MODULATING IMMUNE RESPONSE INSIDE BIOMATERIAL-BASED NERVE CONDUITS TO STIMULATE ENDOGENOUS PERIPHERAL NERVE REGENERATION

A Dissertation

Presented to

The Academic Faculty

by

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in the

School of Materials Science and Engineering

Georgia Institute of Technology

May 2015

MODULATING IMMUNE RESPONSE INSIDE BIOMATERIAL-BASED NERVE CONDUITS TO STIMULATE ENDOGENOUS PERIPHERAL NERVE REGENERATION

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To my parents Soheila Habib and Hossein Mokarram

for all of their love, care and sacrifices for me.

ACKNOWLEDGEMENTS

I consider myself extremely fortunate for the opportunities that given to me during the last 5 years at Georgia Tech. I had an amazing journey that so many people have played part in it. I have been lucky to meet so many talented and supportive people who have helped me immensely in my pursuit of my PhD degree.

First and foremost, I would like to express my deepest gratitude to my advisor, Dr. Ravi Bellamkonda for giving me the opportunity of joining his lab, believing in me, and supporting me to explore my ideas. I will be always thankful of him for his patience and encouragement he has given me as well as pushing me time to time to tackle my weaknesses. His positivity has always encouraged and inspired me to be a better person. I am extremely thankful to him for helping me adapting myself with a new environment and new area of research. I will be always grateful of him for all the opportunities that he gave me and look up to him as my role model.

I am also very thankful of my great committee members. Dr. Andres Garcia for his insightful advices, Dr. Edward Botchwey for his invaluable guidance and for keeping me up-to-date with the literature, Dr. Valeria Milam for her thoughtful comments, and Dr. Art English for all of his help and support that he has given me with the understanding fundamentals of peripheral nerve regeneration. I have enjoyed our conversations and I have learnt a lot from him over the years. They have all provided me with invaluable guidance and mentorship that has driven my project and significantly improved the quality of the science that I have done. It has been my privilege to work with each of them at different aspects of the project and I have learnt significantly from each of them.

I would like to express my gratitude to everyone in Bellamkonda lab over the last five years who have taught me a lot and without their help this thesis would not have been possible: Dr. Lohitash Karumbaiah who introduced me patiently to this new area of research for me. The number of things that I learned from his is simply countless. Dr. Vivek Mukhatyar who initially helped me with my surgeries and though me all the techniques related to *in vivo* work. Dr. Balakrishna Pai for his mentorship, patience, and support on multiple aspect of my project. Dr. Katie Mceeley, Dr. Tarun Saxena, Johnathan Lyon, Kristin Loomis, Jessica Falcone, Yogi Patel, Martha Betancur, Kelly Rockwell, and Nazanin Masoodzadehgan for their support and friendship. I would also like to especially thank Akhil Srinivasan for his countless help with different aspects of my project as well as his friendship. We joined the lab at the same time and have grown together, experienced several activities to expand our horizon outside of the lab, and made lots of good memories. I would also like to thank Inthu Somasuntharam, Tony Awojoodu, and Patiricia Pacheco for numerous great discussions regarding immunomodulation and tissue regeneration as well as for their support and friendship.

I would like to specially acknowledge my undergrads who have worked really hard to help me make deadlines and mentoring them have been one of the most rewarding experiences of my life.

Finally, I would also like to thank my friends and my family who have given me continuous support and made this journey a memorable one.

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LIST OF SYMBOLS AND ABBREVIATIONS

CCL2	Chemokine (C-C Motif) Ligand 2 = MCP-1
CCR2	C-C Chemokine Receptor Type 2
CM	Conditioned Media
CNS	Central Nervous System
CSPG	Chondroitin Sulfate Proteoglycan
CX3CL1	Chemokine (C-X3-C Motif) Ligand 1 = Fractalkine
CX3CR1	CX3C Chemokine Receptor 1
DC	Dendritic Cells
ECM	Extracellular Matrix
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
hMSC	Human Mesenchymal Stem Cell
IFNγ	Interferon Gamma
IL-19	Interleukin-19
IL-4	Interleukin-10
M1	Classically Activated Macrophages
M2	Alternatively Activated Macrophages
M2a	Pro-healing Macrophages-a
M2b	Regulatory Macrophages
M2c	Pro-healing Macrophages-c
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
MS	Multiple sclerosis
NGF	Nerve Growth Factor
PNS	Peripheral Nervous System
PS	Phosphatidylserine
ROS	Reactive Oxygen Species
RSC	Rat Schwann Cells
SC	Schwann Cells
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factors Alpha
VEGF	Vascular Endothelial Growth Factor

SUMMARY

Injuries to the peripheral nervous system (PNS) are major and common source of disability, impairing the ability to move muscles and/or feel normal sensations, or resulting in painful neuropathies. Annually traumatic nerve injuries resulting from collisions, gunshot wounds, fractures, motor vehicle accidents, lacerations, and other forms of penetrating trauma, affected more than 250,000 patients just in the U.S. The clinical gold standard to bridge long non-healing nerve gaps is to use a nerve autograft- typically the patient's own sural nerve. However, autografts are not ideal because of the need for secondary surgery to 'source' the nerve, loss of function at the donor site, lack of appropriate source nerve in diabetic patients, neuroma formation, and the need for multiple graft segments. Despite our best efforts, finding alternative 'nerve bridges' for peripheral nerve repair remains challenging – of the four nerve 'tubes' FDA approved for use in the clinic, none is typically used to bridge gaps longer than 10 mm due to poor outcomes. Hence, there is a compelling need to design alternatives that match or exceed the performance of autografts across critically sized nerve gaps.

Here we demonstrate that early modulation of innate immune response at the site of peripheral nerve injury inside biomaterials-based conduit can favorably bias the endogenous regenerative potential after injury that obviates the need for the downstream modulation of multiple factors and has significant implications for the treatment of long peripheral nerve gaps. Moreover, our study strongly suggests that more than the extent of macrophage presence, their specific phenotype at the site of injury influence the regenerative outcomes. This research will advance our knowledge regarding peripheral nerve regeneration, and help developing technologies that are likely to improve clinical outcomes after peripheral nerve injury. The significant results presented here are complementary to a growing body of evidence showing the direct correlation between macrophage phenotype and the regeneration outcome of injured tissues.

CHAPTER 1 INTRODUCTION

Traumatic injuries during the World Wars in the first half of the last century influenced many of the current surgical techniques that are used for nerve repair. Sydney Sunderland in the 1940s published studies on the intraneural topography of the ulnar, radial, and median nerves as well as of the sciatic nerves and provided the anatomic foundation for peripheral nerve repair [1,2]. With the advent of micro surgical techniques and advancement of technology, several strides in the field of nerve repair have been achieved. For small gap, close to normal functional recovery can be achieved with the current techniques. For longer gaps, we still face some of the same challenges that have been around for the past century. The gold standard to bridging long gaps for the past century has been autographs. However, there are several drawbacks of using autographs that prevent them from achieving full functional recovery after nerve injury.

With the advances made in the field of regenerative medicine, alternatives to using autographs have been explored. Several synthetic and natural guidance channels are currently used in clinical as well as research settings to bridge nerve gaps. These techniques have shown to improve nerve regeneration in small gaps (less than 10 mm in rats and 30 mm in human and primates) but fail when the gaps are longer [3]. Even after the last 150 years of innovation and development of refined surgical techniques and understanding nerve injury, we do not have a suitable replacement for autographs [4]. Even though there were several tissue engineering attempts to provide a scaffold to repair long nerve gaps, match the performance of autograft, none of them have been able to match the performance of autograft yet [3]. Although the search for the minimum critical elements continues, it is fundamentally challenging to replicate individual aspects of a complex biochemical regenerative cascade. This section will discuss the grand challenges of neural tissue engineering.

1.1 Healing or Scarring[1]

Physiological healing, which incorporates the removal of necrotic tissue and its clearance, results in two possible outcomes: 1) "regeneration," which is complete replacement of injured tissue with new fully functional tissue, and 2) "scarring," which is the partial repair of injured tissue with limited or no functionality. The intriguing question that arises is—what determines these outcomes? Generally, after injury of a tissue, several biological pathways become activated and typically local cells undergo changes in phenotype, which consequently determine the physiological healing state of the tissue [5]. Regeneration occurs when this cellular and tissue response acts in concert to facilitate restoration of function. Conversely, scarring occurs when endogenous constraints, which mainly evolve to preserve the more "critical" functions, prevent this orchestration of regenerative healing [5–7].

Regeneration may be defined as the capacity of a tissue to regrow after injury and to restore its original function. Consequently, the regenerative capacity is the probability of regeneration occurring in an organ/tissue for a given injury [8]. Generally, the regenerative capacity of any organ or tissue is limited by endogenous constraints for repair that arise when the injury exceeds the threshold of regenerative capacity of the affected tissue. Although complex tissues retain the capacity for endogenous regeneration to some extent, regeneration is typically constrained by rate-limiting biochemical or cellular processes. Rate-limiting biochemical or cellular processes may be thought of as "knobs," which when turned, help increase or decrease the regenerative capacity of specific tissues. Hence, the critical question in the context of regeneration is: Is it possible to modulate

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[1]Mokarram, N., and Bellamkonda, R. V. Overcoming endogenous constraints on neuronal regeneration. *IEEE Trans. Biomed. Eng*. 58:1900–1906, 2011.

healing pathway of complex tissues or to turn the "knobs" to facilitate alleviation of endogenous constraints and promote regeneration? And if indeed, is this possible, are there some "master knobs" that tip the balance favoring regeneration and away from scarring? To answer these questions, it is necessary to carefully examine both endogenous constraints and healing pathways.

1.2 Endogenous Constraints and the Role of Inflammation

There are a number of endogenous constraints, which determine regenerative capacity of a tissue, and these vary greatly by species, age, pathophysiological state, tissue type, and the extent of injury. For instance, injured fetal tissues, in contrast to adult tissues, can occasionally be completely regenerated [6], [9–11] indicating their greater regenerative capacity. Although the loss of regenerative capacity from young to old is intuitively accepted to be true [12], the differences in the regenerative capacity between age-matched individuals are less well understood.

In the recent years, there is increasing evidence to suggest that inflammation [13–16] and the biochemical pathways [17,18] triggered by the nature and duration of the initial inflammatory response may determine the healing outcomes [19–22].Therefore, it is reasonable to assume that regeneration versus scarring responses may be modulated if mechanisms to influence this inflammatory cascade post-injury existed.

During the inflammation process, an array of complex regulatory pathways operates. Inflammatory pathways include mechanisms that regulate both "local" factors, such as the plasticity of tissue [23,24] and its growth rate [21], and also "long-distance" factors, such as signaling mechanisms for the recruitment of circulating stem and immune cells, and their homing [25–28] (see Figure 1.1). Inflammatory cells (e.g., macrophages and microglia) may secrete either "pro-regenerative" or "pro-scarring" chemokines and cytokines (see blue and red arrows, respectively, in Figure 1.1).

Studies on various immune cell types over the last several years have brought to light the biochemical as well as functional diversities of these immune cells, such as macrophages [29–33], which are extant both in normal and injured tissue [13]. Macrophages as the "first responder" immune cells after injury potentially offer a valuable point of intervention, and strategically employing macrophages could direct the pathway toward predisposing the immune response to a "pro-regenerative" one.

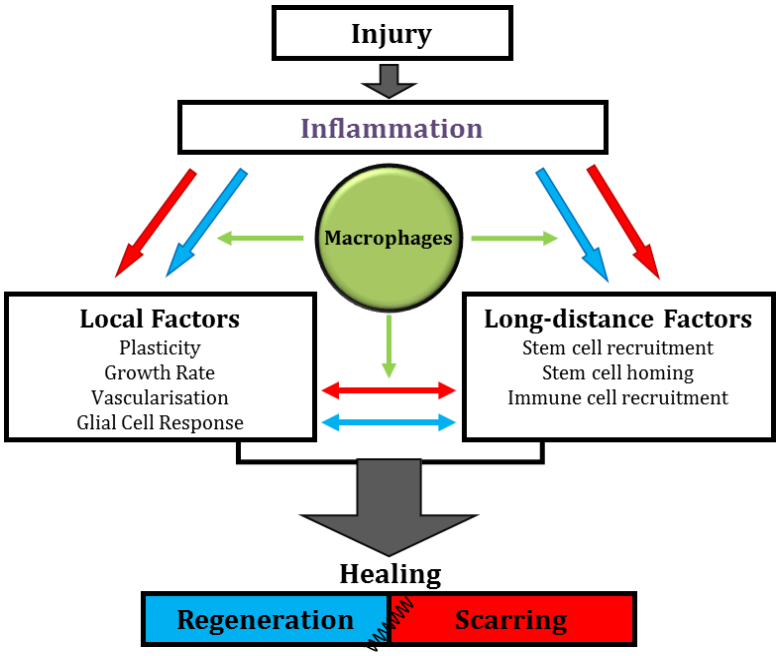

Figure 1.1: Healing vs. Scarring: Potential pathways and interactions that operate in a tissue post-injury.
The sequence of events in the inflammatory pathway may determine the final outcome of healing process (regeneration or scarring) by regulating local and long-distance factors via pro-regeneration (blue arrow) or pro-scarring (red arrow) factors.

1.3 Regeneration and Scarring in the Nervous System

If macrophages as well as glial cells determine differential regenerative capacity of the the central nervous system (CNS) and peripheral nervous system (PNS), then evaluation and characterization of the effects of this response on the processes that thwart regeneration after PNS or CNS injury becomes critical.

1.3.1 Critical Determinants of PNS Regeneration

The PNS, unlike the CNS, has the ability to regenerate in a limited manner. Functional recovery after injuries to the PNS is dependent on factors such as 1) the rate of regeneration, which influences the time to reinnervation of target tissue, 2) the plasticity of the local peripheral nerve and the central cortical neurons in reestablishment of coherent control of target tissue, and 3) the severity of the injury, which may determine the extent to which the "pro-regenerative" glial/inflammatory responses can be modulated.

1.3.1.1 Rate of Regeneration:

An important challenge and determining factor for functional recovery after PNS injury is the time to reinnervation [34]. Based on studies dating back to World War II, delay in repairing an injured peripheral nerve leads to feeble functional outcomes most likely due to a decrease in the ability of the end organ to be reinnervated and also atrophic changes in the regenerating support pathway of Schwann cells [35]. Although the rate of axonal regeneration is fairly constant across mammalian species (~1–4 mm/day) [35], this rate decreases with age and contributes to poor regeneration in older adults [36]. Interestingly, Fu and Gordon demonstrated that regeneration time dependency is not an intrinsic limitation of the neurons, but is controlled extrinsically by signals that originate either in glial cells such as Schwann cells, or target tissue such as muscle [37,38]. In other words, axons maintain their regenerative capacity over time by the promoting mechanisms that stimulate their regeneration. Those promoting mechanisms may decrease over prolonged periods due to Schwann cells or target tissue dysfunction. Based on these observations, the

rate of regeneration is an important rate limiter of success. This may, in turn, be determined by the quality and quantity of supporting healthy Schwann cells.

1.3.1.2 Plasticity:

Plasticity may be defined as the ability of the PNS to sprout and make new connections with target tissues such as muscle. Plasticity may be governed by the presence of inhibitory proteoglycans, such as chondroitin sulfate proteoglycans (CSPGs) found in the basal lamina in the PNS [39,40]. Second, plasticity is also dependent on the ability of the axons to "sprout" multiple neuritis and axonal branches to increase the probability of appropriate target finding and innervation. Neurotrophins such as neurotrophin-3 (NT-3) and nerve growth factor may play an important role in enabling this activity [41,42]. Lastly, plasticity is dependent on the end organ receiving regenerating nerves and its ability to be innervated in a functional manner as nonspecific connections lead to misdirected regeneration [35].

1.3.1.3 Degree of Injury:

As with any tissue, the degree of injury has the potential to overwhelm the regenerative capability. In the case of PNS injury, this translates to the gap length across which regeneration needs to occur. Bringing the two nerve stumps together (coaptation) is typically used to repair short distance nerve defects, but for longer nerve gaps (15 mm or longer), the current clinical gold standard is to use an autograft [4].

1.3.2 Critical Determinants of CNS Regeneration

As is the case with PNS repair, adult CNS regeneration is hostage to the biochemical and cellular processes triggered by neural injury. Functional recovery is dependent on survival of the injured neuronal cell body, regeneration of the damaged axon, remyelination, and functional synapse formation [43].However, contrary to the neurons in the PNS, severed CNS axons fail to regenerate beyond the lesion site [44]. The difference

between regenerative capabilities of these two systems stems from the differential glial response after injury, resulting in vastly different degrees of "permissiveness" of the injury environment to regenerating axons [45]. Hence, nonpermissive regenerative environment after injury stemming from the astroglial scar tissue and inhibitory adult CNS myelin represent formidable barriers to CNS regeneration.

1.3.2.1 Astroglial Scar Tissue:

After CNS injury, astrocytes, oligodendrocyte precursors, meningial cells, and microglia, are recruited to the injury site and form an astroglial scar tissue that "walls off" the CNS lesion [7]. Although some of these events have advantageous effects (like isolating the injury site, minimizing the area of inflammation, and cellular degeneration), many astrocytes in the lesion site become hypertrophic and adopt a "reactive" phenotype, releasing inhibitory extracellular matrix (ECM) molecules such as CSPGs [7].CSPGs are also present in perineural nets that stabilize CNS synapses and limit plasticity in the CNS. Overcoming astroglial scar tissue represents a grand challenge as it negatively impacts regeneration and limits the integration of implanted electrodes or transplanted engineered constructs into the CNS.

1.3.2.2 Myelin-Associated Inhibition and Wallerian Degeneration:

Myelin, a para-crystalline array of Schwann cell plasma membranes or lipid-rich glial, provids a low-capacitance, high-resistance sheath around large axons assisting rapid conduction of nerve impulses [46]. After injury in the CNS, immobilized CNS myelin inhibits axon outgrowth by varied mechanisms [47].Wallerian degeneration, the process in which damaged cells are removed and recycled by glial cells, is slower in CNS in comparison to PNS. The rate of Wallerian degeneration is different in these two systems because of some distinct differences in the glial clearance responses after PNS and CNS injuries. The PNS is more efficient at clearing myelin debris in comparison to the CNS, and it is believed that the Schwann cells are the primary reason for this difference [48].

Schwann cells in the PNS, in contrast to their CNS counterparts, oligodendrocytes, do not require axon signals to survive. In fact, in their developmental stages, oligodendrocytes that fail to make contact to an axon undergo apoptosis [49]. Therefore, unlike Schwann cells, oligodendrocytes are not as capable to clean up the myelin sheaths and their debris after injury. Moreover, oligodendrocytes are also believe to be incapable of recruiting macrophages for debris removal [48].

1.3.2.3 Blood-Brain Barrier:

Another important determinant in CNS regeneration is the blood-brain barrier (BBB). The BBB is the separating system of circulating blood and cerebrospinal fluid in the CNS. One of the main roles of the BBB was believed to be the separation of the systemic immune system from the CNS. Although studies have shown that resident CNS macrophages (microglia) present within the CNS actively interact with peripheral immune cells [50], the CNS is largely thought to be restricted in its capacity to deliver antigens to local lymph nodes and subsequently activation of T cells [48]. In an uninjured tissue, antigens are taken up by antigen presenting cells such dendritic cells, and transported to the lymph nodes. Soluble antigens can also be drained into the lymph nodes. However, in the CNS, although dendritic cells are present in the choroid plexus and meninges, they do not exist in normal parenchymal tissue or perivascular space as the result of BBB [51].

Moreover, in contrast to the PNS, the barrier disruption in the CNS is only limited to the site of injury, whereas in the PNS, the permeability enhances throughout the whole distal stump. The decreased permeability in the CNS could also explain the difference in the number of infiltrated macrophage to the site of injury [48].

1.3.3 Common Determinants of PNS and CNS Regeneration

Besides the aforementioned rate-determining processes in the PNS and CNS, there are some other critical determinants of regenerative fate that are common in both systems. One such factor is the number of available local stem or progenitor cells in the immediate

vicinity of the site of injury. Besides resident neural stem cells [52], stem cells can be either transplanted [53] or endogenously recruited to the site of injury [54]. However, in spite of encouraging data, which indicate that replacement of cells is promising [55–57], the functional impact and reliability have been underwhelming. Moreover, despite intrinsic plasticity of endogenous stem cells, they are incapable of providing complete recovery in severe trauma [4].

Another rate limiter is the ability of an injured tissue to form blood vessels (angiogenesis and vascularization). Angiogenesis is not only a vital process in growth and development, but also play a critical role during wound healing process. Besides vascular endothelial growth factor and the fibroblast growth factors are two long-known effective factors in vessel formation, there are many other critical growth factors which are involved in the process of blood vessel formation and their physiological regulation [58]. These factors should act in concert with each other temporally and spatially in order to form a functioning vascular network [59]. Enhancement in neurogenesis by increasing vascularization has been reported [60].

1.4 New Insights into Regeneration: Immune System as the Key Determinant of Regeneration

There is evidence that secretory products of immune cells are capable of affecting most of the critical determinants of regeneration in the CNS and PNS that makes the immune system the key determinant of regenerative fate. Therefore, the inflammatory cascade post-injury and the role of the immune system has the potential of being the linchpin upon which regeneration versus scarring responses are determined.

Considering the complexity of the biochemical and cellular responses to neural injury, targeting an individual point in the cascade may or may not be the most efficient approach to bias the response to regeneration versus scarring. In this context, the inflammatory response as well as the resulting immune reaction to the injury represent a

promising point of intervention and could potentially represent one of the "master knobs" for eliciting regeneration. Before discussing the effects of different inflammatory responses, a brief introduction to microglia and macrophages as the most studied and diverse inflammatory cells will be provided.

1.4.1 Macrophages and Their Different Activation States

Macrophages are present in all tissues with the different resident names: osteoclasts in bone, histocytes in connective tissue, Kupffer cells in liver, and microglia in the CNS. They migrate as monocytes into the tissue in a steady-state fashion or in response to an inflammation. Although macrophages have been known as the professional phagocytes and the executers of the innate immunity, recent studies illustrate their homeostatic as well as regenerative roles [30,32,33]. In fact, remarkable plasticity of macrophages makes them capable of effectively responding to the different environmental signals by changing their phenotype and physiology. Mosser and Edwards suggest that there exist many shades of activation of macrophages, resulting in a spectrum of macrophage population rather than a few distinct groups [33]. However, there is consensus that macrophage phenotypes roughly fall into three classifications: classically activated, wound-healing, and regulatory (see Table 1-1) [20,30,32,33]. These macrophage phenotypes can sequentially change to each other in response to signals in their respective microenvironments [61,62].

Classically activated macrophages become activated by injury-triggered endogenous inflammatory signals like Th1 cytokine interferon gamma (IFNγ), or by exogenous inflammatory signals like lipopolysaccharide (LPS) [61]. These cells are prototypical immune effector cells, which kill pathogens by production of oxygen, nitrogen radicals as well as phagocytosis. Classically activated macrophages are beneficial for the survival of the organism. However, resolving the injury and restoring normal tissue homeostasis requires an innate immune response that supports replacement of lost and damaged cells and restructuring of the damaged ECM.

Wound healing macrophages represent a second class of macrophages that help in the tissue repair by producing anti-inflammatory cytokines, which mediate angiogenesis as well as ECM deposition. One of the well-established activating signals of this phenotype is interleukin-4 (IL-4). Moreover, the induction of arginase in these cells may lead to polyamine and proline biosynthesis, promoting cell growth and collagen formation.

The third broad category of macrophages is regulatory macrophages that are induced by exposure of macrophages to apoptotic cells and are associated with a robust suppression of the innate immune response. This phenotype allows macrophages to engulf apoptotic cells without inducing a classically activated immune response [19,30,32].

In the nervous system, although other glial cells (like astrocyte [63]) and neurons [64] may also play an immune role, the primary cells involved in the generation of innate immune response are microglia cells. It has been demonstrated that microglia, as the CNS resident macrophages, are also capable of transiting between the three activation states previously described upon receiving an appropriate activating signal (see Table 1-1) [19,20].

Generally, a pro-regenerative bias in activation of these populations in the inflammatory cascade at the appropriate time and location will lead to regeneration, and any inappropriate triggering of pro-scarring macrophages/microglia/glial cells will lead to scarring. For example, in cancer, macrophages inappropriately switch from the classically activated to regulatory phenotype [33], or in autoimmune diseases, such as Alzehimer's or multiple sclerosis (MS), microglia cells become chronically inflammatory (coexistence of wound healing and classically activated phenotypes) in the brain [19,20,65]. Interestingly, many parasitic organisms also alternate the macrophage activation state to the wound healing as a means to enhance their survival within cells or tissues [66].

1.4.2 Harnessing the Immune Response to Enhance Nerve Regeneration

Regenerative biochemical cascade is sometimes halted by lack of critical component, such as resident pluripotent cells, or weak "homing" signals to recruit circulating stem cells, or poor permittivity to regeneration at the site of injury (e.g., gliotic scar). Although the precise effect of each phenotype of microglia is not completely understood, there is strong evidence to support the notion that macrophages can modulate regeneration in the nervous system. The Schwartz laboratory has demonstrated that appropriate activation of microglia by IL-4 or IFNγ differentially induces neurogenesis as well as oligodendrogenesis from adult stem cells [21]. This data suggests that both oligodendrogenesis and neurogenesis of adult neural progenitor cells are blocked by classically activated microglia cells in the mice. However, this process can be altered to be "pro-regenerative" by wound healing associated cytokines (IL-4) in combination with low levels of IFNγ. Therefore, controlled levels of appropriate cytokines can overcome the inhibition of neurogenesis in an inflamed brain. These studies also suggest that the duration, combination, and order of biochemical signals after injury ultimately determine the outcomes [67].

It has also been shown that exposing microglia cells to low concentrations of classically activated cytokine IFNγ (5 ng/mL) enables them to buffer the excitatory neurotransmitter glutamate (a common player in neurodegenerative diseases) and subsequently afford protection to neural tissue [68]. Schwartz group has demonstrated that the cytotoxic effects of microglia exposure to large amounts of IFNγ (>50 ng/mL) is a consequence of upregulated neurotoxic cytokine, tumor necrosis factor-alpha (TNFα) [23], which is a secretory product of classically activated macrophages. However, treatment of the macrophages with TNFα during Wallerian degeneration significantly reduced their phagocytic capacity as well as their ability to ingest myelin debris, and in turn, their regenerative capacity [69].

On the other hand, it has also been demonstrated that lack of TNFα will significantly delay remyelination, which is an important regenerative step in the autoimmune disease such as MS [24]. In addition, the neuroprotective effect of TNFα might be indirectly controlled by astrocytes. Expression of the brain-derived neurotrophic factor by cultured astrocytes is usually elevated by increasing the amount of TNFα [70]. IL-1β, a pro-inflammatory neurotoxic cytokine, has also been shown to have a similar paradoxical effect in promoting regeneration [70]. Thus, although many cytokines such as TNFα and IL-1β are traditionally thought to be neurotoxic, their role is context dependent as their beneficial function was demonstrated in animals deficient in these cytokines [65]. Therefore, macrophages/microglia phenotype has a regulatory effect on the inhibitory environment of CNS as a critical determinant of regeneration. Additionally, microglia exposure to IL-4 stimulates production of insulin-like growth factor (IGF-1) [33] (see Table 1-1), supports neurogenesis [71] and oligodendrogenesis [72], as well as ameliorating the age-related decline of those regenerative process [73].

Macrophages also play a key role in the regeneration process. They actively participate in the cell replacement by the stem cell recruitment and homing, as well as in the resident progenitor cell differentiation and proliferation [74]. Besides the granulocyte-macrophage colony-stimulating factors (GM-CSF), it has been shown that IL-12 (upregulated by classically activated phenotype) also mobilizes hematopoietic stem cells [26,27]. It has also been demonstrated that the monocyte chemoattractant protein-1 (MCP-1), a chemokine produced by classically activated macrophages, has an ability to recruit neural progenitors to the site of injury [25,28]. Moreover, different phenotypes of macrophages by secreting factors like TNF, IL-1, and GM-CSF can cause an increase in the production of granulocyte and monocytes (defensive immune cells) by the bone marrow, which can contribute in healing pathway in several ways. For example, one type of granulocyte, basophils, can release histamine, which leads to dilation and increased permeability of capillaries close to them [58]. It has also been shown that secretory

products of macrophages influence the different phase of angiogenesis both *in vivo* and *in vitro* [63,64]. These participations also support the idea of capability of macrophages in regulating some other rate-determining factors.

Unfortunately, detailed molecular mechanisms and healing pathways of all the different phenotypes of microglia and macrophages in the nervous system are not well characterized yet; preliminary results show that a tightly controlled modulation of these cells can potentially enhance the regeneration in CNS and PNS significantly. Neuroengineering tools involving electrical stimulation, polymeric fibers, hydrogel nanoparticles, and hydrogel microparticles may all offer powerful tools to modulate these intricate inflammatory signaling fates in a manner that is spatially and temporally controlled [3,75–78].

Therefore, in order to meet the challenge of regenerating PNS and CNS nerves, it is important to explore the full spectrum of the microglial and macrophagic cell phenotypes in the inflammatory cascade, and to identify their influence on both local and long-distance critical rate limiters to endogenous regeneration, and where necessary, to use biological and engineering tools to modulate these critical phenotypes to maximize regeneration.

In conclusion, one of the greatest grand challenges in neuro-regeneration is stimulating endogenous repair of injured peripheral and central neural tissues. Specific challenges include bridging long peripheral nerve gaps and overcoming astroglial scar tissue to promote regeneration after spinal cord injury. The particular insight afforded here is the possibility that modulation of the inflammatory cascade after injury may significantly alter the course of healing in the nervous system, thus offering a critical modulation opportunity for promoting regeneration and integration of engineered materials and devices in the nervous system.

Table 1-1: Different activation states of macrophages

Activation state	Classically Activated	Wound Healing	Regulatory
General function	Tissue defense, ROS, and NO production	Tissue repair, fibrosis, ECM reconstruction	Immunosuppression, apoptotic cell uptake
Activating Signals	IFNγ, TNFα	IL-4, IL-13	TGFβ, IL-10, apoptotic cells
Secretory products	TNFα, IL-12, IL-6, IL-1β, MCP-1, ROI	IL-1RA, IL-10, Arginase 1	IL-10, TNFα, IL-6, IL-1β, MHCII
CD markers	CD86, CD80, CCR7	CD163, CD206	CD86, CD163

CHAPTER 2 SPECIFIC AIMS AND HYPOTHESES

Annually over 250,000 Americans suffer from a peripheral nerve injury which results in a loss of function and a compromised quality of life. Peripheral nerve gaps usually are a consequence of traumatic limb injury or collateral damage to peripheral nerves during tumor resection and in more than 50% of cases, no measurable signs of recovery are evident [3,79]. The clinical gold standard to repair critically sized non-healing gaps is to use sural nerve autografts [4,80,81]. However, autografts are not ideal because of the necessity for secondary surgery to 'source' the nerve, lack of appropriate source nerve in diabetic patients, loss of function at the donor site, neuroma formation, and the need for multiple graft segments. Despite our best efforts, finding alternative 'nerve bridges' for peripheral nerve repair remains challenging – of the four nerve 'tubes' FDA approved for use in the clinic, none is typically used to bridge gaps longer than 10 mm due to poor outcomes. Hence, there is a compelling need to design alternatives that match or exceed the performance of autografts in enabling peripheral nerve repair across critically sized gaps. Our *long term goal* is to enhance peripheral nerve regeneration in the critically-sized non-healing gap model.

Several approaches to accelerate regeneration have been explored including the local delivery of neurotrophic factors and cell transplantation [82–85]. Our group has pioneered the use of hydrogels, micro-scale lipid microtubes, and topographical cues to bridge nerves gaps larger than 12 mm [3,4,76,77,86–90]. While these approaches show promise, matching autograft performance in bridging gaps greater than 15 mm remains an elusive goal.

Here, we propose an entirely different and novel approach – instead of trying to augment individual downstream elements of the molecular repair cascade, can we tap into and boost the endogenous molecular repair cascades by intervening upstream? Specifically, are there master knobs or nodes that act as upstream modulators of the healing

cascades/pathways, and if so, is it possible to enhance nerve regeneration by 'biasing' action at these nodes? The reasoning is that successful nerve repair is the result of a complex sequence of cellular events that includes extracellular matrix (ECM) protein secretion and remodeling, trophic factor secretion, and Schwann cell migration. Replicating element by element of this regenerative sequence using tissue engineering approaches is an overly daunting task. The alternative proposed here, is to modulate upstream nodes, which initiate these healing cascades.

Indeed, in multiple animal models and organ systems, the immune system has emerged as the master orchestrator of the healing/non-healing fate [13,91–99]. In fact, in chapter 3, we will discuss a perspective on immunomodulation and tissue repair. Specifically, studies over the past years have shown increasing evidence that the sequence of cellular events associated with nerve regeneration is influenced by immune cells, especially macrophages which are able to play both detrimental and beneficial roles in the tissue regeneration process, depending on their phenotype [95,100,101]. Thus, **our *central hypothesis* is that modulation of the innate immune response early after injury triggers endogenous repair mechanisms that can stimulate nerve repair across long nerve gaps**. We will use the following specific aims to test our hypothesis:

Aim 1: Evaluate the effect of modulating macrophage phenotype on peripheral nerve repair. *Our working hypothesis* was that innate immune cells, especially macrophages, are playing a key upstream role during the regeneration process and early modulation of their phenotype can affect later regeneration outcomes. The *objectives* for this aim were to fabricate nerve conduits containing pro- or anti-inflammatory cytokines, and investigate the effect of their early release inside the nerve conduit for creating a regenerative environment as evident by quantitative molecular signatures in the nerve gap. The *outcomes* of this aim are discussed in Chapter 4.

Aim 2: Investigate the interplay between macrophages phenotype and glial cells function. *Our working hypothesis* was that Schwann cells (SC) play a critical role in helping stimulate nerve growth and modulating macrophages could potentially affect the SC cellular behavior. The *objectives* for this aim were to investigate how modulating macrophage phenotype potentially contributes to the enhancement of nerve regeneration and how that modulation affects SC migration, proliferation, and phenotype *in vitro* and *in vivo.* The *outcomes* of this aim are discussed in Chapter 5.

Aim 3: Determine the effect of monocyte recruiting factor on stimulation of peripheral nerve regeneration across a critically sized nerve gap. *Our working hypothesis* was that there exist monocyte subtypes that may be predisposed to differentiate into macrophages of different phenotypes and recruitment of these precursors (monocyte) could have an impact on the nerve regeneration. Our *objectives* were to fabricate a scaffold containing anti-inflammatory monocyte recruiting factor, and evaluate the effect of its early release, in comparison to the macrophage modulating strategy, in affecting the final regeneration outcome. The *outcomes* of this aim are discussed in Chapter 6.

CHAPTER 3 LITERATURE REVIEW

Repairing peripheral nerves across long gaps is clinically challenging, even despite all the advancement made with autograft transplantation. While scaffolds that present extracellular matrix molecules and trophic factors have been designed, matching the performance of autograft-induced repair has been challenging. Current approaches focus on enhancing axon growth by direct action on nerves, or glial cells, and here we investigate an alternative approach to influencing regenerative outcomes by modulating the initial inflammatory sequence via macrophages. Since the sequence of cellular and molecular events associated with nerve regeneration is influenced by immune cells [95,102], we hypothesize that modulating immune cells upstream of action on nerves or Schwann cells (SC) triggers endogenous repair mechanisms that can stimulate nerve repair across long gaps. In fact, the incredible plasticity of macrophages makes them an interesting case for modulation.

In this chapter, we evaluate the strength of our hypothesis by reviewing the current state of understanding regarding the role that different phenotypes of macrophages and monocytes play following injury and during the course of remodeling in different tissue types. Moreover, we explored recent designs of macrophage modulatory biomaterials for tissue engineering and regenerative medicine applications.

3.1 Introduction[2]

Endogenous healing capacity varies extensively across different species. For example salamanders have the ability to regrow sophisticated body structures even as

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[2] Mokarram, N., and Bellamkonda, R. V. A Perspective on Immunomodulation and Tissue Repair. *Annals of Biomedical Engineering* 42:338–351, 2014.

adults [103] while this capacity is more limited in mammals. The diversity in healing capacity also exists within tissues of the same species. Mammals are much more successful in repairing their dermal tissue, or as the legend of Prometheus suggests, livers, compared to their neural tissue [104,105]. Endogenous healing also varies by age as several studies have demonstrated that the mammalian embryo retains their ability to repair wounds without scar formation, whereas this capability diminishes with age [106,107]. It has been hypothesized that a differential immune response to injury contributes strongly to the differential capacity for healing [108].

3.2 Innate Immune Cells Orchestrating Tissue Repair

It is increasingly being acknowledged that the innate immune system is actively involved during the wound healing by orchestrating a biochemical cascade influencing tissue repair [108,109]. The innate immune system is normally the initial-responder to an injury. It mainly includes myeloid lineage cells that include monocytes/macrophages and neutrophils. Monocytes also falls under the mononuclear phagocyte system, a classification that involves several cell types, including macrophages, that share similar ontogeny at multiple stages of differentiation and play essential roles in inflammation, host defense, and development [31,33]. Circulating monocytes give rise to a diverse number of tissue resident macrophages throughout the body, as well as to specialized cells such as dendritic cells (DCs) [31].

3.3 Monocytes Differentiation to Macrophages

Monocytes are produced from dendritic cell and macrophage progenitors, in the bone marrow [110] and, when matured, they enter the circulation in a chemokine receptor CCR2 dependent process [111]. Released monocytes circulate in the peripheral blood for several days before migrating to tissues and refill the tissue macrophage population [31]. Although most tissue macrophages are derived from peripheral blood circulating monocytes, studies have shown that local proliferation has a considerable role in the

maintenance and renewal of many tissue-resident macrophage populations. In fact, in some of these cases, it has been shown that the recruitment of circulating precursors having little, if any, role in this process [31,112,113]. However inflammatory insults, such as infection or trauma, can results in an augmented dependence on the recruitment of blood-borne circulating precursors to assist with repopulation of the tissue-resident macrophages in the affected tissues [31].

3.4 Many Faces of Macrophages

After injury, the innate immune response is quick and although it lacks the specificity characteristic of adaptive immunity [114], it is regulated by a plenitude of signals and demonstrates spectrum of activation stages and phenotypes [33,115]. Macrophages are important source of both inflammatory and anti-inflammatory signals. They arrive in mammalian wound milieu in the first few days after injury, where they clear dead cells and debris, release pro-inflammatory cytokines, secrete oxygen radicals, produce anti-inflammatory factors that dampen inflammation, stimulate angiogenesis, fibroblast proliferation and migration which are all necessary initial steps toward effective tissue regeneration [116,117]. A clue to better understanding of these contradictory functions can be provided by recognition of the existence of different macrophage and monocyte phenotypes.

Briefly, M1 macrophages have been defined as the classically activated macrophages, whereas M2, or alternatively activated macrophages include almost any macrophage that does not fall under the M1 classification (Figure 3.1). Some experts choose to classify M2 macrophages based on their observed functions—termed ‘‘alternative’’ M2a, ‘‘Type II’’M2b, and ‘‘deactivated’’ M2c, [115,118] while others choose to classify these macrophages based on their functions of classically activated, wound-healing and regulatory macrophages [33]. Despite these alternative approaches to classification, the characteristics of M1 and M2 macrophages are confirmed. M1

macrophages are activated by the well-known pro-inflammatory signals such as IFNγ and LPS; secrete major pro-inflammatory cytokines such as TNFα, IL-1β, , IL-23, IL-12, and IL-6; secrete high levels of reactive oxygen species (ROS); produce low amount of anti-inflammatory cytokines such as IL-10; are efficient antigen presenting cells; and leads to the formation of effector and inducer cells related to the Th1 pathway [31,33,118]. In contrast, M2 cells are activated by molecular cues such as IL-4, IL-13, IL-10 as well as immune complexes; secrete high levels of IL-10; results in expression of mannose, scavenger, and galactose receptors; produce polyamines and ornithine and instead of ROS; and are involved in polarization toward Th2 type response [33,118,119].

It is likely that, with the increase in our appreciation of macrophage activation states, the number of generally recognized phenotypes expands. Moreover, since macrophage phenotype plasticity can be determined by remarkably complex signals from their microenvironments, it is more accurate to be described as a spectrum between the M1 and M2 extreme, in which any given macrophage may express particular components of various M1 or M2 phenotypes [33].

3.5 Different Subtype of Monocytes

Circulating precursors of macrophages, monocytes, also display heterogeneity in both mouse and human [31,120–122]. In mouse, Ly-6C^{high} (Gr1^{high}CCR2+ CX3CR1^{low}) monocytes efficiently infiltrate inflammatory sites and give rise to classically activated macrophages (M1) [31,122] (Figure 3.1). Their counterparts, Ly-6C^{low} (Gr1^{low}CCR2−CX3CR1^{high}) monocytes, accumulate at inflammatory sites less efficiently and are thought to differentiate to resident and alternatively activated macrophages (M2) [31,112,122] (Figure 3.1). Regarding human, studies indicated that monocytes can be categorized into two subsets based on the expression of CD14 and CD16. The dominant subset which expresses high level of CD14 and low or negative level for CD16 (CD162) represents ~85% of the monocyte pool (inflammatory), while the minor subset expresses

low level of CD14 and high level of CD16 (CD16+) (anti-inflammatory) (Figure 3.1) [31,122]. Ly-6C^{high} mouse monocytes roughly correspond to CD14^{high}CD16− human monocytes, which are also CCR2+ CX3CR1^{low}, and Ly6C^{low} mouse monocytes approximately correspond to CD14^{low}CD16+ human monocytes, which also express large amounts of CX3CR1 and low level of CCR2 [31].

3.6 Innate vs. Adaptive Immune Response in the Context of Regeneration

Macrophages, in contrast to lymphocytes which regulate the later phases of regeneration, modulate the early sequence of cellular events which can determine the fate of healing process. Conditional macrophage depletion studies have shown reduction in formation of vascularized granulation tissue and scar formation as well as impairments of epithelialization [123]. In mice, macrophage disruption or depletion of macrophage transcriptional regulation after skeletal muscle injury leads to induction of fibrotic scarring and subsequently incomplete muscle repair [124]. A recent study has shown that systemic macrophage depletion during the first 24 h after salamander limb amputation results in permanent failure of limb regeneration which is associated with extensive fibrosis and dysregulation of gene expression in extracellular matrix (ECM) component [103].

Moreover, macrophages, unlike T-cells, appear to acquire an incredible plasticity. Multiple studies have shown the phenotype switching from M1 to M2 and reverse order [62,125,126]. This plasticity can play a protective role by providing an appropriate response to pathogen as well as effectively resolving such a response without causing excessive local or systemic damage. Although it is still debatable if inflammatory macrophages are changing their phenotype at the site of injury to form regulatory and wound-healing macrophages [127], or if the progressive changes of signals during course of inflammation results in alternation of macrophages functional phenotype [62], the dynamic plasticity of macrophages can be controlled by type, duration, and concentration of plethora of stimulating signals. Therefore, it is not unexpected that analysis of

macrophage phenotype population at different time points may demonstrate a combination of macrophage phenotypes. It is also not surprising that there exist phenotypes that express markers of both M1 and M2 phenotypes. In fact it has been shown that dysfunction in macrophage phenotype transition and plasticity are an underlying mechanism to multiple of diseases including cancer, fibrosis, insulin resistance, and atherosclerosis [128].

In the context of tissue regeneration, macrophage phenotype has also been shown to be a critical player during tissue regeneration process following skeletal muscle, cardiac, heart, skin and nerve injuries as well as others [94,114,129,130]. An increasing body of evidence based on the studies in different animal and organ models have shown that untimely polarization of macrophages, prevention of macrophage polarization, or inability to resolute a chronic polarization can results in a detrimental effect on regeneration outcome [105,128,129,131]. Therefore, macrophages are able to play both detrimental and beneficial roles during the regeneration process, and timely and efficient switch of their phenotype is essential for preventing a harmful or scar tissue outcome, in many cases.

The incredible plasticity of macrophages makes them an interesting target in the context of immunomodulation. In the next section, we provide some examples that explore the potential role of macrophage activation and polarization during the regeneration process of different tissues. We then review examples of macrophage modulatory biomaterials. Finally, in the concluding remarks, we discuss the challenges and opportunities of employing macrophages as a therapeutic target for regeneration.

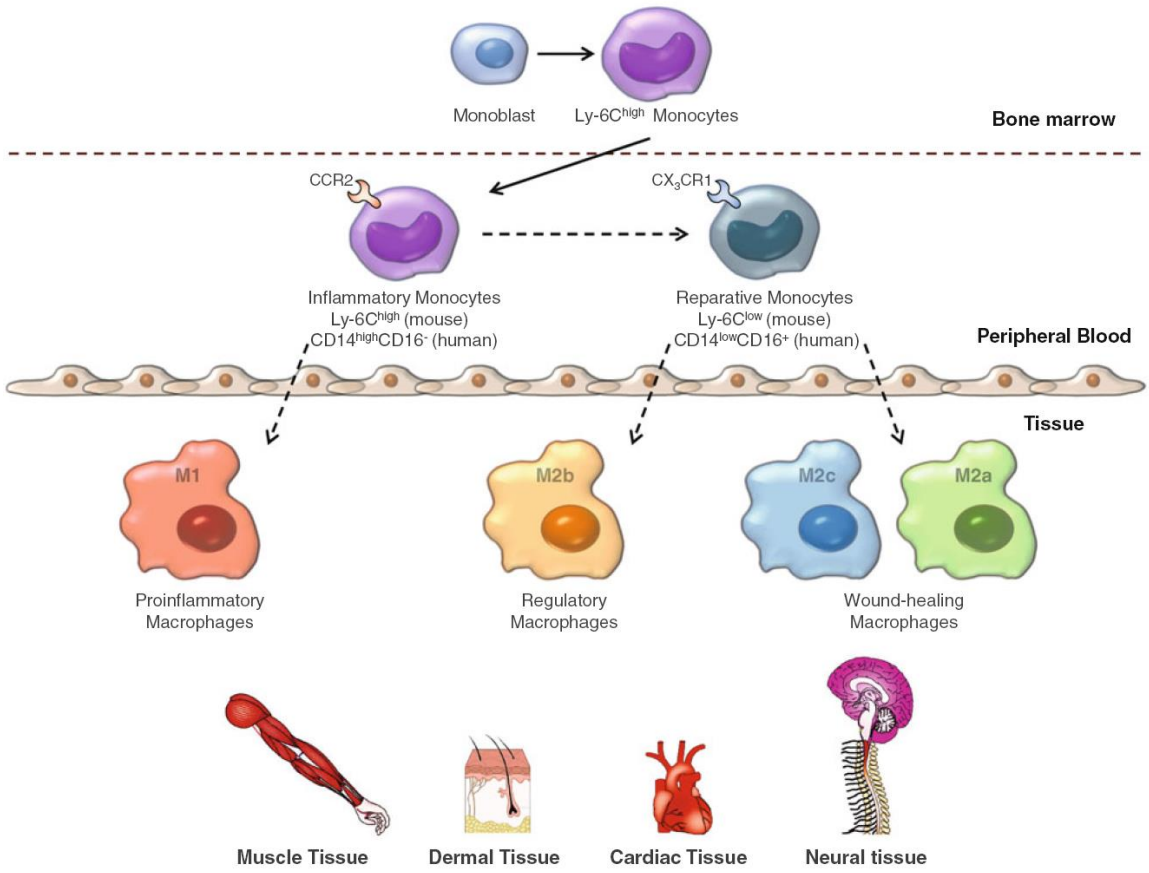

Figure 3.1: Schematic of heterogeneity, source, and potential tissue remodeling targets of monocytes/macrophages.
Monocytes are produced from dendritic cell and macrophage progenitors, in the bone marrow [110] and, when matured, they enter the circulation [111], circulate in the peripheral blood for several days, and finally and especially during an inflammatory insult (such as injury), migrate to tissues to refill the tissue macrophage population [31].

3.7 Macrophage Phenotypes during Tissue Regeneration

3.7.1 Cardiac Tissue

A myocardial infarction (MI) occurs when a coronary artery becomes occluded, resulting in oxygen supply deficiency which leads to necrosis of cardiac myocytes. These events stimulates the complement cascade and initiates an inflammatory response, primarily composed of neutrophil and macrophage infiltration [132]. A potential approach in preventing heart failure is to boost the intrinsic wound healing cascade that happens during the first 2 weeks after MI [133]. During this critical period, the area of infarct is very active with rapid turnover of cells and ECM components; the vulnerable wound is under the mechanical stress of myocardial contraction as well as cycling intraventricular pressure which can result in serious damages in ventricular function as well as geometry [134]. Although studies have confirmed that monocytes and macrophages participate in infarct wound healing and dominate the cellular infiltrate for the first two weeks following MI [114,134], the residence time of cardiac tissue monocytes is very short [135]. Even days after the initial inflammation, there is a rapid turnaround time of about 24 h for the monocyte population to be completely replaced [135]. Surgical removal of the spleen (the main supplier of monocytes after MI) several days after coronary ligation substantially reduced the number of monocytes leading to impaired wound healing and heart failure [135].

A study in a mice model of coronary ligation has shown that there is a temporally biphasic response regarding monocytes involvement in the myocardium. Pro-inflammatory Ly-6C^{high} monocytes arrive first at the site of MI and are the dominant population during the first phase (1-4). These monocytes and promote removal of necrotic debris and digestion of infarcted tissus. In the second phase (days 4-8), reparative Ly-6C^{low} monocytes dominate the wound and results in resolution of inflammation and propagation of repair [114]. This sequential recruitment of monocyte were explained by their different subsets

of chemokine receptors expression [31]. During the first phase, MCP-1 release increases and Fractalkine (CX3CL1) release decreases which leads to recruitment of Ly6C^{high} (CCR2+CX3CR1^{low}) monocytes preferentially [114]. However in the second phase Fractalkine expression is increased, which suggests that recruitment of Ly-6C^{low} (CCR2–CX3CR1^{high}) monocytes during the second phase should preferentially be enhanced [114]. Once recruited, these monocyte subsets mediate distinct biological activities: Ly-6C^{high} monocytes exhibit proteolytic and inflammatory functions, whereas circulating Ly-6C^{low} monocytes have attenuated inflammatory and pro-angiogenic properties [114]. These biological functions are regulated by secretion of cytokines and growth factors: Ly-6C^{high} monocytes express IL-1β, TNF-α, myeloperoxidase, matrix metalloproteinases, cathepsins, and plasminogen activator urokinase and are therefore potently inflammatory, whereas Ly-6C^{low} monocytes express transforming growth factor-β (TGFβ), IL-10, the pro-angiogenic vascular endothelial growth factor (VEGF), and are therefore reparative [114].

The sequential recruitment of monocyte subsets to infarcts correlate well with the time course of tissue healing process and subsets biological properties: The early inflammatory and digestive phase one is followed by active resolution of inflammation and tissue repair in phase two. This well-orchestrated biphasic monocyte response is crucial for appropriate healing. In fact, elimination of the first inflammatory phase impairs the removal of debris and dead cardiac myocytes, whereas annulment of the second phase reduces the deposition of collagen and the generation of microvessels [114]. The non-inflammatory and pro-angiogenic properties of Ly-6C^{low} monocytes and their role in myocardial healing process suggest that the active role of these cells in terminating lingering inflammation.

Although it has been suggested earlier that direct inhibition of macrophage infiltration via the CCL2/CCR2 pathway can contribute to the attenuation of left ventricular remodeling after MI [136,137], recent findings utilizing echocardiography revealed that deletion of CC chemokine receptor 2 (CCR2) which is responsible for recruiting

inflammatory Ly-6C^{high} monocytes to the infarcted region improves left ventricular remodeling [138]. In fact, employing lipid-based nanoparticle as delivery vehicles for CCR2-silencing siRNA prevents inflammatory monocytes accumulation in site of inflammation and subsequently attenuates their number in atherosclerotic plaque and reduces infarct size after coronary artery occlusion [139].

The paradigm shift in the context of monocytes recruitment from a monophasic to biphasic monocyte response after MI, offers new opportunities for therapeutic interventions. For example, in order to accentuate tissue repair, it could be beneficial to modulate the ratio of subsets or timing of recruitment. The discrete mechanisms for recruitment of monocyte subsets (MCP-1-dependent for inflammatory cells, but Fractalkine-dependent for anti-inflammatory cells) offer rational targets to control inflammation and regeneration process, not only after myocardial infarction but also other tissue injuries.

3.7.2 Dermal Tissue

Because of its large area covering the body, dermal tissue is very susceptible to injuries. Therefore, it represents one of the most extensively studied tissues in the context of wound healing. Although the process is known to include macrophages, neutrophils, eosinphils, mast cells, as well as T-cells, macrophages play a central role during the cutaneous wound healing through production of growth factors, cytokines, and matrix metalloproteinases (MMPs) [91,140].

In the context of macrophage phenotypes, changes in the gene expression profile following human patient skin injuries and during cutaneous wound healing have been recently studied [94]. In addition to the up-regulation of a diverse number of genes, during the wound healing process, two transiently up-regulated groups of genes related with M1 and M2 macrophage and their polarization were identified. The first showed an increase of gene expression during the early, inflammatory stage of wound healing and included a

mixture of M1 and M2 related genes (eleven M1 genes and seven M2 genes). The second which happens in the later angiogenesis and tissue remodeling stages of healing demonstrated predominantly M2 associated genes (1 M1 gene and 9 M2 genes). This result simply suggests that there is a correlation between polarization of macrophage subpopulation and cutaneous wound healing process. Although the mechanisms of shift from an M1 to an M2 phenotype during dermal remodeling are not well evaluated, they are thought to be caused by the phagocytosis of debris and apoptotic cells by M1 macrophages [141]. Other studies also demonstrated that depletion of macrophages during the proliferative phase of wound healing leads to a non-complete transition to the remodeling phase which ultimately results in impaired healing [105,123]. Interestingly, advanced aging and diabetes are associated with impaired wound healing, as well as a reduced ability to transition from an M1 to an M2 phenotype [142,143]. Taken together, these studies suggest a correlation exist between the impaired tissue remodeling and defective macrophage polarization.

3.7.3 Musculoskeletal Tissue

In contrast to relatively extensive studies of dermal wound healing, the role of macrophages in tissue regeneration of skeletal muscle has only been explored more recently and more attention was devoted toward the role of different macrophage phenotypes during the remodeling process of injured muscle [130].

After muscle tissue injury, significant number of inflammatory cells infiltrate at the site of injury [144]. In fact, studies have shown that a delay in muscle tissue remodeling and slowed removal of cellular debris is attributed to the reduction of monocytes available following muscle injury due to the depletion of circulating cells [124,131,145]. Moreover, these cells have shown to possess phenotypes that switch from M1 to M2 during the course of muscle regeenration [130,145]. During the initial proliferative stage of muscle remodeling, M1 macrophages are recruited to the site of injury. Later, during the transition

to the early and terminal differentiation stages, these M1 macrophages switch to a more M2 phenotype [130]. Arnold et al. also demonstrated that injured skeletal muscle recruits monocytes exhibiting inflammatory profiles that operate phagocytosis which rapidly convert to anti- inflammatory macrophages that stimulate myogeneinisis and fiber growth [145].

Initial M1 macrophages produce chemokines and cytokines such as IL-6, TNFα, and IL-1β that are mitogenic and chemoattractant for muscle progenitor cells, resulting in the build-up of satellite and mono-nuclear cells at the site of injury [146–149]. When absent, these cytokines have been shown to reduce muscle repair *in vivo* [148]. On the other hand, it has also been demonstrated that these pro-inflammatory cytokines can prevent differentiation of muscle progenitor cells and their presence at the site of remodeling can negatively affect the later stages of regeneration process [150,151]. Therefore, a switch to an M2 environment after initial pro-inflammatory phase is necessary for resolving the inflammatory response. In fact, this transition occurs during the initial stages of differentiation of muscle satellite cells into differentiated skeletal muscle cells [152]. The switch from an M1 to an M2 microenvironment during the early and terminal differentiation stages not only mitigates the inflammation as well as production of cytotoxic nitric oxide, but also supports the fusion and differentiation of cells [124,130]. For example, anti-inflammatory IL-10 secreted by M2 macrophages is believed to both induce a polarization from an M1 to an M2 phenotype and support the fusion and maturation of myotubes [153]. Moreover, additional studies have suggested that transition within M2 phenotypes (from M2a to M2c sub-populations) may also play an important role in affecting later stages of differentiation as well as functional recovery [154].

Similarly, defective macrophage polarization in the process of muscle atrophy which occurs in models of muscular dystrophy causes a continuous recruitment of progenitor cells which are unable to differentiate due to concurrent presence of M1 and M2 population [154,155]. Contrary to the normal process for remodeling of muscle tissue, the

environment of this disease inhibits the switch from M1 to M2 which may explain why ineffective remodeling happens in these models.

3.7.4 Neural Tissue

Nervous system consists of central nervous system (CNS) and peripheral nervous system (PNS) which both have their unique recruitment process in response to injury. Generally, in contrast to CNS, PNS is more capable of repair. Injury to the PNS induces a well-orchestrated cellular process that leads to the complete disintegration of the nerve segment distal to the lesion site, termed Wallerian degeneration (WD) [95].

WD in the distal nerve segment starts within the first 24 h after axotomy, followed by fibrin cable formation, and Schwann cell (SC) and fibroblast infiltration from the both proximal and distal nerve stumps[156]. The SCs migrating from the proximal nerve stump advance along with regenerating axons, which grow through the enriched matrix and span the nerve gap. These regenerating axons reach the distal nerve stump and eventually make connections to the end organ [3]. Almost immediately after peripheral nerve injury, SCs dissociate from axons, dedifferentiate, and, along with fibroblasts, secrete cytokines that promote infiltration of immune cells. Neutrophils, the first immune cells to infiltrate, accumulate in the distal stump within 8 h, but their presence is short-lived [157]. Around the same period of time, circulating monocytes are attracted to the injured nerve where they differentiate into macrophages, and within days they take over as the dominant leukocyte population and play a critical role in ensuring complete WD. They account for the bulk of phagocytosis and debris removal, growth factor production, and remodeling of the ECM of the distal nerve [158,159].

Acute injury in the peripheral nervous system results in triggering of anti-inflammatory and immunosuppressive response, rather a pro-inflammatory response [160]. In fact the signal supporting the alternative macrophage activation is produced immediately after nerve injury and seemed to be established within the nerve, well before

monocytes/macrophages infiltration [160]. Moreover, axotomy of the sciatic nerve in a model of WD leads to the expression of multiple negative regulators of the innate immune system such as Interleukin-1 receptor antagonist (IL-1RA) and myeloid differentiation 88 small (MyD88) which negatively regulated the NF-κB activation [161] within 4 h after injury [160]. It is suggested that stimulation of alternative macrophage response in the nerve seemed to be regulated at the level of IL-13, which is together with IL-4 are the central master knobs for M2 phenotype polarization [33,162]. The main anti-inflammatory cytokines such as IL-4, IL-10, IL- 13, LIF and TGFβ can support axonal outgrowth by modulating the immune cells [163]. For examples IL-4 stimulates immune cells to produce Neurotrophin-3 (NT-3) [164] and Neurotrophin-4 (NT-4) [165] which are both neurotophic factors belong to Nerve Growth Factor (NGF) family of neutrophins. In fact, the facial motoneuron survival after axotomy is significantly lower in IL-4 deficient mice [166]. Moreover, TGFβ increases the chemotactic action of NGF in immune cells [167].

In Central nervous system (CNS), due to the nature of the neural tissue construct, the process and involvement of macrophages and monocytes differs significantly from PNS and other previous examples. Tissue remodeling process in response to injury of the CNS mainly involves mainly the local immune cells. This is due to the existence of the blood brain barrier which is responsible for protecting the central nervous system from the innate immune responders. At early time points following injury of CNS, local tissue innate immune cells called microglia are recruited. Because of microglia's predominantly pro-inflammatory phenotype and the cytotoxic microenvironment developed following injury, tissue damage propagates and scar tissue forms [129]. Although tissue remodeling response in the absence of blood brain barrier breach does involve a small number of blood-derived monocytes at the late time points following injury [168,169], the main pro-inflammatory response differs from the tissue remodeling process involved in the periphery. Circulating monocytes are involved in the peripheral nerve damage leading to spontaneous recovery depending on the extent of the injury. These blood derived monocytes may be capable of

adopting an anti-inflammatory and more regulatory phenotype which promotes functional recovery and resolution of inflammation [170]. This type of response requires a high recruitment of blood-derived monocytes which is unlikely to occur during CNS injury.

In a recent study, the process of tissue repair following spinal cord injury in the context of macrophage polarization has been investigated [129]. It was observed that immediately after injury, predominantly M1 macrophages were induced and remained within the spinal cord lesions. Although at early time points a small number of M2 phenotype were observed, these cells were not seen at later time points. The experimental results suggest the existence of a unique M1 to M2 shift in the spinal cord which differs from that seen in other tissues. In addition, a distinct effect on the survival of neurons and neurite outgrowth was observed in this study [129]. Specifically, M1 conditioned media decreased neural cell viability while M2 conditioned media improved survival of neural cells with greater degree of neurite extension. Therefore, induction of a M2 type response may promote a more constructive remodeling tissue environment. This induction will improve the outcome compared to the default CNS injury where the dominant M1 type decreases neural cell viability [129,171]. Therefore, while in the (PNS), macrophage-mediated phagocytosis of axons was viewed as essential for regeneration, in the CNS, despite infrequent evidence of their potential benefit, the overall role of macrophages was considered to be negative [169].

There are few reports on the impact of injecting blood derived monocytes and polarized macrophages on scar tissue formation and motor function [172–174]. The results show that promoting functional recovery is achievable through injecting the macrophages incubated with sciatic never fragments [174]. Subsequently, a similar approach was tested in clinical trials in human patient, as a therapeutic approach [175]. The Schwartz group's further experiments showed that injecting skin derived macrophages into spinal cord lesions also promoted functional recovery [172]. Although the mechanism involved in the promotion of recovery by these cells in the CNS are not fully known, it is believed that

these cells influence the CNS repair by MMP secretions which can degrade the scar tissue and lead to axonal growth [170]. The optimal injection time of the cells was found to be 1 week post-injury. Similar to the skeletal muscle, the results strongly support the beneficial role of initial M1 macrophages in CNS remodeling [176]. While it is traditionally believed that tissue damage and lesion propagation are a result of recruitment of macrophages and monocytes, these results support the hypothesis that controlled recruitment of appropriate phenotypes of monocytes and macrophages encourage functional recovery in the neural tissue inflammation and remodeling.

3.8 Macrophage Modulatory Biomaterials

As discussed in the previous section, there is significant evidence that during healing process macrophages play a critical role and there is a strong association between immune responses, inflammation and macrophage activation [108,117,128]. Biomaterial experts have focused on the development of materials with ‘‘immunomodulating’’ capabilities, taking advantage of the growing knowledge on the wound healing processes. Brown et al. has demonstrated that the host macrophage response is not only an acceptable but in fact an essential element of a constructive tissue regeneration process for biologically derived implant scaffolds [177]. Therefore, it is preferable to focus on strategies which include, modulate and even promote the immune response rather than inhibit or suppress it.

In general, the main task of an initial M1 type response is to destroy potential pathogens within the wound site and to clean up the wound site from damaged tissue and dead cells. On the other hand, M2 type response is associated with tissue remodeling, which results in either scarring or healing as an outcome, depending on the timing of the phenotype switch [108,170]. In fact, prolonged M1 polarization or overly exuberant transition to an M2 phenotype may lead to excessive scarring or a delay in wound-healing, respectively [128,178]. Therefore, it is important to note that an effective and timely switch

in macrophage polarization is almost always a key component of a positive outcome. However, to date, studies investigating the specific effects of macrophage polarization after synthetic or naturally derived biomaterials implantation have been few.

The first simple and rational approach to modulate macrophage phenotypes is to control physical cues of biomaterials. Recent studies have shown that porous biomaterials elicit less encapsulation and more vascularity compared to non-porous forms [179–181]. To determine the importance of porosity for healing, a series of scaffolds were fabricated in which pores were spherical, uniformly sized, and interconnected. The result has suggested that those materials possessing pores of 30–40 μm healed with very little fibrosis and great degree of vascularity [180]. The reason behind the observed pore size effect is unknown. However, it is hypothesized that by inhibition of spreading (phagocytic) phenotype, large numbers of MØ macrophages in the 30–40 μm pores are being ultimately directed to the regenerative M2 pathway rather than the tissue-destructive M1 and foreign-body giant cell (FBGC) pathway. Although these pores were observed to be heavily infiltrated by macrophages, the ratios of M2/M1 macrophages were significantly higher in comparison to non-porous solid forms, other uniformly sized pores, and non-uniformly sized pores [180,182].

Modification of surface typography is an alternate strategy for physical modulation of macrophages [183–185]. Proliferation, adhesion and migration of cells occur in response to ECM components at the nanoscale. However, Paul et al. showed that micro- structured, rather than nanostructured topography on polyvinylidene fluoride induced human macrophages to an activated state that had the characteristics of both M1 and M2 macrophages [185]. Parallel gratings imprinted on polymeric surfaces with line width ranging from 250 nm to 2 mm were shown to affect macrophage morphology, cytokine secretion, and adhesion independent of the biomaterial surface chemistry [184]. Not only size scale of topographical structure, but also its 3D spatial orientation modulates the macrophage phenotype [186]. In fact, according to a recent study, spatial orientation (2D

vs. 3D) has a much pronounced effect than alteration of surface chemistry on monocyte adhesion and migration as well as on macrophage phenotype and inflammatory mediator profile [186].In addition, independent studies have recently showed that biomaterial surface roughness and stiffness can modulate phenotype of macrophages [187,188].

Together, above studies suggest that modulating macrophage phenotype to M1 or M2 with tuning biomaterial physical and morphological properties is possible. However, besides physical features, there exists a variety of advanced methods for decorating biomaterial surfaces with specific small molecules or biomolecules with known immunomodulatory effect as well as methods for delivering immunomodulatory cytokines. Although the majority of current methods of modulating immune response which includes incorporating bioactive molecules such as adhesion sites, growth factors, and anti-inflammatory mediators or drugs, focus on controlling the number of inflammatory cells at the site of implant (for complete review, refer to [189]), very few studies have investigated the delivery of molecules specifically designed to modulate macrophage phenotype as part of a tissue engineering and regenerative medicine strategy [190–192] .

One study demonstrated that local administration of IL-4 can mitigate polyethylene particle-induced osetolysis through macrophage modulation by reducing the ratio of M1/M2 macrophages [191]. The generation of wear debris is an inevitable result of normal usage of joint replacements which incites chronic macrophage-mediated inflammatory reaction, and therefore leads to implant failure. Moreover, patients requiring revision following total joint replacement had a higher proportion of M1 macrophages than M2 as compared to normal, non-operated osteoarthritic synovial tissues [193]. This is consistent with the hypothesis that particles produced from wear of prosthetic joints activates macrophages and lead to their M1 polarization [193]. These studies strongly suggest that strategies which modulate macrophage polarization toward a more balanced M1/M2 or predominantly M2 phenotype following the implantation can potentially prevent the implant failure [192].

Although immunomodulatory cytokines such as IL-4 are very specific and effective, the clinical challenges presented by protein-based therapies make the use of small molecule alternatives attractive. In a recent study, the role of sphingosine-1-phosphate (S1P), a bioactive signaling lipid, in harnessing endogenous repair mechanisms via macrophages to promote neo- vascularization and tissue regeneration in cranial and long bone critical size defect model was evaluated [190]. S1P signaling is involved in determination of macrophage phenotype and exogenous delivery of S1P promotes macrophages to adopt an M2-like phenotype [194]. *In vivo* delivery of FTY720, a stable analog of S1P, to locally injured tissues in a mandibular bone defect model demonstrated an increase in recruitment of M2 macrophages ($Ly6C^{low}CD206+$) and an enhancement in blood vessel formation which led to a significant bone tissue ingrowth. It is suggested that local delivery of FTY720 from polymer nanofibers scaffolds influenced the phenotype of macrophages at the site of injury by affecting their recruitment, differentiation and/ or proliferation [190]. Local delivery of S1P synthetic analog has also been shown to help with the recruitment of regenerative anti-inflammatory monocytes and subsequently with the enhancement in vascular remodeling [195].

Besides local modulation using immunomodulatory molecules, macrophages can also be modulated by systemic injection of nanoparticles that target circulating or tissue resident macrophages [196]. For example, lipid-based nanoparticles hold great promise as delivery vectors in the treatment of cancer, inflammation, and infections and numerous strategies based on these nanoparticles are being developed to carry drugs into specific target sites [196]. Because liposomes are often first taken up by phagocytic cells, they can simply be designed to target these cells. An elegant example for liposomes, which induce an anti-inflammatory effect, is demonstrated by the phosphatidylserine (PS)-presenting liposomes [120]. This strategy employed intravenous injections of PS-presenting liposomes, mimicking the anti-inflammatory effects of apoptotic cells to modulate the macrophages phenotype at infarct site. In a rat model of acute MI, targeting of PS-

presenting liposomes to infarct macrophages, demonstrated improved infract repair [120]. The treatment promoted angiogenesis, preservation of small scars and prevented ventricular dilatation and remodeling. Following PS-liposome uptake by macrophages *in vitro* and *in vivo*, the cells secreted high levels of anti-inflammatory cytokines (e.g., TGFβ and IL-10) and up-regulated expression of mannose-CD206, concomitant with down-regulation of pro-inflammatory markers such as TNFα and the co-stimulatory molecule CD86 [120]. This PS- presenting liposomes as apoptotic-mimicking particles would act to resolve the inflammation after MI while providing a safe acellular, reproducible, and accessible approach.

Biomaterials can also become immunomodulatory when certain cell types are incorporated within the material. For example, encapsulation of stem cells in the biomaterial can modulate the inflammatory response in a paracrine fashion [197]. MSCs mediate their immunosuppressive effects via a variety of mechanisms. One such mechanism involves the induction of macrophages with immunomodulatory capacities. Macrophages co-cultured with mesenchymal stem cells (MSCs) consistently showed high levels of IL-10 (M2 indicators) and low levels of IL-12 (M1 indicator) compared to controls [197].Intravenous administration of MSCs after MI in mice suppresses the inflammatory response in the myocardium, leading to reduced infarct size, and improved systolic function [198]. Encapsulated hMSCs in alginate hydrogel modulates inflammatory macrophages both *in vitro* and *in vivo*, even in the absence of direct hMSC-macrophage cell contact, to an alternative M2 macrophage phenotype [199]. In a rodent spinal cord injury model, it is demonstrated that even very few hMSCs encapsulated in alginate hydrogel can significantly increase the anti-inflammatory and decrease the pro-inflammatory macrophages and cytokines [200]. These results suggest that conversion of macrophages to the M2 subset as a result of hMSCs effect is responsible, at least in part, for tissue protection and illustrate the potential that encapsulated hMSC has as a powerful *in vivo* immunomodulatory tool after trauma.

Collectively, these studies suggest that approaches which incorporate, modulate, and even encourage the host response rather than inhibit it can be very effective. Future strategies may achieve better regeneration outcome through addition of bioactive surface ligands, incorporation of soluble factors, formation of rational physical cues, and/or modulation of the tissue regeneration environment.

CHAPTER 4 EVALUATE THE EFFECT OF MODULATING MACROPHAGE PHENOTYPE ON PERIPHERAL NERVE REPAIR

4.1 Introduction[3]

The advent of the operating microscope and improved microsurgical techniques have improved clinical results and popularized nerve autograft surgeries after injury [81]. After nerve trauma, the standard clinical operating procedure is to appose the two nerve ends and suture them together without generating tension where possible. If the gap is large such that tensionless apposition is not possible, a nerve autograft - typically the patient's own sural nerve - is used as a bridge. Autografts are biocompatible, non-toxic, provide a support structure to promote axonal adhesion/extension, and currently are the best clinical bridges available. However, besides possibility of neuroma formation and a need for secondary surgery, a serious drawbacks to the use of autografts are that the availability of disposable nerve segments is limited, and multiple lengths of nerve graft are often needed to bridge the gap between the injured nerve stumps [4]. Moreover, only 40-50% of patients regain useful function after receiving autografts [79]. In addition, autografts contain inhibitory chondroitin sulfate proteoglycans (CSPGs), which may reduce their performance as bridges [39,40]. Therefore there is a clear and urgent, unmet clinical need to find an alternative approach to the use of autografts that can match or exceed autograft performance [3,4,201].

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[3] Mokarram, N., A. Merchant, V. Mukhatyar, G. Patel, and Bellamkonda, R. V. Effect of modulating macrophage phenotype on peripheral nerve repair. *Biomaterials* 33:8793–8801, 2012.

Driven by this need, several bioengineering strategies to enhance regeneration have been explored including the design of novel nerve guidance channels [4,77], fillers within nerve guidance channels [202], local delivery of neurotrophic factors [82,83], transplantation of cells [84,85], and application of topographical cues [76,87,203]. While these approaches are promising, the overall success rate in matching autograft performance in bridging long nerve gaps has been limited. Fundamentally, it is challenging to replicate individual aspects of a complex biochemical regenerative cascade although the search for the minimum critical elements continues and current approaches focus on enhancing axon growth by direct action on nerves, or glial cells.

In this chapter, we investigate an alternative approach to influencing regenerative outcomes by modulating the initial inflammatory sequence via macrophages [108]. Since the sequence of cellular and molecular events associated with nerve regeneration is influenced by immune cells [95,102], we hypothesize that modulating immune cells upstream of action on nerves or Schwann cells (SC) triggers endogenous repair mechanisms that can stimulate nerve repair across long gaps [108].

It is evident that the immune response plays an important role during regeneration in many tissues [13,97,204]. Macrophages are quite abundant and phenotypically diverse immune cell populations presented during nerve degeneration and regeneration [95,108,129]. Macrophages arrive at the site of injury within 24 h and their numbers at the site peak within 14-21 days [205], whereas it takes at least one week for lymphocyte influx to occur [95]. Macrophages, which are mainly recruited from circulation, account for the bulk of phagocytosis within days of peripheral nerve injury and play a critical role in debris removal, growth factor production, and remodeling of the extracellular matrix (ECM) of the distal nerve [158,159]. Thus macrophages might represent an upstream ‘lever’ to influence downstream axon and SC regeneration.

Recently, macrophages have been demonstrated to have a spectrum of activation states/phenotypes which has led to categorizing them broadly as classically activated (M1)

and alternatively activated (M2) macrophages [32,33,61]. M1 macrophages, which are activated by injury-triggered endogenous inflammatory signals, such as T-helper 1 (Th1) cytokine IFNγ, considered to be inflammatory, microbicidal, and tumor destructive [32,61]. M1 macrophages produce high levels of oxidative metabolites (e.g. nitric oxide and superoxide) as well as pro-inflammatory cytokines. On the other hand, M2 macrophages, which can be activated by Th2 cytokines such as IL-4, IL-10 and IL-13, support tissue repair by producing anti-inflammatory cytokines which mediate angiogenesis, cell replacement and matrix remodeling while suppressing destructive immunity [32,206]. The M2 phenotype has also been shown to have its own subtypes (M2a, M2b and M2c) [93,115]. While the exact role of each M2 subtype is not well defined, it has been shown that M2a and M2c enhance tissue repair and pro-healing functionality, but the M2b subtype possesses regulatory function with some characteristics of both anti-inflammatory and pro-inflammatory macrophages [93,115].

Despite the fact that macrophages constitute a substantial fraction of the post-injury cellular milieu, very little attention has been devoted to understanding the contribution from different phenotypes of macrophages and the effect of their modulation on regeneration after injury, especially in the context of the peripheral nervous system (PNS) [95,128]. Here, we designed a biomaterial-based conduit to modulate the phenotype of macrophages by employing two principal cytokines (IFNγ or IL-4) for polarizing the early innate immune response toward pro-inflammatory (M1) or pro- healing (M2a and M2c) phenotypes, respectively. Then, we investigated the influence of macrophage phenotype on the peripheral nerve regeneration in a critically-sized, non-healing 15 mm rat sciatic nerve gap (Figure 4.1).

4.2 Materials and Methods

4.2.1 Scaffold Implantation

Polysulfone tubes filled with 0.7% Agarose (SeaPrep Lonza) mixed with 1 mg/ml rat recombinant cytokine IFNγ, IL-4 (Antigenix America Inc.) or Agarose only were prepared (n=4 per group). The scaffolds were kept cooled in 4°C for 1 h. Adult Lewis male rats (250−300 g) were induced to anesthetic depth with inhaled isoflurane at 3−4%. The procedure for fabrication of PAN-MA nano-fiber film scaffolds (n=4) were explained elsewhere [76]. 15 mm nerve autografts, which were resected from the rat tibial nerve being bridged were used as our positive control (n=4). Throughout surgery, the animals were maintained at 1.5−2% isoflurane. Microscissors were used to transect the tibial nerve branch, and the nerve stumps were pulled 1 mm into each end of the 17 mm (Agarose with cytokine) guidance scaffolds (leaving a 15 mm gap) or autografts and fixed into place with a single 10−0 nylon suture (Ethicon). The muscles were reapposed with 4−0 vicryl sutures (Ethicon) and the skin incision was clamped shut with wound clips (Braintree Scientific). After the surgery, the rats were placed under a warm light until sternal, and then housed separately with access to food and water ad libitum at constant temperature (19−22°C) and humidity (40−50%) on a 12:12 h light/dark cycle. To prevent toe chewing, a bitter solution (Grannick's Bitter Apple) was applied twice daily to the affected foot. Marcaine (0.25% w/v) was administered subcutaneously for post-surgical pain relief (0.3 ml/animal). Animals were maintained in facilities approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Institute of Technology and in accordance with the current United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards.

4.2.2 Scaffold Explantation and Analysis

After three weeks, scaffolds or autografts were explanted for histological analysis of nerve regeneration. Explants were fixed in 4% paraformaldehyde in PBS (Sigma

Aldrich), washed, and stored in 30% sucrose in PBS for 24 h. Samples were embedded in O.C.T. gel (Tissue Tek) and frozen for cryosectioning (CM30505, Leica). Scaffold or autografts were sectioned transversely or longitudinally at a thickness of 16 mm and reacted with immunofluorescent markers to quantify the different cell types, using techniques previously described [76]. Briefly, sections were incubated for 1 h at room temperature in a blocking solution of goat serum (Gibco) in PBS, incubated overnight at 4°C in a mixture of primary antibody and blocking solution, washed and incubated for 1 h at room temperature in a solution of secondary antibody mixed in 0.5% triton in PBS. Slides were washed twice more with PBS, incubated with DAPI for 10 min and then dried and cover slipped for evaluation. Primary antibodies NF160 (1:250, mouse IgG1, Sigma), S100 (1:250, rabbit, IgG, Dako Cytomation), CD68 (1:100, mouse IgG1, AbD Serotec (ED1)), CD163 (1:200, mouse IgG1, AbD Serotec (ED2)), CCR7 (1:200, rabbit, IgG, Cell Application), and CD206 (1:100, rabbit, IgG, Santa Cruz Biotechnology) were used. The following secondary antibodies were used: goat anti-rabbit IgG Alexa 488/594 (1:220, Invitrogen), goat anti-mouse IgG1 Alexa 488/594 (1:220, Invitrogen).

4.2.3 Statistical Analysis

Data were analyzed using the Student's t-test for unpaired data where appropriate or one-way ANOVA followed by a Tukey's least significant difference post-hoc test where warranted to determine significant differences between groups with 95% confidence; A p value < 0.05 was considered to represent a statistically significant difference.

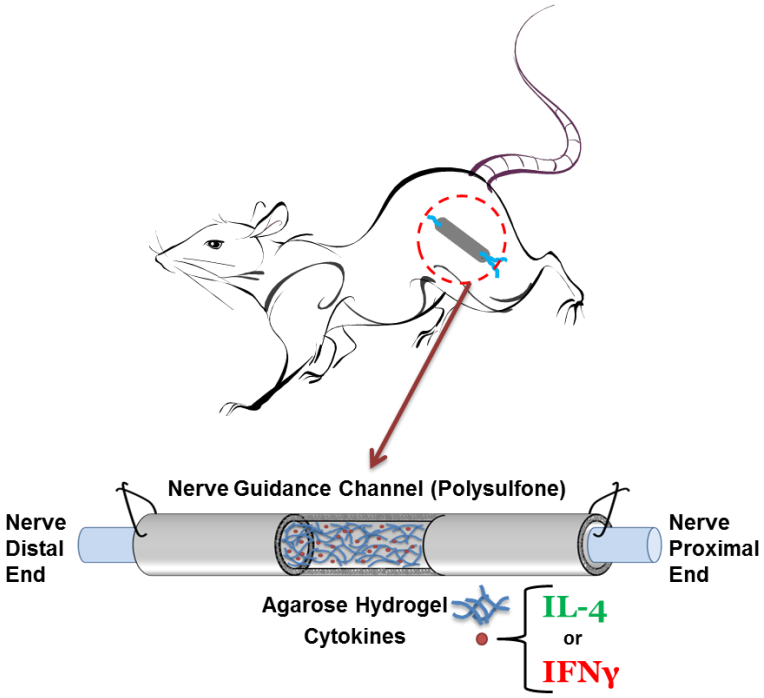

Figure 4.1: Schematic of rat sciatic nerve model and the nerve guidance channel structure.

4.3 Results

4.3.1 Effect of Immunomodulatory Cytokines, IFN-γ or IL-4, on the macrophages within the nerve scaffold, *in vivo*

The influence of delivering either IFNγ or IL-4 on the phenoltypes of macrophages and the subsequent impact on the rate of peripheral nerve regeneration in rodents was assessed *in vivo*. IFNγ or IL-4 (1000 ng/mL) mixed with an Agarose hydrogel was injected inside a 17 mm, semi-permeable, hollow Polysulfone tube that was in turn used to bridge a 15 mm sciatic nerve gap in rats (Figure 4.1). Based on the *in vitro* study, these cytokines diffuse out from the hydrogel within 24 h (Figure 7.2).

After three weeks, animals were euthanized, followed by immunohistochemical staining with CD68, CD163, CCR7 and CD206 to identify total number of activated macrophages, alternatively activated macrophages (M2), pro-inflammatory macro- phages (M1) and anti-inflammatory/pro-healing macrophages (M2a and M2c), respectively. Previously established immunohistochemistry techniques for quantifying fluorescent pixel intensity of aforementioned antibodies were used to quantify macrophages and their subtype [76,92,177,207]. The majority of macrophages remained on the outer layer of the regenerative cable (nerve bundle) and their density decreased approaching the center of the cable (Figure 4.2a). Although both IFNγ and IL-4 increased the total number of macrophages in comparison to non-cytokine treated scaffold (Figure 4.2b), this increase was not significantly different between IFNγ and IL-4 samples. However, the numbers of M1 (CCR7+) and M2 (CD163+) macrophages as well as M2 subtypes, M2a and M2c (CD206+) macrophages, were significantly different between IFNγ and IL-4 treated scaffolds (Figure 4.2Figure 4.3). The ratios of CCR7+ cells to CD68+ cells were significantly lower in both IFNγ and IL-4 containing scaffold ($n= 4$, $P < 0.001$, Student's t-test) (Figure 4.3b). However, the ratios of CD163+ and CD206+ cells to CD68+ cells indicating different subtype of M2 macrophages were higher in IL-4 containing scaffold in

comparison to control (n=4, $P<0.01$ and $P<0.001$, Student's t-test) while in IFNγ containing scaffolds, these numbers were significantly different from the control scaffold (Figure 4.2c andFigure 4.3c).

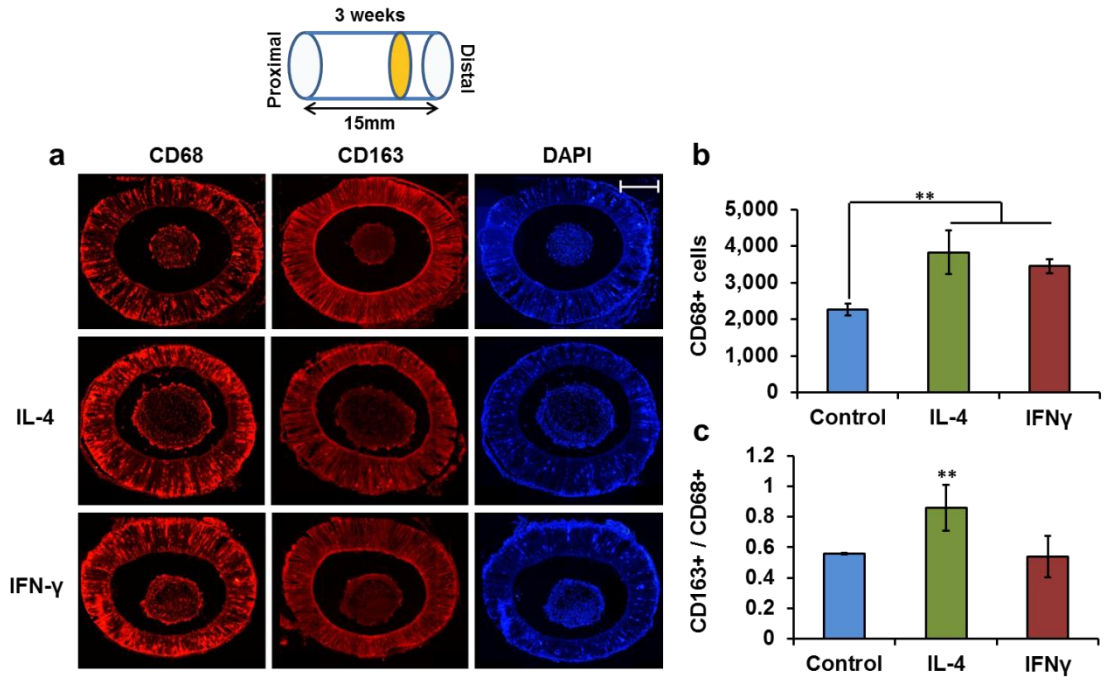

Figure 4.2: Effect of IFNγ and IL-4 on the macrophages and their phenotypes at the site of sciatic nerve injury in the scaffold.

(a) Cross-sectional images of CD68+ cells and CD163+ cells representing number of macrophages and alternatively activated macrophages (M2) at the distal end, respectively. Blue represents the DAPI. Scale bar = 500 μm (b) Quantitative analysis of number of macrophages. Mean ± s.d. n = 4. **P < 0.01 (Student's t-test). (c) Ratio of the $CD163^{+}$ cells to the $CD68^{+}$ cells represents the ratio of alternatively activated macrophages (M2). Asterisks denote a significant difference compared with non-cytokine treated control scaffolds. Both IL-4 and IFN-γ increases the number of macrophages at the distal end of the scaffold.

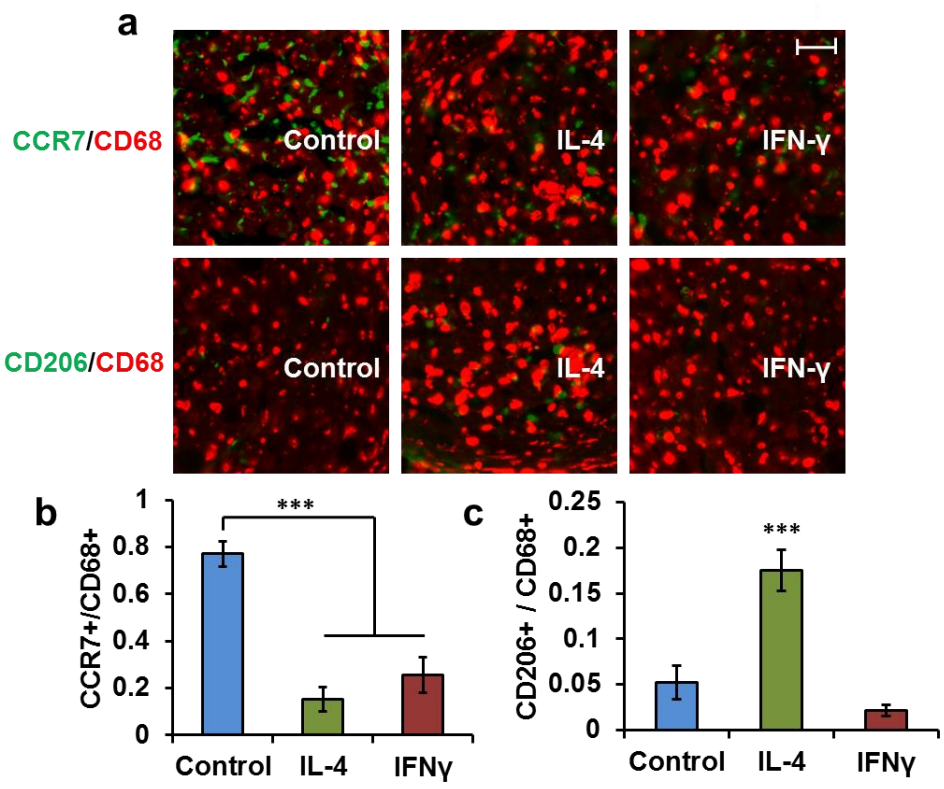

Figure 4.3: Effect of IFNγ and IL-4 on the macrophages and their phenotypes at the site of sciatic nerve injury in the scaffold.
(a) Magnified merged images of CCR7/CCD68 (green/red) and CD206/CD68 (green/red). Scale bar = 40 μm . (b) Ratio of the CCR7+ cells to the CD68+ cells represents the ratio of classically activated macrophages (M1). Mean ± s.d. n = 4. ***P < 0.001 (Student's t-test). (c) Ratio of the CD206+ cells to the CD68+ cells represents the ratio of pro-healing macrophages (M2a and M2c). Mean ± s.d. n = 4. ***P < 0.001 (Student's t-test).

4.3.2 Effect of Immunomodulatory Cytokines, IFN-γ or IL-4, on the SC infiltration and axonal growth, *in vivo*

SC infiltration into the nerve gap was investigated by using immunostaining for SC protein marker (S100) (Figure 4.4). IL-4 treatment considerably enhanced the SC migration toward the middle of the hydrogel as shown in the longitudinally sectioned samples (Figure 4.4a). On the other hand, both IFNγ and non-cytokine treatment resulted in less SC infiltration and thinner regenerative cable (Figure 4.4a, b). Even in the case of the non-cytokine treated control, where the cable formation was usually complete, SC rarely reached the midpoint (Figure 4.4b). A similar trend was found, based on quantitative analysis of fluorescent pixel intensity of immunoreactivity for S100 in the area occupied by SCs at the distal end of the scaffold (Figure 4.4c).

Axons were also visualized by using immunostaining for Neurofilament-160 (NF160) (

Figure 4.5). In contrast to the SC which infiltrate from the both ends of the scaffold, axons grow only from the proximal toward the distal end (

Figure 4.5a). In the case of IL-4 treated samples, axons reached almost the distal end after 3 weeks (

Figure 4.5b). Quantitative analysis using cross-sectional samples at the distal end of the scaffold showed that number of axons (positively stained for NF160) in the IL-4 samples were ~20 times higher in comparison to the non-cytokine treated control ($n = 4$, $P < 0.001$, Student's t-test) (

Figure 4.5c). The number of regenerated axons at the distal end of the scaffold was not significantly different from the control in IFNγ treated scaffold. Sections for all of these analyses were selected at exact same location on the scaffold and based on the observation of biggest difference.

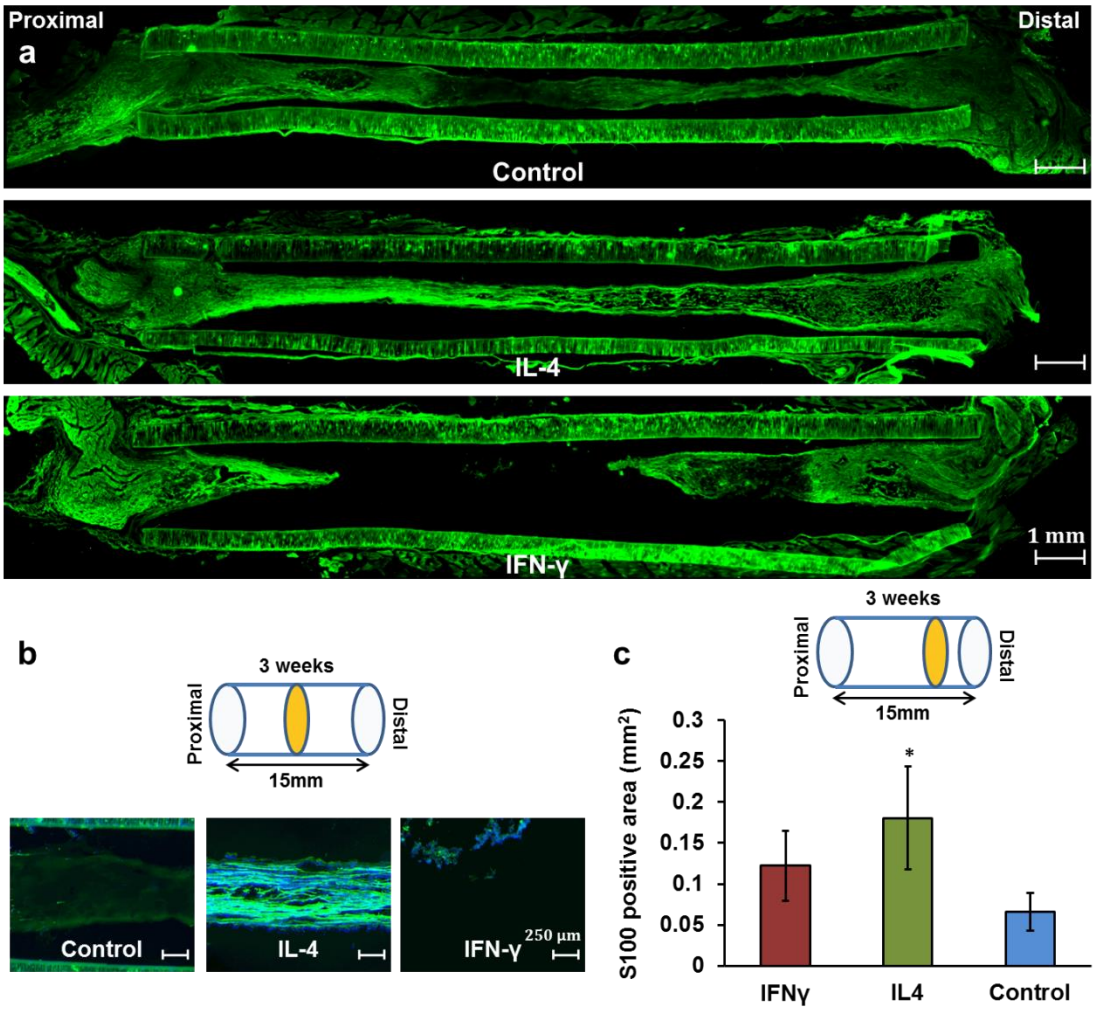

Figure 4.4: Effect of IFNγ and IL-4 release on Schwann cell infiltration inside the scaffold.

(a) Longitudinal section of S100 staining for Schwann cell. Scale bar = 1 mm (b) Merged magnified images with DAPI at the mid-section. Scale bar = 250 μm (c) Quantitative analysis of SCs at the distal end by using S100. Mean ± s.d. n = 3. *P < 0.05 (Student's t-test). Asterisks denote a significant difference compared with non-cytokine treated control scaffold. IL-4 treated scaffold significantly enhances the SC infiltration inside the scaffold in comparison to the non-treated control scaffold.

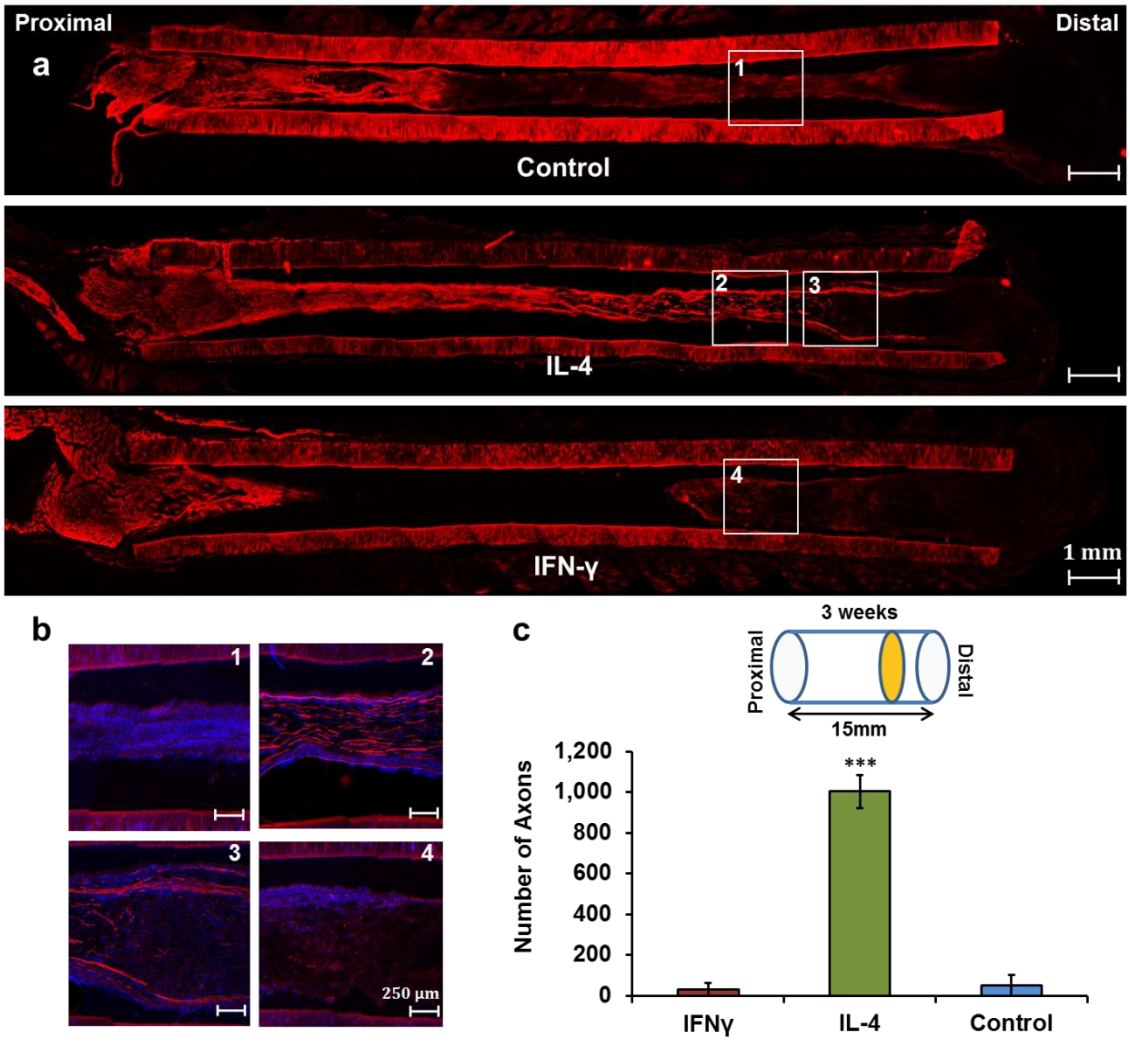

Figure 4.5: Effect of IFNγ and IL-4 release on the axonal growth.
(a) Longitudinal section of NF160 staining for axons. Scale bar = 1 mm (b) Merged magnified images with DAPI magnified images referring to white boxes in panel (a). Scale bar = 250 µm (c) Quantitative analysis of NF160 at the distal ends. Mean ± s.d. n = 4. ***P < 0.001 (Student's t-test). Asterisks denote a significant difference compared with non-cytokine treated control scaffold. Number of axons at the distal end of the IL-4 treated scaffold is ~20 times higher in comparison to the non-treated control scaffold.

4.3.3 Correlation between the Ratio of CD206+ to CCR+ Macrophages and Number of Regenerated Axons, *in vivo*

CD206+ and CCR7+ cells represent the populations of macrophages that have pro-healing (M2a and M2c) and pro-inflammatory (M1) functions, respectively [115,207]. We define the ratio of pro-healing to pro-inflammatory population of macrophages (CD206+/CCR7+) as the regenerative bias. In the IL-4 containing scaffolds, the regenerative bias is approximately twenty times higher than in either the non-cytokine treated control or IFNγ containing scaffolds (Figure 4.6). We calculated the regenerative bias as well as number of axons at the distal ends for two completely separate conditions too: Autograft and Polysulfone scaffold filled with a layer of Poly(acrylonitrile-co-methyl acrylate) (PAN-MA) nano-fiber film [76,86] (Figure 4.6). Combined with this new set of data, we derived a relationship between the regenerative bias and axonal growth (Figure 4.6):

$$A = 833.7 \times RB + 2.088$$

$$R^2 = 0.935$$

where A and RB represent the number of axons and the regenerative bias (the ratio of CD206+ to CCR7+ cells) at the distal, respectively. Figure 4.6 shows that there is a direct correlation between ratio of pro-healing to pro-inflammatory macrophages and number of axons at the distal end of the nerve scaffold in this model. Nevertheless, samples with regenerative bias higher than 1.5, especially autografts, start to deviate from this linear relationship.

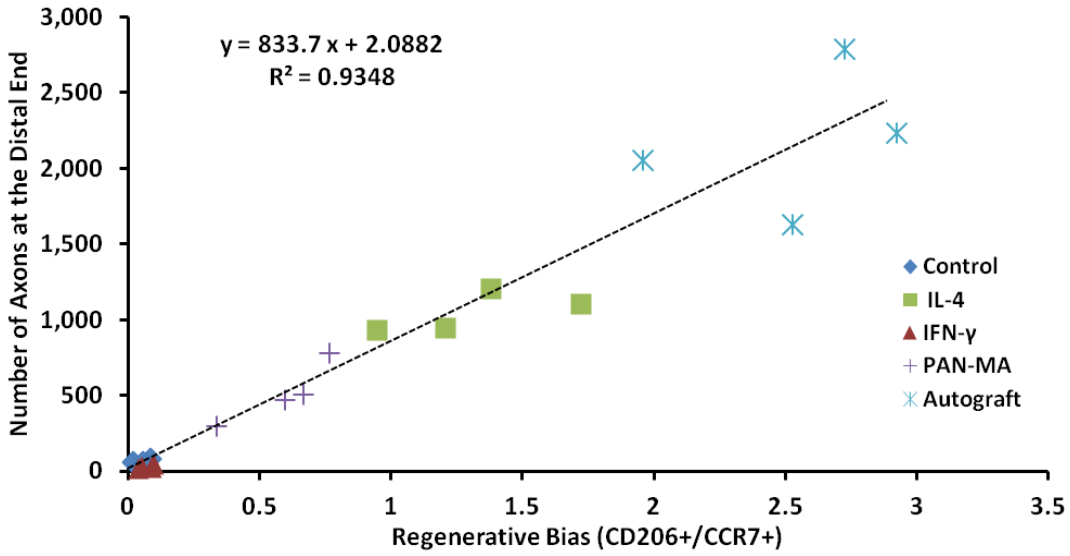

Figure 4.6: Regenerative bias correlates with axonal growth.
At the distal end of nerve stump, the ratio of pro-healing macrophages (CD206+) to pro-inflammatory macrophages (CCR7+), i.e. regenerative bias, directly correlates to (predicts) the final regenerative outcome (axonal growth) with a linear relationship at three weeks post-injury time point. The fact that data from autograft and PAN-MA nano-fiber scaffolds also fit the regenerative bias/axonal growth correlation demonstrates that this model can be universally applied across other nerve regeneration approaches.

4.4 Discussion

Injury to the peripheral nervous system (PNS) induces a well-orchestrated cellular process that leads to the complete disintegration of the nerve segment distal to the lesion site, termed Wallerian degeneration (WD) [208]. WD in the distal nerve segment starts within the first 24 h after axotomy, followed by fibrin cable formation, and Schwann cell (SC) and fibroblast infiltration from the both proximal and distal nerve stumps [156] (Figure 4.7). The SCs migrating from the proximal nerve stump advance along with regenerating axons, which grow through the enriched matrix and span the nerve gap. These regenerating axons reach the distal nerve stump and eventually make connections to the end organ[3]. Almost immediately after peripheral nerve injury, SCs dissociate from axons, dedifferentiate, and, along with fibroblasts, secrete cytokines that promote infiltration of immune cells. Neutrophils, the first immune cells to infiltrate, accumulate in the distal stump within 8 h, but their presence is short-lived [157]. Around the same period of time, circulating monocytes are attracted to the injured nerve where they differentiate into macrophages, and within days they take over as the dominant leukocyte population and play a critical role in ensuring complete WD. They account for the bulk of phagocytosis and debris removal, growth factor production, and remodeling of the ECM of the distal nerve [158,159].

WD and subsequent regeneration are significantly influenced by various immune cells and the cytokines they secrete. Among all immune cells, macrophages are quite abundant during nerve degeneration and regeneration [95,108,129]. Although macrophages have long been known to play a vital role in the degenerative process, recent work has pointed to their importance in influencing the regenerative capacity of peripheral neurons (Figure 4.7) [95,100,101]. Macrophages, in contrast to lymphocytes which regulate the later phases of regeneration, modulate the early sequence of cellular events which can determine the fate of healing process. They are playing at least two important roles during that process: creating a pathway in the distal nerve segment conducive to

axonal regeneration and stimulating the axotomized neuronal cell bodies to switch to a regenerative phenotype [101]. Macrophages recruited from circulation account for the bulk of phagocytosis and play a critical role in debris removal, growth factor production, and remodeling of the ECM of the distal nerve [158,159]. However, an increasing number of studies in multiple animal models and organ systems have shown that untimely macrophage polarization, inhibition of macrophage polarization, or inability to resolve a chronic polarization towards the M1 or M2 extreme has a detrimental effect on regeneration outcome (Figure 4.7) [105,128,129,131]. That is, macrophages can play both beneficial and detrimental roles in the process of tissue remodeling and, in many cases, an efficient and timely phenotypic switch is essential for preventing a deleterious or scar tissue outcome. Thus, macrophages represent an upstream 'lever' to influence downstream axon and SC fates and peripheral nerve regeneration. Figure 4.7 represents our new insight regarding Wallerian degeneration and role of M1 and M2 macrophages during the regeneration process.

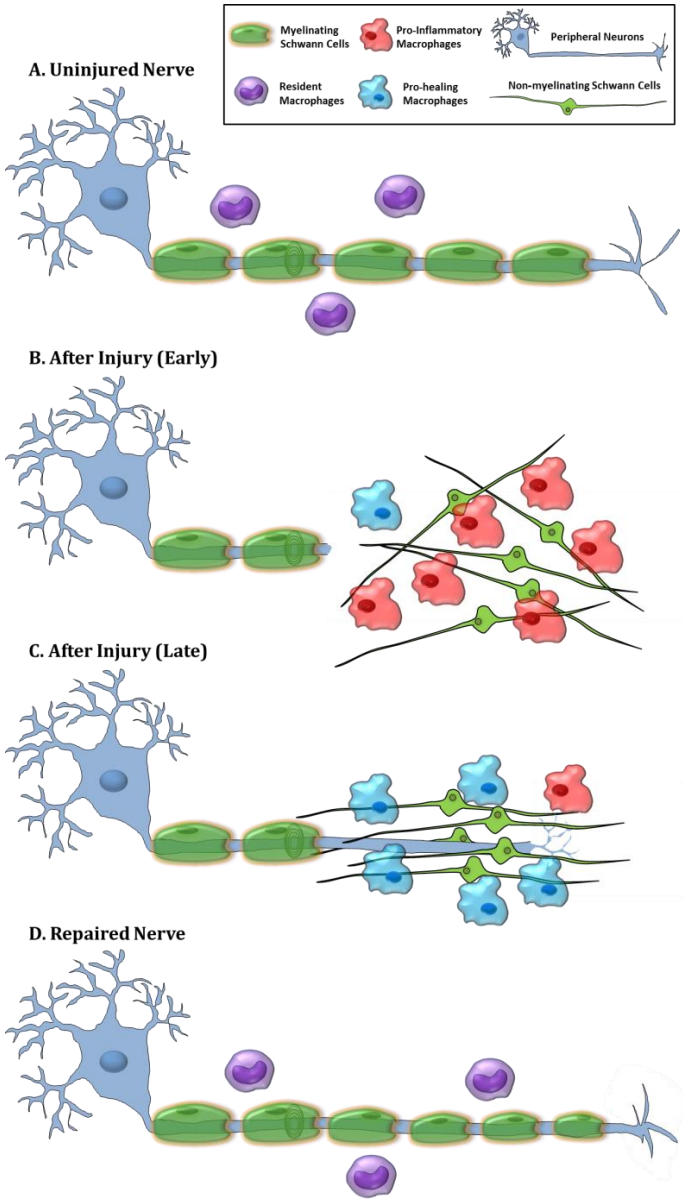
Myelinating Schwann Cells
Pro-Inflammatory Macrophages
Peripheral Neurons
Resident Macrophages
Pro-healing Macrophages
Non-myelinating Schwann Cells
A. Uninjured Nerve
B. After Injury (Early)
C. After Injury (Late)
D. Repaired Nerve

Figure 4.7: New Insight to Wallerian Degeneration and Axonal Regeneration after Injury. A) Uninjured nerve is associated with Schwann cells and resident (inactivated) macrophages. B) Immediately after injury, denervated myelinating Schwann cells release their myelin. These non-myelinating Schwann cells then proliferate and produce cytokines/trophic factors which along with other soluble factors produced from injured axons activate resident macrophages and lead to recruitment of hematogenous macrophages. The activated macrophages clear myelin and axon debris efficiently, change phenotype and produce factors that facilitate Schwann cell migration and alignment, and axon regeneration. C) At the later time points, injured axons form a growth cone and begin to regenerate along aligned Schwann cells. D). Successful regeneration would occur by axonal reinnervation to the peripheral target.

Moreover, although there is strong evidence to support the notion that macrophages modulate regeneration in the nervous system [95,108,129], it is unclear if they support [74,169,204] or hinder [209,210] regenerative biochemical cascades after injury [211]. This apparent lack of clarity might stem from the 'double-edged' characteristics of macrophages [108,212–214]. On one hand macrophages, as the primary phagocytes of the innate immune system, release inflammatory neurotoxin and cytokines leading to degeneration and necrosis. On the other hand, macrophages scavenge debris, stimulate trophic factors such as nerve growth factor (NGF) and IL-6 [206,215], and help remodeling the ECM [92]. These seemingly opposing effects may be explained by the existence of macrophages that are phenotypically and functionally heterogeneous.

It is not known if phenotype-committed macrophages are selectively recruited to the site of injury, or if the phenotype of non-committed macrophages is determined after recruitment by locally-present cytokines [67]. Regardless, macrophage phenotypes can be dynamically regulated by biological cues from the tissue microenvironment [62]. During the course of wound healing in some tissues, such as cutaneous or myocardial tissue, there is a well-defined shift in macrophage activation states: from M1 to M2 and subsequent secreted products [94,114]. However, in other tissues such as the spinal cord, this shift does not occur after injury [129] and hence, it is postulated that this results in impaired recovery of those tissues. Although macrophage phenotypes and their roles in the course of regeneration after traumatically injured central nervous system (CNS) have been explored

[93,129], the effect of macrophage phenotype on PNS regeneration and its associated cells (such as SC and axons) remains to be characterized [95,128].

To address this gap in our understanding, the effects of modulating macrophage phenotype on nerve regeneration were evaluated in a rat sciatic nerve model using Polysulfone/Agarose hydrogel scaffolds mixed with two immunomodulatory cytokines, IL-4 or IFNγ. Although Agarose hydrogels have previously been demonstrated to support regeneration of peripheral nerves [77,216], our study revealed that short-term release of anti-inflammatory cytokine (IL-4) in the Polysulfone/Agarose nerve guidance scaffold substantially accelerates downstream axon regeneration.

Our results strongly suggest that rather than the extent of macrophage presence, their specific phenotype at the site of injury biases the regenerative outcomes. It can be postulated that these results stem from the indirect effect of immunomodulatory cytokines, IL-4 or IFNγ, on SC and axons via modulation of macrophages phenotype since neither of these two cytokines have any significant direct effect on axonal growth [217] [47] and SC migration (see CHAPTER 5). We established that modulating the initial inflammatory sequence via macrophages can bias the regenerative biochemical cascade in a significant manner that obviates the need for the downstream modulation of multiple factors after peripheral nerve injury.

In fact, we defined a new term, regenerative bias, based on the ratio of CD206+/CCR7+ ((M2a + M2c)/M1) macrophages and found a direct correlation between this number and the rate of SC infiltration and axonal growth *in vivo* (Figure 4.4 andFigure 4.6). Our data showed that the number of CD163+ cells (M2 macrophages) and CD206+ cells (M2a and M2c macrophages) were both increased by IL-4 treatment; however this increase was more drastic in the number of CD206+ cells in comparison to CD163+ cells; for this reason, we chose CD206+/CCR7+ ratio to represent inflammatory status of the scaffold as the regenerative bias instead of other potential options such as CD163+/CCR7+ (M2/M1) or CD206+/CD68+ (M2/M) ratios. Moreover, IL-4 polarizes

macrophages toward the M2a subtype, directly, and toward the M2c subtype via M2a secretory products such as IL-10 or TGFβ, indirectly [33,93]. CD163 is a common surface marker among all the subtype of M2 macrophages while CD206 is the surface marker only for M2a and M2c macrophages [93]. Therefore, compared to other potential aforementioned options, the ratio of the CD206+/CCR7+ cells better indicates the effectiveness of the immunomodulatory cytokine delivery at the site of nerve injury. Both CD206 and CCR7 are widely used to characterize the phenotype of macrophages in animal and human models [92,115,128,177,218]. CCR7 is also known to be expressed by certain subtypes of dendritic and T-cells; however, the level of this expression is much smaller as compared to macrophages [92,128,177].

It has already been shown that axons can also be directly affected by secretory products of different phenotypes of macrophages in the CNS [129,171]. Although CM from the unstimulated macrophages has a minimal effect on axonal growth, M1 macrophages are neurotoxic [129,171]. In contrast, M2 macrophages not only have no adverse effect on cortical neurons, but also promote the regenerative response [129]. Additionally, Kigerl et al. demonstrated that M2 macrophages can even overcome axonal growth inhibition caused by CSPG and myelin [129]. Indirect modulation of macrophages toward the M2 phenotype by using fibroblast growth factor (aFGF) can also result in the secretion of growth factors that can potentially help axonal regeneration after spinal cord injury in rats [219]. It has also been shown that certain cytokines that promote M2 macrophage formation, such as IL-10, moderately enhance neuroprotection [220]. The Schwartz laboratory has demonstrated that appropriate activation of microglia by IL-4 or IFNγ differentially induces neurogenesis as well as oligodendrogenesis from adult stem cells [21]. However, to the best of our knowledge, exploiting macrophage phenotype modulation to promote regeneration in the PNS has not yet been reported.

Further, to evaluate our regenerative bias/axonal growth model in other nerve regeneration approaches, PAN-MA nano-fiber film scaffold and autograft were also

studied based on this model. Our data demonstrated that these two completely different approaches for regeneration also fit the regenerative bias model which suggests that this model, independent of the strategy or kind of regenerative scaffold, can be universally applied across other nerve regeneration approaches. However, the noticeable deviation from this linear correlation in high-regenerative-capacity conditions, such as autograft, clearly indicates the involvement of other critical factors in the process of rapid regeneration which this regenerative bias model cannot fully predict.

Although the axonal growth in the IL-4 containing scaffold in this study (as determined by the number of axons at the distal end) is still lower than that for autograft, from our experience with alternate strategies to enhance peripheral nerve repair such as presenting topographical cues or delivering trophic factors, regulating macrophage phenotype using immunomodulatory cytokines resulted in a considerably faster rate of axonal regeneration in this critically-sized nerve gap [4,45]. In fact, the axonal growth rate, as defined by the length of axons in the nerve gap as a function of time, is faster by a factor of 2 compared to the best rate achieved in previous studies [4,32] (Figure 4.6). This data is significant because several studies have already clearly demonstrated that faster axonal growth substantially contributes to higher functional recovery [38,77,221,222].

This approach, by minimizing the need for sophisticated delivery vehicle(s), demonstrates that short and early stimulation of macrophages can significantly influence the longer-term regenerative outcomes. The effectiveness of this approach possibly originates from the fact that besides the direct effects of different macrophage phenotypes on SC infiltration and axonal growth, macrophages play a key role in other aspects of the regenerative process. They actively participate in cell replacement via stem cell recruitment and homing as well as in the resident progenitor cell differentiation and proliferation [74,147]. Their secretory products also influence the different phases of angiogenesis both *in vivo* and *in vitro* [96,223].

Finally, in this study only three surface markers (CD68, CCR7, and CD206) were employed to characterize macrophages and their phenotypes participating in nerve regeneration. It is becoming increasingly evident that other ways of examining macrophage phenotype(s) including protein (cytokine and chemokine) analysis, and gene profiling can help to characterize a specific phenotype better and understand the extent to which each phenotype perform. Furthermore, as with most models, the M1/M2 classification scheme certainly oversimplifies the complexity of the PNS macrophage response. In fact, the present study identified macrophages that co-expressed markers for both or none of the M1 and M2 macrophage. Although the effects of these intermediate macrophages are unknown in the context of PNS regeneration, this model provides a useful conceptual framework. Future studies should examine additional macrophage activation markers and cytokine expression to more extensively characterize the M1/M2 profile and the role these macrophages play in the peripheral nerve regeneration process. Moreover, this study introduces the regenerative bias concept and further studies on the other types of nerve scaffolds need to be done to evaluate the model more comprehensively.

4.5 Conclusion

Our data demonstrates that modulation of macrophage phenotype at the site of peripheral nerve injury can favorably bias the endogenous regenerative potential after injury that obviates the need for the downstream modulation of multiple factors and has significant implications for the treatment of long peripheral nerve gaps. Moreover, the present study strongly suggests that more than the extent of macrophage presence, their specific phenotype at the site of injury influence the regenerative outcomes. These significant results are complementary to a growing body of evidence showing the direct correlation between macrophage phenotype and the regeneration outcome in other tissues.

CHAPTER 5 INVESTIGATE THE INTERPLAY BETWEEN MACROPHAGES PHENOTYPE AND GLIAL CELLS FUNCTION.

5.1 Introduction

Biomaterial-based tubular nerve conduits (or guidance channels) have been used clinically for repairing peripheral nerve injuries [224]. These nerve conduits bridge the injured nerve stumps and help form a fibrin cable which provides a substrate for the ingrowth of SCs and other cells, such as fibroblasts or macrophages. However, this approach is limited in its ability to enable regeneration across nerve gaps that are more clinically relevant, and has been unsuccessful in promoting regeneration across gaps longer than 10-15 mm in rats.

In the PNS, the ability to regenerate in response to a nerve injury is determined by many factors, including the presence or absence of a permissive environment into which axons can elongate, the intrinsic growth capacity of the neuron itself, and the influence of non-neuronal cells [225]. Among all the glial cells, Schwann cells, in particular, are playing a pivotal role to support axonal growth.

Schwann cells are support cells that myelinate the axons, forming a multilamellar sheath around axons. They also play an important role after a nerve injury. The permissiveness of microenvironment at the distal end of nerve is critical for timely and successful repair after an injury [37]. Within the distal nerve stump, SCs go through multiple changes that are necessary to support axonal outgrowth. After the injury, SCs transdifferentiateand become repair cells while losing their myelinating phenotype. Not only the expression of their myelin protein goes down, the expression of their growth-promoting genes, including growth factors and their receptors, and those encoding cell adhesion molecules goes up [222,225]. These changes in Schwann cells after injury might

happen as a result of axonal signal loss, or might trigger actively as a result of an injury cascade [225].

In a healthy nerve, Schwann cells sustain basal lamina tubes; after injury, these tubes (termed bands of Büngner) encourage and guide the growth of regenerating axons. However, chronic denervation detrimentally affect bands of Büngner due to SC atrophy which results in degeneration of and eventual disappearance of basal lamina tubes [225,226].

Following nerve injury, Schwann cells become 'reactive' and produce a number of neurotrophic factors including NGF, BDNF, NT-3, and ciliary neurotrophic factor (CNTF) [227]. They also synthesize and secrete extracellular matrix molecules, such as laminin, which is known to enhance neurite outgrowth [1]. Schwann cells also express a variety of other cell adhesion molecules [228]. For nerve gaps less than the critical length (<10mm) all of these processes occur spontaneously, leading to axonal regeneration. However, for nerve gaps longer than10mm, spontaneous nerve regeneration does not occur, most likely due to failure of the formation of the fibrin cable and subsequent bands of Büngner [229] .

Based on the result from chapter 4, we believe that modulating macrophage phenotype can affect SC function and activity, and in fact, this might be the principal reason for why early modulation of macrophages affect final outcome of axonal growth three weeks after implantation. We believe macrophages can stimulate endogenous SC migration into the nerve gap which can obviate the need for SC transplantation or application of neurotrophic factors. Here we investigated the interplay between macrophages phenotype and SC function both *in vitro* and *in vivo* to shed some light on the potential mechanism of action of macrophage modulation on peripheral nerve regeneration. Figure 5.1 depicts the summary of potential interactions between SC and axons based on the result presented in this chapter and others [129,171].

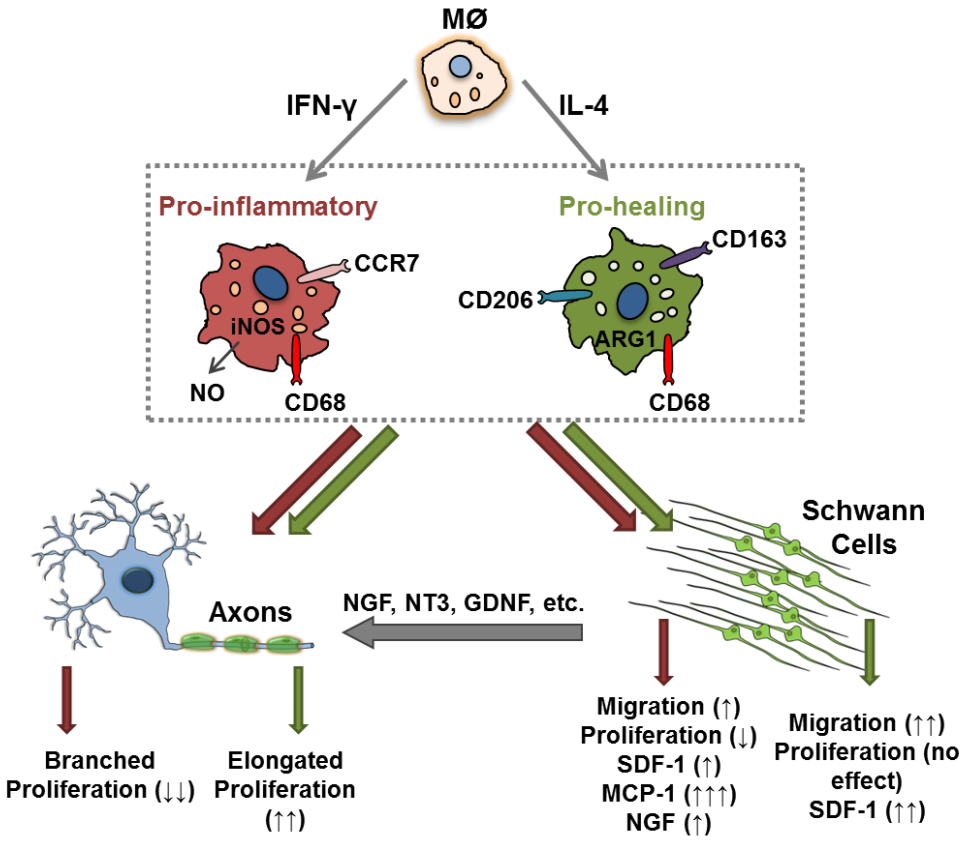

Figure 5.1: Schematic of effects of M1 and M2 macrophages on SC and axons represented by red (dark) and green (light) arrows, respectively.
M1 and M2 macrophages can modulate proliferation and morphology of axons as well as proliferation and secreting products of SCs [129,171,215,230].

5.2 Materials and Methods

5.2.1 SC Proliferation Study

Alveolar rat macrophage cells (NR8383) (2×10^5) were placed in serum-free medium (F12K with 1% P/S; 1% L-glutamine) and incubated, allowing attachment for 4 h (37 °C, 5% CO2). After 4 h, IL-4 or IFNγ (Antigenix America) (50 ng/mL in 1 mL) was added and cells were incubated for 24 h (n=4 per group). Then LPS (100 ng/mL) was added to IFNγ containing medium and all cells were incubated for an additional 7 h. Then medium for all cells was changed to fresh serum-free medium and cells were incubated for another 24 h (37 °C, 5% CO2). After 24 h, medium was collected and frozen at -80 °C. 2×10^5 Schwann cells (RSC96) were added to the complete medium (DMEM with 10% FBS; 1% P/S; 1% L-glutamine) and incubated for 24 h (37 °C, 5% CO_2). After 24 h, the medium was removed upon process extension from RSC96 cells, and 250 µL of serum-free medium (DMEM with 1% P/S; 1% L-glutamine) and 250 µL of CM from macrophages treated with IL-4, IFNγ, or no cytokine (warmed to 37 °C before adding) was added to the cells. Serum-free DMEM medium (250 µL) was added as the control (n=4 per group). The cells were incubated (37°C, 5% CO2) for 72 h. After incubation, medium was removed from top without disturbing cells and CCK8 assay (10% CCK8 in 500 mL serum-free DMEM medium; 450 nm) was performed on cells attached with a standard (31.25K-500K cells). For direct cytokine effect analysis, similar procedure was performed except instead of CM addition, 50 ng/mL of IFNγ or IL-4 or 50, 100, or 500 ng/mL of IL-19 was added directly to the RSC culture.

5.2.2 SC Migration Study

Similar protocol to proliferation study was used to activate the macrophages except after activating macrophages with the cytokines, initial media was changed with fresh serum-free media and 24-well Boyden chambers (BD Falcon FluoroBlok, 8 mm) were inserted into activated macrophage wells and 5 × 104 Schwann cells (RSC96; expressing

Green Fluorescent Protein (see A.3.2 for further details)) in 200 µL serum-free medium (F12K with 1% P/S; 1% L-glutamine) were added to the top insert which does not have any coating. After 72 h incubation, the migrating cells at the bottom of the filters were fixed with paraformaldehyde and counted at five random fields per filter (n=4 per group) (see Figure 7.4). For direct cytokine effect analysis, similar procedure was performed except instead of macrophage culture at the bottom of Boyden Chamber, 50 ng/mL of IFNγ, IL-4, or 500 ng/mL of IL-19 was added directly to the bottom chamber.

5.2.3 RNA Extraction and qRT-PCR Analysis of Macrophages

Total RNA was extracted from cell pellets obtained from the untreated macrophages or macrophages treated with IFNγ + LPS or IL-4 cytokines using the RNEasy Mini Kit (Qiagen). Extracted total RNA was quantified using the Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen). Two micrograms of the total RNA was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). PCR primers were designed (Table 7-2) using Primer Express® software (Applied Biosystems) and obtained from Integrated DNA Technologies (Coralville, IA). Primer validation and qRT-PCR using SYBR green mix (Applied Biosystems) was conducted on a StepOnePlus real-time PCR machine (Applied Biosystems) according to methods previously published [231,232]. All qRT-PCR reactions were performed in triplicates and fold differences for each target gene using comparative CT method were normalized controls (untreated macrophages), and the relative fold change was calculated by normalizing against the endogenous reference, housekeeping, hypoxanthine phosphoribosyltransferase 1 (HpRT1) gene (Figure 5.2).

5.2.4 RNA Extraction and qRT-PCR Analysis of Schwann Cells (SC)

Same method as macrophages was used for SC too. However here the conditions were SC treated with conditioned media (CM) from IL-4 or IFNγ + LPS macrophages and the control was SC treated with CM from untreated macrophages. PCR primers were

designed for the genes of interest (Table 7-3) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene.

5.2.5 Scaffold Implantation

Polysulfone tubes filled with 0.7% Agarose (SeaPrep Lonza) mixed with 1 mg/ml rat recombinant cytokine IL-4 (Antigenix America Inc.) or Agarose only were prepared (n=6 per group). The scaffolds were kept cooled in 4°C for 1 h. Adult Lewis male rats (250−300 g) were induced to anesthetic depth with inhaled isoflurane at 3−4%. Throughout surgery, the animals were maintained at 1.5−2% isoflurane. Microscissors were used to transect the sciatic nerve, and the nerve stumps were pulled 1 mm into each end of the 17 mm guidance scaffolds (leaving a 15 mm gap) and fixed into place with a single 10−0 nylon suture (Ethicon). The muscles were reapposed with 4−0 vicryl sutures (Ethicon) and the skin incision was clamped shut with wound clips (Braintree Scientific). After the surgery, the rats were placed under a warm light until sternal, and then housed separately with access to food and water ad libitum at constant temperature (19−22°C) and humidity (40−50%) on a 12:12 h light/dark cycle. To prevent toe chewing, a bitter solution (Grannick's Bitter Apple) was applied when it was necessary to the affected foot. Marcaine (0.25% w/v (0.3 ml/animal)) and Buprenorphine SR (1 mg/kg) were administered subcutaneously for post-surgical pain relief. Animals were maintained in facilities approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Institute of Technology and in accordance with the current United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards.

5.2.6 Scaffold Explantation and PCR Array Analysis

1 and 2 weeks after implantation (n=6), scaffolds were explanted and snap-frozen and kept frozen at -80°C for gene and protein analysis. The scaffolds were then crushed in liquid nitrogen using mortar and pestle, and treated with RNEasy Mini Kit (Qiagen), RNA

were extracted, and converted to cDNA similar to 5.2.3. Here, we used Rat RT^2 Profiler PCR Array (Qiagen) to do a complete screening on each of our scaffolds. 500ng of cDNA were used for each Qiagen PCR Array following the protocols therein. For the complete list of analyzed genes please see A.4 and A.5.

5.2.7 Statistical Analysis

Data were analyzed using the Student's t-test for unpaired data where appropriate or one-way ANOVA followed by a Tukey's least significant difference post-hoc test where warranted to determine significant differences between groups with 95% confidence; A p value < 0.05 was considered to represent a statistically significant difference.

5.3 Results

5.3.1 Effect of Polarized Macrophages on SC Proliferation/ Migration *in vitro*

Using a Boyden Chamber (BC), the effect of M1 or M2 macrophage phenotype on SC migration was explored *in vitro*. Because some of the secretory products of macrophages are labile, with short half-lives (e.g., oxygen and nitrogen free radicals), media transfer assays may underestimate the migratory potential of conditioned media (CM) from macrophages. Therefore, SCs grown on the trans-well of the BC were placed in the wells of pro-inflammatory and anti-inflammatory plated macrophages. The phenotype of macrophages was confirmed by measuring the amount of nitrite production A.2.1 for method) and the ratio of IL-10 to IL-12 gene expression (see A.2.2 for method) in cells after being exposed to IFNγ and IL-4 cytokines (Figure 5.2). After being primed overnight, these macrophages retained their activation state even after being washed to remove residual stimulating cytokines (Figure 5.2). The rate of SC migration was increased in the presence of CM from either the M1 (treated with IFNγ) or M2 (treated with IL-4) macrophages in comparison to CM from the (control) macrophages treated with no cytokines (Figure 5.3a). The increase in migration in the presence of CM from M2

macrophages was nearly twice that (300% vs. 170%) observed in the presence of CM from M1 macrophages. However, exposing SC directly to IL-4 or IFNγ did not have any effect of the SC migration (Figure 5.3c)

To evaluate whether enhanced SC migration was due to enhanced SC proliferation, CCK8 assays were performed using the same number of cells treated with the CM from M1 and M2 macrophages at 72 h time point. CCK8 measures the metabolic activity of the cells which directly correlates with the number of cells. We have shown that our treatment does not change metabolic activity of individual cells and therefore the overall metabolic activity correlates well with total number of cells and therefore can be used to measure the proliferation rate. Although CM from M2 macrophages did not have any significant effect on the rate of SC proliferation, CM from M1 macrophages marginally, but significantly decreased proliferation rate of the SC compared to the CM from the control ($n=4$, $P < 0.01$, one-way ANOVA followed by a Tukey's post-hoc) (Figure 5.3b). Furthermore, although direct addition of IFNγ and IL-4 to the SC cultures did not have any effect on the SC migration (Figure 5.3c), both of the cytokines significantly decreased the SC proliferation ($n=4$, $P < 0.01$, Student's t-test) (Figure 5.3d).

5.3.2 Effect of Polarized Macrophages on SC phenotype

Based on our literature review, we chose 12 genes that represent the functional status of SC. These genes are playing role in migration, proliferation, myelination, adhesion, growth factor production, and immune functions of SCs [225]. The purpose for this experiment was to investigate if different phenotype of macrophages might affect any of these SC functions and therefore affecting their phenotype.

We observed an up-regulation in SC expression level of 3 genes by exposing SC to CM of M1 macrophages (MCP-1, TGFβ, GFAP), 3 gene by exposing SC to CM of M1 as well as M2 macrophages (SDF1, GDNF, MPZ), and down-regulation in expression level of 4 genes by exposing SC to CM of M2 macrophages (NGF, NGFr, Krox20, Oct6) relative

to CM of MØ macrophage. Among all 12 genes only SDF1 showed more up-regulation in expression when exposed to CM of M2 relative to CM of M1 macrophage (n=5).

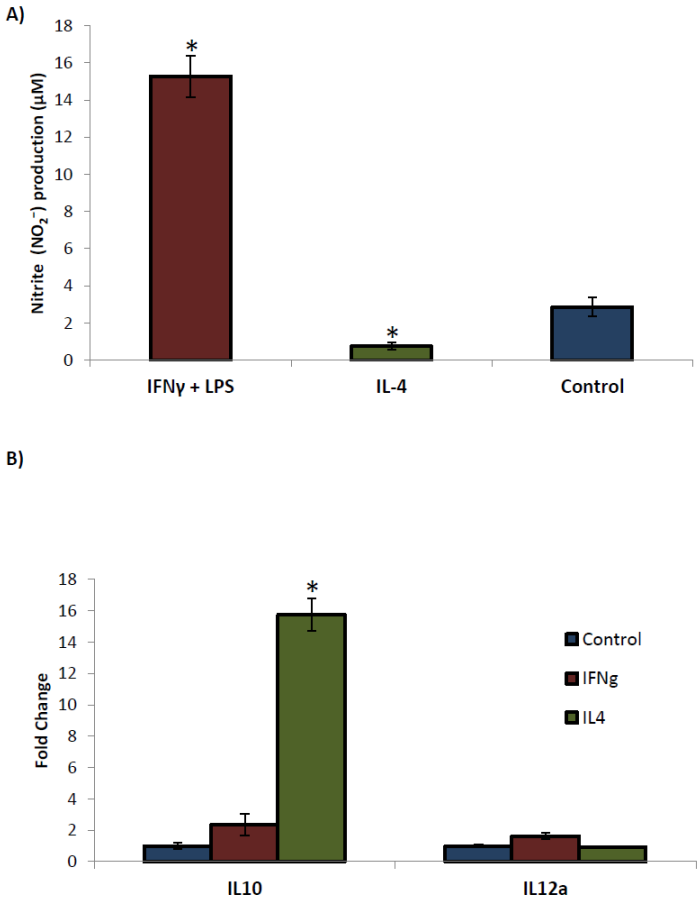

Figure 5.2: Macrophages maintain their phenotype after being exposed to the fresh media.

A) nitrite (NO2-) production in macrophages treated with pro/anti-inflammatory cytokines. B) Gene expression of two main pro/anti-inflammatory characteristic cytokine, IL-10 and IL-12. The ratio of IL10/IL12 indicates the activation state (phenotype) of macrophages. Asterisks denote a significant difference compared with non-cytokine treated control scaffold ($P < 0.05$).

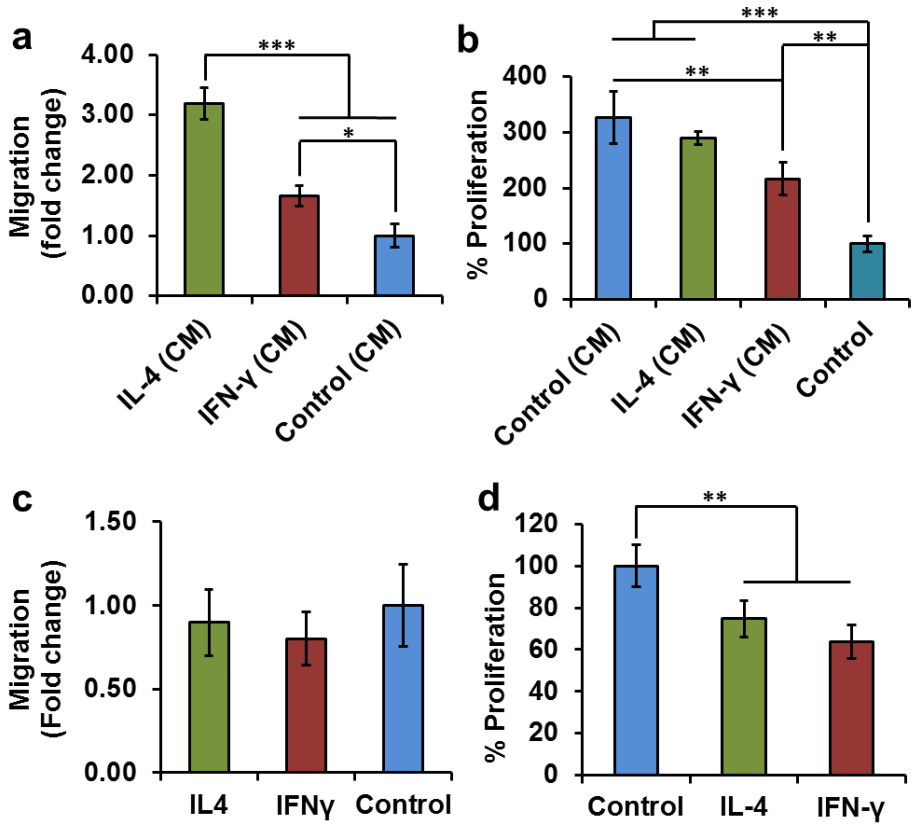

Figure 5.3: Polarizing macrophages to M1 and M2 phenotypes indirectly affect Schwann cells (SC) and axons *in vitro*.
a) Effect of SC migration in CM from macrophages (72 h). Mean ± s.d. n = 4. ***P < 0.001. *P < 0.05 (one-way ANOVA followed by a Tukey's post-hoc) b) SC proliferation in CM from macrophages. Mean ± s.d. n = 4. ***P < 0.001. **P < 0.01 (one-way ANOVA followed by a Tukey's post-hoc) (72h). Direct effect of the cytokines on the Schwann cell, c) proliferation and d) migration. Mean ± s.d. n = 4. **P < 0.01 (Student's t-test). Asterisks denote a significant difference. CM from IL-4 treated macrophages increases the SC migration almost 3 times without affecting the SC proliferation in comparison to the control. Direct addition of cytokines to SC does not have any effect on their migration but decreases their proliferation.

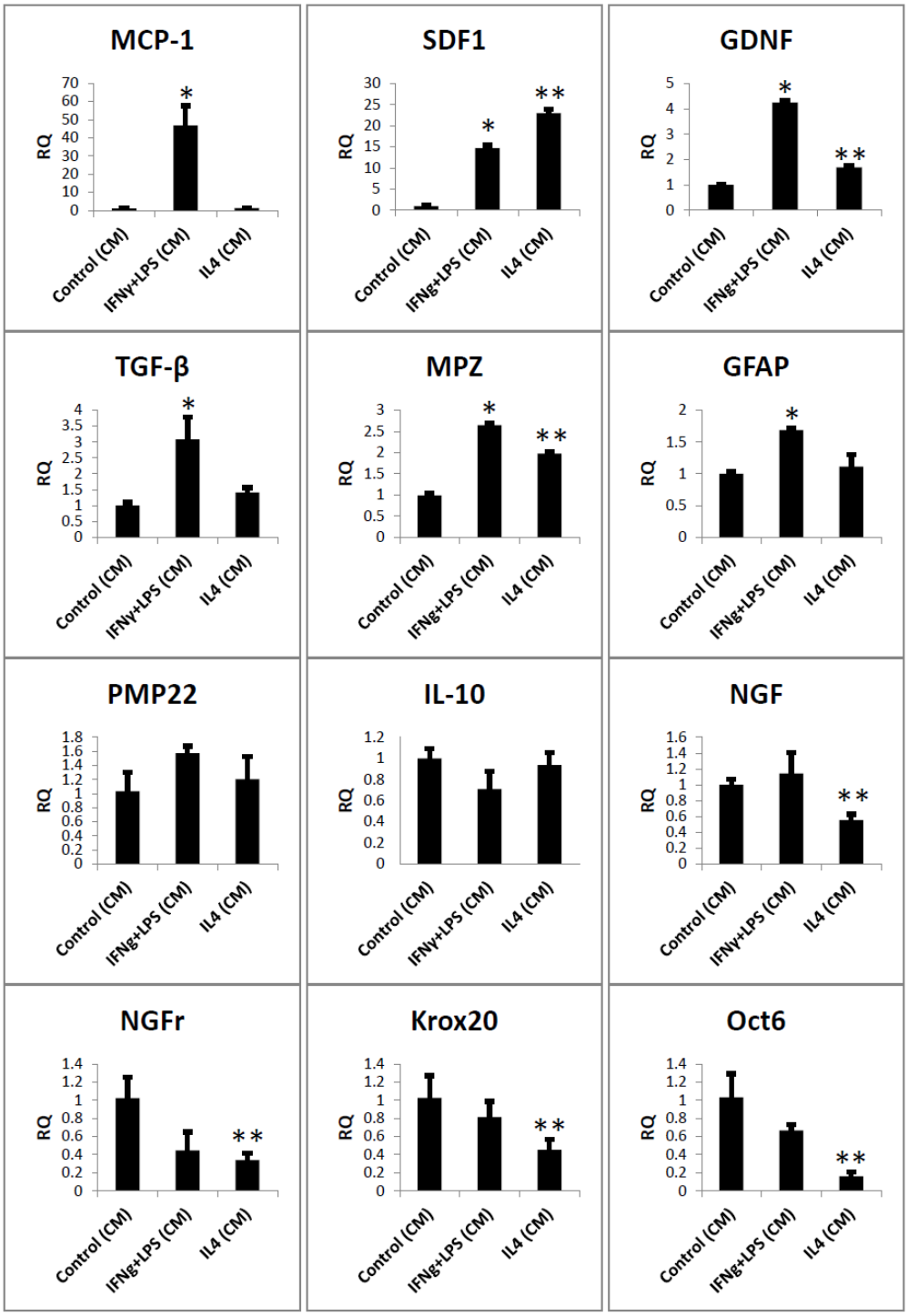

Figure 5.4: Effect of macrophages conditioned media (CM) on SC gene expression level. Asterisks denote a significant difference compared with SC treated with CM from non-cytokine treated macrophage control scaffold ($P < 0.05$).

5.3.3 PCR Array Analysis of Scaffolds

The result presented in CHAPTER 4 and *in vitro* resulted presented in this chapter, convinced us that anti-inflammatory cytokine (IL-4) is able to enhance axonal growth relative to our conditions and controls. Here, we further explored the effect of IL-4 containing scaffold on regenerative environment inside the nerve conduit, 7 and 14 days after nerve implantation (Figure 5.5, Figure 5.6, and Figure 5.7).

The Rat Neurotrophins & Receptors RT² Profiler™ PCR Array and the Rat Common Cytokines RT² Profiler™ PCR Array were used to profile the expression of 84 genes related to neuronal processes, and 84 important cytokine genes, respectively.

Neurotrophic signaling molecules are represented on Neuroptrophins & Receptors Array included neurotrophins and neuropeptides along with their receptors along with genes involved in the normal functions of the neuronal system including neuronal cell growth and differentiation and neuronal regeneration and survival are included. Also the cytokines and receptors involved in neuronal signaling are contained on this array along with genes involved in the transmission of nerve impulses, genes involved in neuronal apoptosis in response to neurotrophic factors and transcription factors and regulators indicative of the activation pathways downstream of the neuronal system.

Rat Common Cytokines PCR Array included interferons and interleukins as well as the bone morphogenetic proteins (BMP) and members of the TGFβ family. Also represented are colony-stimulating factors and various growth factors (fibroblast, insulin-like, platelet-derived, transforming, and vascular endothelial). Tumor necrosis factors were also included as well as other cytokine-related genes.

Expression levels of neurotrophins, neuropeptide, and receptors genes in IL-4 containing scaffolds, relative to the control scaffold (agarose without cytokine), are shown at 7 and 14 days after implantation in Figure 5.5 and Figure 5.6, respectively. Each point corresponds to one gene and the diagonal line in the middle represents no change in expression. Points above the middle diagonal line represent up-regulation and points below

the middle diagonal line represent down-regulation in gene expression of tissue within IL-4 treated scaffold relative to the control. In both Figure 5.5 and Figure 5.6 the dashed lines to either side of the diagonal line represent a two fold change in expression.

At 7 and 14 days after scaffold implantation, a greater than two fold change in expression level was observed for only 5 of 84 genes studied. These genes are Galanin Receptor-1, Galr-2, and Fibroblast Growth Factor Receptor Substrate 3 (FRS3) at the seven day survival time (Figure 5.5), and Gastrin-Releasing Peptide Receptor (GRPR) and Cerebellin 1 Precursor (CBLN1) at the 14 survival time (Figure 5.6).

However when similar analysis was done on tissue sample of 7 day study using common cytokine array, more changes in the expression level of genes were found (Figure 5.7). Again each point corresponds to one gene and the diagonal line in the middle represents no change in expression. Points above the middle diagonal line represent up-regulation and dos below the middle diagonal line represent down-regulation in gene expression of tissue within IL-4 treated scaffold relative to the control. The guide diagonal lines represent 2 folds in change in Figure 5.7.

Our data show that 9 genes affected by IL-4 containing scaffold 7 days after injury. These genes are IL-19, IL-24, and IL-21 which are up-regulated more than two fold in IL-4 treated scaffold relative to the control, and IL-22, IL17f, IL-13, BMP5, IFNα2, and IL36rn which are down-regulated more than two folds due to the treatment (Figure 5.7). Among these genes, 5 of them demonstrate more than 4 folds change (IL-19, IL-24, and IL-21 up-regulation) and (IFNα2 and IL36rn down-regulation) with IL-19 showing the most statistically significant and dramatic up-regulation (statistical analysis was performed using Qiagen online web tool for cytokine arrays).

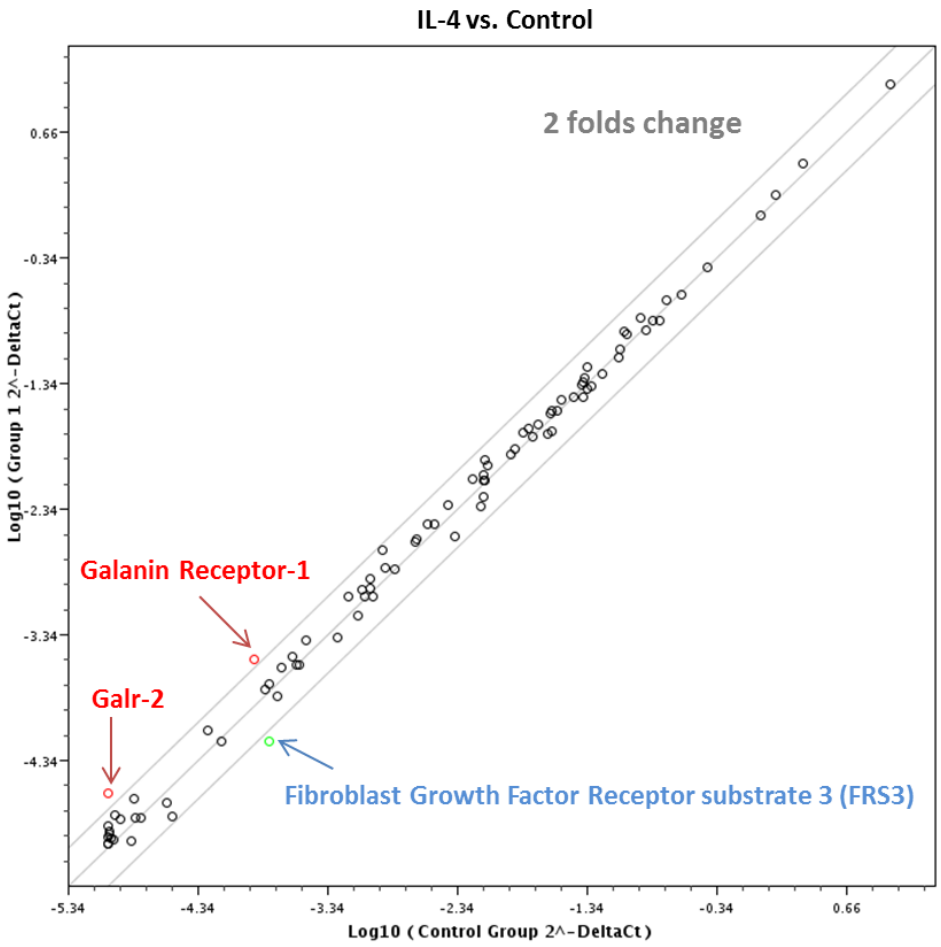

Figure 5.5: The Neurotrophins & Receptors RT² Profiler™ PCR Array-7 days. Each point corresponds to one gene and the diagonal line in the middle represents no change in expression. Points above the middle diagonal line represent up-regulation and points below the middle diagonal line represent down-regulation in gene expression of tissue within IL-4 treated scaffold relative to the control. The guide diagonal lines represent 2 folds in change.

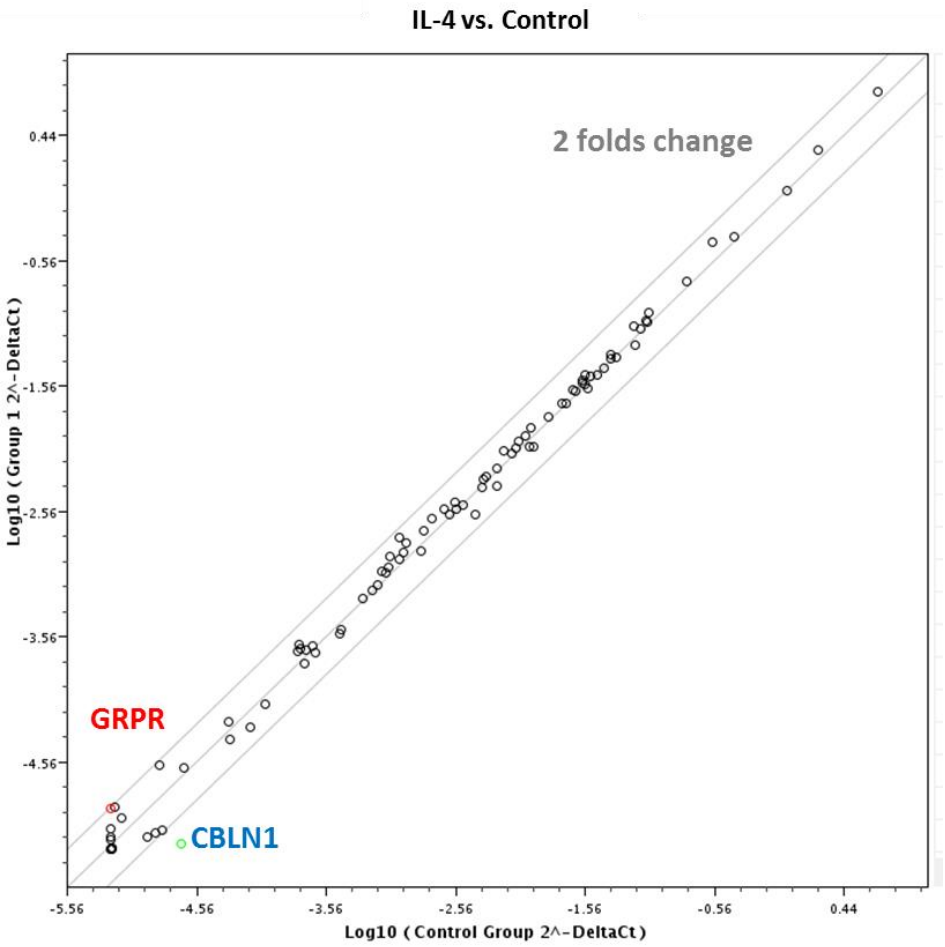

Figure 5.6: The Neurotrophins & Receptors RT² Profiler™ PCR Array-14 days.
Each point corresponds to one gene and the diagonal line in the middle represents no change in expression. Points above the middle diagonal line represent up-regulation and points below the middle diagonal line represent down-regulation in gene expression of tissue within IL-4 treated scaffold relative to the control. The guide diagonal lines represent 2 folds in change.

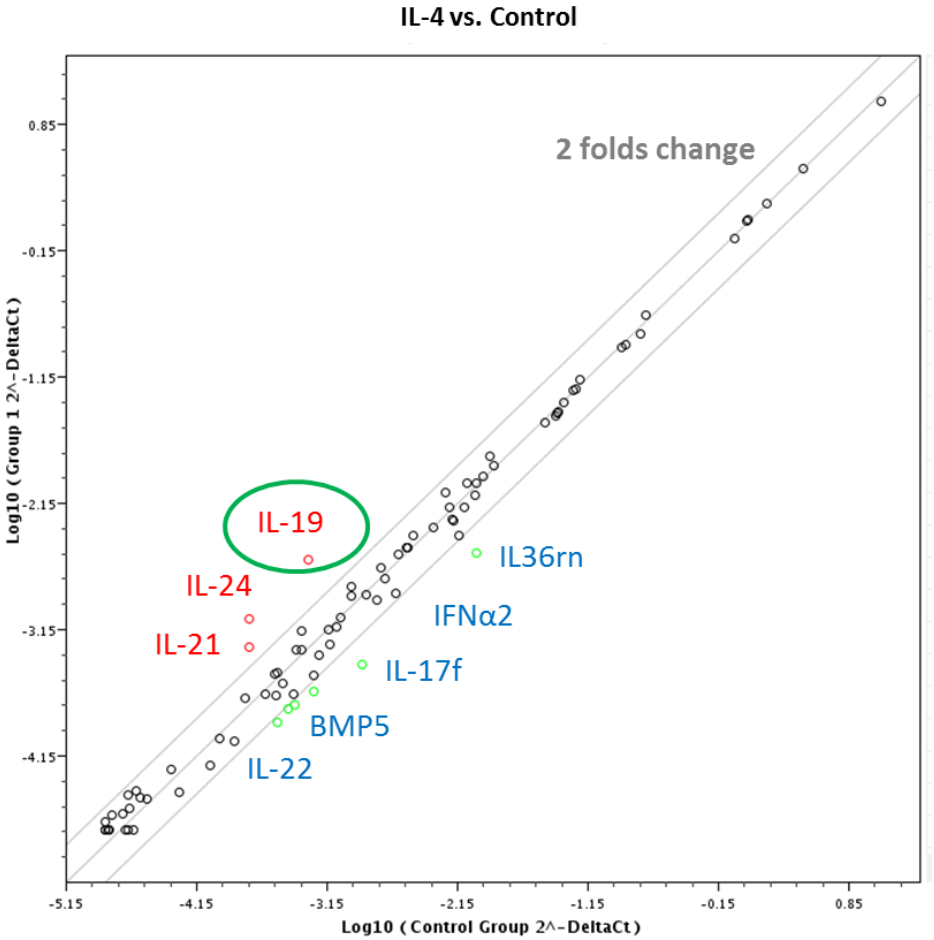

Figure 5.7: The Common Cytokines and Chemokines RT² Profiler™ PCR Array-7 days.

Each point corresponds to one gene and the diagonal line in the middle represents no change in expression. Points above the middle diagonal line represent up-regulation and points below the middle diagonal line represent down-regulation in gene expression of tissue within IL-4 treated scaffold relative to the control. The guide diagonal lines represent 2 folds in change.

5.3.4 Investigate the *in vitro* effect of IL-19 on SC

The result of our gene screening experiment directed us toward the potential role of IL-19. Therefore, we designed an experiment similar to 5.3.1 and 5.3.2 to investigate the effect of IL-19 on SC migration and proliferation (Figure 5.8). Interestingly enough, although IL-19 does not significantly affect SC proliferation in any of tested doses range (50-500 ng/mL) (Figure 5.8c), 500 ng/mL concentration of IL-19 encourages the SC to migrate from the top of the BC insert to the bottom 200% more in comparison to the control (non-treated cells) (Figure 5.8a,b). This correlation matches the correlation that we observed with macrophages conditioned media (Figure 5.3). Similar to 5.3.2, we also studied the effect of IL-19 on SC gene expression level in order to better understand its effect on SCs function (please refer to A.6).

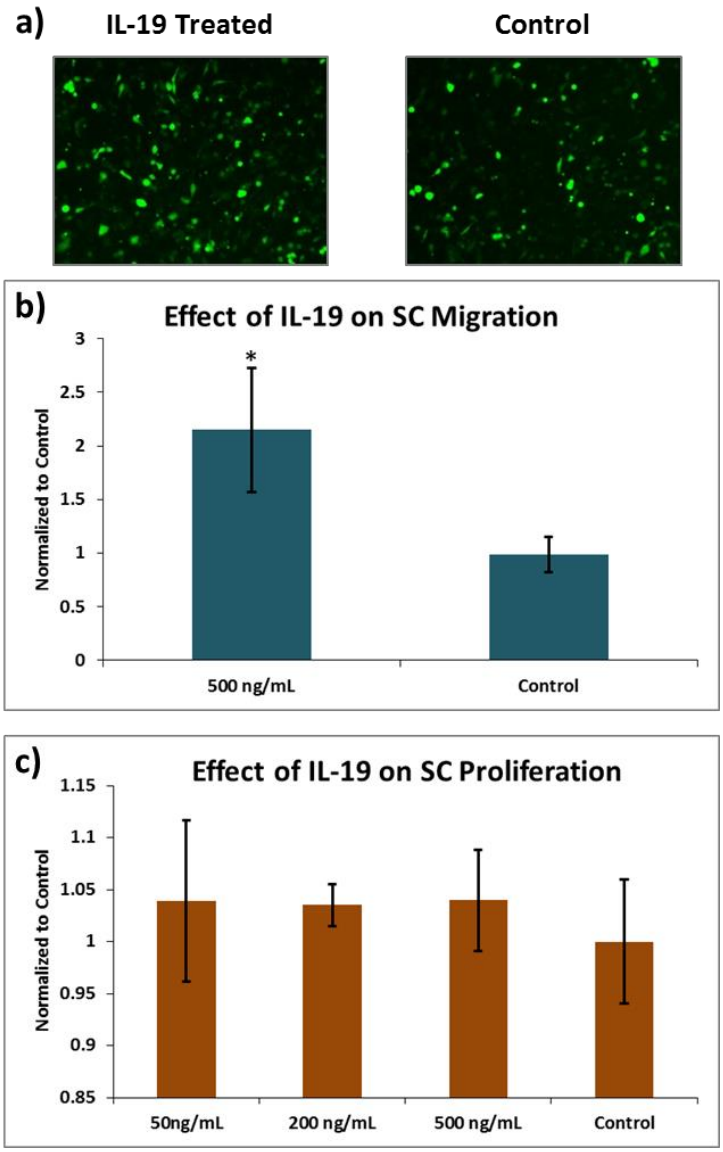

Figure 5.8: Effect of IL-19 on SC migration and proliferation *in vitro.*
a) representative images for GFP-RSC migrated to the bottom of Boyden Chamber insert, b) effect of IL-19 on SC migration, c) effect of IL-19 on SC proliferation.

5.4 Discussion

Nerve regeneration in the PNS is often incorrectly thought to happen fully and with little or no intervention, especially when compared with nerve regeneration in the CNS, which as discussed in CHAPTER 1 is extremely limited. Although peripheral axons are generally capable of regeneration and formation of functional connections, several factors (such as type of injury, delay before intervention, and the patient's age) can decide the extent of functional outcome after repair. We also know that crushed nerves relative to transected nerves have much better outcomes, and also nerve recovery is much worse better after proximal injuries than after distal ones, as axons that need to travel longer distance to reach their target tissue are less likely to reconnect [36,108,225,226]. Recognition of these factors that can affect the final outcome of nerve regeneration has been useful in dissect the underlying cellular mechanisms of regenerating neurons both in in the CNS and PNS. For axon regrowth to happen, the distal environment needs to be supportive of regrowth, and also axons must have the inherent ability to regrow. Schwann cells as mentioned in 5.1 are playing a critical role in the former one.

Recognizing the importance of Schwann cells for axonal sprouting and regeneration, many groups have used Schwann cell seeded nerve conduits [227,233,234]. The use of pre-seeded Schwann cells bypasses the fibrin cable step and accelerates the formation of bands of Büngner. This technique also introduces a persistent source of neurotrophic factors. However, the ability of nerve conduits containing Schwann cells to facilitate nerve regeneration is dependent on the density of pre-seeded Schwann cells. For instance, in repairing an 8 mm long gap in rat sciatic nerve, the required Schwann cell density is 80 million cells per ml [234]. Moreover, a secondary surgery is required to obtain a small nerve segment that can serve as a source of autologous Schwann cells. Additionally, it takes 5-6 weeks to grow a therapeutically significant number of Schwann cells, during which time the injured nerve further degenerates [235]. Therefore, the current techniques for utilizing Schwann cells (SC) for repairing long nerve gaps may not be clinically

acceptable. Besides SC transplantation, multiple neurotrophins, such as NT-3, have also been shown to be capable of enhancing SC infiltration [3]. In fact, NT-3 promotes Schwann cell migration, and subsequently neurite outgrowth.

Here, we showed that M1 and M2 macrophages can also affect SC proliferation and migration. Although it has been documented that CM from classically activated (M1) macrophages increases SC proliferation [215,236], our study extends the known mitogenic effect of activated macrophages on SC to the M2 phenotype. Moreover, since SC infiltration is an integral component of axonal regeneration after sciatic nerve injury, we studied the effect of modulating macrophage activation states on SC migration too. Both M1 and M2 macrophages were shown to promote migration of SC. This result is in agreement with an *in vitro* study showing similar trend of M1 and M2 macrophages in recruitment of vessel associated progenitor cells [147]. The study showed that both M1 and M2 macrophages can induce chemotaxis of progenitor-like cells but with different capability and through different pathways. However, here we also showed that M2 macrophages increased the migration rate of SC 2-fold more than CM from M1 macrophages. These results also parallel another study which investigated the effect of CM from M1 and M2 macrophages on the migration of mouse myoblast-like cell [177].

Although it is clear that factors in the CM from M2 macrophages boost the migration rate, the precise mechanisms by which M2 macrophages trigger such a response are still unknown. However, preliminary gene expression analysis indicates that there are several genes in SC that are affected by macrophage CM migration (Figure 5.4); in particular, stromal cell-derived factor-1 (SDF-1) demonstrates a trend similar to that observed with SC. Recently, it was demonstrated that the SDF-1 receptor, CXCR7, is an active component of SDF-1 signaling in SC [237], suggesting a possible autocrine mechanism. However, further investigation is necessary to identify the precise signaling pathway.

Moreover, although our results also demonstrate the pro-regenerative effect of anti-inflammatory macrophages is mediated through macrophages and subsequently SCs, we wanted to explore the mechanism by which early modulation of innate immune response inside our nerve conduit resulted in significantly improved axonal growth better. As it mentioned also earlier, the ability to regenerate in response to a nerve injury is determined by many factors, including the presence or absence of a permissive environment into which axons can elongate, the intrinsic growth capacity of the neuron itself, and the influence of non-neuronal cells [225]. Since our scaffold modulates the immune response locally and inside the scaffold, the focus was concentrated on the local players which potentially made the environment more permissive for regeneration. Based on this assumption we chose two PCR profiler kits to cover both general wound healing aspect of regeneration as well as neural tissue specific. Among the genes analyzed with the PCR profiler kits, IL-19 expression demonstrated the most statistically significant and dramatic changes (Figure 5.7).

IL-19, a member of the IL-10 cytokine family, is a relatively novel cytokine In addition to IL-19, the IL-10 family encompasses IL-20, IL-22, IL-24, and IL-26 [238]. IL-19 is primarily produced by monocytes [239] but little is known about the effects of this cytokine, although studies to date have shown that it possesses a pleiotropic nature, dependent on the type of tissue and disease with which it is associated (Figure 5.9). IL-19 has been shown to up-regulate the production of IL-6, TNFα, and reactive oxygen species [238,240,241]. However, IL-19 has also been shown to increase the levels of Th2 cytokines in uraemic patients [242], vascular inflammatory diseases [243], inflammatory bowel diseases [244], asthma patients [238], and autoimmune diseases [245]. IL-19 has also been linked to poor prognoses in cancer patients through its promotion of cancer cell proliferation and migration [246,247]. The production of both inflammatory and anti-inflammatory cytokines further proves the dual nature of IL-19. IL-19 has also been shown

to down-regulate the production of IFNγ, a cytokine which has been shown to induce an M1 pathway [244,248,249].

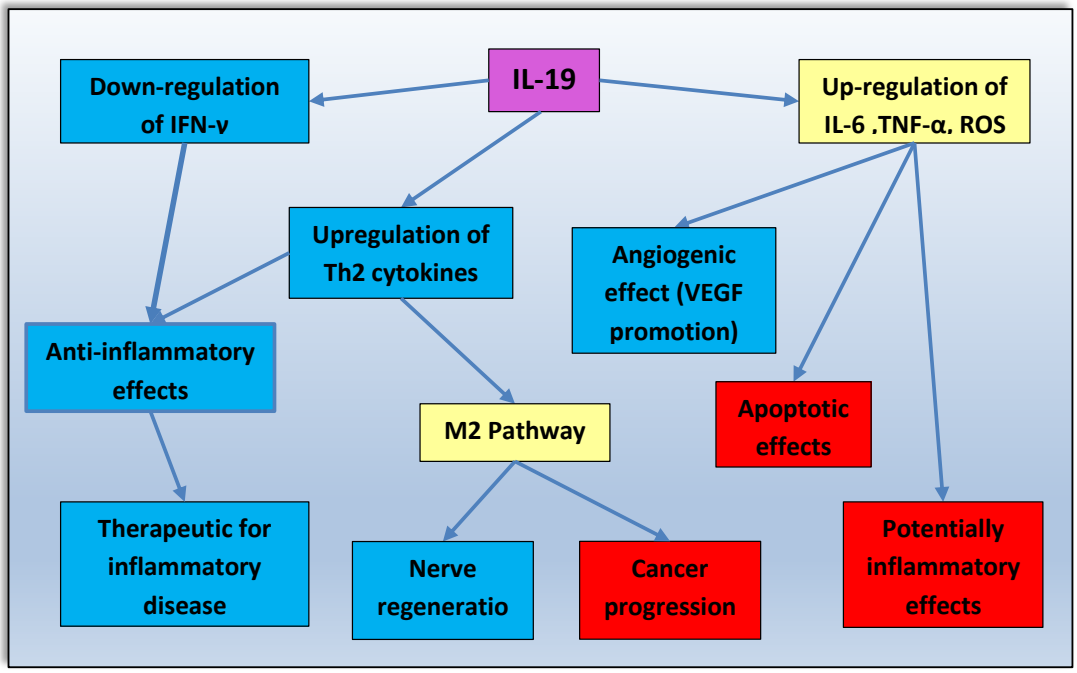

Figure 5.9: Schematic showing pleiotropic nature of IL-19

Although IL-19 has proved to be therapeutic in many different kinds of tissue, its effect in the peripheral nervous system has never been studied. The potential up-regulation of Th2 cytokines by IL-19 stimulation has the potential of promoting an M2 pathway, and thus promoting regeneration.

Recent studies have looked at the role of IL-19 in malignant tumors in promoting tumor growth. When compared to healthy tissue, neoplastic tissue shows significantly more IL-19, and has been stained in many different kinds of carcinoma [246]. A positive correlation between IL-19 and the Th2 pathway has also been observed [247]. On the other hand, LPS stimulation of macrophages leads to the expression of IL-19. This expression was enhanced following treatment with IL-4, but diminished by treatment with IFNγ, suggesting a positive relationship between Th2 cells and macrophages [247]. A study on the regulation of T cells by IL-19 confirmed that IL-19 increased the number of IL-4 producing cells in the presence of an infectious challenge. Not only did the number of IL-4-producing cells increase, but the number of IFNγ-producing cells was also decreased [247] which again confirms the role of IL-19 in promoting the production of Th2 cells. Another study showed that repeated stimulation with IL-19, and other cytokines in the IL-10 family, increased the number of IL-4 and IL-13 producing T cells [250]. This positive correlation between IL-19 and IL-4, as well as the negative correlation between IL-19 and IFNγ, suggest a positive feedback between IL-19 and Th2 cytokines [247].

Furthermore, increased production of IL-19 has also been linked to hyper-proliferation of keratinocytes in psoriatic plaque and, the increased production of IL-4, IL-5, and IL-13 in asthma patients [251]. Although this increase in cell proliferation and Th2 cytokine production can prove detrimental in otherwise healthy patients, these results could have positive results in sites of injury by promoting an M2 response. Both Th2 cytokine production, and an alternatively activated macrophage response occur in response to infection in humans (alternatively activated), and have proven beneficial in reducing inflammation and promoting regeneration.

Moreover, IL-19 is also known to promote the cytokines IL-6 and TNFα [241,248]. Although these cytokines are usually associated with inflammation, they have potentially therapeutic effects. These cytokines are linked to the production of angiogenic factors, such as VEGF, and TGFβ [249].

On the other hand, IL-19 has been linked the production of TNFα as well as cell apoptosis [250,252]. This apoptotic effect of IL-19 could be directly related to the increased production inflammatory mediators such as TNFα, IL-1, and reactive oxygen species [250]. Apoptosis was reduced in monocytes that were stimulated with both IL-19 and TNFα antibody [240]. However, in contrast to this report of the induction of apoptosis by IL-19, studies regarding rheumatoid arthritis and patients with chronic rhinosinusitis/nasal polyps reported a reduction of apoptosis. IL-19 has shown a positive correlation with STAT3 activation in many studies [242,251,253], which is characterized by the prevention of apoptosis, oncogenesis, and cell proliferation. IL-19 in synovial tissue, which originated from both macrophages and fibroblasts, was responsible for the induction of STAT3 in rheumatoid arthritis synovial cells.

Overall, the effects of IL-19 are not completely understood. The potential of IL-19 as a therapeutic treatment for peripheral nervous system injuries remains unexplored, and merits further investigation, both *in vitro* and *in vivo*. Modulation of IL-19 cytokines in the context of nerve regeneration could have a significant implication for advancing regenerative outcomes for patients with PNS injuries.

5.5 Conclusion

Our *in vitro* and *in vivo* data suggest that the beneficiary effect of M2 macrophage on peripheral nerve regeneration might be due to their effect on enhancing the permissiveness of the nerve conduit to growth, mainly though increasing SCs migration. We also believe that IL-19 which is mainly a Th2 cytokine and secreted by monocytes/macrophage and cause angiogenesis and cell proliferation might play a

principal role in enhancing the SC infiltration inside IL-4 containing/M2 encouraging nerve conduit. Therefore IL-19 might be a key factor in the context of enhancing peripheral nerve regeneration by modulating innate immune response using anti-inflammatory IL-4 cytokine.

CHAPTER 6 DETERMINE THE EFFECT OF MONOCYTE RECRUITING FACTOR ON STIMULATING NERVE REGENERATION

6.1 Introduction

Each year, nearly hundred thousand patients undergo peripheral nerve surgeries in Europe and United Sates [254]. Although there are ample microsurgery techniques available for short nerve lesions, our ability to bridge long peripheral nerve gaps remains unsatisfactory. Plastic and hand surgeons as well as neurosurgeons use either autografts, or more recently cadaver derived grafts to bridge peripheral nerve gaps [255]. However, both sources are inadequate, and present challenges of containing inhibitory CSPGs that need removing via processing. These challenges lead to more than 50% of individuals not recovering fully by functional terms [4,256]. Rate of regeneration as well as degree of reinnervation determine the outcome of functional recovery after PNS injuries [3,225]. Several studies have shown that delays in repair after injury contributes to poor functional recovery [35,37,225,257]. Hence, there is critical need to enhance both the extent and rate of regeneration after peripheral nerve injury.

Our laboratory has been investigating the possibility of designing 'tissue engineered' nerve bridges, matching the performance of autografts to functionally bridge 10 mm gaps in rodents [4,87,89]. However, when we applied this strategy to a more challenging and clinically relevant 15 mm nerve gap in rodents, the performance fell short. [77,86]. The best way to approach nerve repair was previously hypothesized to be through recreating the physical and biochemical *milieu* extant in development. Approaches such as hydrogels or aligned polymer fibers and controlled release of NGF or BDNF were no longer the best and the hypothesis needed to be revisited [108].

Our results show that it is more productive to bias the inflammation/immune reaction towards a regenerative biochemical cascade instead of trying to recreate a complex

combination of ECM proteins, angiogenic factors and neurotrophic factors. The biased reaction will lead to the creation of a permissive environment for growth. This approach focuses on upstream modulation of inflammation after injury to attain repair by effective activation of the intrinsic growth capacity of peripheral neurons [108,230,258].

Under homeostatic conditions and depending on the tissue, maintenance of the resident macrophage population occurs by either local proliferation or recruitment and differentiation of blood monocytes [259,260]. However after an inflammatory insult, macrophages that accumulate at the site of injury appear to be largely derived from circulating monocytes [259,260]. The ability of monocytes to mobilize and traffic to where they are needed is central for their function in promoting immune defense as well as tissue regeneration [113]. In the rat, monocytes include two major subsets which have been identified on the basis of chemokine receptor expression and CD43 levels [31,261,262]: a $CD43^{low}CCR2+CX3CR1^{low}$ inflammatory subtype of monocyte recruited to inflamed tissues and a $CD43^{hi}CCR2-CX3CR1^{hi}$ patrolling and anti-inflammatory reparative subtype recruited to non-inflamed tissues [31,195]. Inflammatory monocytes phagocytose debris and clear damaged cells, whereas anti-inflammatory monocytes promote tissue regeneration [195]. Anti-inflammatory monocytes are also the larger population (80-90%) in the rat blood [262].

Monocytes of different subsets appear to be specialized for different functions, but also may be predisposed to differentiate into macrophages of different phenotypes [114,259,260]. In fact, different subtypes of monocytes can be recruited by distinct cues into inflamed tissues, where they can differentiate into classically activated M1 or alternatively activated M2 macrophages, respectively [195]. M1 macrophages produce high levels of oxidative metabolites as well as pro-inflammatory cytokines, and M2 macrophages support tissue repair by producing anti-inflammatory cytokines which mediate angiogenesis and matrix remodeling [32,206]. M1 and M2 subtype of

macrophages represent a simplistic depiction of a continuum spectrum between two activation states [33].

Moreover, recruited monocytes differentiate to macrophages, and recent studies have shown that monocytes may also have cell-specific activities that are yet to be defined. For example, emerging data suggest that monocytes can promote angiogenesis and arteriogenesis [263] and therefore, they themselves might also function as short-lived effector cells within tissues [264]. Thus, therapies that target sources of inflammation, such as monocytes, may lead to favorable results due to the short tissue residence time of monocytes in acute inflammation and the large-scale continuous cell recruitment. After the first hours of injury, manipulating monocyte recruitment could induce resolution of lingering inflammation and help with further tissue repair.

The premise is that preferential recruitment of *anti-inflammatory regenerative monocytes* to the site of injury will bias macrophage response to pro-healing and in turn set off a regenerative biochemical cascade involving Schwann cells and neuronal processes that lead to repair (Figure 3.1). We have identified a chemokine – Fractalkine (CX3CR1 ligand) – that can preferentially recruit anti-inflammatory monocytes to the site of nerve injury and here we investigated if local delivery of Fractalkine within the lumen of a nerve conduit, early after peripheral nerve injury can enhance the regeneration.

6.2 Materials and Methods

6.2.1 Scaffold Implantation

Polysulfone tubes filled with 0.7% Agarose (SeaPrep Lonza) mixed with 10 mg/ml rat recombinant chemokine Fractalkine (CCL2), or IL-4 (Antigenix America Inc.) or Agarose only were prepared (n=12 per group). The scaffolds were kept cooled in 4°C for 1 h. Adult Lewis male rats (250−300 g) were induced to anesthetic depth with inhaled isoflurane at 3−4%. 15 mm nerve autografts, which were resected and flipped from the rat sciatic nerve, were used as our positive control to bridge the 15 mm gap (n=8). Throughout

surgery, the animals were maintained at 1.5−2% isoflurane. Microscissors were used to transect the sciatic nerve, and the nerve stumps were pulled 1 mm into each end of the 17 mm guidance scaffolds (leaving a 15 mm gap) or autografts and fixed into place with a single 10−0 nylon suture (Ethicon). The muscles were reapposed with 4−0 vicryl sutures (Ethicon) and the skin incision was clamped shut with wound clips (Braintree Scientific). After the surgery, the rats were placed under a warm light until sternal, and then housed separately with access to food and water ad libitum at constant temperature (19−22°C) and humidity (40−50%) on a 12:12 h light/dark cycle. To prevent toe chewing, a bitter solution (Grannick's Bitter Apple) was applied when it was necessary to the affected foot. Marcaine (0.25% w/v (0.3 ml/animal)) and Buprenorphine SR (1 mg/kg) were administered subcutaneously for post-surgical pain relief. Animals were maintained in facilities approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Institute of Technology and in accordance with the current United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health regulations and standards.

6.2.2 Clodronate Liposome Study

The suspension of clodronate liposomes was purchased from http://www.clodronateliposomes.com. To prevent precipitation of liposomes, the suspension was shaken before injection. A homogeneous suspension was required in order to warrant an equal concentration of liposomes per ml. For intravenous injection, ca 0.1 ml of the suspension was injected per 10 grams of animal weight, 48 hours before the implantation. Depletion of liver and splenic macrophages is complete after ca 24 hrs.

6.2.3 Scaffold Explantation and Analysis

Three weeks post injection, scaffolds or autografts were explanted for histological analysis of nerve regeneration (n=5). Since axons grow from the proximal to distal end of the scaffold, we only used the distal end (last 2 mm) for histological analysis and the

remaining part of scaffold and autograft were snap frozen in liquid nitrogen and stored in -80^{o}C for further analysis. Explants were fixed in 4% paraformaldehyde (Sigma Aldrich), washed, and stored in 30% sucrose for 24 h. Samples were embedded in O.C.T. gel (Tissue Tek) and frozen for cryosectioning (CM30505, Leica). Scaffolds or autografts were sectioned transversely or longitudinally at a thickness of 16 μm and reacted with immunofluorescent markers to quantify the different cell types, using techniques previously described [76]. Briefly, sections were incubated for 1 h at room temperature in a blocking solution of goat serum (Gibco) in PBS, incubated overnight at 4°C in a mixture of primary antibody and blocking solution, washed and incubated for 1 h at room temperature in a solution of secondary antibody mixed in 0.5% triton in PBS. Slides were washed twice more with PBS, incubated with DAPI for 10 min and then dried and cover slipped for analysis. Primary antibodies NF160 (1:250, mouse IgG1, Sigma), S100 (1:250, rabbit, IgG, Dako Cytomation), RECA (1:250, mouse IgG1, Invitrogen), CD68 (1:100, mouse IgG1, AbD Serotec (ED1)), CD86 (1:100, mouse IgG1, AbD Serotec), Arginase1 (1:250, rabbit, IgG, Cell Application), and CD206 (1:100, rabbit, IgG, Santa Cruz Biotechnology) were used. The following secondary antibodies were used: goat anti-rabbit IgG Alexa 488/594 (1:220, Invitrogen), goat anti-mouse IgG1 Alexa 488/594 (1:220, Invitrogen). Previously established immunohistochemistry techniques for quantifying fluorescent pixel intensity of aforementioned antibodies will be used to quantify each cell [76,92,177,207].

6.2.4 Statistical Analysis

Data were analyzed using the Student's t-test for unpaired data where appropriate or one-way ANOVA followed by a Tukey's least significant difference post-hoc test where warranted to determine significant differences among groups with 95% confidence; A p value < 0.05 was considered to represent a statistically significant difference.

6.3 Results

6.3.1 Effect of Fractalkine Delivery on Number of Regenerated Axons.

In this study, we used the same 15 mm gap model that we studies in CHAPTER 4 but increased the dose of IL-4 to 10 ug/ml and the Agarose hydrogel concentration to 1% wt/vol to increase the release time of IL-4. We also added another group in which instead of IL-4, Fractalkine (anti-inflammatory monocyte recruiting factor) with similar concentration as IL-4 was used. Here, we used IL-4 as our benchmark condition, since we have already established the superiority in performance of IL-4 containing scaffold in several experiments (CHAPTER 4 and CHAPTER 5 . After four weeks, animals were euthanized, and axons were visualized by immunostaining for Neurofilament-160 (NF160). As it is evident in Figure 6.1a, the number of regenerated axons at the distal end of the scaffold is significantly higher than IL-4 treated sample. In fact, the number of regenerated axons at the distal end of Fractalkine scaffold is 2 times more than IL-4 scaffold, only 4 weeks after implantation of nerve conduit. We also observed that increasing the concentration of Agarose hydrogel to 1% has a deterimental effect on distribution hemogenity of regenerated axons as evident by lack of regeneration in the center of nerve cable, most probably due to the higher stiffness of the center (Figure 6.1b) [216].

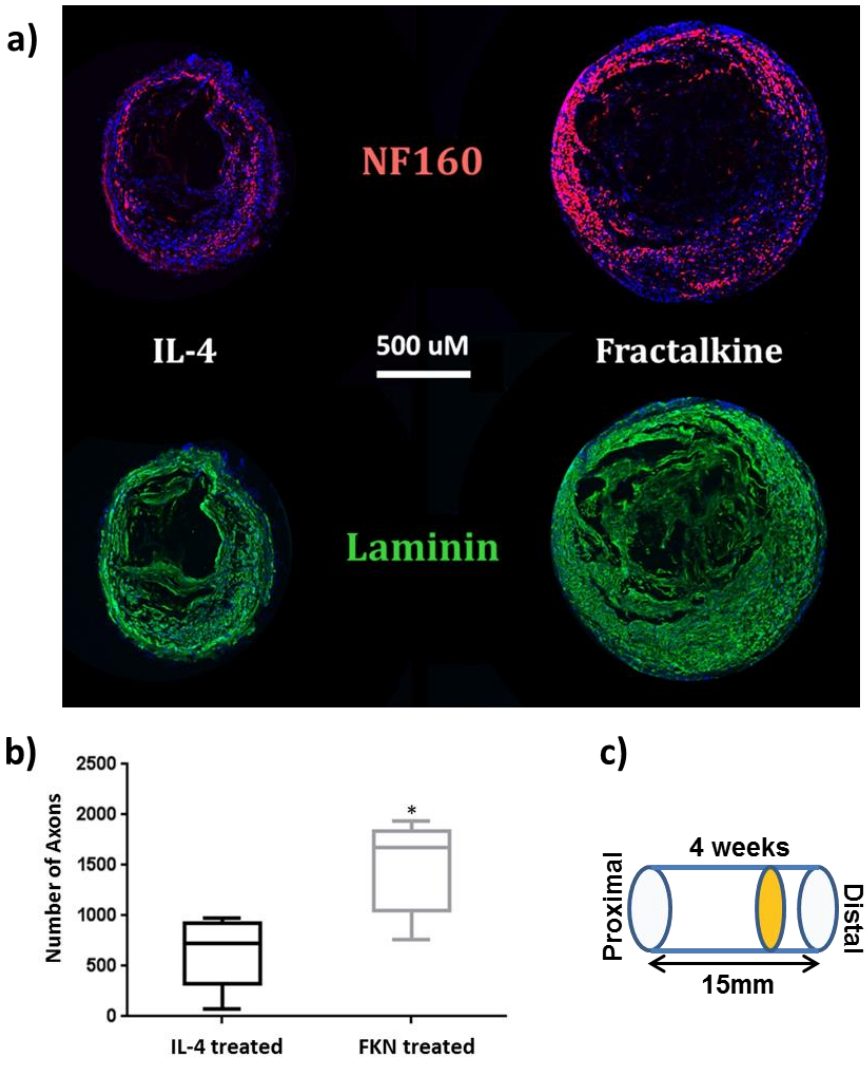

Figure 6.1: Effect of Fractalkine release on the axonal growth.
(a) Immunohistochemical staining of axons (red), laminin (green), and DAPI (blue) at the distal end of nerve stump. (b) Number of regenerated axons at the distal end of Fractalkine vs. IL-4 loaded scaffold four weeks after injury. Immunohistochemical staining of axons (red), laminin (green), and DAPI (blue) at the distal end of nerve stump. (c) Approximate location of sectioning. P value= 0.0114 (two-tailed) (n=5). Fractalkine dramatically increase the number of regenerated axons relative to IL-4.

6.3.2 Effect of Fractalkine Delivery on Number of SC and Endothelial Cells

Based on the result of our study in CHAPTER 5 , we investigated the effect of Fractalkine delivery on affecting the permissiveness of nerve conduit for growth. Here, we used number of infiltrated SC and endothelial cells (early indicator for blood vessel) as our gauge to evaluate the permissiveness of environment. Our data in Figure 6.2 indicated that Fractalkine delivery significantly increased both SC infiltration (Figure 6.2d) and endothelial cells migration inside the nerve conduit (Figure 6.2e), 4 weeks after implantation of nerve conduit.

6.3.3 Effect of Fractalkine Delivery on Number and Phenotype of Macrophages

As we have been investigating the correlation between number of regenerated axons and the profile of immune response at the distal end of nerve conduit, here we also looked at number of macrophages and their phenotype 4 weeks after nerve conduit implantation using already established macrophage markers [76,92,177,207] (Figure 6.3 and Figure 6.4). As it is shown in Figure 6.3d, number of macrophages at the distal end of nerve conduit is significantly lower in the Fractalkine scaffold relative to the IL-4 scaffold. Moreover, although the number of pro-healing macrophages (M2a and M2c) determined by co-staining with CD68 and Arginase is not significantly different between these two conditions (Figure 6.3e), the ratio of pro-healing macrophages to the total number of macrophages (Figure 6.3f) is significantly higher in Fractalkine scaffold relative to the IL-4 scaffold. Also the number and the intensity of mannose receptor (CD206) which is another anti-inflammatory/wound healing marker is significantly higher in Fractalkine scaffold relative to our bench mark control (IL-4 scaffold) (Figure 6.4d). Moreover, our data showed that the number of CD206+CD86+ cells (an indicator for regulatory M2b macrophages) is dramatically higher in Fractalkine scaffold relative to the control IL-4 scaffold. M2b which has both pro- and anti-inflammatory functions can be induced toward the end of regeneration process [93].

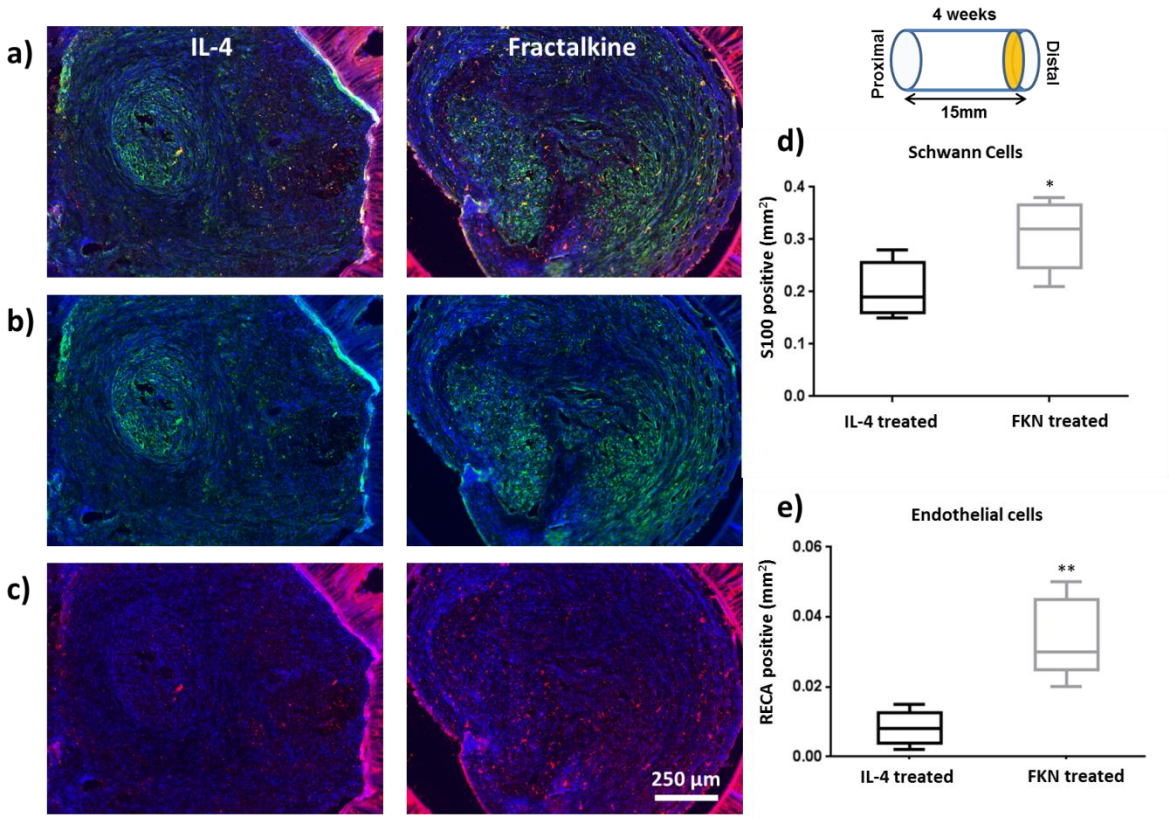

Figure 6.2: Effect of early Fractalkine release on Schwann cells and endothelial cells. a) Combined S100 (green), RECA (red), and DAPI (blue) staining. b) Combined S100 (green) and DAPI (blue) staining. c) Combined RECA (red) and DAPI (blue) staining. d) Quantitative analysis of SCs at the distal end by using S100. P value=0.0243 (two-tailed). e) Quantitative analysis of endothelial at the distal end by using RECA. P value=0.0016 (two-tailed). Fractalkine treated scaffold significantly enhanced both SC infiltration and number of endothelial cells inside the nerve conduit in comparison to the IL-4 treated scaffold.

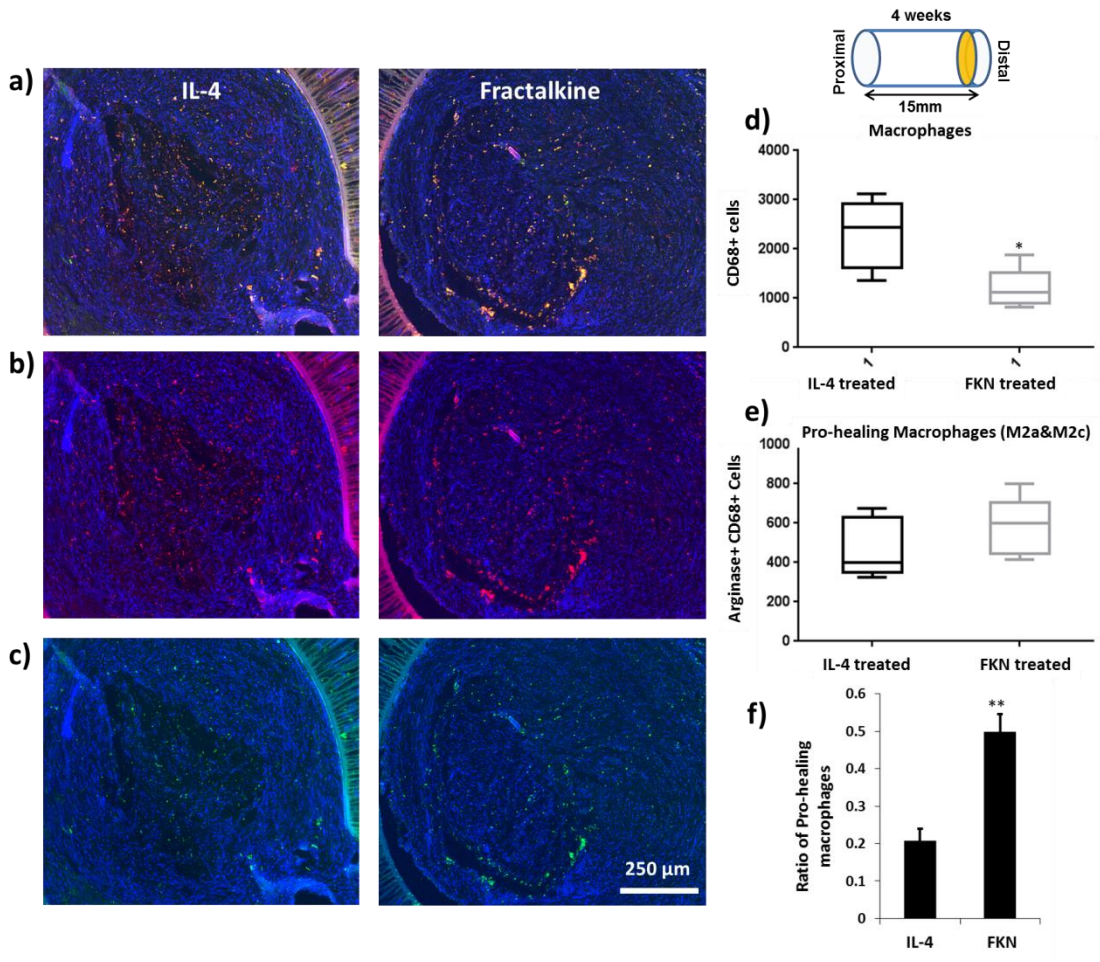

Figure 6.3: Effect of early Fractalkine release on number and phenotype of macrophages.
a) Combined Arginase (green), CD68 (red), and DAPI (blue) staining. b) Combined CD68 (red) and DAPI (blue) staining. c) Combined Arginase (green) and DAPI (blue) staining. d) Quantitative analysis of total macrophage number at the distal end by using CD68 marker. P value=0.015 (two-tailed). e) Quantitative analysis of pro-healing macrophages at the distal end by double staining with CD68 and Arginase. P value=0.288 (two-tailed). f) Calculating the ratio of pro-healing macrophages to the total number of macrophages. P value=0.0019. Fractalkine treated scaffold has significantly lower number of macrophages but higher ratio of pro-healing macrophage relative to IL-4 treated scaffold, 4 weeks after implantation.

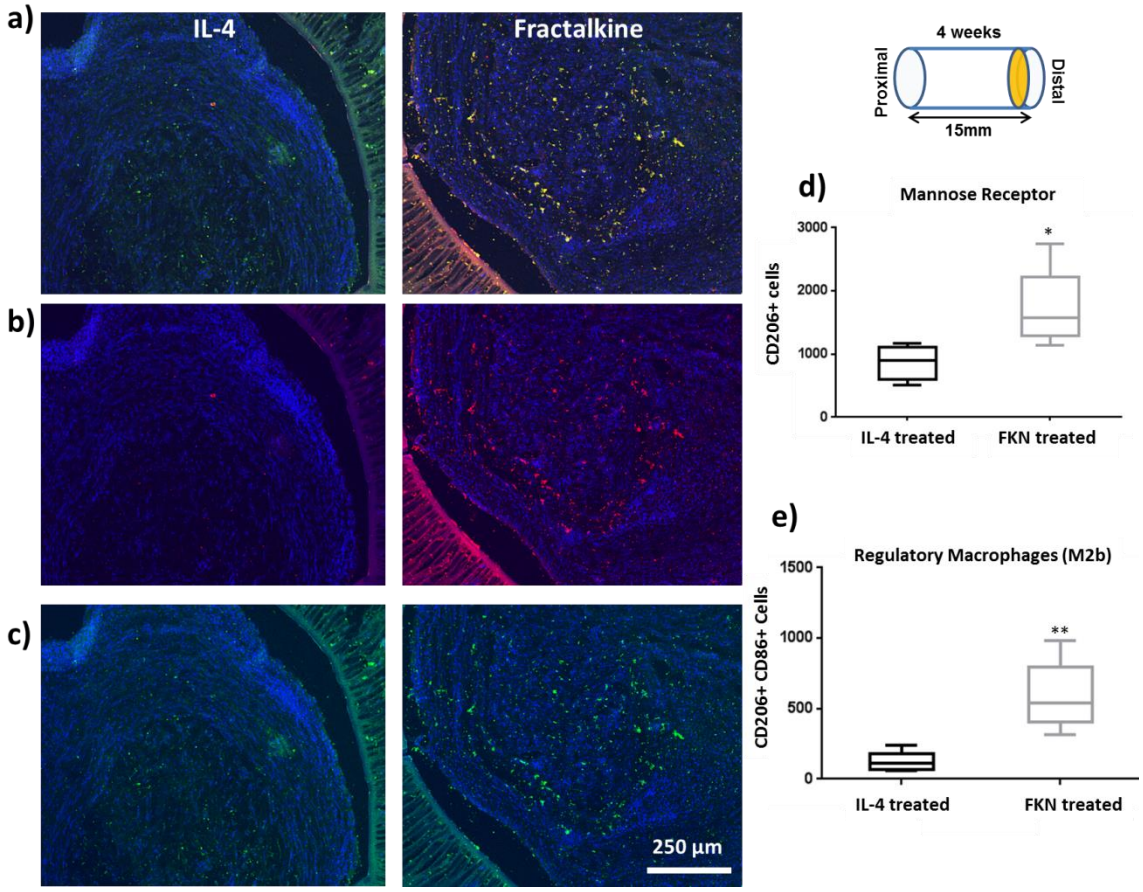

Figure 6.4: Effect of early Fractalkine release on mannose receptor expression and regulatory macrophages.

a) Combined CD206 (green), CD86 (red), and DAPI (blue) staining. b) Combined CD86 (red) and DAPI (blue) staining. c) Combined CD206 (green) and DAPI (blue) staining. d) Quantitative analysis of mannose receptor expressing cells at the distal end assessed by CD206 marker. P value=0.022 (two-tailed). e) Quantitative analysis of regulatory macrophages (M2b) at the distal end by double staining with CD86 and Mannose receptor. P value=0.003 (two-tailed). Fractalkine treated scaffold contains higher number of mannose recptor expressing cells as well as regulatory macrophages relative to IL-4 treated scaffold, 4 weeks after implantation

6.3.4 Effect of Macrophage Depletion on Efficiency of Fractalkine Treatment

Clodronate liposome (Clod-lip) treatment is a well-established methodology to deplete circulating monocytes and therefore significantly reduces the number of infiltrating macrophages at the site of injury [103,131,265]. In order to completely deplete macrophages, several i.v. injection of Clod-lip are required before and right after surgical procedure. However since we were only interested to evaluate the relative effect of macrophage depletion, we injected the animals with Clod-lip only once 48 hr before nerve conduit implantation. Two experimental groups were included: Fractalkine scaffold with Clod-lip injection and Fractalkine scaffold with sham PBS injection. Figure 6.5 clearly demonstrates the negative effect of macrophage depletion due to Clod-lip treatment on axonal regeneration 10 days after implantation in critically sized non-healing 15 mm gap model.

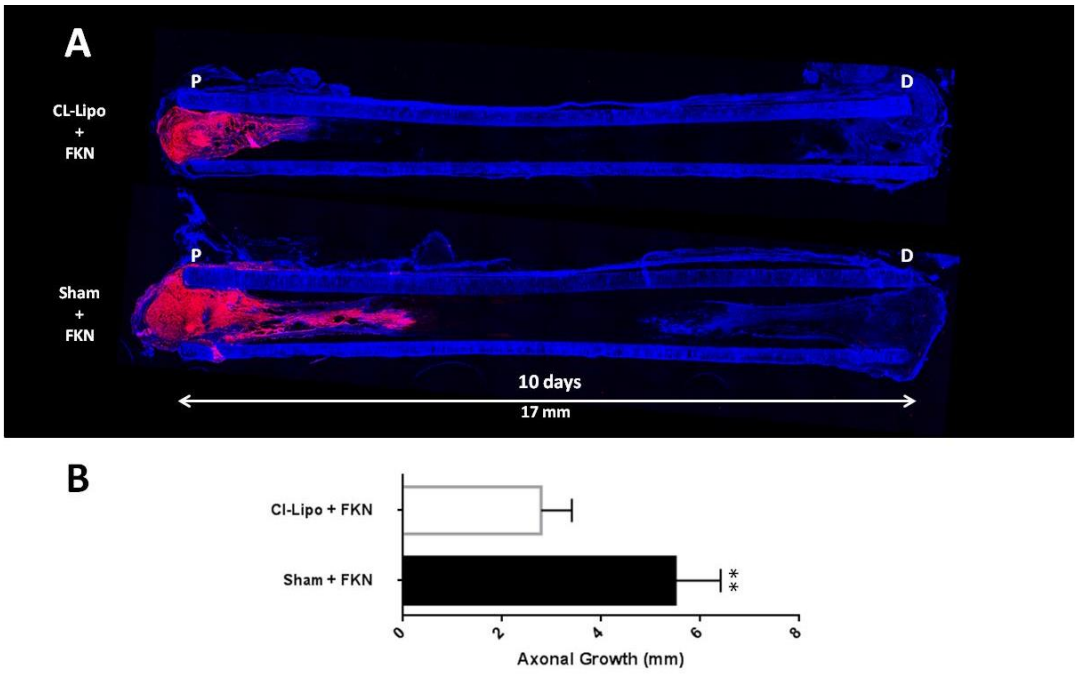

Figure 6.5: Clodronate Liposome Study.
A) Demonstrates the axonal regeneration (stained red with NF160 antibody) using longitudinally sectioned scaffolds in Clodronate liposome treated animal vs. non-treated animal. Both scaffolds contain Fractalkine. B) Quantifies the length of axonal growth 10 days after scaffold implantation. Partial depletion of macrophages using single injection of Clodronate liposome 48 hours before nerve conduit implantation significantly reduced the amount of axonal growth implying the central role that macrophages play in Fractalkine containing nerve conduit. (Blue = Dapi)

6.4 Discussion

After PNS injuries, neurons rapidly respond by changing their activities and promoting a regenerative phenotype. At the distal nerve stump, SCs change their phenotype and adopt a reparative phenotype. SCs as well as infiltrating and resident macrophages remove inhibitory debris which enables new axons sprouting into the degenerated nerve, directed by the bands of Büngner. As the result of these events, a healthy environment is created for regeneration in the PNS.

Extended time without axon regeneration causes the target tissue as well as the distal end of the nerve stump to become chronically denervated. It also causes the neurons to become chronically axotomized which means lacking contact to their target tissue for axons. Both chronic denervation and chronic axotomy hamper regeneration, but studies have shown that chronic denervation is particularly defective [37,225,257]. Chronically denervated distal stump loses its capability to support axonal growth around 8 weeks after the injury, and becomes completely incapable of supporting the growth 6 months after the injury [225]. At the same time period, the chronically denervated muscle tissue starts to go through atrophy. Therefore it is critical for axons to reach their target muscle during this regenerative time window since after this period muscle tissue is less susceptible to support the reinnervation [225,266].

From clinical point of view, it is important to note that some human nerves require extra time to extend to their target tissue due to the long distances, especially in patients with proximal nerve injuries. However, it is also important to mention that Schwann cell atrophy takes longer in humans than in rodents [225]. Nevertheless, to overcome these problems, axons should grow faster, and/or Schwann cells must sustain a regenerative conductive environment for longer period.

Currently, the synthetic biomaterial-based nerve conduits which have already been developed as alternatives to autografts [3,76,77,224,267] are only capable of bridging short gaps (less than 8 mm). These conduits provide a regenerative conductive environment

mainly by supporting fibrin cable formation acting as a migratory substrate for SC and fibroblast FB into the nerve gap [156]. These cells help reorganization of the ECM, but also provide trophic support for regenerating axons which enables the bridging of the nerve gap[229]. However, these nerve conduits have not been very successful in bridging critically sized nerve gaps (that are typically greater than 1.3 cm in rodents and greater than 3 cm in humans). As a result, functional recovery is rarely achieved in those gaps [3]. Here we proposed a new way to maintain the environment permissive to support higher rate as well as degree of axonal growth by releasing of anti-inflammatory monocyte recruiting factor (Fractalkine) inside nerve conduit.

Although monocytes and their descendants (macrophages) have long been known to play an essential role in the degenerative process, recent work has pointed to their importance in influencing the regenerative process [95,101,230]. They are quite abundant during nerve degeneration and regeneration and modulate the early sequence of cellular events which can determine the fate of healing process [95].

Monocyte entry into a nerve, distal to the site of injury, is mediated through upregulation and release of the major monocyte chemokine, Monocyte Chemoattractant Protein (MCP-1) also known as CCL2, by SCs which reaches to its maximum 1 day after injury [268,269]. Once the monocytes enter, they differentiate into macrophages and phagocytose axonal and myelin debris resulted from the ongoing degenerative process. Removal of the debris, which is inhibitory to regenerating axons, is a known prerequisite for successful regeneration *in vivo* [40]. Moreover, Barrette et al. (2008) found that decreasing monocyte infiltration into the sciatic nerve decreased the normal axotomy-induced increase in all four neurotrophins [i.e., NGF, brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4] [270]. Monocytes also enter into peripheral ganglia following nerve injury. Recent work has also suggested that monocyte recruitment around axotomized cell bodies helps mediate the conditioning lesion response [100]. In fact, under conditions in which monocyte do not enter the dorsal root ganglia, there is no enhanced

growth after a conditioning lesion. Thus, monocyte recruitment leads to at least two important effects during degeneration and regeneration process: creating a pathway in the distal nerve segment conducive to axonal regeneration, and stimulating the axotomized neuronal cell bodies to switch to a regenerative phenotype [101]. *Therefore, monocytes could represent an upstream 'lever' to influence downstream axon and SC fates and peripheral nerve regeneration.* Our focus in this chapter was to modulate the former effect of monocytes during nerve regeneration process.

Anti-inflammatory $CD43^{hi}$ monocytes express CX3CR1 receptor [31] and respond to CX3C-chemokine ligand 1 (CX3CL1; also known as Fractalkine). Fractalkine is a membrane-bound chemokine that is expressed in the marginal zone of the spleen but also in different tissues [113]. The chemokine domain of Fractalkine can be cleaved to create a soluble form of the chemokine which acts as a chemoattractant for monocytic cells [271], while the membrane-bound form participates in cell adhesion [272].

In mice, deletion of CX3CR1 leads into dwindled patrolling of anti-inflammatory monocytes [273] and also reduction of the inflammatory monocytes recruitment to splenic sites of bacterial infection [274]. CX3CL1 mainly facilitates early recruitment of anti-inflammatory monocytes to the spleen [274], and CX3CL1/CX3CR1 interaction also provides a survival signal for anti-inflammatory monocytes under both inflammatory and steady state conditions.

In the context of nervous system, it has been shown that following facial nerve axotomy, a marked increase in Fractalkine mRNA was observed [271]. In addition, expression of Fractalkine receptor CX3CR1 in macrophages has been shown to dramatically increase in the sciatic nerve proximal to site of injury but also in the DRG, after sciatic nerve section (axotomy) [275]. In addition, it has been shown than axotomy triggers an anti-inflammatory immune response [160]. In fact, the signal supporting the alternative macrophage environment is produced immediately after nerve damage and appears to be present within the nerve, well before monocytes infiltration [160]. Therefore,

it is rational to hypothesize that mimicking the axotomy immune response (anti-inflammatory) in the synthetic nerve gap by exogenous delivery of Fractalkine early after injury would be an effective way to create a permissive environment for neural regeneration.

In fact, exogenous application of Fractalkine has been proven to be neuroprotective in models of ischemic stroke in rats [276] and to cause significant delay in the development of allodynia [275]. Fractalkine administration also increased neuronal survival, and contributed to angiogenesis through promoting endothelial cell proliferation, thus leading to better functional recovery [276].

It is also important to mention that Fractalkine is the only ligand known to bind and activate the CX3CR1 receptor, and does not bind to any other known mammalian chemokine receptors [277]. Thus, the normal indiscrimination that characterizes the chemokine superfamily is not present for Fractalkine which suggests the roles that are played by Fractalkine and CX3CR1 are non-redundant and probably are critically important for nerve regeneration.

The question of *in situ* conversion versus sequential recruitment of inflammatory or anti-inflammatory monocytes in tissues other than cardiac and skeletal muscle has not been systematically investigated [98] and to the best of our knowledge, the role of Fractalkine and its receptor in monocyte/macrophage accumulation in injured peripheral nerve has not been elucidated. Unraveling the process of monocyte recruitment after peripheral nerve injuries can provide insights for the development of new therapeutics which can manipulate the number and distribution of these cells ,and can subsequently leads into the enhancement of pro-regenerative immune response or reduction of detrimental inflammatory response [113].

Moreover, although it has been determined in some other tissues such as skin, muscle, and cardiac [98], the mechanism by which anti-inflammatory

monocytes/macrophages accumulate in peripheral nerve injury site(selective recruitment or in situ conversion) has not been determined yet.

Finally, it is debatable whether inflammatory macrophages travel to the site of inflammation, and change phenotypes to regulatory and wound-healing macrophages [127], or whether the recruited macrophages change their functional phenotype in response to the dynamic signals of the injured tissue during the course of regeneration [62]. The kinetics and mechanisms of recruitment of blood monocyte populations to injured tissues remain to be elucidated.

Regarding macrophage depletion experiment using Clod-lip, although most investigators prefer PBS or saline liposomes for control experiments and referees are usually asking for such controls if omitted, it is important to note that administration of PBS or saline liposomes does not represent the right control experiment in most cases. Compared to an experiment in macrophage depleted animals, the control animals should have normal healthy, non-blocked, non-suppressed and non-activated macrophages. Liposomes, as most other particulate compounds may block phagocytosis by saturation for certain periods of time. Moreover, it is not known exactly which other macrophage functions will be suppressed or reversely activated and for what duration. To mimic depletion treatments without affecting healthy macrophages, sham operations and injections of e.g. NaCl are sufficient. Overall, our Clod-lip study strongly highlights the central role that macrophages play during the nerve regeneration process enhanced by Fractalkine.

6.5 Conclusion

Although macrophage phenotype is heavily dependent on external stimuli, such as cytokine exposure [33], a contribution of monocyte lineage to macrophage phenotypic determination has also been suggested [260]. Here, we demonstrated that release of anti-inflammatory monocyte recruiting factor (Fractalkine), early after nerve injury, can

dramatically enhance the permissiveness of nerve conduits and stimulate axonal growth 4 weeks after the axotomy. Moreover, we reconfirmed the correlation between number and phenotype of major innate immune cells (macrophages) and nerve repair outcome. While we cannot conclusively confirm that the effect of Fractalkine is due to the recruitment/modulation of anti-inflammatory monocytes, we believe our results (histological and functional) presented here are intriguing enough to warrant investigating this possibility in the future.

CHAPTER 7 CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

Peripheral nerve injuries lead to long term disability and decreased function in approximately 2.8% of all trauma patients [278]. This is followed often by neuropathic pain which significantly affects the quality of life for individuals suffering from peripheral nerve injuries. Even though there have been considerable advances made in microsurgical techniques, bridging of long peripheral nerve gaps remains a continuing clinical challenge. Autografts are the current standard technique to bridge the long gaps but several drawbacks limit their use. Their use can lead to donor site complication such as sensory defect, scar, pain as well as requirements for multiple surgeries if the gaps are long [3]. Furthermore, due to modality mismatch, dimensions, etc., functional recovery approaching closer to pre injury levels is not achieved. These limitations of autografts have led to exploring substitutes to bridging peripheral nerve gaps.

With the advances made in the field of regenerative medicine, alternatives to using autographs have been explored. Several synthetic and natural guidance channels are currently used in clinical as well as research settings to bridge nerve gaps. These techniques have shown to improve nerve regeneration in small gaps but fail when the gaps are longer. Despite the innovative efforts over the last several decades coupled to development of refined surgical techniques and understanding nerve injury, we do not have a suitable replacement for autograft. Historically, advances in the field of regenerative medicine and even in peripheral nerve repair have come due to a better understanding of the physiology and the underlying mechanisms that affect the regeneration process. Tissue engineering strategies to bridge long gaps have shown promise but very little is known with regard to how these technologies interact with the endogenous repair mechanism(s).

Our group has pioneered the use of hydrogels, micro-scale lipid microtubes, and topographical cues to bridge nerve gaps longer than 12 mm [3,4,76,77,86–90]. To create

new and effective therapies that stimulate complete regeneration, we focused on many different constraints of peripheral nerve regeneration. Several strategies could potentially be utilized to support regeneration. These strategies includes but not limited to: enhancing the rate of axonal outgrowth as well as the intrinsic ability of axons to overcome inhibitory signals; increasing neuron survival; preventing atrophy of the target tissue as well as chronic dystrophic changes, so as to promote reinnervation; and inhibiting SC and basal lamina atrophy or replacing atrophied SCs with an appropriate bioengineered scaffold [225].

We have come to realize, and our data attests to this, that instead of trying to recreate a complex combination of ECM proteins, angiogenic factors and neurotrophic factors in the nerve gap, it might be more effective to bias inflammation/immune reaction after injury towards a regenerative biochemical cascade in order to create a permissive environment for growth. This change is a marked shift in approach – rather than identifying the critical factors affecting nerve repair downstream and introducing them in the nerve gap, we focus on upstream modulation of inflammation after injury to attain repair by effective activation of the intrinsic growth capacity of peripheral neurons [108,230,258].

The shift in our understanding of the macrophages role in tissue remodeling after injury is reflected in recent tissue engineering approaches; rather than seeking means for macrophage elimination or prevention of their infiltration to the injured tissue, current efforts have shifted to identifying means for boosting their controlled recruitment and modulation. In contrast to many conventional views regarding tissue inflammation and tissue remodeling, it has been suggested that strategies that promote the spatially, temporally, and phenotypically controlled recruitment of monocytes as well as timely modulation of macrophages may support functional recovery instead of tissue damage and injury expansion. In fact, this study and others confirm that the downstream "host response" to biomaterials is a result of of not simply the presence and number of macrophages but their spatial and temporal phenotype distribution.

The incredible plasticity of macrophages/monocytes makes them an interesting target for modulation in the context of immunoengineering for peripheral nerve repair. Therapies that target source of inflammation, such monocytes, may lead to favorable results due to the short tissue residence time of monocytes in acute inflammation and the large-scale continuous cell recruitment. After the first hours of injury, modulation of macrophage phenotype or preferential recruitment of specific monocyte subtype(s) could induce resolution of lingering inflammation and help with further tissue repair. In fact, dysfunction of macrophage plasticity, has been suggested as an elemental mechanism to a number of diseases including cancer, atherosclerosis, fibrosis, and insulin resistance [128].

As mentioned earlier, it is still debatable if migrated inflammatory macrophages give rise to regulatory and wound-healing [127] or if they alter their phenotype based on receiving progressive signals of inflammatory process [62]. However we know that the dynamic plasticity of macrophages can be regulated based on the type, duration, and concentration of the stimulating signals. Therefore, it is not surprising that examination of the macrophage population at any given time point shows a combination of macrophage phenotypes and/or transient macrophages (that express both M1 and M2 markers).

In addition to potential effects of phagocytosis, macrophage phenotype is regulated by the biochemical *milieu*, including cytokine exposure [33],and by the mechanical properties of the environment, such as strain or substrate stiffness [183,187,188]. Thus, the phenotypic transition that occurs in monocytes/macrophages from the inflammatory to the regenerative phase may be driven, in part, by release of soluble products from neutrophils or other cells [279],autocrine or cell-intrinsic anti-inflammatory feedback mechanisms, and/or changes in the mechanical, cellular, and biochemical makeup of the granulation tissue [98]. The relative contribution of each of these factors and the precise molecules and signaling pathways involved in regulation of macrophage phenotype during *in vivo* tissue repair remain to be determined. Moreover, *in vitro*-differentiated, M1/M2 macrophages are not identical to inflammatory-phase tissue repair monocytes/macrophages [98,280], and

cell culture conditions generally do not accurately mimic the complex tissue repair environment. Thus, caution must be used when interpreting *in vitro* studies of macrophage phenotypic regulation

We know the timing of the switch between M1 and M2 phenotypes is important for repair. While M1 promotes clearing of debris, and the subsequent switch to a more M2-like phenotype facilitates regeneration, some studies have shown committed M1 macrophages hardly switch phenotype to M2 [62,281]. Therefore it might be fundamentally important to intervene with the development of inflammation early on and that might be the reason for dramatic effect of our therapy. In fact, during the course of wound healing in cutaneous or myocardial tissues for example, there is a well-defined shift in macrophage activation states: from M1 to M2 and their secreted products [94,114]. Also interestingly, advanced age and diabetes which have shown to be related with impaired wound healing, have also been shown to have a reduced ability for an M1 to an M2 phenotype transition [142,143]. This is also the case with injuries to other tissues such as the spinal cord, where this shift does not occur after injury [129], thereby contributing to impaired recovery.

In conclusion, we want to emphasize that the current treatment options available for patients with peripheral nerve injury are unsatisfactory. Off the shelf availability of long synthetic grafts would revolutionize the acute management of complex peripheral nerve injuries such as traumatic plexus injuries allowing surgeons to immediately bridge multiple nerves at the time of injury. The same could be said for complex tumor resections in axilla (brachial plexus) or groin (sacral plexus). Here, we described an innovative approach, which uses the endogenous capacity of the body for healing using its own immune cells. We believe our research presented here has the therapeutic potential for making significant progress toward addressing these critical problems. We also believe that the macrophage modulatory scaffold design discussed in this document is a significant advance in the state-of-the-art for biomaterials for immunomodulation and regeneration.

7.2 Future Directions

Understanding the M1/M2 polarization patterns is the first step in furthering our knowledge with regard to the contributions of these cells. Macrophage polarization and their contribution to healing can be fully assessed by examining multiple gene expressions, and cell surface markers in addition to understanding the effects of paracrine interactions with other cells. Also, when determining the outcome of certain conditions, effects of temporal and spatial distribution of macrophages may become very significant. It is highly beneficial to study the exceedingly complex pathways of macrophage phenotypes and their secreted products during tissue regeneration process. Understanding of this pattern will also allow for the development of novel therapeutic strategies. Therapies that target cell production, such as the splenic niche, may lead to favorable results due to the short tissue residence time of monocytes in acute inflammation and the large-scale continuous cell recruitment. After the initial hours of injury, manipulating monocyte phenotype recruitment could induce resolution of lingering inflammation and help with further tissue repair.

Despite the body of evidence that support the difference between the paracrine effects of different phenotype of macrophages, it is still not clear if the conflicting outcomes correlated with distinct phenotype of macrophages are the result of the paracrine or primary functions of these cells. For example, recent studies suggest that macrophages may play a significant role in tissue remodeling process since they might be capable of myeloid to mesenchymal transition [282–284]. These studies have revealed that macrophages assimilate progenitor cells despite their singular pro-inflammatory placement.

Moreover, contradictory outcomes which can be correlated with distinct macrophage phenotypes might be detectable by routine histology. In fact, it is important to determine the function of these cells with a plethora of different tools and not rely only on surface markers. For macrophage classification in contact with biomaterials, cytokine

release is a more reliable criterion in comparison to surface-markers since classification via only surface marker expression may be misleading [186]. For example, scavenger receptors (CD163) have a broad variety of biological activities [285] and their expression by macrophages should not be the only criterion for a proper assessment of the state of inflammatory response. It has been shown that depending on material characteristics, M1 classified macrophages may release anti-inflammatory and pro-angiogenic signals, while M2 classified macrophages may release pro-inflammatory cytokines [186]. Therefore, as cytokines act on various other cell types [286] and tissues [287], cytokine release profile seems more relevant and decisive in evaluating the macrophage response to biomaterials. Thus, a wide array of analyses needs to be performed in order to better understand the mechanisms involved in nerve regeneration with our immunomodulatory nerve conduit.

Finally, although it is known that chemical modification of biomaterials (such as crosslinking with non-degradable agents) can prevent the formation of the beneficial M2 response [92], the effect of biomaterial degradation products on modulating macrophage phenotypes has not been extensively studied yet. Since monocytes/macrophages contribute to biomaterial degradation as well as are affected by degradation products, it is logical to assume that degradation can play a major role in modulating macrophage phenotype. Therefore, an in-depth understanding of events on these lines may provide a better biomaterial design strategy by which a constructive and functional downstream outcome can be promoted.

The question of *in situ* conversion versus sequential recruitment of inflammatory or anti-inflammatory monocytes in tissues other than cardiac and skeletal muscle has not been systematically investigated [98] and to the best of our knowledge, the role of Fractalkine and its receptor in monocyte/macrophage accumulation in injured peripheral nerve has not been elucidated. Therefore, analyzing the process of monocyte recruitment can offer insights for the development of new therapeutics that can modulate the number and distribution of monocytes, and therefore enhancement of pro-regenerative immune

response [113]. Moreover, investigations on these lines have been conducted in some other tissues such as skin, muscle, and cardiac [98], however the mechanism by which anti-inflammatory monocytes/macrophages accumulate in peripheral nerve injury site (selective recruitment or *in situ* conversion) is yet to be determined.

Additionally, studies comparing the efficacy of different modes of macrophage activation, along with an improved understanding of the phenotypic fate of exogenous macrophages, may help to optimize macrophage-based therapies for healing of different tissues and bring these promising treatments closer to widespread clinical reality.

Finally, from a translational perspective there is value in the 'scaffold' not containing biochemical elements so that the scaffold can be classified as a 'device' (instead of a 'biologic' or 'combination' product). However, given that the clinical gold standard, autografts, only achieve full functional recovery in 40% of the cases, it seems beneficial and rational to push the limits of our previously established biomimetic nanofibers-based scaffold by incorporating the studied immunoengineering approach to match or even exceed the performance of autografts. Future designs to combine biomimetic nanofiber approach with the immunoengineering strategy should be considered to investigate whether synergy between biochemical and topographical cues significantly enhances nerve guide function to the point that the need for autografts in bridging long gaps is obviated (Figure 7.1). It is important to note that accelerating regeneration of peripheral nerves, without any change in the nature or extent of regenerated fibers, would have significant clinical implications, as it would lessen the extent of atrophy of the distal muscle. We believe our immunoengineering strategy investigated here is compatible with the nanofiber enhanced nerve guide approach, and if successful, would add a new 'arm' to the current approaches for inducing repair across long gaps.

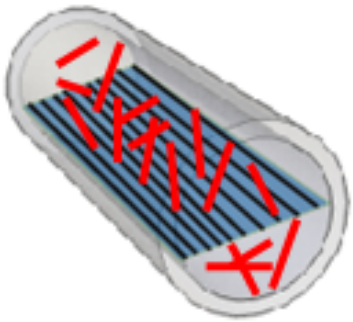

Figure 7.1: Next generation of nerve conduit which includes both topographical and immunomodulatory cues. The black lines on the blue substrate represents the aligned nanofiber film which provides topographical cues for the SCs infiltration and red tubes represent the lipid microtubes containing immunomodulatory factor (e.g. IL-4, Fractalkine, or etc.) which provides biochemical and immunomodulatory cues.

Table 7-1 represents the areas that needs to be studied in future works. There are 3 main categories that require more in-depth studies following the work in this thesis: 1- Cellular analyses; in the context of macrophage modulation using factors such as IL-4, it is necessary to investigate if the observed regenerative effect is due to macrophage polarization or enhancement in *in situ* proliferation of anti-inflammatory macrophages. In the context of reparative monocyte recruitment, it is essential to execute acute studies to show that we preferentially recruited reparative monocytes using chemokine Fractalkine.

2- Functional analyses; in order to show that this study is clinically meaningful, it is important to demonstrate functional data for reinnervated nerves. Electroneurography and Electromyography need to be done at later time points after applying these immunomodulatory molecules in order to assess the functional recovery of damaged nerve.
3- More clinically relevant models; the majority of nerve damages require delayed intervention rather than acute and it is clinically relevant to investigate the capability of our approach for those cases. We believe our intervention represents an upstream approach that can be applied to other injured tissues and maybe even regeneration of the whole organ. Salamanders have the remarkable ability to regenerate complex structures such as limbs, tails, retina, and spinal cord, along with some sections of the heart and brain, during any stage of their life cycle and recently it has been shown that macrophages are the key player in enabling salamander to regenerate itself [103]. Now the question is, can we use the immunomodulatory approach represented here in order to mimic the regenerative capability of salamanders for other mammals and particularly humans?

Table 7-1: Proposed areas for future studies

Cellular	Functional	Clinical
➢ Macrophage polarization vs. *in situ* proliferation ➢ Preferential recruitment of reparative monocyte 	➢ ENG (Electroneurography) ➢ EMG (Electromyography) ➢ Sensory vs. motor neuron regeneration 	➢ Delayed intervention instead of acute ➢ Application for other tissues (*upstream* approach) ➢ Combined traumatic injury 

APPENDIX

A.1. Cytokine Release Profile from Scaffold

Polysulfone tubes filled with 0.7% Agarose (SeaPrep Lonza) mixed with 1 μg/ml rat recombinant cytokine IFN-γ. The two ends of the 17 mm tubes were closed to mimic the effect of suturing *in vivo*. Then the tubes were incubated in 1 L PBS at 37^{o}C and removed from the PBS at 8, 24, 48 and 72 hr (n=4) and kept at -20^{o}C. After the final time point, all the tubes were slowly equilibrated to the room temperature, were cut and put inside the 24-wells plate containing 100k NR8383 macrophages. The amount of nitrite produced by macrophages treated with the tubes was measured using the Griess reagent system. Since macrophages exposed to IFN- γ become inflammatory and produce nitrite, this number were used as an indicator for the residual IFN- γ remained in the tubes after different incubation time in the PBS (Fig S2).

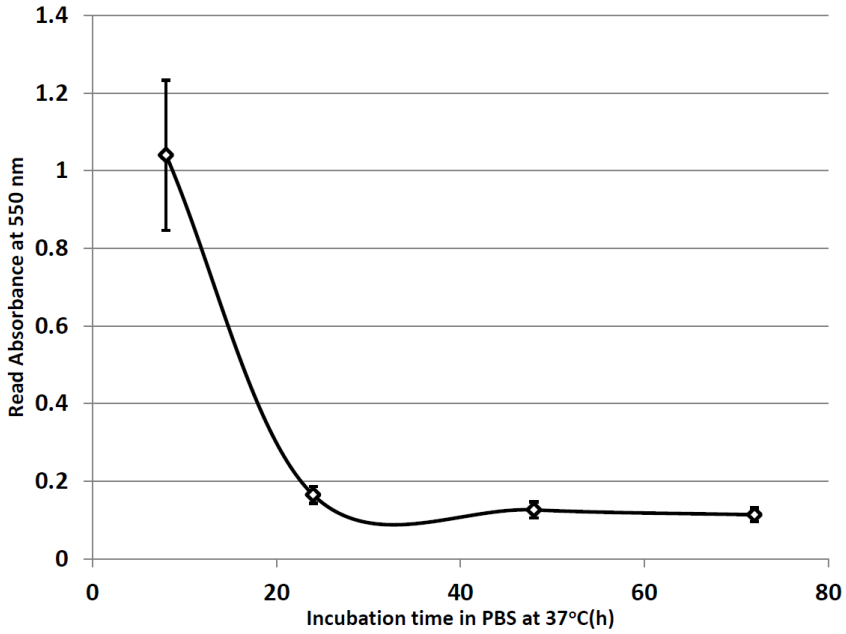

Figure 7.2: Release of IFNγ from the 0.7% based on nitrite production of macrophages. Agarose/Polysulfone tube is measured by adding the tube containing initial 1ug/ml concentration of IFN-γ incubated in 37oC PBS at different time points.

A.2. Macrophage Phenotype Verification

A.2.1. Quantitative Analysis of Nitrite Production of Macrophages

Griess reagent system (Promega) were used to quantify the amount of nitrite production of activated macrophages. The serum-free media of macrophages (F12k) treated with cytokines (20 (or 80) ng/mL IL-4 or 20 (or 80) ng/mL IFN-γ + 100 ng/mL LPS) were changed with fresh media and amount of NO_2^- in the media were measured after 24 hr (Figure 7.3).

A.2.2. RNA Extraction and qRT-PCR Analysis of Macrophages

Total RNA was extracted from cell pellets obtained from the untreated macrophages or macrophages treated with IFN-γ +LPS or IL-4 cytokines using the Qiagen RNEasy Mini Kit (Qiagen, Valencia, CA). Extracted total RNA was quantified using the Quant-iT RiboGreen RNA Reagent and Kit (Invitrogen). Two micrograms of the total RNA was converted to cDNA using a highcapacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). PCR primers were designed (Table 7-2) using Primer Express® software (Applied Biosystems) and obtained from Integrated DNA Technologies (Coralville, IA). Primer validation and qRT-PCR using SYBR green mix (Applied Biosystems) was conducted on a StepOnePlus real-time PCR machine (Applied Biosystems) according to methods previously published [231,232]. All qRT-PCR reactions were performed in triplicates and fold differences for each target gene using comparative CT method were normalized controls (untreated macrophages), and the relative fold change was calculated by normalizing against the endogenous reference, housekeeping, hypoxanthine phosphoribosyltransferase 1 (HpRT1) gene.

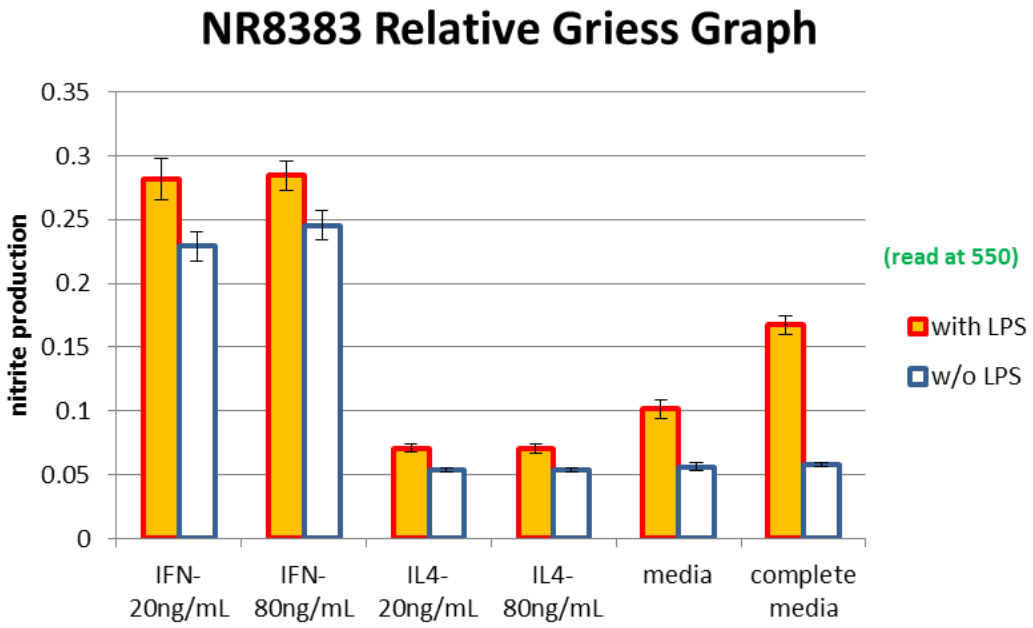

Figure 7.3: Effect of two different concentration of IL-4 and IFNγ on rat aleveloar macrophages to secrete nitrite.

A.3. Schwann Cells Analysis

A.3.1. RNA Extraction and qRT-PCR Analysis of Schwann Cells (SC)

Same method as macrophages were used for SC too. However here the conditions were SC treated with conditioned media (CM) from IL-4 or IFN-γ+ LPS macrophages and the control was SC treated with CM from untreated macrophages. PCR primers were designed for the genes of interest (Table 7-3) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene (Fig. S3).

A.3.2. Transfection of RSC-96 Cells with Green Fluorescent Protein (GFP)

RSC-96 Schwann cells were grown in DMEM supplemented with 10% FBS. For transfection, cells were seeded the previous day to achieve 70% confluence in tissue culture dishes. The cells were transfected with pEGFP-C1(Clontech) using Effectene (Qiagen) as per the manufacturer's protocol. The transfected cells were allowed to grow for an additional 60 h at which point they were dissociated using Trypsin-EDTA and seeded in multi-well culture plates to achieve one cell/well. For selection, the growth medium was supplemented with G418 (Gemini Bio-Products) at a concentration of 500 µg/ml and added to the cultures. The cultures were routinely monitored microscopically for growth as well as for GFP expression. Cultures exhibiting high level of fluorescence were further expanded to generate the cell line that is used in the study.

Table 7-2: Primer sets for qRT-PCR of macrophages

Gene	Forward primer	Reverse primer
IL-10	5'-GCCTGGCTCAGCACTGCTAT-3'	5'-TGGGAAGTGGGTGCAGTTATT-3'
IL-12	5'-TGATGACCCTGTGCCTTGGT-3'	5'-TGCTGATGATTGTGGCTCTGA-3'
HpRT1	5'-TGTTTGTGTCATCAGCGAAAGTG-3'	5'-CTGCTAGTTCTTTACTGGCCACATC-3'

Table 7-3: Primer sets for qRT-PCR of Schwann Cell

Gene	Forward primer	Reverse primer
NGF	5'-GATCGGCGTACAGGCAGAAC-3'	5'-TGTGTCAAGGGAATGCTGAAGT-3'
NGFr	5'-TTGTTCAAGGGCTGGTCCAT-3'	5'-TGAGCGCACTAACAGATTCATCTC-3'
PMP22	5'-TTCAGCGTCCTGTCCCTGTT-3'	5'-GACCAGCAAGGATTTGGAAGAC-3'
GFAP	5'-GCTTCCTGGAACAGCAAAACA-3'	5'-CGAAGTTCTGCCTGGTAAACG-3'
GDNF	5'-CAAGGTAGGCCAGGCATGTT-3'	5'-AGCGGAATGCTTTCTTAGGATATG-3'
Krox20	5'-AGCCTCTACCCGGTGGAAGA-3'	5'-CTCCAGCCACTCCGTTCATC-3'
OCT6	5'-GGGCACCCTCTACGGTAATG-3'	5'-TTGAGCAGCGGTTTGAGCTT-3'
MPZ	5'-AGGCTCAGTGCCATGGAGAA-3'	5'-TGTGGTCCAGCATGGCATAC-3'
SDF1	5'-AGCCAACGTCAAACATCTGAAA-3'	5'-CGGGTCAATGCACACTTGTC-3'
TGF-β	5'-GACTCTCCACCTGCAAGACCAT-3'	5'-GGACTGGCGAGCCTTAGTTTG-3'
MCP-1	5'-TGCAGTTAATGCCCCACTCA-3'	5'-ACACCTGCTGCTGGTGATTCT-3'
IL-10	5'-GCCTGGCTCAGCACTGCTAT-3'	5'-TGGGAAGTGGGTGCAGTTATT-3'
GAPDH	5'- GGTGGACCTCATGGCCTACA-3'	5'- CAGCAACTGAGGGCCTCTCT-3'

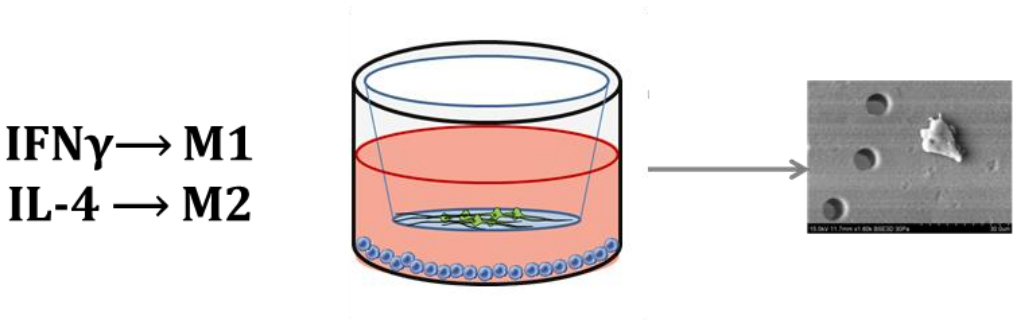

Figure 7.4: Boyden Chamber Schematic. Schematic representing the Boyden Chamber (BC) system used to analyze SC migration. SCs were cultured on the top of the insert (green cells) and polarized macrophages at the bottom of the well. Right image demonstrated the SEM image of SC crawling toward the holes of BC insert.

A.4. List of Genes Profiled by Neurotrophins and Receptor PCR Array

A.4.1. Neurotrophins and Receptors:

Adcyap1r1, Artn, Bdnf, Cntf, Cntfr, Crh, Crhbp, Crhr1, Crhr2, Frs2, Frs3, Gdnf, Gfra1, Gfra2, Gfra3, Gmfb, Gmfg, Hcrtr1, Hcrtr2, Mt3, Ngf, Ngfr, Ngfrap1, Nr1i2, Nrg1 (Hgl), Nrg2, Ntf3, Ntf4, Ntrk1, Ntrk2, Pspn, Ptger2, Tfg, Cd40 (Tnfrsf5), Fas (Tnfrsf6), Ucn, Vgf, Zfp110, Zfp91.

A.4.2. Neuropeptides and Receptors:

Galanin Receptors: Galr1, Galr2.

Neuropeptide Y Receptors: Npy1r, Npy2r, Ppyr1.

Other Neuropeptides and Receptors: Cckar, Grpr, Npffr2 (Gpr74), Ntsr1, Hcrt, Mc2r, Npy, Nrg1 (Hgl), Tacr1.

A.4.3. Neurogenesis:

Central Nervous System Development: Cxcr4, Fgfr1, Ngfr, Ntf3.

Peripheral Nervous System Development: Artn, Gdnf, Gfra3, Ngf, Nrg1 (Hgl), Ntf3.

Axon Guidance: Artn, Gfra3, Ngfr.

Gliogenesis: Fgf2 (bFGF), Nrg1 (Hgl), Ntf3.

Dendrite Morphogenesis: Bdnf, Mt3.

Other Neurogenesis Genes: Bax, Fos, Cbln1, Galr2, Gfra1, Gfra2, Nell1, Ntf4, Ntrk1, Ntrk2.

A.4.4. Cell Growth and Differentiation:

Growth Factors and Receptors: Artn, Bdnf, Fgf2 (bFGF), Fgf9, Fgfr1, Gdnf, Gmfb, Gmfg, Il10, Il1b, Il6, Lif, Mt3, Ngf, Nrg2, Ntf3, Ntf4, Pspn, Tgfa, Tgfb1, Tgfb1i1, Tp53, Vgf.

Cell Cycle: Fgf2 (bFGF), Fgf9, Il1b, Ntrk1, Tgfa, Tgfb1, Tp53.

Cell Proliferation: Bax, Cxcr4, Fgf2 (bFGF), Fgf9, Grpr, Il10, Il1b, Myc, Stat4, Tgfa,

Tgfb1, Tp53.

Cell Differentiation: Cntf, Fgf2 (bFGF), Fgf9, Nf1, Nrg1 (Hgl), Stat3, Tp53, Zfp91.

A.4.5. Cytokines and Receptors:

Cx3cr1, Cxcr4, Il10, Il10ra, Il1b, Il1r1, Il6, Il6r, Il6st (Gp130), Lif, Lifr,.

A.4.6. Apoptosis:

Bax, Bcl2, Bdnf, Cd40 (Tnfrsf5), Hspb1 (Hsp27), Il10, Il6, Myc, Ngfr, Ngfrap1, Fas (Tnfrsf6), Tp53.

A.4.7. Inflammatory Response:

Il10, Il1b, Il6, Stat3, Tgfb1.

A.4.8. Immune Response:

Il10, Cd40 (Tnfrsf5), Lif, Fas (Tnfrsf6).

A.4.9. Transcription Factors and Regulators:

Fos, Fus, Maged1, Myc, Nr1i2, Ntf3, Stat1, Stat2, Stat3, Stat4, Tp53, Tgfb1i1, Zfp110.

A.5. List of Genes Profiled by Common Cytokines and Chemokines PCR Array

A.5.1. Interferons:

Ifna1, Ifna2, Ifna4, Ifnb1, Ifng, LOC690891 (Ifna5).

A.5.2. Interleukins:

Il10, Il11, Il12a, Il12b, Il13, Il15, Il16, Il17b, Il17f, Il18, Il19, Il1a, Il1b, Il1f10, Il1f5, Il1f6, Il1f8, Il1rn, Il2, Il21, Il24, Il3, Il4, Il5, Il6, Il7, Il9, Il17a (Il17), Il20, Il22, Il27, Il1f9.

A.5.3. Bone Morphogenic Proteins (BMPs) and TGF-ß Family:

Bmp1, Bmp10, Bmp2, Bmp3, Bmp4, Bmp5, Bmp6, Bmp7, Gdf1, Gdf10, Gdf11, Gdf15, Gdf5, Mstn, Gdf9, Inha, Inhba, LOC306312 (Gdf2), Gdf3.

A.5.4. TNF Superfamily:

Cd70 (Tnfsf7), Tnfsf13b, Lta, Ltb, Tnf, Tnfrsf11b, Tnfsf10, Tnfsf11, Tnfsf12, Tnfsf13, Tnfsf15, Tnfsf18, Tnfsf4, Cd40lg (Tnfsf5), Faslg (Tnfsf6), Tnfsf9.

A.5.5. Other Growth Factors/Cytokines:

Csf1, Csf2, Ctf1, Ctf2, Fgf10, Lif, Fbrs, Mif, Nodal, Scgb3a1, Aimp1.

A.6. Effect of IL-19 on SC phenotype

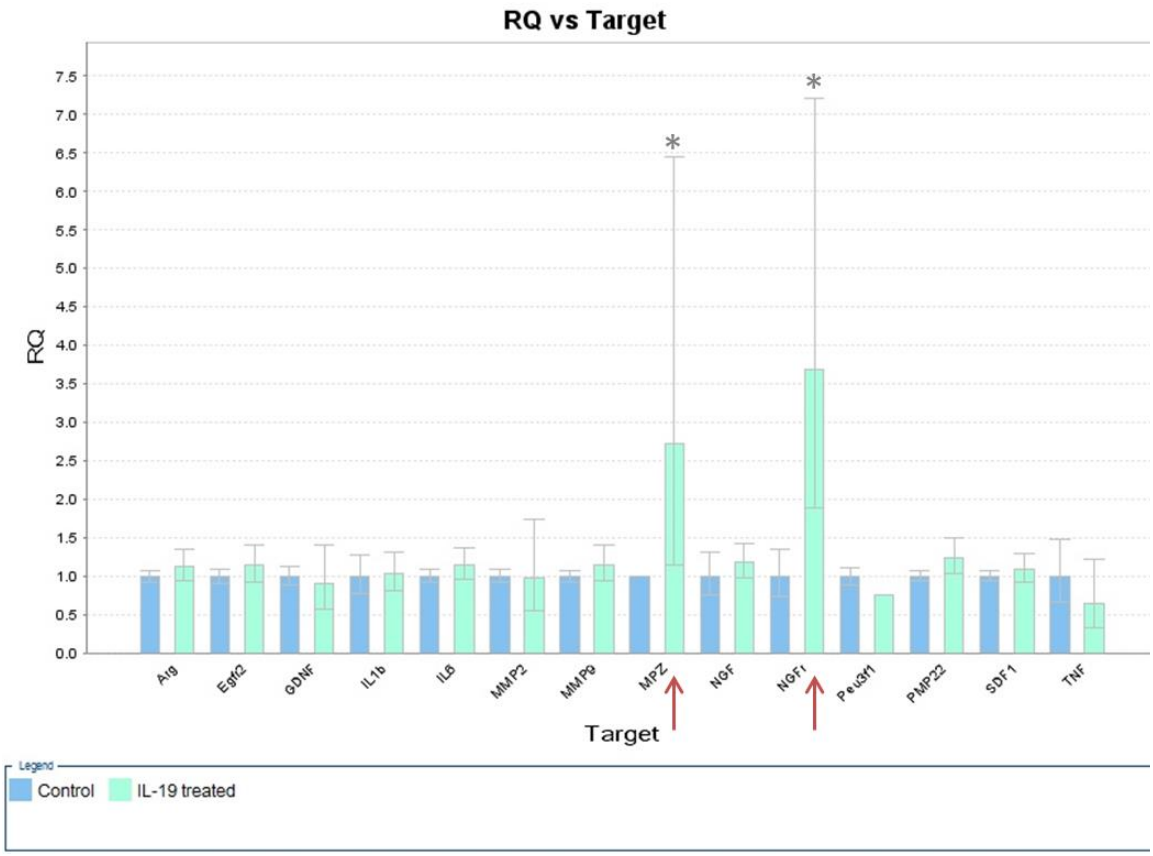

Figure 7.5: Effect of IL-19 treatment (500 ng/mL) on gene expression level of RSCs. IL-19 enhances MPZ which is the myelin precursor gene as well as NGFr which is the Nerve Growth Factor (NGF) receptor gene.

A.7. Correlation Between Regeneration and Macrophage Phenotype in other Scaffolds

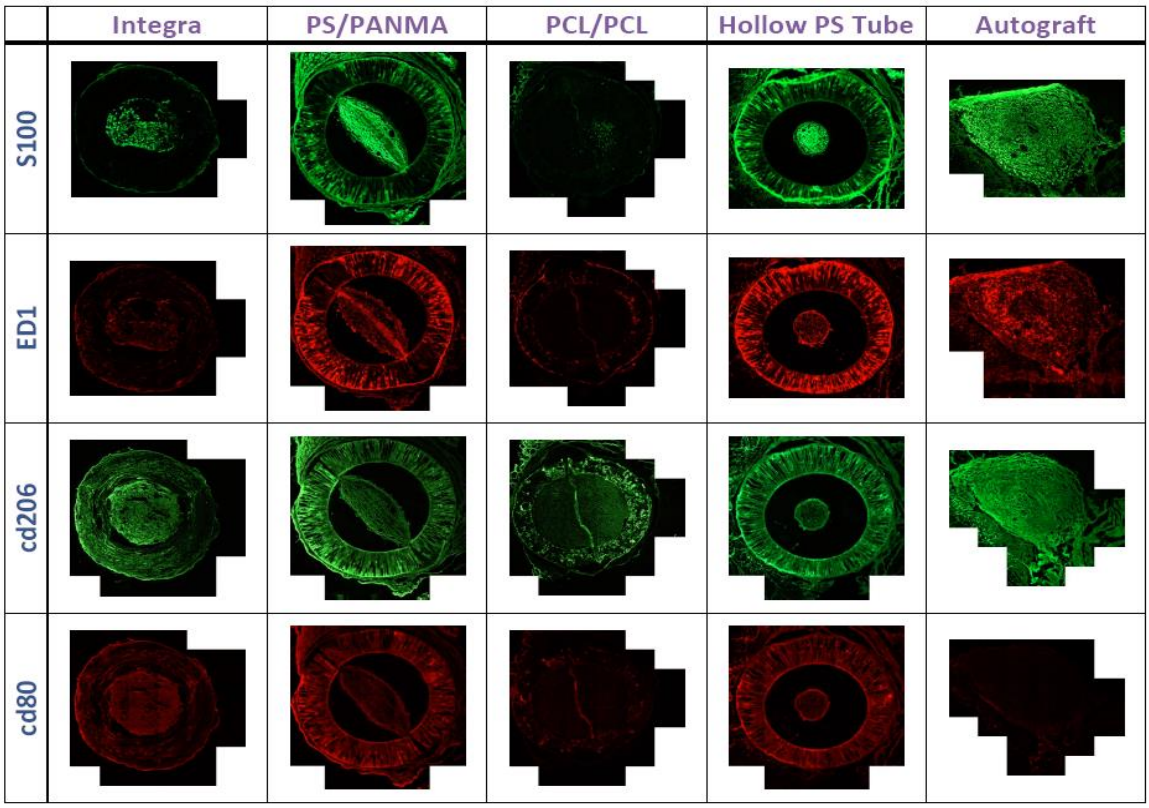

Figure 7.6: The panel of images demonstrating status of immune response and regeneration at the distal end of other scaffolds. S100 stains for SCs, ED1 for macrophages, CD206 for anti-inflammatory, and CD80 for inflammatory cells. All images are captured from the sections at the distal end of the scaffold. Autograft and Integra represent the clinical gold standard and commercially available tube, respectively.

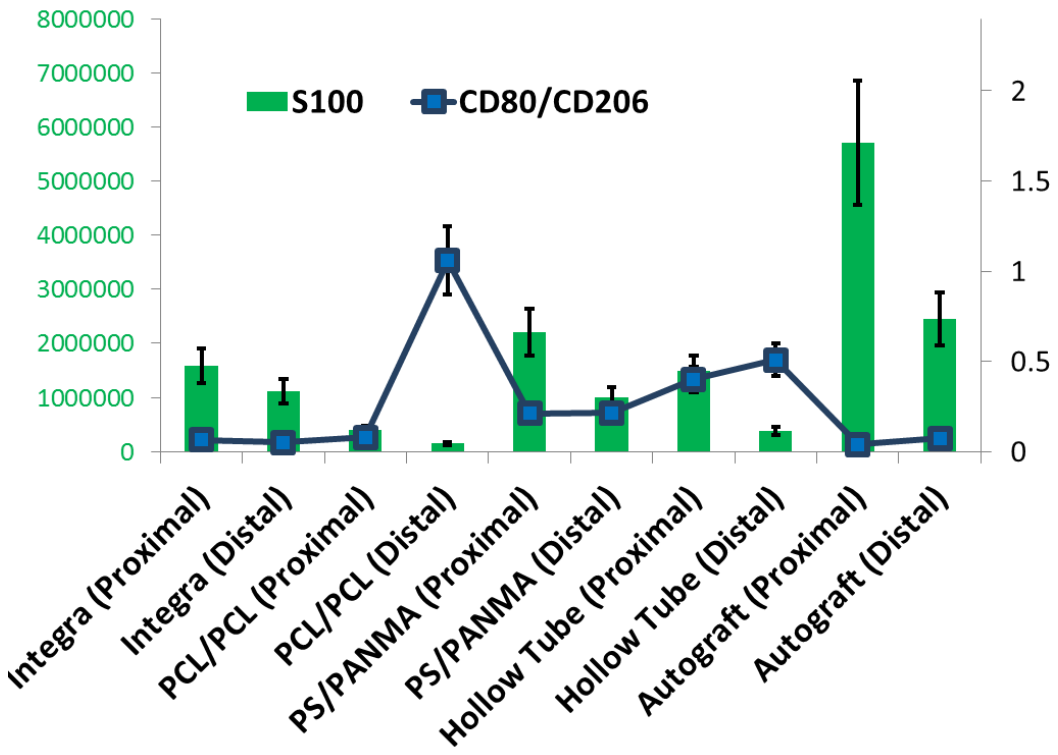

Figure 7.7: The correlation between number of SCs at the distal end of the scaffold and ratio of pro- to anti-inflammatory immune cells. SCs number demonstrates an inverse correlation to the ratio of pro- to anti-inflammatory immune cells both at the distal and proximal end of the scaffold. The left axis represent total fluorescently positive area in μm^2.

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