T cell regulation of regenerative environment in acellular nerve allograft repaired peripheral nerves

Deng Pan
Washington University in St. Louis

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T Cell Regulation of Regenerative Environment in Acellular Nerve Allograft Repaired Peripheral Nerves
by
Deng Pan

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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St. Louis, Missouri
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Deng Pan

Washington University in St. Louis

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Peripheral nerve injury (PNI) is common and has debilitating long term sequelae. Development of new therapies to improve regeneration following PNI is therefore critical. Acellular nerve allografts (ANAs) are increasingly utilized in the clinic for the repair of PNI and an improved mechanistic understanding of nerve regeneration through ANA is important for developing better therapies. Inflammation is an important aspect of regeneration, and the role of macrophage has been increasingly documented. Other aspect of inflammation has not been well-defined. In particular, there is limited understanding on the role of T cells in the regeneration of peripheral nerve. However, evidence in other organ systems, including brain, spinal cord, muscle, and liver support the hypothesis that T cells can play in role in regeneration. Therefore, the overall aim of this work was to identify the impact of T cells on regeneration of peripheral nerve through acellular nerve allograft scaffolds. My thesis is therefore divided into two parts.

In the first part, I examined the leukocyte populations in short and long ANAs to identify the changes in leukocyte populations following nerve repair. Two (2) cm ANAs were used as model
scaffold that supported robust axon regeneration, while 4 cm ANAs were used as model that did not support axon regeneration. To quantify regeneration, both histology of regenerated ANAs and gene expression from Schwann cells were quantified. Utilizing transgenic rats, I also examined if the altered leukocyte population may contribute to regeneration through ANAs. This study suggested that nerve graft length affected the accumulation of T cells, which in turn contributed to the regeneration of nerve repaired with ANAs.

In the second part, I determined how T cells contributed to the regeneration of nerves repaired with ANAs. While T cells have been associated with regulating IL-4 within sites of injury, this has not been demonstrated in nerve repair. Furthermore, what role, if any, IL-4 plays in the regeneration of peripheral nerve is not clear. Finally, how T cells may be regulating IL-4 is also not known. Previous studies have shown that T cells secrete IL-4 or regulate other cells, such as eosinophils, to provide IL-4 indirectly. Thus, identifying the mechanism of IL-4 regulation by T cells is important. In this part of my thesis, a mouse sciatic nerve repair model was used to take advantage of the transgenic animals available. Flow cytometry and gene expression analysis were used to quantify inflammation and its association to T cells. Knockout animals and antibody depletion strategies were used to selectively deplete specific cells or cytokines related to T cells. Regeneration were quantified using histological and immunofluorescence methods. This work demonstrated that T cells recruit eosinophils that express IL-4, which are important for regeneration by promoting myelination.

Overall, my thesis work demonstrated the importance of T cells in the regeneration of peripheral nerve and showed that T cells are critical for the regulation of IL-4 within regenerating ANAs by recruiting IL-4 expressing eosinophils.
Chapter 1 Overview of peripheral nerve injury and regeneration

1.1. Anatomy of the peripheral nervous system
Peripheral nerve injuries (PNIs) represent important public health concerns as it affects 1 per 5000 persons each year. Importantly, PNIs frequently occur in young men and women, with resulting long-term disability, and loss of productivity due to loss of muscle function (Jaquet et al., 2001). Furthermore, left unrepaired, PNI can induce the formation of neuroma, with devastating side effects such as chronic pain (Wall & Gutnick, 1974). Despite advances in microsurgical techniques, complete functional recovery has been elusive. Currently, improvements in surgical techniques have reached a plateau, and research is critical for improvements in treating patients with PNI (Bellamkonda, 2006).

In mammals, the peripheral nervous system (PNS) is a complex system that comprises of neurons, glia cells, and extracellular matrix (ECM) (Fig. 1). The PNS consists of two broad categories: the sensory-somatic nervous system and the autonomic nervous system. The autonomic nervous system consists of the parasympathetic and sympathetic nervous system, which regulates the

Figure 1 Macroscopic anatomy of the peripheral nerve
“fight-or-flight” response. The sensory-somatic nervous system consists of motor and sensory neurons that direct volunteer responses. The motor neurons originate in the spinal cord, and project their axons to innervate muscles. Sensory neurons originate in the dorsal root ganglion (DRG) and innervate sensory organs such as the skin. Nervous tissue of the PNS consist primarily of the axons of these motor and sensory neurons bundled into a structure that includes a variety of support cells and ECM. Within the intact peripheral nerve, the most common type of cells are Schwann cells which wrap the axons allowing for electrical conduction of signals. Other support cell types include neural fibroblasts, resident macrophages, endothelial cells, and pericytes. Both ECM and the support cells play a critical role in maintaining and repairing the nerve after damage or injury.

1.2 Structure and ECM of peripheral nerve

Within peripheral nerves, the axons are bundled into fascicles. A nerve fascicle consists of three distinct layers: the innermost endoneurium, which enclose a single axon and its supporting Schwann cells; a middle perineurium, and the outermost epineurium that consists of dense
connective tissue surrounding the entire nerve (Fig. 2). The epineurium consists of connective
tissues that delimit the nerve from the surrounding tissue. In the perineurium, neural fibroblasts
lay collagen bundles, while endothelial cells form microvessels that support nerve function.
Endothelial cells here are tightly coupled via tight junction to strictly regulate the diffusion of
substrates from the blood. The relative impermeability of this barrier protects the enclosing cells
and axons (which are especially sensitive to changes in the microenvironment) from fluctuations
of blood chemistry or other potentially harmful blood-born materials. Within the endoneurium,
Schwann cells and neural fibroblasts support the axons and produce and remodel ECM (Podratz,
Rodriguez, & Windebank, 2001). During homeostasis, the main function of Schwann cells is to
myelinate axons. Myelination allows increased conduction velocity of signals in the nerve.

The major ECM found in the peripheral nerve include laminin, collagen, fibronectin and
glycosaminoglycans. Laminins are an essential ECM component of peripheral nerves. Laminins
consists of heterotrimers of α, β, and γ chains, and as many as 18 subtypes of laminins have been
described. Laminin-2, composed of α2, β1, and γ1 chains, is the major laminin isotype found
within the peripheral nerve (Z.-L. Chen & Strickland, 2003). Collagen composes the majority of
ECM of the peripheral nerve. Within the nerve, the most abundant collagens are Type I, Type III,
Type IV, and type V collagens (Koopmans, Hasse, & Sinis, 2009). Type I, Type III, and type V
collagens are fibril forming, while Type IV collagens are network-forming. Among the collagens,
Type V collagen has garnered most interest due to its impact on Schwann cell behavior. Together
with laminin, Type V collagen forms a tube like structure that are required for Schwann cell
myelination of axons (Chernousoy, 2006). On the other hand, type IV collagen is a basement
membrane component that likely acts as a support for Schwann cells. Interestingly, some collagens
have an inhibitory effect towards nerve regeneration and likely have a regulatory function. Interestingly, type VI collagen, another network collagen, appear to inhibit Schwann cell myelination of axons. In mouse lacking Type VI collagen, myelin thickness is increased (P.Chen, Cescon, Megighian, &Bonaldo, 2014). Fibronectin is a lesser component of nerve based on quantity. Fibronectin within the peripheral nerve is deposited by endoneural fibroblasts and Schwann cells. However, the role of fibronectin is unclear. Finally, glycosaminoglycans within the peripheral nerve include heparan, keratin, chondroitin, dermatan, and their respective sulfates. In particular, chondroitin sulfate proteoglycans (CSPGs) have received particular attention due to its inhibitory effect on axon outgrowth and regeneration (Zuo, Hernandez, &Muir, 1998). In healthy nerve, CSPGs bind to laminin and reduce cellular binding to laminin to reduce proliferation.

1.3 Injury of peripheral nerves

Unlike the central nervous system, the peripheral nervous system is capable of regeneration (Z.-L.Chen, Yu, &Strickland, 2007). While neurons in the peripheral nervous system (PNS) do not replicate, damaged axons of the neurons in PNS can regenerate and reestablish lost connections with end-organs resulting from the injury. The efficacy of regeneration depends greatly on the type of injury (Seddon, 1943).

Clinical categorization of nerve injury depends on the extent of injury. The Sunderland scale (Fig. 3) is the most commonly used clinical classification. It categorizes nerve injury based on the
anatomy of injury. Type I and II injuries are localized within the endoneurium and have good outcome. Type III and higher injuries are more extensive and require greater surgical intervention.

While the Sunderland scale breaks down clinical injuries into 5 categories, two types of nerve injury are most commonly studied, especially in animal models. The first is compression injury, which is similar to a Type II injury. In this type of injury, the endoneurial tube, perineurium, and epineurium are all intact (Seddon, 1943). Nerve conduction is blocked due to the initial force from the trauma as well as resultant ischemia. When this injury is modeled in rodents by a single crush, function is recovered within weeks to months (Omura et al., 2004). The second type of injury is neurotmesis, or transection injury, in which laceration created by knife or sharp objects create interruption of nerves and the associated connective sheath. Because nerves are naturally under tension, such disruption causes retraction of the nerves proximal to the cut, further lengthening the distance between the proximal and distal stumps, creating greater gaps that axons must traverse to

Figure 3 Clinical classification of nerve injury

The grading of nerve injury is based on the anatomical location and extent of injury. Type I injury is focal demyelination. Type II injury is limited loss of continuity of axons and myelin but the ECM is not disrupted. Type III injury is disruption of axons but loss is limited within the perineurium. In Type IV, only the epineurium is intact while all other part of nerve is disrupted. In Type V, there is complete transection.

While the Sunderland scale breaks down clinical injuries into 5 categories, two types of nerve injury are most commonly studied, especially in animal models. The first is compression injury, which is similar to a Type II injury. In this type of injury, the endoneurial tube, perineurium, and epineurium are all intact (Seddon, 1943). Nerve conduction is blocked due to the initial force from the trauma as well as resultant ischemia. When this injury is modeled in rodents by a single crush, function is recovered within weeks to months (Omura et al., 2004). The second type of injury is neurotmesis, or transection injury, in which laceration created by knife or sharp objects create interruption of nerves and the associated connective sheath. Because nerves are naturally under tension, such disruption causes retraction of the nerves proximal to the cut, further lengthening the distance between the proximal and distal stumps, creating greater gaps that axons must traverse to
reach the distal stump and distal end-organs. Unlike compression injury, transection injuries have very poor regenerative outcomes (GuidoStoll & Müller, 2006).

1.4 Regeneration of injured nerves

Following nerve transection, a cascade of events occurs at all levels of the peripheral nerve (Fig. 3). The composition and dynamics of cells within the nerves are also drastically changed as a result. The diversity of cell types that is involved in the regeneration of injured PNS underscores the complexity of this process. Broadly, the repair mechanism can be broken down into 3 categories based on the timing of regeneration including i) Activation, ii) extension, and iii) remyelination. Within each of the categories, distinct cells are involved in these processes.

1.4.1 Activation
This is the earliest stage following injury. The injury signal triggers a response throughout the injured nerve both proximally and distally, with distinct effects. At the most proximal location, the injury signal triggers transcriptional changes at neuronal cell bodies to activate transcription of regeneration associated genes (RAGs) (Bomze, Bulsara, Iskandar, Caroni, & Skene, 2001; Schmitt et al., 2003; Skene, 1989). These RAG genes, which include BDNF, CCL2 and GAP43, which are repressed during homeostasis, are rapidly upregulated within hours of injury. Translated proteins of RAGs have diverse function. For example, BDNF is a potent growth factor that promote proliferation of glial cells (Lindsay, 1988; Jian-Yi Zhang, Luo, Xian, Liu, & Zhou, 2000). GAP43 is an adaptor protein that brings actin and other signal proteins together to facilitate axonal regeneration (Bomze et al., 2001). CCL2 helps to promote macrophage recruitment both locally
near the neuron cell body, and distally at the site of injury (Kwon et al., 2015; Niemi et al., 2013). Together, translated product of these genes promote neuronal survival following injury and promote a pro-regenerative environment in which axons may regenerate. Interestingly, when these genes are induced to express at supraphysiological level, a further enhancement of regeneration was observed, suggesting a graded response of regeneration to the expression of RAGs (Niemi, DeFrancesco-Lisowitz, Cregg, Howarth, & Zigmond, 2016).

More distally, at the site of injury, glial cells become activated. Schwann cells, the majority of which normally myelinates axons, adopts a unique “repair” phenotype (Arthur-Farraj et al., 2012). Previous work from other groups has demonstrated that both Notch signaling and ERK-mediated signaling induce a repair Schwann cell phenotype. Thus, multiple signals are likely capable of inducing such reversion from mature to immature (Napoli et al., 2012; Woodhoo et al., 2009). Ultimately, these signals must induce the phosphorylation of the regulatory gene C-Jun to turn on the repair-related gene expression in Schwann cells. Following C-Jun phosphorylation, it begins to down-regulate myelination related genes, including EGR2, MBP, PMP2 and MPZ. At the same time, it up-regulates genes such as GDNF, Olig1, and Shh. Activated repair Schwann cells have multiple functions. First, it begins to phagocytose myelin debris around the site of injury. Clearance of this debris will eventually allow axons and Schwann cells to migrate into the site of injury. Secondly, the activated Schwann cells begins to synthesize chemokines and cytokines, such as IL-6 and CCL2, which recruits macrophages and other immune cells which promote regeneration. While hematogenous macrophages are being recruited, resident macrophages within the nerve begin to proliferate (Mueller et al., 2003). Within the peripheral nerve injury, macrophage perform many functions at each temporal sequence. During the early activation stage,
Macrophages are important for phagocytosis of myelin debris (Perry, Brown, & Gordon, 1987; GStoll, Griffin, Li, & Trapp, 1989). In addition to macrophages and Schwann cells, a variety of other cell types have been shown to be important at the early of regeneration as well. Neutrophils and fibroblasts assist in phagocytosis of myelin debris (Lindborg, Mack, & Zigmond, 2017). Circulating myelin specific antibodies, which are produced by B cells, bind to myelin at the site of injury and promote their phagocytosis by macrophages (Vargas, Watanabe, Singh, Robinson, & Barres, 2010). Distal to the injury, axons become fragmented and begin to disintegrate. Disintegration of axons allow them to be cleared to facilitate eventual growth of new axons (Hirata & Kawabuchi, 2002; GStoll et al., 1989). Finally, at the most distal location—target muscle, the axons within the neuromuscular junction begin to disintegrate, and a unique type of Schwann cells—terminal Schwann cells, begin to migrate out of the NMJ in preparation of new innervations from regenerated axons (Koirala, Qiang, & Ko, 2000).

1.4.2 Axon extension

Following activation, axons and Schwann cells begin to migrate into the site of injury. Recent studies have shown that this process is highly regulated, both temporally and spatially. Prior to migration of Schwann cells into the site of injury, it must first be vascularized because Schwann cells migrate poorly in 3-D ECM matrix without proper guidance and support (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Calavia, et al., 2015). The major driver of vascularization is macrophage (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Garcia Calavia, et al., 2015). Macrophages can originate from the circulating monocyte or peripheral nerve resident macrophages. While earliest (<4 days) macrophages are tissue resident macrophages, subsequent macrophages are mostly hematogenously derived (Mueller et al., 2003). Due to the hypoxic nature
of non-vascularized site of injury, macrophages begin expression of HIF-1α. Activation of HIF1-α then leads to the transcription of downstream genes, including VEGF. The release of VEGF recruits endothelial cells into the injured nerve (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Calavia, et al., 2015). Indeed, loss of VEGF impedes angiogenesis within the site of injury. Interestingly however, long term regeneration is not affected with the loss of VEGF in myeloid cells, suggesting that other compensatory mechanism of angiogenesis is available in the absence of macrophage derived VEGF (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Calavia, et al., 2015).

Once blood vessels become fully formed, it allows the migration of glial cells. In addition to the cell surface provided by endothelial cells, other cell intrinsic and extrinsic factors also modulate Schwann cell migration. For example, the kinase Cdc2 is activated following injury and promotes Schwann cell migration both in vivo and in vitro (Han et al., 2007). Loss of Schwann cell surface receptor p75 also impedes Schwann cell migration (Yamauchi, Chan, & Shooter, 2004). Neuronal signals also impact Schwann cell migration. For example, neuronal neuregulin is a powerful signal that triggers Schwann cell proliferation (Li, Tennekoon, Birnbaum, Marchionni, & Rutkowski, 2001; Maurel & Salzer, 2000; Perlin, Lush, Stephens, Piotrowski, & Talbot, 2011). However, it also promotes Schwann cell migration independent of their proliferation (Perlin et al., 2011). This is done through the activation of Schwann cell MEK pathway and its downstream factors. Unfortunately, while many factors have been identified to be important for Schwann cell migration, there is limited information on mechanism by which these specific factors increase Schwann cell migration.
In addition to the temporal regulation of migration, the regeneration of injured nerve is also spatially regulated. It has been long understood that injured nerves form a band of Bungner – a tube like structure in which Schwann cells and other ECM materials bridges the injured nerve together so that axons can traverse the site of injury. Recent evidence shows that nerve fibroblasts is an important player in regulating the migration of Schwann cells. Fibroblasts express EphrinA, a cell surface receptor that repels EphrinB, which Schwann cells express. This allows fibroblasts to repel Schwann cells, while Schwann cells attract each other (Parrinello et al., 2010). Macrophage is also involved in the spatial regulation of Schwann cell migration. The expression of Slit3 on macrophages causes Schwann cells to migrate away from the macrophages (Dun et al., 2019). These push and pull forces promote the sorting of Schwann cells into cords, which in turn organizes the regenerating axon bundles. Indeed, loss of EphrinB or Robo, through genetic deletion or antibody mediated blockade, significantly inhibited nerve regeneration and disrupted organization of regenerating axons. Interestingly, recent research has shown that the interaction of Schwann cells and fibroblasts can be further modulated by other factors. For example, TGF-β enhanced the expression of EphrinB on Schwann cells through induction of N-cadherin. This then leads to increased cell-cell repulsion between fibroblasts and Schwann cells (Clements et al., 2017).

As Schwann cells migrate into the site of injury, local ECM also undergoes remodeling to facilitate this process. Laminin and collagen in particular, are known to play important role in this process. Laminin has long been demonstrated as a critical ECM for nerve regeneration. In vitro studies have demonstrated that neurite extension from dorsal root ganglion (DRG) is greater on laminin compared to collagen, fibronectin or fibrinogen, suggesting that it plays important role in
promoting axonal repair (Deister, Aljabari, & Schmidt, 2007; Pittier, Sauthier, Hubbell, & Hall, 2005). When antibodies to laminin were used to inhibit binding of cells to laminin within a nerve graft, regeneration is severely retarded (Toyota, Carbonetto, & David, 1990). Previous studies have also demonstrated that laminin is needed to induce Schwann cell proliferation. Schwann cells that lose laminin have a defect in proliferation due to loss of G-protein coupled receptor signaling, leading to loss of phosphorylation of ErbB, and reduction in expression of downstream pro-proliferation genes (Yang et al., 2005; Wei-Ming Yu, Feltri, Wrabetz, Strickland, & Chen, 2005). Additionally, loss of laminin was also shown to inhibit Schwann cell myelination due to loss of signaling mediated by Rac1, a downstream signaling molecule related to integrin (W.-M. Yu, Chen, North, & Strickland, 2009). Type I and Type III collagens are secreted by immature Schwann cells and neural fibroblasts following injury. These collagens provide tensile strength, and once nerve has regenerated, collagens continue to provide cellular support.

Following the migration of Schwann cells into the site of injury, they begin to facilitate the regeneration of axon across the injury. Axonal extension is regulated by the receptors on the growth cone of axons. The receptors modulate axonal signaling based on the molecular cues within the environment (Letourneau, Shattuck, Roche, Takeichi, & Lemmon, 1990; Song et al., 1998). Axons use Schwann cell surface and processes to direct its elongation of growth cone (Son & Thompson, 1995). In addition to providing substrate onto which axons can migrate and elongate, Schwann cells also express growth factors such as GDNF and BDNF that promote axonal elongation (Baloh et al., 1998; Bamber et al., 2001). Evidence also suggest that the ability of Schwann cells to promote axonal elongation is type dependent. Schwann cells from sensory nerves express higher level of BDNF and NGF to promote sensory axons, while Schwann cells from
motor nerves express pleiotrophin and GDNF (Höke et al., 2006). In addition to promoting angiogenesis, macrophage is another source of factors that promote axonal elongation. In vitro experiments showed that IL-4 or IFN-γ has limited effect on neuron growth. However, both IL-4 and IFN-γ can promote macrophages to secrete factors that promote axonal regeneration. Interestingly, they promote different patterns of axonal elongation. Macrophages polarized by IFN-γ promote short but arborizing growth while IL-4 polarized macrophages induced longer, unipolar growth pattern (Kristina AKigerl et al., 2009).

1.4.3 Remyelination
As axons regenerate across the site of injury, Schwann cells must then begin to myelinate axons. The regulation of myelination is under tight control by mechanisms within neurons, Schwann cells, and possibly others.

Axonal size has long been shown to be a factor in the initiation of myelination/remyelination (Mayoral, Etxeberria, Shen, & Chan, 2018). Anatomical studies showed that axon diameter of greater than 1 µm is required for myelination (Rushton, 1951). This may reflect either a distinct distribution of axonal signaling for axons of different sizes or concentration of signaling molecules when axons reach critical size. Indeed, larger diameter fibers that are destined for myelination express Nrg1 that are critical for the initiation of myelination. Nrg1 signals to ErbB on the Schwann cells. Loss of Nrg1 severely impacts myelination, and overexpression of Nrg1 increases myelin thickness (Michailov et al., 2004; Taveggia et al., 2005). Appropriate myelination is controlled by proteases which removes neuronal Nrg1 when myelination is not required, such as during early phase of nerve regeneration (Luo et al., 2011). Unlike Nrg1 which is expressed on the
surface of axons, BDNF is released by axons to facilitate myelination. BDNF is synthesized in the neuron cell body and transported anterograde to the site of injury. While the exact mechanism of how BDNF modulate myelination is not yet clear, it exerts its impact partly through the activation of TrkB signaling cascade.

In Schwann cells, one of the major regulator of myelination is gpr126 (Monk et al., 2009; Monk, Oshima, Jörs, Heller, & Talbot, 2011). The ligand of Gpr126 is unclear but collagen and laminin appear to signal to gpr126 (Petersen et al., 2015). In mice, gpr126 is not required for myelination during development, however, loss of gpr126 prevents proper myelination following injury (Mogha et al., 2016).

1.5. Other leukocytes and their role to regeneration

As detailed in the sections above, macrophages and neutrophils play important roles in regeneration of nerve throughout the different stages. While macrophages and neutrophils have been increasingly studied for their role on nerve regeneration, the role of other leukocyte populations are less known. Leukocytes that have been less characterized include basophils, eosinophils and cells of adaptive immunity (T and B cells). Unlike macrophages and neutrophils, latter cells of the innate immune response, including basophils and eosinophils, secrete cytokines and release cytotoxic granules to fight off helminth. Cells of adaptive immunity also respond to injuries at a slower rate. Rather than directly acting on regeneration, cells of adaptive immunity are important for regulation of cells of the innate immunity.
1.5.1 Eosinophils
Eosinophils are leukocytes most commonly associated with allergy and helminth infection. Following their infiltration to site of allergy/infection, they degranulate to release cytotoxic molecules including major basic protein, cationic proteins, and peroxidase. These cytotoxic molecules are important for fighting off infections but can cause tissue damage in the case of allergy (Butterworth & David, 1981; Butterworth, Wassom, Gleich, Loegering, & David, 1979; Gleich, Frigas, Loegering, Wassom, & Steinmuller, 1979).

More recently eosinophils have found extended roles in tissue regeneration and homeostasis. In various models, eosinophils are found to accumulate within the site of injury and have been found to promote tissue regeneration and repair. Much of the beneficial effect of eosinophils stem from their capacity of produce IL-4 (Goh et al., 2013). In fact, eosinophils have been found to be major source of IL-4 in many organ systems (Gessner, Mohrs, & Mohrs, 2005). The ability of eosinophils to express IL-4 does not require an injury trigger or other signaling. Rather, eosinophils express IL-4 even while in circulation (Voehringer, Shinkai, & Locksley, 2004). IL-4 has been shown to be an important signal in the regeneration of tissue, and can modulate function of a variety of cells (see below). In addition to IL-4, eosinophils has also been found to express other neurotropic factors including GDNF, NGF and NT-3 (Kobayashi, Gleich, Butterfield, & Kita, 2002). Indeed, conditioned media of eosinophils promotes neurite extension. Thus, eosinophils may play an underappreciated role in the regeneration of peripheral nerve (Foster et al., 2011).

1.5.2 B cells
B cells, together with T cells, form the body’s adaptive immunity. B cells are the body’s major producer of antibodies. While they normally produce IgM, which have anti-helminth properties,
under activation they can also produce IgGs, which when bind to target antigen, can trigger phagocytosis by macrophages. B cells have been shown to be a minor constituent of cells following peripheral nerve injury. However, they have been shown to be important to promote Wallerian degeneration within the peripheral nerve after nerve injury. Antibodies that are specific to myelin proteins are produced by B cells at homeostasis. These antibodies circulate at low concentration during homeostasis but can bind to damaged myelin. Binding can then trigger myelin phagocytosis by macrophages followed by recruitment of additional macrophages to the site of injury. Indeed, loss of B cells significantly delay nerve repair following crush injury (Vargas et al., 2010). Interestingly, reconstitution of antibody to the myelin protein P0 can partially ameliorate the deficit. On the other hand, reconstitution of antibody to irrelevant epitope have no such effect. Therefore, antibodies specific to myelin proteins secreted by B cells are necessary for optimal regeneration.

1.5.3 T cells
T cells are the other members of the adaptive immunity. T cells can be broadly categorized as cytotoxic T cells, or effector T cells. Cytotoxic T cells, which express CD8, perform immune-surveillance by binding to cells that express MHC I molecules that are not part of “self”. On the other hand, effector T cells regulate inflammation by secreting factors that may either encourage, dampen, or modify inflammation. For example, IFN-γ, secreted by Th1 cells, are known to further drive inflammation. On the other hand, IL-4 secreted by Th2 cells can trigger alternative activation of macrophages. Finally, TGF-β can inhibit macrophage activation.
Traditionally, T cells have mainly been considered as part of cell-based immunity. In such immunity, T cells surveil the cells of the host by binding to their MHC molecules. This then allows T cells to differentiate between self, and non-self. Cells that are deemed non-self are either cells that are foreign to the host, or cells that have been infected or otherwise mutated. This will then trigger T cell activity based on the specific T cell sub-types. CD8+ T cells will release cytotoxic molecules such as perforin, granzyme, and granulysin that triggers cell apoptosis via activation of the caspase enzymes. On the other hand, CD4+ T cells can secrete factors such as IL-4 and IFN-γ which amplify the response of cells of innate immunity such as macrophage. In addition to macrophages, T cells can also modulate the recruitment of other myeloid cells including eosinophils and basophils through IL-5 expression.

In addition to regulating immunity, recent studies also demonstrated the ability of T cells to contribute to regeneration. T cells can contribute to regeneration through a variety of means. They can recruit and polarize macrophages through regulation of IL-4 (K.Sadtler et al., 2016). They can directly secrete factors to promote regeneration and healing (Dombrowski et al., 2017). They can promote an acute inflammatory environment within the site of injury to activate leukocytes (JingZhang et al., 2014). For example, Sadtler et. al found that in the absence of adaptive immunity or CD4 T cells, recruitment of macrophage to muscle injury was reduced. Furthermore, the reduction of macrophage was due to reduced accumulation of CD206+ macrophages. Lack of T cells altered the macrophage phenotype to be more pro-adipose tissue formation rather than ECM deposition (KaitlynSadtler et al., 2016). Walsh et. al, using a spinal cord injury model, found that the lack of IL-4 secreting T cells reduced recovery following spinal cord injury (Walsh et al., 2015). IL-4 from T cells was found to be essential for regeneration and acts directly on the neurons
to promote their axonal elongation and survival. Dombrowski et al. found that T regulatory cells, representing a specific subset of T cells, promotes axonal myelination through its production of CCN3 which stimulate oligodendrocyte maturation (Dombrowski et al., 2017).

While T cells have been shown to be largely beneficial in regeneration of other organs, studies on the role of T cells in peripheral nerve regeneration have found conflicting results. For example, in facial nerve regeneration, it was found that lack of adaptive immunity affected survival of motor neuron following injury (Serpe, Kohm, Huppenbauer, Sanders, & Jones, 1999). Further study demonstrated that CD4, but not CD8 T cells or B cells were responsible for exacerbated neuronal cell death following injury (Serpe, Byram, Sanders, & Jones, 2005; Serpe, Coers, Sanders, & Jones, 2003). Similarly, it was shown that loss of CD4+ T cells led to increased neuronal cell death following the injury of either retinal ganglions (neuronal cell body of the retina nerve) or spinal cord. Furthermore, MHCIId+ T cells support neuronal cell survival in part through its release of IL-4 to promote survival (Walsh et al., 2015). On the other hand, Rag2 knockout mice, which also lacks B and T cells, showed enhanced functional recovery following femoral nerve injury compared to wildtype (Mehanna, Szpotowicz, Schachner, & Jakovcevski, 2014). Yet another group found that Rag1 knockout mice that lack B and T cells have early jump start in its regeneration of crushed sciatic nerve (Bombeiro et al., 2016). Some of the discrepancies observed in these studies may be attributed to systemic compensation in mice without adaptive immunity. For example, mice with B and T cells have higher level of circulating macrophage/monocytes, which are known to promote regeneration. An early jump start in regeneration may therefore be attributed to the higher level of macrophages found in these mice.
In conclusion, the inflammatory response to nerve regeneration is intricately coordinated (Fig. 4). There is early and rapid influx of neutrophil that participates in myelin clearance. Subsequently, macrophages and T cells are recruited. Macrophage in particular has diverse functions in peripheral nerve regeneration. On the other hand, the role of T cells in nerve regeneration is less known.

### 1.6 Cytokines and their impact on regeneration

One of the way leukocytes may regulate nerve regeneration is through their secretion of cytokines, which in turn can trigger signaling transduction in variety of cells including neurons, Schwann cells, endothelial cells or others. The same cytokines can originate from variety of cell sources and may have multiple cell targets. Below, I have highlighted IL-4, IL-10, and IFN-γ, three cytokines that are associated with inflammation and regeneration. IL-4 is a classic Th2 cytokine, while IFN-γ is a classic Th1 cytokine. IL-10 is an anti-inflammatory cytokine that is not part of the Th1/Th2 classification.
1.61 IL-4

IL-4 is mainly produced by Th2 cells, basophils and eosinophils. Classically, IL-4 is important for defense against helminth by recruiting anti-helminth granulocytes such as eosinophils and basophils. One way that IL-4 exert its effect on regeneration is through its signaling in macrophages. Macrophages have been found to be critical for regeneration (see above). IL-4 potently stimulates macrophages to proliferate (Jenkins et al., 2013). This increases macrophage accumulation within tissue, which can promote regeneration. IL-4 also modulate the gene expression of macrophages which can have beneficial effect on regeneration (Italiani & Boraschi, 2014; Rőszer, 2015). In vitro studies showed that IL-4 primed macrophages can potently stimulate axonal extension of neurons (Kristina AKigerl et al., 2009). Similarly, IL-4 primed macrophages have increased potency to promote Schwann cell migration. In vivo studies have shown that exogenous IL-4 can promote axonal extension and Schwann cell migration though its effect on macrophages (Mokarram, Merchant, Mukhatyar, Patel, & Bellamkonda, 2012). IL-4 has also been shown to have a direct impact cells other than leukocytes. For example, recent evidence showed that IL-4 can promote calcium influx within neurons which promotes axonal survival and neurite extension (Vogelaar et al., 2018). Finally, IL-4 can also regulate Schwann cell phagocytic activity by upregulating CD209, which is important for phagocytosis (Teles et al., 2010). Additional evidence exist in other organ systems. Following liver injury, IL-4 from eosinophils promote proliferation of hepatocytes to facilitate tissue regeneration (Goh et al., 2013). In the muscle, eosinophil derived IL-4 promotes the maturation of progenitor cells into muscle cells (Horsley, Jansen, Mills, & Pavlath, 2003). While IL-4 has beneficial effect in promoting neuron survival and regeneration in the CNS, how IL-4 can impact PNS regeneration in vivo is not certain. Exogenous levels of IL-4 have been found to be beneficial to regeneration by promoting an M2 macrophage phenotype which can promote Schwann cell migration (Mokarram et al., 2012). On the other hand,
loss of IL-4 receptor had negligible effect on regeneration of nerve through silicone tube (Tomlinson, Žygelytė, Grenier, Edwards, & Cheetham, 2018). However, loss of function studies on the effect of IL-4R has been limited to the final extent of axon elongation. Other aspects of regeneration, such as myelination, have yet to be investigated.

1.62 IL-10
Within T cell population, T regulatory cells are the main source of IL-10. However, IL-10 can be produced by other cell types including macrophages and dendritic cells. Within the peripheral nerve, IL-10 can also be produced by Schwann cells, and macrophages (Taskinen, H. S., Olsson, T., Bucht, A., Khademi, M., Svelander, L., & Röyttä, M. (2000). The most prominent target of IL-10 is macrophage. It is classically associated with downregulation of inflammation through inhibition of macrophage proliferation, and altering its activation in part by down-regulating glycolysis to turn down reactive oxygen species production (Taskinen et al., 2000). Indeed, loss of IL-10 is often associated with unchecked inflammation and tissue injury. Within the peripheral nerve, IL-10 also plays a role in modulating macrophage phenotype. Nerve infiltrating macrophages play an important role in phagocytosing myelin. Interestingly, myelin phagocytosis also changes macrophage phenotype to become more anti-inflammatory. In IL-10 knockout mice however, the phenotype switching is blunted, and these mice express higher level of inflammatory cytokines including IL-10, CCL2, CCL5 and TNF-α throughout the course of nerve regeneration, leading to poor regeneration (Taskinen et al., 2000).
1.63 IFN-γ

Th1 cells are a major source of INF-γ. However, nerve injury may also trigger INF-γ production in fibroblasts. Classically, INF-γ modulates macrophage to upregulate its classical inflammation related genes such as iNOS, which mediates production of reactive oxygen species. Activated macrophages are important for defense against tissue insults from pathogens. However, such reactive oxygen species can also induce tissue damage. Surprisingly, in vitro studies using macrophage and INF-γ demonstrated that activated macrophages did not inhibit neurite extension, instead, it triggered neurite branching (K. A. Kigerl et al., 2009). Similar studies showed that INF-γ activated macrophages also increased Schwann cell proliferation in vitro. In vivo study using exogenous INF-γ found that it inhibited axonal regeneration and Schwann cell migration (Mokarram et al., 2012). However, study in which INF-γ receptors are depleted from the macrophages did not show changes in nerve regeneration following transection and repair with silicone conduits (Tomlinson et al., 2018). Indeed, studies in CNS showed that INF-γ may even have neuroprotective role (Carlson et al., 1999; Fontaine et al., 2002). Thus, the role of IFN-γ in nerve regeneration is so far unclear. One reason for the divergent impact of IFN-γ may due to its dose dependence. Exogenously added IFN-γ may be supraphysiologial, and thus produce impacts that are not observed at more physiological doses.

1.7. Repair of nerve gaps

As just described, following a nerve transection injury, the cellular response can be adequate to allow for axon growth from the proximal nerve end to the distal nerve end for short distances. However, for most nerve transection injuries, additional tissue damage and scarring prevents this
cellular response without surgical intervention. It is not surprising then, that surgical repair of peripheral nerves has been extensively studied.

Early strategies of repairing PNI relied on suturing together the proximal and distal stumps of the peripheral nerve. However, for larger nerve gaps, such techniques place undue tension on the nerve and results in poor regeneration with a high level of intraneural fibrosis (Ijkema-Paassen, Jansen, Gramsbergen, & Meek, 2004). The current gold standard (which has remained for the past 50 years) for reconstructing nerve injuries relies on using autografts – autologous nerves taken from the patient – to replace injured nerves. Generally, sensory donor nerves are used as grafts for the repair. However, autografts are limited by the availability of suitable nerves. Furthermore, harvest of donor nerves leads to loss of sensation at the donor site and can lead to complications including infection and the formation of a painful neuroma. In addition, it has been previously shown that sensory nerves are ill-suited for repair of motor nerves due to mismatch of axon size and difference between the phenotype of Schwann cells within the sensory and motor nerves (Scheib & Höke, 2013). Currently, autografts are used to bridge nerve defects of up to 6 cm. For larger defects, allografts of motor nerves from a donor is typically used. However, motor allograft often requires the use of immunosuppressants following repair, and come with its associated side effects such as opportunistic infections, and potentially cancer due to loss of immune surveillance.

Due to the disadvantages of autografts and allografts, there has been a long-standing interest in developing bioengineered substitutes for autografts. In fact, as early as 1950, some authors have proposed utilizing scaffolds to promote nerve regeneration. For example, Danny-Brown utilized epineurium of distal nerve stump as a scaffold to bridge nerve gaps in a cat ulnar nerve injury.
model (Denny-Brown, 1946). Three months following the surgery, axons were seen sprouting into the distal stump, demonstrating that a scaffold alone is sufficient to allow nerve regeneration. This strategy has recently been re-discovered and continues to be investigated (Karacaoğlu, Yüksel, Peker, & Güler, 2001). With the rising prominence of biomaterials, there is now increasing options for bioengineered nerve grafts. Modern day substitutes for autografts include cadaver allografts, synthetic nerve conduits, and acellular nerve allografts (ANAs).

Synthetic nerve conduits are nerve grafts fabricated using biomaterials. As one of the earliest clinically utilized nerve conduits, poly glycolic-acid tubes were used to repair peripheral nerve injury in patients (Dellon & Mackinnon, 1988). The emergence of the second generation of engineered nerve grafts followed the realization that biodegradability is critical to their design. Premature degradation of nerve grafts prior to migration of axons into grafts prevents the formation of conducive regenerative environment (Sundback et al., 2005). Delayed degradation can cause nerve irritation and compression, which inhibits nerve growth and can cause pain. More recently, there has been greater interest in using extracellular matrix or its individual components as the material for these grafts. Purified ECM materials such as hyaluronan, collagen or fibrin have been used to form conduits (Ahmed, Dare, & Hincke, 2008; Ceballos et al., 1999; Seckel et al., 1995). These materials have advantages over the traditional polymeric material due to their greater biocompatibility and lower toxicity, as well as their ability to promote proliferation and migration of Schwann cells, which are important for nerve regeneration. Others have co-opted them to function synergistically with polymer conduits by injecting them as gels (Wood et al., 2009). Additional strategies includes changing the microstructure within the nerve grafts (Kim, Haftel, Kumar, & Bellamkonda, 2008), inclusion of growth factors or drugs (Abidian, Kim, & Martin, 2006;
Acellular nerve allografts (ANAs) are grafts derived from peripheral nerves of allogeneic donors, without the associated cellular components. In ANAs, isolated nerves (typically taken from cadavers) have been subjected to decellularization processes to remove cells and other possibly immunogenic components. The post-processing scaffold contains the total peripheral nerve matrix and structure which then acts as the scaffold for repopulation of cells (Sondell, Lundborg, & Kanje, 1998). Decellularization can be done with 1) freezing, 2) hyper or hypotonicity, 3) detergents or a mix of above. Work from the lab of Susan Mackinnon as well as others have exhaustively characterized the methods of decellularization, and currently, a combinatorial approach that includes detergent treatment with hypotonicity has been shown to give rise to ANAs with highest regenerative potential (Hudson, Zawko, et al., 2004; Hudson, Liu, & Schmidt, 2004). The loss of allogenic cellular substrates results in lower immunogenicity and eliminated the need for immunosuppression. ANAs are emerging as a popular alternative to autografts due to their ease of manufacturing and more robust regenerative potential compared to other products such as empty conduits. Recently, the FDA has approved ANAs for the repair of peripheral injury, further asserting the clinical importance of ANAs. Unfortunately, ANAs also have limited regenerative capacity. Specifically, in rat sciatic nerve injury, regeneration in which the distance between the proximal and distal stumps is equal or greater than 3 cm is limited for ANAs. As the length of the
nerve graft or construct increases, axonal regeneration decreases until few or no axons reach the distal nerve.

1.9. Factors affecting nerve regeneration

While peripheral nerve can be very efficient in regeneration following injury, suboptimal recovery following repair is still a significant problem. There are many factors contributing to poor regeneration. However, the most clinically relevant factors include age of patients, quality of repair, and delay in treatment. Pertinent to my thesis topic, the size of injury is a major factor and the focus of my thesis.

1.91 Age
Aging has been shown to be a factor that have wide range of effects on a variety of health metrics. Regeneration in particular, has long been shown to be worse with age (Ballard & Edelberg, 2007; Sadeh, 1988; Timchenko, 2009). Poor regeneration of peripheral nerve following injury has been well documented in mice, rats, and human. This has been hypothesized to be caused by decline in the responsiveness of immune systems, as well as changes in the reparative phenotype of Schwann cells (Komiyama & Suzuki, 1991, 1992). Axons from aged animals regenerate in a more tortuous manner, indicative of altered regenerative environment within sites of injury. However, the transcriptions of neurons themselves are not altered in aged animals. Rather, studies have shown that Schwann cells alter their transcription of injury related genes (Painter et al., 2014). This led to delayed activation of Schwann cells. Macrophages are early responders to injury, and in older animals and humans, macrophages showed reduced ability infiltrate to the site of injury in an
appropriate manner. For example, they overactivate in nerves that are not under injury, and have delayed activation when injuries actually occur (Yuan et al., 2018).

1.92 Delay in treatment
While nerve pair in most peripheral nerve injury model in the lab occurs immediately after, presentation of nerve injury in the clinic often follows a more delayed course. Often, patients may present to the clinic up to months after the initial nerve injury. Unfortunately, delays in the treatment of injury is also associated with adverse outcomes. Research has shown that changes in the distal stump following prolonged injury is the cause for the impaired regeneration (Gordon, Tyreman, & Raji, 2011). For example, Schwann cells in the distal stump may reduce their ability to support axonal regeneration after long term denervation. Fibrosis also occurs in the distal stump due to the increase in the proportion of fibroblasts and reduced Schwann cells. The increased collagen deposition due to fibrosis reduces area for which axons can grow across. More distally, NMJs become less receptive for reinnervation of axons, leading to reduced functional recovery (Fischbach & Robbins, 1971).

1.93 Size of injury
Despite its significant capacity for regeneration, peripheral nerve injury with large size has long confronted clinicians for its difficulty to heal. Such “critical size” effect is also seen in other organ systems such as muscle, skin, and bone. When autografts were used for repair, there is no consensus on the maximum gap that can be bridged, and in fact, widely varying degrees of success have reported in autografts up to 200 mm (Lenoble et al., 1989). However, there is a general consensus that any regenerative success declines as autografts go beyond 60 mm (Mackinnon
As an example, meaningful sensory recovery from a digital nerve defect repair using autografts was observed in 100% of patients with defect lengths less than 21 mm, while this recovery rate fell to 67% for lengths between 21 and 49 mm and only 9% for lengths greater than 49 mm (Kallio, 1993). This relationship regarding defect length and recovery rates has also been observed for conduits.

While the issue of critical gap of ANAs has been reported in animal models, current clinical data suggest that length up to 6 cm can be effective (Rinker et al., 2017). There is also limited understanding on why they fail to regenerate. However, conduits have significantly smaller critical gap length than autografts. The difference in critical size gap likely originate from the unique ways different nerve grafts facilitate regeneration. For example, ANAs and conduits require repopulation by Schwann cells, while autografts do not. Changes in Schwann cell phenotype as they migrate may be one of the factors which limits ANAs at greater extent than autografts. Indeed, recent finding shows that Schwann cells within long ANAs that regenerate poorly can display an altered phenotype characterized by reduced proliferation, and increased expression of protein p16, consistent with senescence (Saheb-Al-Zamani et al., 2013). While Schwann cells in poorly regenerated long ANAs have high level of senescence, the Schwann cells in nerves repaired with autografts/isografts or short ANAs that promote good regeneration do not (Poppler et al., 2016; Saheb-Al-Zamani et al., 2013). In senescence, cells stop replication and instead adopt a unique secretory phenotype that express cytokines, chemokines and proteases (Rodier & Campisi, 2011). Interestingly, when long ANAs that poorly support regeneration is replaced with autografts with a second surgery, or when autografts that sandwiched between long ANAs, regeneration improved. This suggest that local environment within long ANAs, including Schwann cells with senescence
phenotype is likely the major cause of poor regeneration. Indeed, when DRG neurons from poorly regenerated nerves were grown in vitro, they extend axons normally. Thus, the local environment of ANAs, rather than neurons are likely the culpable reason for poor regeneration (Poppler et al., 2016). Altered Schwann cell phenotype represent one such changes in the local environment, but there may also be other changes. For example, angiogenesis may be impaired or delayed in critical sized defects, leading to poor repopulation of Schwann cells within nerve grafts (Carlier, vanGastel, Geris, Carmeliet, &VanOosterwyck, 2014).

1.10 Ongoing experimental strategies to design alternatives to overcome critical gap length

1.10.1 Scaffolding and topology

The use of synthetic conduits containing internal scaffolding, which has included ECM materials, such as collagen, laminin, and fibrin, has demonstrated advantages in improving regeneration compared to empty conduits (Madison, daSilva, Dikkes, Sidman, &Chiu, 1987; Wood et al., 2010). However, it is unlikely these internal scaffolds could promote regeneration that surpasses acellular nerve. These scaffolds develop with a random arrangement of the molecular fibers, which differs significantly from the organized and longitudinal arranged structure of nerve. Instead, recent advances that arrange the topology of the scaffold fibers holds promise, and the most interesting developments are the use of scaffolds with a longitudinal organization. This scaffolding can be achieved through a variety of means, including through the use of electromagnetic fields and photolithography, but electrospinning techniques are becoming increasingly well-developed for this approach (Mu, Wu, Lu, Wei, &Yuan, 2014; Xie, MacEwan,
Schwartz, & Xia, 2010). These techniques allow for aligned fibers, synthetic or “naturally-derived”, to be deposited resulting in longitudinally oriented pores or channels as small as 1 µm, which are comparable to endoneurial tubes, ranging from 1-20 µm. These designs allow for scaffolds that not only more closely mimic nerve’s structure but could lead to more rapid cell migration and polarization of cells. As endothelial cell polarization is important for both SC migration and then axon outgrowth (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Calavia, et al., 2015), this could lead to a major translational development where these conduits match the regenerative outcomes of more “naturally-derived” alternatives, such as acellular nerve. However, the use of synthetic fibers and/or conduits also holds an advantage whereby drugs for improving regeneration (detailed in the next section) could be readily incorporated into the underlying scaffold.

1.10.2 Local drug delivery
The delivery of biologically active molecules locally during regeneration is another strategy that has been extensively pursued for several decades. Nerve scaffolds or the conduit itself can be incorporated with bioactive molecules via chemical interactions, via crosslinking or affinity-based interactions, or physically encapsulated. While chemical interactions can provide drugs immediately to cells or as cells proceed to grow within a scaffold, physical encapsulation of drugs provides a greater range of delivery options, such as long-term or sustained release. Numerous molecules or drugs have been delivered to nerve for regeneration across a nerve defect, but the most researched category for delivery have been neurotrophic factors (Kemp, Walsh, & Midha, 2008; Sarker, Saman, McInnes, Schreyer, & Xiongbiao, 2018). Of the neurotrophic factors, glial cell line-derived derived neurotrophic factor (GDNF) is a promising example that promotes
regeneration because it targets both axons and SCs. Both sensory and motor neurons express receptors for GDNF (Ret/GFRα1), where GDNF signaling promotes axon outgrowth and neuronal survival (Gavazzi, Kumar, McMahon, & Cohen, 1999; Matheson et al., 1997; Trupp et al., 1995). In addition, SCs also express receptors for GDNF (NCAM/GFRα1), where GDNF signaling activates pathways in SCs implicated in cell migration, differentiation, and trophic factor production (Iwase, Jung, Bae, Zhang, & Soliven, 2005). Exogenous GDNF delivered to nerve has been shown to improve not only axon regeneration but functional recovery (Kokai, Bourbeau, Weber, McAtee, & Marra, 2011; Wood et al., 2010).

More recently, the local delivery of drugs that accelerate axon outgrowth have been investigated. FK-506, an immunosuppressive drug, enhances nerve regeneration, as it increases axon growth rate and functional recovery in animal models (Gold, Katoh, & Storm-Dickerson, 1995; Gold, Storm-Dickerson, & Austin, 1994). Approaches have been developed to provide FK506 locally to enhance axon regeneration given its abilities to stimulate more rapid axon outgrowth (Labroo et al., 2016; Tajdaran, Chan, Shoichet, Gordon, & Borschel, 2019; Tajdaran, Chan, Zhang, Gordon, & Borschel, 2019). This reduces the potential for any systemic toxicity from its immunosuppression. Furthermore, while this delivery strategy has demonstrated its potential to improve nerve regeneration in animal models, the sustained local delivery of FK506 from a nerve graft alternative bridging a nerve defect could translate to benefits even after axons cross the bridged region, as it could continue to stimulate accelerated axon growth through the distal nerve.

Alternatively, based on our increasing knowledge of the immune response during regeneration across nerve defects, recruiting immune cells, such as macrophages, is a promising strategy. While
angiogenic factors, such as vascular endothelial growth factor (VEGF) have been locally delivered from conduits to promote improved angiogenesis within bridged nerve gap, tuning the local immune system to recruit cells to promote endogenous angiogenesis could have advantages, such as a greater degree of endothelial cell polarization (Hoben et al., 2015; Hobson, Green, & Terenghi, 2000). Furthermore, the immune system has a critical role in resolving inflammation following injury, where macrophages also have additional roles in this aspect of tissue regeneration. Macrophages alter their secreted cytokines based on their phenotype. While a simplification, macrophages are broadly classified as classically activated (M1) or alternatively activated (M2) phenotypes. The M1 macrophage response predominates during the onset of injury while the M2 polarization or subtypes generally promotes healing, remodeling, and resolution of regeneration (Mills, 2012; Rószer, 2015). Studies using conduits releasing either factors promoting a more M1-like phenotype (IFNγ) versus a more M2-like phenotype (IL-4, collagen VI, or fractalkine) have demonstrated improved nerve regeneration when factors promoting a greater accumulation of M2-like phenotype macrophages were used to repair a nerve defect compared to conduits lacking this ability (Mokarram et al., 2017, 2012).

### 1.10.3 Supplementing cells

To match and possibly even surpass the outcomes of the autograft will likely entail the development of alternative that includes a cellular component. SCs and stem cells have been shown to be the most promising sources for this supplementation, as their supplementation to nerve graft alternatives bridging defects in animals has been shown to be advantageous. However, several challenges remain toward translation that include improving the survival of transplanted cells and determining the best choice of cells. Supplementing nerve graft alternatives, such as ANAs, with
SCs has been shown to result in very low survival of the transplanted cells, which can be as low as 2% of transplanted cells surviving up to 7 days (Gambhir et al., 2016; Jesuraj et al., 2011). While SCs in culture have also been shown to not transform or reach a proliferative limit that would alter their utility for later transplantation (Mathon, Malcolm, Harrisingh, Cheng, & Lloyd, 2001), the difficulty in SC isolation and their culture has significantly impeded clinical translation despite decades of concerted interest and effort (Gambhir et al., 2016; Ogden et al., 2000). Instead, stem cell transplantation seems a more promising avenue as these cells can differentiate into multiple cell types and self-renew in culture. While a more extensive review of stem cells used for nerve graft alternatives can be found in Johnson et al. (Johnson, Wood, Moore, & Mackinnon, 2013), recent work from Shin and colleagues has progressed the field further. They developed an approach to seed mesenchymal stromal (stem) cells upon ANAs with improved viability and greater reproducibility. A bioreactor was used to seed the mesenchymal stromal cells onto ANA, whereby this technique has led to cells surviving up to 29 days (Rbia, Bulstra, Bishop, van Wijnen, & Shin, 2018). Overall, supplementation of cells to nerve graft alternatives seems an inevitable translational advance once the processes to reliably seed these cells to the alternatives are robust.

1.11 Concluding remarks

There has been much discovery on the fundamental basics of nerve regeneration. However, the limited ability of ANAs to facilitate regeneration of large gap injuries represent significant clinical challenge. While many strategies have been proposed and tested to overcome the “critical length” barrier, there is limited understanding of the mechanisms that underpinn the size limitation of regeneration. Understanding the specific deficits in regeneration across long gaps can further
inform rational design and development of therapies. Previous studies have found altered Schwann cells likely contributed to poor regeneration in long ANAs. However, other changes likely predated the changes in Schwann cell function. Inflammatory response has been found to pre-date the migration of Schwann cells, and could be one of the factors that is altered in poor regeneration across critical gaps (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Calavia, et al., 2015). Among leukocytes, T cells are especially understudied because of our limited understanding on the role of T cells in nerve regeneration. However, T cells may be especially important for regeneration through acellular tissue scaffolds due to their ability to modulate leukocytes, which is critical for scaffold regeneration. These data suggest that T cells may be important in promoting regeneration across ANAs.

Thus, it is likely that inflammation, especially T cell associated inflammation may be a factor that is altered in the regeneration across long ANAs. Experiments are needed to identify if these factors are present. Furthermore, mechanistic experiments are needed to determine if the absent factors indeed contribute to the defective regeneration in long ANAs.
Chapter 2 The accumulation of T cells within acellular nerve allografts is length-dependent and critical for nerve regeneration

The content of this chapter has been published under the title “The accumulation of T cells within acellular nerve allografts is length-dependent and critical for nerve regeneration”, at Experimental neurology, 318, 216-231., DOI: 10.1016/j.expneurol.2019.05.009
2.1 Abstract

Repair of traumatic peripheral nerve injuries can require graft material to bridge a nerve defect. While autologous nerve grafts remain the gold standard, the use of alternatives, such as acellular nerve allografts (ANAs), is common. Although ANAs support axon regeneration across short nerve defects (<3cm), axon regeneration across longer defects (>3cm) is limited. It is unclear why alternatives, including ANAs, are functionally limited by length. After repairing Lewis rat nerve defects using short (2 cm) or long (4 cm) ANAs, we showed that, consistent with previous data, long ANAs have severely reduced axon regeneration across the grafts, and contain Schwann cells with a unique phenotype. Additionally, we found that long ANAs have disrupted angiogenesis and altered leukocyte infiltration compared to short ANAs as early as 2 weeks after repair. In particular, long ANAs contain fewer T cells compared to short ANAs. These outcomes are accompanied with reduced expression of inflammatory and anti-inflammatory cytokines, including IFN-γ and IL-4, within long versus short ANAs. While T cells within ANAs do not express IL-4, they express elevated levels of IFN-γ. We then assessed the contribution of T cells to regeneration across nerve grafts using athymic rats. Interestingly, T cell deficiency had minimal impact on axon regeneration across nerve defects repaired using autografts. Conversely, T cell deficiency reduced axon regeneration across nerve defects repaired using ANAs. Our data suggest that a factor responsible for limited axon regeneration across long ANAs is due to reduced T cell accumulation.

Keywords: Regeneration, T cells, peripheral nerve, acellular nerve allograft, Schwann cells
2.2 Introduction

Nerve injuries continue to be a significant public health concern, with more than 300,000 patients requiring nerve surgery annually (Jaquet et al., 2001; Taylor, Braza, Rice, & Dillingham, 2008). Severe traumatic injuries frequently require surgical intervention and may generate a significant tissue defect or gap between nerve ends. In these instances, a material is often necessary to bridge the nerve gap to facilitate axon growth to the distal nerve end. Autologous nerve grafts are widely considered the gold standard for surgical repair of nerve injuries resulting in a gap (Ijkema-Paassen et al., 2004). However, the use of autologous nerve grafts requires the harvest of donor nerves from patients themselves, which is undesirable. Thus, there is an unmet clinical need for “off-the-shelf” alternatives to autografts. A clinically-available alternative is the acellular nerve allograft (ANA), which provides an extracellular matrix (ECM) scaffold with structural elements similar to native nerve. During regeneration, cells repopulate and remodel the scaffold to promote axon regeneration. While ANAs have been increasingly used for the repair of short gap or small diameter nerve injuries with results often comparable to autologous nerve grafts (Isaacs and Browne, 2014; Zhu et al., 2017), the repair of long gap or large diameter nerve injuries with these alternatives is still tenuous as regeneration across these longer or larger alternatives can fail.

Previous animal and clinical studies have shown that repair of nerve gaps using long ANAs (>3 cm) can result in limited axon regeneration and functional recovery (Poppler et al., 2016; Saheb-Al-Zamani et al., 2013; Xeroulis et al., 2007; S.Zhu et al., 2017). This length-dependent decline in regeneration across autograft alternatives is not limited to ANAs. Graft length limitations exist for collagen conduits and silicone tubes (Mokarram et al., 2012; Whitlock et al., 2009). These results suggest that there is an intrinsic limitation in regeneration
across long or large, cell-free scaffolds. Specifically, our previous studies have demonstrated that failure to regenerate across long nerve gaps bridged by acellular alternatives (like ANAs) is due to how cells repopulate ANAs rather than the intrinsic failure of motor and sensory neurons to regenerate their axons across long distances (Poppler et al., 2016).

While our previous study suggests that altered Schwann cell functions or phenotypes could be causal to limited axon regeneration across long ANAs, there are a variety of cells that repopulate ANAs in addition to Schwann cells. Leukocytes, such as macrophages, are some of the first cells to respond to tissue injury and play a critical role in facilitating tissue regeneration in acellular scaffolds. The disruption of leukocyte repopulation or functions can impact regeneration. Specifically, the dysfunction of macrophages or T cells has been implicated in poor regeneration of skin, heart, and central nervous system tissues (Hesketh, Sahin, West, &Murray, 2017; Lavine et al., 2014; Loots et al., 1998; Sindrilaru et al., 2011).

Macrophages are required for proper angiogenesis following injury. The depletion of macrophages in the context of peripheral nerve injury can lead to deficient angiogenesis and poor Schwann cell migration (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Calavia, et al., 2015). Additionally, recent studies demonstrate that macrophages play a role in modulating Schwann cell functions (Stratton et al., 2018). Other studies have found that nerve repaired with a conduit containing a greater proportion of macrophages with CD206 expression is associated with better regenerative outcomes, suggesting macrophage phenotype can impact nerve regeneration through cell-free scaffolds (Mokarram et al., 2017, 2012). While these data suggest that macrophages play important roles in nerve regeneration, limited data are available regarding how macrophage functions might differ based on scaffold length.
Similar to macrophages, T cells are critical for regeneration in many organ systems (Burzyn et al., 2013; Epelman, Liu, & Mann, 2015; Walsh et al., 2015; Jing Zhang et al., 2014). In general, T cells can modulate regeneration through a variety of mechanisms including secretion of cytokines, release of growth factors, or direct cell-cell interaction (Burzyn et al., 2013; Ishii et al., 2012; Pull, Doherty, Mills, Gordon, & Stappenbeck, 2005). However, the role of T cells in peripheral nerve regeneration is less established. Interestingly, T cell deficiency during nerve regeneration following a recoverable nerve crush can actually enhance early axon regeneration but then delay long term recovery (Bombeiro et al., 2016). Furthermore, the majority of these studies have not yet considered the role of T cells during nerve regeneration across a scaffold, such as during nerve defect repair. In this scenario, there is a high demand for cell migration and repopulation, including extensive angiogenesis, to bridge the scaffold and facilitate axon regeneration. As an example of T cells’ role in this context, T cells were critical to robust muscle regeneration when acellular scaffolds were used to repair muscle defects (Kaitlyn Sadtler et al., 2016). Therefore, T cells may play a more prominent role in nerve regeneration across acellular scaffolds, such as ANAs.

In this study, we focused on determining regenerative differences between ANAs based on their length. We specifically focused on leukocytes (i.e. macrophages and T cells) repopulating these ANAs, where we uncovered novel differences in adaptive immunity responses between ANAs based on length. Furthermore, from these findings we confirmed a role for T cells in mediating regeneration across ANAs.
2.3 Materials and Methods

Reagents and Chemicals

All reagents, consumables, and chemicals, unless otherwise stated, are purchased from Sigma-Aldrich (St. Louis, MO)

Surgical procedures

Adult rats (200-250g, Charles River Laboratories, Wilmington, MA) were randomized to groups destined for either donor (graft) or experimental treatments. Surgical procedures and peri-operative care measures were conducted in compliance with the AAALAC accredited Washington University Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health guidelines. All animals were housed in a central animal care facility and provided with food (PicoLab rodent diet 20, Purina Mills Nutrition International, St. Louis, MO) and water ad libitum.

For procedures, rats were anesthetized using a cocktail of ketamine (75 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and dexmedetomidine (0.5 mg/kg; Pfizer Animal Health, Exton, PA). For rats serving as graft donors, following euthanization, their sciatic nerves were transected at the level of exiting nerve roots to the cruciate ligaments. In experimental rats, the right sciatic nerve was exposed and transected 5 mm proximal to the distal trifurcation, sparing the sural nerve. The indicated grafts were reversed (distal end of donor graft facing proximal nerve stump of recipient) sutured into the nerve gap with 9-0 nylon microsuture (Sharpoint,
Reading, PA). A two-layer closure of muscle and skin was performed using 6-0 vicryl and 4-0 nylon suture, respectively. Atipamezole solution (0.1mg/kg; Zoetis, Florham Park, NJ) was administered for anesthesia reversal. The animals were recovered on a warming pad and monitored for postoperative complications before returning them to a central animal care facility. Postoperative pain was managed using Buprenorphine SR™ (0.05 mg/kg; ZooPharm, Windsor, CO). Animals were monitored daily post-operatively for signs of infection and/or distress. At the appropriate endpoints, animals were euthanized with injection of Somnasol (150 mg/kg; Delmarva Laboratories) and their nerves collected for respective studies (see below).

ANA processing

ANAs were decellularized using a modified series of detergents in the method described previously (Poppler et al., 2016). Nerves isolated from donor animals the nerves were repeatedly washed in deionized water and three detergents in a sodium phosphate buffer: Triton X-100, sulfobetaine-16 (SB-16), and sulfobetaine-10 (SB-10). All grafts were washed and stored in 10 mM phosphate-buffered 50 mM sodium solution at 4°C and used within 3 days.

Sciatic function index

Starting from 6 weeks after surgery, rats underwent walking track assessment to determine their functional recovery. Rat hind feet were glazed with water-soluble, non-toxic paint before the animal was placed on a plexiglass track lined with construction paper. Each animal walked down the track until at least 3 pairs of footprints with clear markings were
obtained. On each footprint, the following three parameters were measured using a digital caliper: external toe spread, measuring the distance between the first and fifth toes on the experimental (ETS) and contralateral sides (NTS); intermediary toe spread, measuring the distance between the second and fourth toes on the experimental (EIT) and contralateral sides (NIT); and print length, measuring the distance of the print from the distal end of the third toe to the heel on the experimental (EPL) and contralateral sides (NPL). These variables were used to calculate the tibial functional index (TFI) using the following formula, developed by De Medinaceli and modified by Bain:

\[
SFI = -38.3 \frac{(EPL - NPL)}{NPL} + 109.5 \frac{(ETS - NTS)}{NTS} + 13.3 \frac{(EIT - NIT)}{NIT} - 8.8
\]

Histology and histomorphometry

Histology and histomorphometric analysis of nerve were performed as previously described (Hunter et al., 2007). Briefly, tissue was collected and fixed via immersion in 3% glutaraldehyde (Polysciences, Inc., Warrington, PA), then post-fixed in 1% osmium tetroxide, and serially dehydrated. These tissues were embedded in epoxy resin (Polysciences) and sectioned on an ultramicrotome for 1.5 µm cross sections. Slides were then counterstained with 1% toluidine blue dye and analyzed at 1000x overall magnification on a Leitz Laborlux S microscope. A semi-automated digital image-analysis system linked to morphometry macros developed for peripheral nerve analysis (Clemex Vision Professional, Clemex
Technologies, Longueuil, Québec), was used. Six random fields per histological section were imaged by person blinded to the groupings of the experiments. Binary histomorphometry analysis of the digitized information based on gray and white scales allowed measurements of total fascicular area and total fiber number in the relevant nerve sections. Further counting of myelinated motor fibers across multiple randomly selected fields per nerve permitted calculation of nerve fiber density (fibers/mm²), total number of myelinated fibers, distribution of myelinated fiber width (μm²), percent myelinated fiber and percent fiber debris. These numbers were averaged across the six images to obtain counts for a single animal (n=1). Total average across all animals in the group were presented.

Relative Muscle Mass

After nerve harvest, the gastrocnemius muscles were harvested from the experimental and contralateral sides. Wet muscle weight was recorded on each side, and the ratio of the ipsilateral to contralateral muscle weight was calculated.

Immunohistochemistry

Nerve samples were explanted and immediately placed in 4% paraformaldehyde in phosphate-buffer overnight followed by immersion in 30% sucrose in PBS solution for 24–48 h. Samples were then frozen in OCT Compound (VWR, Radnor, PA) and sectioned at 15 μm onto pretreated charged glass slides. Sections were rehydrated with PBS and blocked using 5% normal goat serum diluted in PBS before primary antibody staining. Primary antibody in 5%
serum buffer was applied and incubated at 4°C overnight with antibodies outlined in table 1. Sections were then washed in PBS and stained for the appropriate fluorochrome-conjugated secondary antibodies for 1 h at room temperature. All sections were mounted with Fluoroshield mounting medium with DAPI (Abcam, Boston, MA) and then imaged using the Fluoview FV1000 confocal microscope and acquisition system (Olympus, Waltham, MA) at overall 200x (20x water immersion objective) or 600x magnification (60x oil immersion objective). A minimum of three sections were analyzed and averaged for each tissue area using ImageJ (NIH). For cell counts, field size was kept standard at a 60x objective (600x magnification overall), and colocalization of the primary marker(s) with DAPI was considered a positive cell.

Cell sorting

ANAs were explanted from rats at described endpoints after nerve repair and finely minced. Each sample was then incubated with 1 mL of digestion buffer (0.1% collagenase, 0.05% DNase in 2% fetal bovine serum (FBS)/ Dulbecco’s Modified Eagle Media (DMEM)). Samples were incubated for 20 minutes at 37°C with constant agitation. Following digestion, the samples were re-suspended in FACS buffer (2% FBS, 0.1% EDTA in phosphate buffered saline) and filtered through a 70 µm sized cell strainer. Cells were then incubated with anti-CD32 for 10 minutes and then incubated with a cocktail of CD45-PE-Cy7, Thy1-PerCP, and O4-PE for sorting of Schwann cells. (Irigoyen et al., 2018; Lutz, 2014). For staining of T cells, cells were incubated with a cocktail of CD45-PE, CD11bPE/Cy7, CD3 PerCP, CD4 FITC, CD8 Pacific blue. Cells were then washed and resuspended in FACS buffer. Cells were sorted on Aria II (BD
Bioscience, San Jose, CA) at the Washington University Flow Cytometry core. Schwann cells were sorted by CD45−Thy1−O4+. T cells were sorted by CD45+CD11b−CD3+.

RT-PCR

Total RNA was prepared from either flow sorted cells or ANA explants. RNA was extracted using Trizol (Life Technologies), chloroform and an RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. RNA concentration was determined on a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The cDNA was generated with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed using a Step One Plus thermocycler (Applied Biosystems, Foster City, CA) using Taqman Master Mix (Applied Biosystems) reagents with specific oligonucleotide primer pairs (Table 2). The PCR conditions were 50°C for 2 min and then 95°C for 15 s and 60°C for 1 min, repeated for 40 cycles, with a hot start at 95°C for 10 min. The expression levels of each gene were normalized to those of the internal control gene (Actb). Data was analyzed using Step One Software v2.2.2 (Applied Biosystems, Foster City, CA).

Cell culture

Rat Schwann cells are isolated from adult Lewis rat sciatic nerves. Isolated nerves were desheathed and digested with digestion cocktail of 0.1% collagenase in DMEM and 2% FBS. Isolated cells were then cultured on PLL-laminin coated plates until confluence in DMEM, 10% FBS with 2 μM forskolin. To purify the Schwann cells, cells were treated with rabbit anti-thy1
IgM at room temperature followed by complement mediated lysis using rabbit complement. Schwann cells purity was determined by staining with S100. When indicated, Schwann cells were plated onto PLL coated cell culture dish with serum free medium (DMEM, 0.1% BSA, 1x N2 supplement) with or without 10 nM IFN-γ (Peprotec, Rock Hill, NJ).

Statistical analysis

Statistical analyses were performed using GraphPad Prism. All data were compiled as mean ± standard deviation. Data were tested for normality using the Kolmogorov–Smirnov test. Student’s t test was performed for analysis between 2 groups. For larger grouped analysis, Two-way ANOVA with Bonferroni correction is used in conjunction with multiple comparison. A significance level of p < 0.05 was used in all statistical tests performed.

2.4 Results

Regenerative capacity of ANAs depends on ANA length

Previously, we demonstrated that axon regeneration across ANAs is length dependent (Saheb-Al-Zamani et al., 2013), and cellular repopulation of longer ANAs (i.e. >3 cm) differs compared to shorter ANAs (<3 cm) (Poppler et al., 2016). In our present studies, we wished to expand the comparisons of regenerative differences between short (<3 cm) versus long (>3 cm) ANAs. Therefore, we first evaluated regenerative outcomes from nerve repaired using 2 cm (short) compared to 4 cm (long) ANAs.
In male Lewis rats, 2 cm or 4 cm ANAs were used to repair a sciatic nerve gap, and the extent of axon regeneration evaluated at eight (8) weeks following repair (n=6 for both groups). As shown in Fig. 5A, and consistent with previous data, we found robust myelinated axon regeneration to the mid-graft when nerves were repaired using 2 cm ANAs, where nearly 10,000 myelinated axons were found to have regenerated. Conversely, regeneration across 4 cm ANAs was inferior, where the mid-graft of nerve repaired with 4 cm ANAs contained less than 1,000 myelinated axons (p<0.0001). Furthermore, as we examined the distal nerves for axonal regeneration (Fig. 5B), we found that while nerve repaired with 2 cm ANAs maintained almost 10,000 myelinated axons, there were almost no observable myelinated axons in the distal stump when repaired with 4 cm ANAs (p<0.0001). Relative gastrocnemius muscle mass is indicative of reinnervation of denervated muscles. Relative gastrocnemius muscle mass was significantly greater for nerve repaired using 2 cm compared to 4 cm ANA (p<0.0001) (Fig. 5C). We also compared regenerative outcomes among male and female rats receiving nerve repair using 2 cm ANAs (n=6 for both groups). Female rats repaired with ANAs yielded similar axon regeneration and recovery of muscle atrophy compared to males (Fig. 6). These results demonstrate that ANAs can support robust axon regeneration up to a certain length, suggesting that mechanisms within longer ANAs limit axon regeneration.

Schwann cells repopulating ANAs differ based on ANA length

The profoundly reduced axon regeneration across long ANAs suggests disrupted regenerative processes that would normally promote axon growth. Previously, we found Schwann cells (SCs) repopulating long (>3 cm) ANAs exhibited altered phenotypes (Poppler et
al., 2016). We therefore further characterized the SCs repopulating ANAs at the mid-graft region four weeks following repair (n=4 for both groups). In line with previous results, the mid-graft of 2 cm ANAs contained approximately 3-fold greater S100⁺ area (an indication of SC number) compared to 4 cm ANAs (p=0.0195, Fig. 7A-B). Furthermore, we found that nearly 10% of SCs in the mid-graft of 2 cm ANAs expressed markers of proliferation (Ki-67⁺), while less than 2% of SCs in the 4 cm ANAs expressed these markers (p=0.0067, Fig. 7C).

To further understand the phenotype of SCs, we isolated SCs from within these ANAs and characterized their gene expression (n=3 for both groups). Overall, there were no significant differences in the number of Schwann cells isolated per cm from either 2 cm or 4 cm ANAs (Fig. 7D-E). Despite the similar density of SCs, the gene expression of SCs from 2 cm ANAs differed from those within 4 cm ANAs. We found the SCs contained within 2 cm vs 4 cm ANAs expressed greater levels of genes related to maturation and myelination (Fig. 7F). These genes include the master myelination regulatory gene Krox20, and other myelination related genes include Mbp, Mpz, and Pmp2 (Ghislain and Charnay, 2006). As SCs down regulate their myelination genes in response to injury entering into a more “repair” phenotype status (Arthur-Farraj et al., 2012). We therefore probed for genes indicative of SC activation, including C-Jun, Mmp9, Gdnf, and Calca. Surprisingly, we did not find higher levels of these genes in SCs taken from 4 cm ANAs. Instead, select genes related to activation, including C-Jun and Mmp9, were expressed to a greater extent in SCs from 2 cm ANAs (Fig. 7G). Taken together, these data suggest that at 4 weeks post repair, the SCs in 2 cm ANAs are becoming mature while the SCs in 4 cm ANAs fail to mature. Additionally, these results suggest that long 4 cm ANAs may be experiencing earlier regenerative events (i.e. prior to week 4 post repair) that may in turn alter SC functions and phenotype.
Angiogenesis is affected by ANA length

As robust and precise angiogenesis contributes to SC functions (Cattin et al., 2015; Cattin and Lloyd, 2016), we assessed if longer ANAs (4 cm) undergo altered angiogenesis compared to shorter ANAs (2 cm). Four weeks after nerve repair using either 2 cm or 4 cm ANAs, FITC-conjugated lectin was intravenously injected into rats prior to ANA harvest to visualize functional vessels (n=4 for both groups). Quantification of FITC+ area demonstrated that while both 2 and 4 cm ANAs contained functional vessels, the mid-graft of 2 cm ANAs had 2-fold greater FITC+ area compared to 4 cm ANAs (p=0.0315, Fig. 8A) suggesting reduced numbers of functional blood vessels within longer ANAs. To further explore this outcome, we assessed the extent of endothelial cell quantities within long and short ANAs. At both 2 weeks (p=0.0153), and 4 weeks (p=0.0126) post-repair, at the mid-graft 2 cm ANAs had approximately 2-fold greater RECA-1+ (endothelial cells) area compared to 4 cm ANAs, suggesting a greater quantity of endothelial cells in 2 vs 4 cm ANAs (Figs. 8B-C). Overall, these data demonstrate long ANAs have disrupted angiogenesis, as compared to shorter ANAs.

Leukocyte response is affected by ANA length

Angiogenesis and tissue regeneration are highly dependent on the local wound immune response. Macrophages are one of the first leukocytes to respond to injury, where they mediate angiogenesis following nerve injury (Cattin et al., 2015; Cattin and Lloyd, 2016) and can be generally characterized based on their activation status (i.e. M1 versus M2). Given the
differences in angiogenesis observed as early as 2 weeks post repair in mid-grafts of short vs long ANAs, we compared macrophage (CD68+ cells) quantities and phenotype within 2 vs 4 cm ANAs (n=4 for each group). Using immunofluorescence, we found that 2 weeks after repair with ANAs of either length, macrophages begin to accumulate within ANAs. Macrophages represented about 35-40% of total cells in ANAs. However, despite differences in SCs and angiogenesis, there were no differences in macrophage quantities among 2 vs 4 cm ANAs at 2 weeks, nor differences in the proportion of CD206+ macrophages (CD206+CD68+ cells) (Fig. 9A-C). At 4 weeks, macrophage quantities contained in either length ANA at the mid-graft were substantially reduced compared to 2 weeks. However, the relative proportion of CD206+ macrophages of total macrophages increased in ANAs of either length. Then, comparing 2 and 4 cm ANAs to one another at 4 weeks, the total number of macrophages in 2 cm ANAs were almost double compared to 4 cm ANAs (p=0.0289, 8% vs 5% of total cells for 2 cm and 4 cm, respectively; Fig. 9D-F). In turn, 4 cm ANAs also accumulated fewer total CD206+ macrophages compared to 2 cm ANAs. However, the relative proportion of CD206+ macrophages were not different between 2 cm and 4 cm ANAs. Overall, these data suggest that initial macrophage accumulation among ANAs does not differ until 4 weeks. Therefore, there may be processes prior to 4 weeks within long ANAs that reduce macrophage numbers.

T cells infiltrate into sites of injury following initial macrophage migration, where T cells can be an integral to cell functions during the regeneration various tissues (Burzyn et al., 2013; Nishimura et al., 2009; Zhang et al., 2014; Sadtler et al., 2016). Considering 2 cm vs 4 cm ANAs, infiltration of T cells (CD3+) into ANAs was observed as early as 2 weeks following repair (Fig. 10A-B). At 2 weeks, the total number of T cells in the mid-graft of 2 cm ANAs (~7% of total cell numbers) was nearly 3-fold greater than that of 4 cm ANAs (p=0.0096, Fig. 10A-B).
While the total number of T cells greatly differed between 2 vs 4 cm ANAs, both 2 cm and 4 cm ANAs contained nearly identical proportions of CD4+ T cells of the total T cell populations, representing almost 30% of all T cells. At 4 weeks, the total proportion of T cells in 2 cm ANAs remained consistent to the values at 2 weeks (~7-8% of total cells; Fig. 10D-F). However, T cells represented only about 2% of total cells in 4 cm ANAs (p=0.0142). Again at 4 weeks, both 2 cm and 4 cm ANAs contained nearly identical proportions of CD4+ T cells of the total T cell populations, now representing approximately 40% of all T cells.

Cytokine expression is altered based on ANA length

To further examine 2 vs 4 cm ANAs at 2 weeks, we examined gene expression of cells contained in ANAs. We focused on a panel of inflammatory cytokines related to T cell functions based on our observations regarding T cells. We approached the characterization via two different ways. First, we measured and compared the gene expression of inflammatory cytokines in the bulk 2 cm and 4 cm ANAs (n=4 for both groups). Two cm ANAs contained upregulated expression of a variety of pro- and anti-inflammatory cytokines including IL-2, IL-4, and IFN-γ compared to 4 cm ANAs (p=0.0024, 0.0446, and 0.01 respectively, Fig. 11A). Next, to further elucidate the characteristics and gene expression of T cells, we isolated and examined the proportion of CD8 cytotoxic and CD4 effector T cells from 2 cm ANAs (n=3 for both groups). We focused on 2 cm ANAs since immunofluorescence did not show differences in T cell proportion between 2 cm and 4 cm ANAs, only their number. At 2 weeks, we found that CD4+ T cells represent a five-fold greater majority of T cells found within ANAs compared to CD8+ T cells (p=0.0002, Figs. 11B-C). Since T cells may regulate cytokines expressed by other cells, such as macrophage, or directly secrete cytokines, we determined T cell cytokine expression directly, where gene expression was compared to splenic, non-activated T cells (n=3 for both...
groups). Despite the overall upregulation of IL-4 in 2 cm ANAs compared to 4 cm ANAs, T cells within ANAs showed no change in the expression of IL-4, while IFN-γ was upregulated compared to splenic T cells. (Fig. 11D).

ANA length limits angiogenesis and T cell accumulation within ANAs

Previously, we determined that nerves repaired with long ANAs accumulate factors within the ANA that limit axonal regeneration (Poppler et al., 2016). As the extent of angiogenesis and T cell accumulation are reduced within long ANAs, we assessed if ANA length contributed to this outcome. The ANAs used from nerve repaired initially using 2 or 4 cm ANAs was harvested and trimmed to equal lengths (1.5 cm) at 2 weeks post-repair. These 1.5 cm pre-grafted ANAs were then used to repair freshly transected nerves (i.e. “re-grafting”; Fig. 12A). This approach retained the environments within these 2 or 4 cm ANAs while eliminating length as a factor in nerve regeneration (n=4 for both groups). We found that 2 weeks after “re-grafting,” there was no significant differences in the proportion of RECA-1+ area between re-grafts derived from 2 cm or 4 cm ANAs (Fig. 12B). Similarly, we found that there were no significant differences in the proportion of macrophages (Fig. 12C) or T cells (Fig. 12D) within the re-grafted ANAs. This outcome suggests that early differences in angiogenesis and the immune response between short and long ANAs is due to ANA length.

T cell deficiency impairs nerve regeneration across ANAs
Given that T cell quantity is reduced within long ANAs, we next assessed whether T cells contribute to regeneration of peripheral nerves. We examined axonal regeneration in T cell deficient (rnu/rnu) and T cell-sufficient (Lewis or rnu+/+) rats. First, we assessed if T cells contributed to axonal regeneration when sciatic nerves are repaired with nerve isografts. Lewis or athymic (rnu/rnu) rats were grafted with 4 cm isografts derived from their respective strains. Eight weeks after nerve repair, we examined the extent of nerve regeneration using histomorphometry (n=4 for both groups). Despite a T cell deficiency, there were no significant differences in the number of regenerated myelinated axons at the mid-graft or distal nerve in athymic rats compared to Lewis rats eight weeks after repair with isografts (Figs. 13A-C). Furthermore, examination of the ratio of axonal myelination suggest that Lewis rats or rnu/rnu rats had similar G-ratios in distal stump (Fig. 13D). These sets of experiments suggest that T cells have limited impact on axonal regeneration when regeneration across the graft does not require substantial cell repopulation of the graft.

Next, we assessed whether T cell contributed to axonal regeneration when sciatic nerves are repaired with ANAs. Initially, to determine if T cells affected regeneration across ANAs, we repaired nerve defects using 4cm ANAs and measured axon regeneration at an 8 weeks endpoint (n=4 for both groups). However, in long (4 cm) ANAs, axon regeneration to even the mid-graft is limited (<1000 myelinated axons) in Lewis rats. Therefore, while athymic rats contained no axons within even the mid-graft, these results in the 4 cm ANA model were not statistical due to the large variability in regeneration in the Lewis rat 4 cm ANA model (Fig 14). Therefore, we assessed nerve regeneration across short (2cm) ANAs, which normally support robust axon regeneration across this gap length (Fig. 5) (n=8 for RNU/RNU, n=6 for Lewis). While axon regeneration across 2 cm ANAs was robust in Lewis rats, axon regeneration was decreased by
~50% in athymic rats in the mid-graft, and almost 4 fold in the distal nerve (mid-graft, p=0.0118, distal p<0.0001 Figs. 15A-C). To ensure the allogeneic nature of the ANAs was not a factor, whereby T cells were artificially being recruited to ANAs where they contribute to regeneration, we repaired sciatic nerves in Lewis rats with either 2 cm ANAs (derived from Sprague Dawley) or 2 cm acellular nerve isografts (ANIs), where nerve was derived from Lewis rats (n=4 for both groups). Eight weeks after repair, there were no differences in the number of regenerated myelinated axons within mid-graft or across ANAs to the distal nerve (Fig. 16). This outcome suggests that allogenicity has limited impact in regeneration, and that T cells could indeed be contributing to normal regeneration across acellular scaffolds, such as ANAs.

Based on these findings, we performed additional experiments comparing T cell-sufficient rats derived from a genetic background matching athymic rats: Heterozygous Foxn1 mutation RNU rats (rnu/+). This comparison minimizes confounding factors associated with any potential differences in nerve recovery between rat strains (n=8 for RNU/RNU, n=7 for RNU/+). Even in the rnu/+ heterozygotes, axon regeneration across the ANA was robust. Compared to rnu/rnu T cell deficient rats, the rnu/+ rats had significantly more myelinated axons in both the mid-graft and distal nerve (mid-graft p=0.024, distal p=0.0015 Figs. 17A-C). Furthermore, we found the myelination degree to be significantly greater (lower G ratio) in the distal stump of T cell sufficient rnu/+ rats than their T cell deficient counterparts (p=0.0088, Fig. 17D). We also assessed if T cell deficiency contributed to aspects of functional recovery. Comparing relative gastrocnemius muscle mass, recovery was reduced in athymic rats compared to their heterozygous counterpart (Fig. 17E). Behavioral assessment was performed using walking track analysis quantified by the sciatic functional index (SFI). Consistent with the outcome of other regenerative metrics, T cell-sufficient rats demonstrated improved SFI scores compared to
athymic rats (p=0.013, **Fig. 17F**). Overall, these results strongly suggest a causal relationship between T cells and regeneration across ANAs, where T cell deficiency impairs nerve regeneration across ANAs.

### 2.4 Discussion

While autografts remain the gold standard for repair of peripheral nerve injuries resulting in a gap between the nerve ends, ANAs have been increasingly used as an alternative to autografts (Isaacs and Browne, 2014; Karabekmez et al., 2009). Yet, despite adequate efficacy for ANAs to repair small gap or small diameter nerve injuries, the capabilities of ANAs to promote regeneration across long nerve gaps has been limited (S.Zhu et al., 2017). In order to rationally design improvements of alternatives, such as ANAs, there is a need to better understand factors that limit or promote nerve regeneration across ANAs. To that end, we utilized a rat model of sciatic nerve transection with ANA repair to investigate differences in regeneration across short or long ANAs, as short ANAs support regeneration while long ANAs fail to support adequate regeneration. Through these studies, we identified several factors and cell types that could limit nerve regeneration across long ANAs, and provide causal evidence that suggests T cells are critical to promoting nerve regeneration across ANAs.

Our previous studies primarily suggested that differences in axon regeneration across longer ANAs was mainly attributable to differences in the ANA environment rather than an innate deficiency of neurons to regenerate their axons across longer nerve grafts (Poppler et al., 2016). Our present studies are consistent with these findings. We saw that the SCs within shorter ANAs, which supported robust axon regeneration, displayed a phenotype distinct from SCs in
longer ANAs. Unlike those in the longer ANAs, the SCs in the shorter ANAs are more proliferative and express higher levels of myelination related genes. SCs are known to acquire a “repair” phenotype following nerve injury in which they down regulate myelination genes and upregulate repair related genes, such as growth factors (Arthur-Farraj et al., 2012). Interestingly, in our study, the SCs within longer ANAs expressed lower levels of myelination genes, yet did not express higher level of repair related genes such as C-Jun, Mmp9, Gdnf and Gap43. This finding suggests that SCs in longer ANAs may adopt a phenotype that is distinct from either repair or myelination.

To more thoroughly explore why SCs were different between ANAs based on length, we examined key regenerative processes prior to the substantial repopulation of ANAs by SCs. Blood vessels are essential to axonal regeneration and the migration of SCs into sites of injury (Cattin, Burden, VanEmmensis, Mackenzie, Hoving, Calavia, et al., 2015). Given the reduced number of SCs within longer ANAs, it is possible that delayed angiogenesis contributed to reduced SC number and altered phenotype within long ANAs. Previous studies have shown that compared to the gold-standard autografts, acellular scaffolds used to repair nerve have reduced revascularization, suggesting an innately slow process of endothelial cell repopulation of the scaffolds (Fansa, Schneider, &Keilhoff, 2001). Thus, longer length ANAs may require a greater amount of time for angiogenesis to complete, leading to delayed or disrupted revascularization. Our data here demonstrated that longer ANAs have disrupted angiogenesis compared to shorter ANAs.

Since macrophages are critical to promoting angiogenesis, we initially hypothesized that reduced angiogenesis was due to either a reduced infiltration of macrophages or an altered phenotype of macrophages in long ANAs. Surprisingly, we did not observe obvious differences
in macrophages between 2 cm vs 4 cm ANAs at 2 weeks. In fact, macrophage quantities were not impacted until week 4, even though changes to angiogenesis were evident by week 2. Furthermore, the proportion of macrophages that express CD206, a prominent marker of their anti-inflammatory phenotype, were not different between 2 cm and 4 cm ANA despite higher IL-4 gene expression detected in 2 cm ANAs. This outcome was counterintuitive given the known role of IL-4 promoting a CD206+ macrophage phenotype (Bosurgi et al., 2017). However, the simultaneous elevated expression of IFN-γ in 2 cm ANAs may have prevented alternative activation of macrophages in vivo (Appelberg, Orme, Pinto de Sousa, & Silva, 1992). Given similar infiltration of macrophages into ANAs of either length early during regeneration (i.e. 2 weeks), the reduced angiogenesis observed in long ANAs is unlikely initially caused by macrophages alone.

The most significant finding of the studies was our observation of reduced T cell numbers within long ANAs compared to short ANAs. Concomitant with reduced T cell accumulation at week 2 in 2 cm compared to 4 cm ANAs, we also found significantly reduced cytokine expression with 4 cm ANAs (IFN-γ, IL-2, IL-4, and IL-13). These results suggest that T cells provide or regulate the level of inflammatory cytokines within ANAs. Interestingly, IFN-γ, IL-4, IL-10, and IL-13 have all been shown to be important for regeneration in a variety of organ systems (Cheng, Nguyen, Fantuzzi, & Koh, 2008; Goh et al., 2013; Horsley et al., 2003; Mokarram et al., 2012). While anti-inflammatory cytokines have been shown to be important for activating gene expression in macrophages to promote tissue regeneration resolution after injury, inflammatory cytokines have been found to promote cell infiltration to sites of injury. Therefore, it is possible that T cells mediate nerve regeneration across ANAs via regulation of inflammatory cytokines. Interestingly, we found that while cells in 2 cm ANAs have greater expression of IL-4
and IFN-γ, T cells did not upregulate their expression of IL-4 compared to splenic T cells. Rather, our data demonstrated that ANA infiltrating T cells, at least early on, upregulate their expression of IFN-γ, a Th1 cytokine that promotes inflammation. While high levels of exogenous IFN-γ have been shown to be detrimental to nerve regeneration, physiological levels of IFN-γ have been found to be beneficial to regeneration. Furthermore, previous studies have found that low levels of IFN-γ promote SC proliferation. Indeed, when SCs are cultured with IFN-γ at these low levels, the SCs become more proliferative, and express higher levels of GDNF, a growth factor important for nerve regeneration (Fig. 18).

Based on these findings, we directly examined the role of T cells on nerve regeneration across cellular and acellular grafts. When nerve was repaired using an isograft (cellular), the absence of T cells did not significantly deter axonal regeneration. This finding is consistent with previous studies showing that a lack of adaptive immunity did not inhibit regeneration following a nerve crush injury (Bombeiro et al., 2016). Conversely, when nerve was repaired using ANA, axonal regeneration and recovery was significantly diminished when T cells were absent. For nerves repaired with ANAs, the loss of T cells is associated with reduced number of myelinated axons, reduced axonal myelination, and reduced functional recovery. It is unlikely that T cells directly act on neurons to significantly promote their survival and regeneration of axons. If they did, we would have expected reduced axonal regeneration when athymic rats were repaired with isografts. Given that loss of T cells significantly reduced nerve regeneration from ANA repair, it suggests that the neuronal impact of T cells is limited. Rather, the significant differences in nerve regeneration across ANAs in athymic rats compared to their heterozygous counterparts suggest that T cells may play a greater role in the repopulation or regulation of cells within the ANA environment.
In conclusion, our study demonstrated that long compared to short ANAs are repopulated with Schwann cells having an altered phenotype, contain reduced angiogenesis and T cell accumulation, and contain reduced expression of inflammatory cytokines. T cells likely play a major role in nerve regeneration across ANAs, as a T cell deficiency resulted in reduced regeneration of sciatic nerve through even short ANAs. This finding was unique to ANAs, as regeneration across cellular isografts was unaffected. Our data therefore provides additional mechanistic insight into the cause for failed regeneration in long nerve defects, and may have relevance to regeneration of other tissues where acellular scaffolds are used to promote healing.
Figure 5. ANAs facilitate axon regeneration in a length dependent manner.

Eight weeks after nerve repair using either 2 cm or 4 cm ANAs, the extent of axon regeneration to the A) mid-graft of ANA and B) distal nerve was quantified. At this same endpoint, C) relative gastrocnemius muscle weight was also measured. Representative histological images of nerve are shown, where white scale bar is 20 μm. Data represented as mean ± SD; n=6 for both groups; p<0.001 for both mid-graft and distal nerve; Solid line with grey area represents number of myelinated axons when nerves are repaired with 2 cm isografts. p<0.05 for muscle weights.
Figure 6. ANAs facilitated axon regeneration independent of biological sex

Eight weeks after nerve repair using 2 cm ANAs, the extent of axon regeneration to the A) mid-graft of ANA and B) distal nerve was quantified. At this same endpoint, C) relative gastrocnemius muscle weight was also measured. Representative histological images are shown, where white scale bar is 20 μm. Data represented as mean ± SD; n=6 for each group; ns: not significant; p<0.05 for muscle weights.
Figure 7. Schwann cell phenotypes differ in nerves repaired with short (2 cm) or long (4 cm) ANAs

Four weeks after nerve repair using ANAs, SC quantity and phenotype within mid-graft of 2 cm and 4 cm ANAs were assessed. A) Representative IHC for S100 (red) and Ki-67 (green). Scale bar represent 20 μm. B) Quantification of S100+ area and C) proportion of Ki-67+ Schwann cells. Data represented as mean ± SD; n=4 for each group; * indicates p<0.01. Similarly, SCs were isolated from the entirety of these ANA using FACS. D) Representative flow cytometry showing gating of CD45-Thy1-O4+ Schwann cells, and E) quantification of Schwann cells per cm of graft from either 2 cm or 4 cm grafts. SCs sorted from FACS were used to probe for gene expression using real time RT-PCR. Quantification of SC F) activation related and G) maturation related gene expression. Data represented as mean ± SD; n=3 for both groups; * indicates p<0.01; ns: not significant.
Figure 8. Angiogenesis differs among short (2 cm) or long (4 cm) ANAs

After nerve repair using ANAs, angiogenesis within mid-graft of 2 cm and 4 cm ANAs was assessed. A) Representative IHC images of perfused FITC-conjugated lectin (green) with quantification 4 weeks after ANA repair. At B) 4 weeks and C) 2 weeks, angiogenesis was assessed using IHC RECA-1 (green) to identify endothelial cells. Representative IHC images of mid-graft for 2 cm or 4 cm ANA with quantification of RECA-1+ area. Data represented as mean ± SD; n=4 for all groups; * indicates p<0.05. Scale bars represent 40 μm.
Figure 9. Macrophage accumulation has minimal changes among short (2 cm) or long (4 cm) ANAs

After nerve repair using ANAs, macrophage accumulation within mid-graft of 2 cm and 4 cm ANAs was assessed A-C) 2 weeks and D-F) 4 weeks following repair. Two weeks after repair, representative IHC images of CD206 (red) and CD68 (green) from mid-graft of A) 2 cm ANAs, and B) 4 cm ANAs. C) The proportion of CD68+ cells, CD206+ CD68+ cells, and proportion of CD206+ cells among CD68+ cells was quantified. Four weeks after repair, representative IHC images of CD206 (red) and CD68 (green) from mid-graft of D) 2 cm ANAs, and E) 4 cm ANAs. F) The proportion of CD68+ cells, CD206+ CD68+ cells, and proportion of CD206+ cells among CD68+ cells was quantified. Data represented as mean ± SD; n=4 for groups; * indicates p<0.05; ns: not significant.
Figure 10. T cell accumulation is altered among short (2 cm) or long (4 cm) ANAs

After nerve repair using ANAs, T cell accumulation within mid-graft of 2 cm and 4 cm ANAs was assessed A-C) 2 weeks and D-F) 4 weeks following repair. Two weeks after repair, representative IHC images of CD206 (red) and CD68 (green) from mid-graft of A) 2 cm ANAs, and B) 4 cm ANAs. C) The proportion of CD3+ cells, CD3+ CD4+ cells, and proportion of CD4+ cells among CD3+ cells was quantified. Four weeks after repair, representative IHC images of CD206 (red) and CD68 (green) from mid-graft of D) 2 cm ANAs, and E) 4 cm ANAs. F) The proportion of CD3+ cells, CD3+ CD4+ cells, and proportion of CD4+ cells among CD3+ cells was quantified. Data represented as mean ± SD; n=4 for groups; * indicates p<0.05; ns: not significant.
Figure 11. Cytokine expression differed based upon ANA length

A) Two weeks after nerve repair using ANA, gene expression from cells contained within 2 cm and 4 cm ANAs was measured using RT-PCR. n=4 for both groups, * indicates p< 0.05. Additionally, T cells were isolated from the entirety 2 cm ANA using FACS. B) Representative flow cytometry of 2 cm ANAs gated CD45+CD11b-CD3+ cells. C) Quantification of the proportion of CD4+ and CD8+ cells among CD3+ cells. D) Quantification of IFN-γ and IL-4 gene expression from T cells isolated from 2 cm ANAs. n=3. All data represented as mean ± SD; * indicates p<0.01.
Figure 12. ANA length functionally limited angiogenesis and T cell accumulation within ANAs

A) Schematic of experimental procedures for “re-grafting” short or long ANAs. Two weeks after re-grafting, mid-graft was assessed using IHC. B) Representative images for RECA-1 (green) and C) quantification of RECA-1+ area. D) Representative images for CD68 (green) and CD206 (red) and E) quantification of the proportion of CD68+ cells and CD68+CD206+ cells. F) Representative images for CD3 (red) and CD4 (green) and G) quantification of the proportion of CD3+ cells and CD3+CD4+ cells. Data represented as mean ± SD; n=4 for all groups; ns: not significant. Scale bars represent 40 μm.
Figure 13. T cell deficiency does not affect axon regeneration across long (4cm) isografts

Eight weeks after nerve repair using 4 cm isografts in Lewis or rnu/rnu rats, the extent of nerve regeneration to the mid-graft of ANA and distal nerve was quantified. Representative histological images of nerve at A) mid-graft and B) distal nerve are shown, where white scale bar is 20 μm. C) Quantification of myelinated axons in the mid-graft and distal nerve. D) Quantification of G-ratio in the distal nerve of Lewis or rnu/rnu rats. Data represented as mean ± SD; n=4 for both groups; ns: not significant.
Eight weeks after nerve repair using 4 cm ANAs in Lewis or rnu/rnu rats, the number of myelinated axons in the mid-graft was quantified. Data represented as mean ± SD; n=4 for each group; ns: not significant.
Figure 15. T cell deficiency affects regeneration across short (2cm) ANAs

Eight weeks after nerve repair using 4 cm isografts in Lewis or rnu/rnu rats, the extent of nerve regeneration to the mid-graft of ANA and distal nerve was quantified. Representative histological images of nerve at A) mid-graft and B) distal nerve are shown, where white scale bar is 20 μm. C) Quantification of myelinated axons in the mid-graft and distal nerve. D) Quantification of G-ratio in the distal nerve of Lewis or rnu/rnu rats. Data represented as mean ± SD; n=4 for Lewis rats and 8 for rnu/rnu rats; * indicates p<0.05. ns: not significant.
Figure 16. T cell deficiency affects regeneration across short (2cm) ANAs

Eight weeks after nerve repair using 2 cm ANAs in rnu/+ or rnu/rnu rats, the extent of nerve regeneration to the mid-graft of ANA and distal nerve was quantified. Representative histological images of nerve at A) mid-graft and B) distal nerve are shown, where white scale bar is 20 μm. C) Quantification of myelinated axons in the mid-graft and distal nerve. D) Quantification of myelination ratio in distal nerve. E) Relative gastrocnemius muscle weight was measured at 8 weeks. F) Sciatic function index was measured from 6 to 8 weeks after repair. Data represented as mean ± SD; n= 8 for both groups; * indicates p<0.05.
Figure 17. Repair of nerve gaps using acellularized nerve from isogenic donors did not affect regeneration

Eight weeks after nerve repair using 2 cm ANAs and ANIs, the extent of axon regeneration to the mid-graft of ANA and the distal nerve was quantified. G ratio of myelinated axons in the distal nerve was also quantified. Data represented as mean ± SD; n=6 for each group; ns: not significant.
A

Control

+ IFN-γ

Ki-67

DAPI

B

Fold change in gene expression Relative to SC

SC

SC + IFN-γ (n=3)

(n=3)

C-Jun

GAP43

BDNF

GDNF

MBP

% Ki-67+

Control

+ IFN-γ

n=3

n=3
Figure 18. IFN-γ increase Schwann cell proliferation and activate GDNF

A) Two days after 20,000 Schwann cells were treated with control media or media with IFN-γ, the proliferation of the Schwann cells are determined by staining for Ki-67. Data represented as mean ± SD; n=3 for each group, * denote p<0.05. B) Schwann cells were also cultured in the same condition and used for real time RT-PCR. Data represented as mean ± SD; n=3 for each group, * denote p<0.05.
## Table 1. Antibodies and their dilutions used in Chapter 2

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Chapter 3 T cells and IL-4 have critical roles in promoting nerve regeneration across acellular nerve allografts

The content of this chapter has been submitted under the title “T cells and IL-4 have critical roles in promoting nerve regeneration across acellular nerve allografts” to Glia
3.1 Abstract
Acellular nerve allografts (ANAs) are increasingly used to bridge a nerve gap to facilitate regeneration. The adaptive immune system, specifically T cells, plays a role in promoting regeneration in this context. However, how T cells promote regeneration across ANAs is not clear. Here, we show that T cells accumulate within ANAs repairing nerve gaps resulting in regulation of cytokine expression within the ANA environment. This in turn ultimately leads to robust nerve regeneration and functional recovery. Nerve regeneration across ANAs and functional recovery in Rag1KO mice, which lack adaptive immunity, was significantly hampered compared to wildtype (WT) mice. Prior to appreciable nerve regeneration, ANAs from Rag1KO mice contained not only minimal T cells, but fewer eosinophils and reduced gene expression of Type 2 cytokines, including IL-4, compared to ANAs from WT mice. Furthermore, during this early regenerative period, both T cells and eosinophils regulated IL-4 expression within ANAs. Eosinophils represented the majority of IL-4 expressing cells within ANAs, while T cells regulated IL-4 expression. Specifically, depletion of CD4 T cells from WT mice limited both IL-4 expressing cell and eosinophil accumulation within ANAs. Finally, an essential role for IL-4 during nerve regeneration across ANAs was confirmed as suggested by this data. Nerves repaired using ANAs had reduced regeneration of myelinated axons and functional recovery in IL-4 KO mice compared to WT mice. Our data suggest T cells regulate the expression of IL-4 within the ANA environment to promote regeneration of myelinated axons.

**Keywords:** Regeneration, T cells, IL-4, acellular nerve allograft, myelination
3.2 Introduction

Severe damage to a nerve can result in a defect requiring repair of the nerve ends with a “bridge,” which enables axon regeneration from the proximal to distal nerve end (Grinsell & Keating, 2014). The best approach to repair defects is still a major unmet clinical challenge. While nerve autografts remain the gold standard for repair of gap injuries (Whitlock et al., 2009), the autograft has disadvantages including a need for additional surgery and loss of feeling at the donor site. Therefore, there has been a considerable interest for decades to develop alternatives to autografts (Karabekmez et al., 2009). Clinically, “off-the-shelf” alternatives have ranged from empty polymer conduits to more recent tissue-engineered materials, such as processed or acellular tissue scaffolds. However, as promising as these newer alternatives are for treating nerve defects, these alternatives still need further improvement. Review of the clinical literature considering nerve repair using alternatives still finds that alternatives can yield inadequate and inconsistent functional recovery (Rbia & Shin, 2017). To develop improved alternatives and further advance the field, a better understanding of the underlying biology that drives regeneration across a repaired nerve defect is needed.

Acellular nerve allografts (ANAs) are a promising alternative to autografts that can promote some level of regeneration across nerve defects (Karabekmez et al., 2009). Their use in the clinic for nerve repair is increasingly common. But, as ANAs, as well as many other alternatives, are initially acellular, these alternatives require extensive angiogenesis and cell repopulation to promote axon regeneration. Recent studies have demonstrated that the innate immune system has an essential role during nerve regeneration driving both angiogenesis and cell repopulation. Specifically, the cells of the innate immune system (primarily macrophages) infiltrate small, unrepaired defects recruiting endothelial cells to form vessels, which in turn
drive Schwann cell (SC) repopulation and axon regeneration (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Calavia, et al., 2015). Therefore, the immune system has a critical role in ultimately coordinating the processes that facilitate nerve regeneration across defects.

We recently described similar processes involving the immune system for ANAs (Pan et al., 2019). However, we found additional, undescribed processes that contributed to regeneration across ANAs. Long ANAs (> 4 cm in length), which poorly promote axon regeneration across these scaffolds (Poppler et al., 2016; Saheb-Al-Zamani et al., 2013), accumulated few T cells during ongoing regeneration, while abundant T cells accumulated within shorter ANAs that promoted robust nerve regeneration and some level of functional recovery. Furthermore, T cell deficiency in the context of regeneration across short ANAs reduced axon regeneration across the ANA (Pan et al., 2019). T cells have been shown to play important roles in the regeneration of spinal cord, brain, and muscle. However, their role in regeneration of peripheral nerve is less defined. While we showed that in a rat model of nerve repair with ANAs, T cells are important for efficient regeneration, how T cells may promote nerve regeneration was not considered.

One of the ways T cells may promote regeneration is via regulation of cytokines and inflammation through interaction with the innate immune system (Murphy & Reiner, 2002; Seder & Paul, 1994). In our previous studies considering the role of T cells in regeneration across ANAs, ANAs which contained fewer T cells also contained lower gene expression of interleukin-4 (IL-4), suggesting T cells and IL-4 levels in ANAs may be linked (Pan et al., 2019). However, mechanistic studies are needed to determine if loss of T cells and low IL-4 expression is correlative or causative. Furthermore, how IL-4 is expressed and maintained within nerve injuries is also unknown. While T cells and eosinophils are two important cell types known to express high levels of IL-4, other cells, including Schwann cells, have been hypothesized to be
sources of IL-4 (Ozaki, Nagai, Lee, Myong, & Kim, 2008). Where IL-4 is expressed, and how it is regulated, during nerve regeneration across an alternative are important questions that need addressing.

The contributions of IL-4 to successful nerve regeneration are also unclear. One of the major roles of IL-4 is modulation of inflammation. Specifically, IL-4 is generally considered an anti-inflammatory or Type 2 cytokine that can promote resolution of inflammation, and in turn, tissue regeneration. In line with this endogenous role of IL-4, exogenous delivery of IL-4 during regeneration across a nerve defect injury leads to elevated levels of macrophages and greater number of CD206 macrophages in regenerating nerve (Mokarram et al., 2017, 2012). These CD206 macrophages are generally considered “pro-regenerative” and have been shown to promote Schwann cell migration both in vitro and in vivo in the context of nerve (Mokarram et al., 2012). While this overall evidence would suggest that macrophages are the primary target of IL-4 during nerve regeneration, IL-4 could also signal to the nervous system during regeneration.

Studies suggest endogenous IL-4 signaling can have profound effects on the nervous system. Previous studies have shown that loss of IL-4 in mice with experimental autoimmune encephalomyelitis (EAE) contributed to worsening demyelination (Falcone, Rajan, Bloom, & Brosnan, 1998). Furthermore, exogenous IL-4 administration to mice with EAE can improve their functional recovery, in part through its direct action with IL-4 receptors expressed by neurons resulting in cytoskeleton remodeling and axonal repair (Shaw et al., 1997; Vogelaar et al., 2018). In our previous studies considering ANAs and T cells, while ANAs that had less T cell accumulation facilitated reduced axon regeneration across the ANA, SCs within these ANAs also expressed lower levels of myelination related genes (Pan et al., 2019). Thus, our previous data suggests a link between myelination and IL-4, whereby it is possible that IL-4 may promote
myelination during nerve regeneration. Myelination is a critical step in the regeneration of peripheral nerve to achieve functional recovery (Michailov et al., 2004; Ogata et al., 2004; Stratton et al., 2018).

Therefore, we undertook experiments to further delineate the role of T cells on nerve regeneration across alternatives (i.e. ANAs) repairing nerve defects. Using mouse models, we asked how T cells may contribute to the regeneration of peripheral nerve across a defect. We specifically considered whether T cells had a role in IL-4 regulation. And, based on those results, we also asked if IL-4 signaling contributed to specific aspects of nerve regeneration across an ANA, including nerve myelination and functional recovery.

### 3.3 Materials and Methods

**Reagents and Chemicals**

All reagents, consumables, and chemicals, unless otherwise stated, are purchased from Sigma-Aldrich (St. Louis, MO)

**Animals and experimental design**

Commercially-available adult mice (20-25g, Jackson Laboratories, Bar Harbor, ME) were utilized for all experiments. Mice were randomized to groups for experimental treatments. Randomized C57BL/6J mice were used as donor mice to derive ANAs. Surgical procedures and
peri-operative care measures were conducted in compliance with the AAALAC accredited Washington University Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health guidelines. All animals were housed in a central animal care facility and provided with food (PicoLab rodent diet 20, Purina Mills Nutrition International, St. Louis, MO) and water ad libitum.

For all experimental groups, mice sciatic nerve was transected and repaired using ANAs as described in detail in the “Surgical procedures” section that follows. Studies were performed using C57BL/6J mice (wild-type or WT) to first determine the extent of T cell accumulation within ANAs utilizing immunohistochemistry (IHC) and flow cytometry. To assess the role of the adaptive immune system during nerve regeneration across ANAs, Rag1KO mice (homozygous for the $\textit{Rag}^{1tm1Mom}$ mutation, which produce no mature T cells or B cells) (Mombaerts et al., 1992) were used to compare to WT mice. WT and Rag1KO mice were evaluated using IHC, histology, relative muscle mass, and grid walk analysis to determine the extent of nerve regeneration across nerve repaired using ANAs. In turn, during ongoing nerve regeneration, the regenerative environment within the ANA was compared between WT and Rag1KO mice using immunohistochemistry, flow cytometry, and gene analysis (qRT-PCR). To identify IL-4 expressing cells, IL-4/GFP-enhanced transcript (4Get) mice were utilized, in which the allele has a bicistronic IRES-EGFP reporter cassette inserted between the translational stop codon and the 3' UTR of the interleukin 4 gene ($\textit{Il}4$) (Mohrs, Shinkai, Mohrs, & Locksley, 2001). Thus, IL-4-expressing cells also express the fluorescent reporter. During nerve regeneration across ANAs, 4Get mice were evaluated using IHC and flow cytometry to determine IL-4-GFP expressing cells within ANAs. Then, 4Get mice were depleted of CD4 T cells and evaluated for IL-4-GFP expressing cells and changes to immune cell populations using IHC and flow.
cytometry. To confirm a role for CD4 T cells and IL-4, WT mice were depleted of these factors and evaluated using IHC to assess early nerve regeneration metrics. Finally, IL-4KO mice (homozygous for the Il4tm1Cgn targeted mutation) (Metwali et al., 1996) were compared to an independent set of WT mice to evaluate the extent of nerve regeneration across ANAs using histology, relative muscle mass, and grid walk analysis.

*Surgical procedures*

For surgical procedures, mice were anesthesized using a cocktail of ketamine (100 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and dexmedetomidine (0.5 mg/kg; Pfizer Animal Health, Exton, PA). For mice serving as ANA donors, following euthanization, their sciatic nerves were transected proximally at the level of exiting nerve roots, and distally just beyond the sciatic trifurcation. In experimental mice receiving the ANAs, the right sciatic nerve was exposed and transected 5 mm proximal to the distal trifurcation. The indicated grafts were reverse oriented (distal end of donor graft facing proximal nerve stump of recipient) sutured into the nerve gap with 11-0 nylon microsuture (Sharpoint, Reading, PA). A two-layer closure of muscle and skin was performed using 6-0 vicryl and 4-0 nylon suture, respectively. Atipamezole solution (0.1mg/kg; Zoetis, Florham Park, NJ) was administered for anesthesia reversal. The animals were recovered on a warming pad and monitored for postoperative complications before returning them to a central animal care facility. Postoperative pain was managed using Buprenorphine SR™ (0.05 mg/kg; ZooPharm, Windsor, CO). Animals were monitored daily post-operatively for signs of infection and/or distress. At the appropriate endpoints, animals were
euthanized via cervical dislocation under anesthesia, and their tissues collected for respective studies (see below).

**ANA processing**

ANAs were decellularized using a modified series of detergents in the method described previously (Poppler et al., 2016). Nerves isolated from donor animals the nerves were repeatedly washed in deionized water and three detergents in a sodium phosphate buffer: Triton X-100, sulfobetaine-16 (SB-16), and sulfobetaine-10 (SB-10). All grafts were washed and stored in 10 mM phosphate-buffered 50 mM sodium solution at 4°C and used within 3 days.

**Antibody-mediated depletion of cells or IL-4 neutralization**

In select mice as indicated in the results, CD4 T cells were depleted or IL-4 neutralized of its activity through the administration of neutralizing antibodies (Bio X Cell, Lebanon, NH) given until a 2 week endpoint. Seven days following surgery, to allow for recovery following surgery, rat anti-mouse CD4 antibody (clone GK1.5) or its corresponding control isotype (clone LTF-2), or rat anti-mouse IL-4 antibody (clone 11B11) or its corresponding control isotype (clone TNP6A7), were given intraperitoneally. Each mouse was given 250 µg of antibody diluted to 100 µL using PBS. These were continued once every other day until the endpoint.

**Immunohistochemistry (IHC)**
To assess cell populations and protein expression within ANAs, nerve samples were explanted at indicated endpoints and immediately placed in 4% paraformaldehyde in phosphate-buffer overnight followed by immersion in 30% sucrose in PBS solution for 24–48 hours. Samples were then frozen in OCT Compound (VWR, Radnor, PA) and sectioned at 15 µm onto pretreated charged glass slides. Sections were rehydrated with PBS and blocked using 5% normal goat serum diluted in PBS before primary antibody staining. Primary antibodies were used to stain for T cells (CD3), axons (NF200), Schwann cells (S100 or SOX10), myelin basic protein (MBP), macrophages (CD68, CD206), and eosinophils (Siglec F). Primary antibody in 5% serum buffer was applied and incubated at 4°C overnight with specific antibodies and concentrations outlined in Table 1. Sections were then washed in PBS and stained for the appropriate fluorochrome-conjugated secondary antibodies for 1 h at room temperature. All sections were mounted with Fluoroshield mounting medium with DAPI (Abcam, Boston, MA) and then imaged using the Fluoview FV1000 confocal microscope and acquisition system (Olympus, Waltham, MA) at overall 200x (20x water immersion objective) or 600x magnification (60x oil immersion objective). A minimum of three sections were analyzed and averaged for each tissue area using ImageJ (NIH) to obtain a value for each single animal (n=1). For percent area, an ImageJ macro was used to quantify the percentage of the area in a standardized field that was positive for the marker measured. For cell counts, field size was kept standard at a 60x objective (600x magnification overall), and colocalization of the primary marker(s) with DAPI was considered a positive cell.

Flow cytometry
To identify and quantify immune cell populations, at the indicated endpoints after surgery, mice were sacrificed and their ANAs, blood (collected via cardiac puncture), or spleen harvested. Nerve tissues and spleen were digested using a cocktail of 1 mL digestion buffer (0.1% collagenase, 0.05% DNase in 2% fetal bovine serum (FBS)/ Dulbecco`s Modified Eagle Media (DMEM)). Samples were incubated for 20 minutes at 37°C with constant agitation, and following digestion, the samples were re-suspended in FACS buffer (2% FBS, 0.1% EDTA in phosphate buffered saline). RBCs from blood or spleen were removed using RBC lysis buffer. After filtering through 75 um membrane, the cells were incubated with Fc block for 10 minutes followed by an antibody cocktail specific to the cell of interest (Supplemental Table 1) for 1 hour before analysis with flow cytometry using BD Fortessa. At least 50,000 events were acquired for flow cytometry. Data was analyzed using FlowJo. Macrophages were gated by CD45+, CD11b+, CD64+, F4/80+. Eosinophils were gated by CD45+, CD11b+, CD64-, SiglecF+. Neutrophils were gated by CD45+, CD11b+, Ly6G+. T cells were gated by CD45+, CD11b-, CD3+. B cells were gated by CD45+, CD11b-, CD3-, CD19+. Single color stained compensation beads were used as control.

Gene analysis (qRT-PCR)

To quantify gene expression from all cells within ANAs, total RNA was prepared from ANA explants. RNA was extracted using Trizol (Life Technologies), chloroform and a RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. RNA concentration was determined on a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The cDNA was generated with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-
time PCR was performed using a Step One Plus thermocycler (Applied Biosystems, Foster City, CA) using Taqman Master Mix (Applied Biosystems) reagents with specific oligonucleotide primer pairs (Supplemental Table 2). The PCR conditions were 50˚C for 2 min and then 95˚C for 15 s and 60˚C for 1 min, repeated for 40 cycles, with a hot start at 95˚C for 10 min. The expression levels of each gene were normalized to those of the internal control gene (Actb). Data was analyzed using Step One Software v2.2.2 (Applied Biosystems, Foster City, CA).

**Histology and histomorphometry**

To quantify the extent of nerve regeneration at a 4 week endpoint in indicated mice, histology and histomorphometric analysis of nerve were performed as previously described (Hunter et al., 2007). Briefly, tissue was collected and fixed via immersion in 3% glutaraldehyde (Polysciences, Inc., Warrington, PA), then post-fixed in 1% osmium tetroxide, and serially dehydrated. These tissues were embedded in epoxy resin (Polysciences) and sectioned on an ultramicrotome for 1.5 µm cross sections. Slides were then counterstained with 1% toluidine blue dye and analyzed at 1000x overall magnification on a Leitz Laborlux S microscope. A semi-automated digital image-analysis system linked to morphometry macros developed for peripheral nerve analysis (Clemex Vision Professional, Clemex Technologies, Longueuil, Québec), was used. At least six random fields per histological section of a single animal were imaged by person blinded to the groupings of the experiments. Binary histomorphometry analysis of the digitized information based on gray and white scales allowed measurements of the myelinated axons. From counting myelinated fibers across the randomly selected fields, calculation of an average nerve fiber density (fibers/mm²) was used to quantify the total number of myelinated
fibers based on the total nerve fascicular area. Also from the averaged fields, the average myelinated fiber width (μm²), percent myelinated fiber, and percent myelin debris was calculated. The values for an animal’s experimental nerve thus reflected a single ‘n’ value, despite multiple fields averaged for a given animal nerve.

**Relative Muscle Mass**

To further quantify the extent of nerve regeneration, relative gastrocnemius muscle mass was measured as it is indicative of reinnervation of denervated muscles. After nerve harvest at 4 weeks, the gastrocnemius muscles were harvested from the experimental and contralateral sides. Wet muscle weight was recorded on each side, and the ratio of the ipsilateral to contralateral muscle weight was calculated.

**Grid walk assessment**

Prior to surgery and measured every two weeks until week 8 post-operative, mice underwent a grid walk assessment to determine their functional recovery. Mice were placed on an elevated mesh or grid with a grid size measuring 3.5cm by 3.5cm. After mice have acclimated to the grid for at least 5 mins, they were recorded with a video camera for at least 4 minutes moving upon the grid. From the video considering the injured limb, the total number of steps with that foot and steps that resulted in a foot placement missing the mesh and going through the grid (slipped steps) was measured. Foot fault was calculated as the proportion of slipped steps to total steps.
Retrograde labeling of motor neurons

Two weeks after a sciatic nerve injury without repair in Rag1KO or WT mice, mice underwent a second procedure under anesthesia as described before. The proximal nerve was transected to expose the axoplasm and placed in a silicone gel (Tyco, Mansfield, MA) well to isolate it. The well was filled with 4% Fluoro-Gold in 0.9% saline to immerse the nerve and label it for 30 mins. The dye was then removed, wound irrigated with saline, and the incision closed in 2 layers as before. After 7 days, the animals were euthanized as before and the lumbar region of the spinal cord was removed and placed in 4% paraformaldehyde. After 24 hours, the spinal cords were placed in 30% sucrose until frozen in OCT Compound (VWR) and cut into 30 µm thick longitudinal sections on a cryostat (Leica, Buffalo Grove, IL). The total number of Fluoro-Gold-positive cell nuclei in the lumbar ventral horns from all slides collected from each animal was counted on an Olympus IX81 microscope using both 10X and 20X objectives (100-200X overall magnification) with a DAPI (365nm) filter (Omega Optical, Brattleboro, VT).

Statistical analysis

Statistical analyses were performed using GraphPad Prism. Each animal was considered an ‘n’ value. All data were compiled as mean ± standard deviation. Data were tested for normality using the Kolmogorov–Smirnov test. Student’s t test was performed for analysis between 2 groups. A significance level of p < 0.05 was used in all statistical tests performed.
3.4 Results

*T cells accumulate within ANAs*

We first determined the extent of T cell (CD3) accumulation within ANAs that were grafted into mice subjected to sciatic nerve injury, which revealed a temporal relationship regarding T cell accumulation within ANAs. Immune fluorescent analysis revealed that few CD3 cells accumulated within ANAs by day 10, but CD3 cell numbers increased substantially by day 14, which was sustained and even slightly increased through day 18. This accumulation of CD3 cells was specific to the ANA, as CD3 cell accumulation in the distal nerve stump was less than 1/3 of those in the ANA throughout all time points examined (*Fig. 19A*).

Then, to further characterize the role of the adaptive immune system during regeneration across ANAs, flow cytometry was used to analyze first the T cell populations and compare them relative to other leukocytes (CD45 cells) within cells isolated from ANAs. Similar to the results from immunofluorescence analysis, the proportion of CD3 cells within ANAs increased over time as regeneration proceeded, where CD3 cell proportions reached 11% of total CD45 cells within the ANA by day 18 (*Fig. 19B*). Furthermore, CD4 T cells were present at ~50% higher proportion compared to CD8 T cells after 10 days post repair. The extent of B cell (CD19) accumulation within ANAs was also assessed. The proportion of CD19 cells was substantially smaller (<1.5% of CD45 cells; *Fig. 19C*) compared to CD3 cells at a 14-day endpoint. Since it was revealed that CD3 cell numbers increase locally within ANAs, we also analyzed the proportion of CD3 and CD19 cells in the spleen at this 14-day endpoint to determine if nerve repair using ANAs also affected systemic cell numbers. However, there was no increase in the
proportion of CD3 or CD19 cells in spleen relative to total CD45 cell numbers (Fig. 19D-E). These results demonstrate that T cells are actively recruited into ANAs during nerve regeneration in a mouse model, where their local numbers within the nerve environment increase as regeneration proceeds.

*Rag1KO models have reduced nerve regeneration across ANAs*

Given the influx of T cells into ANAs, we assessed whether T cells also contributed to nerve regeneration using Rag1KO mice. Two weeks after ANA repair, Rag1KO had no reduction in initial axon growth into ANAs compared to WT, as revealed by NF200, which labels axons (Fig. 20A). However, in Rag1KO almost all of the NF200 axons were non-myelinated, as evidenced by the qualitative lack of co-staining with MBP (Fig. 20A). Conversely, WT had robust MBP co-staining with NF200 axons. Consequently, WT ANAs contained increased MBP expression compared to Rag1KO, based on MBP area. By four weeks after ANA repair, Rag1KO exhibited myelinated axon regeneration, but Rag1KO had reduced numbers of regenerating myelinated axons, both in the ANA (Fig. 20B) as well as at the distal nerve (Fig. 20C), compared to WT. Further histological assessment of axon regeneration revealed that while the G ratio was not different between Rag1KO and WT, Rag1KO had higher levels of myelin debris within ANAs, suggesting a disruption of remyelination during ongoing regeneration (Fig. 20D, Fig 23A for larger images of histology). Furthermore, regenerated myelinated fiber size and myelin area were reduced within Rag1KO compared to WT ANAs (Fig. 20D). Downstream from regenerating nerve, relative gastrocnemius muscle mass in Rag1KO was reduced compared to WT (Fig. 20E). To determine if these changes to nerve
regeneration affected functional recovery, grid walk analysis revealed that Rag1KO had a persistent increase in the proportion of foot faults compared to WT starting at 4 weeks after repair, demonstrating delayed functional recovery (Fig. 20F). Finally, we addressed whether adaptive immune deficiency affected neuron survival. Rag1KO and WT sciatic nerve retrograde traced 2 weeks after the initial nerve injury demonstrated no differences in motoneuron counts (Figure 21). Overall, Rag1KO mice revealed that the adaptive immune system contributes to promoting nerve regeneration across ANAs.

*Rag1 KO models have altered immune environments within ANAs during early stages of nerve regeneration*

To determine how the adaptive immune system, including T cells, might contribute to nerve regeneration across ANAs, we analyzed the ANA environment within Rag1KO compared to WT during early stages of nerve regeneration (i.e. before substantial myelinated axon regeneration cross the ANA). From assessing gene expression from all cells contained within ANAs at 14 days after repair, Rag1KO ANAs contained lower levels of transcripts encoding Th2 cytokines, including IL-4 and IL-13, compared to WT ANAs (Fig. 24A). Based on these cytokine expression patterns, immune cell populations within ANAs were also assessed at this endpoint. IHC analysis confirmed no differences in CD68 cell quantities within ANAs among groups (Fig. 24B). However, when considering the proportion of macrophages, CD68 cells represented a greater proportion of cells within ANAs from Rag1KO compared to WT. Yet, there still remained no change in the proportion of CD68, CD206 cells (M2 macrophages) among groups. Flow cytometry analysis of ANAs also revealed no changes in CD64, F4/80 total
macrophages cell quantities relative to total CD11b cells, nor the proportion of CD64 F4/80 cells expressing CD206, among Rag1KO compared to WT (Fig. 24C). Considering other immune cell populations, Rag1KO ANAs contained a decreased quantity of Siglec F cells, representing eosinophils (Fig. 24D) and no differences in Ly6G neutrophils (Fig. 24E) relative to total CD11b cells compared to WT ANAs. However, despite these local changes within ANAs, there were no systemic changes in CD64 F4/80 or Ly6G cell quantities from Rag1KO compared to WT (Fig. 22). Conversely, Rag1KO did contain increased Siglec F cell proportion within the spleen compared to WT (Fig. 22). Overall, these data demonstrate that the ANA environment derived from Rag1KO models is altered compared to WT and suggest that T cell loss could be a primary reason for the changes based on the shift in Th2 cytokines and corresponding immune cell populations.

*T cells regulate IL-4 expression within ANAs*

Based on the shift/difference in *Il4* transcript levels contained within Rag1KO vs WT ANAs, the cell sources of IL-4 were identified using IL-4/GFP (4Get) mice. A correlation between T cell accumulation and IL-4 expressing cell accumulation was identified. Ten days following nerve repair with ANAs, there were minimal numbers of CD3 cells, nor IL-4-GFP expressing cells, within ANAs. By day 14, both cell populations substantially increased within ANAs compared to day 10 (Fig. 25A). This increase of CD3 cells and IL-4-GFP expressing cells were sustained up to 18 days post repair. However, only a minority of the IL-4-GFP cells colocalized with CD3 cells (Fig. 25B). Instead, the majority of IL-4-GFP cells colocalized with Siglec F cells. Furthermore, flow cytometry analysis verified these findings demonstrating that
Siglec F cells were the majority of IL-4-GFP cells, with over 50% of Siglec F cells expressing GFP while <5% of other immune cells express GFP (Fig. 25C). Overall, this data demonstrated that T cells were not the primary cells expressing IL-4, but instead, their accumulation within ANAs is associated with the accumulation of IL-4 expressing eosinophils. Thus, the data establishes a link between T cells and IL-4 expressing eosinophils.

**CD4 T cells impact eosinophil accumulation within ANA**

To then explore the association between T cells, IL-4 expressing cells, and eosinophils, 4Get mice nerves repaired with ANAs were subsequently depleted of CD4 T cells. Depletion of CD4 cells starting from 7 days post repair up until a 2 week endpoint led to a substantial reduction in the number of CD3 cells that accumulated within ANAs (Fig. 26A). As well, this depletion lead to reduction in IL-4-GFP expressing cells within ANAs compared to isotype control antibody treated mice at 2 weeks (Fig. 26A). To further consider the scope of changes in eosinophil accumulation within ANAs, flow cytometry analysis of major leukocyte populations was performed considering both the ANA and systemic levels in spleen and blood at 2 weeks. Based on these findings, flow cytometry quantified the number of immune cells expressing GFP to further corroborate the sources of IL-4 expression. While CD4 cell depletion reduced Siglec F cell quantities within ANAs compared to the isotype control (Fig. 26B), CD4 depletion did not change the proportion of CD11b myeloid cells within ANAs. As well, despite the reduction in Siglec F cell quantities within ANAs, the proportion of Siglec F cells that expressed GFP within the ANA was not altered. However, considering these effects at a systemic level, while CD4 antibody depletion led to a loss of ~50% of total CD3 cells from blood (Fig. 26C) and spleen
(Fig. 26D), it did not alter the proportion of Siglec F cells relative to CD11b myeloid cells contained in blood or spleen, nor the proportion of Siglec F cells expressing IL-4-GFP. Taken together, these data demonstrate that CD4 T cells regulate eosinophil accumulation within ANAs during early stages of nerve regeneration, but CD4 T cells likely do not affect the abilities of eosinophils to express IL-4.

Depletion of CD4 T cells or neutralization of IL-4 impacts early myelin expression

Given our data demonstrating a role for CD4 T cells, we assessed whether depletion of CD4 T cells in the setting of WT mice impacted nerve regeneration across ANAs. Two weeks after ANA repair, mice that had CD4 depletion showed no changes in axonal regeneration, based on NF200 area (Fig. 27A). However, there was greater degree of myelin expression within ANAs of isotype treated compared to the CD4 depleted mice, based on MBP area. This reduction in myelin expression was likely not caused by a deficiency of SC accumulation within the graft as S100 area was not different between CD4 depleted compared to isotype treated (Fig. 27B). Thus, loss of CD4 cells contributed to delayed myelination of regenerated axons. Given this outcome and the link between CD4 T cells and IL-4 expression, WT mice were also treated with IL-4 antibody to neutralize IL-4 activity within ANAs. Similar to the results of CD4 depletion, ANAs from mice with IL-4 neutralization for two weeks showed no changes in axonal regeneration, based on NF200 area (Fig. 27C), but myelin expression was reduced, based on MBP area. And, similar to CD4 depletion, IL-4 neutralization did not have effects on SC numbers that accumulated within ANAs, based on S100 area (Fig. 27D). Thus, loss of IL-4 contributed to delayed myelination of regenerated axons.
**IL-4 contributes to nerve regeneration across ANAs**

IL-4KO mice were then used to determine if IL-4 alone substantially contributed to nerve regeneration across ANAs. Nerve histology at 4 weeks after ANA repair revealed that IL-4KO had fewer myelinated axon numbers within ANAs compared to WT (Fig. 28A), but no differences in axon numbers across ANAs in distal nerve (Fig. 28B). IL-4KO also had significantly greater level of myelin debris within ANAs compared to WT (Fig. 7C, Fig. S3B for larger images of histology). And while an IL-4 deficiency did not affect the G ratio or Axon area, it did reduce Fiber area, again suggesting a role in myelination. Finally, IL-4KO also demonstrated differences in downstream recovery. IL-4KO had reduced relative gastrocnemius muscle mass compared to WT (Fig. 28D). And critically, IL-4KO had reduced functional recovery compared to WT. Specifically, IL-4KO had increased foot slippage compared to WT during grid walk assessment starting by week 4 (Fig. 28E), mirroring a major finding from the Rag1KO mice studies. Overall, IL-4KO mice models regenerating nerve across ANAs demonstrated deficiencies in nerve regeneration, including myelination and functional recovery.

### 3.5 Discussion

The role of T cells during peripheral nerve regeneration, and in the context of regeneration upon scaffolds, is unresolved. Previously, we found that T cells were associated with successful regeneration across an ANA repairing nerve defects, and in fact, a T cell deficiency decreased regeneration and recovery across ANAs used to repair nerve defects.
However, we did not explore the mechanism by which T cells may promote such effects. Here, we demonstrate that T cells regulate IL-4 during nerve regeneration across ANAs, specifically by recruiting IL-4 expressing eosinophils. In turn, T cells and IL-4 both promoted nerve regeneration across ANAs, as their deficiencies reduced early myelin protein expression and the quantity of myelinated axons regenerating across ANAs, and ultimately reduced functional recovery.

After a nerve injury repaired using ANAs in mice, we observed a sustained accumulation of T cells within the ANA environment. The presence of T cells within ANAs was slightly delayed compared to myeloid cells. It is possible that cells arriving early to the ANAs, such as macrophages, may be required to recruit T cells. For example, macrophage may promote angiogenesis which promotes T cell accumulation within tissue (Lim et al., 2003). Furthermore, T cells accumulated within the graft environment likely are specific to the injury. Indeed, fewer T cells were observed in the injured distal nerve, demonstrating that T cells specifically accumulate within the ANA wound environment, suggesting their potential importance to resolving regeneration in this context. Recent studies have found that the wound environment of a nerve defect is unique compared to the injured distal nerve (Clements et al., 2017). SCs and macrophages within this defect wound environment play unique roles and express different genes compared to those in the distal nerve (Cattin, Burden, VanEmmenis, Mackenzie, Hoving, Garcia Calavia, et al., 2015; Clements et al., 2017). T cells may therefore be recruited into the ANA due to its unique environment.

We determined that nerve regeneration across ANAs and functional recovery in Rag1KO mice, which lack functional B and T cells, was reduced compared to wild-type mice. These results are similar to our previous studies comparing nerve regeneration across ANAs in athymic
rats, which lack functional T cells, to heterozygous T cell sufficient rats (Pan et al., 2019). Rag1KO mice exhibited both reduced regeneration of myelinated axons across the ANA, and reduced functional outcomes. Previous work demonstrated T cells have a role in promoting motoneuron survival following a facial nerve injury (Serpe et al., 2005). Therefore, motoneuron death could account for changes in regeneration in our sciatic nerve injury model in Rag1KO mice. However, our results does not support this mechanism, as Rag1KO and WT mice retrograde tracing revealed no differences in motoneuron counts after a sciatic nerve injury. Furthermore, analysis of the ANA environment of Rag1KO mice revealed evidence of a T cell role within the ANA. We observed reduced expression of Th2 cytokines, in particular Il-4, contained within ANAs of Rag1KO mice. Thus, this led us to hypothesize that reduced IL-4 levels within the ANA environments of T cell deficient mice may be one of the reasons for their reduced regeneration and recovery.

Surprisingly, despite a higher level of IL-4 expression levels contained within the ANAs of in WT mice, which can drive macrophage proliferation and M2 polarization, no differences in macrophage numbers or their polarization was observed compared to Rag1KO mice. It is possible that in WT mice, their ANA environments may contain elevated levels of inflammatory cytokines, which thus dampen the impact of IL-4 on polarization. At present, our cytokine analysis was limited as we primarily focused on anti-inflammatory cytokines given our previous work that demonstrated a relationship between T cells and Th2 cytokine expression (Pan et al., 2019). While no broad differences in macrophages were observed, we did observe an increase in eosinophils in WT mice ANAs compared to Rag1KO mice ANAs, consistent with the elevated IL-4 gene expression, for which eosinophils were a major source. This deficiency of eosinophils in ANAs of RAG1KO mice is likely due to the lack of T cells within the ANA environment,
rather than systemic changes. And in support of this, Rag1KO mice did have greater proportion of eosinophils among their myeloid cells in their spleen, suggesting that the potential for eosinophils to migrate and infiltrate ANAs was not dampened per se.

We also identified sources of IL-4 within ANAs, which is a novel finding regarding the source of IL-4 expression within ANAs, or nerve in general. Using 4get mice, which allowed tracking of all cells expressing IL-4, we determined that eosinophils, but not T cells, are the major source of IL-4. Given the reduced number of eosinophils and lower expression of IL-4 in Rag1KO mice, we hypothesized that lack of T cells may contribute to deficient eosinophil accumulation. This was supported by our observation following CD4 T cell depletion. Loss of CD4 T cells drastically reduced the number of IL-4 expressing cells within ANAs. This general relationship regarding CD4 T cells and IL-4 regeneration is consistent with studies in other organ systems (Gavett, Chen, Finkelman, & Wills-Karp, 1994; Nakajima et al., 1992; Voehringer et al., 2004). While loss of CD4 T cells reduced eosinophil accumulation within ANAs, it did not inhibit IL-4 expression of the few eosinophils that did accumulate. Furthermore, loss of CD4 T cells did not change systemic eosinophil numbers since similar numbers of eosinophils were found in blood of CD4 T cell depleted or isotype control treated mice. Nor did it broadly prevent migration of eosinophils, as splenic eosinophils did not become diminished. Taken together, these data present a novel mechanistic finding, whereby CD4 T cells regulate IL-4 expression by recruiting eosinophils into ANAs.

Consistent with data that CD4 T cells regulate IL-4, we found that depletion of either CD4 T cells or neutralization of IL-4 through antibodies significantly reduced myelin protein expression (MBP) within the ANAs. This similar deficiency in myelin expression during nerve regeneration across ANAs strongly suggests IL-4 has a positive impact on nerve regeneration.
Furthermore, while the number of regenerating myelinated axons and proportion of myelin debris within ANAs was affected in IL-4KO mice, SC accumulation within ANAs was not affected by IL-4 neutralization. These data suggest that glial or macrophage are not the only targets of IL-4, but rather, suggest that IL-4 may directly contribute to the axon-myelination process itself. For example, a possible explanation that will need to be explored is whether IL-4 targets axons leading to changes in axonal expression of signals to SCs, which then promote SC interactions with axons, such as myelin expression.

In the context of nerve regeneration across a defect, the role of IL-4 has been unclear. While supraphysiological levels of IL-4 provided through sustained drug release have been found to promote nerve regeneration across a defect (1 cm) bridged by conduits (Mokarram et al., 2017, 2012), loss of endogenous IL-4 signaling during regeneration across a small nerve defect (~3 mm) had limited impact on the extent of final motor axon regeneration (Tomlinson et al., 2018). Specifically, the loss of IL-4 signaling through IL-4RKO mice resulted in no differences to WT mice regarding the final number of motoneurons regenerating their axons to distal nerve by 8 weeks following a repaired nerve injury (Tomlinson et al., 2018). However, this outcome does not rule out a role for IL-4 signaling in the rate of nerve regeneration, the degree of myelination, or the ultimate functional outcome. We showed, using IL-4KO mice, that loss of IL-4 significantly reduced the number of myelinated axons actively regenerating within the ANA environment by 4 weeks, as well as reduced functional recovery even until 8 weeks. Therefore, our results do not conflict with, but add to, previous evidence on the role of endogenous IL-4 signaling during nerve regeneration.

In conclusion, our data demonstrate that T cells promote nerve regeneration across ANAs. Our data suggest this T cell role is mediated in part through their ability to recruit...
eosinophils, which secrete IL-4 within the ANA environment. Our data also suggests that IL-4 signaling subsequently promotes the myelination of regenerating axons, as well as promotes functional recovery. As a number of studies have focused on using exogenous IL-4 delivery during tissue regeneration as a therapeutic agent due to its action on macrophages, future studies should also consider the effect of IL-4 on other cell types, including neurons and their axons. The ability of T cells to regulate IL-4 and eosinophils also suggest possible strategies to modulate local environments by promoting T cell accumulation or targeting T cells therapeutically.
A

Day 10

Day 14

Day 18

ANA graft

Distal nerve

B

Day 10

CD8+ 23.4

CD4+ 36.7

Day 14

CD8+ 26.3

CD4+ 52.7

Day 18

CD8+ 29.8

CD4+ 41.7

C

Day 14

CD19+

SSC

D

Day 10

CD8+ 39.5

CD4+ 46.6

Day 14

CD8+ 39.8

CD4+ 50.0

Day 18

CD8+ 41.2

CD4+ 42.0

E

Day 14

CD19+

SSC

114
Figure 19. T cells temporally accumulate within ANAs repairing sciatic nerve of WT mice

A) Representative immunofluorescence images of T cells (CD3, red) within ANA and distal nerve at 10, 14, and 18 days after repair with corresponding quantification. B) Representative flow cytometry analysis for CD4 and CD8 T cells within ANA. Cells were gated on CD45⁺CD11b⁻CD3⁺. Total T cells relative to hematopoietic cells (CD3/CD45) and the proportion of CD4 to CD8 T cells were quantified. C) Representative flow cytometry and quantification of B cells (CD19) within ANA. Cells were gated on CD45⁺CD11b⁻CD3⁻. D) Representative flow cytometry and quantification of CD4 and CD8 T cells from spleen. Cells were gated on CD45⁺CD11b⁻CD3⁺. E) Representative flow cytometry and quantification of B cells from spleen. Cells were gated on CD45⁺CD11b⁻CD3⁻. Mean ± SD, n=3/group; p values shown.
The extent of nerve regeneration across ANAs in WT vs Rag1KO mice was assessed at multiple endpoints. A) Representative immunofluorescence and quantification of neurofilament (NF200, green) and myelin (MBP, red) in the mid-graft of ANA at 2 weeks. At 4 weeks after ANA repair, representative histological images and quantification of myelinated axon regeneration in the mid-graft of ANA (B) and distal nerve (C). D) Additional quantification of histomorphometric data from the mid-graft of ANA. E) Relative (to uninjured side) weight of gastrocnemius muscle at 4 weeks. F) Functional assessment (behavior) following ANA repair over time. Mean ± SD, n=5/group IHC and functional assessment; n=8/group histology and muscle assessment at 4 weeks; p values shown.
Figure 21. Rag1KO mice does not have diminished neuron survival after injury

Retrograde traced motor neurons from the proximal nerve 2 weeks after an initial sciatic nerve injury. No differences in traced neurons from sciatic nerve injury resulted in RAG1KO vs WT. Mean ± SD, n=4/group; p values shown.
Figure 22. Quantification of immune cell populations from spleen of Rag1KO and WT mice

Macrophages (F4/80, CD64) among CD11b myeloid cells, CD206 macrophages among all macrophages, eosinophils (Siglec F), and neutrophils (Ly6G) were quantified using flow cytometry after dissociation of cells from spleen. Mean ± SD, n=4/group; p values shown.
Figure 23. High magnification histological images of regeneration.

A). Representative histological images of mid-graft of ANAs from Rag1KO or wildtype control.

B) Representative histological images of mid-graft of ANAs from IL-4KO or wildtype control.

Arrowhead, myelin debris. Arrow, aberrantly myelinated axons.
Figure 24. Cytokine expression and myeloid cell repopulation within ANAs is altered in Rag1KO mice at 2 weeks

A) Gene expression of select cytokines from all cells contained within ANAs. B) Representative immunofluorescence and quantification of macrophages (CD68, green) and CD206 macrophages (CD206, red) from the mid-graft of ANAs. C) Representative flow cytometry and quantification of macrophages (F4/80 CD64) among myeloid cells (CD11b), and proportion of CD206 macrophages among all macrophages. Cells were gated on CD45^+CD11b^+F4/80^+CD64^+.

Representative flow cytometry and quantification of neutrophils (Ly6G, D) and eosinophils (Siglec-F, E) among myeloid cells. Cells were gated on CD45^+CD11b^+. Mean ± SD, n=5/group; p values shown.
Figure 25. Eosinophils are the major source of IL-4 within ANAs during early regeneration

IL-4-GFP mice sciatic nerve were repaired using ANA to identify sources of IL-4. A) Representative images and quantification of T cells (CD3, red) and IL4-GFP cells (green) within ANAs at 10, 14, and 18 days after ANA repair. B) Representative images and quantification of T cells and eosinophils (Siglec F) colocalization with GFP expressing cells at 14 days. C) Representative flow cytometry and quantification of proportion of neutrophils, macrophages, eosinophils, and T cells from spleen expressing GFP at 14 days after ANA repair. Neutrophils were gated on CD45+CD11b+Ly6G+. Macrophages were gated on CD45+CD11b+F4/80+CD64+. Eosinophils were gated on CD45+CD11b+SiglecF+. T cells were gated on CD45+CD11b+CD3+. Mean ± SD, n=3/group; p values shown.
**Figure 26. CD4 T cells affect IL-4 expression within ANAs via their regulation of eosinophils**

IL-4-GFP mice sciatic nerve were repaired using ANA and treated with antibodies to deplete CD4 T cell accumulation within ANAs up to the 14 day endpoint. A) Representative images and quantification of IL4-GFP cells (green) and T cells (CD3, red) within ANAs. B) Representative flow cytometry and quantification of eosinophils (Siglec F) and IL4-GFP expression among eosinophils within ANAs, including the proportion of myeloid cells (CD11b) among CD45 cells, eosinophils among myeloid cells, and IL4-GFP cells among eosinophils. Quantification of flow cytometry of blood (C) and spleen (D) from treated mice for the proportion of T cells among hematopoietic cells (CD45), eosinophils among myeloid cells, and IL4-GFP cells among eosinophils. Mean ± SD, n=3/group; p values shown.
Figure 27. CD4 T cells and IL-4 are critical to early myelination of axons within ANAs

WT mice sciatic nerve were repaired using ANA and treated with antibodies to deplete CD4 T cells or neutralize IL-4 up to the 14 day endpoint. A&B) Representative immunofluorescence and quantification of myelin (MBP, red), neurofilament (NF200, green), and Schwann cells (S100, green) within ANAs for CD4 depletion groups. C&D) Representative immunofluorescence and quantification of myelin (MBP, red), neurofilament (NF200, green), and Schwann cells (S100, green) within ANAs for IL-4 neutralization groups. Mean ± SD, n=5/group; p values shown.
**Figure 28. Nerve regeneration across ANAs in IL-4KO mice is impaired**

The extent of nerve regeneration across ANAs in WT vs IL-4KO mice was assessed at multiple endpoints. At 4 weeks after ANA repair, representative histological images and quantification of myelinated axon regeneration in the mid-graft of ANA (A) and distal nerve (B). C) Additional quantification of histomorphometric data from the mid-graft of ANA. D) Relative (to uninjured side) weight of gastrocnemius muscle at 4 weeks. E) Functional assessment (behavior) following ANA repair over time. Mean ± SD, n=5/group histology and muscle assessment at 4 weeks; n=5/group functional assessment; p values shown.
Table 3. Antibodies for immunofluorescence and their dilutions used in chapter 3

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Table 4. Antibodies for flow cytometry and their dilutions chapter 3

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Table 5. Real time RT-PCR primers and their Thermo Fisher Assay ID used in chapter 3

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Chapter 4 Summary of findings

The overall findings from my work demonstrated that T cells are important for the regeneration of peripheral nerve in both rats and mice through regulation of the cytokine IL-4. In Chapter 2 I set out to identify factors that differ between short ANAs, that facilitated robust regeneration, and long ANAs, that failed to support axon regeneration. We found that long ANAs had few myelinated axons within both the ANA graft, as well as in the distal stump. We further demonstrated that Schwann cells within long ANAs have distinctly altered gene expression where both genes related to Schwann cell maturation, and Schwann cell activation are down-regulated. By comparing cells that infiltrated the ANAs early during the regenerative process (at 2 and 4 weeks), we were able to identify a T cell deficiency, in addition to changes in macrophage and angiogenesis as factors that are different between short and long ANAs during the regenerative process. We subsequently determined that lack of T cells reduced both functional recovery and regeneration of myelinated axons when nerves are repaired using ANAs. Surprisingly, we found that lack of T cells did not contribute significantly to regeneration when nerves were repaired with isografts, suggesting a unique role of T cells in facilitating regeneration through acellular gaps resulting from nerve injury.

In Chapter 3 we further defined the role of T cells in the regenerative process. We confirmed that adaptive immunity, in general, (using Rag1KO mice) and CD4 T cells specifically (using antibody-mediated depletion of CD4 T cells in WT mice) are important for regeneration of myelinated axons in mice models of nerve injury repaired using ANA. Lack of adaptive immunity led to reduced expression of Th2 cytokines including IL-4 and IL-13. Surprisingly, we
found that lack of T cells did not change the accumulation of macrophages in the ANAs or the expression of CD206 (an indicator of IL-4 polarization) on these macrophages. Instead, we found that lack of T cells reduced the accumulation of eosinophils. Using transgenic mice, we found that eosinophils are the major source of IL-4 within the ANA environment. Using an antibody-mediated depletion approach, we found that depletion of CD4 T cells reduced eosinophil accumulation, similar to those observed in Rag1KO mice. Interestingly, the depletion of CD4 reduced eosinophil accumulation but did not change their IL-4 expression, suggesting that CD4 T cells are important for the recruitment of eosinophils but did not change the regulation of IL-4 expression within eosinophils. Finally, utilizing IL-4KO mice and antibody-mediated neutralization of IL-4 in WT mice, we determined that IL-4 is important for the regeneration of myelinated axons within ANAs.

As summarized in Fig 29, my work here demonstrated the importance of T cells in regulating the recruitment of eosinophils, which express pro-regenerative cytokines, such as IL-4, within the ANA environment. IL-4 promotes regeneration by promoting myelination of axons within the ANAs to facilitate regeneration and functional recovery.
Figure 29 Summary of finding

Current understanding of nerve regeneration following transection injury proceeds via the following steps: A) Macrophages are recruited to the site of injury and express VEGF due to the local hypoxia. B) VEGF allows vascularization of the injury, and Schwann cells, guided by fibroblasts and macrophages, migrates into the site of injury. C) Aided by the Schwann cells, axons start to extent into the injured site. Our work shows that in addition, when the nerve injury is repaired using ANAs, D) T cells migrate to the ANA graft. E) There, these T cells begin to recruit eosinophils that express IL-4. IL-4 subsequently promotes the regeneration of myelinated axons. F) In contrast, large nerve grafts that failed to regenerate have low quantities of T cells and subsequently limited regeneration of myelinated axons.
Chapter 5 Future work

While my data has demonstrated the role of T cells in regeneration of ANA repaired nerves, it also shed light on additional questions that are unanswered.

Firstly, my current work demonstrated the importance of T cells in the regeneration of peripheral nerve. However, the role of another group of leukocytes—macrophages—was not investigated. My data (see appendix) demonstrated that macrophages, contributes to the regeneration within ANAs. However, how macrophages may be recruited into ANAs is not yet clear. Previously, Schwann cells have been found to produce IL-6, CCL2, and LIF in vitro. These factors in turn, have been shown to recruit macrophage in vitro. In particular, I have found that CCL2 contributes to the recruitment of macrophages. Mice deficient in CCL2 have reduced macrophage accumulation within ANAs after 2 weeks of surgery (see appendix). CCL2 is expressed by diverse population of cells within the peripheral nerve, including Schwann cells, macrophages, and neurons. How cell specific CCL2 may contribute to regeneration needs to be investigated to clarify the role of CCL2.

Secondly, my current work demonstrated the important role of T cells in regulating IL-4 through recruitment of IL-4 expressing eosinophils. We studied the specific roles of T cells in regeneration of peripheral nerve when repaired with ANAs. However, what role, if any, T cells play in the regeneration of peripheral nerve when repaired with autografts is not clear. In the rat model, when nerve injury was repaired with autograft, the lack of T cells did not impact regeneration. This may suggest that both T cells and IL-4 are not required for regeneration when autografts are used. Alternatively, eosinophils can be recruited without T cells when injuries are repaired with
autografts. Further elucidating the role of T cells in different types of nerve injury and repair scenarios will further clarify the role of T cells.

Finally, while we have shown that IL-4 is important for promoting myelination of axons, we did not identify the specific target(s) of IL-4. Previous studies have shown IL-4 signaling to neurons or macrophages can promote regeneration, suggesting that these can cells may be the target of IL-4. For example, in vitro addition of IL-4 to neuron induced increased calcium transient and promoted neuron survival (Vogelaar et al., 2018). On the other hand, loss of macrophage IL-4 receptor reduced their local proliferation and prevented timely conversion to pro-regenerative macrophages (Knipper et al., 2015). While these data suggest that macrophage and neurons are possible candidate for IL-4 within the peripheral nerve, it has been shown that Schwann cells also express receptors for IL-4 (Ozaki et al., 2008). When exogenous IL-4 were added in vitro, it altered Schwann cell expression of CD209, a phagocytic receptor (Teles et al., 2010). Thus, there are multiple possible candidates for the role of IL-4 and isolating the cell type responsible will be critical for deeper mechanistic understanding of how IL-4 promotes regeneration of myelinated axons.

Based on the current work I have presented and the questions that arise from my results, I therefore present the following future aims:

5.1. Dissecting the role of macrophages in regeneration of ANAs
Previous studies have demonstrated the importance of macrophages in promoting angiogenesis, which is required for migration of Schwann cells into site of injury. My preliminary (see appendix) data shows that angiogenesis is also required for efficient migration of Schwann cells into ANAs following their repair, and that macrophage, recruited by the chemokine CCL2, are mediators of efficient angiogenesis. Blockage of VEGF receptor, a key signaling mediator of angiogenesis, abolishes vascularization of the ANAs as well as Schwann cell migration. Interestingly, while T cell accumulation was abolished in the absence of blood vessels, macrophages continue to accumulate. Depletion of macrophages using clodronate liposome, a drug that specifically depletes macrophages, led to reduced angiogenesis and Schwann cell accumulation. To determine if hematogenously derived macrophage was necessary for angiogenesis, nerve repair was performed in CCL2KO mice, which has no monocyte accumulation in the circulation. CCL2KO mice developed reduced vasculature compared to the wildtype control, and reduced accumulation of Schwann cells in the ANAs. Thus, hematogenously derived macrophages are likely the major contributor to vascularization of ANAs.

To further elucidate the role of macrophages, future work will need to consider the role of tissue resident macrophages on the regeneration of peripheral nerve when repaired with ANAs. To deplete tissue resident macrophages, a tamoxifen inducible CX3CR1-DTR mice could be used. As described in published work, mice can be given tamoxifen followed by diptheria toxin to deplete CX3CR1+ macrophages (Diehl et al., 2013). Two weeks of lead time will allow circulating monocytes and macrophages to return to normal level due to high level of proliferation in the bone marrow while keeping the tissue resident CX3CR1+ macrophages depleted. To then examine the role of tissue resident macrophages, sciatic nerves can be transected and repaired using ANAs.
Two weeks later, the regeneration across ANAs can be quantified by examining the area of CD31+ blood vessels, S100+ Schwann cells, and NF200+ axons.

5.2. Examining the role of IL-4 in other models of nerve injury and repair

In Chapter 2 we demonstrated that T cells promote regeneration of peripheral nerve across ANAs. However, T cells had limited effect on the regeneration of autografts. In Chapter 3, we identified regulation of IL-4 is one avenue of how T cells promote regeneration of myelinated axons. Therefore, why T cells are dispensable in the context of autograft mediated repair is worth exploring. One possibility is that IL-4 is not required for regeneration. Another possibility is that recruitment of IL-4 expressing cells, such as eosinophils, can occur independent of T cells. Indeed, recruitment of eosinophils in response to injury can occur without T cell mediation.

To explore these questions, we could utilize commercially available CD4 KO mice to investigate the role of IL-4 and T cells in autograft. Repair of nerve injury in CD4KO or WT mice can be repaired with isogenic nerve grafts, and the regeneration followed to examine the role of CD4 T cells in nerve regeneration. Similar experiments can be done using IL-4KO mice. Quantification of axonal extension and myelination could allow us to determine the role, if any, of IL-4 in facilitating regeneration of autografts. If axonal regeneration is reduced in IL-4KO mice but not in CD4KO mice, it would suggest that IL-4, but not T cells, are required for regeneration. We could then utilize flow cytometry and immunofluorescence to determine the proportion and accumulation of eosinophils within the autografts following injury in CD4KO mice and WT
control. If there are no differences in the eosinophil number in CD4KO and WT control, it would suggest that CD4 T cells are not necessary for the recruitment of IL-4 expressing eosinophils. On the other hand, if eosinophil numbers are different, we could then utilize IL-4gfp mice to identify the source of IL-4 within autograft repaired nerves.

5.3. Dissecting the mechanism of IL-4 mediated regeneration of myelinated axons

In Chapter 3 we demonstrated that IL-4 promotes the regeneration of myelinated axons within ANAs. However, how IL-4 is able to achieve this effect was not identified. Using cell-specific depletion of IL-4 receptor could allow us to identify the cell population that is most critical for regeneration of myelinated axons. At present, we hypothesize that the changes in myelination in IL-4 deficient mice are due to altered signaling within neuron itself. This is because we did not observe changes in Schwann cell number or macrophage number within the ANAs, suggesting that IL-4 may have limited impact on these cells. Furthermore, axons express IL-4 receptor following injury, suggesting that it may be a target of IL-4. To identify how neurons respond to IL-4 during regeneration across ANAs, we could utilize commercially available transgenic Cre mice specific to motoneurons (Hb9-Cre) or sensory neurons (BAF53b-Cre). We can knockout IL-4R from neurons by crossing these lines with the established floxed IL-4R mice (Knipper et al., 2015). The IL4R floxed mice harbor loxP-flanked sequences that span the IL-4R gene enabling the conditional disruption of IL-4R via Cre-induced recombination. Under control of the endogenous upstream elements, cre expression is directed to motor or sensory neurons. Using these conditionally depleted IL-4R mice will allow us to determine if myelination was
delayed in these mice following nerve repair with ANAs. Further functional studies can be performed to determine if lack of IL-4R within the neurons also impacted functional recovery. However, if specific depletion of IL-4R within the neurons did not impact changes in myelination, we can also cross the IL4R floxed mice into MPZ-cre or LysM-Cre to deplete IL-4 receptor in Schwann cells or myeloid cells respectively, to determine if loss of IL-4 signaling in these cells impacted regeneration of myelinated axons.
Appendices

Introduction

Peripheral nerve injuries are common, and severe damage to a nerve can result in a gap between the nerve ends causing a potentially devastating loss of function (Robinson, 2000). While small nerve defects can be repaired directly, longer defects require the use of nerve grafts to bridge the gap (Ijkema-Paassen et al., 2004; Rbia and Shin, 2017). Off-the-shelf alternatives to nerve autografts are increasingly desired and used in the clinic. Yet, a prevailing concern when using alternatives clinically is the general variability and inconsistency in outcomes (Kallio, 1993). Furthermore, as the length of alternatives increases to repair longer gap, axon regeneration becomes limited, a phenomenon that has been observed across many autograft alternatives (Mokarram et al., 2017; Saheb-Al-Zamani et al., 2013). Therefore, further knowledge regarding the biology of regeneration across alternatives is critical toward understanding these shortcomings.

Acellular nerve allografts (ANAs) are one such clinically-available alternative, where nerves are obtained from allogenic donors and decellularized to minimize antigenicity. ANAs also represent a useful model to understand the processes mediating nerve regeneration across a clinically-relevant nerve gap. Because ANAs are initially acellular, they require repopulation by host cells, including migration of Schwann cells, but first undergo angiogenesis and vascularization by endothelial cells (Pan et al., 2019; Poppler et al., 2016). In previous studies, we identified decreased angiogenesis and effective vascularization within long (4 cm) compared to short (2 cm) ANAs. This decreased vascularization of long ANAs was followed by decreased Schwann
cell or T cell accumulation within ANAs, and ultimately failed axon regeneration across the ANA (Pan et al., 2019). Insufficient angiogenesis and lack of proper vascularization are widely known to inhibit tissue regeneration (Hankenson et al., 2011; Madeddu, 2005). Therefore, while our observations did not demonstrate reduced angiogenesis was the cause of failure, these associations suggest that link. Furthermore, they serve as strong motivation to further understand the role of vascularization in cell repopulation of ANAs, as well as how angiogenesis is promoted within ANAs.

Understanding what promotes angiogenesis could be key to understanding why the extent of angiogenesis differed between ANAs based on length. The process of angiogenesis is largely promoted by sprouting of vessels to the hypoxic region, whereby existing vessels allow for endothelial cell proliferation and migration to the hypoxic regions (Risau, 1997). Subsequently, the newly arrived endothelial cells at the site of injury form vessels that connect to the existing vasculature. In the case of a small nerve gap, the innate immune system is responsible for promoting angiogenesis within the gap region (Cattin et al., 2015; Cattin and Lloyd, 2016). Specifically, macrophages infiltrate the nerve injury site, and produce pro-angiogenic factors, such as vascular endothelial cell growth factor (VEGF). However, it is not known if macrophages also promote angiogenesis within ANA used to repair clinically-relevant sized nerve gaps. Furthermore, because of the larger volume requiring angiogenesis, there may be roles for other cells of the innate immune response that are known to aid macrophage functions, such as neutrophils. One such example is neutrophil. Neutrophils are the earliest leukocytes to arrive at site of injury, and can promote macrophage recruitment or otherwise alter gene expression in macrophages (Beck-Schimmer et al., 2005; Warnatsch et al., 2015). Thus, it is
possible that neutrophils arrive at ANAs prior to macrophages and their accumulation in ANA is required for macrophages to migrate into ANAs.

In addition, while it has been shown that macrophages are important for angiogenesis, the sources of macrophage, as well as the chemokines for recruiting macrophages are not clear. Two major sources of macrophages have been described. Tissue resident macrophages are niche specific macrophages that resides in various tissues including nerve. Given specific signals, these macrophages proliferate and carry out tissue specific functions. Hematogenously derived macrophages are monocytes that became mature following recruitment to specific site. Previously, CCR2 signaling has been found to be important for recruitment of macrophages to sciatic nerve following injury (Lindborg et al., 2017). Multiple ligands can engage CCR2 receptor to signal macrophage influx including CCL2, CCL7, and CCL8 (Crown et al., 2006). In particular, chemokine (C-C motif) ligand 2 (CCL2), also referred to as monocyte chemoattractant protein 1 (MCP1), is known to be produced in sciatic nerve following injury, and recruits macrophages both in vitro (Martini et al., 2008; Van Steenwinckel et al., 2015). Thus, CCL2 represent a possible candidate for the recruitment of macrophages into ANAs following repair. Defining what macrophage populations and how these macrophages are recruited may shape the immune environment within the ANAs, and can thus better inform our understanding of regeneration across ANAs.

Therefore, in this paper, we explored the contribution of angiogenesis to nerve regeneration following repair with ANAs. We then dissected the role of macrophages and neutrophils, if any, in promoting angiogenesis and subsequent nerve regeneration. In addition, we asked how macrophages accumulate within the ANAs and if CCL2 contributes to this process.
Materials and methods

Reagents and Chemicals

All reagents, consumables, and chemicals, unless otherwise stated, are purchased from Sigma-Aldrich (St. Louis, MO)

Animals care and use

Commercially-available male mice 7 to 10 weeks old (20-25g, Jackson Laboratories, Bar Harbor, ME) were utilized for all experiments. Mice were randomized to groups for experimental treatments. Randomized C57BL/6J mice were used as donor mice to derive ANAs. Surgical procedures and peri-operative care measures were conducted in compliance with the AAALAC accredited Washington University Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health guidelines. All animals were housed in a central animal care facility and provided with free access to food (PicoLab rodent diet 20, Purina Mills Nutrition International, St. Louis, MO) and water.

Surgical procedures

For surgical procedures, mice were anesthetized using a cocktail of ketamine (100 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and dexmedetomidine (0.5 mg/kg; Pfizer Animal Health, Exton, PA). For mice serving as ANA donors, following euthanization, their
sciatic nerves were transected proximally at the level of exiting nerve roots, and distally just beyond the sciatic trifurcation. In experimental mice receiving the ANAs, the right sciatic nerve was exposed and transected 5 mm proximal to the distal trifurcation. The indicated grafts were reverse oriented (distal end of donor graft facing proximal nerve stump of recipient) sutured into the nerve gap with 11-0 nylon microsuture (Sharpoint, Reading, PA). A two-layer closure of muscle and skin was performed using 6-0 vicryl and 4-0 nylon suture, respectively. Atipamezole solution (0.1mg/kg; Zoetis, Florham Park, NJ) was administered for anesthesia reversal. The animals were recovered on a warming pad and monitored for postoperative complications before returning them to a central animal care facility. Postoperative pain was managed using Buprenorphine SR™ (0.05 mg/kg; ZooPharm, Windsor, CO). Animals were monitored daily post-operatively for signs of infection and/or distress. At the appropriate endpoints, animals were euthanized via cervical dislocation under anesthesia, and their tissues collected for respective studies (see below).

**ANA processing**

ANAs were decellularized using a modified series of detergents in the method described previously (Poppler et al., 2016). Nerves isolated from donor animals the nerves were repeatedly washed in deionized water and three detergents in a sodium phosphate buffer: Triton X-100, sulfobetaine-16 (SB-16), and sulfobetaine-10 (SB-10). All grafts were washed and stored in 10 mM phosphate-buffered 50 mM sodium solution at 4°C and used within 1 week.
Immunohistochemistry (IHC)

To assess cell populations and protein expression within ANAs, nerve samples were explanted at indicated endpoints and immediately placed in 4% paraformaldehyde in phosphate-buffer overnight followed by immersion in 30% sucrose in PBS solution for 24–48 hours. Samples were then frozen in OCT Compound (VWR, Radnor, PA) and sectioned at 15 µm onto pretreated charged glass slides. Sections were rehydrated with PBS and blocked using 5% normal goat serum diluted in PBS before primary antibody staining. Primary antibodies were used to stain for T cells (CD3), axons (NF200), Schwann cells (S100 or SOX10), myelin basic protein (MBP), and macrophages (CD68, CD206). Primary antibody in 5% serum buffer was applied and incubated at 4°C overnight with specific antibodies and concentrations outlined in Supplementary Table 1. Sections were then washed in PBS and stained for the appropriate fluorochrome-conjugated secondary antibodies for 1 h at room temperature. All sections were mounted with Fluoroshield mounting medium with DAPI (Abcam, Boston, MA) and then imaged using the Fluoview FV1000 confocal microscope and acquisition system (Olympus, Waltham, MA) at overall 200x (20x water immersion objective) or 600x magnification (60x oil immersion objective). A minimum of three sections were analyzed and averaged for each tissue area using ImageJ (NIH) to obtain a value for each single animal (n=1). For percent area, an ImageJ macro was used to quantify the percentage of the area in a standardized field that was positive for the marker measured. For cell counts, field size was kept standard at a 60x objective (600x magnification overall), and colocalization of the primary marker(s) with DAPI was considered a positive cell.
Flow cytometry

To identify and quantify immune cell populations, at the indicated endpoints after surgery, mice were sacrificed and their ANAs, blood (collected via cardiac puncture), or spleen harvested. Nerve tissues and spleen were digested using a cocktail of 1 mL digestion buffer (0.1% collagenase, 0.05% DNase in 2% fetal bovine serum (FBS)/ Dulbecco’s Modified Eagle Media (DMEM)). Samples were incubated for 20 minutes at 37°C with constant agitation, and following digestion, the samples were re-suspended in FACS buffer (2% FBS, 0.1% EDTA in phosphate buffered saline). RBCs from blood or spleen were removed using RBC lysis buffer. After filtering through 75 um membrane, the cells were incubated with Fc block for 10 minutes followed by an antibody cocktail specific to the cell of interest (Supplemental Table 2) for 1 hour before analysis with flow cytometry using BD Fortessa. At least 50,000 events were acquired for flow cytometry. Data was analyzed using FlowJo. Macrophages were gated by CD45+, CD11b+, CD64+, F4/80+. Neutrophils were gated by CD45+, CD11b+, Ly6G+. Single color stained compensation beads were used as control.

Relative Muscle Mass

To further quantify the extent of nerve regeneration, relative gastrocnemius muscle mass was measured as it is indicative of reinnervation of denervated muscles. After nerve harvest at 6 weeks, the gastrocnemius muscles were harvested from the experimental and contralateral sides. Wet muscle weight was recorded on each side, and the ratio of the ipsilateral to contralateral muscle weight was calculated.
**Grid walk assessment**

Prior to surgery and measured every two weeks until week 6 post-operative, mice underwent a grid walk assessment to determine their functional recovery. Mice were placed on an elevated mesh or grid with a grid size measuring 3.5cm by 3.5cm. After mice have acclimated to the grid for at least 5 mins, they were recorded with a video camera for at least 4 minutes moving upon the grid. From the video considering the injured limb, the total number of steps with that foot and steps that resulted in a foot placement missing the mesh and going through the grid (slipped steps) was measured. Foot fault was calculated as the proportion of slipped steps to total steps.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism. Each animal was considered an ‘n’ value. All data were compiled as mean ± standard deviation. Data were tested for normality using the Kolmogorov–Smirnov test. Student’s t test was performed for analysis between 2 groups. A significance level of p < 0.05 was used in all statistical tests performed.

**Results**

*Endothelial cell accumulation and angiogenesis occurs prior to appreciable Schwann cell repopulation of ANAs*
Previously, we determined that processes involved in nerve regeneration across ANAs repairing a sciatic nerve gap occurred in a temporal manner, whereby significant axon growth, Schwann cell and T cell repopulation within ANAs occurred starting at two weeks post-repair (Pan et al., 2019). Therefore, first we established a timeline of endothelial cell migration and vessel formation within ANAs, and this relationship was described with respect to Schwann cell repopulation of ANAs.

Based on the extent of CD31+ area (endothelial cells), CD31 cells, and some vessel formation, were present, but their presence was limited within ANAs 10 days following repair. Subsequently, the proportion of CD31+ cells increased significantly over time. CD31+ area increased to nearly 5% of total area within ANAs by day 14, and further increased to 8% by day 18 (Supplemental Fig. 1). Concurrently, the formation of elongated vessels became prominently clear over this same time period. Similarly, based on the extent of S100+ area (Schwann cells), Schwann cell accumulation within ANAs closely followed endothelial cell accumulation and increased in proportion to the extent of vessel formation. While no S100+ cells were detectable on day 10 within ANAs, S100+ area increased by day 14 onward (Supplemental Fig. 1). Overall, this data demonstrates appreciable angiogenesis only closely precedes Schwann cell repopulation of ANAs, and continues to progress during ongoing nerve regeneration across ANAs.

*Blockade of angiogenesis inhibits Schwann cell and T cell repopulation of ANAs*

Given the association between increased endothelial cell quantities and angiogenesis closely preceding increased Schwann cells quantities within ANAs, we next assessed whether endothelial cells and the formation of vessels was responsible for cell migration and repopulation.
of ANAs. WT mice given cabozantinib to inhibit VEGF signaling showed drastically reduced endothelial cells quantities, based on decreased CD31+ area, within ANAs compared to untreated mice (Fig. 2A). Furthermore, no vessels could be discerned within the ANAs. As a result of cabozantinib and decreased endothelial cells and vessels, these ANAs also had severely reduced Schwann cell accumulation, based on decreased S100+ area (Supplemental Fig. 2A), and essentially no T cell accumulation (CD3+ area) compared to ANAs from untreated mice (Supplemental Fig. 2B). Conversely, macrophages were only modestly impacted by cabozantinib. While the total number of CD68+ cells (macrophage) within ANAs was reduced in cabozantinib treated compared to untreated mice, there was also an increase in the proportion of CD68+ macrophages compared to other cell types (Supplemental Fig. 2C). Finally we assessed axonal regeneration and found similar to Schwann cells, that inhibition of angiogenesis drastically reduced axonal regeneration (Supplemental Fig 2D). This data demonstrated that VEGF signaling was critical to endothelial cell accumulation and angiogenesis within ANAs, which in turn promoted Schwann cell and T cell repopulation but had only moderate influence on macrophage recruitment.

*Cells of the innate immune system accumulate prior to endothelial cells and remain during ongoing angiogenesis*

Given the presence of CD68 macrophages in ANAs despite blocking VEGF signaling, we reasoned that macrophages, and other cells of the innate immune system, could accumulate within ANAs before endothelial cell migration and angiogenesis. Thus, leukocytes likely are major contributors of angiogenesis. To determine the extent of innate immune system
accumulation within ANAs, flow cytometry was used to analyze these cell populations relative to total leukocytes (CD45 cells). While CD64+ F4/80+ macrophages consisted of the majority of CD11b+ myeloid cells, proportion of neutrophils were highest early during regeneration (day 10 post surgery) and declined throughout the regeneration process (Supplemental Fig. 3A). On the other hand, the proportion of CD11b+ cells among all leukocytes, or the proportion of CD45+ cells among all cells did not change over time (Supplemental Fig. 3B). To further visualize the changes in macrophages over time, we then stained CD68+ macrophages within the ANAs 10, 14, and 18 days post repair, and found that their highest number occurs at day 10, 14, and was reduced at day 18 (Supplemental Fig. 3C).

*Macrophages promote substantial angiogenesis within ANAs, while neutrophils have limited contributions to angiogenesis within ANAs*

Based on the observations that the innate immune system is recruited to ANAs prior to angiogenesis, and the limited disruption to macrophage recruitment despite blockade of VEGF signaling, the role of the innate immune system in endothelial cell migration and angiogenesis was determined.

Macrophage were depleted through administering clodronate liposomes to WT mice, which severely reduced macrophage accumulation within the ANAs in a dose dependent manner. Longer duration of clodronate liposome administration led to greater depletion of CD68+ macrophages (Supplemental Fig. 4A). Regardless of clodronate liposome dose, however, endothelial cell quantities (CD31+ area) and vessel formation was reduced within these ANAs from treated mice compared to ANAs from untreated mice. In turn, Schwann cell accumulation
within ANAs from clodronate liposome treated mice were also reduced compared to ANAs from untreated mice (Supplemental Fig. 4B). Altogether, these data demonstrate that macrophages, and not neutrophils, have essential contributions driving endothelial cell migration and angiogenesis within ANAs. As well, the concurrent accumulation of Schwann cells within ANAs associated with angiogenesis suggests that, again, angiogenesis is responsible for their accumulation within ANAs.

The role of neutrophils was assessed by their depletion in WT mice administered Ly6G antibody. Despite this substantial neutrophil depletion, no differences in endothelial cell quantities (CD31+ area) or vessel morphology were observed within ANAs from these mice compared to ANAs derived from isotype antibody treated mice (isotype control, Supplemental Fig 5A). Likewise, Schwann cell accumulation within the ANAs of these neutrophil depleted mice was not different than ANAs from control mice (Supplemental Fig. 5B). We also examined CD68+ macrophage accumulation within ANAs and found no difference between isotype treated and Ly6G depleted mice (Supplemental Fig 5C).

**Hematogenous macrophages impact regeneration**

Given the impact of macrophage on promoting regeneration, we wondered next what impact hematogenous macrophage has on regeneration. CCR2KO mice has reduced circulating monocytes(Boring et al., 1998). We therefore examined regeneration of ANA repaired nerve in WT and CCR2KO mice. CCR2KO mice had significantly reduced CD68+ macrophages (Supplemental Fig 6C). Consistently, we found that CCR2KO mice had significantly reduced CD31+ blood vessel. Similarly S100+ Schwann cells, and reduced NF200+ axons (Supplemental Fig. 6A-B).
Macrophages are recruited to ANAs via CCL2, which also regulates their phenotype

Given the importance of monocyte derived macrophages on regeneration in ANAs, we wondered what signaling is important for the recruitment of these monocytes. CCL2 is a chemokine produced by Schwann cells, neurons, and endothelial cells following injury. We therefore assessed if CCL2, the primary chemokine responsible for recruiting CCR2 monocytes, contributed to their recruitment within ANAs.

Following nerve repair with ANAs, the accumulation of CD68 macrophages, based on CD68+ area, was greater within ANAs from WT mice compared to CCL2KO mice (Supplemental Fig. 7A). To determine if difference in macrophage accumulation between ANAs from WT vs CCL2KO mice was due to recruitment vs proliferation, we assessed the quantity of macrophages expressing proliferation marker Ki-67. Comparing the proportion of Ki-67 expression among CD68 macrophages, no differences between ANAs from these mice were found (Supplemental Fig. 7B). Next, given that CCL2 has been known to promote macrophage polarization to an M1 inflammatory phenotype, we also examined the proportion of CD206 (M2) CD68 macrophages through immunofluorescence. Fewer CD206 CD68 macrophages accumulated within ANAs from WT compared to CCL2KO mice, a proportion of CD206+ cells of CD68 macrophages (Supplemental Fig. 7A). To further confirm the macrophage phenotype changes between WT and CCL2KO mice, we utilized flow cytometry to analyze the macrophages within ANAs. Similarly, CCL2KO mice ANAs had greater proportion of CD206 macrophages compared to WT ANAs (Supplemental Fig. 7C). Finally, we examined the proportion of Ly6C+ monocytes in blood of WT and CCL2KO mice and found significant but small differences (Supplemental Fig. 7D), suggesting that changes in the macrophages within ANAs are most likely due to CCL2 mediated recruitment of monocytes into the ANAs (Supplemental Fig. 7B). On the other hand,
we did not observe difference in neutrophil proportion in the blood of CCL2KO and WT mice (Supplemental Fig. 7D). These data taken together with data derived from clodronate liposome experiments demonstrates that macrophage accumulation within ANAs is largely driven by hematogenous-derived monocytes/macrophages.

Loss of CCL2 impedes regeneration

Given the role of CCL2 in macrophage recruitment within ANAs, we assessed the impact of CCL2 loss on regeneration. Fourteen days after nerve transection and repair with ANAs, we found that the ANAs from WT mice had significantly more endothelial cells than those of CCL2KO mice based on CD31+ area (Supplemental Fig. 8A). Consistent with the observation that endothelial cells and blood vessel formation is associated with Schwann cell accumulation, Schwann cell accumulation (based on S100+ area) was also reduced within ANAs of CCL2KO vs WT mice (Supplemental Fig. 8A). Overall, these data suggest that CCL2 is required for effective regeneration.

Discussion

There has been increasing realization of the importance of macrophages for peripheral nerve regeneration. Macrophages has been shown to be important for promoting Schwann cell maturation, clearance of myelin, and orchestrating angiogenesis(Cattin et al., 2015; Lindborg et al., 2017; Stratton et al., 2018). However, the role of macrophages in graft repaired nerve injury is less understood. Given the acellular nature of ANAs, angiogenesis is required to facilitate
regeneration. Our work here demonstrated that macrophages are important for angiogenesis, macrophages that promote angiogenesis within ANAs are primarily hematogenous-derived macrophages, and that efficient SC and T cell repopulation and regeneration only occurs following vascularization of ANAs. Furthermore, we demonstrated that CCL2 is one of the chemokines that promotes recruitment of macrophages.

Following nerve repair with ANAs, we observed a drastic increase in angiogenesis within the nerve graft. Surprisingly, this is a relatively delayed process as there was limited angiogenesis even 10 days after injury, and as well, angiogenesis continues for at least another 8 days concurrent with the accumulation of key regenerative cells, such as Schwann cells. Thus, angiogenesis is tightly linked to regeneration of peripheral nerve through ANAs. Furthermore, unlike small gap injuries (~2-3 mm) where angiogenesis occurs rapidly (<4 days)(Cattin et al., 2015), larger gaps (10 mm) repaired by ANAs require substantially more time to vascularize.

Unsurprisingly, angiogenesis was necessary for nerve regeneration across ANAs. Inhibition of angiogenesis almost completely abolished Schwann cell accumulation. Previously, it was shown that in a nerve transection model resulting in a small minor gap, endothelial cells are required as substrate for Schwann cell migration(Cattin et al., 2015). Despite the well-organized ECM matrix provided by ANAs between a nerve gap, we found that endothelial cells are still required for Schwann cells to accumulate within the ANAs. Furthermore, we found that T cells, which contribute to axonal regeneration in ANA repaired nerves(Pan et al., 2019), were absent in ANAs without angiogenesis, suggesting that blood vessels are required for T cells to accumulate. Finally, while the proportion of macrophages increased in ANAs without blood vessel – likely due to the decrease in number of Schwann cells and T cells – their total number decreased. It is possible that macrophages that first arrive within ANAs are independent of blood vessels, but
subsequent recruitment of additional macrophages may require blood vessels. For example, additional monocytes may be recruited to ANAs, where they mature into macrophages (Shi and Pamer, 2011).

We also explored which myeloid (CD11b+) populations are required for angiogenesis. Neutrophils and macrophages have both been suggested to contribute to angiogenesis, either independently or in conjunction (Cattin and Lloyd, 2016; Lin et al., 2006; Shojaei et al., 2008). However, their contribution to angiogenesis in ANAs or other scaffold repaired nerves have not been assessed. Here, using antibody-mediated depletion of systemic neutrophils, we found that loss of neutrophils had limited impact on angiogenesis. On the other hand, loss of macrophages drastically impacted regeneration. This is consistent with previous findings that macrophages are a major source of VEGF, and loss of VEGF from myeloid cells impacted angiogenesis (Cattin et al., 2015). Here, we demonstrated that loss of macrophage led to both a drastic reduction in angiogenesis and Schwann cell accumulation.

Macrophages in injured nerve can be derived via local proliferation of resident macrophages, or recruitment of monocytes that mature into macrophages. Both sources of macrophages occur within the injured nerves (Mueller et al., 2003). Utilizing CCR2KO mice, we demonstrated that monocyte derived macrophages are important for angiogenesis. Various chemokines signals through CCR2, including CCL2, CCL7. Among them, CCL2 is expressed by Schwann cells and neurons, making it a likely candidate for macrophage recruitment within the peripheral nerve (Kwon et al., 2015; Niemi et al., 2016; Van Steenwinckel et al., 2015). Indeed, we found that loss of CCL2 reduced macrophage accumulation by had no impact on the proliferation of macrophages, suggesting that it promotes the recruitment of hematogenous macrophages. Furthermore, loss of CCL2 resulted in reduced angiogenesis and Schwann cell accumulation.
This is consistent with our data that loss of macrophage accumulation within ANAs contributed to reduced regeneration.

In conclusion, our data demonstrated that CCL2/CCR2 promotes the recruitment of hematogenous macrophages to ANAs, and that macrophages are important for promoting angiogenesis and subsequent accumulation of Schwann cells within ANAs.
Appendix Figure 1. Regeneration within ANAs are time dependent.
Representative immunofluorescence images and quantification of ANAs 10, 14, and 18 days post sciatic nerve repair. ANAs are stained for endothelial cells (CD31, green), and Schwann cells (S100, red).
Appendix Figure 2 Inhibition of angiogenesis disrupts regeneration.

A) Representative immunofluorescence staining, and quantification of CD31 and S100 of ANA repaired nerves in mice receiving cabozantinib or water control. B) Representative immunofluorescence staining and quantification of CD31 and CD3 of ANA repaired nerves in mice receiving cabozantinib or water control. C) Representative immunofluorescence staining and quantification of CD68 and CD206 of ANA repaired nerves in mice receiving cabozantinib or water control. D) Representative immunofluorescence staining and quantification of NF200 of ANA repaired nerves in mice receiving cabozantinib or water control.
Appendix Figure 3 Macrophages accumulate in ANAs in a time dependent fashion. Quantification from flow cytometry of CD64+F4/80+ macrophages, Ly6G+ neutrophils, Ly6C monocytes, CD11b+ myeloid cells, and CD45+ leukocytes within ANAs 10, 14, and 18 days post repair. B) Representative immunofluorescence staining and quantification of CD68+ area of ANA repaired nerves in mice 10, 14, and 18 days post repair.
Appendix Figure 4 Depletion of macrophages disrupts regeneration

A) Representative immunofluorescence staining and quantification of CD68 and CD206 of ANA repaired nerves in mice receiving PBS or clodronate liposome. B) Representative immunofluorescence staining and quantification of CD31 and S100 of ANA repaired nerves in mice receiving PBS or clodronate liposome.
Appendix Figure 5 Neutrophil is dispensable for regeneration within ANAs

A) Representative immunofluorescence staining and quantification of CD31 and S100 of ANA repaired nerves in mice receiving PBS or clodronate liposome. B) Representative immunofluorescence staining and quantification of NF200 of ANA repaired nerves in mice receiving PBS or clodronate liposome. C) Representative immunofluorescence staining and quantification of CD68+ area of ANA repaired nerves in mice receiving PBS or clodronate liposome.
Appendix Figure 6 CCR2 signaling is required for regeneration within ANAs

A) Representative immunofluorescence staining and quantification of CD31 and S100 of ANA repaired nerves in CCR2KO mice and WT control. B) Representative immunofluorescence staining and quantification of NF200 of ANA repaired nerves in CCR2KO mice and WT control. C) Representative immunofluorescence staining and quantification of CD68 and CD206 of ANA repaired nerves in CCR2KO mice and WT control.
Appendix Figure 7 CCL2 signaling modulate macrophage recruitment

A) Representative immunofluorescence stainining and quantification of CD68 and CD206 of ANA repaired nerves in CCL2KO mice and WT control. B) Representative immunofluorescence stainining and quantification of CD68 and CD206 in ANA repaired nerves in CCL2KO mice and WT control. C) Representative flow cytometry showing proportion of CD206+ cells among CD64+F4/80+ macrophages in CCL2KO mice and WT control and proportion of CCR2+ macrophages among all CD64+F4/80+ macrophages. D) Quantification of proportion of Ly6C+ monocytes and Ly6G+ neutrophils in blood of WT and CCL2KO mice.
Appendix Figure 8 CCL2 is required for optimal regeneration

A) Representative immunofluorescence staining and quantification of CD31 and S100 of ANA repaired nerves in CCL2KO mice and WT control. B) Representative immunofluorescence staining and quantification of NF200 of ANA repaired nerves in CCL2KO mice and WT control.


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