THE ROLE OF INTERLEUKIN-10 IN CD4+ T CELL-MEDIATED NEUROPROTECTION AFTER FACIAL NERVE INJURY

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Anatomy and Cell Biology,
Indiana University

May 2019

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I dedicate this work to my parents, Frederick and Karen Runge. This work would not have been possible without your support and love.

ACKNOWLEDGMENTS

I am grateful for the contributions of many individuals who made this work possible. I sincerely thank Dr. Kathryn Jones, my Ph.D. mentor. Her guidance has influenced my growth as a scientist tremendously. I am deeply grateful for her commitment to supporting the members of her laboratory as they pursue their professional goals. Her approachability, communicativeness, inquisitive nature, and compassion for her students exemplify what a good mentor should be. I would also like to thank the members of my research committee, Drs. Michelle Block, Virginia Sanders, Dale Sengelaub, and Xiao-Ming Xu. The expertise of each individual member provided diverse perspectives that shaped the trajectory of my research project in truly invaluable ways. I am thankful for the Anatomy and Cell Biology department graduate student advisor, Dr. Joseph Bidwell, for his commitment to ensuring the success of each student by always looking out for our best interests. I would also like to thank the department administrative staff, including Kate McMillan, Marthe Augustin, and Tracy McWilliams, for all of their hard work in keeping the department up and running. I also sincerely thank the National Institutes of Health and the Indiana Clinical and Translational Sciences Institute, which generously provided funding for my graduate studies.

I am deeply thankful for the members of the Jones laboratory who have helped me along the way. Dr. Deborah Olmstead Setter taught me nearly everything I needed to know to get my projects off the ground. I am truly lucky to have had such a talented individual act as both mentor and friend to guide me through the unique pressures of graduate school. I thank Felicia Kennedy for her instrumental work in assisting with my projects and for being a wonderful friend in the laboratory. I am grateful for Drs. Todd

Brown, Chandler Walker, and Abhirami Iyer, who have been invaluable sources of help and advice. I would also like to thank Eric Regele for his hard work and dedication in contributing to the neuroimmune project.

I am thankful for the members of the Indiana University Medical Scientist Training Program, including co-directors Dr. Raghu Mirmira, Maureen Harrington, Brittany-Shea Herbert, and Rebecca Chan, and our program administrator Janice Receveur. The importance of the supportive and nurturing environment that the MSTP provides for its students cannot be overstated. I am also deeply thankful for my colleagues and friends in the MSTP, especially Katharine Andrews, Alexander Kiel, Brittani Bungart, Eric Hawley, and Benjamin Ulrich, who began their MSTP careers with me in 2013, as well as Victoria Alexe' Engel and Mohammad Aref, who joined our cohort along the way. The challenges of medical and graduate school are easier to meet in the company of good friends.

I would also like to extend special thanks to my undergraduate research mentor at Loyola University Chicago, Dr. M. William Rochlin, who inspired me to apply to a graduate program in the first place. Dr. Rochlin fostered a rigorous scientific environment in which undergraduate students could attain mastery and autonomy over their research projects to a degree that is rarely seen. The skills I gained under his tutelage have served me well throughout graduate school, and I am sure they will continue to benefit me throughout my career.

I am deeply grateful for my parents and best friends, Karen and Frederick Runge, for their endless love and encouragement. I am extremely thankful for all of the sacrifices they made to give me the best education possible. They instilled in me at an early age a

love of reading, learning, and discovery that laid the foundation for all of my future academic and personal achievements. I would not be the person I am today without their support. Finally, I thank Daniel Duzenbury, whose love and boundless optimism have provided me with motivation and solace while I navigate the challenges of graduate school. This dissertation would have taken twice as long to write without his gentle reminders to stay on track.

Elizabeth Marie Runge

THE ROLE OF INTERLEUKIN-10 IN CD4+ T CELL-MEDIATED NEUROPROTECTION AFTER FACIAL NERVE INJURY

The adaptive arm of the immune system is necessary for facial motoneuron (FMN) survival after facial nerve axotomy (FNA). CD4+ T cells mediate FMN survival after FNA in an interleukin-10 (IL-10) dependent manner, but are not themselves the cellular source of neuroprotective IL-10. The aims of this study are to elucidate the neuroprotective capacity of cell-specific IL-10 expression, and to investigate the manner in which CD4+ T cells participate in IL-10 signaling after FNA.

Immunohistochemistry revealed that FMN themselves were constitutive producers of IL-10, and astrocytes were induced to make IL-10 after FNA. *Il10* mRNA co-localized with microglia before and after axotomy, but microglial production of IL-10 protein was not detected. To determine whether any single source of IL-10 is critical for FMN survival, Cre/Lox mouse strains were utilized to selectively knock out IL-10 in neurons, astrocytes, and microglia. In agreement with the localization data reflecting concerted IL-10 production by multiple cell types, no single cellular source of IL-10 was necessary for FMN survival.

Gene expression analysis of wild-type, immunodeficient, and immune cell-reconstituted animals was performed to determine the role of the immune system in modulating the central IL-10 signaling cascade. This revealed that CD4+ T cells were necessary for full upregulation of central IL-10 receptor (IL-10R) expression after FNA, regardless of their own IL-10R beta (IL-10RB) expression or IL-10R signaling capability. Surprisingly, the ability of CD4+ T cells to respond to IL-10 was critical for their ability

to mediate neuroprotection. Adoptive transfer of IL-10RB-deficient T cells resulted in increased central expression of genes associated with microglial activation, antigen presentation, T cell co-stimulation, and complement deposition in response to injury. These data suggest that IL-10RB functions on the T cell to prevent non-neuroprotective immune activation after axotomy.

The conclusions drawn from this study support a revised hypothesis for the mechanisms of IL-10-mediated neuroprotection, in which IL-10 serves both trophic and immune-modulating roles after axotomy. This research has implications for the development of immune-modifying therapies for peripheral nerve injury and motoneuron diseases.

Kathryn J. Jones, Ph.D., Chair

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AKT	Ak strain transforming, also known as protein kinase B
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
APC	Antigen presenting cell
Ax	Axotomized
B2m	β2-microglobulin (gene)
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-x_L	B-cell lymphoma extra large
BDNF	Brain-derived neurotrophic factor
bp	Base pair
BSA	Bovine serum albumin
C	Control
CCL_	Chemokine C-C motif ligand _
CCR_	Chemokine C-C motif receptor _
CD_	Cluster of differentiation _
CD40L	CD40 ligand
cDNA	Coding DNA
CIPN	Chemotherapy-induced peripheral neuropathy
cKO	Conditional knockout
CNS	Central nervous system
CR3	Complement receptor 3
CSF	Cerebrospinal fluid
CT	Cycle threshold
CX3CL_	Chemokine C-X3-C motif ligand _
CX3CR_	Chemokine C-X3-C motif receptor _
DAPI	4′,6-diamidino-2-phenylindole
ddH_2O	Distilled, deionized water
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dpo	Days post operation
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinases
FISH	Fluorescent *in situ* hybridization
FMN	Facial motoneuron
FMNuc	Facial motor nucleus
FNA	Facial nerve axotomy
GAP-43	Growth-associated protein 43
GLAST	Glutamate aspartate transporter

GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GSK-3β	Glucose synthase kinase 3 beta
h	Hour
H2ab1	histocompatibility 2, class II antigen A, beta 1 (gene)
HIV-1	Human immunodeficiency virus 1
HRP	Horseradish peroxidase
IBA1	Ionized calcium binding adaptor molecule 1
IFNγ	Interferon gamma
IHC	Immunohistochemistry/immunohistochemical
IL-_	Interleukin-_
IL-10R	IL-10 receptor
Il10ra	IL-10R alpha (gene)
IL-10RA	IL-10R alpha (protein)
Il10rb	IL-10R beta (gene)
IL-10RB	IL-10R beta (protein)
iNOS	Inducible nitric oxide synthase
IRES	Internal ribosome entry site
JAK1	Janus kinase 1
JNK	c-Jun N-terminal kinases
LCM	Laser capture microdissection
LIF	Leukemia inhibiting factor
LMN	Lower motoneuron
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein 1
MHC_	Major histocompatibility complex class _
min	Minutes
mRNA	Messenger RNA
MS	Multiple sclerosis
NeuN	Neuronal nuclei
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMJ	Neuromuscular junction
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
Nos2	Nitric oxide synthase 2 (gene name for iNOS)
NTF	Neurotrophic factor
OCT	Optimum cutting temperature
PACAP	Pituitary adenylyl cyclase activating polypeptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PECAM-1	Platelet/endothelial cell adhesion molecule 1

PEN	Polyethylene naphthalate
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
qPCR	Quantitative polymerase chain reaction
RAG-2	Recombinase activating gene 2
RER	Rough endoplasmic reticulum
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT	Reverse transcription
SC	Schwann cell
Scid	Severe combined immunodeficient
sec	Seconds
SEM	Standard error of the mean
SOCS_	Suppressor of cytokine signaling _
SSC	Saline-sodium citrate
STAT_	Signal transducer and activator of transcription _
T-BET	T-box expressed in T cells
Tbx21	T-box transcription factor 21 (gene name for T-BET)
TCR	T cell receptor
TEA	Triethanolamine
TGFβ	Transforming growth factor beta
Th_	T helper cell _
Thy1	Thymocyte antigen 1 (gene)
TLR	Toll-like receptor
TNFR1	TNF receptor 1 (protein)
Tnfrsf1	TNF receptor superfamily 1 (gene)
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
TYK2	Non-receptor tyrosine-protein kinase 2
UMN	Upper motoneuron
WT	Wild-type
μMT	IgM heavy chain μ mutation

CHAPTER 1: INTRODUCTION

Damage to peripheral nerves results in significant disability due to impairment of motor, sensory, and autonomic functions. The National Institute of Neurological Disorders and Stroke estimates that 20 million Americans suffer from some form of peripheral neuropathy (*NINDS,* 2018). Although nerve damage due to direct trauma is rarer than diabetes- or chemotherapy-induced neuropathy, it is estimated that between two and five percent of patients admitted to Level I trauma centers have some form of peripheral nerve injury (PNI) (Noble et al., 1998). Among military personnel, the incidence is much higher. Approximately 15% of medically retired Army service members have sustained some form of PNI (Rivera et al., 2014). Healthcare costs for the treatment of PNI can range from a median of $18,000/case for single nerve injuries to over $34,000/case for complex brachial plexus injuries (Lad et al., 2010). In spite of the substantial costs of treating these injuries, clinical outcomes are poor. Only 10% of nerve injuries that require surgical repair have outcomes comparable to their pre-injury functional capacity (Portincasa et al., 2007). Improved therapies that promote better outcomes after nerve injury are greatly needed. Understanding the mechanisms of recovery after PNI will likely also aid in the discovery of therapies for other axonopathies such as amyotrophic lateral sclerosis (ALS). Similar to PNI, a precipitating insult on the distal portion of the motor unit is theorized to be a significant contributing factor in the pathogenesis of ALS (Fischer et al., 2004; Mariotti & Bentivoglio, 2000; Morgan & Orrell, 2016).

There are three basic requirements for functional recovery after PNI: 1) central neuron survival, 2) axon regeneration, and 3) reconnection to target. Because

motoneurons are post-mitotic and cannot be replaced after injury, promoting neuron survival is paramount for successful recovery. Neuro-immune interactions promote neuron survival after injury. After facial nerve axotomy (FNA), immunodeficient mice that lack the adaptive arm of the immune system have decreased facial motoneuron (FMN) survival in the facial motor nucleus (FMNuc) compared to wild-type (WT) mice with functioning immune systems (Serpe et al., 1999; Serpe et al., 2000). This neuroprotective effect has been determined to be specific to CD4+ T cells (Serpe et al., 2003). CD4+ T cells must encounter major histocompatibility complex class II (MHCII) on antigen presenting cells (APCs) in the periphery to obtain their neuroprotective ability (Byram et al., 2004). Most major subsets of CD4+ T cells expand in the draining deep cervical lymph nodes after FNA (Xin et al., 2008). Only the T helper cell 2 (Th2) subset has been identified as critical for motoneuron survival after FNA, although both the Th1 and Th2 subsets may be important for axon regeneration (Beahrs et al., 2010; Deboy et al., 2006b). Having encountered MHCII and multiplied peripherally, T cells are next attracted to the FMNuc by centrally-derived chemokines (Wainwright et al., 2009a; Wainwright et al., 2009b; Wainwright et al., 2009c), where they must again encounter MHCII on a central APC in order to confer neuroprotection (Byram et al., 2004).

The precise manner in which CD4+ T cells mediate motoneuron survival in the FMNuc after axotomy is unknown. The secretion of neurotrophic factors (NTFs) is one potential mechanism, although studies of the FMNuc have failed to find a T cell-specific NTF that is necessary for FMN survival (Serpe et al., 2005; Xin et al., 2012). Furthermore, axotomy triggers FMN to upregulate expression of molecules that are important for axonal regeneration, regardless of the presence of CD4+ T cells. Recent

studies have indicated that CD4+ T cells may rather exert their neuroprotective influence by regulating the glial response to axotomy (Setter et al., 2018b).

The anti-inflammatory cytokine interleukin-10 (IL-10) may be an important mediator of the neuroprotective capability of CD4+ T cells. IL-10 was first discovered as a Th2-produced cytokine capable of suppressing pro-inflammatory cytokine production by Th1 cells (Fiorentino et al., 1989). Its sources and actions are now understood to be much more diverse (Moore et al., 2001). CD4+ T cells cannot mediate FMN survival when IL-10 is absent; however, they themselves are not the requisite source of IL-10. It is likely that a central cell type in the FMNuc mediates FMN survival via IL-10 production (Xin et al., 2011). Although IL-10 is required for CD4+ T cells to confer neuroprotection, the central cellular source of IL-10 as well as the details of T cell involvement in the IL-10 signaling cascade in the axotomized FMNuc have not been previously identified. This information is necessary to understand the process of endogenous immune-mediated neuroprotection and thereby identify potential mechanisms that can be exploited for the treatment of nerve injury and disease. *The central hypothesis for this study is that CD4+ T cells are critical for potentiating microglial-derived IL-10 signaling and facilitating its neuroprotective capacity in the FMNuc.*

This hypothesis was tested in the following aims:

Aim 1: Determine the central cellular sources of IL-10 in the facial motor nucleus and the respective contributions of these sources to motoneuron survival after axotomy. *The hypothesis for this aim is that microglia are the obligate source of neuroprotective IL-10 after axotomy.* After ruling out a peripheral immune contribution of neuroprotective IL-10, the first part of this aim was tested using two methods of

immunohistochemical (IHC) identification of IL-10-producing cells, as well as fluorescent *in situ* hybridization (FISH) for *Il10* mRNA in the FMNuc. The results reveal that FMN produce IL-10 constitutively, whereas IL-10 expression is induced by axotomy in astrocytes. Microglia co-localize with *Il10* mRNA, but protein translation is not apparent. For the second part of this aim, IL-10 was selectively knocked out in neurons, astrocytes, and microglia using Cre/Lox technology. Concordant with the localization data, no single cellular source of IL-10 is necessary for FMN survival. It is concluded that the multiple central sources of IL-10 have redundant roles for FMN survival after axotomy.

Aim 2: Characterize adaptive immune cell participation in neuroprotective IL-10 receptor signaling after facial nerve axotomy. *The hypothesis for this aim is that CD4+ T cells are necessary for central IL-10 receptor expression and directly respond to IL-10 themselves to confer neuroprotection.* IHC labeling was utilized to determine that CD4+ T cells are capable of interacting directly with central cells in the FMNuc and thus may regulate changes in central gene expression via cell-cell interactions. Quantitative polymerase chain reaction (qPCR) analysis for IL-10 receptor (IL-10R) gene expression was performed in the FMNuc of WT, immunodeficient, and CD4+ T cell-reconstituted animals. The results indicate that CD4+ T cells are necessary for full induction of IL-10R gene expression after axotomy, although this induction does not appear to depend upon IL-10 signaling by the T cells themselves. However, IL-10 signaling by CD4+ T cells is necessary for their ability confer neuroprotection. CD4+ T cells deficient in IL-10R expression promote a gene expression signature in the FMNuc suggestive of enhanced microglial activation, antigen presentation, T cell co-stimulation, and complement

deposition, indicating that T cells unresponsive to IL-10 may promote a state of immune activation detrimental to FMN survival. A future direction will be to determine whether this failure to mediate FMN survival is due simply to loss of normal Th2-mediated neuroprotective mechanisms, or to counteraction of these mechanisms by an opposing T cell process that is actively harmful to FMN survival.

CHAPTER 2: LITERATURE REVIEW

2.1. Peripheral nerve injury

2.1.1. Anatomic organization and basic principles

In simplified terms, the role of the nervous system is to receive information from the internal or external physical environment, process and integrate these signals, and convey a response of the body to stimuli. Neurons and glia are two main cell types constituting the nervous system. Neurons are highly specialized cells that are optimized for the transmission of signals. Glial cells, which are the more abundant cell type in the nervous system, have numerous and complex roles that fundamentally maintain the environment necessary for neurons to perform their functions. Anatomically, the nervous system can be divided into central and peripheral compartments. The central nervous system (CNS) consists of the brain and spinal cord, and the peripheral nervous system (PNS) consists of ganglia and nerves. The PNS can be further subdivided on a functional basis into sensory, motor, autonomic, and enteric cell populations.

Voluntary movement requires coordinated activity by both upper and lower motoneurons (UMNs and LMNs). UMN cell bodies reside in the cortex of the brain and project axons that travel in white matter bundles called tracts. These axons synapse on LMNs that reside in brainstem nuclei and spinal cord columns. LMNs project axons that exit the CNS and form peripheral nerves that terminate at specialized synapses called neuromuscular junctions (NMJ). At the NMJ, motoneuron impulses that are sent to target muscle fibers trigger a cascade of events that ultimately results in muscle contraction.

Trauma to peripheral nerves results in interruption of these signals and failure of voluntary movement. Types of nerve injuries are categorized according to their severity.

Neurapraxia is the mildest form of nerve injury in which the myelin sheath is damaged but axons are left intact, resulting in a temporary conduction block followed by complete and rapid recovery. This type of injury typically occurs due to nerve compression. *Axonotmesis*, which occurs after crush or contusion injury, is more severe than neurapraxia because it constitutes axotomy (severing of the axon) followed by subsequent degeneration. However, the connective tissue sheaths surrounding the injured axons, fascicles, and nerves (termed the endoneurium, perineurium, and epineurium, respectively) remain intact to guide subsequent regeneration. With this neural sheath intact, regeneration can occur at a rate of 1–5 mm/day, depending on the size of the nerve. *Neurotmesis* is the most severe form of injury characterized by complete transection of the axon and nerve sheath (Seddon, 1942; Seddon et al., 1943; Sunderland, 1951). This form of injury is most likely to result in death of the central cell body, although the amount of neuronal death that occurs after axotomy is variable (Moran & Graeber, 2004).

Because motoneurons are terminally differentiated, they cannot multiply to replace neurons that have died after axotomy. Promoting central neuron survival is critical for subsequent axon regeneration, reconnection to target, and functional recovery. The development of therapies for nerve injury will depend on elucidation of the mechanisms that determine whether a neuron survives or dies after axotomy. A neurotmesis injury was utilized in this study to investigate these mechanisms.

2.1.2. The facial nerve axotomy model

2.1.2.1. Anatomy and topographic organization

The facial nerve receives its motor contribution from neurons in the FMNuc, which is located in the dorsal pons in humans and the ventral pons in rodents. Axons from the FMNuc travel dorsally and loop around the abducens nucleus, forming a structure called the *genu*, prior to exiting the ventral pons. This unusual course is due to migration of facial motoneurons around the abducens nucleus during embryogenesis (Chandrasekhar, 2004). The intracranial portion of the nerve is joined by the nevus intermedius, which is composed of parasympathetic fibers from the superior salivary nucleus in the pons and both general and special sensory fibers from the geniculate ganglion *en route* to brainstem sensory nuclei. As a mixed nerve, the facial nerve enters the internal acoustic meatus located in the petrous portion of the temporal bone and travels through the facial canal of the skull. In the facial canal, most non-motor fibers diverge to join the greater petrosal nerve and the chorda tympani. A short motor branch also emerges to innervate the stapedius muscle of the inner ear. Immediately after the facial nerve exits the stylomastoid foramen, the posterior auricular nerve and branches to the stylohyoid and digastric muscles diverge from the main nerve trunk. Some reports indicate that the posterior auricular nerve also contains a sensory component (Semba & Egger, 1986). The remaining main trunk of the facial nerve consists only of motor fibers. The facial nerve then enters the parotid gland and splits into its five main terminal branches: temporal, zygomatic, buccal, marginal mandibular, and cervical. These branches innervate the muscles of facial expression and the platysma.

Because none of the muscles that the facial nerve innervates cross a joint, the FMNuc consists of a pure α-motoneuron population without any γ-motoneurons necessary for innervation of muscle spindles. The number of FMN in the FMNuc vary according to species, and estimates range from 2000–3000 in mouse (Ashwell, 1982) to 3000–5000 in rat (Semba & Egger, 1986; Watson et al., 1982). The FMNuc is divided into three main columns (Watson et al., 1982) and six subnuclei (Ashwell, 1982). These divisions follow a "mirror-image" somatotopic organization. The dorsal and ventral regions of the FMNuc innervate the upper (frontalis and orbicularis oculi) and lower (mentalis) facial muscles, respectively. Medial-lateral organization of the FMNuc is reversed: the lateral subnuclei consists of neurons that project somewhat to the midline (vibrissae and nasolabial muscles), the intermediate subnuclei project to the buccal muscles/facial platysma, and the medial to the temporal and auricular muscles (Ashwell, 1982; Komiyama et al., 1984; Semba & Egger, 1986; Watson et al., 1982).

2.1.2.2. The facial nerve axotomy as a model of target disconnection-induced motoneuron death

FNA is a useful model of motoneuron injury for a number of reasons. If lesioned close to its emergence from the stylomastoid foramen, only motor fibers will be damaged. The mostly homogenous population of α-motoneurons housed in the FMNuc allow for their study without dilution by other uninjured neuron populations. There are also no interneurons or cross-talk between contralateral FMNuc; thus, the uninjured side may be used as a paired internal control (Isokawa-Akesson & Komisaruk, 1987). FNA does not cause any physical disruptions affecting blood-brain barrier permeability

(Raivich et al., 1998), and infiltration of the FMNuc by peripheral macrophages is not observed after axotomy (Graeber et al., 1988c). Finally, FNA is a relatively mild injury that does not result in loss of vital functions of the animal (Jones et al., 2015).

The extent of FMN death that occurs after FNA is dependent on a number of variables. In general, FMN death as well as time to regeneration is greater after proximal compared to distal lesions (Dai et al., 2000; Mattsson et al., 1999; Sharma et al., 2009). Avulsion injury causes the most severe phenotype, although this is probably due to disturbance of the blood-brain barrier, which is unique to this form of axotomy (Soreide, 1981a). There is also significant variability in the amount of FMN death between species. Hamsters have virtually no FMN death after axotomy at the stylomastoid foramen (Huppenbauer et al., 2005), whereas mice have approximately 15% FMN death at four weeks post lesion and 50% death at ten weeks post lesion (Serpe et al., 2000; Setter et al., 2018a). The age at which the axotomy is performed is also a significant factor to FMN death. Embryonic and neonatal animals have significantly greater rates of FMN death after axotomy than adult animals, possibly due to the reliance of motoneurons on target-derived growth factors during the developmental period (Huppenbauer et al., 2005; Lavelle & Lavelle, 1959; Soreide, 1981b). Different subnuclei within the FMNuc also exhibit different amounts of FMN death. Although the overall average amount of FMN death at one month post axotomy in mice is approximately 15%, the ventrolateral subnucleus has the greatest amount of cell death at 30%, whereas the ventromedial subnucleus has the least amount of death at 3% (Canh et al., 2006). This discrepancy may be due to the greater distance of the lateral nuclei from NTFs provided by penetrating blood vessels from the basilar artery, which is located at the ventral midline of the

brainstem, or perhaps due to intrinsic differences in motoneurons that innervate different muscle groups.

2.1.3. Central responses to axotomy

The responses of neurons, astrocytes, and microglia to axotomy constitute a CNS reaction historically referred to as the "axon reaction" (Lieberman, 1971) or "nerve cell body response to injury" (Grafstein, 1975). The individual elements that constitute this reaction are discussed below.

2.1.3.1. Motoneuron response

In 1892, renowned neuropathologist Franz Nissl described using aniline dyes to stain granular basophilic structures in FMN cell bodies (Danilevicius, 1968; Nissl, 1892). These structures came to be known as "Nissl bodies" or "Nissl substance". Nissl substance was later determined to be the ribosome-studded rough endoplasmic reticulum (RER), which stains with basic dyes due to an abundance of ribonucleic acid (RNA) (Palay & Palade, 1955). In axotomized FMN, one of the earliest changes observable by light microscopy is dispersion of the large Nissl bodies into finer particles (Cammermeyer, 1955; Cragg, 1970). This dispersion of the Nissl bodies causes paling of the perinuclear cytoplasm, known as *chromatolysis*, which can be observed after axotomy in both rodents and humans (Graeber et al., 1993; Lavelle & Lavelle, 1959). Conversely, an overall increase in general cytoplasmic basophilia has also been reported after axotomy (Cammermeyer, 1955; Torvik & Skjorten, 1971a). Electron microscopy revealed that these phenomena are due to segmentation and dispersion of RER cisterns

and a complementary shift in the predominant location of ribosome clusters from the RER to the cytoplasm (Torvik & Skjorten, 1971a). These events are accompanied by eccentric displacement and swelling of the motoneuron nucleus and nucleolus, indicating an overall increase in RNA transcription, manufacture of ribosomes, and downstream protein synthesis (Cammermeyer, 1955; Cragg, 1970; Grafstein, 1975; Lieberman, 1971). These changes reflect a shift in the prioritization of neuronal resources away from neurotransmission (RER membrane-associated translation) toward anabolic regeneration (cytoplasmic translation), and in some ways recapitulates the pattern of translation that occurs during the developmental period (Griffith & La Velle, 1971).

The precise trigger for the axon reaction is unknown. Loss of axoplasm volume, depolarization of the neuron, and failure of action potentials have been proposed as causative factors (Cragg, 1970; Grafstein, 1975). However, these do not explain the discrepancy in onset of nucleolar swelling after distal versus proximal lesions, a difference attributable to some retrograde signal that travels at a rate of approximately 4–5 mm/day. If the retrograde transport of trophic factors in the proximal portion of the severed axon continues after axotomy, the eventual cessation of these trophic factors reaching the cell body may constitute this signal (Cragg, 1970). It is most likely a combination of signals that prompts the motoneuron to switch from a neurotransmission-focused to a regeneration-focused metabolic state.

Differential production of specific molecules in the pre- and post-axotomy state demonstrates this shift of focus. After axotomy, smooth endoplasmic reticulum proliferates and increases synthesis of lipids, presumably to regenerate the axonal membrane (Grafstein, 1975; Lieberman, 1971). Increased expression of mRNA encoding

growth-associated protein GAP-43, which is enriched in the growth cones of regenerating axons, co-localizes with FMN (Ide, 1996; Jones et al., 1997a). Cytoskeletal components important for longitudinal axon growth including βII-tubulin and actin increase in expression on both the mRNA (Hoffman & Cleveland, 1988; Jones et al., 1999; Tetzlaff et al., 1991) and protein levels (Tetzlaff et al., 1988a). Conversely, transcription and translation of neurofilament, which is important for radial axon growth, decreases after axotomy (Bisby & Tetzlaff, 1992; Hoffman & Cleveland, 1988; Tetzlaff et al., 1991; Tetzlaff et al., 1988a). Translation of proteins important for neurotransmission, such as neurotransmitter precursors and their modifying enzymes, also decreases (Grafstein, 1975; Lieberman, 1971).

2.1.3.2. Microglial response

Microglia proliferate within 1–4 days after axotomy (Graeber et al., 1988c; Torvik & Skjorten, 1971b). They increase expression of the motility-associated cytoskeletal protein vimentin and migrate toward the injured FMN, occupying perineuronal positions for at least the first week after injury (Graeber et al., 1988b; Graeber et al., 1988c). This migration may be mediated by complement receptor 3 (CR3), which is upregulated on microglia within 24 hours of axotomy (Graeber et al., 1988a; Svensson et al., 1993). Neuronal release of chemokine C-X3-C motif ligand 1 (CX3CL1, also known as fractalkine) may be another chemotactic signal via its cognate receptor CX3CR1 expressed on microglia (Harrison et al., 1998). FNA also induces expression of monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) in FMN, which can trigger migration of microglia (Flugel et al., 2001). Despite (or perhaps due to) being

completely ensheathed by microglia, most neurons remain viable (Torvik & Skjorten, 1971b). Microglia do not phagocytize neurons or their synaptic terminals at this time (Blinzinger & Kreutzberg, 1968). Eventually the perineuronal microglia migrate back into the neuropil, and their former positions are taken up by astrocytes (Graeber & Kreutzberg, 1988). However, activated microglia are still present in the FMNuc, instead forming clusters and phagocytizing neurons that have already degenerated (Torvik & Skjorten, 1971b). Microglia in the injured FMNuc increase their expression of MHC classes I and II, on which they may present antigen derived from degenerating neurons (Bohatschek et al., 2004; Streit et al., 1989b).

2.1.3.3. Astrocyte response

Astrocytes in the FMNuc appear to increase in number by three days post axotomy, peak in number at 21 days, and may remain elevated for more than six months (Cammermeyer, 1955). However, this increase is not due to astrocyte proliferation, but rather due to local migration and hypertrophy (Graeber & Kreutzberg, 1986; Graeber et al., 1988c). After FNA, astrocytes undergo a non-mitotic transformation from a protoplasmic to a fibrous reactive type characterized by the presence of cytoplasmic filaments (Torvik & Skjorten, 1971b); (Graeber & Kreutzberg, 1986). Appearance of these filaments correlates with strong upregulation of glial fibrillary acidic protein (GFAP) mRNA and protein within 24 hours of axotomy (Jones et al., 1997b; Svensson et al., 1993; Tetzlaff et al., 1988b). This initial reaction is followed several weeks later by a second astrocyte transformation that is characterized by the appearance of thin, lamellar astrocyte processes covering the surfaces of injured motoneurons (Graeber & Kreutzberg,

1988). GFAP immunoreactivity resolves if target reconnection is allowed to occur, but may persist for a year or more if axon regeneration is mechanically blocked by a barrier (Laskawi & Wolff, 1996). This indicates that persistent loss of retrograde transport in neurons constitutes a signal for astrocyte activation. Unlike microglia, astrocytes do not express MHCI or MHCII in the FMNuc after axotomy (Streit et al., 1989b).

2.1.3.4. Oligodendrocyte response

Considerably less attention has been given to the role of oligodendrocytes after axotomy. A transient increase in oligodendrocyte number has been observed at 3–5 days post FNA (Cammermeyer, 1955). However, several other studies report that there is no change in oligodendrocyte number or morphology due to injury (Barron et al., 1990; Torvik & Skjorten, 1971b).

2.1.3.5. Synaptic stripping

Numerous electron microscopy studies have observed microglial processes extending along neuronal surfaces and separating UMN synaptic contacts from the injured LMN dendrites and soma after FNA (Blinzinger & Kreutzberg, 1968; Torvik & Skjorten, 1971b). Some studies note that after this initial reaction subsides, astrocyte processes interpose themselves between the microglia and FMN and continue to insulate neurons from synaptic input (Graeber & Kreutzberg, 1988; Svensson et al., 1993). In human postmortem tissue, astrocyte processes ensheathing injured FMN that have lost synaptic markers such as synaptophysin have also been observed (Graeber et al., 1993). Synaptic stripping and glial association with injured FMN are likely to be neuroprotective

mechanisms that shield injured FMN from excitotoxic inputs (Lopez-Redondo et al., 2000).

It is important to note that the interposition of glial processes between the pre- and post-synaptic neuron terminals does not necessarily represent active disruption of the synaptic structure, but rather may reflect opportunistic movement of glia into the negative space created by independent retraction of UMN axons and LMN dendrites (Grafstein, 1975). Indeed, inhibiting microglial proliferation after hypoglossal axotomy has no effect on UMN synaptic retraction or subsequent peripheral nerve regeneration (Svensson & Aldskogius, 1993). Neuronal nitric oxide synthase (nNOS) activity increases after axotomy and is sufficient for synapse loss, suggesting that synaptic stripping is a motoneuron-autonomous process (Perry & O'Connor, 2010; Sunico et al., 2010). However, microglia *do* play a role in pathological forms of synaptic stripping that are implicated in neurodegenerative diseases such as Alzheimer's disease (Rajendran & Paolicelli, 2018).

2.1.4. Peripheral responses to axotomy

A tightly regulated peripheral response to axotomy occurs concomitantly with the central reaction of motoneurons and glia already discussed. Both innate and adaptive immune mechanisms become active in the periphery after FNA and are important for neuroprotection.

2.1.4.1. Wallerian degeneration

In 1850, neurophysiologist Augustus Waller described the degeneration of myelin and axons in the distal nerve segment after hypoglossal and glossopharyngeal nerve transection in frogs (Waller, 1850). The regulated sequence of events occurring in the distal nerve segment is now known as Wallerian degeneration. After axotomy, Schwann cells (SCs) de-differentiate and decrease their myelin synthesis (LeBlanc & Poduslo, 1990). Rather than insulating axons, SCs proliferate and actively phagocytize myelin (Fernandez-Valle et al., 1995). They also secrete MCP-1/CCL2 and leukemia inhibiting factor (LIF) in an IL-6-dependent fashion, which together act as chemoattractant molecules for macrophages (Bolin et al., 1995; Tofaris et al., 2002). Macrophages in the vicinity then become activated, infiltrate the nerve, and phagocytize the degenerating nerve segment (Bruck, 1997; Perry et al., 1987). In the periphery, production of complement proteins is also important for macrophage participation in Wallerian degeneration. Failure of these innate immune responses to clear away debris after axotomy impedes subsequent axon regeneration (Dailey et al., 1998).

2.1.4.2. Peripheral adaptive immune response

Along with the innate immune responses that are essential for Wallerian degeneration, adaptive immune responses also become activated after axotomy. Axotomy causes self-antigen (likely neuronal or myelin-derived) to drain into peripheral lymphoid tissues, and CD4+ T cells encounter this antigen on MHCII presented by APCs in the periphery (Byram et al., 2004). All major CD4+ T cell subsets investigated (Th1, Th2, Th17, Tr1, and Foxp3+ regulatory T cells) expand in the draining deep cervical lymph

nodes after FNA. Peak cellular expansion occurs by seven days post operation (dpo) and declines by nine dpo, indicating mobilization of T cells out of the lymph nodes (Xin et al., 2008). The generation of the adaptive immune response after axotomy is critical for neuroprotection and has been the focus of extensive study by our laboratory.

2.1.5. Importance of the immune response after axotomy

2.1.5.1. Immunodeficient mice have increased axotomy-induced FMN death

Investigation into the adaptive immune contribution to neuroprotection after FNA was first performed using severe combined immunodeficient (*Scid*) mice. These mice lack a DNA repair enzyme critical for V(D)J recombination to occur, resulting in failed generation of diverse B and T cell receptor repertoires. Whereas WT mice with normal immune systems do not show a statistically significant decrease in FMN survival after axotomy until 28 dpo, *Scid* mice show accelerated early neuronal loss, with significantly decreased FMN survival as early as 7 dpo (Serpe et al., 2000). By 28 dpo, FMN survival in *Scid* mice decreases to 52–55% of the control side, compared to WT mice which have 85–87% FMN survival (Serpe et al., 1999; Serpe et al., 2000). FMN survival continues to decline in both WT and *Scid* through ten weeks post axotomy, although survival in *Scid* remains depressed relative to WT (45% compared with 60% FMN survival) (Serpe et al., 2000). Furthermore, functional recovery is delayed after crush axotomy in *Scid* mice (Serpe et al., 2002). Nerve crush is a relatively mild injury that is permissive for target reconnection and causes minimal FMN death. The delayed recovery in *Scid* mice indicates that the adaptive immune system is necessary not only for FMN survival, but may also be important for FMN regenerative capacity.

Because the *Scid* mutation produces defects in DNA repair, it could theoretically affect all cells in the body, not only the adaptive immune cells. Furthermore, it has been shown that the *Scid* mutation can become "leaky" as mice age (Bosma et al., 1988). This led to the adoption of the recombinase activating gene 2 knockout (RAG-$2^{-/-}$) mouse model of immunodeficiency, in which V(D)J recombination is prevented in a more stable and specific manner. Similar to the *Scid* mouse, FMN survival is significantly decreased in the RAG-$2^{-/-}$ mouse relative to WT at 28 dpo (64% versus 87% FMN survival) (Serpe et al., 2000). Reconstitution of either the *Scid* or RAG-$2^{-/-}$ immune system with WT whole splenocytes, which contain both B and T cells, restores FMN survival to WT levels (Serpe et al., 1999; Serpe et al., 2000). Because target reconnection would have occurred by approximately four weeks after axotomy under conditions ideal for regeneration (Kujawa et al., 1989), these data suggest that the adaptive immune system supports FMN survival during the early post-axotomy phase prior to target reconnection, after which target-derived NTF would presumably take the primary supportive role. However, this neuroprotective immune response cannot be sustained indefinitely if target reconnection is prevented.

2.1.5.2. CD4+ T cells mediate neuroprotection after FNA

The adaptive immune milieu is a heterogenous cell population. To determine if a particular adaptive immune cell population is necessary for FMN survival, axotomy was superimposed on a variety of immunodeficient mouse models. Neither mice deficient for the immunoglobulin heavy constant μ_m (μMT mice), which lack mature B cells, nor *Cd8*$^{-/-}$ mice have decreased FMN survival after axotomy. However, *Cd4*$^{-/-}$ mice exhibit

decreased FMN survival after FNA similarly to what is seen in RAG-$2^{-/-}$ mice, indicating that the neuroprotective capacity of the adaptive immune system is specific to the CD4+ T cell. Furthermore, adoptive transfer of isolated CD4+ T cells is sufficient to rescue FMN survival in RAG-$2^{-/-}$ mice (Serpe et al., 2003).

CD4+ T cells are themselves a diverse population and can be further subdivided into a number of T helper subsets that have differential expression of transcription factors and cytokines. Surprisingly, CD4+ T cells depleted of the CD25+ regulatory T cell subset, which exerts immunosuppressive and anti-inflammatory effects, are still capable of mediating neuroprotection. The CD4+CD1d-restricted NKT cell, which may be protective in experimental autoimmune encephalitis (EAE), is also not important for neuroprotection after FNA (DeBoy et al., 2006a; Teige et al., 2004). The importance of the Th1 and Th2 subsets for neuroprotection was assessed by performing FNA on mice deficient for signal transducer and activator of transcription (STAT)4, STAT6, interferon gamma (IFNγ), and IL-4. STAT4 is important for differentiation of the IFNγ-producing Th1 subset, and STAT6 is important for differentiation of the IL-4-producing Th2 subset. This series of experiments determined that Th2 cells and their associated cytokine IL-4 are necessary for FMN survival after axotomy (Deboy et al., 2006b). Interestingly, mice lacking expression of *Tbx21* (also known as T-BET), the Th1 master transcription factor, also have slowed functional recovery after nerve crush. This suggests that both Th1 and Th2 cells may be important for peripheral regeneration, while Th2 cells alone support central FMN survival (Beahrs et al., 2010).

2.1.5.3. Dynamics of T cell-mediated neuroprotection

In order for CD4+ T cells to effect their functions, they must presumably demonstrate specificity against antigen and migrate from the peripheral lymph nodes to the CNS. Chimeric mice that have MHCII expression restricted to either the central or peripheral compartments were employed to determine if the location of antigen presentation was important for CD4+ T cells to confer neuroprotection. Irradiated WT mice given *H2ab1*$^{-/-}$ (histocompatibility 2, class II antigen A, beta 1 knockout) bone marrow only express MHCII on central APC and have decreased FMN survival after axotomy unless given pre-activated, but not naïve, CD4+ T cells. Irradiated *H2ab1*$^{-/-}$ mice given WT bone marrow only express MHCII in the periphery and also have decreased FMN survival after axotomy, although FMN survival in this mouse is not amenable to rescue by either naïve or pre-activated CD4+ T cells (Byram et al., 2004). These experiments show that CD4+ T cells must sequentially encounter MHCII expressed on peripheral and central APCs in order to confer neuroprotection. Furthermore, the central APC is most likely to be microglia, as they are resistant to irradiation unlike perivascular monocytes or infiltrating macrophages (Hickey & Kimura, 1988).

Having encountered antigen presented on MHCII in the periphery, T cells must then migrate to the centrally-located APC. Th2-associated, but not Th1-associated chemokines increase by seven dpo in the axotomized FMNuc (Wainwright et al., 2009a). Pituitary adenylyl cyclase activating polypeptide (PACAP) is expressed by injured FMNs after axotomy and is capable of eliciting Th2-associated chemokine CCL11 expression by microglia *in vitro* (Armstrong et al., 2003; Wainwright et al., 2008). CCL11 is

expressed constitutively in FMN *in vivo*, but axotomy induces a shift in its expression from motoneurons to astrocytes (Wainwright et al., 2009c). Mice deficient for CCR3, a cognate receptor for CCL11, have decreased FMN survival after axotomy, and *Ccr3*$^{-/-}$ CD4+ T cells fail to confer neuroprotection in RAG-2$^{-/-}$ mice (Wainwright et al., 2009b). This indicates that axotomy induces production of chemotactic molecules in the FMNuc that enable recruitment of neuroprotective Th2 cells.

An illustrated summary of these proposed dynamics of CD4+ T cell-mediated neuroprotection is shown in Picture 1.

2.1.5.4. Potential mechanisms of T cell-mediated rescue

The overall extent of FMN death after axotomy is not uniformly distributed across the FMNuc, being more predominant in the lateral nuclei in the WT animal. The subpopulation of motoneurons that dies under conditions of immunodeficiency and is amenable to CD4+ T cell-mediated rescue is fairly evenly distributed across FMNuc subnuclei, although the dorsomedial subnucleus has the greatest FMN loss and recovery before and after immune cell reconstitution (Canh et al., 2006). This suggests that infiltrating T cells secrete an NTF that sustains FMN survival after injury. Brain-derived neurotrophic factor (BDNF) supports FMN survival in RAG-2$^{-/-}$ mice, but its production by CD4+ T cells is not necessary for neuroprotection (Serpe et al., 2005; Xin et al., 2012). A crucial NTF produced by CD4+ T cells after FNA has not been identified. It is therefore possible that CD4+ T cells exert their neuroprotective effects through other mechanisms, such as by modulating the glial response to axotomy. This is supported by a recent finding that the inherent regenerative gene program in injured FMN is not altered

by immune status; rather, the CD4+ T cell is important for expression of genes associated with astrocytic and microglial activation (Setter et al., 2018b).

IL-10 is an anti-inflammatory cytokine that has received much attention in CNS research due to its pro-survival and glial-modulating effects (Lobo-Silva et al., 2016). IL-10 is critical for FMN survival after axotomy. Although IL-10 derived directly from CD4+ T cells is not required after FNA, a source of IL-10 is necessary for CD4+ T cells to mediate their neuroprotective effects (Xin et al., 2011). This neuroprotective IL-10 likely derives from a central source, as it cannot cross the blood-brain barrier (Kastin et al., 2003). Studies differ as to whether IL-10 levels increase or remain stable in the FMNuc after axotomy, but central expression of its receptor increases after FNA and localizes to neurons and/or astrocytes (Villacampa et al., 2015; Xin et al., 2011). IL-10 may therefore have both trophic and glial-modulating roles in the FMNuc after FNA.

2.2. Interleukin 10

2.2.1. Discovery and characterization of IL-10 and its receptor

The discovery and early characterization of IL-10 is interwoven with those foundational studies in immunology that first supported the existence of different CD4+ T cell subsets. In 1971, Christopher Parish determined that T cells can mediate a delayed-type hypersensitivity reaction in the absence of B cell interactions. For the first time, the generation of cell-mediated and antibody-mediated immunity were distinguished as separate and often antagonistic phenomena (Parish, 1971). These differential effects were later traced to the existence of two helper T cell subsets, Th1 and Th2, which Tim Mosmann and Robert Coffman functionally characterized in 1986 based on cytokine

production. They determined that Th1 cells produce IL-2 and IFNγ, and Th2 cells produce IL-3 and "mast cell growth factor" (later renamed IL-4) (Mosmann et al., 1986). Furthermore, these T cell subsets were capable of cross-regulation. The discovery that IFNγ produced by Th1 cells suppresses proliferation of Th2 cells directly supported Parish's observations from the previous decade (Fernandez-Botran et al., 1988). Shortly thereafter followed the discovery of a new Th2-associated cytokine that suppresses Th1 cytokine synthesis (Fiorentino et al., 1989). This Th2-associated suppressive cytokine was cloned in 1990 and renamed IL-10 (Moore et al., 1990).

Rapid characterization of the IL-10R soon followed these discoveries. The IL-10R is a heterotetrameric type II cytokine receptor composed of two alpha and two beta subunits (Kotenko et al., 1997; Liu et al., 1994; Pestka et al., 2004). IL-10 binds as a dimer to the alpha subunit, and the beta subunit is consequently engaged for signaling (Pestka et al., 2004; Tan et al., 1993). Both receptor subunits are necessary for IL-10 signal transduction (Kotenko et al., 1997). The IL-10R beta (IL-10RB) subunit is shared with some interferon receptors and other IL-10 family cytokine receptors. Despite this promiscuity, cells lacking IL-10RB respond normally to both type I interferons and IFNγ, but are completely unresponsive to IL-10 (Spencer et al., 1998). IL-10R alpha (IL-10RA) and IL-10RB recruit Janus kinase 1 (JAK1) and non-receptor tyrosine-protein kinase (TYK2), respectively. Following their autophosphorylation, these kinases recruit STAT proteins that translocate to the nucleus to regulate transcriptional events. IL-10 is known to increase transcription of suppressor of cytokine signaling (SOCS)1 and SOCS3, which inhibit NF-κB and downstream cytokine production. IL-10 signaling also increases expression of mitochondrial proteins Bcl-2 and Bcl-x_L, which inhibit cytochrome *c*

release and subsequent apoptosis (Moore et al., 2001; Pestka et al., 2004). Other pro-survival and proliferative pathways activated by IL-10 include PI3K/AKT and MAPK/ERK. IL-10 inhibits pro-apoptotic kinases including JNK and p38 MAPK (Strle et al., 2001).

2.2.2. Characterization of IL-10 function in periphery

Loss of IL-10 leads to the development of spontaneous enterocolitis due to uncontrolled cell-mediated autoimmunity (Kuhn et al., 1993). Loss of only the IL-10RB subunit is sufficient to replicate this phenotype (Spencer et al., 1998). The dramatic increases in inflammation seen in these disease models support the role of IL-10 as an important regulator of immune responses in the periphery. In addition to Th2 cells, other common peripheral sources of IL-10 include regulatory T cells (Tregs), monocytes/macrophages, and dendritic cells. Many additional cell types, such as cytotoxic T cells, natural killer cells, B cells, mast cells, neutrophils, eosinophils, and epithelial cells can also make IL-10 under special conditions (Iyer & Cheng, 2012).

One of the earliest characterized roles of IL-10 in the periphery was the inhibition of Th1-secreted cytokine production, as has been described previously (Fiorentino et al., 1989). IL-10 was found to have this effect mainly by its actions on macrophage and dendritic APCs (Fiorentino et al., 1991b). IL-10 suppresses the production of pro-inflammatory cytokines by APC (particularly monocytes/macrophages) that are responsible for both Th1 activation and acute inflammatory responses. Th2 responses are not significantly affected by this mechanism (Fiorentino et al., 1991a). In fact, IL-10-

primed dendritic cells may preferentially promote Th2 subset differentiation (Liu et al., 1998).

IL-10 also inhibits the ability of APC to participate in antigen presentation and T cell co-stimulation by decreasing expression of MHCII and CD80/86 (also known as B7.1/7.2) (de Waal Malefyt et al., 1991; Ding et al., 1993; Ding & Shevach, 1992). Through these mechanisms, IL-10 is capable of converting immature dendritic cells into "tolerogenic" APCs that promote CD4+ T cell anergy (Steinbrink et al., 1997). IL-10 also tends to favor the development of humoral, rather than cell-mediated, immunity by promoting B cell differentiation and proliferation (Itoh & Hirohata, 1995). Unlike macrophages and dendritic cells, the antigen-presenting ability of B cells is not affected by IL-10 (Fiorentino et al., 1991b).

Although IL-10 was originally believed only to act on T cells indirectly via the APC, it was later determined that IL-10 can indeed have direct effects on T cells. IL-10 directly inhibits T cell proliferation via suppression of IL-2 signaling (de Waal Malefyt et al., 1993). IL-10 also directly suppresses T cell activation and cytokine secretion that is dependent on CD28-CD80/86 interactions (Schandene et al., 1994; Taylor et al., 2006). It has this effect by blocking CD28 phosphorylation and PI3K binding in T cells (Akdis et al., 2000; Joss et al., 2000). Inhibition of CD28 in T cells may be downstream of IL-10RB and TYK2 activation specifically (Verma et al., 2016). Loss of CD28 co-stimulation results in specific T cell tolerance to antigen (Akdis & Blaser, 2001). Anergic T cells may be capable of inhibiting antigen presentation by dendritic cells and suppressing the proliferation of other T cells in an antigen-specific and contact-dependent fashion (Chai et al., 1999; Vendetti et al., 2000).

2.2.3. Potential roles of IL-10 in the CNS

Based on the signaling pathways and mechanisms described above, IL-10 can theoretically promote neuroprotection in three ways: 1) suppression of pro-inflammatory cytokine secretion from monocytes/macrophages (which may include CNS microglia); 2) suppression of cellular autoimmunity through inhibition of MHCII and co-stimulatory molecule expression on APCs and CD28 transduction in T cells; and/or 3) direct promotion of pro-survival and suppression of pro-apoptotic pathways in neurons. While studies that employ exogenous application of IL-10 in the CNS produce interesting insights into the neuroprotective capacity of IL-10, such insights may represent non-physiologically relevant mechanisms of IL-10 function. Important clues about the role of IL-10 in the CNS are perhaps better afforded by studying endogenous IL-10 production as well as the consequences of IL-10 reduction or elimination in the context of injury or disease.

Loss of endogenous IL-10 is associated with increased neuroinflammation, cerebral edema, and intracerebral hemorrhage in *Listeria monocytogenes* brain infections, possibly due to over-recruitment of peripheral lymphocytes into central tissues (Deckert et al., 2001). Endogenous IL-10 limits cerebral ischemia-induced infarct volume *in vivo* and protects primary cortical neurons against death due to excitotoxicity and oxygen-glucose deprivation *in vitro* (Grilli et al., 2000). T cells are likely involved in this process, as the success of myelin peptide immunization in reducing ischemic infarct volume after middle cerebral artery occlusion is dependent on IL-10-producing CD4+ T cells (Frenkel et al., 2005). Regulatory T cells also have beneficial effects on inflammation and neuron survival after hemorrhagic stroke, in which they modulate microglial polarization via IL-

10 production (Zhou et al., 2017). Suppressed IL-10 production by Tr1 regulatory T cells and decreased CD4+ T cell responsiveness to IL-10 are observed in patients with multiple sclerosis (MS) (Martinez-Forero et al., 2008). First-degree family members of patients with MS are at greater risk of developing disease if their innate production of IL-10 is low (de Jong et al., 2000). Enhancing the induction of Trl regulatory T cells limits neuroinflammation in a mouse model of MS by reducing glial activation, suppressing recruitment of peripheral lymphocytes, and preserving blood-brain barrier integrity in an IL-10 dependent manner (Mayo et al., 2016).

In addition to T cell production of IL-10, microglial production of IL-10 in response to inflammatory stimuli is also important for neuroprotection. *In vivo* lipopolysaccharide (LPS) challenge promotes IL-10 production by microglia, and blocking this IL-10 signaling increases neuronal death and production of pro-inflammatory mediators including IL-1β, tumor necrosis factor alpha (TNFα), and reactive oxygen species (Park et al., 2007). In the $SOD1^{G93A}$ mouse model of ALS, blocking IL-10 signaling causes an increase in microglial activation in the spinal cord and precipitates the onset of disease symptoms, although lifespan is not affected (Gravel et al., 2016). Overall, these studies indicate that endogenous IL-10, frequently produced by CD4+ T cells and microglia, is important for CNS health by modulating inflammatory activation of glia, limiting the recruitment of peripheral immune cells to central tissues, and/or providing direct trophic support to neurons.

2.3. Aims of this study

2.3.1. Aim 1: Determine the central cellular sources of IL-10 in the facial motor nucleus and the respective contributions of these sources to motoneuron survival after axotomy

IL-10 is necessary for CD4+ T cells to mediate neuroprotection after FNA, but CD4+ T cells are not the requisite source of IL-10 in the FMNuc. Additionally, IL-10 applied directly to the proximal nerve stump, which lies outside of the CNS, does not improve FMN survival (Xin et al., 2011). These data suggest that a central source of IL-10 is necessary for neuroprotection. This hypothesis is supported by the fact that IL-10 cannot cross the blood-brain barrier (Kastin et al., 2003). However, it is possible that the neuroprotective role of IL-10 is purely due to its ability to favor the development of Th2 rather than Th1 responses in the context of antigen presentation in the peripheral lymph nodes (Fiorentino et al., 1991a; Liu et al., 1998). Only Th2 cells are neuroprotective after axotomy (Deboy et al., 2006b). Therefore, IL-10 production both by peripheral APC as well as by central cells in the FMNuc such as microglia, neurons, and astrocytes must be evaluated.

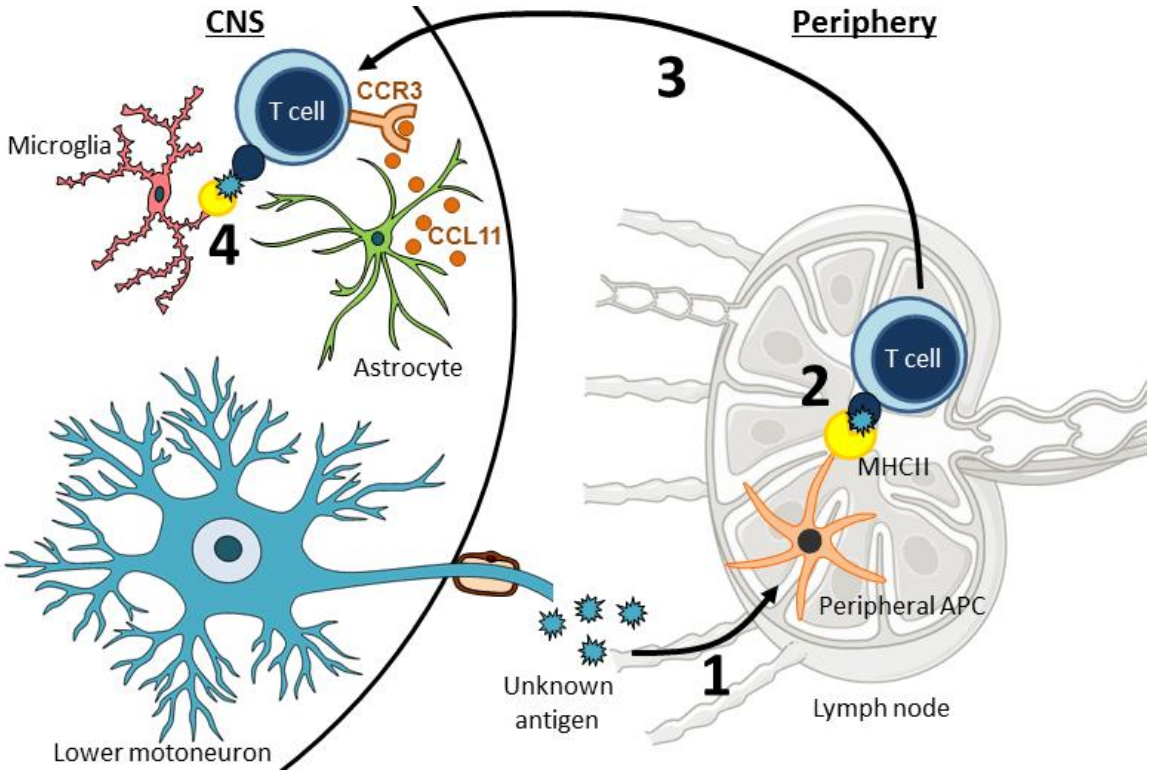
In CNS injury and disease, neuroprotective IL-10 production is typically associated with regulatory T cells and microglia (see section 2.2.3). Because an IL-10 contribution from T cells has been ruled out in the FNA model, microglia are the most likely source of IL-10 in the FMNuc (Xin et al., 2011). Microglia are known to produce IL-10 in response to a number of inflammatory stimuli (Gravel et al., 2016; Jack et al., 2005; Ledeboer et al., 2002; Seo et al., 2008). Additionally, Th2 cells produce IL-4, which promotes the polarization of microglia toward the “M2” anti-inflammatory phenotype characterized in part by IL-10 production (Chabot et al., 1999; Chhor et al.,

2013; Zhang et al., 2014) Therefore, the hypothesis for this aim is that microglia are the obligate source of neuroprotective IL-10 after axotomy. This hypothesis will be tested using a combination of IHC localization and Cre/Lox recombination to reduce IL-10 expression in a cell-specific manner.

2.3.2. Aim 2: Characterize adaptive immune cell participation in neuroprotective IL-10 receptor signaling after facial nerve axotomy

FMN survival after axotomy is dependent on both CD4+ T cell-mediated and IL-10-mediated mechanisms. Although CD4+ T cells are not responsible for IL-10 production after FNA, it is not known whether they regulate downstream IL-10 signaling. Neuroprotective actions of IL-10 after FNA are likely effected through increased expression of the IL-10R (Xin et al., 2011), which may be mediated by CD4+ T cells. IHC will be performed to determine whether CD4+ T cells are capable of interacting directly with central cells in the FMNuc to influence their gene expression. qPCR analysis will be performed in WT and RAG-2$^{-/-}$ mice to determine whether the adaptive immune system is necessary for IL-10R subunit expression in the FMNuc after axotomy. Because IL-10 can have direct actions on T cells, the ability of IL-10R-deficient CD4+ T cells to regulate central IL-10R expression and confer neuroprotection will also be assessed. The hypothesis for this aim is that CD4+ T cells are necessary for central IL-10 receptor expression and must respond to IL-10 themselves to confer neuroprotection. Loss of IL-10R signaling on T cells may result in failed acquisition of tolerance to self-antigen (Akdis & Blaser, 2001). This possibility will be explored via analysis of genes

associated with inflammatory immune activation, including general microglial activation markers, antigen presentation and co-stimulation molecules, and complement deposition.



Picture 1: Schematic illustrating hypothesized dynamics of CD4+ T cell-mediated neuroprotection.

1) After FNA, an unknown antigen (potentially neuronal, myelin-derived, or glial in origin) drains into the deep cervical lymph nodes, is taken up and processed by peripheral APCs (likely dendritic cells), and displayed on their MHCII. 2) Peripheral CD4+ T cells recognize antigen displayed on MHCII and undergo clonal expansion. 3) Attracted by chemokines such as CCL11 produced by astrocytes, CD4+ T cells migrate to the FMNuc. 4) CD4+ T cells encounter antigen and MHCII on a centrally located APC, likely microglia. T cell encounter with MHCII in both the peripheral and central compartments is necessary for neuroprotection.

CHAPTER 3: MATERIALS AND METHODS

3.1. Animals used in this study

See Table 1 for a list of animal strains used in this study. For animals obtained from The Jackson Laboratory, only female mice were used due to the tendency of male non-littermates to engage in fighting behavior after axotomy, leading to infections at the surgery site. These animals were obtained at six or seven weeks of age and allowed to acclimate for at least one week prior to use. For animals bred in house ($IL10^{flox/flox}$ strain crossed with all Cre-expressing strains), equal numbers of male and female mice were used in experiments. Mice were housed in sterilized microisolater cages equipped with a laminar flow system to maintain a pathogen-free environment and allowed access to autoclaved food pellets and water *ad libitum*. IL-$10^{-/-}$ and *Il10rb*$^{-/-}$ mice were also given access to wet food to prevent possible complications from the development of enterocolitis, although no symptoms of enterocolitis (such as loss of body weight) were noted in any animals during the duration of the study. Animal rooms operated on a 12 hour light/dark cycle. All animal procedures complied with National Institutes of Health guidelines on the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine.

3.2. Preparation of genomic DNA and polymerase chain reaction (PCR)

Genomic DNA was extracted from tail snips (for genotyping) and spleen (to confirm adoptive transfer engraftment) using Gentra® PureGene® Core Kit A (QIAGEN Sciences, cat. no. 1042601) following manufacturer instructions. PCR was performed on genomic DNA using primers listed in Table 2 and illustra™ PuReTaq Ready-To-Go™

PCR Beads (GE Life Sciences, cat. no. 27955701). PCR amplicons were run on a 1.5-2% agarose gel at 120-150 V for 20–40 minutes and were subsequently imaged using a Cell Biosciences FluorChem E system.

PCR for IL10 exon 1 yielded a 133 base pair (bp) sequence in WT. PCR for IL10 floxed sequence and exon 2 yielded a 480 bp sequence in WT and 514 bp sequence in floxed animals. All IL10 PCR was performed using the following program: 95°C for 2 min; 30 cycles of 95°C for 30 sec, 60° for 45 sec, 72°C for 45 sec; and 72°C for 5 min.

PCR for *Thy1*^{Cre} yielded a 300 bp sequence in animals expressing Cre recombinase under control of the *Thy1* promoter. PCR for *Gfap*^{Cre} yielded a 200 bp sequence in animals expressing Cre recombinase under control of the *Gfap* promoter. PCR for *Cx3cr1*^{Cre} yielded a 300 bp sequence in animals expressing Cre recombinase under control of the *Cx3cr1* promoter. All Cre PCR was performed using the following program: 94°C for 2 min; 10 cycles of 94°C for 20 sec, 65°C for 15 sec with -0.5°C per cycle decrease, 68°C for 10 sec; 28 cycles of 94°C for 15 sec, 60°C for 15 sec, 72°C for 10sec; and 72°C for 2 min.

3.3. Adoptive transfer

Donor animals were utilized at a 1:1 ratio to recipients. Following CO_2 euthanasia and cervical dislocation, spleens were removed and placed in 1–2 mL cold working buffer (0.5% BSA + 2 mM EDTA in 1X PBS). Spleens were mechanically dissociated using the back of a sterile syringe, and the cell suspensions were filtered through a 70 μm cell strainer and pelleted via centrifugation for 10 min at 350 *g*. When adoptive transfer of whole splenocytes was desired, the cell pellet was resuspended in 1 mL red blood cell

lysis buffer (150 mM NH_4Cl + 10 mM $KHCO_3$ + 0.1 mM EDTA in ddH_2O) and left to rest for 5 min at room temperature prior to leveling up with cold working buffer and centrifugation for 10 min at 350 *g*. When adoptive transfer of isolated CD4+ T cells was desired, pellets were resuspended in 90 µl working buffer/10^7 cells. 10 µl CD4 magnetic beads (Miltenyi Biotec, cat. no. 130-117-043)/10^7 cells were added and cells were incubated for 10 min on ice. Cells were manually sorted using LS columns (Miltenyi Biotec, cat. no. 130-042-401) following manufacturer instructions. The eluent was then passed through a new column a second time to enhance cell purity. Flow cytometry revealed sorted CD4+ T cell purity to be 96% (data available upon request). Cells were resuspended in working buffer for counting and subsequently washed with 1X PBS. Cells were resuspended in a suitable volume of 1X PBS to yield $50 \cdot 10^6$ whole splenocytes or $5 \cdot 10^6$ sorted CD4+ T cells per 100 µl injection. Recipient mice received donor cells via tail vein injection one week prior to FNA to allow for engraftment of transferred cells.

3.4. Induction of Cre recombinase

For Cre/Lox experiments, animals expressing at least one copy of Cre recombinase and homozygous for floxed IL-10 sequences ($Cre^{+/+}IL10^{fl/fl}$ or $Cre^{+/-}IL10^{fl/fl}$) were used in the experimental group. Animals negative for either Cre or floxed IL-10 sequences ($Cre^{-/-}IL10^{any}$ or $Cre^{any}IL10^{wt/wt}$) were used as littermate controls. One week prior to axotomy (seven weeks of age), animals in both groups were given once daily intraperitoneal injections of 75 mg/kg tamoxifen (Sigma-Aldrich, cat. no. T5648-5G) suspended in corn oil (Sigma-Aldrich, cat. no. C8267) at a concentration of 20 mg/mL

for five consecutive days. To maintain Cre expression, injections were administered on a biweekly basis until euthanasia.

To evaluate extent of IL-10 knockdown, mice were given once daily intraperitoneal injections of tamoxifen for five consecutive days, followed by one week of biweekly injections. Two weeks after tamoxifen induction of Cre was started, LPS was injected at 5 mg/kg to stimulate IL-10 production in glia. LPS was not administered to animals used for evaluation of neuronal IL-10 production. Animals were euthanized at either 3 h (for evaluation of microglia) or 12 h (for astrocytes) post LPS injection.

Following euthanasia and brain removal, brains were dissociated using the MACS Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, cat. no. 130-092-628) following manufacturer's protocol for manual dissociation, and cells were sorted using anti-CD11b (Miltenyi Biotec, cat. no. 130-093-634), anti-GLAST (130-098-803), or neuron isolation kit (130-115-390) microbeads per manufacturer instructions. Sorted cells were pelleted, washed with PBS, and lysed with Pierce™ RIPA Lysis and Extraction Buffer (Thermo Scientific™, cat. no. 89900). Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific™). For evaluation of IL-10 in glia, 25–100 ng protein was blotted onto a nitrocellulose membrane and probed with rat anti-mouse IL-10 antibody (Invitrogen, cat. no. 12-7101-81) at 1:1000 dilution followed by goat anti-rat HRP (Abcam, cat. no. ab97057) at 1:25000 dilution. HRP signal was detected using GE Healthcare Amersham™ ECL™ Prime Western Blotting Detection Reagent (Fisher Scientific, cat. no. 45-002-401) following manufacturer instructions. Integrated density measurements were performed on dot blot images in ImageJ. IL-10 in microglia was determined to be 14–34% reduced in cKO compared to littermate controls, and IL-10 in

astrocytes was determined to be 51-83% reduced (data available upon request). For evaluation of IL-10 in neurons, protein lysates were analyzed using a Mouse IL-10 ELISA MAX™ Deluxe kit (BioLegend, cat. no. 431414). IL-10 was reduced by approximately 27% in neurons isolated from cKO animals compared to littermate controls (data available upon request).

3.5. Facial nerve axotomy

FNA was performed when mice were eight weeks old. The detailed surgical procedure has been published previously (Olmstead et al., 2015). Briefly, animals were anesthetized with 2.5% isoflurane in oxygen at a flow rate of 0.9 L/min. The incision site was wetted with 70% ethanol, shaved, and alternately swabbed with 70% ethanol and iodine solution three times. All surgical instruments were sterilized prior to use. Using aseptic techniques, a small (~4 mm) incision was made just posterior to the ear protuberance. The underlying fascia was dissected bluntly until the facial nerve was visible. The facial nerve was traced posteriorly and severed just distal to its emergence from the stylomastoid foramen, taking care not to apply traction to the nerve to avoid its avulsion from the brainstem. The distal nerve stump was either pushed away from the proximal stump, or a small segment of the distal nerve stump was resected to prevent reconnection. The wound site was closed with a metal wound clip, which was removed between 7–10 days after surgery. Following surgery, successful transection of the facial nerve was confirmed by behavioral assessment of eye blink and whisking reflexes.

3.6. Motoneuron counting

For motoneuron counting, animals (n = 3–6) were euthanized at 28 dpo using CO_2 inhalation and cervical dislocation. Brains were removed and flash-frozen at the interface of a pre-chilled 37.5% 2-methylbutane/62.5% 1-bromobutane biphasic solution on dry ice for at least five minutes. Frozen brains were stored at -80°C. One hour prior to sectioning, brains were thawed at -20°C and embedded in optimum cutting temperature (OCT) medium. Brainstem sections spanning the caudal-rostral extent of the FMNuc were cryosectioned at 25 μm, collected onto Superfrost Plus slides (Thermo Scientific), and stored at -20°C. For thionin staining, sections were fixed in 4% paraformaldehyde (PFA) for 15 min and washed 2x5 min in ddH_2O. Sections were stained in working thionin solution (described below) for 15 min and dipped in ddH_2O for 30 sec, followed by dehydration in a 50%, 70%, 95%, 100% ethanol series for 30 sec each. Sections were cleared in CitriSolv overnight or up to three days and subsequently coverslipped using Permount toluene-based mounting medium.

For counting, an impartial investigator coded all slides. A separate blinded investigator used a Leica DMRB light microscope and Neurolucida software (version 10.31) to manually count motoneurons in the FMNuc. To avoid double-counting, only FMN profiles with a nucleus and nucleolus were counted. Mean percentage FMN survival was quantified by dividing the total number of FMN on the axotomized side by the total number on the control side and multiplying by 100%. A student's t-test was performed for experiments containing ≤2 groups, and one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc test was performed for experiments containing ≥2 groups with an alpha of 0.05.

To prepare thionin staining solutions, a stock solution of 1% thionin acetate (Sigma Aldrich, cat. no. 861340) was prepared in ddH_2O, stirred overnight, and filtered. Acetate buffer was prepared by mixing 180 mL 1 M glacial acetic acid and 20 mL sodium acetate and adjusting pH to 3.5. To prepare the working thionin solution, 38 mL thionin stock was combined with 200 mL acetate buffer and 560 mL ddH_2O.

3.7. Immunohistochemistry

3.7.1. IL-10/GFP reporter

For IL-10/green fluorescent protein (GFP) reporter mice (n = 3/time point), brains were removed and flash-frozen as described in section 3.6. Various time points (3, 7, 10, 14, and 28 dpo) were used. Brainstem sections spanning the caudal-rostral extent of the FMNuc were cryosectioned at 8 μm, collected onto Superfrost Plus slides (Thermo Scientific), and stored at -20°C. Sections were fixed in 4% PFA for 15 minutes and washed 3x5 minutes in PBS. Sections were blocked in 10% donkey serum, 1% BSA, and 0.01% Triton X-100 in PBS for one hour prior to incubation with primary antibodies (see Table 3 for antibodies and concentrations used) diluted in the same blocking medium overnight at 4°C. Sections were washed 3x5 minutes in PBS prior to incubation with secondary antibody for 1 h at room temperature. Excess secondary antibody was removed with 3x5 min washes in PBS, and slides were mounted with DAPI Fluoromount-G® (Southern Biotech, cat. no. 0100-20). Images were captured with an Olympus BX50 inverted fluorescent microscope using Olympus cellSens Entry 1.9 software, and brightness/contrast adjustments to reduce background were performed in Adobe Photoshop.

3.7.2. Immunohistochemistry performed on wild-type tissue

Animals (n = 3/time point) were euthanized at 3, 7, 10, 14, and 28 day time points via ketamine-xylazine overdose and exsanguination followed by perfusion with 2% buffered PFA. Brains were removed, post-fixed in 2% PFA 1 h, and equilibrated in 30% sucrose. Brains were embedded in OCT medium, and 8 μm brainstem sections containing the FMNuc were collected. Sections were first blocked for 1 h at room temperature in 10% donkey serum, 1% bovine serum albumin, and 0.01% Triton X-100 in PBS, followed by incubation in primary antibody (see Table 3) for 2 h at room temperature or 16 h at 4°C. Secondary staining and imaging proceeded as described in section 3.7.1. When fluorescent Nissl staining was desired, NeuroTrace™ 435/455 Blue Fluorescent Nissl Stain (Thermo Fisher, cat. no. N21479) was diluted 1:100 in PBS, applied to sections for 20 min after removing the secondary antibody, and washed 3x5 min in PBS prior to mounting in ProLong™ Gold Antifade medium (Invitrogen, cat. no. P36930).

3.8. Fluorescent *in situ* hybridization

For FISH, care was taken to use only reagents prepared in DEPC-treated H_2O to avoid RNase contamination. Animals (n = 3/time point) were euthanized, and brains were removed, flash-frozen, and sectioned at 8 μm as described in section 3.6. FISH was performed using Stellaris® RNA FISH probes (LGC Biosearch Technologies) following manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols with the following changes. Sections were fixed for 4 h in 4% PFA, washed 2x10 min in PBS, dipped in H_2O, and equilibrated in 1X triethanolamine (TEA) buffer for 10 min. Next, slides were immersed in 1X TEA + 0.25% acetic anhydride for 10 min (stirring)

and washed in 2X SSC for 10 min. Slides were then dehydrated following the Stellaris® protocol. After air drying, slides were hybridized with 1000 nM Stellaris® RNA FISH probes diluted in Stellaris® hybridization buffer in a humidified chamber at 55°C overnight. Ready-made probes were used for *Cx3cr1* Quasar® 570 (cat. no. VSMF-3102-5), and custom probes for *Il10* Quasar® 670 were designed using the Stellaris® FISH Probe Designer for sequence NM_010548.2. Day 2 of the protocol followed manufacturer's instructions, with the exception of using DAPI Fluoromount-G® (Southern Biotech) mounting medium instead of separate DAPI labeling and slide mounting. Imaging was performed using a Nikon A1 confocal microscope, and post-processing to remove background was performed in ImageJ.

3.9. Laser capture microdissection (LCM)

Brains were removed and flash-frozen as described in section 3.6. 25 μm sections spanning the FMNuc were collected onto PEN membrane slides (Leica Microsystems, cat. no. 11505158). Slides were stored at -80°C until ready to use. For staining and lasering, care was taken to use only reagents prepared in DEPC-treated H_2O to avoid RNase contamination. Slides were thawed at -20°C for one hour prior to LCM and were removed and processed one at a time. Each slide was stained with the following series: 1 min 100% ethanol, 2x15 sec H_2O, 30 sec thionin stain (4X thionin concentration as described in section 3.6), 2x15 sec H_2O, 20 sec 70% ethanol, 20 sec 90% ethanol, 20 sec 100% ethanol. Slides were air-dried 1–2 min after staining. A Leica ASLMD microscope was used to collect the right and left FMNuc into tubes containing 60 μl RNA extraction buffer (Arcturus PicoPure® RNA Isolation Kit, Applied Biosystems, cat. no. KIT0204).

Tubes were incubated at 42°C for 30 min, centrifuged at 800 *g* for 2 min, and stored at -80°C until RNA extraction.

3.10. RNA extraction and reverse transcription (RT)

RNA was extracted from LCM samples following the Arcturus PicoPure® RNA Isolation Kit protocol with an elution volume of 14 µl. For the spleen standard used for *Tnf* qPCR, RNA was extracted from WT spleen tissue using the QIAGEN RNeasy Mini Kit (cat. nos. 74104 and 74106) and QIAGEN RNase-Free DNase Set (cat. no. 79254) following manufacturer instructions. RNA was quantified using a NanoDrop 2000 spectrophotometer and stored at -80°C until RT. 60 ng of RNA was used for RT, which was performed using SuperScript® VILO Master Mix (Invitrogen, cat. no. 11755050) following the manufacturer's protocol. cDNA was stored at -20°C until ready for qPCR.

3.11. qPCR

Reaction volumes for qPCR used 1 µl cDNA, 1 µl TaqMan® FAM gene expression assay (see Table 4), 10 µl TaqMan Gene Expression Master Mix (Thermo Fisher, cat. no. 4369016), and 8 µl RNase-free H_2O. qPCR was performed using a Bio-rad Bio-Plex® 200 real-time PCR system using the following program: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. To maintain consistency with previous studies using custom-made primers for *Cd68*, the following custom TaqMan primers and probes were ordered: F 5'-CCCAAATTCAAATCCGAATCC-3', R 5'-GGTACCGTCACAACCTCC-3', probe 5'-

AAAGTGAGTGCGTCCCTTGCAGCC-3'. All other probes are commercially available and are listed in Table 4.

CT values were normalized to the reference gene *Gapdh* and the percent and fold changes in mRNA expression between axotomized and control FMNuc were calculated following the Pfaffl method (Pfaffl, 2001). Because *Tnf* is only expressed on the axotomized FMNuc, the ΔΔCT value was calculated using a spleen cDNA standard, and its levels in axotomized FMNuc are expressed relative to WT spleen. The same spleen standard was used for comparison across all samples. Outliers were detected using the Grubbs' test (also called extreme studentized deviate method). One-way ANOVA followed by Student-Newman-Keuls multiple comparisons or two-way ANOVA followed by Bonferroni multiple comparisons (as applicable depending on number of groups and conditions) was performed with an alpha of 0.05 to determine significance.

Strain	**Alternately named**	**Source**
B6(Cg)-Il10$^{tm1.1Karp}$/J	IL-10/GFP reporter	The Jackson Laboratory
B6(Cg)-*Rag2*$^{tm1.1Cgn}$/J	RAG-2$^{-/-}$	The Jackson Laboratory
B6.129P2(Cg)-Cx3cr1$^{tm2.1(cre/ERT2)}$Litt/WganJ	*Cx3cr1*^{Cre}	The Jackson Laboratory
B6.129P2-Il10^{tm1Cgn}/J	IL-10$^{-/-}$	The Jackson Laboratory
B6.129S2-*Il10rb*^{tm1Agt}/J	*Il10rb*$^{-/-}$	The Jackson Laboratory
B6.Cg-Tg(GFAP-cre/ERT2)505Fmv/J	*Gfap*^{Cre}	The Jackson Laboratory
C57BL/6J	WT	The Jackson Laboratory
IL10$^{flox/flox}$		Dr. Gang Huang, Cincinnati Children's Hospital; see Roers et al. (2004)
Tg(Thy1-cre/ERT2,-EYFP)HGfng/PyngJ	*Thy1*^{Cre}	The Jackson Laboratory

Table 1: Mouse strains utilized in this study.

Gene	**5' primer sequence**	**3' primer sequence**
IL10 exon 1 (133 bp in WT)	ATG CCT GGC TCA G	CCA CAT GCT CCT AGA GCT GC
IL10 floxed sequence and exon 2 (480 bp WT, 514 bp floxed)	CCA GCA TAG AGA GCT TGC ATT ACA	GAG TCG GTT AGC AGT ATG TTG TCC AG
Thy1^{Cre} transgene (300 bp)	TCT GAG TGG CAA AGG ACC TTA GG	CGC TGA ACT TGT GGC CGT TTA CG
Thy1^{Cre} internal control (200 bp)	CAA ATG TTG CTT GTC TGG TG	GTC AGT CGA GTG CAC AGT TT
Gfap^{Cre} transgene (200 bp)	GCC AGT CTA GCC CAC TCC TT	TCC CTG AAC ATG TCC ATC AG
Gfap^{Cre} internal control (324 bp)	CTA GGC CAC AGA ATT GAA AGA TCT	GTA GGT GGA AAT TCT AGC ATC ATC C
Cx3cr1^{Cre} transgene (300 bp)	AAG ACT CAC GTG GAC CTG CT	CGG TTA TTC AAC TTG CAC CA
Cx3cr1^{Cre} internal control (695 bp)	AAG ACT CAC GTG GAC CTG CT (common)	AGG ATG TTG ACT TCC GAG TTG

Table 2: Primer sequences for PCR.

Primary antibodies	**Manufacturer and Catalog Number**	**Dilution**
Mouse anti-GFAP 594 (used in IL-10/GFP reporter)	Thermo Fisher A-21295	1:500
Rabbit anti-GFP 488	Thermo Fisher A-21311	1:100
Rabbit anti-IBA1	Thermo Fisher 019-19741	1:500
Mouse anti-NeuN 555	Millipore MAB377A5	1:200
Rabbit anti-GFAP (used in perfused WT tissue)	Thermo Fisher PA3-16727	1:1000
Goat anti-IL-10	R&D Systems AF519	1:200
Rat anti-CD4 488	Biolegend 100423	1:100
Rabbit anti-CD3	Abcam ab16669	1:200
Rabbit anti-CD31	Abcam ab124432	1:500

Secondary antibodies	**Manufacturer and Catalog Number**	**Dilution**
Donkey anti-rabbit 568 (used for GFAP, IBA1, CD3, CD31)	Abcam ab175470	1:1000
Donkey anti-goat 488 (used for IL-10)	Abcam ab150129	1:1000
Donkey anti-rat 488 (used for CD4)	Thermo Fisher A21208	1:1000

Table 3: Antibodies utilized for immunohistochemistry.

Gene	TaqMan ID	RefSeq Accession Number
B2m	Mm00437762_m1	NM_009735.3
C3	Mm01232779_m1	(GenBank–no RefSeq numbers available) BC029976.1, BC043338.1, DQ408205.1, EU868829.1, HM856604.1, K02782.1
Cd40	Mm00441891_m1	NM_011611.2, NM_170702.2, NM_170703.2, NM_170704.2, NR_027852.1
Cd80	Mm00711660_m1	NM_009855.2
Cd86	Mm00444543_m1	NM_019388.3
Gapdh	Mm99999915_g1	NM_001289726.1, NM_008084.3
H2ab1	Mm00439216_m1	NM_207105.3
Nos2	Mm00440502_m1	NM_010927.3
Tnfrsf1	Mm00441883_g1	NM_011609.4
Tnfα	Mm00443260_g1	NM_013693.3

Table 4: TaqMan® assays utilized in this study.

CHAPTER 4: RESULTS

4.1. Aim 1: Determine the central cellular sources of IL-10 in the facial motor nucleus and the respective contributions of these sources to motoneuron survival after axotomy

4.1.1. Contribution of IL-10 from peripheral immune cells to central facial motoneuron survival

Previous studies have shown that although IL-10 is necessary for CD4+ T cells to mediate neuroprotection, CD4+ T cells themselves are not the required IL-10 producers in this injury model (Xin et al., 2011). Prior to operating under the assumption that the source of neuroprotective IL-10 must be centrally located, it was first essential to eliminate other peripheral sources of IL-10, such as APCs, as potential candidates. This is due to the possibility that IL-10 produced by peripheral APCs in the lymph nodes may be necessary for CD4+ T cells to differentiate to a neuroprotective subtype after axotomy. To confirm that a peripheral APC was not the necessary source of IL-10, FNA was performed on WT, IL-10$^{-/-}$, and IL-10$^{-/-}$ mice reconstituted with WT whole splenocytes (a heterogeneous population of T cells, B cells, and APCs). FMN survival was determined at 28 dpo. In accordance with previously published findings (Xin et al., 2011), FMN survival in IL-10$^{-/-}$ mice (Figure 1A, 73.4 ± 2.77%) as well as in WT whole splenocyte-reconstituted IL-10$^{-/-}$ mice (69.8 ± 3.11%) was significantly reduced compared to WT (84.4 ± 2.94%, $p = 0.01$), indicating that IL-10 produced by any peripheral immune source is not sufficient for FMN survival after axotomy.

To confirm that transplanted WT splenocytes were able to successfully engraft into the IL-10$^{-/-}$ host, PCR was performed on genomic DNA extracted from WT, IL-10$^{-/-}$,

and reconstituted IL-10$^{-/-}$ spleens using primers flanking a 133 bp sequence within exon 1 of the IL10 gene. In the IL-10$^{-/-}$ animal, codons 5-55 of exon 1 are replaced with a termination codon followed by a neomycin cassette (Kuhn et al., 1993). Gel electrophoresis of the resulting PCR amplicons revealed that the 133 bp fragment was present in WT, absent in IL-10$^{-/-}$ mice, and restored in the IL-10$^{-/-}$ mice reconstituted with WT whole splenocytes, indicating that transplanted WT splenocytes were capable of engrafting into host lymphatic tissue and furthermore survived within the host until 28 dpo (Figure 1B).

4.1.2. IL-10/GFP reporter characterization[1]

With a peripheral immune contribution of neuroprotective IL-10 effectively ruled out, detection of the central source of IL-10 could proceed. A transgenic IL-10/GFP reporter mouse line was utilized to detect central sources of IL-10. This line was created by Dr. Christopher Karp's laboratory via the insertion of an internal ribosome entry site (IRES) downstream of exon 5 of the *Il10* locus (Madan et al., 2009). Transcription of the resultant IL-10/GFP fusion mRNA occurs under control of the endogenous IL-10 promoter, but separate IL-10 and GFP proteins are translated. The result is independent, yet spatiotemporally coupled translation of IL-10 and GFP proteins, the former capable

[1] The experiment contained within section 4.1.2 is reflective of a collaborative effort between Elizabeth Runge and Dr. Deborah Olmstead Setter. Data resulting from this experiment and presented here may overlap with those presented in Dr. Setter's thesis (Setter, 2017) but are otherwise unpublished. Data to which this statement applies are presented here with permission from Dr. Setter. Future publication of these data will include Elizabeth Runge and Dr. Setter as co-authors.

of being secreted from the producing cell, and the latter remaining behind to label the cell body.

To confirm that the IL-10/GFP reporter represents a biological system capable of synthesizing and secreting functional IL-10 in a manner similar the WT animal, FNA was performed on WT and IL-10/GFP mice. Quantification of FMN survival at 28 dpo revealed no significant difference between WT and transgenic reporter (Figure 2, 83.5 ± 3.24% and 79.83 ± 5.27% respectively, $p = 0.57$), indicating that IL-10 was functioning normally in the IL-10/GFP mice.

Determination of optimum conditions for detection of GFP in the IL-10/GFP reporter mouse revealed a detrimental effect of extensive PFA fixation on the ability to detect GFP signal (data not shown). It is possible that perfusion fixation denatured the GFP structure, which is a known issue when working with PFA (Kiernan, 2006). As both IL-10 and GFP expression in the reporter mouse are controlled by the endogenous IL-10 promoter and thus are likely to be present at relatively low levels, the presence of aldehyde residue induced-autofluorescence may also obscure the GFP signal (Madan et al., 2009; Swenson et al., 2007). Therefore to preserve GFP labeling, brains were flash-frozen immediately after removal, sectioned, and lightly post-fixed on the slide, thereby avoiding potential over-fixation due to perfusion-based methods. GFP was also amplified with an anti-GFP antibody to improve signal.

4.1.3. Central cellular localization of IL-10[2]

Neurons, microglia, and astrocytes were investigated as potential sources of neuroprotective IL-10 in the axotomized FMNuc. It was hypothesized that microglia are the major central cellular source of IL-10, as it has been documented that microglia are capable of producing IL-10 *in vitro* (Ledeboer et al., 2002; Mizuno et al., 1994), particularly upon interacting with T cells (Chabot et al., 1999), as well *in vivo* in response to CNS injury (Ma et al., 2015). The cellular source of IL-10 was investigated in the FMNuc at 3, 7, 10, 14, and 28 days after axotomy.

4.1.3.1. Neurons

The possibility of neuronal production of IL-10 was investigated utilizing IHC co-labeling of GFP (as proxy for IL-10 production) with NeuN (*neu*ronal *n*uclei marker) in the IL-10/GFP reporter mouse. NeuN is an RNA-binding protein involved in alternate mRNA splicing. It is associated with a mature neuronal phenotype; alteration of this phenotype in response to axotomy frequently results in diminished NeuN immunoreactivity, which is documented extensively in the literature (Darlot et al., 2017; Duan et al., 2016). This effect of axotomy on NeuN expression can be appreciated in Figure 3, particularly at 10 days post injury. NeuN labeling of axotomized FMN was nonetheless sufficient for detection. GFP labeling was detected in both the left and right

[2] The IL-10/GFP reporter experiments contained within section 4.1.3 are reflective of a collaborative effort between Elizabeth Runge and Dr. Deborah Olmstead Setter. Data resulting from these experiments and presented here may overlap with those presented in Dr. Setter's thesis (Setter, 2017) but are otherwise unpublished. Data to which this statement applies are presented here with permission from Dr. Setter. Future publication of these data will include Elizabeth Runge and Dr. Setter as co-authors.

FMNuc of the unoperated (sham) animal, as well as in the control and axotomized FMNuc of operated animals at all time points investigated (Figure 3). Across all conditions, GFP localized strongly to the cytoplasm of cells with NeuN-labeled nuclei. This GFP-neuronal co-localization did not change appreciably regardless of axotomized condition or time point, indicating that FMN may be an important constitutive source of IL-10.

4.1.3.2. Microglia

A microglial source of IL-10 was next investigated utilizing the marker IBA1. IBA1 is specific to microglia in the CNS and is a commonly employed marker of microglial activation (Hoogland et al., 2015; Ito et al., 1998). It functions as an actin binding protein that is critical for microglial membrane ruffling and phagocytosis (Ohsawa et al., 2000; Sasaki et al., 2001). Although IBA1 reliably labels microglia in perfusion-fixed tissue, attempts to utilize the IBA1 marker in the flash-frozen IL-10/GFP reporter tissue failed to label microglia (data not shown). Antibody labeling for other microglia-associated markers, such as CD68 and F4/80, also failed to yield sufficient labeling of microglia, suggesting that microglial structures may be exquisitely sensitive to fixation methods.

As an alternate method of identifying IL-10 production by microglia, IBA1 labeling was utilized alongside a direct antibody against IL-10 protein in WT tissue. This avoidance of the IL-10/GFP reporter permitted the use of perfusion fixation, which restored IBA1 labeling of microglia in the FMNuc (Figure 4). IL-10 antibody robustly labeled cells having a classic motoneuron morphology in both the control and axotomized

FMNuc at all time points. This IL-10 labeling appeared to be restricted to the cytoplasm and left a central clearing occupied by DAPI, which intercalates with DNA and labels cell nuclei. These data support the previous conclusion utilizing the IL-10/GFP reporter mouse that neurons are constitutive producers of IL-10 in the FMNuc.

IBA1 labeling in the control FMNuc revealed small numbers of microglia processes and the occasional resting microglia cell body (see Figure 4, 3 dpo C). After axotomy, increased IBA1 immunoreactivity was observed, with microglia appearing to ensheath injured motoneurons at 3, 7, and 10 dpo. This likely represents the movement of microglia into the perineuronal space after synaptic stripping of the FMN (Blinzinger & Kreutzberg, 1968; Torvik & Skjorten, 1971b). Microglia appeared in close proximity to the FMN and therefore also to FMN-associated IL-10 immunoreactivity, making discernment of neuronal- and microglial-associated expression of IL-10 difficult to distinguish. In some instances microglia appeared to co-localize with IL-10 signal (Figure 4, arrowheads at 7 and 10 dpo), but upon close observation this apparent "co-localization" was likely due to intimate association of microglia with neuronal processes. Most observed microglia appear not to co-localize with IL-10 labeling. At later time points (14 and 28 dpo), IBA1 labeled nodular structures which are likely clusters of microglia phagocytizing debris from dead neurons (Figure 4, arrows) (Raivich et al., 1998; Torvik & Skjorten, 1971b). These clusters did not co-localize with IL-10 labeling.

Although IL-10 labeling of neurons appeared to be consistent between the IL-10/GFP reporter and IHC on WT tissue, antibody labeling of a secreted ligand such as IL-10 nonetheless presents a number of uncertainties. If a ligand is secreted rapidly, antibody may not label the cellular source; it is also possible that the antibody could label

the ligand bound to its extracellular receptor on another cell. Therefore, a second method of localization was desired to further elucidate whether microglia are a potential source of IL-10 in the FMNuc after axotomy. FISH was utilized to co-localize *Il10* and *Cx3cr1* transcripts in the facial motor nucleus at 3 days post injury. *Cx3cr1* encodes the fractalkine receptor, which is primarily expressed on microglia in the CNS and has important roles for intracellular calcium mobility and chemotaxis, particularly in response to neuronal damage (Harrison et al., 1998). FISH revealed the presence of scattered punctate areas of co-localized *Il10* and *Cx3cr1* probes in the control (left) FMNuc, which appeared to increase in number after axotomy (Figure 5). This raises the possibility that microglia make *Il10* mRNA in the facial motor nucleus, but it is unknown whether this is translated into functional protein.

4.1.3.3. Astrocytes

To determine whether astrocytes are a source of IL-10 in the facial motor nucleus, IHC labeling of the astrocyte-associated intermediate filament protein GFAP was utilized. Because it was found that reliable GFAP labeling can be achieved using both perfusion-fixed and lightly post-fixed flash frozen tissue, both the direct IHC method using IL-10 antibody on WT tissue as well as GFP labeling of IL-10/GFP reporter were utilized. Using direct IL-10 IHC, IL-10 again robustly labeled cells on both the control and axotomized FMNuc in a characteristic motoneuron-like pattern (Figure 6). GFAP labeling in the control FMNuc was mainly restricted to astrocytes lining penetrating blood vessels and along the *glia limitans* on the ventral edge of the brainstem. GFP co-localized occasionally with GFAP-labeled astrocytes adjacent to blood vessels (Figure 6,

7 dpo C). GFAP-labeled astrocytes were detected within the FMNuc parenchyma in response to axotomy beginning at 3 dpo and persisting through 28 dpo. Weak IL-10 co-localization with GFAP was detectable at early time points after axotomy (Figure 6, arrowheads at 3, 7, and 10 dpo) and increased in strength at later time points as GFAP-labeled astrocytes became increasingly hypertrophied (arrowheads at 14 and 28 dpo). IL-10 labeling appeared mainly in astrocyte cell bodies, but occasionally also labeled processes.

IL-10/GFP reporter also displayed a progressive increase in GFAP immunoreactivity in the time points following axotomy, but unlike in the perfusion-fixed WT tissue, GFAP-labeled astrocytes were scarce at 3 dpo (Figure 6, right pane). This delay in the onset of GFAP immunolabeling is likely attributable to the fixation method used to preserve GFP signal in the reporter mouse. GFP labeling representing IL-10 production began to co-localize with GFAP by 10 dpo. By 14 and 28 dpo, nearly all GFAP-labeled astrocytes observed in the FMNuc were also positive for GFP. GFP labeling other structures in the FMNuc, likely motoneurons, could be observed through all time points.

In summary, axotomy induced expression of IL-10 by astrocytes. This expression was detectable beginning at early (3 dpo) time points when utilizing direct IL-10 antibody in perfused tissue, and at later (10 dpo) time points using the IL-10/GFP reporter. This expression appeared to persist through 28 dpo using both labeling methods. Neuronal expression of IL-10 appeared to be constitutive and did not noticeably change after axotomy. Microglial production of IL-10 was not detected by immunohistochemical methods, although there is evidence that microglia may transcribe *Il10* mRNA.

4.1.4. Cell-specific conditional IL-10 knockout[3]

With at least two potential sources of IL-10 in the FMNuc identified, it was next important to determine whether any single source was necessary for FMN survival after axotomy. Three mouse strains were created to selectively knock out IL-10 expression in single central cell populations using tamoxifen-inducible Cre recombination of floxed IL-10 sequences. Cre recombinase expression was driven by the promoters for *Thy1*, *Cx3cr1*, and *Gfap* for targeted deletion of IL-10 in neurons, microglia, and astrocytes, respectively. The *Thy1*^{Cre} mouse contains a modified *Thy1* promoter region restricting its expression to neuronal cells (Caroni, 1997). Tamoxifen induction of Cre began one week prior to axotomy and continued until 28 dpo, at which time FMN survival was assessed. Littermates lacking either Cre or floxed IL-10 sequences were used as controls and received identical treatments to experimental mice.

Conditional knockout (cKO) of IL-10 did not significantly reduce FMN survival in any of the Cre-expressing mouse lines compared to littermate controls, indicating that IL-10 production by neurons, microglia, or astrocytes alone was not critical for neuroprotection after axotomy (Figure 7A, 85.7 ± 5.22% in control vs. 86 ± 6.27% in cKO, $p = 0.97$; 7B, 83.3 ± 3.65% vs. 88.1 ± 2.54%, $p = 0.30$; 7C, 81.3 ± 6.77%, vs 85.3 ± 7.22%, $p = 0.71$, respectively). This is in agreement with the localization data showing IL-10 production by both neurons and astrocytes at minimum. It is likely that conditional

[3] The CX3CR1^{Cre} and GFAP^{Cre} experiments contained within section 4.1.4 are reflective of a collaborative effort between Elizabeth Runge and Dr. Deborah Olmstead Setter. Data resulting from these experiments and presented here may overlap with those presented in Dr. Setter's thesis (Setter, 2017) but are otherwise unpublished. Data to which this statement applies are presented here with permission from Dr. Setter. Future publication of these data will include Elizabeth Runge and Dr. Setter as co-authors.

knockdown of IL-10 from one of these sources induces compensatory production by another.

4.2. Aim 2: Characterize adaptive immune cell participation in neuroprotective IL-10 receptor signaling after facial nerve axotomy

4.2.1. Immunohistochemical identification of potential CD4+ T cell-neuron-glia interactions in the axotomized facial motor nucleus

It is unknown whether the CD4+ T cells responsible for mediating neuroprotection after axotomy are capable of infiltrating into the CNS parenchyma, or alternatively whether they must communicate with central cells across the blood-brain barrier. Immunohistochemical labeling was utilized to determine whether T cells are capable of entering the FMNuc after axotomy where they may potentially interact with centrally located cells.

4.2.1.1. Detection of CD4+ T cells in FMNuc after axotomy

Utilization of an antibody against CD4 antigen revealed no infiltrate in the unoperated FMNuc (Figure 8A). In the contralateral axotomized FMNuc, small CD4+ cells appeared beginning at 10 dpo and persisting through 14 and 28 dpo (Figure 8B, 14 dpo pictured). These cells were approximately 5–10 μm in diameter and consisted of a bright rim of CD4+ immunoreactivity surrounding a central DAPI-labeled nucleus in most sections. Many cells appeared elongated rather than perfectly circular in shape (Figure 8C). To ensure that these cells were true T cells as opposed to other CD4+ immune cells such as monocytes/macrophages, sections were also labeled with anti-CD3

antibody, which binds the T cell co-receptor. All CD4+ cells observed infiltrating the FMNuc were CD3+, but not all CD3+ T cells were CD4+, indicating that CD8+ T cells may also infiltrate the FMNuc after axotomy (Figure 8D–F). Collectively these data support findings in the literature indicating the presence of T cells in the FMNuc after axotomy (Bohatschek et al., 2004; Ha et al., 2007b; Ha et al., 2006; Huang et al., 2012; Raivich et al., 1998).

4.2.1.2. Determination of CD4+ T cell infiltration into CNS parenchyma

Although CD4+ T cells were detected in the FMNuc after axotomy, this observation alone was not sufficient to determine whether they are capable of interacting directly with cells in CNS parenchyma, or whether they are restricted to the vascular compartment. In combination with anti-CD4 to label T cells and a fluorescent Nissl stain to label neurons, an antibody against CD31 was used to visualize vasculature in the FMNuc. CD31, also known as PECAM-1 (platelet/endothelial cell adhesion molecule 1), is enriched in cell-cell junctions in the vascular endothelium. This triple stain revealed the presence of small penetrating blood vessels in the control FMNuc without evidence of T cell infiltration (Figure 9A). CD4+ T cells were detectable in the axotomized FMNuc at 14 dpo. These T cells appeared not to be contained within CD31-labeled blood vessels, indicating that they are capable of extravasating into the CNS parenchyma (Figure 9B–D). Furthermore, some T cells appeared to be in close proximity to injured motoneurons (Figure 9C–D, arrowheads).

4.2.1.3. Evaluation of CD4+ T cell association with microglia after axotomy

CD4+ T cells must interact with MHCII on a central APC to mediate neuroprotection after axotomy (Byram et al., 2004). With the ability of CD4+ T cells to extravasate into the FMNuc parenchyma established, potential associations of CD4+ T cells with microglia, a prominent candidate for APC in the FMNuc, were next evaluated. Double labeling for CD4 and IBA1 revealed the presence of small resting microglia in the unaxotomized FMNuc that were not associated with any T cell infiltrate (Figure 10A). At 14 dpo, microglia increased expression of IBA1, and a CD4+ T cell infiltrate into the injured FMNuc was observed (Figure 10B-D). These CD4+ T cells often appeared to be in close proximity with activated microglia and clustered around microglial nodules (Figure 10C-D).

In summary, IHC showed that CD4+ T cells infiltrate the FMNuc after axotomy. These T cells appear capable of crossing the blood-brain barrier and entering the CNS parenchyma where they may interact directly with central cells. Close associations of T cells with neurons and microglia in particular were observed. It is therefore possible that immune-mediated neuroprotection (as well as any other central molecular response to axotomy that is influenced by peripheral immune status) is effected by local paracrine signaling from infiltrating T cells or by direct contact between T cells and central cells in the FMNuc.

4.2.2. Effect of immune status on central IL-10 receptor expression

To determine whether the adaptive immune system has an effect on central upregulation of the IL-10R after axotomy, qPCR was performed for IL-10R subunit gene

expression in the FMNuc at 7, 14, 28, and 56 dpo in WT and RAG-2$^{-/-}$ mice. Gene expression was calculated as percent increase in expression in the axotomized FMNuc relative to paired control FMNuc. Two-way ANOVA revealed that *Il10ra* expression was significantly different between WT and RAG-2$^{-/-}$, although post hoc tests failed to detect a specific time point at which this effect was significant (Figure 11A, $p = 0.0159$). The percent increase in *Il10rb* expression was also significantly different between WT and RAG-2$^{-/-}$ (Figure 11B, $p < 0.0001$) ; RAG-2$^{-/-}$ mice had decreased *Il10rb* expression relative to WT particularly at 7 (114.2 ± 30.57% compared to 198.1 ± 16.86%, $p = 0.0019$) and 14 dpo (100.7 ± 9.89% compared to 174.3 ± 15.18%, $p = 0.0062$). These data indicate that the adaptive arm of the immune system is necessary for full upregulation of IL-10R subunits in the FMNuc after axotomy.

To determine whether the CD4+ T cell specifically regulates IL-10R expression, the 14 dpo time point was selected for all subsequent analyses as this coincides with the peak of T cell infiltration into the FMNuc after FNA (Raivich et al., 1998) as well as the peak of microglial and astrocyte activation-associated gene expression (Setter et al., 2018b). Gene expression was calculated as fold change of mRNA expression in axotomized FMNuc relative to control FMNuc. Fold change in *Il10ra* expression (Figure 12A) was significantly reduced in RAG-2$^{-/-}$ (3.97 ± 0.26) compared to WT (5.70 ± 0.35, $p < 0.05$) and was restored to WT levels after adoptive transfer of WT CD4+ T cells (6.12 ± 0.38). Fold change in *Il10rb* expression (Figure 12B) was likewise significantly reduced in RAG-2$^{-/-}$ (2.06 ± 0.10) compared to WT (2.74 ± 0.15, $p < 0.01$), and adoptive transfer of WT CD4+ T cells restored normal *Il10rb* expression (2.74 ± 0.07).

This regulation of IL-10R expression by CD4+ T cells could be due to 1) T cell-triggered induction of IL-10R expression on central cells, 2) infiltration of IL-10R-expressing T cells into the FMNuc after axotomy, or 3) a combination of both mechanisms. To elucidate which scenario occurs after FNA, RAG-2$^{-/-}$ mice were reconstituted with CD4+ T cells lacking IL-10RB (*Il10rb*$^{-/-}$), which is necessary for IL-10 signal transduction (Kotenko et al., 1997; Spencer et al., 1998). *Il10ra* fold change in RAG-2$^{-/-}$ reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells (Figure 12A, 7.54 ± 0.65) was significantly increased relative to RAG-2$^{-/-}$ ($p < 0.001$), indicating that IL-10 signaling within T cells was not necessary for T cells to regulate *Il10ra* gene expression in the FMNuc. In fact, upregulation of *Il10ra* in RAG-2$^{-/-}$ mice given *Il10rb*$^{-/-}$ CD4+ T cells was significantly greater than in WT or RAG-2$^{-/-}$ given WT CD4+ T cells ($p < 0.05$). *Il10rb* fold change in RAG-2$^{-/-}$ reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells (Figure 12B, 2.64 ± 0.09) was significantly increased relative to RAG-2$^{-/-}$ ($p < 0.001$) and was not significantly different from WT or RAG-2$^{-/-}$ + WT CD4+ T cells. Due to complete absence of *Il10rb* expression by the transgenic T cells, this restoration of *Il10rb* gene expression could only be due to a T cell-mediated induction of *Il10rb* in central cells in the FMNuc. Therefore it appears that CD4+ T cells mediate IL-10R expression on central cells in the FMNuc after axotomy regardless of their own IL-10R expression or function, although the presence and signaling capability of IL-10R on WT infiltrating T cells could not be ruled out by this experiment.

4.2.3. Impact of IL-10 receptor-deficient CD4+ T cells on facial motoneuron survival after axotomy

Athough it was found that *Il10rb*$^{-/-}$ CD4+ T cells were capable of triggering an increase in IL-10R expression by central cells in the FMNuc, the neuroprotective capacity of these T cells remained unknown. It has previously been established that RAG-2$^{-/-}$ mice exhibit decreased FMN survival after axotomy relative to WT, and that WT CD4+ T cells restore FMN survival in RAG-2$^{-/-}$ mice to WT levels (Serpe et al., 2003; Serpe et al., 2000). The ability of *Il10rb*$^{-/-}$ CD4+ T cells to mediate neuroprotection was assayed by counting FMN survival in WT, RAG-2$^{-/-}$, and RAG-2$^{-/-}$ reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells at 28 dpo. In accordance with the literature, survival in RAG-2$^{-/-}$ was significantly decreased compared to WT (Figure 13, 69 ± 1.3% compared to 81 ± 3%, p <0.05). However, *Il10rb*$^{-/-}$ CD4+ T cells failed to rescue FMN survival; survival in reconstituted animals remained low compared to WT (61.8 ± 3.5%, $p < 0.01$).

To confirm that adoptively transferred *Il10rb*$^{-/-}$ T cells were still viable *in vivo* at 28 dpo, IHC was performed using spleens from WT, RAG-2$^{-/-}$, and RAG-2$^{-/-}$ mice reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells. WT spleens showed expression of CD3+/CD4+ T cells within follicles; CD3 labeling (specific for T cells) was lost in RAG-2$^{-/-}$ splenic follicles but was restored in RAG-2$^{-/-}$ given *Il10rb*$^{-/-}$ CD4+ T cells (Figure 14).

Overall, these data suggest that the increase in IL-10R expression by central cells, although mediated by T cells, is insufficient for FMN survival after FNA; rather, IL-10R signaling by the T cell itself may be necessary for neuroprotection.

4.2.4. Central gene expression profile changes after facial nerve axotomy in mice reconstituted with wild-type or IL-10 receptor-deficient CD4+ T cells

IL-10R signaling in T cells is known to cause suppression of CD28 co-stimulation in the context of antigen presentation, resulting in T cell tolerance to antigen (Akdis et al., 2000). Tolerized T cells can suppress the activation and proliferation of other T cells and inhibit APC expression of MHCII and co-stimulatory molecules in an antigen-specific manner (Chai et al., 1999; Vendetti et al., 2000). It is therefore likely that loss of IL-10R signaling in T cells permits them to become reactive against self-antigen. Once armed, effector T cells (particularly Th1 and Th17 cells) can activate APCs (including microglia) to a pro-inflammatory state (Chen et al., 2017; Dasgupta et al., 2002; Li et al., 2013; Murphy et al., 2010). Additionally, loss of tolerogenic T cells could disinhibit APC expression of MHCII and co-stimulatory molecules, thereby potentiating adaptive immune responses against self-antigen.

To investigate the role of T cell IL-10R signaling in modulating central APC activation after FNA, qPCR for genes associated with microglial/macrophage activation, antigen presentation, T cell co-stimulation, and complement deposition/synaptic pruning was performed in the FMNuc of WT, RAG-2$^{-/-}$, and RAG$^{-/-}$ mice reconstituted with either WT CD4+ T cells (which confer neuroprotection after axotomy) or *Il10rb*$^{-/-}$ CD4+ T cells (which fail to confer neuroprotection). The hypothesis was that loss of IL-10 receptor signaling on T cells would promote a pro-inflammatory, autoimmune microglial/macrophage gene signature in the FMNuc.

4.2.4.1. Genes associated with activated microglia: CD68, CD40, TNFα, TNF receptor, Nos2

After FNA, microglia proliferate in the FMNuc and express the pan-macrophage marker CD68 (Blinzinger & Kreutzberg, 1968; Graeber et al., 1988c; Torvik & Skjorten, 1971b; Wainwright et al., 2010). At 14 dpo, *Cd68* mRNA increased approximately six fold in the WT axotomized FMNuc; there was no difference in *Cd68* expression between WT, RAG-2$^{-/-}$, and RAG-2$^{-/-}$ mice reconstituted with WT CD4+ T cells (Figure 15A, 6.078 ± 0.88, 5.809 ± 0.25, 6.785 ± 0.29, respectively). RAG-2$^{-/-}$ reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells had significantly increased *Cd68* expression (9.37 ± 0.69) relative to WT ($p < 0.01$), RAG-2$^{-/-}$, and RAG-2$^{-/-}$ + WT CD4+ T cells ($p < 0.05$), indicating that *Il10rb*$^{-/-}$ T cells promote an increase in general microglial reactivity after axotomy.

CD40 is a co-stimulatory protein found on APCs that is necessary for their activation (Banchereau et al., 1994). At 14 dpo, *Cd40* mRNA was modestly increased in the axotomized FMNuc relative to control across all conditions, and there were no significant differences between WT, RAG-2$^{-/-}$, and RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4+ T cells (Figure 15B, 1.57 ± 0.11, 1.46 ± 0.08, 2.17 ± 0.24, respectively). There was also no significant difference between RAG-2$^{-/-}$ reconstituted with WT CD4+ T cells (2.40 ± 0.25) and those reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells, although the former showed significantly increased *Cd40* expression relative to WT and RAG-2$^{-/-}$ ($p < 0.05$).

TNFα is a pro-inflammatory cytokine secreted by classically activated microglia as part of the innate immune response to eliminate pathogens (Lull & Block, 2010). TNFα also acts on microglia in an autocrine fashion via ligation of TNF receptor 1 (TNFR1, gene name *Tnfrsf1*) to promote further TNF- and other pro-inflammatory

cytokine-associated signaling (Kuno et al., 2005). No *Tnf* transcript was detected in the control FMNuc, in agreement with previous studies (Haulcomb et al., 2014; Setter et al., 2018b). Therefore to compare changes in *Tnf* mRNA expression, the differences between *Tnf* and *Gapdh* cycle threshold values were normalized to a standardized spleen control, which was the same for each axotomized FMNuc sample. As shown in Figure 15C, *Tnf* mRNA in axotomized FMNuc at 14 dpo ranged between 1–3% of spleen standard, and no differences were observed between WT, RAG-2$^{-/-}$, RAG-2$^{-/-}$ + WT CD4+ T cells, and RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4+ T cells (0.015 ± 0.003, 0.017 ± 0.004, 0.024 ± 0.005, 0.023 ± 0.004, respectively). *Tnfrsf1* mRNA approximately doubled relative to control FMNuc across all conditions; no significant differences were detected (Figure 15D, WT: 2.20 ± 0.08, RAG-2$^{-/-}$: 2.01 ± 0.13, RAG-2$^{-/-}$ + WT CD4: 2.11 ± 0.08, RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4: 2.10 ± 0.10).

Also upregulated in pro-inflammatory microglia, inducible nitric oxide synthase (iNOS, gene name *Nos2*) facilitates production of free radical nitric oxide (NO), which is cytotoxic to invading pathogens (Lull & Block, 2010; Moss & Bates, 2001). iNOS activity is also associated with neuroinflammation and neurodegenerative diseases such as MS (Dasgupta et al., 2002; Liu et al., 2002). At 14 dpo, *Nos2* mRNA did not appear to be upregulated in the axotomized FMNuc and in fact maybe have been slightly decreased relative to control in WT and RAG-2$^{-/-}$ mice receiving *Il10rb*$^{-/-}$ CD4+ T cells (Figure 15E, results of one-sample t test against fold change of 1: WT, 0.76 ± 0.05, $p = 0.008$; RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4, 0.71± 0.09, $p = 0.033$). There were no significant differences detected between these groups and RAG-2$^{-/-}$ or RAG-2$^{-/-}$ mice receiving WT CD4+ T cells (0.87 ± 0.17 and 0.78 ± 0.11, respectively).

In summary, RAG-2$^{-/-}$ mice given *Il10rb*$^{-/-}$ CD4+ T cells showed increased expression of *Cd68*, indicating greater levels of microglial activation and/or proliferation. However, more specific markers alluding to a pro-inflammatory microglial phenotype were not significantly different between RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4+ T cells and other conditions, indicating that the failure of *Il10rb*$^{-/-}$ CD4+ T cells to mediate neuroprotection is likely not due to their direct activation of microglia to an overtly cytotoxic state.

4.2.4.2. Genes associated with antigen presentation and T cell co-stimulation: MHCII, CD80, and CD86

As innate immune cells and APCs of the CNS, microglia present antigen on MHCII to infiltrating CD4+ T cells (Yang et al., 2010). In order to become activated against antigen, T cells must also receive co-stimulatory signals from APCs via interactions of T cell CD28 antigen with APC CD80 and CD86 (also known as B7.1 and B7.2) (Janeway & Bottomly, 1994). However, this activation is not unidirectional; the interaction of T cells and microglia/macrophages also induces upregulation of microglial MHCII and co-stimulatory molecules to optimize their antigen-presenting capability, sustaining the adaptive immune response (Aloisi et al., 2000a; Yang et al., 2010). Both MHCII and CD86 are also upregulated on microglia in response to stimulation by classical activators such as IFNγ and LPS (Aloisi et al., 1998; Menendez Iglesias et al., 1997; Satoh et al., 1995).

H2ab1 encodes the predominant MHCII haplotype expressed in C57BL/6J mice. Axotomy robustly induced *H2ab1* in WT animals; expression increased approximately five fold relative to control FMNuc at 14 dpo (Figure 16A). RAG-2$^{-/-}$ mice that received

adoptive transfer of *Il10rb*$^{-/-}$ CD4+ T cells exhibited dramatically higher induction of *H2ab1* expression after axotomy compared to WT, RAG-2$^{-/-}$, and RAG-2$^{-/-}$ + WT CD4+ T cells (12.5 ± 1.88 vs. 4.9 ± 0.71, 6.1 ± 1.12, 5.8 ± 0.89, respectively; $p < 0.01$).

Gene expression of the co-stimulatory molecule CD80 was modestly increased (approximate two-fold induction) in WT after axotomy; expression of *Cd80* trended higher in RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4+ T cells but was not significantly different from other conditions (Figure 16B, WT: 2.0 ± 0.28; RAG-2$^{-/-}$: 1.3 ± 0.25; RAG-2$^{-/-}$ + WT CD4: 2.6 ± 0.56; RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4: 3.2 ± 0.45). Expression of *Cd86*, however, increased much more robustly in response to axotomy, with approximate nine-fold induction in WT (Figure 16C). RAG-2$^{-/-}$ mice given *Il10rb*$^{-/-}$ CD4+ T cells exhibited significantly increased expression of *Cd86* after axotomy compared to WT, RAG-2$^{-/-}$, and RAG-2$^{-/-}$ + WT CD4+ T cells (13.7 ± 1.10 vs. 8.9 ± 0.67, 7.9 ± 1.13, 10.1 ± 1.24, respectively, $p < 0.05$).

Collectively these data show that loss of IL-10R signaling in T cells results in increased expression of genes encoding antigen presentation and T cell co-stimulatory molecules in the FMNuc after axotomy.

4.2.4.3. Genes associated with synaptic pruning: B2m and C3

MHC class I is a cell surface molecule with complex roles in synaptic plasticity during development (Shatz, 2009), microglial phagocytic nodule formation (Bohatschek et al., 2004), and synaptic stripping after peripheral nerve injury (Oliveira et al., 2004; Sabha et al., 2008). Unlike MHCII, MHCI can be displayed on any nucleated cell in the body. *B2m* encodes the MHCI subunit β2-microglobulin. At 14 dpo, *B2m* expression

increased fivefold in the WT axotomized FMNuc and was not significantly different in RAG-2$^{-/-}$ mice (Figure 17A, 5.0 ± 0.28, 7.2 ± 0.85, respectively). Adoptive transfer of WT CD4+ T cells into RAG-2$^{-/-}$ resulted in significantly increased *B2m* expression relative to WT and RAG-2$^{-/-}$ (RAG-2$^{-/-}$ + WT CD4: 11.0 ± 1.02, vs WT, $p < 0.001$; vs. RAG-2$^{-/-}$, $p < 0.01$). RAG-2$^{-/-}$ mice given *Il10rb*$^{-/-}$ CD4+ T cells had significantly increased expression of *B2m* after axotomy compared to WT, RAG-2$^{-/-}$, and RAG-2$^{-/-}$ + WT CD4+ T cells (RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4: 14.9 ± 0.81, vs. WT and RAG-2$^{-/-}$, $p < 0.0001$; vs. RAG-2$^{-/-}$ + WT CD4, $p < 0.01$).

The complement protein C3 is involved in microglial-mediated synaptic pruning (Schafer et al., 2012) as well as APC and T cell activation (Strainic et al., 2008). *C3* mRNA increased modestly in the FMNuc of WT mice after axotomy and was not significantly different in RAG-2$^{-/-}$ mice (Figure 17B, 2.6 ± 0.39, 0.9 ± 0.35, respectively). Adoptive transfer of WT CD4+ T cells into RAG-2$^{-/-}$ mice resulted in increased *C3* expression (5.3 ± 1.24, vs. WT, $p < 0.05$; vs. RAG-2$^{-/-}$, $p < 0.01$). Adoptive transfer of *Il10rb*$^{-/-}$ CD4+ T cells resulted in even greater *C3* expression than in WT, RAG-2$^{-/-}$, or RAG-2$^{-/-}$ + WT CD4+ T cells (9.4 ± 0.86, vs. WT and RAG-2$^{-/-}$, $p < 0.0001$; vs. RAG-2$^{-/-}$ + WT CD4, $p < 0.01$).

In summary, adoptive transfer of *Il10rb*$^{-/-}$ CD4+ T cells increased central expression of genes associated with microglial activation (*Cd68*), antigen presentation (*H2ab1* and *B2m*), T cell co-stimulation (*Cd86*), and synaptic pruning (*B2m* and *C3*) after axotomy, without increasing markers that would indicate an overtly pro-inflammatory microglial phenotype (*Cd40, Tnf, Tnfrsf1, Nos2*). These data suggest that loss of IL-10R signaling in T cells results in enhancement of antigen presentation by central APC

(probably microglia), which may facilitate ongoing adaptive immune activation against neuronal self-antigen and prevent the neuroprotective actions of CD4+ T cells.

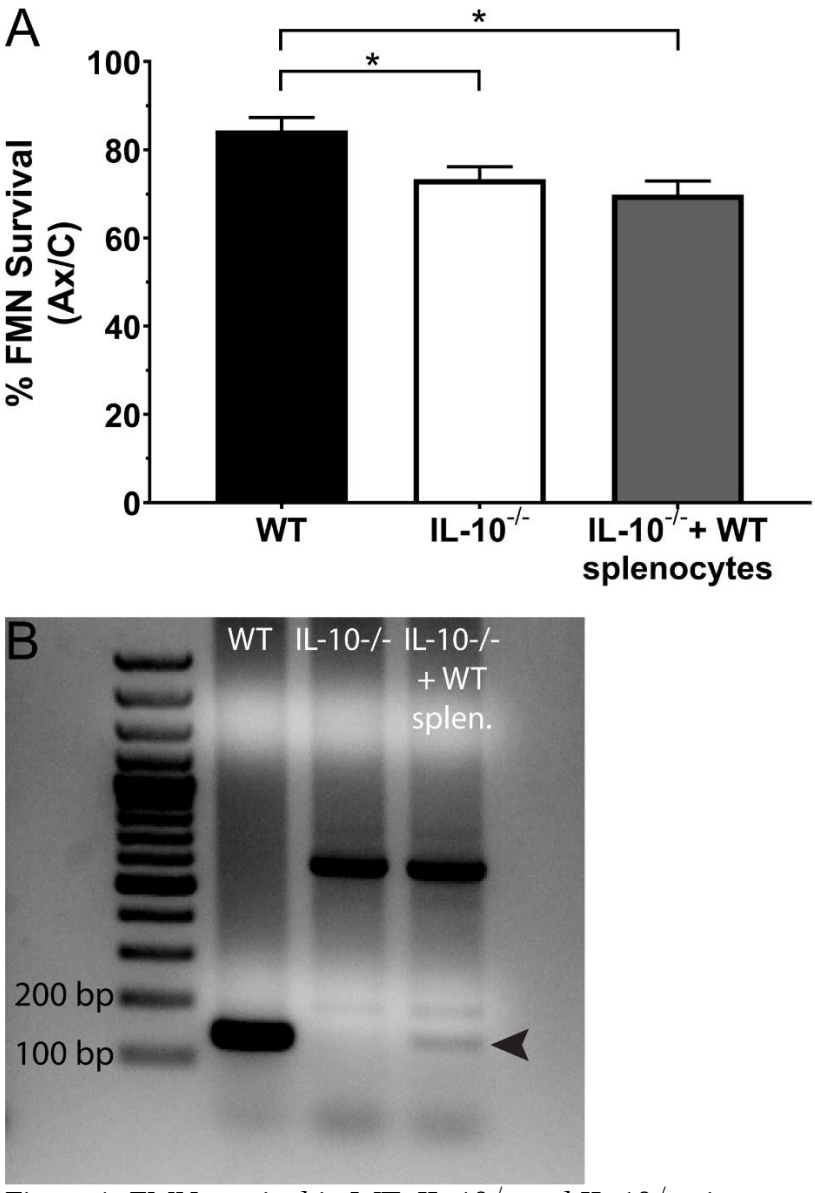


Figure 1: FMN survival in WT, IL-$10^{-/-}$, and IL-$10^{-/-}$ mice reconstituted with WT whole splenocytes and splenocyte engraftment confirmation.

A) Average percent survival of axotomized FMN relative to control ± SEM at 28 dpo. No significant difference was detected between IL-$10^{-/-}$ and IL-$10^{-/-}$ receiving splenocytes; survival in both groups was significantly decreased relative to WT (* $p < 0.05$). B) PCR amplicon gel showing a 133 bp fragment (arrow) of exon 1 of the IL10 coding sequence which is present in WT, absent in IL-$10^{-/-}$, and restored in IL-$10^{-/-}$ reconstituted with WT whole splenocytes.

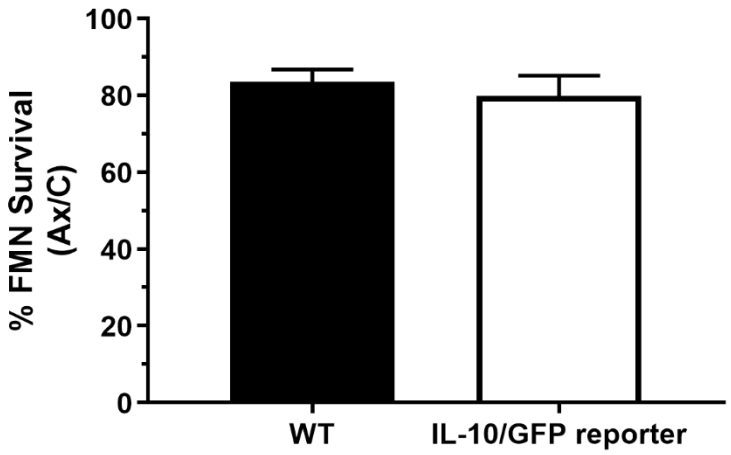


Figure 2: FMN survival in WT and IL-10/GFP reporter mice.

Average percent survival of axotomized FMN relative to control ± SEM at 28 dpo. No significant difference was detected between WT and reporter.

E. M. Runge and D. O. Setter (Setter, 2017) contributed equally to this figure. Reproduced with Dr. Setter's permission.

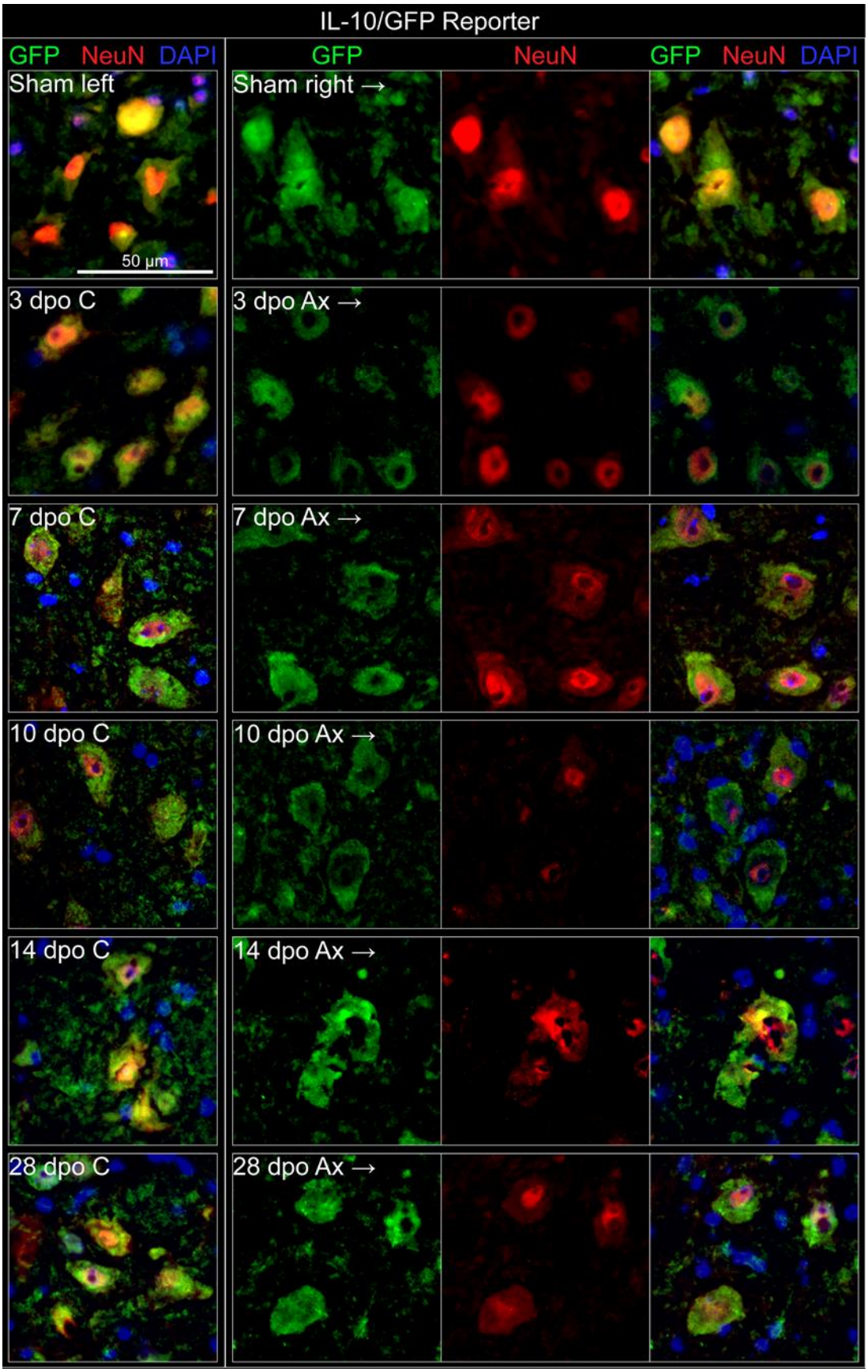

IL-10/GFP Reporter
GFP NeuN DAPI
GFP
NeuN
GFP NeuN DAPI
Sham left
50 µm
Sham right →
3 dpo C
3 dpo Ax →
7 dpo C
7 dpo Ax →
10 dpo C
10 dpo Ax →
14 dpo C
14 dpo Ax →
28 dpo C
28 dpo Ax →

Figure 3: IHC co-localization of neurons (NeuN) with GFP in IL-10/GFP reporter mouse.

Dpo = days post operation, C = control (left) FMNuc, Ax = axotomized (right) FMNuc.

E. M. Runge and D. O. Setter (Setter, 2017) contributed equally to this figure. Data reproduced with Dr. Setter's permission.

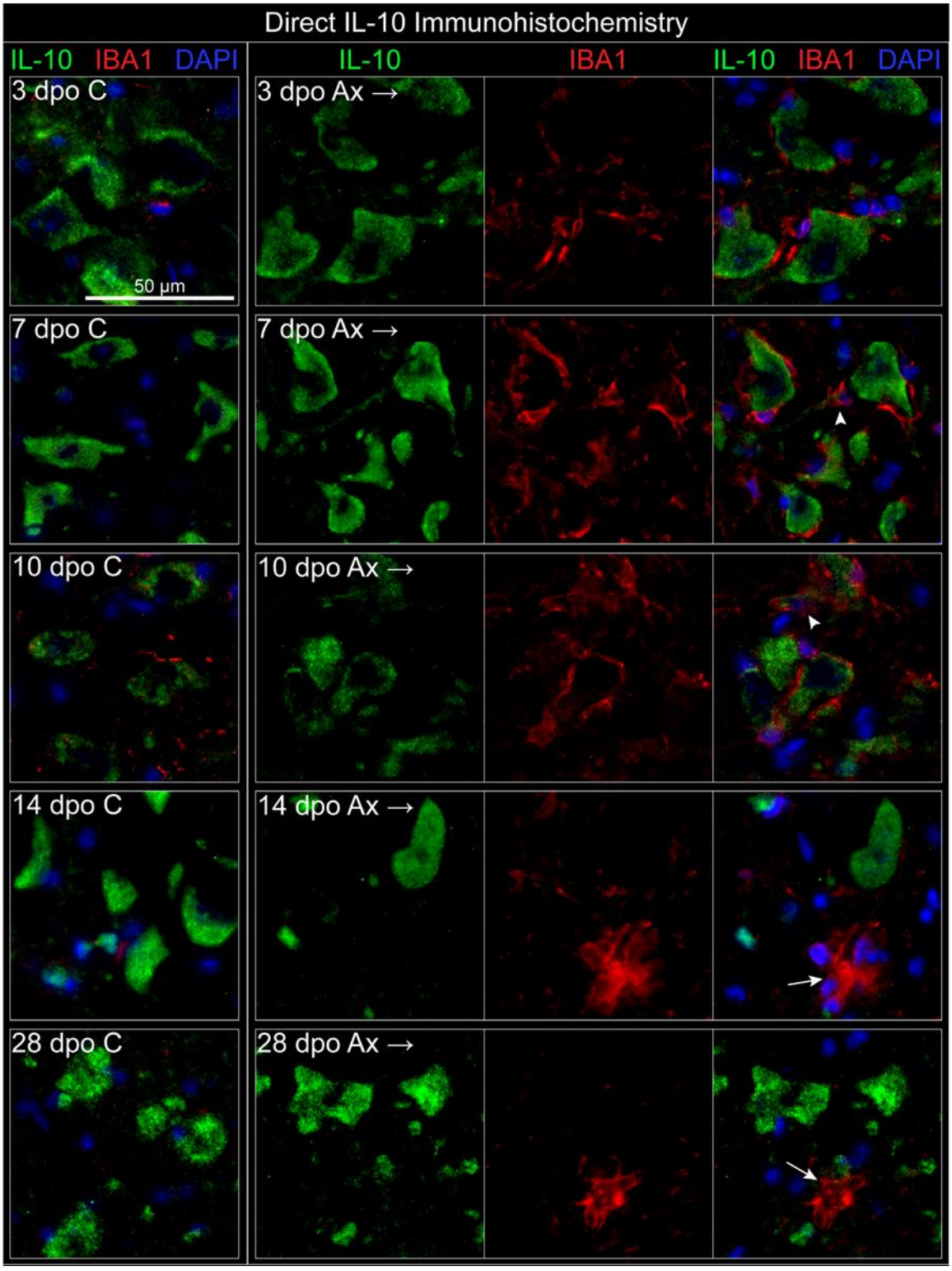

Direct IL-10 Immunohistochemistry
IL-10 IBA1 DAPI
IL-10
IBA1
IL-10 IBA1 DAPI
3 dpo C
3 dpo Ax →
50 µm
7 dpo C
7 dpo Ax →
10 dpo C
10 dpo Ax →
14 dpo C
14 dpo Ax →
28 dpo C
28 dpo Ax →

Figure 4: Direct IHC localization of microglia (IBA1) and IL-10 in WT mice.

Arrowheads at 7 and 10 dpo demonstrate microglia lying in close proximity to IL-10-positive neuronal processes. Arrows at 14 and 28 dpo indicate microglial nodules.

Dpo = days post operation, C = control (left) FMNuc, Ax = axotomized (right) FMNuc.

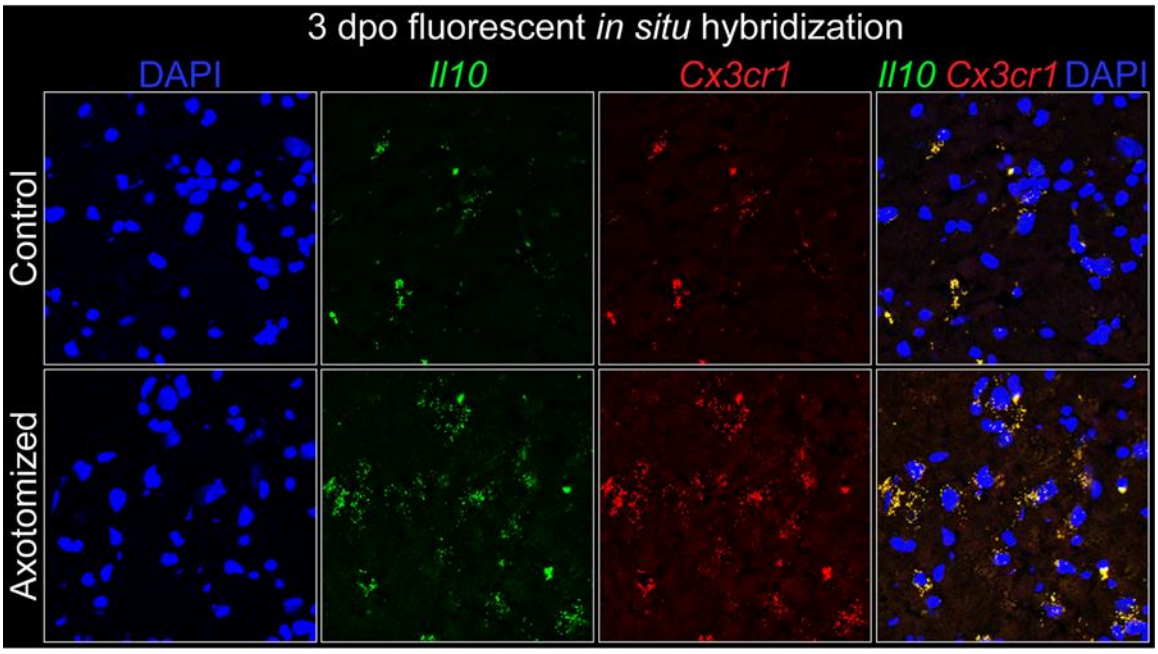


Figure 5: Fluorescent *in situ* hybridization for *Il10* and *Cx3cr1* mRNA transcripts at 3 dpo.

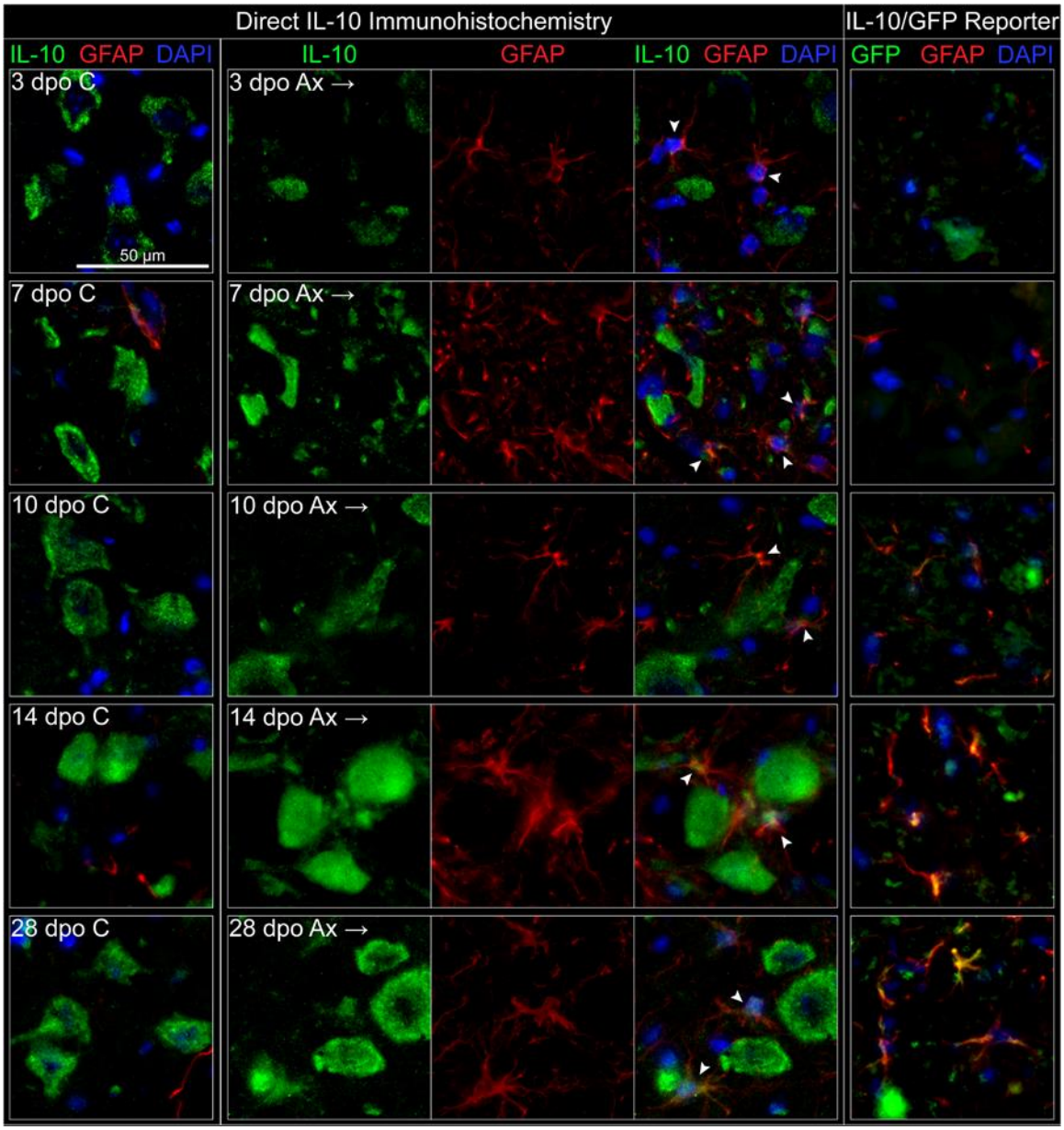


Figure 6: IHC co-localization of astrocytes (GFAP) with IL-10 using both direct IL-10 antibody and IL-10/GFP reporter.

Left panels depict direct IL-10 antibody, right panels depict reporter. Arrowheads indicate areas of co-localization.

Dpo = days post operation, C = control (left) FMNuc, Ax = axotomized (right) FMNuc.

E. M. Runge and D. O. Setter (Setter, 2017) contributed equally to the IL-10/GFP reporter panels in this figure. Data reproduced with Dr. Setter's permission.

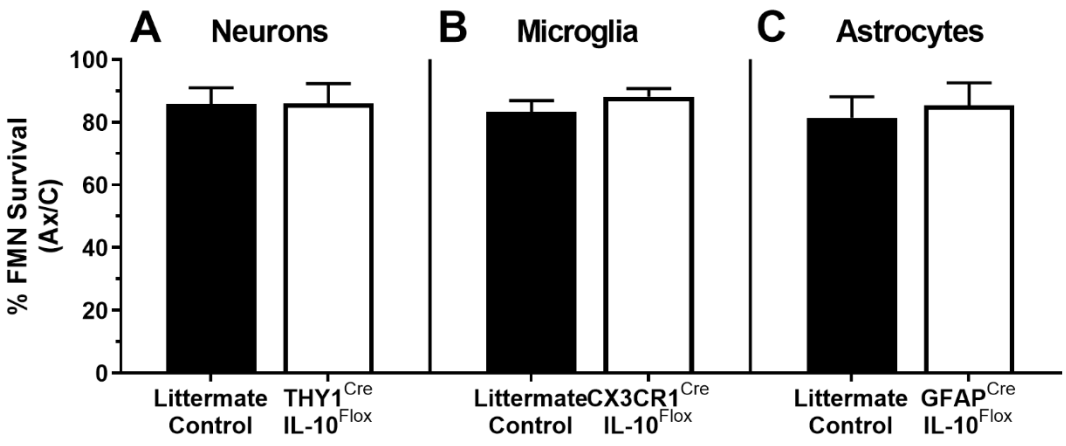


Figure 7: FMN survival in IL-10 cKO mice compared to littermate controls.

Average percent survival of axotomized FMN relative to control ± SEM at 28 dpo in mice lacking IL-10 in A) neurons, B) microglia, and C) astrocytes. No significant differences were seen between littermates and cKO.

E. M. Runge and D. O. Setter (Setter, 2017) contributed equally to panels B and C in this figure. Data reproduced with Dr. Setter's permission.

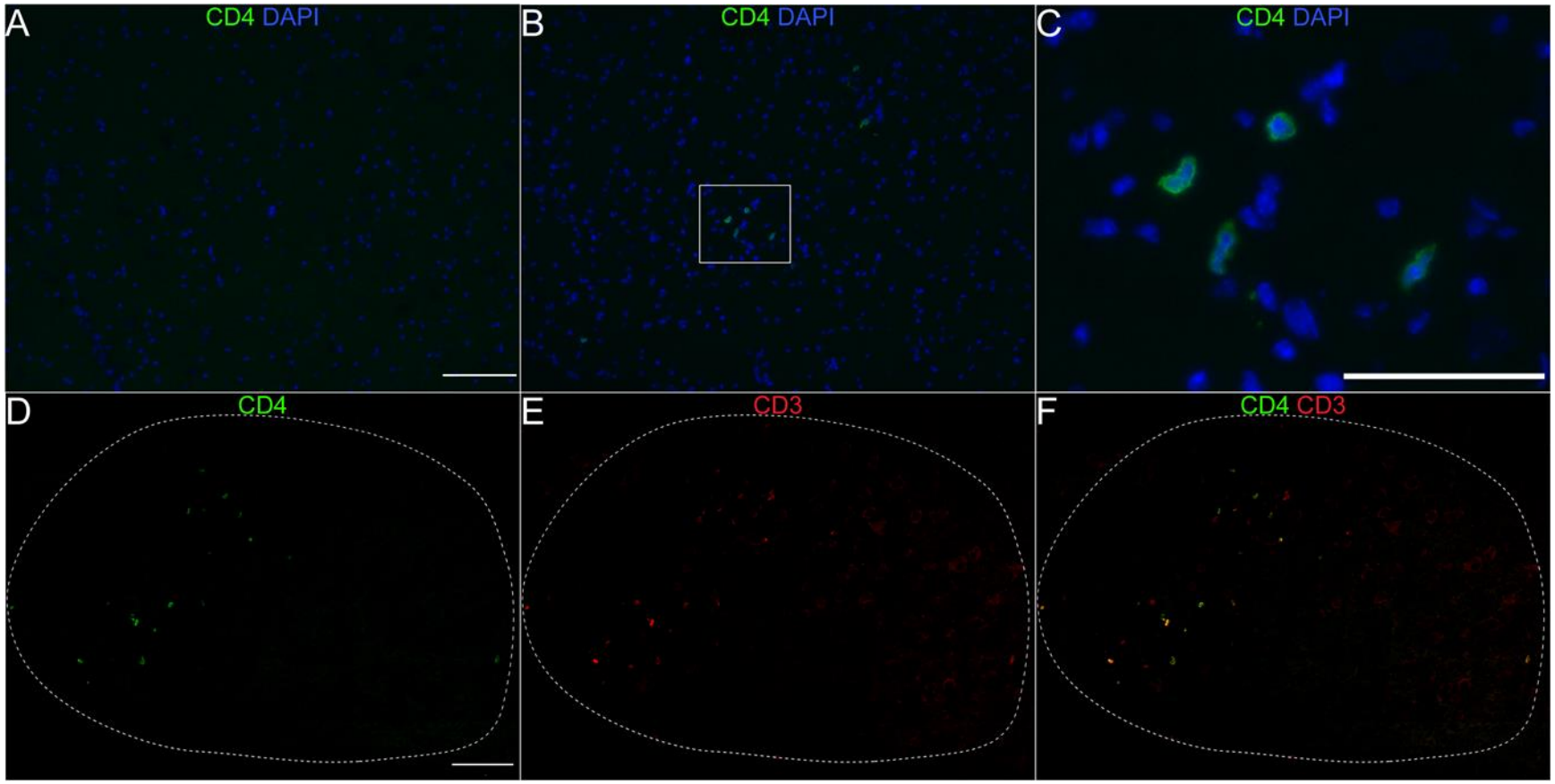


Figure 8: IHC identification of CD4+ T cells in the FMNuc.

A) 20X image of control FMNuc at 14 dpo with no CD4+ cells. B) 20X image of axotomized FMNuc at 14 dpo with CD4+ cell infiltration. C) 40X image of box in B. D) 20X composite image of axotomized FMNuc at 14 dpo with CD4+ cells. White dashed line indicates boundary of FMNuc. E) Same field as D showing CD3+ cells. F) Overlaid channels in D and E showing CD3+/CD4- and CD3+/CD4+ T cells. Scale bar in A = 100 μm and applies to A and B. Scale bar in C = 50 μm. Scale bar in D = 100 μm and applies to D, E, and F.

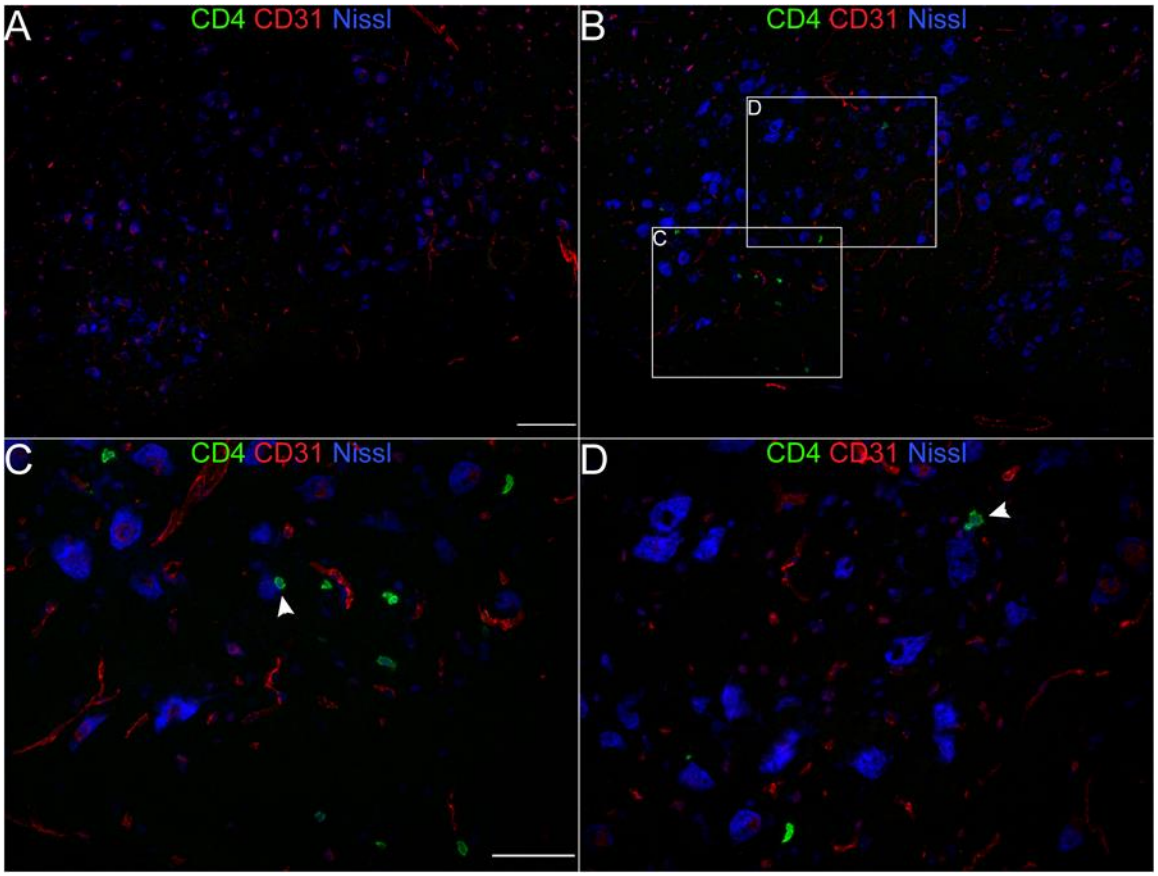


Figure 9: IHC of CD4+ T cells and CD31-labeled endothelial layer of blood vessels penetrating the FMNuc.

A) 20X composite image of control FMNuc at 14 dpo showing no infiltrating T cells. B) 20X composite image of axotomized FMNuc at 14 dpo. C,D) 40X magnified fields from boxes in B showing CD4+ T cells not contained within CD31-labeled blood vessels. Note CD4+ T cells marked with arrowheads in close proximity to FMN labeled with fluorescent Nissl stain. Scale bar in A = 100 μm and applies to A and B. Scale bar in C = 50 μm and applies to C and D.

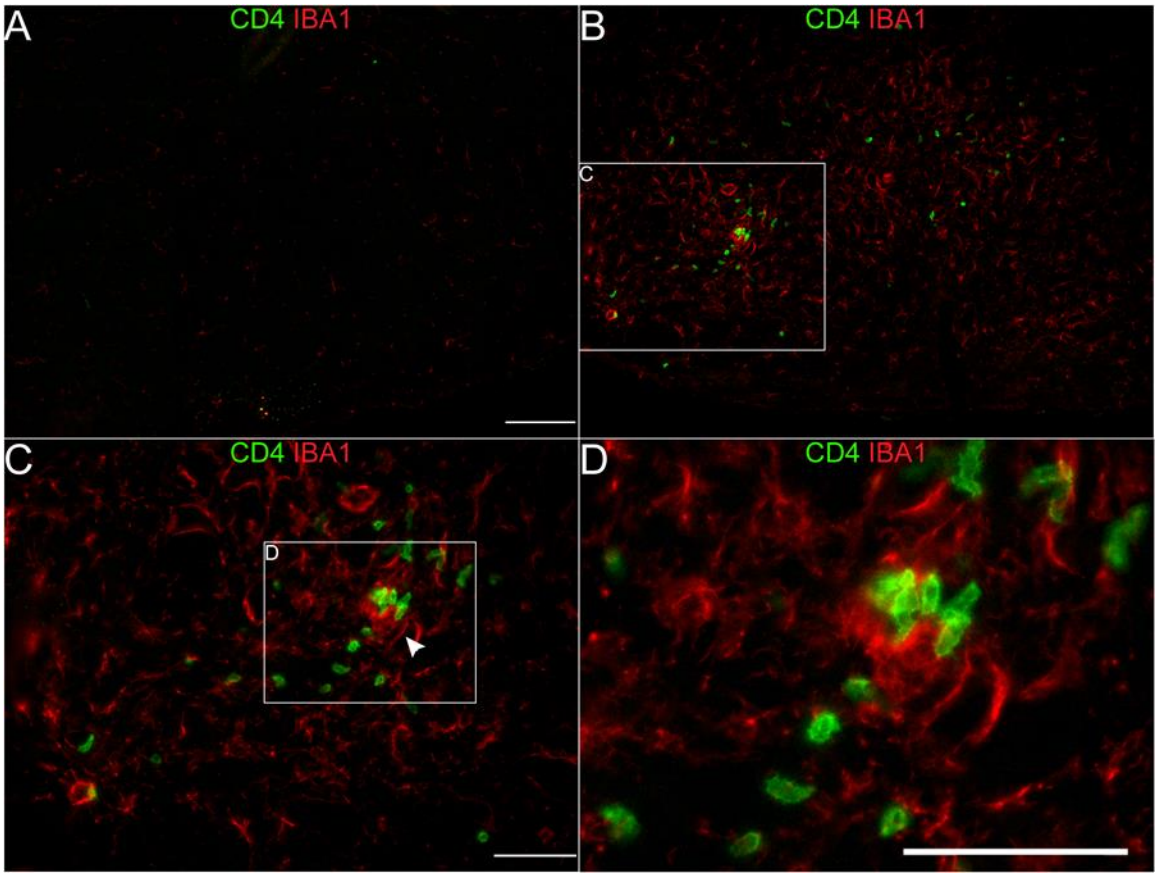


Figure 10: IHC of CD4+ T cells and IBA+ microglia in the FMNuc.

A) 20X composite image of control FMNuc at 14 dpo. Small resting microglia can be observed. B) 20X composite image of axotomized FMNuc at 14 dpo with infiltrating CD4+ T cells and microglial activation indicated by increased IBA1 immunoreactivity. C,D) Magnified fields showing close association of CD4+ T cells and microglia. Note cluster of microglia and T cells in nodule-like structure. Scale bar in A = 100 μm and applies to A and B. Scale bars in C and D = 50 μm.

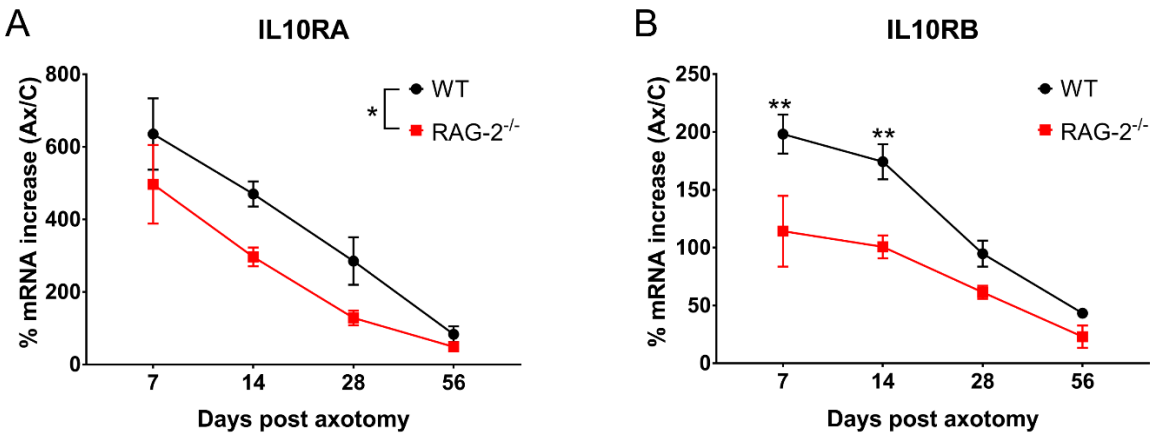


Figure 11: Effect of immune status on central IL-10 receptor expression.

mRNA percent increase in expression of genes in the axotomized FMNuc relative to control FMNuc. Bars represent mean percent change ± SEM. For *Il10ra*, two-way ANOVA revealed a significant overall difference between WT and RAG-2$^{-/-}$, but post-hoc tests failed to determine at which specific time points differences existed. * $p < 0.05$, ** $p < 0.01$

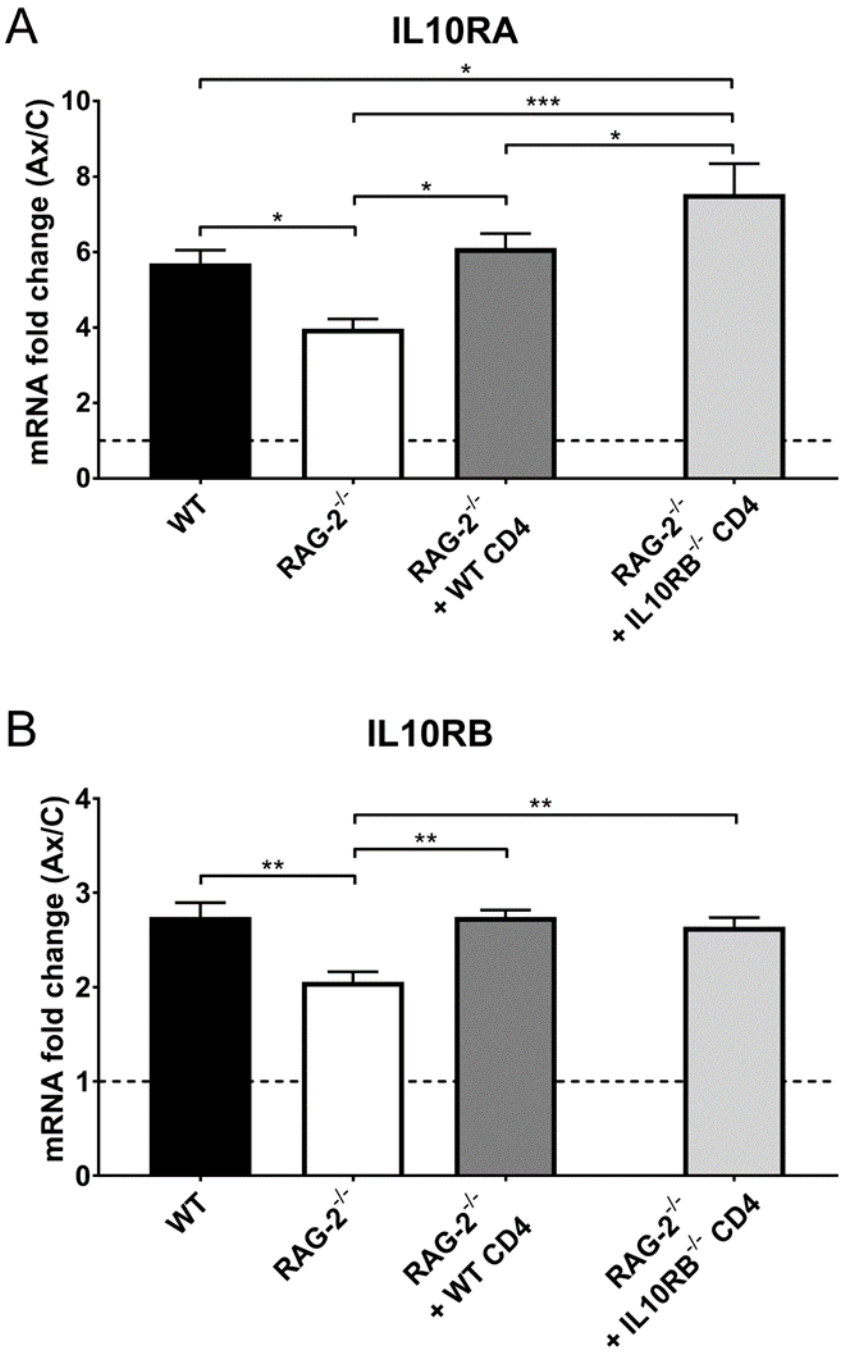


Figure 12: Expression of IL-10R subunit genes at 14 days post FNA in WT, RAG-2$^{-/-}$, RAG-2$^{-/-}$ + WT CD4+ T cells, and RAG-2$^{-/-}$+ *Il10rb*$^{-/-}$ CD4+ T cells.

mRNA fold change of genes in the axotomized FMNuc relative to control FMNuc. Bars represent mean fold change ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

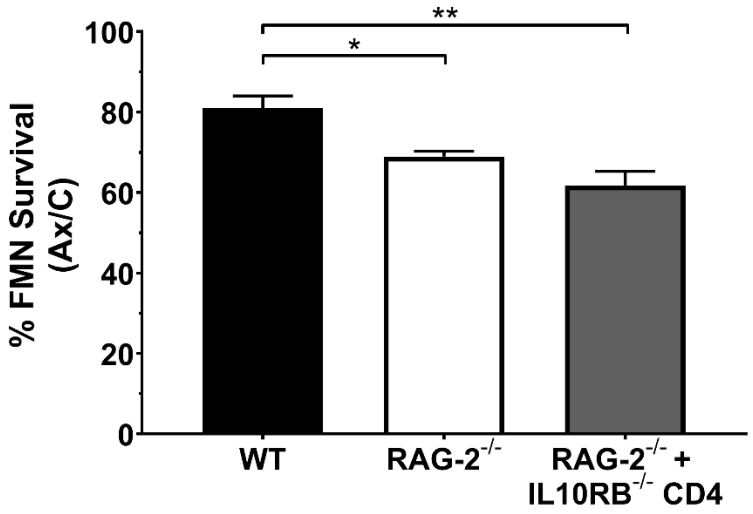


Figure 13: FMN survival in WT, RAG-$2^{-/-}$, and RAG-$2^{-/-}$ reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells.

Average percent survival of axotomized FMN relative to control ± SEM at 28 dpo. Survival in both RAG-$2^{-/-}$ and RAG$^{-/-}$ reconstituted with CD4+ T cells was decreased relative to WT, indicating that *Il10rb*$^{-/-}$ T cells fail to confer neuroprotection. * $p < 0.05$, ** $p < 0.01$

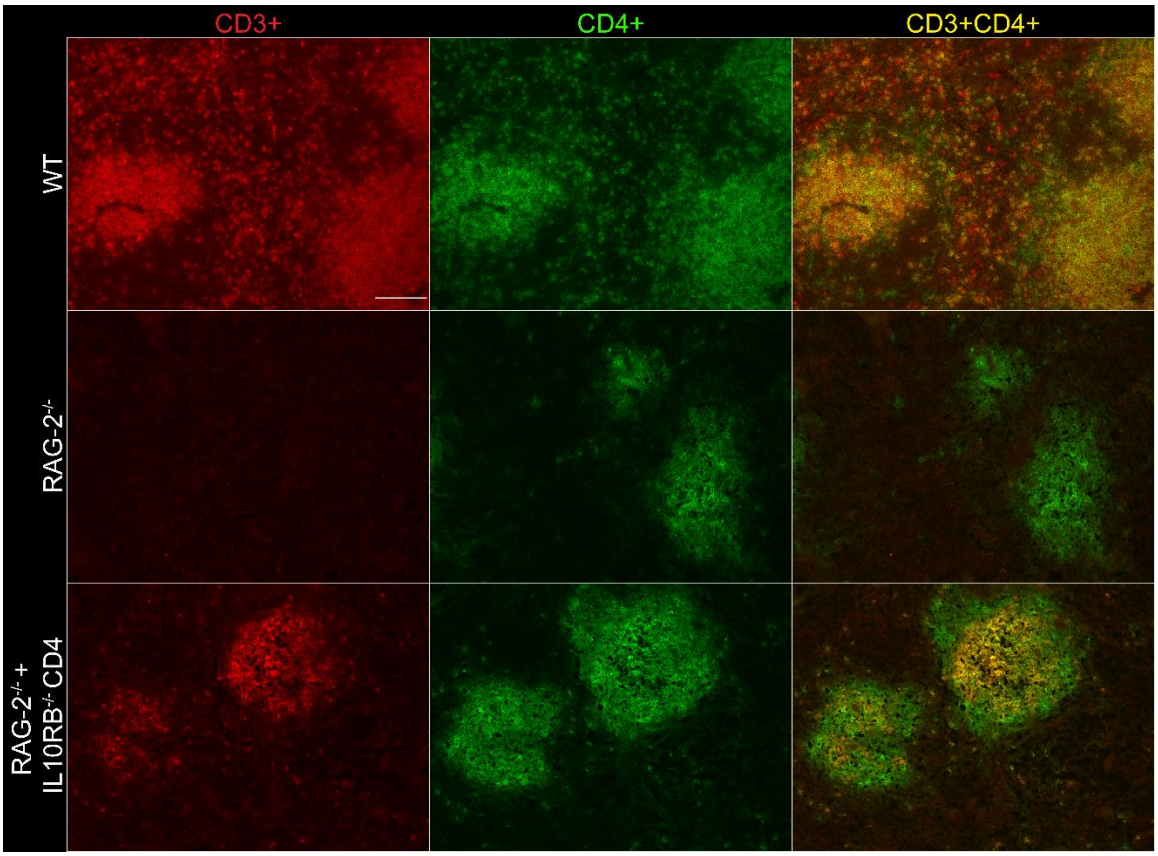


Figure 14: Confirmation of IL10RB$^{-/-}$ CD4+ T cell engraftment at 28 dpo.

In WT animals, splenic follicles are CD3+/CD4+. In RAG-2$^{-/-}$, splenic follicles are negative for CD3+ T cells. Adoptive transfer of *Il10rb*$^{-/-}$ CD4+ T cells restores CD3+ expression in splenic follicles, indicating that *Il10rb*$^{-/-}$ CD4+ T cells are were successfully transplanted and remain viable *in vivo* until 28 dpo. Scale bar = 100 μm and applies to all panels.

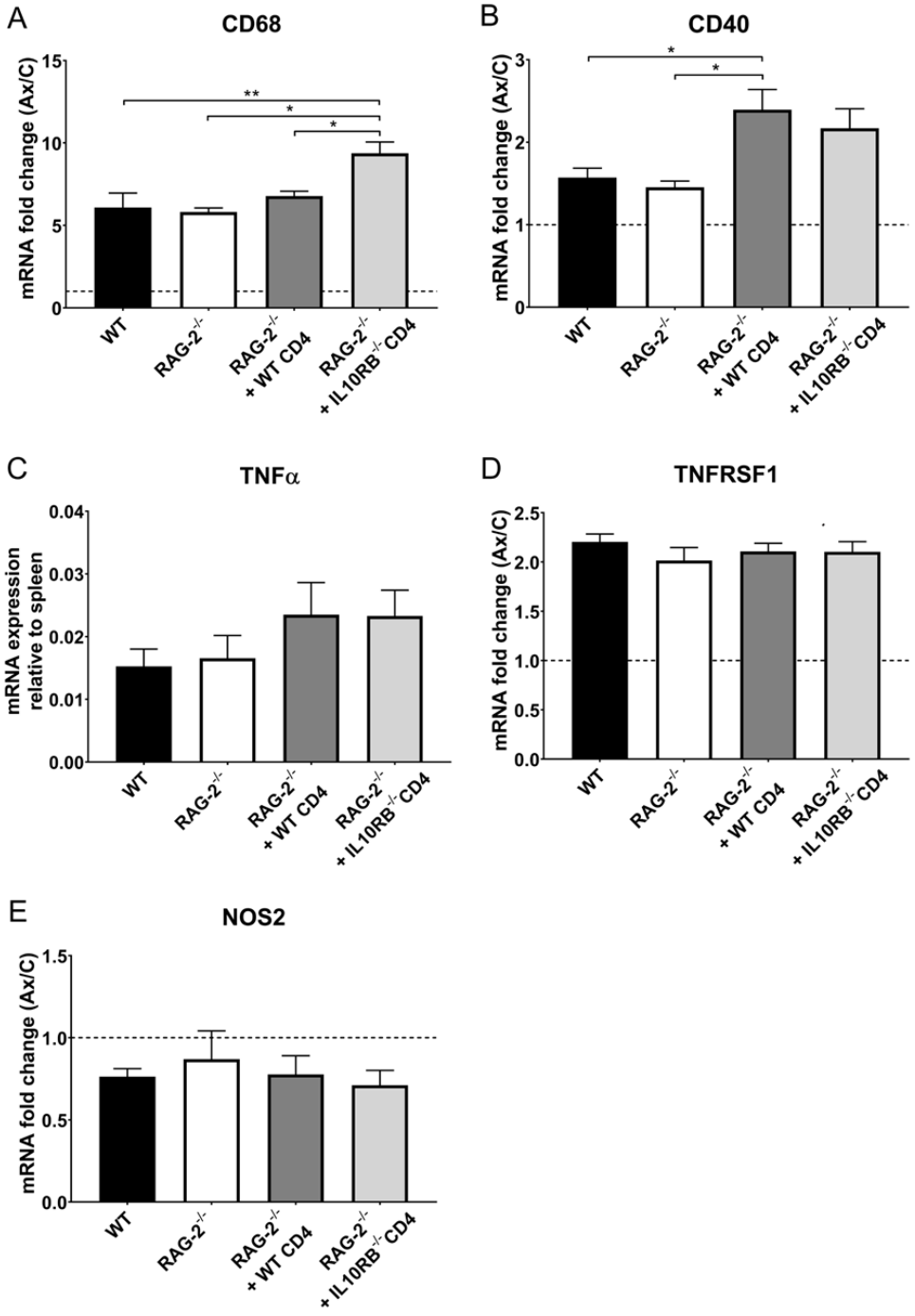


Figure 15: Expression of genes association with microglial activation at 14 days post FNA in WT, RAG-2$^{-/-}$, RAG-2$^{-/-}$ + WT CD4+ T cells, and RAG-2$^{-/-}$ + *Il10rb*$^{-/-}$ CD4+ T cells.

mRNA fold change of genes in the axotomized FMNuc relative to control FMNuc (except for C, which reflects relative expression in axotomized FMNuc relative to spleen standard.) Bars represent mean fold change ± SEM. * $p < 0.05$, ** $p < 0.01$

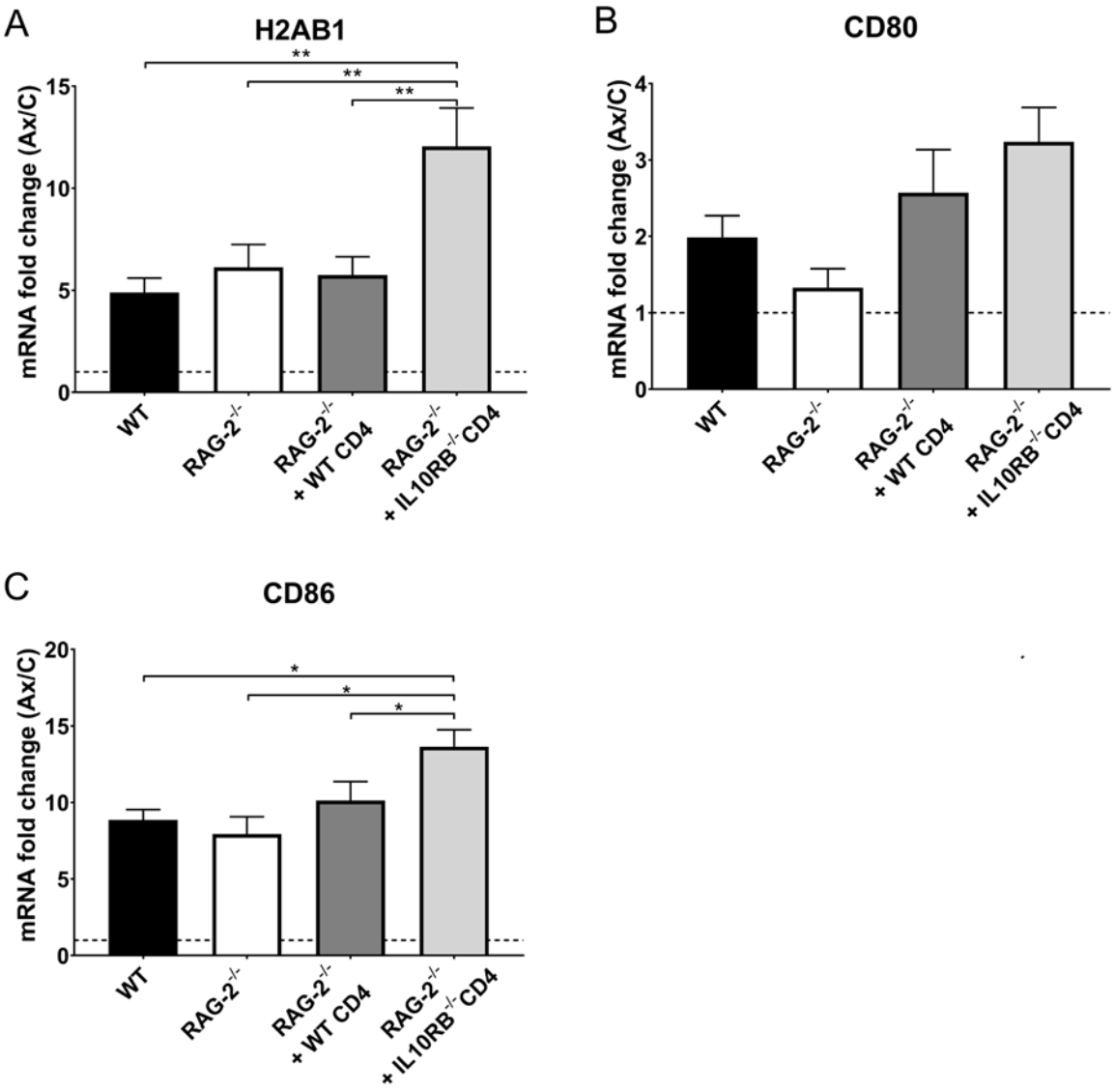


Figure 16: Expression of genes association with antigen presentation and T cell co-stimulation at 14 days post FNA in WT, RAG-$2^{-/-}$, RAG-$2^{-/-}$ + WT CD4+ T cells, and RAG-$2^{-/-}$+ *Il10rb*$^{-/-}$ CD4+ T cells.

mRNA fold change of genes in the axotomized FMNuc relative to control FMNuc. Bars represent mean fold change ± SEM. * $p < 0.05$, ** $p < 0.01$

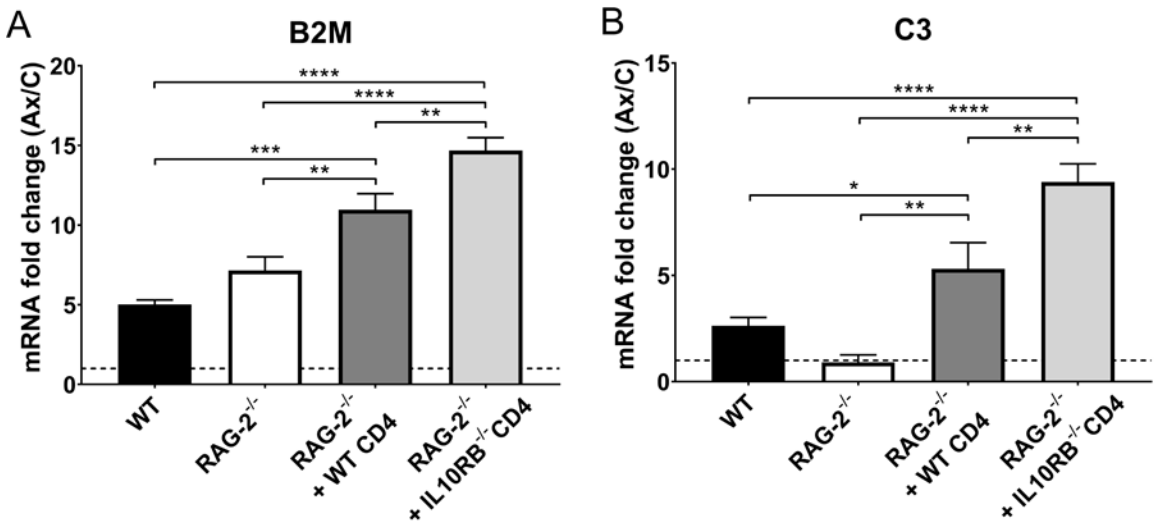


Figure 17: Expression of genes associated with synaptic pruning at 14 days post FNA in WT, RAG-2$^{-/-}$, RAG-2$^{-/-}$ + WT CD4+ T cells, and RAG-2$^{-/-}$+ *Il10rb*$^{-/-}$ CD4+ T cells.

mRNA fold change of genes in the axotomized FMNuc relative to control FMNuc. Bars represent mean fold change ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

CHAPTER 5: DISCUSSION

5.1. Aim 1 Discussion

The goal of this aim was to determine the central cellular sources of IL-10 in the FMNuc and the respective contributions of these sources to FMN survival after axotomy. The anti-inflammatory cytokine IL-10 was first discovered as a Th2-secreted factor that suppresses IFNγ production by activated Th1 cells (Fiorentino et al., 1989). IL-10 has since been recognized as a major immunoregulator with roles in T cell development, macrophage and dendritic cell activation, and antigen presentation (Fiorentino et al., 1991a; Kuhn et al., 1993; MacNeil et al., 1990; Steinbrink et al., 1997). IL-10 is also important for the resolution of inflammation after CNS injury and disease, an effect which is frequently mediated by IL-10-producing CD4+ T cells and microglia (Frenkel et al., 2005; Gravel et al., 2016; Mayo et al., 2016; Park et al., 2007; Zhou et al., 2017).

CD4+ T cells are critical for WT levels of FMN survival after FNA (Serpe et al., 2003). IL-10 is necessary for CD4+ T cells to mediate neuroprotection after axotomy, but surprisingly, CD4+ T cells are not the source of IL-10 in this injury model (Xin et al., 2011). IL-10 is incapable of crossing the blood-brain barrier; because FNA does not cause mechanical damage to the blood-brain barrier, it is most likely that the IL-10 important for neuroprotection derives from a central source in the facial motor nucleus (Kastin et al., 2003; Raivich et al., 1998). However, it is also possible that IL-10 production by a peripheral APC is important for the differentiation of neuroprotective Th2 cells. Therefore, it was first necessary to rule out an IL-10 contribution from the peripheral immune system (see discussion section 5.1.1).

In the CNS, microglia are the most likely producers of IL-10 after axotomy. Microglia are capable of IL-10 production following IFNγ and LPS challenge *in vitro* and

in vivo (Ledeboer et al., 2002; Mizuno et al., 1994; Park et al., 2007; Williams et al., 1996). The expression of pro- and anti-inflammatory markers by microglia *in vitro* enables crude categorization of microglia into "M1" and "M2" subtypes, although microglial polarization *in vivo* exists along a more nuanced spectrum (Martinez & Gordon, 2014). IL-4 induces the polarization of M2-type microglia, which produce IL-10 and are characterized by decreased expression of inflammatory mediators such as NO and co-stimulatory molecules such as CD86 (Chhor et al., 2013; Zhang et al., 2014). IL-4 is the signature cytokine of the Th2 cell subset critical for neuroprotection after axotomy, and microglia produce more IL-10 after interacting with T cells *in vitro* (Chabot et al., 1999; Deboy et al., 2006b). After spinal cord injury, M2-type macrophages can also reciprocally promote neuroprotective Th2-predominant immune responses (Ma et al., 2015). In light of this evidence, it was hypothesized that IL-4-producing Th2 cells travel into the FMNuc after axotomy, where they promote microglial production of IL-10 that is necessary for neuroprotection.

5.1.1. The contribution of IL-10 from the peripheral immune system is not sufficient for motoneuron survival after axotomy

Although IL-10 production by CD4+ T cells was effectively excluded as being necessary for neuroprotection after axotomy (Xin et al., 2011), IL-10 production by other peripheral immune cells, such as dendritic cells or macrophages, remained a potential neuroprotective alternative given the ability of peripheral IL-10 to modulate T cell subset activation. After facial nerve axotomy, most CD4+ T cell subsets–including Th1, Th2, Th17, Tr1, and FOXP3+ Tregs–expand in the draining lymph nodes (Xin et al., 2008).

However, only the IL-4-producing Th2 subset in particular mediates neuroprotection (DeBoy et al., 2006a; Deboy et al., 2006b). IL-10 suppresses APC-induced IFNγ secretion by Th1 cells, but largely does not affect IL-4 production by Th2 cells (Fiorentino et al., 1989; Fiorentino et al., 1991b). IL-10 suppresses Th1 function primarily by decreasing MHCII and co-stimulatory molecule expression on APCs (de Waal Malefyt et al., 1991; Ding et al., 1993; Ding & Shevach, 1992). There is also evidence that IL-10 can prime dendritic cells to skew the differentiation of naïve T cells toward the Th2 subset, which is supported by studies indicating that IL-$10^{-/-}$ mice have Th1-predominant immune responses (Berg et al., 1996; Liu et al., 1998). The predominance of pro-inflammatory Th1/Th17 responses over anti-inflammatory Treg/Th2 responses is characteristic of several neurodegenerative diseases, including ALS and MS (Mantovani et al., 2009; Rentzos et al., 2012; Saresella et al., 2013).

IL-10 produced by APCs in the periphery could set up conditions ideal for Th2 subset differentiation and function, which would have implications for overall adaptive immune neuroprotective capacity after axotomy. To rule out IL-10 from a peripheral APC as being necessary for central neuroprotection after FNA, IL-$10^{-/-}$ mice were adoptively transferred with WT whole splenocytes. The whole splenocyte milieu includes innate (granulocytes, monocytes/macrophages, and dendritic cells), adaptive (T and B cells), and stromal cells. After adoptive transfer, only cells in the peripheral immune compartment express the WT IL-10 gene. The observed failure of whole splenocytes to rescue FMN survival indicates that IL-10 from any peripheral immune cell, including APCs, is not sufficient for neuroprotection after axotomy. This result reinforces the hypothesis that a central source of IL-10 is critical for FMN survival.

It has been previously demonstrated that adoptive transfer of whole splenocytes into RAG-2$^{-/-}$ mice results in restoration of normal splenic architecture, indicating successful engraftment of transplanted cells into host tissue (Serpe et al., 1999). However, it was unknown whether the intact (and hyper-reactive) peripheral immune system of the IL-10$^{-/-}$ mouse would permit adoptive transfer of foreign splenocytes. Failure of transplanted immune cells to rescue FMN survival could simply indicate immune rejection. PCR amplification of genomic DNA revealed the presence of the WT IL-10 gene in the spleens of IL-10$^{-/-}$ animals that had received WT splenocytes, indicating that these cells survived in the IL-10$^{-/-}$ spleen up to four weeks after axotomy.

Although IL-10 production by peripheral immune cells appears not to be sufficient for FMN survival after axotomy, a peripheral immune source of IL-10 does have a role in axon regeneration in the periphery. IL-10-expressing macrophages infiltrate the sciatic nerve after crush injury and appear to be important for resolution of the pro-inflammatory phase of Wallerian degeneration, which is important for clearance of tissue debris (Sawada et al., 2007; Siqueira Mietto et al., 2015). IL-10$^{-/-}$ mice have slower axon regeneration and delayed recovery of sensory and motor function after sciatic crush (Siqueira Mietto et al., 2015). Exogenous application of IL-10 after sciatic nerve transection and suture reduces collagen deposition and improves transduction of compound action potentials across the injury site (Atkins et al., 2007). These data suggest dual location-dependent roles for IL-10 after axotomy, both of which are essential for functional recovery: central IL-10 is critical for FMN survival, whereas peripheral IL-10 from immune sources is important for axon regrowth.

5.1.2. Facial motoneurons constitutively express IL-10, and this expression is not affected by axotomy

Use of an IL-10/GFP reporter mouse revealed GFP co-localization with NeuN-labeled neurons in both the control and injured FMNuc of animals that received axotomy, as well as in un-operated sham control animals. Supporting these results, an antibody specific for IL-10 labeled the cytoplasm of cells in the FMNuc having a distinct motoneuron morphology (large, multipolar cells with prominent central nuclear clearings). This labeling was also present in both control and axotomized FMNuc and did not appreciably change in response to axotomy. These results were unexpected, given the relative dearth of studies in the literature showing IL-10 production by neurons compared to glia. One report on IL-10 expression in the context of cerebral ischemia showed strong co-localization of both IL-10 and its receptor with healthy cortical, striatal, and hippocampal neurons (Fouda et al., 2013). In postmortem spinal cord samples, IL-10 co-localizes with neurons in both control and ALS patients, but in the latter appears to be restricted to intracellular TDP-43 inclusions, suggesting that neurons make IL-10 that can become sequestered in disease (Berjaoui et al., 2015).

In vitro studies of primary neuron cultures reveal insights both into the possibility of neuronal production of IL-10 as well as potential reasons for its constitutive expression. Low basal production of IL-10 that increased after prolonged toll-like receptor (TLR) stimulation was observed in one study of primary neurons cultured in serum-free media (Chistyakov et al., 2018). Another found that application of an IL-10 neutralizing antibody in the absence of exogenous IL-10 or serum-containing media suppressed JAK1/STAT3 activation, increased apoptosis, decreased neurite extension by

suppressing Netrin-1 signaling, suppressed synaptophysin expression, and reduced the density of dendritic spines in primary neuron cultures subjected to oxygen-glucose deprivation (Chen et al., 2016). These data suggest that endogenous IL-10 production by neurons may have an autocrine role in conferring resistance to ischemia and metabolic stress and potentially is important for maintenance of synapses. However, conclusions drawn from studies of primary neuron cultures must be conservative, as glial contamination of cultures is a possibility. An autocrine role for neuronal IL-10 is supported *in vivo* by reports that FMN and healthy cortical neurons also express the IL-10R constitutively (Fouda et al., 2013; Villacampa et al., 2015; Xin et al., 2011).

5.1.3. Astrocyte production of IL-10 is induced by axotomy

Both IL-10/GFP reporter and direct IL-10 immunohistochemistry revealed induction of GFAP-labeled astrocyte expression that co-localized with IL-10 production in the axotomized FMNuc. Using the direct labeling method, co-localization of astrocytes with IL-10 occurred as early as 3 dpo and persisted through 28 dpo. IL-10/GFP reporter exhibited a delayed astrocyte response compared to direct IL-10 IHC, with prominent GFAP expression not observed until 7 dpo and GFP co-localization delayed until 10 dpo. This difference is likely due to more thorough fixation of proteins in the WT mouse tissue used for direct IL-10 IHC. WT tissue was perfusion-fixed prior to sectioning, whereas IL-10/GFP reporter mouse tissue was flash-frozen, sectioned, and fixed on the slide in order to preserve GFP signal.

Astrocyte production of IL-10 has been characterized both *in vitro* and *in vivo*. Reports of IL-10 production by resting or unstimulated astrocytes are conflicted. Some

studies indicate that IL-10 mRNA and protein expression is negligible in unstimulated primary cultured astrocytes (Wirjatijasa et al., 2002), especially in comparison with microglia (Ledeboer et al., 2002). Others report low constitutive production of IL-10 by primary astrocytes in plain culture media (Chistyakov et al., 2018) and greater production when exposed to cerebrospinal fluid (CSF) in the absence of additional stimulation (Mishra et al., 2016). Regardless of their constitutive expression of IL-10, cultured primary astrocytes are known increase expression of both IL-10 mRNA and protein in response to LPS and TLR stimulation in a dose-dependent fashion (Chistyakov et al., 2018; Ledeboer et al., 2002; Mizuno et al., 1994). It is also noteworthy that the fold-change in astrocyte production of IL-10 is greater than that in microglia in glial co-cultures, suggesting that astrocytes are more sensitive to stimuli that trigger IL-10 production (Ledeboer et al., 2002). Interestingly, IL-10 production by astrocytes is suppressed when they are exposed to CSF from patients with ALS, suggesting the presence of a pro-inflammatory factor that shuts down normal IL-10 secretion (Mishra et al., 2016).

In vivo, astrocyte production of IL-10 has been documented in both human patients with MS and rodent models of experimental autoimmune encephalomyelitis (EAE). In human postmortem brain tissue samples, IL-10, IL-4, and their receptors co-localize with reactive astrocytes in active demyelinating MS lesions as well as in areas of brain infarction (Hulshof et al., 2002). Another study in human MS tissue detected prominent co-labeling of IL-10 and astrocytes around blood vessels adjacent to acute lesions and in the hypercellular rim of chronic active lesions (Cannella & Raine, 1995). These locations suggest a role for astrocytic IL-10 in restricting the area of inflammatory

cell infiltration. IL-10 mRNA and protein also co-localize with perivascular astrocytes that lie in close proximity to peripheral immune infiltrates in EAE. Interesting, microglia as well as infiltrating macrophages and T cells also express IL-10 in this model (Jander et al., 1998). These perivascular astrocytes form glial-scar-like barriers that restrict inflammatory adaptive immune cell infiltration (Voskuhl et al., 2009).

Targeted overexpression of IL-10 by astrocytes also modifies the cellular responses to FNA. This increase in astrocytic IL-10 induces greater upregulation of IL-10R expression on FMN, suggesting a feed-forward role of IL-10 in the FMNuc that promotes its own signaling (Villacampa et al., 2015). These data support findings from our laboratory showing that while total IL-10 protein levels do not significantly change in the FMNuc after axotomy, there is increased IL-10R expression and therefore likely increased IL-10 signaling events overall (Xin et al., 2011). The adaptive immune system is also essential for full upregulation of IL-10R expression in the FMNuc after axotomy (Aim 2 of this study). In the aforementioned study, greater production of astrocyte IL-10 is paradoxically associated with increased microglial nodule formation and MHCII expression as well as a two-fold increase in CD3+ T cell infiltration into the FMNuc. FMN survival is also improved in this model, however, the authors report much lower FMN survival in WT mice than what is typically observed in our laboratory (65% at 21 dpo vs. 85% at 28 dpo in our laboratory), and survival in their IL-10-overexpressing animals (81%) was comparable to what we typically observe in WT animals (Villacampa et al., 2015).

Altogether, these data indicate roles for astrocyte production of IL-10 in modulating the immune response to injury or disease as well as promoting

neuroprotective IL-10 signaling in neurons. This immune modulation may take the form of an immunosuppressive role, as is seen in MS (Cannella & Raine, 1995; Hulshof et al., 2002), or it potentially may favor the infiltration of neuroprotective T cell subsets, as is implied by Villacampa et al. (2015). As previously described, overall IL-10 protein levels do not significantly change in the FMNuc after axotomy (Xin et al., 2011); therefore, the amount of IL-10 produced by astrocytes may be small in comparison with that produced constitutively by neurons, or perhaps there is a slight decrease in IL-10 production by neurons after axotomy that is undetectable by IHC and compensated for by astrocytes. As astrocytes are both highly mobile and frequently optimally situated for surveillance of the blood-brain barrier, a shift in IL-10 production toward the astrocyte suggests a more immunomodulatory role after axotomy in comparison with constitutive IL-10 expression in neurons.

5.1.4. Microglia appear not to produce IL-10 protein after facial nerve axotomy

The IL-10/GFP reporter mouse could not be utilized for co-labeling of microglia as all microglial antigens tested were undetectable by antibody labeling in the flash-frozen tissue. Use of perfusion-fixed tissue enabled excellent detection of IBA1 positive microglia after axotomy. At early time points after axotomy, microglia could be observed ensheathing injured FMN, while at later time points they formed phagocytic nodules. At no point was definite co-labeling of IBA1 and IL-10 detected, suggesting that microglia do not make IL-10 after axotomy. Microglial production of IL-10 after axotomy therefore remains somewhat ambiguous, as the IL-10 antibody may not bind IL-10 within its cellular source if it is rapidly secreted after synthesis. The particular antibody utilized in

this study neutralizes IL-10 and prevents it from binding to its receptor, indicating that it should only label free or intracellular IL-10 and not IL-10 bound to its receptor a target cell (Xie et al., 2015). Other studies have been published utilizing antibody detection of IL-10 with successful labeling of microglia and macrophages/monocytes (Hulshof et al., 2002; Jander et al., 1998; Park et al., 2007; Siqueira Mietto et al., 2015), but it is likely that the dynamics of IL-10 production by microglia differs in different injury and disease models. Despite this finding, *Il10* mRNA co-localized with *Cx3cr1* mRNA both in the control and axotomized FMNuc. It has been reported that production of IL-10 is heavily regulated on a post-transcriptional level, as many cell types express *Il10* mRNA constitutively but do not translate IL-10 protein (Powell et al., 2000; Tone et al., 2000). This may represent a mechanism by which microglia can rapidly synthesize IL-10 only after the correct stimulus.

IL-10 production by microglia in culture is similar to what is seen in peripheral macrophages. Some reports indicate constitutive production of microglia in primary culture (Ledeboer et al., 2002), while others report trace or negligible production (Chabot et al., 1999; Hulshof et al., 2002; Mizuno et al., 1994). LPS stimulation triggers increased IL-10 mRNA and protein production by microglia in a dose-dependent manner (Ledeboer et al., 2002; Mizuno et al., 1994). Microglia derived from presymptomatic ALS mice produce more IL-10 in response to LPS challenge, suggesting a compensatory response to ongoing neuroinflammation (Gravel et al., 2016). TLR stimulation (Jack et al., 2005) and purinergic receptor activation (Seo et al., 2008) also stimulate IL-10 production by microglia *in vitro*. Microglial production of IL-10 is dramatically increased when co-cultured with T cells, a phenomenon which is reversible via CD40 and CD80/86 blockade

(Chabot et al., 1999); along similar lines, IFNγ (produced by Th1 cells and a potent activator of macrophages) also promotes IL-10 production by microglia *in vitro* (Williams et al., 1996).

In vivo demonstrations of microglial IL-10 production are somewhat scarcer. LPS injection stimulates microglial production of IL-10 *in vivo*; interestingly, these microglia are also immunopositive for IL-1β, TNFα, and iNOS, suggesting a microglial phenotype that is not strictly pro- nor anti-inflammatory. Blockade of IL-10 signaling causes a significant increase in neuron cell death in the cerebral cortex after LPS injection (Park et al., 2007). IL-10 production by spinal cord parenchymal microglia is seen in rodent models of EAE (Jander et al., 1998) as well as in active MS lesions in humans (Hulshof et al., 2002), although the effect is not as robust as what is observed in astrocytes. In an ALS mouse model, targeting IL-10 overexpression to microglia results in delayed disease onset and a modest increase in survival (Gravel et al., 2016).

Altogether, these data suggest that while microglia are capable of producing IL-10 in response to overt inflammatory stimuli *in vitro* and *in vivo*, the relatively mild inflammation induced by FNA may not severe enough to trigger IL-10 production to a degree that is detectable by IHC. Alternate methods of detection, such as flow cytometry, may be better suited to detecting subtle changes in microglial IL-10 synthesis if influenced by axotomy.

5.1.5. No single central source of IL-10 in the facial motor nucleus is critical for neuroprotection

To determine whether IL-10 production by neurons, astrocytes, or microglia is necessary for CD4+ T cell-mediated neuroprotection, a Cre/Lox system was employed to selectively knock out IL-10 production in each of these populations prior to axotomy. Selective deletion of IL-10 revealed that production by any of these single central sources was not critical for neuroprotection. These data are in accordance with previous findings that IL-10 is produced by at least two cell populations in the FMNuc (neurons and astrocytes), with microglial production of IL-10 ambiguous after axotomy. After knockout, it is likely that the IL-10 remaining from one source is sufficient to mediate neuroprotection on its own; similarly, selectively knocking out IL-10 production from a single source may induce enhanced compensatory production from another.

Compensation of a cytokine after knockout by an alternative source is a common phenomenon. For example, IL-17 is typically produced by Th17 cells in a renal ischemia-reperfusion model, but can also be produced by natural killer cells when the model is imposed on a T cell-deficient background (Mehrotra et al., 2017). Compensation of cytokine knockout by an alternative redundant cytokine is also frequently observed in the literature (Khader et al., 2005; McMahon et al., 2001; Nedvetzki et al., 2004), although such redundancy is unlikely to be occurring in our model as IL-$10^{-/-}$ mice have reduced FMN survival (Xin et al., 2011). It is likely that both neuronal and astrocyte production of IL-10 in tandem is necessary for FMN survival after axotomy, and future studies will explore the impact of IL-10 knockout in both of these populations simultaneously.

5.1.6. Summary of results and revised hypothesis

These data demonstrate that IL-10 from a peripheral immune source is insufficient to support FMN survival after axotomy, indicating that a central source of IL-10 is critical for neuroprotection. IL-10 is produced constitutively by motoneurons in the FMNuc and is induced in astrocytes after axotomy. Microglia may constitutively transcribe *Il10* mRNA, but there is currently no evidence for translation of IL-10 protein in microglia after FNA. Neuroprotective IL-10 does not derive exclusively from neurons, astrocytes, or microglia. From these data, a revised hypothesis of IL-10 action in the FMNuc after FNA is that neuronal production of IL-10 serves a homeostatic role for neuron resilience to stress by supporting neuron survival and synaptogenesis in an autocrine fashion. IL-10 produced by astrocytes after FNA, in addition to exerting trophic influences on neurons, may also modulate the infiltrating immune milieu in a neuroprotective manner. A trophic role for IL-10 is supported by data indicating that there is constitutive neuronal expression of the IL-10R in the FMNuc which changes after axotomy, especially in response to IL-10 produced by astrocytes (Villacampa et al., 2015; Xin et al., 2011; see section 5.2.2 for further discussion). The importance of peripheral immune responsiveness to IL-10 signaling after axotomy is crucial to elucidate and is addressed by Aim 2 of this study.

5.1.7. Limitations of this study and future directions

Although this study thoroughly investigates expression of IL-10 by neurons, astrocytes, and microglia in the FMNuc, other central cell types have gone relatively unexplored. Oligodendrocytes, ependymal cells, pericytes, and perivascular macrophages

are also candidates for IL-10 production after axotomy. Perivascular macrophages in particular may produce IL-10 in the context of MS (Hulshof et al., 2002). Perivascular macrophages in the FMNuc also upregulate MHCII after axotomy, raising the possibility of interactions with CD4+ T cells at the blood-brain barrier that could potentially be vulnerable to IL-10 modulation (Villacampa et al., 2015). These cells are likely not replenished by circulating monocytes and thus would evade influence by the adoptive whole splenocyte transfer performed in this study (Faraco et al., 2017).

In the periphery, SCs and endoneurial cells are alternative sources of IL-10 that would also not be reconstituted by WT whole splenocyte transfer into the IL-$10^{-/-}$ animal. Sciatic nerve crush causes upregulation of *Il10* mRNA in the distal nerve portion within one day of axotomy, which persists up to two weeks (Gillen et al., 1998). Another study showed that *Il10* mRNA localizes to both the proximal and distal nerve endoneurium as early as 14 hours after sciatic nerve transection and lasts through four weeks (Taskinen et al., 2000). SCs strongly upregulate *Il10* mRNA within two to four days after axotomy (Jander et al., 1996). However, exogenous application of IL-10 to the proximal nerve stump does not promote neuron survival, suggesting that SC- and endoneurium-derived IL-10 is unlikely to be important for neuron survival via a retrograde mechanism (Xin et al., 2011). The ineffectiveness of exogenous IL-10 application may be attributable to its short half-life, estimated at 2.7 to 4.5 hours (Huhn et al., 1997). Given that production of IL-10 in the peripheral nerve is a temporally regulated process, a bolus of IL-10 applied in the periphery may not be dynamically favorable for neuroprotection.

There is also the question of systemic inflammation having an impact on FMN survival in a nonspecific manner due to the spontaneous development of enterocolitis in

IL-10$^{-/-}$ mice (Kuhn et al., 1993). However, the IL-10$^{-/-}$ mice utilized in this study were backcrossed on a C57BL/6J background, which confers resistance to the colitis phenotype (Berg et al., 1996; Grilli et al., 2000). No pathological weight loss was observed in the IL-10$^{-/-}$ mice used in this study. Therefore it is likely that the phenotype observed in this study and others (Grilli et al., 2000; Xin et al., 2011) results from the lack of endogenous IL-10 in the CNS rather than nonspecific gut inflammation.

Given the results of this study and reports in the literature, a new hypothesis has been formulated for the neuroprotective role of IL-0 in the FMNuc. The first part of this hypothesis emphasizes constitutive production of IL-10 in the FMN and inducible production of IL-10 from astrocytes as being dually important for FMN survival after axotomy. Future experiments will focus on dual elimination of IL-10 production by both neurons and astrocytes for observation of effects on FMN survival. This could be achieved by employing CRISPR/Cas9-mediated deletion of IL-10 from one cellular source imposed on a Cre/Lox background for deletion of IL-10 from the other. Additionally, while it has been shown that IL-10 from a peripheral immune source in the context of antigen presentation and T cell differentiation is not *sufficient* for FMN survival, the question of whether it is *necessary* for neuroprotection has not been answered. Central expression of IL-10 may not be capable of mediating neuroprotection if a lack of IL-10 in the periphery (such as from an APC) leads to failure of neuroprotective T cell development. IL-10$^{-/-}$ bone marrow transplantation into irradiated WT donors would restrict IL-10 expression to the central compartment while eliminating it from APCs and other immune effectors in the peripheral compartment.

The second part of the new hypothesis proposes two main neuroprotective mechanisms of IL-10 action: 1) direct trophic influences on the neuron and 2) immunomodulatory effects on infiltrating adaptive immune effectors and resident glia. To evaluate direct trophic influences on the neuron, Cre recombination of the floxed *Il10ra* locus could be targeted to the *Thy1* promoter for inducible knockdown (as was performed for IL-10 in this study) or to the motoneuron differentiation gene *Hb9* for constitutive knockdown (Arber et al., 1999). This would evaluate whether IL-10 receptor signaling in neurons is necessary for their neuroprotection. The role of IL-10 signal transduction in infiltrating T cells is explored in Aim 2 of this study and will be further discussed in section 5.2.

5.2. Aim 2 Discussion

Both CD4+ T cells and IL-10 are necessary for FMN survival after axotomy (Serpe et al., 2003; Xin et al., 2011). Although regulatory T cells make IL-10 and are neuroprotective in many injury and disease models (Groux et al., 1997; Mayo et al., 2016; Taylor et al., 2006; Xie et al., 2015; Zhao et al., 2012; Zhou et al., 2017), CD4+ T cells depleted of the CD25+ regulatory subset are sufficient to rescue FMN survival when transferred into immunodeficient mice (DeBoy et al., 2006a). In fact, IL-10 derived from a CD4+ T cell source is not required for neuroprotection in this injury model at all, as IL-10$^{-/-}$ CD4+ T cells are as efficacious as WT CD4+ T cells at rescuing FMN survival when adoptively transferred into RAG-2$^{-/-}$ mice (Xin et al., 2011). Additionally, total IL-10 protein levels do not significantly change in the FMNuc after axotomy; rather, it is likely that a cellular and spatial shift in IL-10 production (Aim 1 of this study) as well as

an increase in IL-10R signaling is the main driving force for IL-10-mediated neuroprotection after FNA (Xin et al., 2011). While immunodeficiency causes transient depression of IL-10 levels in the FMNuc during the early post-injury phase (Xin et al., 2011), the effect is modest and occurs prior to the significant T cell infiltration observed by others (Raivich et al., 1998; Villacampa et al., 2015). Therefore it remains unclear as whether these two neuroprotective pathways, IL-10-mediated and adaptive immune-mediated, are linked or act independently of one another. *Aim 2 of this study sought to determine whether such a link exists by characterizing adaptive immune cell participation in neuroprotective IL-10 receptor signaling after facial nerve axotomy*.

For the first part of this aim, it was hypothesized that the adaptive arm of the immune system, being critical for neuroprotection, is also necessary for normal levels of IL-10R expression in the FMNuc. If true, this would bridge a gap between CD4+ T cell-mediated neuroprotection and the central neuroprotective IL-10 cascade. Prior to testing this hypothesis, it was necessary to determine whether T cells must communicate with cells in the FMNuc remotely (i.e., across the blood-brain barrier) or are capable of traveling into the FMNuc parenchyma and interacting directly with resident cells there. This would indicate whether central expression of IL-10R and other markers are potentially effected by direct T cell contact. The second part of Aim 2 investigated IL-10R expression by infiltrating T cells themselves. In the discussion for Aim 1, it was hypothesized that the induction of IL-10 expression by astrocytes could inhibit or otherwise modulate the infiltrating adaptive immune response to axotomy. For Aim 2, it was hypothesized that IL-10R expression by infiltrating T cells (and therefore their susceptibility to IL-10 signaling in the FMNuc) was necessary for immune-mediated

neuroprotection, potentially by influencing their activation against self-antigen and subsequent modulation of the glial milieu after axotomy.

5.2.1. CD4+ T cells are capable of extravasation into the FMNuc parenchyma after axotomy where they may interact with central cells directly to mediate neuroprotection

After their initial activation in the periphery, T cells must interact with MHCII on a central APC, mostly likely microglia, to confer neuroprotection after axotomy (Byram et al., 2004). In order to interact with central microglia, CD4+ T cells must be capable of homing to the FMNuc after axotomy, extravasating across the blood-brain barrier to enter the CNS parenchyma, and migrating toward regions of high microglial activation. Previous work using single-label IHC has reported the presence of CD3+, CD4+, and CD8+ cells in the FMNuc after axotomy (Bohatschek et al., 2004; Ha et al., 2007a; Huang et al., 2012; Raivich et al., 1998; Villacampa et al., 2015). However, given the limitations of single-cell IHC as well as the potential expression of CD4 on non-T cells, including microglia and macrophages (Almolda et al., 2009), it was necessary to perform co-IHC of CD3 with CD4 to demonstrate that true CD4+ T cells infiltrate the FMNuc after FNA. Furthermore, the ability of CD4+ T cells to leave the blood vessels and enter the FMNuc parenchyma has not been demonstrated definitively. In the aforementioned studies, lack of co-IHC to label the penetrating blood vessels of the FMNuc left the question of whether the T cells remain restricted to the vascular compartment unanswered. The 14 dpo time point was selected for analysis as this represents the peak of T cell infiltration in the literature (Bohatschek et al., 2004; Raivich et al., 1998).

The results in this study indicate that true CD3+CD4+ T cells are found within the FMNuc after axotomy. The remaining CD3+CD4- cells are likely CD8+ T cells. Importantly, it appears that CD4+ T cells are not restricted to the lumen of blood vessels in the FMNuc. In fact, some CD4+ T cells are observed lying in close proximity to or extending pseudopod-like structures toward Nissl-stained FMN cell bodies. Therefore CD4+ cells are capable of both homing to the FMNuc after axotomy and extravasating across the blood-brain barrier to interact with central cells. CD4+ T cell localization near FMN cell bodies is likely mediated at least in part by the Th2-associated chemotactic cytokine CCL11, which is induced in astrocytes after axotomy (Wainwright et al., 2009c). T cells must express CCR3, one of the receptors for CCL11, in order to confer neuroprotection (Wainwright et al., 2009b).

Having translocated into the FMNuc parenchyma, CD4+ T cells must next interact with a central source of MHCII in order to confer neuroprotection (Byram et al., 2004). Microglia are the most likely candidates for central APC due to their established roles as central innate immune players expressing MHCII and T cell co-stimulatory molecules, which are weakly expressed or absent in activated astrocytes (Aloisi et al., 2000b; Cross & Ku, 2000; Hayes et al., 1987; Kreutzberg, 1996; Satoh et al., 1995). One study in the axotomized FMNuc showed that only perivascular macrophages, but not microglia, express MHCII (Liu et al., 2005); however, this observation is contradicted by numerous other studies demonstrating FMNuc MHCII expression in both perivascular macrophages and microglia, but not astrocytes or FMN (Hurley & Coleman, 2003; Petitto et al., 2003; Raivich, 2002; Streit et al., 1989a; Villacampa et al., 2015). Our laboratory has also shown that MHCII expressed on microglia, not perivascular macrophages, is

necessary for CD4+ T cell-mediated neuroprotection (Byram et al., 2004). In the current study, CD4+ T cells appear to associate with microglial nodules, which is in agreement with other studies showing accumulation of CD3+ T cells near microglial clusters after FNA (Raivich et al., 1998; Villacampa et al., 2015). Microglial nodules in the FMNuc are associated with phagocytosis of dead neurons and are characterized by strong MHCII immunoreactivity, making them potential "hotspots" for presentation of neuronal antigen to infiltrating T cells (Huang et al., 2012; Villacampa et al., 2015).

5.2.2. CD4+ T cells are necessary for normal upregulation of IL-10 receptor expression after axotomy, regardless of their own IL-10 receptor expression or signaling capability

The ability of the peripheral immune system to regulate central gene expression in the FMNuc is well established (Setter et al., 2018b). CD4+ T cells likely regulate gene expression via direct interactions with resident central cells, as it has now been demonstrated that they are capable of translocation across the blood-brain barrier into the FMNuc. Because CD4+ T cells are critical for IL-10-mediated neuroprotection despite not being the obligate IL-10 source (Xin et al., 2011), it was hypothesized that T cells potentiate IL-10R gene expression and thus may promote downstream IL-10 signaling in the FMNuc. To determine whether the adaptive arm of the immune system affects central expression of the IL-10R, analysis of both IL-10R subunit genes, *Il10ra* and *Il10rb*, was performed after FNA. IL-10RA is the ligand-binding subunit expressed preferentially on immune cells and is often referred to as the inducible subunit, whereas IL-10RB is expressed on a broader population of immune and non-immune cells and often has constitutive expression (Moore et al., 2001). Although IL-10RB is considered a

promiscuous receptor subunit that is shared with other IL-10 family cytokines, it is necessary for IL-10 signal transduction, and loss of IL-10RB recapitulates the pathology observed in IL-10$^{-/-}$ animals (Kotenko et al., 1997; Spencer et al., 1998; Zdanov, 2010). Axotomy induced upregulation of both receptor subunit genes, although induction of *Il10ra* was greater than that of *Il10rb*. Induction of both subunits was significantly decreased under immunodeficient conditions. This effect was specific to the CD4+ T cell, as adoptive transfer of isolated CD4+ T cells restored IL-10R subunit expression. These data indicate that there are two components of IL-10R induction after axotomy: an intrinsic response of the FMNuc to injury that triggers partial induction of IL-10R expression, and a CD4+ T cell-dependent component required for full induction.

The change in IL-10R expression that is dependent on CD4+ T cells could be accounted for by 1) induction of IL-10R expression on central cells, such as neurons, that is triggered by the presence of CD4+ T cells, 2) infiltration of IL-10R-expressing T cells into the FMNuc, or 3) a combination of both mechanisms. To answer this question, CD4+ T cells deficient in IL-10RB were transferred into immunodeficient mice. Despite lacking functional IL-10R, these CD4+ T cells were nonetheless able to restore IL-10R subunit expression in the FMNuc. *Il10rb*$^{-/-}$ CD4+ T cells actually produced a greater increase in *Il10ra* expression after axotomy than did WT CD4+ T cells. This may have been a compensatory increase in response to a failure of IL-10R signaling in transgenic T cells. It is unknown whether transcription of *Il10ra* is dependent upon *Il10rb*; therefore, expression of *Il10ra* by infiltrating *Il10rb*$^{-/-}$ CD4+ T cells could theoretically account for the entirety of the change observed in *Il10ra* expression, although this is unlikely. The change in *Il10rb* triggered by the presence of *Il10rb*$^{-/-}$ T cells, however, can only be

explained by an induction on central cells. Therefore, it appears that CD4+ T cells regulate expression of IL-10R (at least the RB subunit) by inducing its expression on central cells in the FMNuc. However, the presence of IL-10R on infiltrating T cells cannot be ruled out by this study. Expression of IL-10R on naïve T cells is low, but is induced upon T cell activation (Akdis & Blaser, 2001; Joss et al., 2000). It has been observed in our laboratory that expression of other T cell receptors (such as CD3, CD4, and CD8) is rarely detectable by qPCR amplification in the FMNuc after axotomy (unpublished observations), despite the presence of these cells being confirmed by IHC. The failure of qPCR to detect T cell markers is likely due to the relatively low numbers of T cells that actually infiltrate the FMNuc compared to the populations of resident neurons and glia; the relatively low expression of receptors on T cell surfaces (nonetheless important for T cell function [Kamanaka et al., 2011]); and/or a lack of active transcription of receptor subunits in T cells due to their stable expression on the cell membrane. In conclusion, CD4+ T cells are necessary for full induction of IL-10R expression in the FMNuc, and they perform this function at least in part by triggering gene expression in central cells.

A previous study from our laboratory showed no difference in IL-10R expression between WT and RAG-2$^{-/-}$ mice; however, only the seven dpo time point was investigated (Xin et al., 2011). It makes sense that analysis of later time points would be necessary to detect differences in central receptor expression that are mediated by the peripheral immune system, because at seven days the majority of CD4+ T cells are still located within the draining cervical lymph nodes and have not yet mobilized to the FMNuc (Xin et al., 2008). One study has shown that IL-10R immunoreactivity (receptor

subunit not specified) localizes to neurons in the FMNuc constitutively and actually decreases at 7 dpo, with at least partial restoration of expression occurring by 14 dpo (Villacampa et al., 2015). The same study did not detect IL-10R expression on astrocytes after axotomy, which conflicts with results from our laboratory (Xin et al., 2011); however, both report IL-10R expression on neurons. Combined with the gene expression data in this study, the transient decrease in IL-10R immunoreactivity described by Villacampa et al. (2015) suggests that axotomy causes an early loss of IL-10R protein displayed on the FMN cell membrane that is accompanied by a compensatory increase in IL-10R mRNA production for restoration of IL-10R signaling at later time points. The immune system may therefore have an instrumental role in promoting recovery of IL-10R expression on injured FMNs, as CD4+ T cells are necessary for full induction of IL-10R gene expression in the FMNuc after axotomy.

A recent study on immune-mediated neuroprotection in the context of chemotherapy-induced peripheral neuropathy (CIPN) bears striking similarities with our observations after FNA. In this study, both CD8+ T cells and IL-10 are necessary for recovery of normal sensation after CIPN; however, the T cell is not the requisite source of IL-10. Rather, the presence of CD8+ T cells is necessary for upregulation of *Il10ra* mRNA in dorsal root ganglion neurons after paclitaxel-induced injury (Krukowski et al., 2016). This study as well as ours support a role for T cell regulation of the IL-10 signaling cascade via induction of IL-10R expression on neurons after peripheral nerve injury.

IL-10 has direct trophic actions on neurons. Both primary cortical and spinal cord neurons express IL-10R *in vitro* (Chen et al., 2016; Zhou et al., 2009b). Application of

exogenous IL-10 to primary cortical neurons undergoing oxygen-glucose deprivation downregulates expression of pro-apoptotic Bax while increasing expression of Bcl-2, promoting neurite outgrowth, and enhancing synaptogenesis in a JAK1/STAT3/Netrin-1-dependent fashion (Chen et al., 2016). Treating primary spinal cord neurons with IL-10 activates a number of pathways in these neurons, including the JAK1/STAT3, PI3K-AKT, and GSK-3β pathways. Interestingly, activation of AKT in these neurons causes NF-κB activation (rather than inhibition downstream of the JAK1/STAT3 pathway, the typical mechanism in immune cells) which promotes expression of Bcl-2 and Bcl-x_L and prevents release of cytochrome *c*. By these mechanisms, IL-10 prevents glutamate-induced excitotoxicity in cultured neurons (Zhou et al., 2009b). Exogenous IL-10 treatment also improves neuron survival *in vivo* after spinal cord injury through regulation of the same pro- and anti-apoptotic factors (Zhou et al., 2009a).

It is possible that the IHC labeling described in previous studies of the FMNuc (Villacampa et al., 2015; Xin et al., 2011) failed to capture the expression of IL-10R on other relevant cell types after axotomy. Both astrocytes and microglia can express IL-10R, and IL-10 has dramatic effects on glial inflammation in the context of injury and disease. Therefore, the consequences of IL-10 signaling through glia is worthy of discussion. IL-10R is present on both primary astrocytes and microglia *in vitro*, but is preferentially expressed by astrocytes when the two are co-cultured (Ledeboer et al., 2002; Norden et al., 2014). Astrocytes that have been stimulated with IL-10 produce TGFβ *in vitro*, which suppresses pro-inflammatory cytokine section by microglia and increases microglial expression of CX3CR1 and the IL-4 receptor (Norden et al., 2014; Norden et al., 2016). IL-10-stimulated astrocytes also downregulate expression of genes

associated with macrophage activation and chemotaxis (Mayo et al., 2016). These studies support a role for IL-10R signaling in astrocytes as a mechanism of indirect microglial regulation. However, the opposite has also been demonstrated: one study showed that IL-10 suppresses astrocyte proliferation and hypertrophy after corticectomy, but does not have this effect on isolated astrocytes *in vitro*. Therefore, the observed effects were hypothesized to be downstream of a suppressive influence of IL-10 on microglia (Balasingam & Yong, 1996).

In microglia, IL-10 suppresses genes associated with pro-inflammatory macrophage activation, such as *Il1b*, *Il6*, *Nos2*, and *Cd40* (Mayo et al., 2016), as well as the production of IL-12, TNFα, NO, and other reactive oxygen species (Lodge & Sriram, 1996; Qian et al., 2006). Similar to its effects on macrophages in the periphery, IL-10 decreases MHCII expression by microglia and inhibits their ability to activate CD4+ T cells (Mizuno et al., 1994; Williams et al., 1996). In combination with IL-4 and TGF-β, IL-10 promotes the conversion of microglia toward a neuroprotective M2 phenotype (Kiyota et al., 2012; Zhang et al., 2014; Zhou et al., 2017).

In conclusion, CD4+ T cells are necessary for full induction of IL-10R expression in the FMNuc after axotomy. IL-10 signaling in neurons is known to have direct trophic and anti-apoptotic effects, whereas IL-10 signaling through glia suppresses glial inflammation and antigen presentation. Studies that have attempted to localize IL-10R expression in the FMNuc after axotomy are concordant in their identification of neurons as constitutive expressers of IL-10R (Villacampa et al., 2015; Xin et al., 2011). CD4+ T cells may therefore have an important role in maintaining this expression to promote neuron survival after injury.

5.2.3. CD4+ T cells must express functional IL-10 receptor in order to confer neuroprotection

Il10rb$^{-/-}$ CD4+ T cells regulate overall IL-10R gene expression in the FMNuc similarly to WT CD4+ T cells, which rescue FMN survival after axotomy (Serpe et al., 2003). Therefore, it was surprising that *Il10rb*$^{-/-}$ CD4+ T cells were incapable of rescuing FMN survival in RAG-2$^{-/-}$ mice to WT levels. This was not due to a failure of *Il10rb*$^{-/-}$ T cell survival until 28 dpo, as viable T cells were detectable in splenic follicles in the immunodeficient host. A failure of *Il10rb*$^{-/-}$ T cells to home to the FMNuc is also unlikely, due to their aforementioned ability to regulate central gene expression. Therefore the loss of the ability of these CD4+ T cells to confer neuroprotection is presumably specific to the loss of IL-10R signaling within the T cell itself.

IL-10 can act on T cells via direct and indirect mechanisms. Indirectly, IL-10 acts on APCs to suppress Th1-associated cytokine secretion in the context of antigen presentation and T cell activation (Fiorentino et al., 1989; Fiorentino et al., 1991b). There is also evidence that IL-10 can cause APCs to skew T cell differentiation toward the Th2 phenotype (Liu et al., 1998). Loss of this indirect pathway can lead to pathogenic Th1 responses (Berg et al., 1996). Directly, IL-10 signaling in T cells inhibits CD28 phosphorylation (Akdis et al., 2000; Joss et al., 2000; Schandene et al., 1994; Taylor et al., 2006). CD4+ T cells must receive at least two forms of stimulation by an APC in order to become activated against antigen: their T cell receptors and CD4 co-receptor must engage with antigen presented on MHCII on the APC, and the T cell co-stimulatory molecule CD28 must also engage with APC CD80/86 (B7.1/7.2). Loss of either of these cues causes T cell anergy, or the loss of immune response against antigen (Janeway &

Bottomly, 1994). Therefore, IL-10 acts directly on the T cell to inhibit the CD28-dependent co-stimulatory pathway and induce T cell tolerance. This loss of tolerance to self-antigen after axotomy in *Il10rb*$^{-/-}$ CD4+ T cells could be the cause for their failure to mediate neuroprotection, despite the fact that their presence still triggers expression of IL-10R in central cells in the FMNuc.

The harmful role of autoreactive T cells is supported by studies finding that immunization with or without neural antigen exacerbates motoneuron loss after spinal cord injury and FNA, indicating an overall detrimental effect of enhancing adaptive immune reactivity on neuron survival. The immunization strategies employed by these studies likely optimized a Th1 response to injury (Ankeny & Popovich, 2007; Jones et al., 2004). These findings initially appear to contradict a previous study from our laboratory, which concluded that T cells must be activated both peripherally and centrally in order to confer neuroprotection after axotomy (Byram et al., 2004). However, this study investigated the importance of MHCII expression on microglia in the central compartment after axotomy, and not the need for central T cell re-activation *per se*. Therefore, the need for CD4+ T cells to encounter MHCII centrally in order to confer neuroprotection could actually represent the necessity of MHCII-induced anergy in the absence of CD28 co-stimulation, rather than T cell re-activation. Alternatively, the neuroprotective capacity of Th2 cells may benefit from central re-activation by microglia, whereas IL-10 may be necessary to suppress Th1 or Th17 responses that are not neuroprotective.

Other studies have demonstrated the importance of IL-10R signaling specifically in T cells. Adoptive transfer of CD4+ T cells overexpressing a dominant-negative form of

IL-10RA into immunodeficient mice causes the development of spontaneous enterocolitis (Kamanaka et al., 2011). In mouse models and in humans, T cells nonresponsive to IL-10 escape Treg control, proliferate to a greater degree, and produce higher amounts of inflammatory cytokines including Th17 and IFNγ, both of which activate macrophages and promote cellular autoimmunity (Kamanaka et al., 2011; Shouval et al., 2017). Interestingly, IL-10R signaling and downstream STAT3 phosphorylation are also defective in CD4+ T cells from patients with systemic lupus erythematosus and MS, suggesting that a failure of T cell suppression by IL-10 may play an important role in autoimmune disease, including in the CNS (Cui et al., 2011; Martinez-Forero et al., 2008).

5.2.4. CD4+ T cells lacking IL-10R induce greater expression of genes associated with microglial activation, antigen presentation, T cell co-stimulation, and synaptic pruning after axotomy

Anergic T cells are not merely inactive cells without immunological functions. They can suppress the proliferation of other effector T cells and prevent cell-mediated toxicity against skin grafts *in vivo* (Chai et al., 1999). Anergic T cells also actively suppress APCs *in vitro* by downregulating their expression of MHCII, CD80, and CD86, thereby preventing antigen presentation and co-stimulation for other T cells (Vendetti et al., 2000). Both of these actions are antigen-specific and mediated by cell-cell contact. Conversely, effector T cells induce greater expression of MHCII and co-stimulatory molecules on macrophages/monocytes largely via IFNγ signaling (Schroder et al., 2004). Therefore it was hypothesized that loss of IL-10R signaling in CD4+ T cells would lead

to increased activation of central macrophages (i.e. microglia). The 14 dpo time point was analyzed according to the rationale described in previous experiments; additionally, this time point represents the peak of microglial gene expression in the FMNuc after axotomy (Setter et al., 2018b). Genes relating to microglial activity were selected for investigation and divided into three categories based on associations with: 1) general activation and pro-inflammatory activity, 2) antigen presentation and T cell co-stimulation, and 3) complement deposition and synaptic pruning.

5.2.4.1. Genes associated with activated microglia

To investigate general microglial activation and pro-inflammatory cytokine activity, the following genes were selected: *Cd68*, *Cd40*, *Tnf*, *Tnfrsf1*, and *Nos2*. After FNA, microglia proliferate in the FMNuc (Blinzinger & Kreutzberg, 1968; Graeber et al., 1988c; Torvik & Skjorten, 1971b). These proliferating cells lack expression of markers for perivascular macrophages such as ED2 and therefore are likely a true microglial population (Graeber et al., 1988a). mRNA for CD68, a pan-macrophage marker, increases in the FMNuc after axotomy (Haulcomb et al., 2014; Setter et al., 2018b) and corresponds with the appearance of CD68+ microglia (Wainwright et al., 2010). Therefore *Cd68* mRNA may be used as a general marker of microglial activation (and potentially proliferation) in the FMNuc after axotomy. In immunodeficient mice reconstituted with *Il10rb*$^{-/-}$ CD4+ T cells, the level of *Cd68* in the axotomized FMNuc was significantly greater than in animals having WT CD4+ T cells, indicating increased microglial activation due to the loss of T cell IL-10R expression.

Cd40, *Tnf*, *Tnfrsf1*, and *Nos2* are genes associated with pro-inflammatory microglia. CD40 ligation is one of three steps critical for macrophage activation by effector T cells (the other two being MHCII activation and IFNγ signaling) (Banchereau et al., 1994). In resting microglia or in microglia that have been redirected toward an M2 phenotype, expression of CD40 is low (Aloisi et al., 2000a; Aloisi et al., 1998; Tierney et al., 2009). Microglia strongly increase CD40 expression in response to LPS, IFNγ, and upon interacting with Th1 (but not Th2) cells (Aloisi et al., 2000a; Aloisi et al., 1998). Increased expression of CD40 is associated with CNS autoinflammatory disease such as MS and HIV-1 encephalitis (D'Aversa et al., 2008; Ponomarev et al., 2006; Tan et al., 1999). *Tnf* and *Nos2* (gene name for inducible nitric oxide synthase, iNOS) are also associated with pro-inflammatory microglial activation and are induced by LPS stimulation (Chhor et al., 2013; Lodge & Sriram, 1996; Park et al., 2007). Although TNFα and NO are essential for the innate immune response to pathogens, they exhibit toxic bystander effects toward neurons and glia (Lodge & Sriram, 1996; Lull & Block, 2010; Moss & Bates, 2001; Smith et al., 2012). TNFR1 is upregulated in response to TNFα signaling on microglia to promote further TNF- and other pro-inflammatory cytokine-associated signaling in an autocrine fashion (Kuno et al., 2005). There was no difference in FMNuc *Cd40* expression when comparing RAG-2$^{-/-}$ mice reconstituted with WT or *Il10rb*$^{-/-}$ CD4+ T cells, although *Cd40* expression in the former was significantly elevated compared to WT. There were also no differences detected in *Tnf*, *Tnfrsf1*, and *Nos2* gene expression. These data indicate that while *Il10rb*$^{-/-}$ T cells increase overall microglial activation, they do not necessarily do so in an overtly pro-inflammatory fashion compared to WT CD4+ T cells. It is possible, however, that alterations in

inflammatory markers were missed due to the time point selected for this analysis, as *loss* of *Tnf* expression is observed as early as 7 dpo in immunodeficient mice and is regulated by CD4+ T cells (Setter et al., 2018b).

5.2.4.2. Genes associated with antigen presentation and T cell co-stimulation

To investigate the hypothesis that *Il10rb*$^{-/-}$ T cells enhance antigen presentation and co-stimulation in the FMNuc after axotomy, the genes for MHCII (*H2ab1*) and co-stimulatory molecules CD80 and CD86 were analyzed in the axotomized FMNuc. *Il10rb*$^{-/-}$ T cells induced significantly higher *H2ab1* and *Cd86* expression after axotomy compared to WT or immunodeficient mice given WT CD4+ T cells. This increase in gene expression is likely specific to microglia, as microglia have been demonstrated to express MHCII in the FMNuc (Hurley & Coleman, 2003; Petitto et al., 2003; Raivich, 2002; Streit et al., 1989a; Villacampa et al., 2015). MHCII and CD86 are both expressed at low levels in resting microglia, but increase upon LPS and IFNγ stimulation, TLR activation, or when interacting with Th1/17 cells (Aloisi et al., 2000a; Aloisi et al., 1998; Lodge & Sriram, 1996; Menendez Iglesias et al., 1997; Olson & Miller, 2004; Satoh et al., 1995). Astrocytes rarely express MHCII or co-stimulatory molecules (De Simone et al., 1995; Kreutzberg, 1996; Satoh et al., 1995), even in the course of massive peripheral immune infiltration such as during EAE (Cross & Ku, 2000; Hoftberger et al., 2004).

Th1 cells induce upregulation of MHCII on microglia, but Th2 cells suppress its expression on microglia via an IL-4-dependent mechanism, implicating MHCII as an important mediator of Th1/Th2 cross-regulation (Aloisi et al., 2000a; Suzumura et al., 1994). Therefore, the increase in *H2ab1* expression caused by *Il10rb*$^{-/-}$ CD4+ T cells in

this study may be a consequence of Th1 subset skewing and loss of the neuroprotective Th2 subtype. Low CD86 is associated with tolerogenic APC and M2 anti-inflammatory microglia (Ma et al., 2015; Steinbrink et al., 1997; Tierney et al., 2009; Zhang et al., 2014), whereas increased MHCII and CD86 on microglia is associated with neurodegenerative and autoimmune conditions (De Simone et al., 1995; Hayes et al., 1987; Ponomarev et al., 2006; Tooyama et al., 1990). Expressing MHCII on microglia at specific loci prior to EAE induction results in lymphocytic infiltration and lesion formation at the same loci (Konno et al., 1990), while blocking CD86 co-stimulation reduces EAE incidence and severity (Girvin et al., 2000). Overall, these data suggest that *Il10rb*$^{-/-}$ CD4+ T cells promote greater antigen presentation and T cell co-stimulation by microglia after FNA, which may facilitate harmful (or at least non-neuroprotective) autoimmune mechanisms effected by greater infiltration of Th1 cells.

5.2.4.3. Genes associated with complement deposition and synaptic pruning

This study showed that expression of *B2m*, which encodes the MHC class I subunit β2-microglobulin, was significantly greater in RAG-2$^{-/-}$ mice reconstituted with WT CD4+ T cells compared to WT or non-reconstituted RAG-2$^{-/-}$. However, expression of *B2m* in RAG-2$^{-/-}$ mice given *Il10rb*$^{-/-}$ CD4+ T cells was the greatest of all conditions investigated. MHCI is an antigen presenting molecule important for cell-mediated immunity and neuronal plasticity during development (Shatz, 2009). Unlike MHCII, which is predominantly expressed on professional APCs, MHCI has the potential to be expressed on all nucleated cells in the body.

There is some evidence that FMN cell bodies express MHCI protein after axotomy (Maehlen et al., 1988), although this is contradicted by other studies in the FMNuc (Bohatschek et al., 2004; Streit et al., 1989b). However, multiple studies have indicated that MHCI mRNA also co-localizes with motoneurons (Lidman et al., 1999; Linda et al., 1998). This discrepancy indicates that motoneuron MHCI is likely translated or transported away from the cell body and expressed on distal processes. MHCI at synapses appears to promote axon regeneration by regulating the stripping of upper motoneuron synaptic inputs from the dendrites and cell bodies of axotomized lower motoneurons, although studies conflict as to whether MHCI has this effect by promoting (Sabha et al., 2008) or restricting (Berg et al., 2013; Oliveira et al., 2004) the extent of synaptic stripping. However, conclusions drawn from studies showing impaired axon regeneration in MHCI-deficient rodents (Akdagli et al., 2016; Oliveira et al., 2004) must be tempered by the fact that genetic MHCI deficiency will also result in loss of CD8+ T cells, complicating the phenotype. It is also worth noting that activated microglia can induce expression of MHCI on neuron cell bodies, resulting in cytotoxic T cell attack and subsequent neurodegeneration (Cebrian et al., 2014); however, this does not explain the failure of *Il10rb*$^{-/-}$ CD4+ T cells to mediate neuroprotection, as the reconstituted RAG-2$^{-/-}$ mice in this study lack CD8+ T cells.

Microglial upregulation of *B2m* is another explanation for the changes observed in this study. Multiple studies indicate that microglial expression of MHCI accounts for much of its total upregulation in the FMNuc after axotomy (Bohatschek et al., 2004; Lidman et al., 1999; Streit et al., 1989b). In the FMNuc, MHCI immunoreactivity is highly specific to microglia and appears to be important for their ability to form

phagocytic nodules and interact with infiltrating CD8+ T cells (Bohatschek et al., 2004; Streit et al., 1989b). Microglia are capable of presenting exogenous antigen on MHCI to CD8+ T cells (Beauvillain et al., 2008; Jarry et al., 2013). Furthermore, MHCI expression by microglia may be necessary for recruitment of CD8+ T cells to the central compartment (Malo et al., 2018). As with MHCII, IFNγ produced by CD4+ T cells can induce expression of MHCI on macrophages (King & Jones, 1983; Wong et al., 1983). It is likely that infiltrating CD4+ T cells instruct microglia to upregulate MHCI and thereby prime microglia for CD8+ T cell interactions. The observed increase in *B2m* expression in immunodeficient mice given WT CD4+ T cells in this study may therefore simply indicate a greater degree of microglial activation. This potentially becomes *hyper*activation in immunodeficient mice given *Il10rb*$^{-/-}$ CD4+ T cells, as has been suggested by changes in other markers such as *Cd68*, *H2ab1*, and *Cd86*.

Similarly to *B2m*, expression of complement protein *C3* was also increased in RAG-2$^{-/-}$ given WT CD4+ T cells relative to WT or RAG-2$^{-/-}$, and RAG-2$^{-/-}$ given *Il10rb*$^{-/-}$ CD4+ T cells had the greatest *C3* expression compared across all conditions. Peripherally, complement is integral to the innate immune response by facilitating opsonization of pathogens and promoting macrophage chemotaxis and phagocytosis (Lubbers et al., 2017). Complement can also promote T cell activation, differentiation, and proliferation (Strainic et al., 2008). After axotomy, complement in the periphery is beneficial for efficient removal of myelin and proper Wallerian degeneration (Ramaglia et al., 2009; Ramaglia et al., 2007; Ramaglia et al., 2008). Centrally, complement has a role in tagging synapses for microglial-mediated synaptic pruning (Schafer et al., 2012). Activated microglia in the FMNuc express high levels of complement receptor after

axotomy (Berg et al., 2013; Graeber et al., 1988a). In the axotomized FMNuc, the complement components C1, C1q, C3, and C3d co-localize with microglia located in close proximity to neuronal membranes, but not with neurons or astrocytes. *C3* mRNA expression is most concentrated in microglia. mRNAs for clusterin and CD59, both of which are complement cascade inhibitors, co-localize with FMN cell bodies (Mattsson et al., 1998). Therefore it is likely that the increase in *C3* expression in this study is due to production by activated microglia.

In neurodegenerative disease, microglia can become inappropriately activated to phagocytize synapses labeled with C3 (Hong et al., 2016). High levels of complement components, including C3, and exaggerated pruning are associated with synapse loss in neurodegenerative and psychiatric diseases. These include Alzheimer's disease, ALS, and schizophrenia (Chiu et al., 2009; Forsyth & Lewis, 2017; Heurich et al., 2011; Hong et al., 2016; Kawamata et al., 1992; Shen et al., 1997; Sta et al., 2011). Complement can also promote the release of superoxide anions by microglia (Colton & Gilbert, 1987). Blocking the complement cascade improves neuron survival after traumatic brain injury by suppressing the microglial/macrophage response to injury (Fluiter et al., 2014). Because synapse elimination can be either protective (by shielding injured FMN from excitotoxic inputs) or destructive (as occurs in neurodegenerative disease), the moderate increase in *C3* caused by adoptive transfer of WT CD4+ T cells in this study likely represents a "Goldilocks zone" for optimum synaptic stripping after axotomy, whereas the greater increase observed after transfer of *Il10rb*$^{-/-}$ CD4+ T cells may reflect harmful microglial activation.

To summarize, both *B2m* and *C3* expression were elevated in RAG-2$^{-/-}$ mice given CD4+ T cells, but *Il10rb*$^{-/-}$ CD4+ T cells elicited greater increases in mRNA expression than WT CD4+ T cells. If the observed changes in *B2m* expression are taken to represent neuronal rather than microglial induction, then this has interesting implications for synaptic stripping. MHCI and complement are believed to have opposing roles, respectively inhibiting and promoting synapse loss (Berg et al., 2013). Induction of both after axotomy suggests that the increase in MHCI is a compensatory response by the injured FMN to resist synaptic loss effected by activated microglia and complement. It is interesting that expression of *B2m* and *C3* are increased in RAG-2$^{-/-}$ reconstituted with WT CD4+ T cells, which confer neuroprotection after axotomy, as well as in those given *Il10rb*$^{-/-}$ CD4+ T cells, which fail to support FMN survival. However, expression of both markers was increased to a greater degree after transfer of *Il10rb*$^{-/-}$ CD4+ T cells, indicating that there is a threshold of "safe" innate immune activation that is exceeded when *Il10rb*$^{-/-}$ CD4+ T cells are present.

5.2.5. Summary of results

These data show that CD4+ T cells are capable of crossing the blood-brain barrier and entering the FMNuc parenchyma after axotomy, where they may interact directly with central cells, including microglia. It is likely through this direct cell-cell contact that they modulate gene expression in the FMNuc. Because overall levels of IL-10 in the FMNuc do not change in response to axotomy (Xin et al., 2011), induction of neuroprotective IL-10 signaling depends on increased IL-10R expression. The presence of CD4+ T cells triggers upregulation of IL-10R gene expression on central cells in the

FMNuc after axotomy, indicating that CD4+ T cells are necessary for full induction of neuroprotective IL-10 signaling. Despite regulating IL-10R gene expression on other cells normally, T cells that are unresponsive to IL-10 themselves fail to confer neuroprotection after axotomy. This may be due to a loss of IL-10-mediated T cell tolerance to self-antigen. T cells lacking IL-10RB increase gene expression associated with greater microglial activation (*Cd68*), antigen presentation (*H2ab1* and *B2m*), T cell co-stimulation (*Cd86*), and synapse elimination (*C3* and *B2m*) in the FMNuc after axotomy. This suggests that one neuroprotective mechanism of IL-10 in the FMNuc is to prevent neurotoxic autoimmunity by inducing T cell tolerance to self-antigen.

Although the percentages of FMN survival are approximately the same between non-reconstituted RAG-2$^{-/-}$ and RAG-2$^{-/-}$ given *Il10rb*$^{-/-}$ CD4+ T cells, the gene signatures in the FMNuc differ dramatically. For example, immunodeficient mice exhibit a blunted glial response to axotomy (Setter et al., 2018b), whereas the microglial reaction in RAG-2$^{-/-}$ given *Il10rb*$^{-/-}$ CD4+ T cells appears to be hyper-responsive. The amount of FMN death that occurs after FNA is likely also due to differing mechanisms. In non-reconstituted immunodeficient mice, the increase in FMN death is due to loss of Th2 cells, which may normally promote neuroprotection in part by triggering the upregulation of IL-10R in the FMNuc. In immunodeficient mice given *Il10rb*$^{-/-}$ CD4+ T cells, the neuroprotective effect of CD4+ T cells triggering central IL-10R expression is still present, but the benefit may be nullified by the actions of a neurotoxic autoreactive T cell that is unresponsive to IL-10. In the spectrum of immune responses that can occur after axotomy, either extreme is not favorable, and optimum FMN survival likely depends on balanced activation of immune effectors.

5.2.6. Limitations of this study and future directions

In this study and others, *in vivo* demonstrations of microglia presenting antigen to CD4+ T cells have relied on indirect measures. For example, MHCII co-localizes with phagocytic microglial nodules in the literature (Huang et al., 2012; Villacampa et al., 2015), and this study has shown that CD4+ T cells also accumulate in association with IBA1+ microglial nodules; therefore, the conclusion is drawn indirectly that microglia interact with CD4+ T cells via MHCII-T cell receptor interactions. Triple immunohistochemistry for CD4, IBA1, and MHCII in the axotomized FMNuc in combination with confocal microscopy would definitively demonstrate CD4+ T cell-microglia interactions in the context of antigen presentation. Similar analysis should also be performed with astrocytes to determine their antigen presentation capacity. Baseline antigen presentation events should then be compared to conditions of immunodeficiency and the presence of defective T cells lacking IL-10R expression. Utilization of IHC or Western blot to confirm that the observed mRNA changes coincide with alterations in protein levels should also be performed. IHC or FISH would also be useful to localize markers that the literature indicate may be expressed in different cell types, such as *B2m* as described in section 5.2.4.3.

In the literature, loss of IL-10R signaling in T cells causes predominant Th17 responses (Kamanaka et al., 2011; Shouval et al., 2017). In this study, increased expression of genes associated with microglial activation in the FMNuc suggests that loss of IL-10RB may cause CD4+ T cell differentiation to become skewed toward a non-neuroprotective subtype rather than the Th2 subtype, which confers neuroprotection (Deboy et al., 2006b). The increase in MHCII gene expression in the presence of *Il10rb*$^{-/-}$

T cells after FNA supports this, as MHCII is induced by Th1 cells and suppressed by Th2 cells (Aloisi et al., 2000a; Suzumura et al., 1993). To determine whether loss of IL-10RB affects T cell subset differentiation, adoptive transfer of WT and *Il10rb*$^{-/-}$ CD4+ T cells could be performed one week prior to axotomy, followed by collection of draining cervical lymph nodes at one week post axotomy for flow cytometric analysis. Additionally, although equal numbers of T cells were transferred into immunodeficient mice, loss of IL-10RB on T cells may influence how many T cells actually infiltrate the FMNuc after axotomy. Quantification of T cell numbers in the FMNuc would be informative.

Finally, the exact mechanisms behind the failure of *Il10rb*$^{-/-}$ T cells to mediate FMN survival are unknown. Although increased microglial activation has been implicated, *Il10rb*$^{-/-}$ CD4+ T cells do not cause increased expression of canonical pro-inflammatory genes such as *Nos2* or *Tnf* in microglia. However, microglia can induce neuronal death by other mechanisms, such as excitotoxic glutamate release (Brown & Vilalta, 2015). The increase in complement component *C3* expression triggered by *Il10rb*$^{-/-}$ CD4+ T cells in this study also suggests that increased synaptic elimination (or even C3-mediated phagocytosis of stressed neurons) may play a role in neuronal survival after axotomy. Further exploration of these mechanisms will be necessary to fully characterize the role of IL-10 in shaping the adaptive immune response to axotomy.

5.4. Significance of findings

5.4.1. Feasibility of IL-10 as a therapeutic

In agreement with the literature, the results from this study demonstrate neuroprotective roles of IL-10 in the injured nervous system. The application of IL-10 as a therapeutic drug is made difficult by the fact that it has a short half-life of only a few hours (Huhn et al., 1997). Studies utilizing systemic IL-10 for the treatment of peripheral autoimmune diseases have attempted to circumvent this with more frequent administration, but this results in undesirable side effects (Saxena et al., 2015). Furthermore, IL-10 does not cross the blood-brain barrier, complicating its application for the treatment of CNS injury and disease (Kastin et al., 2003). Many studies showing CNS benefits of IL-10 have administered the cytokine via intraspinal or intracerebroventricular injection, which is highly invasive (Bluthe et al., 1999; Brewer et al., 1999).

Even if a significant amount of IL-10 administered systemically cannot reach the injury site, it may still be capable of attenuating inflammation if the CNS pathology is mediated by infiltrating peripheral cells. However, the timing of systemic IL-10 administration is important. After contusive spinal cord injury, systemic administration of IL-10 is capable of suppressing TNFα production by activated monocytes and reducing lesion volume if administered acutely (30 minutes after injury) (Bethea et al., 1999). To surmount these difficulties, many studies have utilized viral vectors or other transgenic means of increasing and maintaining IL-10 expression centrally (Ayers et al., 2015; Cua et al., 2001; Joniec-Maciejak et al., 2014; Kiyota et al., 2012; Koeberle et al., 2004; Zhou et al., 2009a), but these methods entail significant ethical issues for use in human

patients. Finally, enhancing IL-10 levels is unlikely to be effective for conditions in which the IL-10R is hypo-responsive, as has been observed in some MS patients (Martinez-Forero et al., 2008).

By elucidating the effects of IL-10 on the ability of CD4+ T cells to mediate neuroprotection, conclusions from this study support a rationale for using cell-based immunomodulatory therapies in peripheral nerve injury. T cells that are autoreactive to self-antigen released by axotomy may be harmful for neuron survival, but complete elimination of T cells altogether is also detrimental. Tolerizing T cells to self-antigen *ex vivo* in the presence of exogenously applied IL-10, or alternatively by exposing T cells to self-antigen in the context of CD28 blockade, followed by re-infusion into patients may confer enhanced neuroprotective benefits. Similar therapeutic approaches have been explored for the treatment of graft versus host disease and demonstrate great promise (Blazar et al., 1998; Chen et al., 2003; Dillinger et al., 2017; Zeller et al., 1999).

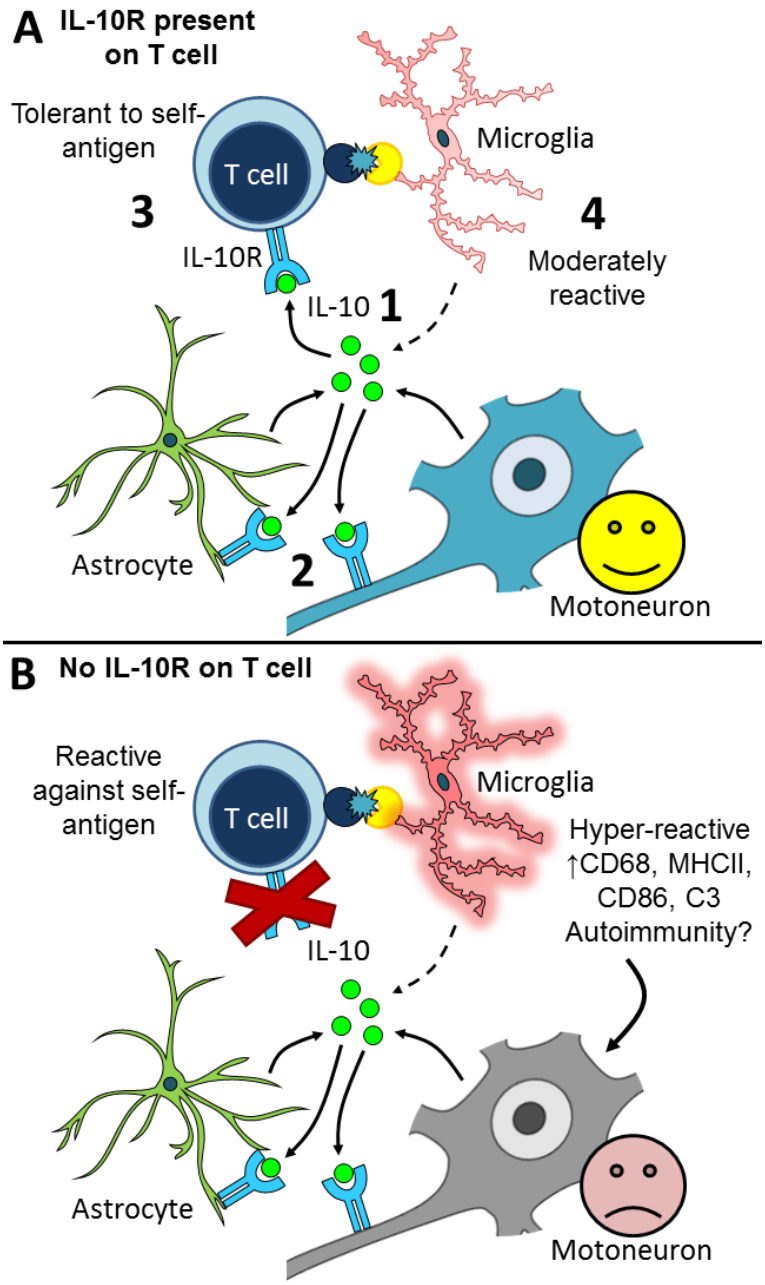
5.4.2. A new theory of IL-10-mediated neuroprotection after axotomy

Collectively, the conclusions from this body of work support dual roles for IL-10-mediated neuroprotection that involve: 1) trophic support for injured FMN and 2) suppression of neurotoxic immune activation. An illustration summarizing this new theory of IL-10-mediated neuroprotection is shown in Picture 2. Constitutive neuronal expression of IL-10 could likely serve a homeostatic role for maintenance of synapses and resistance to stress via JAK1/STAT3 signaling (Chen et al., 2016), while also suppressing apoptosis in response to injury (Chen et al., 2016; Zhou et al., 2009a, 2009b). Injury-induced IL-10 production by astrocytes presumably also contributes to trophic

influences on injured FMN, and may additionally restrict harmful immune infiltration, as is observed in MS (Cannella & Raine, 1995; Hulshof et al., 2002; Voskuhl et al., 2009). The redundancy of both neuronal and astrocytic production of IL-10 after FNA means that neither source is strictly necessary for FMN survival, as compensation can occur when production by one of these sources is lost.

In order for IL-10 to perform these functions, its receptor must also be expressed on the necessary target cells. For a direct trophic role of IL-10 to be efficacious, this means IL-10R must be expressed on neurons, which is supported by the literature (Fouda et al., 2013; Villacampa et al., 2015; Xin et al., 2011). An indirect trophic role could be mediated via IL-10R expression on other FMNuc resident cells, potentially including astrocytes (Xin et al., 2011). CD4+ T cells are required for normal induction of IL-10R expression in the FMNuc after axotomy, bridging the gap between IL-10-mediated and immune-mediated neuroprotection. Importantly, for IL-10 to mediate suppression of potentially harmful immune responses to axotomy, its receptor must also be expressed on infiltrating immune effectors. The failure of IL-10R-deficient CD4+ T cells to confer neuroprotection after FNA supports this hypothesis. Normal IL-10 signaling in T cells results in their tolerance to antigen (Akdis & Blaser, 2001; Akdis et al., 2000; Joss et al., 2000; Taylor et al., 2006); this tolerance can be passed along to other T cells and APCs in a cell contact-dependent and antigen-specific fashion (Chai et al., 1999; Vendetti et al., 2000). Loss of tolerance to self-antigen through loss of IL-10 signaling could potentially result in dis-inhibition of a harmful autoimmune response to nerve injury, given that T cells unresponsive to IL-10 promote a microglial gene signature indicative of enhanced antigen presentation, T cell co-stimulation, and synaptic elimination. These discoveries

contribute to the growing body of knowledge characterizing the mechanisms of immune-mediate neuroprotection and support the use of immunotherapies after nerve injury.



Picture 2: Summary illustration for proposed mechanism of IL-10-mediated neuroprotection.

A) IL-10 function in animal with WT CD4+ T cells. 1) FMN make IL-10 constitutively, astrocytic production of IL-10 is inducible, and microglial production of IL-10 is unknown. 2) The appearance of CD4+ T cells triggers greater induction of central IL-10R expression, possibly potentiating the effects of IL-10 after FNA. 3) The IL-10R must also be expressed on T cells to induce their tolerance to self-antigen. 4) Tolerized T cells promote benign microglial reactivity. B) When T cells do not express the IL-10R, they promote a microglial gene signature indicative of autoimmunity, which may contribute to the observed failure of these T cells to confer neuroprotection.

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CURRICULUM VITAE

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EDUCATION

2013–2021 (projected)	M.D. Indiana University School of Medicine, Indianapolis, IN
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RESEARCH EXPERIENCE

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2011–2012	Undergraduate research Research advisor: Dr. M. William Rochlin Loyola University, Chicago, IL

MANUSCRIPTS

1. Functional and Anatomical Outcomes of Facial Nerve Injury With Application of Polyethylene Glycol in a Rat Model. Brown BL, Asante TJ, Welch HR, Sandelski MM, Drejet SM, Shah K, **Runge EM**, Shipchandler TZ, Jones KJ, Walker CL. JAMA Facial Plast Surg. 2018 May 17. doi: 10.1001/jamafacial.2018.0308.
2. Impact of peripheral immune status on central molecular responses to facial nerve axotomy. Setter DO, **Runge EM**, Schartz ND, Kennedy FM, Brown BL, McMillan KP, Miller WM, Shah KM, Haulcomb MM, Sanders VM, Jones KJ. Brain Behav Immun. 2018 Feb;68:98-110. doi: 10.1016/j.bbi.2017.10.005.
3. Ephrin-B/EphB signaling is required for normal innervation of lingual gustatory papillae. Treffy RW, Collins D, Hoshino N, Ton S, Katsevman GA, Oleksiak M, **Runge EM**, Cho D, Russo M, Spec A, Gomulka J, Henkemeyer M, Rochlin MW. Dev Neurosci. 2016;38(2):124-38. doi: 10.1159/000444748.
4. Neurotrophin-4 is more potent than brain-derived neurotrophic factor in promoting, attracting and suppressing geniculate ganglion neurite outgrowth. **Runge EM**, Hoshino N, Biehl MJ, Ton S, Rochlin MW. Dev Neurosci. 2012;34(5):389-401. doi: 10.1159/000342996.

ABSTRACTS AND PRESENTATIONS

Oral Presentations

1. The role of IL-10 signaling in CD4+ T cell-mediated neuroprotection after facial motoneuron injury. 2nd Annual Purdue-Indiana University Symposium

on Brain and Spinal Cord Injury Research, 24 September 2018, West Lafayette, IN

2. The role of IL-10 signaling in CD4+ T cell-mediated neuroprotection after facial nerve injury. 2018 Midwest Motoneuron Consortium, 22 September 2018, Indianapolis, IN
3. CD4+ T cell-mediated neuroprotection and the role of IL-10 after facial motoneuron injury. Richard L. Roudebush VA Research Seminar, 28 February 2018, Indianapolis, IN
4. The role of IL-10 in T cell-mediated neuroprotection. Indiana University Medical Scientist Training Program Student Seminar, 23 March 2017, Indianapolis, IN

Poster Presentations

1. Cellular sources and immune regulation of a neuroprotective interleukin-10 signaling pathway in the facial motor nucleus after axotomy. Runge EM, Setter DO, Iyer AK, Kennedy FM, Regele EJ, Sanders VM, Jones KJ. 8th Annual Les Turner ALS Symposium, November 2018, Chicago, IL
2. Cellular sources and immune regulation of a neuroprotective interleukin-10 signaling pathway in the facial motor nucleus after axotomy. Runge EM, Setter DO, Iyer AK, Kennedy FM, Sanders VM, Jones KJ. Society for Neuroscience Annual Meeting 2018, San Diego, CA
3. CD4+ T cells must respond to neuroprotective IL-10 to mediate motoneuron survival after facial nerve axotomy. Runge EM, Setter DO, Regele EJ, Kennedy

FM, Sanders VM, Jones KJ. Anatomy Fall Research Forum, October 2018, Indianapolis, IN

4. A CD4+ T cell-dependent IL-10 signaling pathway is neuroprotective after facial motoneuron injury. Runge EM, Setter DO, Regele EJ, Kennedy FM, Sanders VM, Jones KJ. 2018 Midwest Motoneuron Consortium, September 2018, Indianapolis, IN
5. The role of IL-10 in CD4+ T cell-mediated neuroprotection after facial nerve injury. Runge EM, Setter DO, Kennedy FM, Iyer AK, Regele EJ, Sanders VM, Jones KJ. 2018 Indiana CTSI Annual Meeting, September 2018, Indianapolis, IN
6. Neuroprotective IL-10 is made by multiple central sources and signals via a T cell-regulated pathway after axotomy. Runge EM, Setter DO, Iyer AK, Kennedy FM, Sanders VM, Jones KJ. The 33rd MD/PhD National Student Conference, July 2018, Keystone, CO
7. Dynamics of interleukin-10 signaling in the facial motor nucleus after axotomy. Runge EM, Setter DO, Iyer AK, Kennedy FM, Sanders VM, Jones KJ. 2018 Scientific Spring Symposia hosted by the MODEL-AD Center and the IADC, Indianapolis, IN
8. Interleukin-10 expression in the facial motor nucleus: Roles for motoneuron survival after axotomy. Runge EM, Setter DO, Iyer AK, Kennedy FM, Sanders VM, Jones KJ. 2017 IU/PU Joint Symposium on Spinal Cord & Brain Injury Research, Indianapolis, IN

9. Interleukin-10 expression in the facial motor nucleus: Roles for motoneuron survival after axotomy. Runge EM, Setter DO, Kennedy FM, Sanders VM, Jones KJ. Society for Neuroscience Annual Meeting 2017, Washington, D.C.
10. Benefits of combinatorial therapies for improving functional recovery in a rat model of facial nerve injury. Runge EM, Brown BL, Welch HR, Muldoon JL, Martinez D, Drejet SM, Asante TJ, Best AR, Walker CL, Jones KJ. Experimental Biology 2017, Chicago, IL
11. Benefits of combinatorial therapies for improving functional recovery in a rat model of facial nerve injury. Runge EM, Brown BL, Welch HR, Muldoon JL, Martinez D, Drejet SM, Asante TJ, Best AR, Walker CL, Jones KJ. American Physician Scientists Association Annual Meeting 2017, Chicago, IL
12. Benefits of combinatorial therapies for improving functional recovery in a rat model of facial nerve injury. Runge EM, Drejet SM, Brown BL, Asante TJ, Welch HR, Walker CL, Best AR, Muldoon JL, Jones KJ. Society for Neuroscience Annual Meeting 2016, San Diego, CA
13. Neurotrophin-4 is more potent than brain-derived neurotrophic factor in promoting, attracting and suppressing geniculate ganglion neurite outgrowth. Runge EM, Hoshino N, Biehl MJ, Ton S, Rochlin MW. Biology Department Fall Poster Fair 2012, Loyola University, Chicago, IL
14. Blocking the p75 receptor inhibits neurite outgrowth in developing geniculate and posterior trigeminal ganglia. Runge EM, Rochlin MW. Biology Department Summer Poster Fair 2011, Loyola University, Chicago, IL

GRANT SUPPORT, HONORS, AND AWARDS

2018–2019	TL1 Predoctoral Award, Indiana Clinical and Translational Sciences Institute, Grant Numbers TL1 TR002531 and UL1 TR002529 (A. Shekhar, PI)
2016	Graduate Student Travel Award, Indiana University School of Medicine
2014–2016	Ruth L. Kirschstein Institutional National Research Service Award (T32) Appointee, Indiana University-Purdue University at Indianapolis
2013	University Fellowship, Indiana University School of Medicine
2013	Biology Honors Award, Loyola University
2011, 2012	Summer Biology Research Fellowship, Loyola University
2009–2012	National Merit Scholarship
2009–2012	Presidential Scholarship, Loyola University

TEACHING EXPERIENCE

2018	Co-instructor, *Principles of Neuroimmunology* Indiana University School of Medicine, Indianapolis, IN
2016–2017	Teaching assistant, *Neuroscience and Clinical Neurology* Indiana University School of Medicine, Indianapolis, IN
2015	Tutor, *Medical Physiology* Indiana University School of Medicine, Indianapolis, IN
2012–2013	Course instructor, *Biology for the MCAT* The Princeton Review, Chicago, IL

LEADERSHIP

2018–Present	American Physician Scientists Association Undergraduate Mentorship Program
2015–Present	Combined Degree Student Council Representative Indiana University School of Medicine, Indianapolis, IN

PROFESSIONAL MEMBERSHIPS

2018–Present	American Academy of Physical Medicine and Rehabilitation
2018–Present	American Academy of Neurology
2017–Present	American Association of Anatomists
2015–Present	Society for Neuroscience
2013–Present	American Physician Scientists Association
2011	Alpha Epsilon Delta, Loyola University
2010–2011	American Medical Student Association, Loyola University