly happens in young adults and the patients usually live with disability for their lifetime, the total cost of SCI is estimated to reach $1.5 million for a paraplegic and $3 million for a tetraplegic patient [121]. Despite extensive research, no effective treatment has evolved for SCI yet. The only approved treatment in clinical setting is methylprednisolone sodium succinate (MPSS) [122-126]. MPSS has limited therapeutic efficacy for SCI patients and inflicts numerous morbidities due to its side-effects and complications [122-126]. Patients treated with MPSS are five times more likely to require intensive care in the course of hospitalization [127]. They are also at a greater risk for developing various types of infections [127]. Altogether, MPSS side effects increase the risk and cost of SCI management for the patients and the health care system [127]. Given the socioeconomic impact of SCI and lack of appropriate treatment strategy, it is critical to develop new effective treatments for this devastating condition.

In our current study, we have utilized a clinically relevant model of compressive-contusive SCI in the rat, which is designed to mimic the pathophysiology of human SCI [128-130]. Our model simulates the most common mechanism of human SCI in which a burst fracture primary injury is followed by a persistent compression by the fractured vertebral fragments [131, 132]. Due to the complexity of SCI pathophysiology, it is now well established that an effective treatment strategy needs to target multiple aspects of the secondary injury processes to foster meaningful repair and functional recovery [5, 133, 134]. Thus, developing multifaceted therapeutic approaches is a top priority in SCI therapeutic development. Nrg-1 is a growth factor with multiple essential roles in neurodevelopment [135, 136]. In recent years, compelling evidence from our laboratory and others has demonstrated multiple positive roles for Nrg-1 in brain and spinal cord injuries [1-3]. Our group has shown that Nrg-1 treatment, enhances endogenous oligodendrocyte preservation, replacement and remyelination [1, 18]. Progressive demyelination following SCI

contributes to axonal dysfunction and neurological deficits in SCI patients [137-142]. The imbalanced glial activation and hostile inflammatory milieu with a host of inhibitory and toxic components such as CPSGs, pro-inflammatory cytokines, ROS, and autoantibodies create an anti-regenerative microenvironment that hinders the ability of exogenous and endogenous cells in repair and recovery after SCI [2, 15, 143, 144]. Of note, clinical trials aiming to improve functional recovery by transplanting exogenous NPCs in humans have gained suboptimal results due to the impermissible microenvironment of SCI [5, 139, 145, 146]. Interestingly, our therapeutic approach with Nrg-1 treatment addresses multiple aspects of SCI pathophysiology (Fig. 4.1) [2, 3]. Our research has demonstrated the beneficial outcomes of Nrg-1 treatment in SCI by attenuating the glial scar and particularly CSPGs after SCI [2]. CSPGs are well-known for their inhibitory properties in endogenous and exogenous cell replacement and axonal regeneration [5, 11, 15, 147]. Previous studies from our laboratory and others have shown significant improvements in endogenous and exogenous cell replacement and functional recovery following degradation of CSPGs or inhibition of their signaling pathways [4, 5, 15]. Hence, the ability of Nrg-1 treatment in reducing CSPGs provides a great therapeutic potential for optimizing cell replacement in endogenous or cell therapeutic strategies for SCI.

Importantly, Nrg-1 presents a viable treatment strategy for SCI due to its safety and feasibility. A clinical grade of Nrg-1 is approved by the food and drug administration (FDA) for use in clinical trials on congestive heart failure [148, 149]. Moreover, Nrg-1 is shown to readily pass the blood-brain and blood-spinal cord barriers making it feasible for systemic delivery [150]. However, although we have not observed any obvious side effects caused by Nrg-1 in our animal models, studies have reported side effects such as nausea and vomiting following administration of higher doses of Nrg-1 in humans [148]. To avoid any potential off-target side effects of Nrg-1

in systemic deliveries, our laboratory has recently developed a novel method for local intraspinal delivery of Nrg-1 using poly lactic-co-glycolic acid (PLGA) microparticles [151]. This system has shown to be a promising approach for sustained and controlled delivery of Nrg-1 in SCI.

In summary, this thesis has provided novel scientific evidence that supports a multifaceted positive role for Nrg-1 in moderating neuroinflammation and glial scar formation following traumatic SCI (Fig. 4.1). We demonstrated that intrathecal and systemic administration of Nrg-1 can induce a pro-regenerative response in glial and immune cells that fosters a supportive microenvironment for repair and regeneration. Importantly, our study has introduced a novel clinically relevant therapeutic approach to address multiple aspects of SCI pathophysiology that can be potentially used in other CNS neuroinflammatory conditions such as multiple sclerosis.

4.7 Study Limitations

Similar to any scientific research, our work is not free of limitations. Identifying these limitations aids in better interpretation of our findings and provides a roadmap for future investigations.

One limitation in this study is the inherent complexity of Nrg-1/ErbB signaling cascade. ErbB receptors work as hetero- and homodimers, and share structural and functional similarities with overlapping downstream signaling pathways [152], which makes study of this network a challenging task. While we attempted pharmacological inhibition of ErbB receptors as well as genetic manipulation of these receptors with siRNA and CRISPR approaches, it was challenging to study ErbB receptors individually as changes in one receptor altered other members of this network. To address this problem more specific loss or gain of function of these pathways needs to be assessed in genetically engineered rodent models and in cell-specific *in vitro* settings to further elucidate the mechanisms behind Nrg-1 signaling in different immune and glial cell types.

Another limitation of our study is that Nrg-1 treatment was delivered shortly (within 15 minutes) after the injury. It would be more clinically relevant to study the effects of Nrg-1 delivery at later time-points to better simulate the timing of such therapies in SCI management in clinical settings. While delayed Nrg-1 administration was not tested in this thesis, based on our findings, we anticipate that delayed Nrg-1 would be effective as well.

A technical limitation of our flow cytometry approach is the low number of immune cells detected in the spinal cord tissue. This limitation becomes more prominent in detection of immune cell sub-types such as IL-10 expressing T_{reg} cells that are normally less frequent among T cell populations. We addressed this limitation by implementing proper negative and positive controls such as unstained, uninjured and isotype controls in our study to ensure that the detected cells in the injured tissue are specific. However, further studies using fluorescence minus one (FMO) negative controls, fluorescence-activated cell sorting (FACS) followed by culturing and stimulation of cytokine expressing cells can be conducted to increase the specificity of the detection of cytokine expressing cells using flow cytometry.

Last but not least, studies have shown that Nrg-1 can modulate neuronal excitability [153]. The functional recovery observed in our study could be partially due to an increased excitability of spinal locomotor centers responding to tactile stimulus. This necessitates further electrophysiological studies to elucidate the effects of Nrg-1 on the excitability of spinal neural circuitry and its contribution to the improvement of motor function.

4.8 Future Directions

My PhD thesis has identified the promise of Nrg-1 therapy in moderating glial scar formation and neuroinflammation, two major pathophysiological events in SCI. Multifaceted effects of Nrg-1 in

fostering a permissive microenvironment in SCI for repair and regeneration can be used to optimize endogenous cell replacement. Based on the results of this thesis and our previous studies [1], it is anticipated that Nrg-1 treatment would provide a supportive environment to improve survival of oligodendrocytes and promote the differentiation of endogenous precursor cells in the injured spinal cord. Moreover, our study showed the beneficial effects of systemically delivered Nrg-1 treatment in rats, which represents a feasible route for Nrg-1 delivery to patients. Moreover, inflammation and glial scarring are shared pathologies among several CNS conditions. Given our findings in SCI, we propose that Nrg-1 encompasses several characteristics that makes it a potential immunotherapy for CNS autoimmune conditions such as multiple sclerosis.

Therapeutically, to take this novel treatment one step closer to clinical application, it would be reasonable to validate our results in larger animal models such as pigs or primates. Functional outcomes in these larger animal models could be assessed using electrophysiological approaches such as transcranial magnetic stimulation [154] and electromyography (EMG) as well as behavioural tests such as Nout's scale or modified Tarlov scale [155].

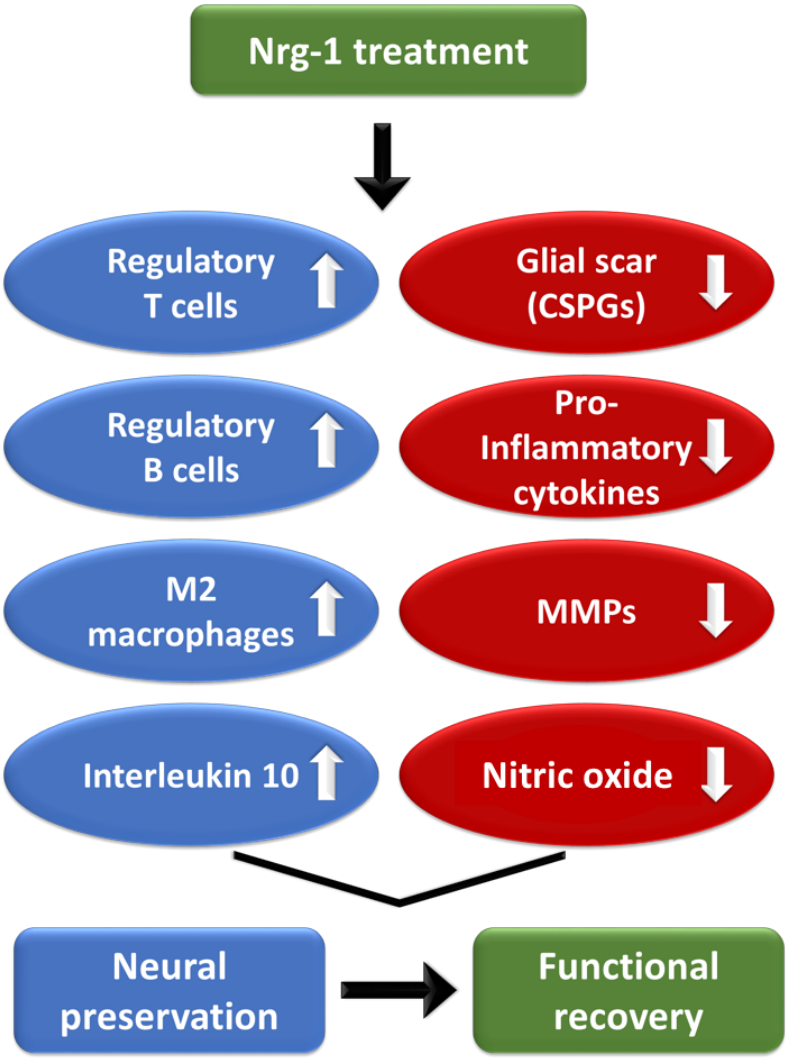


Fig. 4.1: Summary of Nrg-1 effects in modulating secondary injury processes following SCI. We have shown that Nrg-1 improves neural preservation and functional recovery through a multifaceted mechanism which is depicted above and includes fostering a pro-regenerative immune response and moderating glial scar formation.

4.9 References

1. Gauthier, M.-K., et al., *Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury.* Eur. J. Neurosci, 2013. **38**(5): p. 2693-2715.
2. Alizadeh, A., et al., *Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury.* Glia, 2017. **65**(7): p. 1152-1175.
3. Alizadeh, A., et al., *Neuregulin-1 elicits a regulatory immune response following traumatic spinal cord injury.* Journal of Neuroinflammation, 2018. **15**(1).
4. Dyck, S.M. and S. Karimi-Abdolrezaee, *Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system.* Exp Neurol, 2015. **269**: p. 169-87.
5. Karimi-Abdolrezaee, S., et al., *Synergistic Effects of Transplanted Adult Neural Stem/Progenitor Cells, Chondroitinase, and Growth Factors Promote Functional Repair and Plasticity of the Chronically Injured Spinal Cord.* J. Neurosci, 2010. **30**(5): p. 1657-1676.
6. Fitch, M.T. and J. Silver, *CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure.* Exp Neurol, 2007.
7. Buss, A., et al., *NG2 and phosphacan are present in the astroglial scar after human traumatic spinal cord injury.* BMC Neurol, 2009. **9**: p. 32.
8. Grimpe, B. and J. Silver, *A Novel DNA Enzyme Reduces Glycosaminoglycan Chains in the Glial Scar and Allows Microtransplanted Dorsal Root Ganglia Axons to Regenerate beyond Lesions in the Spinal Cord* J Neurosci, 2004. **24**(6): p. 1393-1397.

9. Levine, J.M., *Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury.* J Neurosci, 1994. **14**(8): p. 4716-30.

10. Asher, R.A., et al., *Neurocan is upregulated in injured brain and in cytokine-treated astrocytes.* J Neurosci, 2000. **20**(7): p. 2427-38.

11. Barritt, A.W., et al., *Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury.* J Neurosci, 2006. **26**(42): p. 10856-67.

12. Alilain, W.J., et al., *Functional regeneration of respiratory pathways after spinal cord injury.* Nature, 2011. **475**(7355): p. 196-200.

13. Tom, V.J., et al., *Combining Peripheral Nerve Grafts and Chondroitinase Promotes Functional Axonal Regeneration in the Chronically Injured Spinal Cord.* J. Neurosci, 2009. **29**(47): p. 14881-14890.

14. Lang, B.T., et al., *Modulation of the proteoglycan receptor PTPsigma promotes recovery after spinal cord injury.* Nature, 2015. **518**(7539): p. 404-8.

15. Dyck, S.M., et al., *Chondroitin Sulfate Proteoglycans Negatively Modulate Spinal Cord Neural Precursor Cells by Signaling through LAR and RPTPsigma and modulation of the Rho/ROCK Pathway.* Stem Cells, 2015.

16. Karimi-Abdolrezaee, S., et al., *Chondroitinase and Growth Factors Enhance Activation and Oligodendrocyte Differentiation of Endogenous Neural Precursor Cells after Spinal Cord Injury.* Plos One, 2012. **7**(5): p. 1-16.

17. Lau, L.W., et al., *Chondroitin sulfate proteoglycans in demyelinated lesions impair remyelination.* Ann Neurol, 2012. **72**(3): p. 419-32.

18. Kataria, H., et al., *Neuregulin-1 promotes remyelination and fosters a pro-regenerative inflammatory response in focal demyelinating lesions of the spinal cord.* Glia, 2017.

19. Cui, M., et al., *FOXO3a inhibits TNF-alpha- and IL-1beta-induced astrocyte proliferation:Implication for reactive astrogliosis.* Glia, 2011. **59**(4): p. 641-54.

20. Ito, Y., et al., *Structural characterization of the epitopes of the monoclonal antibodies 473HD, CS-56, and MO-225 specific for chondroitin sulfate D-type using the oligosaccharide library.* Glycobiology, 2005. **15**(6): p. 593-603.

21. Cregg, J.M., et al., *Functional regeneration beyond the glial scar.* Exp Neurol, 2014. **253**: p. 197-207.

22. Bush, T.G., et al., *Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice.* Neuron, 1999. **23**(2): p. 297-308.

23. Faulkner, J.R., et al., *Reactive Astrocytes Protect Tissue and Preserve Function after Spinal Cord Injury.* J Neurosci, 2004. **24**(9): p. 2143-2155.

24. Cannella, B., et al., *Neuregulin and erbB receptor expression in normal and diseased human white matter.* J Neuroimmunol, 1999. **100**(1-2): p. 233-42.

25. Ryzhov, S., et al., *ERBB signaling attenuates proinflammatory activation of nonclassical monocytes.* Am J Physiol Heart Circ Physiol, 2017. **312**(5): p. H907-H918.

26. Tynyakov-Samra, E., et al., *Reduced ErbB4 Expression in Immune Cells of Patients with Relapsing Remitting Multiple Sclerosis.* Mult Scler Int, 2011. **2011**: p. 561262.

27. Ren, X., et al., *Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke.* J Neurosci, 2011. **31**(23): p. 8556-63.

28. Walsh, J.T. and J. Kipnis, *Regulatory T cells in CNS injury: the simple, the complex and the confused.* Trends Mol Med, 2011. **17**(10): p. 541-7.

29. Shechter, R., et al., *Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice.* PLoS Med, 2009. **6**(7): p. e1000113.

30. Calvo, M., et al., *Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury.* J Neurosci, 2010. **30**(15): p. 5437-50.

31. Calvo, M., et al., *Following nerve injury neuregulin-1 drives microglial proliferation and neuropathic pain via the MEK/ERK pathway.* Glia, 2011. **59**(4): p. 554-68.

32. David, S. and A. Kroner, *Repertoire of microglial and macrophage responses after spinal cord injury.* Nat Rev Neurosci, 2011. **12**(7): p. 388-99.

33. Brewer, K.L., J.R. Bethea, and R.P. Yezierski, *Neuroprotective effects of interleukin-10 following excitotoxic spinal cord injury.* Exp Neurol, 1999. **159**(2): p. 484-93.

34. Zhou, Z., et al., *IL-10 promotes neuronal survival following spinal cord injury.* Exp Neurol, 2009. **220**(1): p. 183-90.

35. Zhang, B., et al., *Age decreases macrophage IL-10 expression: Implications for functional recovery and tissue repair in spinal cord injury.* Exp Neurol, 2015. **273**: p. 83-91.

36. Puntambekar, S.S., et al., *Interleukin-10 is a critical regulator of white matter lesion containment following viral induced demyelination.* Glia, 2015. **63**(11): p. 2160-2120.

37. Fiorentino, D.F., et al., *IL-10 inhibits cytokine production by activated macrophages.* J Immunol, 1991. **147**(11): p. 3815-22.

38. Balasingam, V. and V.W. Yong, *Attenuation of astroglial reactivity by interleukin-10.* J Neurosci, 1996. **16**(9): p. 2945-55.

39. Miron, V.E., et al., *M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination.* Nat Neurosci, 2013. **16**(9): p. 1211-8.

40. Lang, R., et al., *Shaping gene expression in activated and resting primary macrophages by IL-10.* J Immunol, 2002. **169**(5): p. 2253-63.

41. Corraliza, I.M., et al., *Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages.* Biochem Biophys Res Commun, 1995. **206**(2): p. 667-73.

42. Chang, C.I., J.C. Liao, and L. Kuo, *Arginase modulates nitric oxide production in activated macrophages.* Am J Physiol, 1998. **274**(1 Pt 2): p. H342-8.

43. Zhou, R., et al., *The living eye "disarms" uncommitted autoreactive T cells by converting them to Foxp3(+) regulatory cells following local antigen recognition.* J Immunol, 2012. **188**(4): p. 1742-50.

44. Yu, W.R. and M.G. Fehlings, *Fas/FasL-mediated apoptosis and inflammation are key features of acute human spinal cord injury: implications for translational, clinical application.* Acta Neuropathol, 2011. **122**(6): p. 747-61.

45. Jones, T.B., *Lymphocytes and autoimmunity after spinal cord injury.* Exp Neurol, 2014. **258**: p. 78-90.

46. Tsuda, M., et al., *IFN-gamma receptor signaling mediates spinal microglia activation driving neuropathic pain.* Proc Natl Acad Sci U S A, 2009. **106**(19): p. 8032-7.

47. Bate, C., et al., *Interferon-gamma increases neuronal death in response to amyloid-beta1-42.* J Neuroinflammation, 2006. **3**: p. 7.

48. Yilmaz, G., et al., *Role of T lymphocytes and interferon-gamma in ischemic stroke.* Circulation, 2006. **113**(17): p. 2105-12.

49. Yong, V.W., et al., *Gamma-interferon promotes proliferation of adult human astrocytes in vitro and reactive gliosis in the adult mouse brain in vivo.* Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7016-20.

50. Walsh, J.T., et al., *Regulatory T cells in central nervous system injury: a double-edged sword.* J Immunol, 2014. **193**(10): p. 5013-22.

51. Hsu, P., et al., *IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1.* J Immunol, 2015. **195**(8): p. 3665-74.

52. Schmidt, A., et al., *Human macrophages induce CD4(+)Foxp3(+) regulatory T cells via binding and re-release of TGF-beta.* Immunol Cell Biol, 2016. **94**(8): p. 747-62.

53. Gensel, J.C., et al., *Macrophages promote axon regeneration with concurrent neurotoxicity.* J Neurosci, 2009. **29**(12): p. 3956-68.

54. Tiemessen, M.M., et al., *CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19446-51.

55. Arpaia, N., et al., *A Distinct Function of Regulatory T Cells in Tissue Protection.* Cell, 2015. **162**(5): p. 1078-89.

56. Ankeny, D.P. and P.G. Popovich, *B cells and autoantibodies: complex roles in CNS injury.* Trends Immunol, 2010. **31**(9): p. 332-8.

57. Bouaziz, J.D., et al., *IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro.* Eur J Immunol, 2010. **40**(10): p. 2686-91.

58. Blair, P.A., et al., *CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients.* Immunity, 2010. **32**(1): p. 129-40.

59. Iwata, Y., et al., *Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.* Blood, 2011. **117**(2): p. 530-41.

60. Pennati, A., et al., *Regulatory B cells induce formation of IL-10 expressing T cells in mice with autoimmune neuroinflammation.* J Neurosci, 2016.

61. Oropallo, M.A., R. Goenka, and M.P. Cancro, *Spinal cord injury impacts B cell production, homeostasis, and activation.* Semin Immunol, 2014. **26**(5): p. 421-7.

62. Lucin, K.M., V.M. Sanders, and P.G. Popovich, *Stress hormones collaborate to induce lymphocyte apoptosis after high level spinal cord injury.* J Neurochem, 2009. **110**(5): p. 1409-21.

63. Ankeny, D.P., et al., *Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis.* J Neurochem, 2006. **99**(4): p. 1073-87.

64. Ankeny, D.P., Z. Guan, and P.G. Popovich, *B cells produce pathogenic antibodies and impair recovery after spinal cord injury in mice.* J Clin Invest, 2009. **119**(10): p. 2990-9.

65. Noble, L.J., et al., *Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events.* J Neurosci, 2002. **22**(17): p. 7526-35.

66. Maeda, K., et al., *IL-6 increases B-cell IgG production in a feed-forward proinflammatory mechanism to skew hematopoiesis and elevate myeloid production.* Blood, 2010. **115**(23): p. 4699-706.

67. Mizoguchi, A. and A.K. Bhan, *A case for regulatory B cells.* J Immunol, 2006. **176**(2): p. 705-10.

68. Nguyen, D.H., et al., *Immunoglobulin G (IgG) attenuates neuroinflammation and improves neurobehavioral recovery after cervical spinal cord injury.* J Neuroinflammation, 2012. **9**: p. 224.

69. Tarassishin, L., H.S. Suh, and S.C. Lee, *LPS and IL-1 differentially activate mouse and human astrocytes: role of CD14.* Glia, 2014. **62**(6): p. 999-1013.

70. Esposito, E. and S. Cuzzocrea, *Anti-TNF therapy in the injured spinal cord.* Trends Pharmacol Sci, 2011. **32**(2): p. 107-15.

71. Lawrence, T., *The nuclear factor NF-kappaB pathway in inflammation.* Cold Spring Harb Perspect Biol, 2009. **1**(6): p. a001651.

72. Yao, A.H., et al., *Early Blockade of TLRs MyD88-Dependent Pathway May Reduce Secondary Spinal Cord Injury in the Rats.* Evid Based Complement Alternat Med, 2012. **2012**: p. 591298.

73. Kielian, T., *Toll-like receptors in central nervous system glial inflammation and homeostasis.* J Neurosci Res, 2006. **83**(5): p. 711-30.

74. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

75. Kigerl, K.A., et al., *Toll-like receptor (TLR)-2 and TLR-4 regulate inflammation, gliosis, and myelin sparing after spinal cord injury.* J Neurochem, 2007. **102**(1): p. 37-50.

76. Babcock, A.A., H. Toft-Hansen, and T. Owens, *Signaling through MyD88 regulates leukocyte recruitment after brain injury.* J Immunol, 2008. **181**(9): p. 6481-90.

77. Pineau, I., et al., *Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion.* Brain Behav Immun, 2010. **24**(4): p. 540-53.

78. De Filippo, K., et al., *Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation.* Blood, 2013. **121**(24): p. 4930-7.

79. Roy, M., et al., *CXCL1 can be regulated by IL-6 and promotes granulocyte adhesion to brain capillaries during bacterial toxin exposure and encephalomyelitis.* J Neuroinflammation, 2012. **9**: p. 18.

80. Nakamura, M., et al., *Role of IL-6 in spinal cord injury in a mouse model.* Clin Rev Allergy Immunol, 2005. **28**(3): p. 197-204.

81. Guptarak, J., et al., *Inhibition of IL-6 signaling: A novel therapeutic approach to treating spinal cord injury pain.* Pain, 2013. **154**(7): p. 1115-28.

82. Terao, S., et al., *Blood cell-derived RANTES mediates cerebral microvascular dysfunction, inflammation, and tissue injury after focal ischemia-reperfusion.* Stroke, 2008. **39**(9): p. 2560-70.

83. De Paola, M., et al., *Chemokine MIP-2/CXCL2, acting on CXCR2, induces motor neuron death in primary cultures.* Neuroimmunomodulation, 2007. **14**(6): p. 310-6.

84. Luster, A.D., J.C. Unkeless, and J.V. Ravetch, *Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins.* Nature, 1985. **315**(6021): p. 672-6.

85. Gonzalez, R., et al., *Therapeutic neutralization of CXCL10 decreases secondary degeneration and functional deficit after spinal cord injury in mice.* Regen Med, 2007. **2**(5): p. 771-83.

86. Swanson, B.J., et al., *RANTES production by memory phenotype T cells is controlled by a posttranscriptional, TCR-dependent process.* Immunity, 2002. **17**(5): p. 605-15.

87. Choi, S.S., et al., *Human astrocytes: secretome profiles of cytokines and chemokines.* PLoS One, 2014. **9**(4): p. e92325.

88. Liu, F., et al., *Role of IL-10-producing regulatory B cells in modulating T-helper cell immune responses during silica-induced lung inflammation and fibrosis.* Sci Rep, 2016. **6**: p. 28911.

89. Chung, E.Y., et al., *Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1.* Immunity, 2007. **27**(6): p. 952-64.

90. Didangelos, A., et al., *Regulation of IL-10 by Chondroitinase ABC Promotes a Distinct Immune Response following Spinal Cord Injury.* J. Neurosci, 2014. **34**(49): p. 16424-16432.

91. Bethea, J.R., et al., *Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats.* J Neurotrauma, 1999. **16**(10): p. 851-63.

92. Thompson, C.D., et al., *The therapeutic role of interleukin-10 after spinal cord injury.* J Neurotrauma, 2013. **30**(15): p. 1311-24.

93. Adzemovic, M.Z., et al., *Expression of Ccl11 associates with immune response modulation and protection against neuroinflammation in rats.* PLoS One, 2012. **7**(7): p. e39794.

94. Kovac, A., M.A. Erickson, and W.A. Banks, *Brain microvascular pericytes are immunoactive in culture: cytokine, chemokine, nitric oxide, and LRP-1 expression in response to lipopolysaccharide.* J Neuroinflammation, 2011. **8**: p. 139.

95. Parajuli, B., et al., *CCL11 enhances excitotoxic neuronal death by producing reactive oxygen species in microglia.* Glia, 2015. **63**(12): p. 2274-84.

96. Yang, L.P., X.A. Zhu, and M.O. Tso, *Minocycline and sulforaphane inhibited lipopolysaccharide-mediated retinal microglial activation.* Mol Vis, 2007. **13**: p. 1083-93.

97. Wang, F., et al., *CCL11 promotes migration and proliferation of mouse neural progenitor cells.* Stem Cell Res Ther, 2017. **8**(1): p. 26.

98. Chanteux, H., et al., *LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms.* Respir Res, 2007. **8**: p. 71.

99. Liu, B.S., et al., *TLR-mediated STAT3 and ERK activation controls IL-10 secretion by human B cells.* Eur J Immunol, 2014. **44**(7): p. 2121-9.

100. Saraiva, M., et al., *Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose.* Immunity, 2009. **31**(2): p. 209-19.

101. Hutchins, A.P., D. Diez, and D. Miranda-Saavedra, *The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges.* Brief Funct Genomics, 2013. **12**(6): p. 489-98.

102. Hedrich, C.M. and J.H. Bream, *Cell type-specific regulation of IL-10 expression in inflammation and disease.* Immunol Res, 2010. **47**(1-3): p. 185-206.

103. Marchant, A., et al., *Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia.* Eur J Immunol, 1994. **24**(5): p. 1167-71.

104. Braun, D.A., M. Fribourg, and S.C. Sealfon, *Cytokine response is determined by duration of receptor and signal transducers and activators of transcription 3 (STAT3) activation.* J Biol Chem, 2013. **288**(5): p. 2986-93.

105. Xu, Z., et al., *Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke.* Biochem Biophys Res Commun, 2004. **322**(2): p. 440-6.

106. Li, Y., et al., *Neuregulin-1 is neuroprotective in a rat model of organophosphate-induced delayed neuronal injury.* Toxicol Appl Pharmacol, 2012. **262**(2): p. 194-204.

107. Guo, W.P., et al., *Neuroprotective effects of neuregulin-1 in rat models of focal cerebral ischemia.* Brain Res, 2006. **1087**(1): p. 180-5.

108. Croslan, D.R., et al., *Neuroprotective effects of neuregulin-1 on B35 neuronal cells following ischemia.* Brain Res, 2008. **1210**: p. 39-47.

109. Iaci, J.F., et al., *Glial growth factor 2 promotes functional recovery with treatment initiated up to 7 days after permanent focal ischemic stroke.* Neuropharmacology, 2010. **59**(7-8): p. 640-9.

110. Shyu, W.C., et al., *Neuregulin-1 reduces ischemia-induced brain damage in rats.* Neurobiol Aging, 2004. **25**(7): p. 935-44.

111. Kovacs, T., et al., *Neuregulin 1-induced AKT and ERK phosphorylation in patients with fragile X syndrome (FXS) and intellectual disability associated with obstetric complications.* J Mol Neurosci, 2014. **54**(1): p. 119-24.

112. Liu, T., et al., *Upregulated Ras/Raf/ERK1/2 signaling pathway: a new hope in the repair of spinal cord injury.* Neural Regen Res, 2015. **10**(5): p. 792-6.

113. Yu, C.G., et al., *Involvement of ERK2 in traumatic spinal cord injury.* J Neurochem, 2010. **113**(1): p. 131-42.

114. Fahlman, C.S., et al., *Activation of the neuroprotective ERK signaling pathway by fructose-1,6-bisphosphate during hypoxia involves intracellular Ca2+ and phospholipase C.* Brain Res, 2002. **958**(1): p. 43-51.

115. Ashii, A., et al., *ERK1/ERK2 MAPK Signaling is Required to Increase Myelin Thickness Independent of Oligodendrocyte Differentiation and Initiation of Myelination.* J. Neurosci, 2012. **32**(26): p. 8855-8864.

116. von Gise, A., et al., *Apoptosis suppression by Raf-1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals.* Mol Cell Biol, 2001. **21**(7): p. 2324-36.

117. Yang, K., et al., *Up-regulation of Ras/Raf/ERK1/2 signaling impairs cultured neuronal cell migration, neurogenesis, synapse formation, and dendritic spine development.* Brain Struct Funct, 2013. **218**(3): p. 669-82.

118. Tao, F., et al., *Role of Neuregulin-1/ErbB Signaling in Stem Cell Therapy for Spinal Cord Injury-induced Chronic Neuropathic Pain.* Stem Cells, 2012.

119. WHO, *WHO | Spinal cord injury.* WHO, 2013. **Fact sheet N°384**.

120. Noonan, V.K., et al., *Incidence and prevalence of spinal cord injury in Canada: a national perspective.* Neuroepidemiology, 2012. **38**(4): p. 219-26.

121. Singh, A., et al., *Global prevalence and incidence of traumatic spinal cord injury.* Clin Epidemiol, 2014. **6**: p. 309-31.

122. Hugenholtz, H., *Methylprednisolone for acute spinal cord injury: not a standard of care.* CMAJ, 2003. **168**(9): p. 1145-6.

123. Fehlings, M.G., *Summary statement: the use of methylprednisolone in acute spinal cord injury.* Spine, 2001. **26**(24 Suppl): p. S55.

124. Bracken, M.B., et al., *Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study.* Jama, 1997. **277**(20): p. 1597-604.

125. Bracken, M.B., et al., *Methylprednisolone or naloxone treatment after acute spinal cord injury: 1-year follow-up data. Results of the second National Acute Spinal Cord Injury Study.* J Neurosurg, 1992. **76**(1): p. 23-31.

126. Bracken, M., et al., *A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study.* New England Journal of Medicine, 1990. **322**(20): p. 1405-1411.

127. Aguayo, J.M.D.T., *Side effects of steroid use in patients with traumatic spinal cord injury.* Coluna/Columna, 2015. **14**(1): p. 45-49.

128. Cheriyan, T., et al., *Spinal cord injury models: a review.* Spinal Cord, 2014. **52**(8): p. 588-95.

129. Rivlin, A.S. and C.H. Tator, *Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat.* Surg Neurol, 1978. **10**(1): p. 38-43.

130. Poon, P.C., et al., *Clip compression model is useful for thoracic spinal cord injuries: histologic and functional correlates.* Spine (Phila Pa 1976), 2007. **32**(25): p. 2853-9.

131. Dumont, R.J., et al., *Acute spinal cord injury, part I: pathophysiologic mechanisms.* Clin Neuropharmacol, 2001. **24**(5): p. 254-64.

132. Fehlings, M.G., et al., *Perioperative and delayed complications associated with the surgical treatment of cervical spondylotic myelopathy based on 302 patients from the*

AOSpine North America Cervical Spondylotic Myelopathy Study. J Neurosurg Spine, 2012.

133. Alluin, O., et al., *Examination of the Combined Effects of Chondroitinase ABC, Growth Factors and Locomotor Training following Compressive Spinal Cord Injury on Neuroanatomical Plasticity and Kinematics.* PLoS One, 2014. **9**(10): p. e111072.

134. Marsh, B.C., et al., *Movement rehabilitation after spinal cord injuries: emerging concepts and future directions.* Brain Res Bull, 2011. **84**(4-5): p. 327-36.

135. Falls, D.L., *Neuregulins: functions, forms, and signaling strategies.* Exp Cell Res, 2003. **284**(1): p. 14-30.

136. Birchmeier, C. and D.L. Bennett, *Neuregulin/ErbB Signaling in Developmental Myelin Formation and Nerve Repair.* Curr Top Dev Biol, 2016. **116**: p. 45-64.

137. Alluin, O., et al., *Kinematic study of locomotor recovery after spinal cord clip compression injury in rats.* J Neurotrauma, 2011. **28**(9): p. 1963-81.

138. Sinha, K., et al., *Functional changes in genetically dysmyelinated spinal cord axons of shiverer mice: role of juxtaparanodal Kv1 family K+ channels.* J Neurophysiol, 2006. **95**(3): p. 1683-95.

139. Karimi-Abdolrezaee, S., et al., *Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury.* J Neurosci, 2006. **26**(13): p. 3377-89.

140. Eftekharpour, E., et al., *Structural and functional alterations of spinal cord axons in adult Long Evans Shaker (LES) dysmyelinated rats.* Exp Neurol, 2005. **193**(2): p. 334-49.

141. Karimi-Abdolrezaee, S., E. Eftekharpour, and M.G. Fehlings, *Temporal and spatial patterns of Kv1.1 and Kv1.2 protein and gene expression in spinal cord white matter*

after acute and chronic spinal cord injury in rats: implications for axonal pathophysiology after neurotrauma. Eur J Neurosci, 2004. **19**(3): p. 577-89.

142. Nashmi, R. and M.G. Fehlings, *Mechanisms of axonal dysfunction after spinal cord injury: with an emphasis on the role of voltage-gated potassium channels.* Brain Res Brain Res Rev, 2001. **38**(1-2): p. 165-91.

143. Karimi-Abdolrezaee, S. and R. Billakanti, *Reactive Astrogliosis after Spinal Cord Injury-Beneficial and Detrimental Effects.* Mol. Neurobiol, 2012.

144. Fitch, M.T. and J. Silver, *CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure.* Exp. Neurol, 2008. **209**(2): p. 294-301.

145. Badner, A., A.M. Siddiqui, and M.G. Fehlings, *Spinal cord injuries: how could cell therapy help?* Expert Opin Biol Ther, 2017. **17**(5): p. 529-541.

146. Fehlings, M.G., et al., *A Phase I/IIa Clinical Trial of Recombinant Rho Protein Antagonist in Acute Spinal Cord Injury.* J. Neurotrauma, 2011. **28**: p. 787-796.

147. Cafferty, W.B., et al., *Functional axonal regeneration through astrocytic scar genetically modified to digest chondroitin sulfate proteoglycans.* J Neurosci, 2007. **27**(9): p. 2176-85.

148. Gao, R., et al., *A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure.* J Am Coll Cardiol, 2010. **55**(18): p. 1907-14.

149. Rupert, C.E. and K.L. Coulombe, *The roles of neuregulin-1 in cardiac development, homeostasis, and disease.* Biomark Insights, 2015. **10**(Suppl 1): p. 1-9.

150. Kastin, A.J., V. Akerstrom, and W. Pan, *Neuregulin-1-beta1 enters brain and spinal cord by receptor-mediated transport.* J Neurochem, 2004. **88**(4): p. 965-70.

151. Santhosh, K.T., A. Alizadeh, and S. Karimi-Abdolrezaee, *Design and optimization of PLGA microparticles for controlled and local delivery of Neuregulin-1 in traumatic spinal cord injury.* J Control Release, 2017. **261**: p. 147-162.

152. Mei, L. and K.A. Nave, *Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases.* Neuron, 2014. **83**(1): p. 27-49.

153. Li, K.X., et al., *Neuregulin 1 regulates excitability of fast-spiking neurons through Kv1.1 and acts in epilepsy.* Nat Neurosci, 2011. **15**(2): p. 267-73.

154. Petrosyan, H.A., et al., *Transcranial magnetic stimulation (TMS) responses elicited in hindlimb muscles as an assessment of synaptic plasticity in spino-muscular circuitry after chronic spinal cord injury.* Neurosci Lett, 2017. **642**: p. 37-42.

155. Kim, W.R., et al., *Functional Test Scales for Evaluating Cell-Based Therapies in Animal Models of Spinal Cord Injury.* Stem Cells Int, 2017. **2017**: p. 5160261.