T CELL-MEDIATED NEUROPROTECTION IN THE INJURED CENTRAL NERVOUS SYSTEM: GAINING INSIGHT WITH THE FACIAL NERVE AXOTOMY MODEL

By

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To my dad, whose fleeting presence in my life imparted everlasting moments of laughter and comfort. And to my mom, who courageously fulfilled the unexpected role of "dad."

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LIST OF ABBREVIATIONS

CNS	Central nervous system
BBB	Blood brain barrier
FMN	Facial motor nucleus
IL-15	Interleukin-15
MHC	Major histocompatibility complex
NGS	Normal goat serum
NO	Nitric oxide
PBS	Phosphate buffered saline
PF	Paraformaldehyde
RAG-2 KO	Recombinase activating gene-2 knockout
scid	Severe combined immunodeficiency

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T CELL-MEDIATED NEUROPROTECTION IN THE INJURED CENTRAL NERVOUS SYSTEM: GAINING INSIGHT WITH THE FACIAL NERVE AXOTOMY MODEL

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Although T cells aid in maintaining homeostasis of the normal central nervous system (CNS), the significant trafficking of T cells to the CNS under conditions of pathology, infection, or injury presents a dichotomy. The presence of T cells in the CNS under certain contexts is considered detrimental, where T cells have been shown to cause or exacerbate neuropathology. Under certain experimentally-induced conditions, however, T cells have been shown to confer neuroprotection. In the interest of developing immune-based strategies to treat neurodegenerative disease or CNS injury, it is important to understand the conditions that drive T cells to act in a bi-directional manner in the CNS. To begin to address this issue, we used the well-characterized facial nerve axotomy model to study the interactions between T cells, microglia, and injured neurons. We demonstrated that the level of neuronal death can influence the magnitude and rate of T cell accumulation to the injured CNS. Moreover, T cells that are exposed to neuronal injury in early adulthood possess the ability to exhibit memory and increase their responsiveness to the same form of injury induced later in life. Although we found that the presence of T cell memory in the injured FMN was associated with modest effects on functional recovery, there was no obvious impact on measures of neuronal survival. The lack of effect on neuronal survival may have been due to the inability to detect a population of neurons that have

been shown in previous studies to undergo severe atrophy and shrinkage following axotomy. It was also shown in those studies that the atrophic neurons possess the capacity to regenerate (i.e., reverse atrophy as demonstrated by the increase in neuronal cell number and size) following nerve re-injury. Using this nerve re-injury model, we found that immunodeficiency impaired the regenerative response of injured facial motor neurons but that T cells were not associated with the reversal of atrophy in wild-type mice. We propose that T cells respond to the neuronal death induced by injury and aid in promoting the long-term survival of the surrounding neurons by maintaining them in an atrophied state where they can be prompted to regenerate. Our findings provide intriguing information regarding the impact of T cells on the status of injured neurons, which may have important implications for the future development of treatment strategies that could aid in the prolonged survival of the neuron following injury.

CHAPTER 1
INTRODUCTION TO THE IMMUNE SURVEILLANCE IN THE CNS

Immune Privilege in the CNS

The concept of "immune privilege" in the central nervous system (CNS) once referred to the failure of the CNS to initiate adaptive immune responses. This concept was derived from the inability of the CNS to 1) form lymph organs allowing for lymphatic drainage of CNS antigen to peripheral immune organs, 2) express detectable levels of major histocompatibility complex (MHC), a molecule required for T cells to recognize their cognate antigen, and 3) reject grafted foreign tissue (Carson et al., 2006). Moreover, the presence of a blood brain barrier (BBB), a structural boundary composed of endothelial cells separated by tight junctions, restricts the passage of blood-borne molecules to nutrients necessary for CNS maintenance while facilitating the transport of metabolites and excitatory neurotransmitters considered toxic out of the CNS and was thought to prevent peripheral immune cell entry to the CNS (Han and Suk, 2005; Hickey et al., 1991; Ohtsuki, 2004).

It is now appreciated that CNS immune privilege refers not to the absolute exclusion of peripheral immune responses but rather to unique mechanisms that protect the complex and delicate tissue of the CNS from the intrinsic consequences of inflammatory reactions (Carson et al., 2006). The brain and spinal cord, confined within a an inelastic skull and vertebral column, respectively, have limited capacity to tolerate the swelling that is associated with inflammation Moreover, inflammation in the CNS can induce neuronal cell death and regeneration. Thus, the removal and/or addition of neurons that are part of critical circuitry may be detrimental. The BBB is restrictive but not impermeable to the entry of T lymphocytes even under nonpathological conditions (Cose et al., 2006; Hickey et al., 1991). The presence of perivascular macrophages at the BBB interface, however, perform immune regulatory functions that serve as

a primary point of regulation, providing a hostile environment for pathogens as well as for peripheral immune cells (Aloisi, 2001; Williams et al., 2001). Moreover, the constitutive expression of MHC proteins in the CNS remains low under normal conditions thus further restricting the initiation of autoimmune responses but can be upregulated following injury, with aging, and also during specific times during neurodevelopment (Boulanger, 2004; Corriveau et al., 1998; Goddard et al., 2007). While the random circulation of T cells in the normal CNS occurs at low levels, the persistence and accumulation of T cells in the compromised CNS occurs when T cells detect their cognate MHC molecule expressed on an antigen presenting cell (APC) (Hickey et al., 1991).

Following extravasation to the CNS parenchyma, T cells have the potential to interact with a variety of cells. Astrocytes and microglia have been been shown to produce various cytokines that can impact T cell function and activation (Aloisi et al., 2000). The two glial cell types differ, however, in their ability to function as APCs, with microglia being more efficient in their capacity to present antigen. Microglia, the resident immune cells of the CNS, phagocytose foreign pathogens and neuronal debris. Moreover, MHC molecules that are constitutively expressed by microglia can be upregulated following CNS insult or infection. (Kreutzberg, 1996). Conversely, T cells can regulate the function of microglia by promoting their activated phenotype (Aloisi et al., 2000). The recent finding that neurons possess the ability to upregulate the expression of T cell regulatory molecules *in vitro* suggests that T cells may also directly interact with neurons (Liu et al., 2006).

Context-Dependent Effects of T Cells in the Compromised CNS

Although T cells have been shown to maintain CNS health under normal conditions, their presence can exert effects that are both detrimental and beneficial in the compromised CNS. It is well-agreed upon that the presence of T cells in CNS can lead to deleterious effects, resulting in

the demyelination, axonal damage, and neuronal loss that has been seen in some forms of CNS disease and injury (). Under certain experimentally-induced conditions, however, T cells have been shown to confer neuroprotection to injured neurons. Studies across various animal models of injury have demonstrated the potential of T cells to promote neuronal survival, remyelination, and functional recovery, regardless of the status of the BBB (Bieber et al., 2003; Hofstetter et al., 2003; Schwartz and Hauben, 2002; Serpe et al., 2000; Serpe et al., 2002).

Despite these findings, there remain discrepancies regarding the ability of T cells to exert neuroprotection. The finding by Hauben et al. that passive and active immunization to CNS antigen improves recovery following spinal cord injury is equivocal, with a different study demonstrating adverse effects following spinal cord contusion using similar immunization protocols (Hauben et al., 2000; Jones et al., 2004). Moreover, T cells appear to worsen acute damage following aseptic cerebral injury, as indicated by the greater extent of tissue damage and increased apoptosis seen in recombinase activating gene-2 knockout (RAG-2 KO) mice lacking mature, functional T and B cells (Fee et al., 2003). Whether the apoptosis was specific to neuronal or glial cells was not examined. Taken together, these studies clearly demonstrate the need to define the conditions in the CNS that drive T cells to act in a positive vs. negative manner.

Facial Nerve Axotomy Model

The well-characterized facial nerve axotomy model is commonly used to study the molecular mechanisms underlying neuronal and axonal regeneration following peripheral nerve injury. The facial nerve projects from the motor neurons of the facial motor nucleus (FMN) to the facial musculature and controls various functions, including the eyeblink reflex and whisker response (Moran and Graeber, 2004; Serpe et al., 2002). The location of the facial nerve in the rodent model and a schematic of the facial nerve axotomy model is shown in Figure 1-1A and 1-

1B, respectively. Peripheral nerve injury induces a retrograde response that results in various cellular and molecular changes affecting the neuronal cell bodies (Makwana and Raivich, 2005). These changes are thought to occur as part of the regeneration program of the neuron and may help to maintain its survival following injury (Moran and Graeber, 2004).

The experimental virtues of the facial nerve axotomy model are manifold. Because the facial nerve is injured at its exit from the stylomastoid foramen, the BBB remains intact, thus preventing the trafficking of non-specific cells into the brain. Moreover, conditions of regeneration and degeneration can be examined in the same type of lesion model by inducing injuries of varying degrees of severity. Nerve crush injury, the mildest form of injury, results in the regeneration of damaged axons within an intact neural sheath intact. Sheer transection of the nerve results in an intermediate form of injury and variable rates of nerve regeneration (Moran and Graeber, 2004; Raivich et al., 2004). Nerve resection, considered the most severe form of nerve injury, involves removing a portion of the nerve to prevent reconnection and produces profound neuronal loss and atrophy. Finally, as will be discussed in detail below, a notable feature of the model is the site-specific microglial activation and T cell trafficking that occurs in the injured FMN. The close physical apposition of the various cells of interest – T cells, microglia, and neurons – renders the model ideal for studying their interactions.

Neuroimmune Response in the Injured FMN

One of the earliest changes to occur in the FMN following nerve axotomy is the activation and proliferation of microglia. Changes in microglial reactivity occur as early as one day following axotomy, where the highly ramified morphology of resting microglia become stout and deramified and with the peak of proliferation occurring at 3 days post-axotomy (Moller et al., 1996; Raivich et al., 1999). Moreover, microglial activation is accompanied by an upregulation of MHC I expression (Raivich et al., 1998). Over time, the presence of neuronal

death in the FMN following axotomy results in the formation of microglial phagocytic clusters, which represent a dead or dying neuron being engulfed by group of microglial cells and have been used as an indirect marker of neuronal death. The peak rate of the neuronal death response in the FMN following axotomy has been shown to occur at 14 days. In addition to producing a variety of factors that can be toxic to neurons, including immune complement proteins, and nitric oxide (NO), microglia have been shown to express a number of factors that may be essential for neuronal survival (Banati et al., 1993; Batchelor et al., 1999; Chamak et al., 1994; Giulian, 1999).

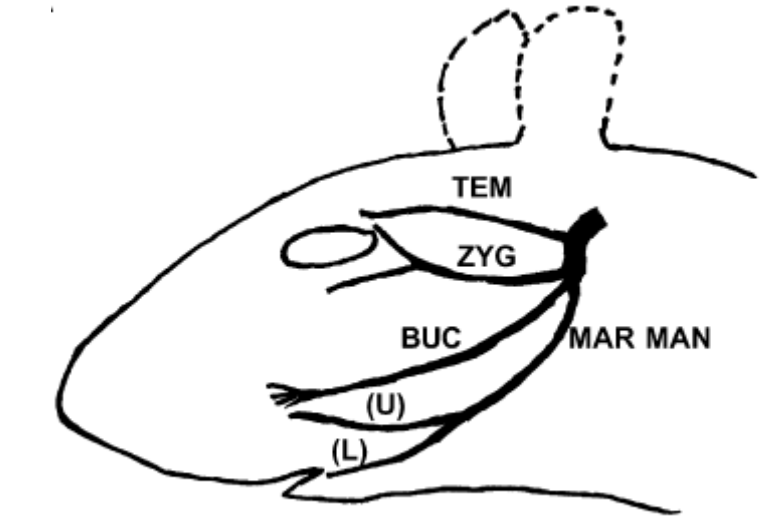
In a landmark study, Raivich and colleagues demonstrated that T cells enter brain parenchyma and specifically traffic to the site of the injured FMN following nerve axotomy (Raivich et al., 1998). Notably, the trafficking of T cells to the injured FMN occurred in the presence of an intact BBB. The initial phase of T cell infiltration between 2-4 days post-axotomy was followed by a more intense phase that peaked at 14 days post-axotomy. The two phases of infiltration appear to be mediated by specific cytokines, where interleukin-6 (IL-6), macrophage colony-stimulating factor (MCSF) is thought to mediate the initial antigen-independent response to axotomy and interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) affect the later, antigen-independent phase (Galiano et al., 2001; Raivich et al., 2003; Raivich et al., 1998; Streit et al., 1998). The trafficking of T cells to the injured FMN is a species-dependent phenomenon, as the T cell response following axotomy is more prominent in mice compared to rats. Interestingly, these differences may be attributed to the extent of neuronal loss that occurs as a result of axotomy in these two species, where mice generally exhibit greater neuronal loss compared to rats (Moran and Graeber, 2004).

Role of T Cells Following Facial Nerve Axotomy

T cells have been shown to prevent the initial neuronal loss or slow the rate of neurodegeneration in the FMN following facial nerve axotomy (Serpe et al., 1999; Serpe et al., 2000). It was shown that severe combined immunodeficient (*scid*), which lack functionally mature T and B cells, exhibited a greater rate of neuronal loss following facial nerve transection than wild-type mice and that immune reconstitution of *scid* mice by adoptive transfer of normal splenocytes restored the rate of neuronal loss to wild-type levels. Interestingly, by 10 weeks post-axotomy, the latest time point examined in that study, the levels of neuronal survival appeared to converge between the two groups of mice, suggesting that the ability of T cells to rescue neurons was transient. Similarly, it was shown in a different study that *scid* mice exhibited greater neuronal death, as demonstrated by the increased number of microglial phagocytic clusters, in the injured FMN than wild-type mice at 14 days post-transection (Petitto et al., 2003). Additionally, functional recovery of the whisker response was delayed in *scid* mice following facial nerve crush injury (Serpe et al., 2002). That immunodeficiency exacerbates neuronal survival was confirmed in recombinase activating gene-2 (RAG-2 KO) mice, a similar mouse model deficient in peripheral T and B cells (Serpe et al., 2000). Subsequent studies suggest that interactions between $CD4^+$ T cells and $MHC2^+$ microglia are important in conferring neuroprotection in the injured FMN (Byram et al., 2004).

By comparison, there was no clear association between endogenous T cell responses and measures of neuronal survival and functional recovery following facial nerve axotomy in other studies. Studies in transgenic mice with marked decreases in T cell trafficking to the injured FMN resulted in no changes in the level of neuronal survival or in the rate of nerve regeneration (Galiano et al., 2001; Kalla et al., 2001; Raivich et al., 2003; Raivich et al., 2002; Werner et al., 2001). Moreover, despite marked decreases in T cell trafficking to the injured FMN in

interleukin-15 knockout (IL-15 KO) mice compared to wild-type mice, neuronal death levels were comparable (Huang et al., 2007). Notably, Ankeny and Popovich (2007) showed that immunization with T cells primed to either CNS or non-CNS antigen prior to facial nerve transection in mice exacerbated neuronal loss. Taken together, these conflicting data suggest that there may be intrinsic differences between T cell responses derived from adoptively transferred cells, cells primed in vitro, and endogenous T cell responses, differences in the physiology of mice completely devoid of peripheral immune cells and those that have some level of endogenous cells, and/or methodological differences (i.e., surgery protocol, background strain of animals).



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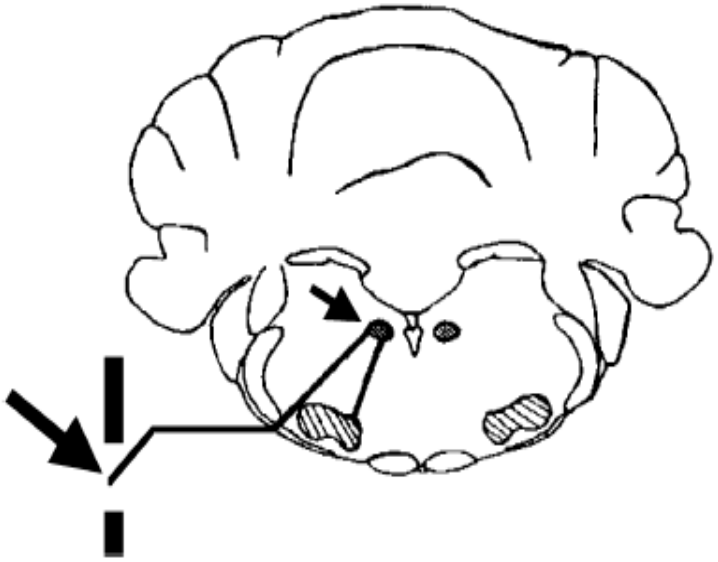

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Figure 1-1. Location of the facial nerve in rodents (A) and schematic of the facial nerve axotomy paradigm (B) (Moran and Graeber, 2004).

CHAPTER 2
MATERIALS AND METHODS

Animals

All animals used in the following studies were cared for in accordance to the NIH Guide for the Care and and Use of Laboratory Animals. Mice were housed in microisolator cages under specific pathogen free conditions. The following strains of mice were used in this study:

- C57BL/6 (Jackson Laboratories, Bar Harbor, ME)
- C57BL/6 (Taconic, Hudson, NY)
- 129 (Taconic)
- B6x129 (F1) (Taconic)
- B6/RAG-2 KO (Taconic)
- 129/RAG-2 KO (Taconic)

Animal Surgery

Animals were anesthetized at a rate of 4% isoflurane, and maintained at a rate of 2% isoflurane. For nerve crush injuries, the main branch of the facial nerve was exposed and micro-tipped forceps were used to apply compression to the nerve for a period of 10 seconds. For nerve transection injuries, the main branch of the facial nerve was exposed and cut. For nerve resection injuries, the main branch of the facial nerve was exposed and a portion of the nerve removed to prevent reconnection. The whisker response was checked immediately following surgery to ensure complete paralysis.

For the double injury paradigm described in Chapter 4, the right facial nerve was exposed and transected in sensitized mice while naive mice received a sham transection (nerve was exposed but not transected). Sixty-six days following the initial surgery, the left facial nerve was exposed and transected in both groups. In the functional recovery study, the left facial nerve was exposed and crushed 70 days after the initial surgery. Mice were sacrificed at 14 days post-second injury.

For the re-injury paradigm described in Chapters 5 and 6, we compared two groups of mice, referred to as "chronic resection + sham re-injury" and "chronic resection + re-injury." Both groups received a resection of the right facial nerve. Ten weeks later, the right facial nerve was re-exposed and in the "chronic resection + re-injury" group, the neuroma that had formed at the proximal nerve stump was removed. In the "chronic resection + sham re-injury" group, the neuroma remained intact. Both groups were sacrificed at 14 days post-second surgery.

For animal perfusion, mice were anesthetized by intraperitoneal injection of a 0.5 mg/ml ketamine cocktail solution (ketamine/xylazine/acepromazine in a 3:3:1 ratio) and were perfused transcardially with 4% paraformaldehyde (PF, Fisher) or 1 X phosphate buffered saline (PBS). Brains were collected, post-fixed in 4% PF for 2 hours at room temperature, and cryoprotected by immersion in 30% sucrose (Fisher) overnight at 4°C. Following cryoprotection, brains were snapfrozen in isopentane (Fisher) and stored at -80°C.

Light Immunohistochemistry

Using the ambiguus nucleus and the facial nerve root as the starting and ending points, respectively, approximately forty 15um sections were cut throughout the caudal-rostral extent of the facial motor nucleus. Sections were collected on Superfrost/Plus slides (Fisher) and stored at -80°C. For immunohistochemistry, tissue sections were incubated in normal goat serum (NGS) (Vector) for 1 hour at room temperature followed by overnight incubation with the primary antibody of interest at 4°C (Table 1). Prior to incubation with the primary antibodies, CD4 and CD8, saline perfused tissue was post-fixed in zince fixative overnight. Sections were washed in 1 × PBS after each incubation step. Visualization of the primary antibodies was performed by incubation of sections in goat anti-rat secondary antibody (1:2000, Vector Labs) for 1 hour at room temperature followed by incubation in avidin-peroxidase conjugates (1:500, Sigma) for 1

hour. No signal was obtained with each of the primary or secondary antibodies alone. The chromagen reaction was revealed by incubation in 3,3′-diaminobenzidine (DAB)-H_2O_2 solution (Sigma; 0.07% DAB/0.004% H_2O_2). Sections were counterstained with cresyl violet, dehydrated in ascending alcohol washes, cleared in xylenes, and coverslipped.

Table 1. Summary of primary antibodies used.

Antigen	Antibody	Dilution	Cellular IR	Source
CD3	CD3 (17A2, RtPC)	1:500	T lymphocytes	Pharmingen
CD4	CD4 (L3T4, RtPC)	1:500	T lymphocytes microglia	Pharmingen
CD8	CD8 (H35-17.2, RtPC)	1:500	T lymphocytes	Pharmingen
CD11b	CD11b (5C6, RtPC)	1:500	Microglia	Serotec
MHC II	I-A/I-E (C-18, RtPC)	1:500	Microglia	Pharmingen
NeuN	NeuN (MsMC)	1:500	Neurons	Chemicon
Rt, Rat; Ms, Mouse; PC, polyclonal; MC, monoclonal				

Nissl stain

Eight representative sections throughout the FMN were stained with cresyl violet (Sigma) following immunohistochemistry. Slides were placed in cresyl violet for 10-20 minutes, rinsed in distilled water, dehydrated in ascending alcohol washes, cleared in xylenes, and coverslipped.

Assessment of Functional Recovery

Mice were scored for the intensity of the whisker response each day following crush injury for 14 days, using a scale ranging from 0-3 that was previously described by Raivich et al. () (Raivich et al., 2004). A score of 0 represents complete paralysis while a score of 3 represents complete recovery. Each mouse was placed in a clear plexiglass box and the whisker response was recorded for a period of 2 minutes using a Panasonic high definition digital camera. Mice were scored each day by 2 independent investigators and the number of days to reach each recovery score was compared between groups.

Image Analysis

Optical density of CD11b staining in the injured FMN was measured in 8 sections per animal using the MCID image analysis software. The average ratio of CD11b staining intensity of the injured to uninjured FMN was calculated for each animal and subject groups were compared using ANOVA.

Quantification and Statistical Analysis

The number of $CD3^+$ T cells or $CD11b^+$ microglial phagocytic clusters was quantified in sections throughout the FMN by an experimenter under blind conditions. Eight sections per mouse (approximately 1/5 of the entire FMN) were used to assess each variable. Mean counts per section were calculated for statistical analysis and analysis of variance (ANOVA) was used to make comparisons between subject groups. Where applicable, Fisher's least significance difference test was used to make pair-wise post-hoc comparisons between groups.

Neuronal survival and neuronal cell size were quantified using ImageJ software (National Institutes of Health). For the assessments of neuronal survival, the number of Nissl-stained neurons in the injured FMN containing a nucleolus that were greater than 20 um in diameter

were counted and expressed as a percentage of the number of neurons in the contralateral, uninjured FMN (% neuronal survival). Neuronal cell size was measured in 3 representative sections of the FMN each spaced 90 μm apart. The medial sub-nucleus, which is innervated by the auricular branch of the facial nerve, remains uninjured and was excluded from our analyses (McPhail et al., 2004).

CHAPTER 3
ENDOGENOUS T CELL AND MICROGLIAL RESPONSE TO FACIAL NERVE AXOTOMY: EFFECT OF GENETIC BACKGROUND AND THE RAG-2 KO GENE

Introduction

Emerging data suggest that injury-induced neuroglial reactivity may be genetically mediated (Lidman et al., 2003; Olsson et al., 2000; Piehl et al., 1999). In response to facial nerve axotomy, microglia are the glial cell type that undergo mitosis in the injured FMN and are thought to play a critical role in motor neuron regeneration (Graeber et al., 1998; Streit, 2002). Robust differences in astroglial reactivity and overall MHC I expression have been described between inbred mouse strains following facial nerve axotomy, where high and low phenotypic responses are seen in C57BL/6 (B6) and 129 mice, respectively (Lidman et al., 2002). Although measures of microglial reactivity were not compared between these high and low reactive inbred mouse strains, other research indicates that microglial responsiveness to facial nerve transection also appears to be influenced by genetic background (Werner et al., 2001).

Several lines of evidence suggest that levels of microglial reactivity induced by facial nerve axotomy may be modulated, in part, by peripheral T cells that migrate to the injured facial motor nucleus. In support of this, we have shown in a previous study that measures of microglial cell reactivity induced by facial nerve axotomy were modified significantly by the presence of T cells in the injured FMN of interleukin-2 knockout mice (Petitto et al., 2003). Interactions between T cells and microglia may be critical in mediating neuroimmunological processes associated with neuronal regeneration in mice (Byram et al., 2004; Moran and Graeber, 2004). Once thought to be detrimental to the CNS, the presence of peripheral T lymphocytes in the CNS have been shown to be neuroprotective following certain types of injury to the brain (Hauben et al., 2000; Moalem et al., 1999). In the mouse facial nerve axotomy model, T cells infiltrate the CNS through an intact blood brain barrier (BBB) and home to affected motoneurons (Moran and

Graeber, 2004; Raivich et al., 1998). T lymphocytes appear to confer neuroprotection upon a selective population of facial motoneurons, as severe combined immunodeficient (*scid*) mice, which lack mature T and B cells, show decreased neuronal survival following nerve injury when compared to wild-types (Jones et al., 2005a; Serpe et al., 1999). Adoptive transfer of functional T cells into *scid* mice restores the neuroregenerative capacity of these animals to the levels of wild-type mice (Serpe et al., 1999; Serpe et al., 2000). Given the degree of neuroprotection conferred by T cells in the injured FMN that was

It was previously demonstrated that B6 and 129 mice exhibited high and low levels of astroglial reactivity and MHC1 expression, respectively, following facial nerve transection (Lidman et al., 2002). In this study, we compared the T cell and microglial response following facial nerve transection in B6 and 129 mice to test the hypothesis that these strains would also exhibit high and low levels of axotomy-induced T cell infiltration in the injured FMN and that these differences would be associated with changes in microglial reactivity (Lidman et al., 2002). Both strains of mice display the H2b haplotype, thus controlling for MHC genetics. To examine these hypotheses, we compared the following axotomy-induced dependent variables in four strains of mice, B6, 129 and recombinase activating gene-2 knockout (RAG2 KO) mice on their respective background strains. In addition, we followed the inheritance pattern of the aforementioned neuroimmune measures in the F1 generation produced by an outcross of B6 and 129 mice. Since RAG-2 KO mice lack mature T and B cells due to their inability to undergo V(D)J recombination, comparisons made between these mice with their respective background controls allowed us to determine if the peripheral response (i.e., T cell trafficking to the injured FMN) modifies the central response (i.e., microglial activity, neuronal death).

Results

Strain-dependent Differences in the T Cell Response to Injured Facial Motor Neurons

As shown in Figure 3-1, the number of T cells in the FMN following facial nerve transection was greater in B6 compared to 129 mice. Because T cell counts in the 129 mice were at zero, accurate variance estimates could not be generated. Thus, T cell counts from these subject groups were not subjected to parametric or nonparametric statistical analysis to avoid violating mathematical assumptions used in these two inferential statistics models. Representative photomicrographs of the T cell response in the injured FMN are shown in Figure 3-2. Note the robust T cell response in the injured FMN of B6 (Figure 3-3A) compared to 129 mice (Figure 3-3B). The lack of T cells in the FMN of 129 mice could not be attributed to a deficient source of peripheral T cells, as $CD3^+$ T cells were present in the spleen (data not shown).

Quantitative counts of $CD3^+$ cells in the injured FMN of the F1 cross are shown in Figure 3-1. Again, because the 129 strain did not display $CD3^+$ T cells, accurate variance estimates could not be assessed for statistical analyses. It is apparent, however, that the level of T cell infiltration in the F1 cross is comparable to that of the C57 mice and markedly different from the 129 mice.

Effect of Strain Differences on the Microglial and Neuronal Death Response to Injured Facial Motor Neurons

To determine whether the T cell response influences microglial reactivity, we compared the number of $MHC2^+$ microglia in the injured FMN between C57 and 129 mice. As shown in Figure 3-3, the 129 mice exhibited significantly greater numbers of $MHC2^+$ microglia than the B6 mice [$F(1,10)=5.561$; $p<0.05$]. The absence of T cells did not impact the level of $MHC2^+$

microglia in either the B6 or 129 strain, as seen in the comparisons between each RAG-2 KO strain and their respective background strain.

We also compared the number of perineuronal $CD11b^+$ microglial phagocytic clusters, an indirect measure of neuronal death, in B6 and 129 mice. As shown in Figure 3-4, significantly higher numbers of phagocytic microglial clusters were present in the injured FMN of B6 mice than in 129 mice [$F(1,10)=14.072$; $p<0.01$]. Image analysis revealed no differences in overall CD11b staining intensity in the ratio of the optical density measure of the injured vs. uninjured FMN between the strains.

Effect of RAG-2 Gene Deletion on the Microglial and Neuronal Death Response to Axotomy

As expected, CD3+ T cells were not detectable in the injured FMN of RAG-2 KO mice on either background (data not shown). As shown in Figure 3-2, the number of $MHC2^+$ microglial counts between the B6-RAG2 KO and the 129-RAG2 KO mice revealed a strain-dependent difference, as 129-RAG2 KO mice possessed significantly higher cell counts than B6-RAG2 KO mice [$F(1,10)=9.461$; $p<0.05$]. Photomicrographs of MHC II^+ microglia in B6-RAG2 KO and 129-RAG2 KO mice are shown in Figures 1G-1H, respectively. A comparison of MHC II^+ microglia in B6-RAG2 KO mice and B6 wild-type mice showed no significant differences (Figures 3-2). Similarly, the number of MHC II+ microglia did not differ between 129-RAG2 KO mice and 129 wild-type mice. Representative photomicrographs are shown in Figure3-3E to Figure 3-3F.

In Figure 3-4, the number of microglial phagocytic clusters was significantly greater in the B6-RAG2 KO mice than in the 129-RAG2 KO mice [$F(1,10)=7.628$; $p<0.020$] but did not differ between the RAG2 KO animals of each strain compared to their respective background controls. Previously we had found that B6-*scid* mice exhibited substantially greater neuronal

loss than B6 mice at 14 days post-axotomy (Petitto et al., 2003). Since we did not observe the expected increased loss of motoneurons in either RAG2 KO strains compared to their respective background controls, we compared B6-RAG1 KO mice with B6 mice to examine whether the effects due to the loss of the RAG-1 gene were similar to those seen with the loss of the RAG-2 gene or the SCID mutation. Quantification of $CD11b^+$ microglial phagocytic clusters between B6- RAG1 KO mice and its wild-type control revealed no significant differences (data not shown).

Discussion

In this study, we observed high and low levels of T cell trafficking to the injured FMN in B6 and 129 mice, respectively. The marked differences T cell infiltration were not correlated with differences in overall microglial activity, as demonstrated by MHC2 expression by microglia and image analysis of CD11b staining intensity. The high and low patterns of T cell infiltration in B6 and 129 mice, respectively, were, however, positively correlated with differences in astroglial reactivity and MHC1 expression previously demonstrated in these mice (Lidman et al., 2002). Since astrocytes play a role in maintaining the BBB and have been implicated in the recruitment of T cells, it is plausible that the increased astrocytic reactivity that was observed previously in C57 mice may allow T cells greater access to the injured FMN.

Contrary to our hypothesis, a greater endogenous T cell response in the axotomized FMN of C57 mice was not associated with less neuronal death. Although C57 mice exhibited more prominent T cell trafficking to the injured FMN compared to the 129 strain, they also exhibited a significant increase in the number of microglial phagocytic clusters compared to the 129 strain. While we did not compare long-term neuronal survival between these two strains of mice, a previous study by Raivich et al. showed that neuronal loss was 30% and 20% in C57 and 129 mice, respectively, at 28 days post-transection. That a greater T cell response was associated

with greater levels of neuronal death and less neuronal survival indicates that T cells may be a response to the degree of neuronal loss. This notion is supported by the observation that facial nerve axotomy generally results in more profound neuronal cell loss in adult mice than rats, which typically exhibit less neuronal loss in the absence of a notable T cell response following facial nerve axotomy (Graeber and Moran, Graeber et al., 1990, Streit and Kreutzberg, 1988). Alternatively, greater neuronal death and subsequent loss may be a result of the increased presence of T cells. Other compensatory mechanisms, such as the increased expression of MHC2 by microglia observed in the 129 mice, may account for the reduced neuronal death levels observed in those mice. Under certain conditions, microglia have been shown to produce brain derived neurotrophic factor (BDNF), a potent survival factor for motor neurons (Batchelor et al., 1999; Serpe et al., 2005).

Interestingly, the absence of peripheral T cells in RAG-2 KO mice of either background strain was not associated with changes in the levels of neuronal death or measures of microglial reactivity, as demonstrated by the number of $MHC2^{+}$ microglia. This finding is in contrast to the studies discussed previously that demonstrated decreased neuronal survival in RAG-2 KO mice following facial nerve axotomy (Serpe et al., 2000). The differential outcomes may reflect methodological differences in assessing neuronal survival and loss, where surviving neurons were quantified by Serpe et al. and dead neurons being cleared by microglia were assessed in the current study.

The identification of the genes involved in regulating the T cell and microglial response following CNS injury may be informative since the interaction between these two events appears to mediate neuroprotection following injury to motor neurons. Moreover, T cells appear to promote the antigen-presenting phenotype of microglia by upregulating the expression of MHC2

on microglia and influencing microglial proliferation (Aloisi et al., 2000, Carson, 2002; Sedgewick et al., 1998). Conversely, microglia have been shown to alter T cell function. Thus, it is important to determine whether those genes regulating the peripheral response (i.e., infiltrating T cells) can influence the central response (i.e., microglial activation, neurodegeneration), which may be influenced by a separate set of genes. To begin addressing this issue, we compared the neuroimmune response in the B6x129 F1 generation with the strain-dependent responses of the parental strains, B6 and 129. We showed that the expression of T cell infiltration and the neuronal death response were dominant phenotypes, as T cell levels in the injured FMN of the F1 generation resembled the B6 strain while levels of microglial phagocytic clusters resembled the 129 strain. By contrast, both parental strains contributed to the phenotype for MHC2 expression by microglia. Similar patterns of inheritance were observed in the F1 generation of two rat strains with demonstrated differences in the neuroimmune response following nerve root avulsion and suggest that the genetic regulation of T cells and microglial MHC2 expression following injury is conserved across species and injury models. Moreover, that the complete absence of T cells in RAG-2 KO mice resulted in no differences in MHC2 expression by microglia or in the neuronal death response when compared to wild-type controls, suggests that the genetic regulation of the central response to injury may be independent of peripheral immune responsiveness.

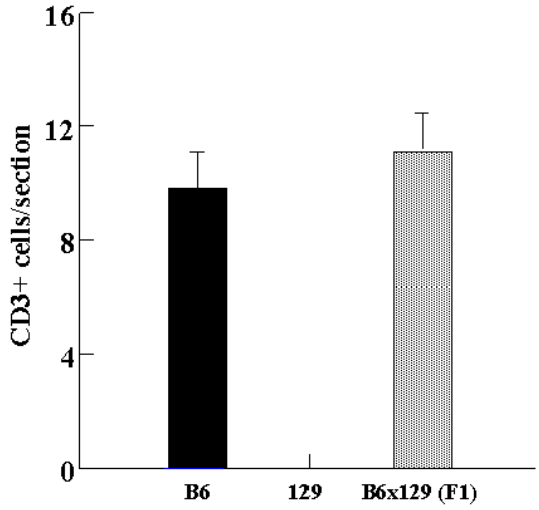

Figure 3-1. Comparison of the number of CD3+ T cells in the injured FMN between B6, 129, and B6x129 (F1) mice. Each bar represents the mean±S.E.M. of 6 mice/group.

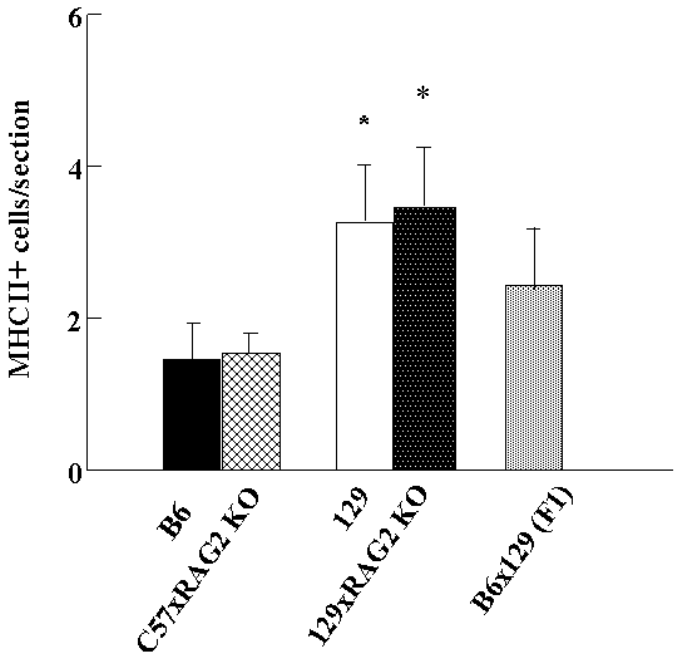

Figure 3-2. Comparison of MHC2+ microglia in the axotomized FMN between subject groups. Each bar represents the mean S.E.M. of 6 mice/group. *$p<0.05$.

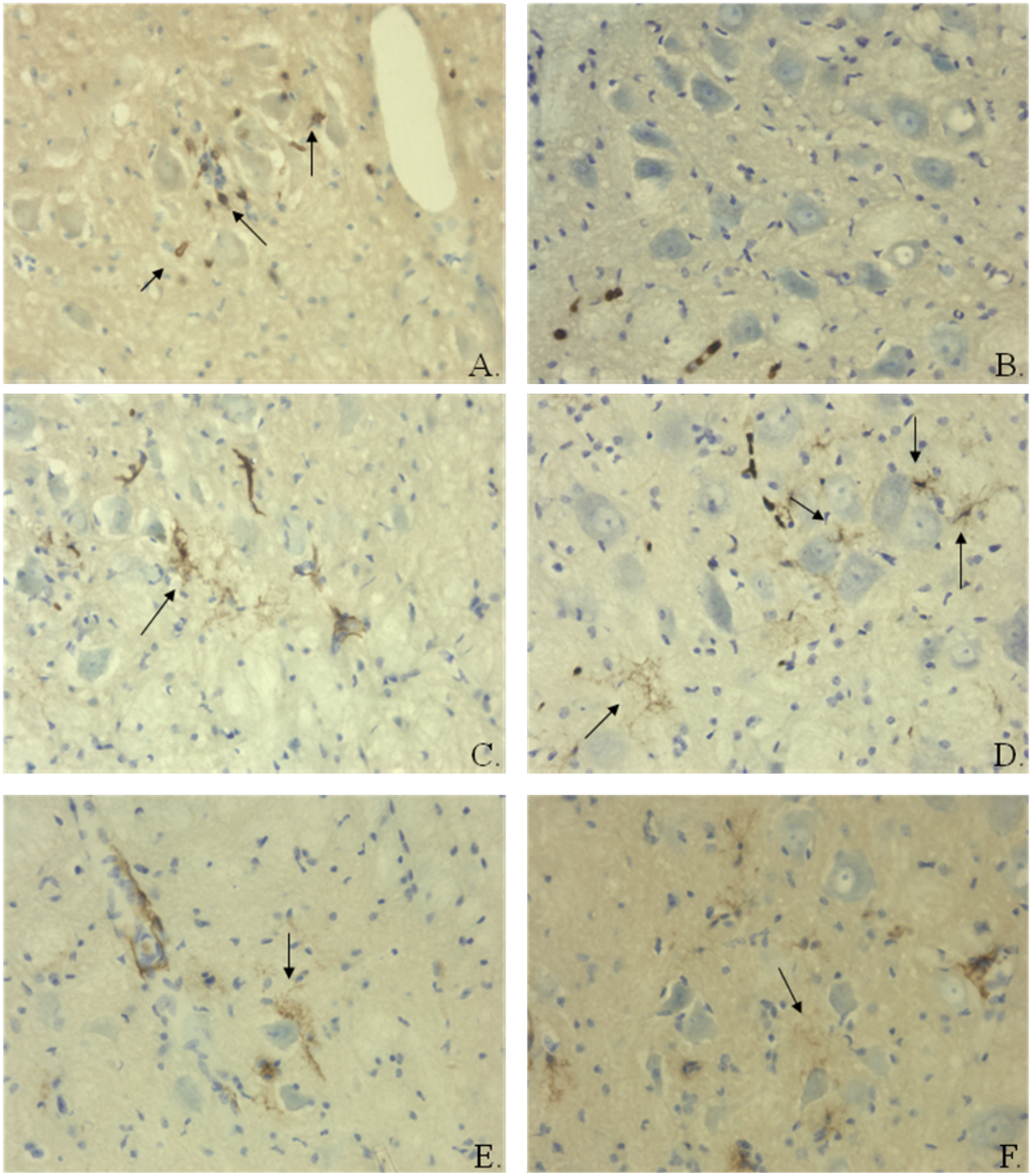

Figure 3-3. Representative photomicrographs of CD3+ T cells and MHC2+ microglia in the FMN of B6, B6/RAG-2 KO, 129, and 129/RAG-2 KO mice 14 days after facial nerve transection. A-H x220.

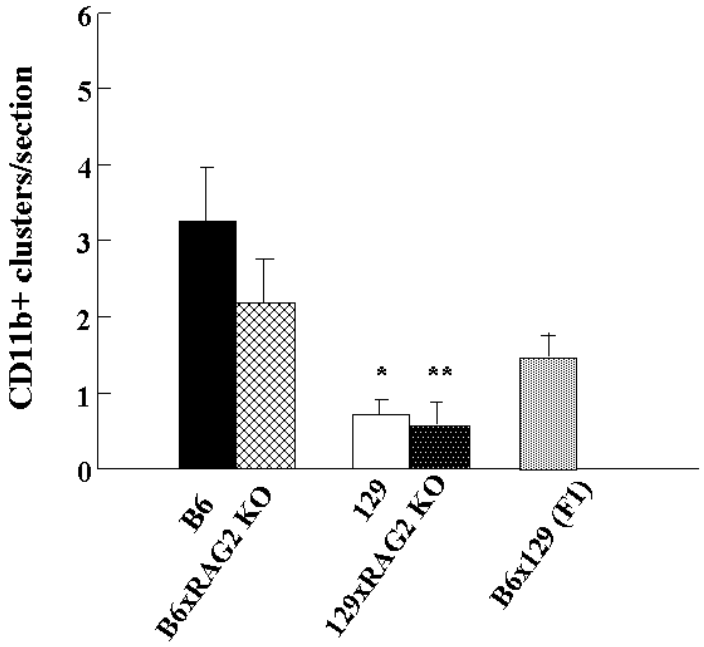

Figure 3-4. Comparison of perineuronal CD11b+ microglial phagocytic clusters in the axotomized FMN between subject groups. Each bar represents the mean S.E.M. of 6 mice/group. *$p<0.01$, **$p<0.05$.

CHAPTER 4
T CELL MEMORY IN THE FACIAL MOTOR NUCLEUS: EFFECT ON NEURONAL SURVIVAL AND FUNCTIONAL RECOVERY FOLLOWING INJURY

Introduction

An important feature of T lymphocytes is their ability to encode long-term memory to a previously encountered antigen such that re-exposure to the antigen results in a response that is greater in magnitude and more rapid (Ahmed and Gray, 1996; Rogers et al., 2000). Although T cell memory responses to pathogens in the periphery are well-characterized, it was unknown whether the immue system of immunologically unmanipuated mice could be sensitized to neuronal injury in the brain and exhibit memory when the same form of injury is induced later in life. Using the facial nerve axotomy model, we tested the hypothesis that previous injury to the FMN on one side of the brain would elicit a more robust T cell response when a second injury is induced in the contralateral FMN later in adulthood. For this study, we compared two groups of mice, referred to here as "sensitized" and "naïve." For the first surgery, the right facial nerve was exposed and the main branch was transected in sensitized mice. In naïve mice, the main branch of the facial nerve was exposed but not transected. Ten weeks later, the contralateral facial nerve was exposed and transected in both groups. The experimental paradigm is depicted in Figure 4-1. We compared sensitized and naïve mice at 14 days post-second injury for differences in the number of 1) $CD3^+$ T cells, 2) perineuronal microglial phagocytic clusters, a measure of neuronal death, and 3) $MHC2^+$ microglia. Since T cells appear to provide neuroprotection to axotomized facial motor neurons under certain conditions in this injury model, a secondary hypothesis we sought to test was that the predicted increase in T cell trafficking to the injured FMN of sensitized mice would be associated with greater levels of neuronal survival. To test this hypothesis, we compared long-term neuronal survival at 49 days following the second injury in a different cohort of sensitized and naïve mice. Neuronal survival

was examined at 49 days post-axotomy in this study because of the substantial loss that has been shown to occur at this time point, allowing us to detect the predicted increase as well as potential decrements in neuronal survival (Serpe et al., 2000).

A second aim of the study was to determine whether T cell memory is associated with improved functional recovery. Because nerve transection results in variable rates of recovery, we used a modified version of the double injury paradigm where the injury was a crush instead of a transection injury. The modified paradigm is shown in Figure 4-6. As discussed in Chapter 1, the facial nerve innervates muscles involved in the whisker and eyeblink response. Functional recovery of the whisker response was assessed by scoring the intensity of the whisker response for each mouse using a scale ranging from 0-3, where 0 represents complete paralysis and a 3 represents full recovery.

Results

Effect of Prior Exposure to Neuronal Injury on the T Cell and Microglial Response to Repeated Injury

To determine whether prior exposure to neuronal injury can elicit a T cell memory response to subsequent injury, we compared the number of $CD3^+$ T cells in the injured FMN of sensitized and naïve mice at 14 days following the second injury, as shown in Figure 4-2A. As mentioned previously, we performed the transection of the contralateral (left) nerve 66 days following the first transection (right nerve) in the sensitized mice, as Raivich et al. (1998) have shown that T cells are cleared from the injured FMN by this time. This finding was confirmed by our lab where we examined animals at 50 days post-axotomy and could not detect T cells in the injured FMN (data not shown). In sensitized mice, there was nearly a two-fold increase in the number of $CD3^+$ T cells in the injured FMN compared to naïve mice [$F(1,30)=12.61$, $p<0.01$]. There was no significant sex effect. Figure 4-3 shows photomicrographs of representative

sections of the FMN following axotomy of the contralateral nerve in naïve and sensitized mice. Note the robust T cell response in sensitized mice compared to naïve mice. By 49 days post-axotomy, the number of T cells in the injured FMN of both subject groups was markedly reduced (1–2 $CD3^+$ T cells/section) with no significant differences in the number of T cells between groups. An occasional T cell was observed in the uninjured FMN (control side) of naïve mice.

To determine whether the increased T cell response observed in sensitized mice results in an increase in microglial activation, we compared the number of $MHC2^+$ microglia in the injured FMN between subject groups at 14 days post-axotomy, as shown in Figure 4-2B. The number of $MHC2^+$ microglia did not significantly differ between subject groups. By day 49 post-axotomy, MHC2 expression on microglia was not apparent in either group. MHC2 positivity could not be detected in the uninjured FMN (control side) of naïve mice. There were no differences in the intensity of CD11b staining in the injured FMN between subject groups (data not shown).

Effect of T Cell Memory in the Injured FMN on the Neuronal Response to Facial Nerve Transection

Measures of neruonal outcome were examined at two distinct time points following axotomy. In Figure 4-4, we compared the number of neurons undergoing cell death, as represented by the number of $CD11b^+$ perineuronal microglial phagocytic clusters in the injured FMN of sensitized and naïve mice at day 14 post-axotomy and showed that the number of dead/dying neurons identified by counting microglial phagocytic clusters did not differ significantly between subject groups. At day 49 post-axotomy, we were able to detect only an occasional neuron undergoing cell death. Microglial phagocytic clusters were undetectable in the uninjured FMN (control side) of naïve mice. NeuN immunoreactivity in the injured FMN also did not differ between subject groups at 14 days post-axotomy (data not shown).

To examine the relationship between T cell memory and long-term neuronal survival, we also quantified the number of Nissl-stained neuronal cell bodies at 49 days following the second surgery in a different cohort of naive and sensitized mice. Since sensitized mice were injured in both FMN, assessments of % neuronal survival (injured vs. uninjured FMN) could not be made. Thus, comparisons were made between the absolute number of neurons/section in the injured FMN of sensitized and naïve mice. Although there was a significant decrease in the number of neurons counted in the injured compared to uninjured side, there were no significant differences in the number of Nissl-stained neurons between sensitized and naïve mice, as shown in Figure 4-5A. Since neurons have been shown to shrink significantly by 11 weeks post-injury, we also assessed the cross-sectional area of motor neurons in three representative sections (each 90um apart) as an additional measure of neuronal status. In Figure 4-5B, the groups did not differ significantly in this measure. Moreover, neuronal cell size in the injured FMN of naïve mice did not decrease significantly compared to the uninjured FMN, suggesting that neurons have not yet begun to shrink at this time point or that nerve reconnection may have occurred.

Effect of T Cell Memory in the Injured FMN on Functional Recovery Following Facial Nerve Crush

In Figure 4-7A, sensitized mice showed an earlier onset of recovery and reached a score of 1 by 3.28±0.18 days compared to 4.63±0.32 days in naïve mice [$F(1,13)=11.92$, $p<0.01$]. There was no significant difference in the number of days for both groups to reach a score of 2 or 3. In Figure 4-7B, the overall rates of recovery for both groups are depicted with differences in the rates of recovery occurring early between 3-4 days post-crush (arrow). Between 6-14 days post-crush, the rates of recovery between groups were comparable.

To determine whether the early onset of modest recovery in sensitized mice was associated with a T cell memory response in the FMN following crush injury, we compared the T cell

response in naive and sensitized mice that reached a score of 3 by day 14. As shown in Figure 4-8A , the number of T cells in the injured FMN following crush injury in naïve and sensitized mice was 3.75±0.90 and 8.30±1.88 T cells/section, respectively [$F(1,13)=5.20$, $p<0.05$]. In Figure 4-8B, the presence of microglial phagocytic clusters was negligible in both subject groups.

Discussion

Our findings suggest that peripheral T cells can encode long-term T cell memory to prior exposure to neuronal injury and respond more robustly to a similar form of CNS injury elicited later in adulthood. Sensitized mice given prior exposure to neuronal injury exhibited nearly a two-fold increase compared to naive mice. Although Raivich demonstrated that the T cell response in the FMN at 3 days post-injury was not affected by previous injury to the contralateral side, the spacing between injuries in that experiment was 11 days (Raivich et al., 1998). By contrast, the injuries in our studies were separated by a period of 10 weeks, allowing for greater potential for T cell sensitization to occur. Though T cell migration into the CNS has been examined more extensively in infection, little is known about whether long-lived T memory cells can be generated to endogenous brain antigens. Interactions between T cells and APCs are required for antigen-specific T cell responses. Whereas naïve T cells appear to interact with antigen presenting cells in lymphoid tissues, antigen experienced T cells exhibit memory that enables them to interact with antigen presenting cells in non-lymphoid tissues that are typically associated with lymphoid organs where T cells first encountered antigen (Campbell and Butcher, 2002; Masopust et al., 2001; Mora et al., 2003; Weninger et al., 2002; Williams and Butcher, 1997). In keeping with what is known from the immunological literature with regards to the facial nerve axotomy model in mice, initial antigen experience of effector T cells may be acquired in the draining cervical lymph nodes, and subsequently MHC2-bearing microglia may

present antigen to experienced T cells at the site of injury in the injured FMN (Byram et al., 2004; Olsson et al., 1992). Given the considerable time period between the injury that induced sensitization and the contralateral injury later in life, long-lived memory T cells may reside in the splenn, rather than the draining lymph node. The vibrissa motor cortex has been shown to send bilateral projections to both facial motor nuclei in rats (Grinevich et al., 2005). Although unlikely, an alternative explanation for the increased T cell response following the second axotomy in the sensitized mice could be attributable to some form of supramotor activation that affected the contralateral facial motor nucleus, and in turn leading to an upregulation of factors (e.g., chemokines, chemoattractive cytokines) responsible for T cell homing to the FMN (Raivich et al., 1998).

Contrary to our initial hypothesis, the presence of T cell memory in the injured FMN of sensitized mice was not associated with changes in the rate of neuronal death at 14 days post-axotomy, as shown by the number of microglial phagocytic clusters, or in the level of neuronal survival at 49 days post-axotomy. Moreover, neuronal cell size, which has been shown to decrease significantly following resection, was not impacted by the presence of increase T cell responses in the injured FMN (McPhail et al., 2004). In fact, we did not detect significant cell shrinkage following transection when compared to neurons in the contralateral, uninjured side. It is possible that the T cell memory response in the sensitized mice, although greater in magnitude, was not sufficient to increase neuronal survival. Alternatively, the number of T cells responding to neuronal injury in naive mice may be sufficient to provide the necessary neuroprotection afforded by infiltrating lymphocytes, and additional cells above such a critical threshold (e.g., levels seen in naïve mice) may not confer added benefit. The findings in this study corroborate with those in Chapter 3 as well as with findings by Raivich et al. in which there was no

association between greater T cell response and improvements in measures of the rate of neuronal death or long-term neuronal survival (2001). A previous study by Yoles et al. showed that the survival of ganglion cells was increased when optic nerve crush injury was preceded by spinal cord contusion injury (2001). It is important to note, however, that experimentally-induced injuries initiated within the CNS, such as that described in the Yoles study, result in significant BBB breakdown, allowing non-specific cells from the periphery to traffic to the CNS (Pan W, 2001, Raivich G, 2004, Schnell, L, 1999). Our studies used a peripheral nerve injury model where T cells have been shown to traffic across an intact BBB (Raivich et al., 1998).

With regards to functional recovery following facial nerve crush, we observed a modest recovery effect following crush injury in sensitized mice that was associated with a two-fold increase in the number of T cells trafficking to the injured FMN. Contrary to our hypothesis, however, the time to full recovery did not differ between subject groups. Although the appearance of weak whisker movement occurred between 3-5 days post-crush in sensitized mice, approximately 1.5 days earlier than what was observed in naive mice, the modest recovery effect was unlikely due to axonal regeneration. Previously, it was shown that whisker reinnervation in the facial nerve crush model is not apparent until 9 days post-crush, which is consistent with the second phase of recovery that we observed in both groups between 8-9 days post-crush (Werner et al., 2001). Moreover, by 4 days post-crush, it was shown that the fastest growing axons at the injured site extended approximately 6-7 mm, a distance that would be unlikely to bridge the gap between the proximal and distal ends of the crushed nerve. Although the spontaneous recovery could have been due to the degeneration-induced release of neurotransmitters at the neuromuscular junction, we speculate that the effect seen at 3 days post-crush may have been due to the early sprouting of spared axons to their target. In studies where fluorogold was

injected distal to the site of injury following facial nerve crush, few fluorogold-positive neurons were observed as early as 3 days post-crush (Kamijo et al., 2003). It was suggested that nerve compressions of different duration and magnitude can produce crush injuries of varying severity, resulting in a mixed population of damaged and spared axons (Kobayashi et al., 2003). While the regeneration of damaged axons may be unlikely at early post-injury time points, the early sprouting of spared axons in the injured FMN is plausible. With the whisker response being crucial for sensory processing in the mouse, the early onset of recovery that we observed in sensitized mice may, for example, be beneficial to their survival in the wild. Moreover, the association between T cell memory and functional recovery may be more significant in severe models of CNS injury, in which spontaneous recovery occurs despite the failure of damaged axons to regenerate (Barritt et al., 2006; Bradbury et al., 2002; Gage et al., 1983a; Gage et al., 1983b).

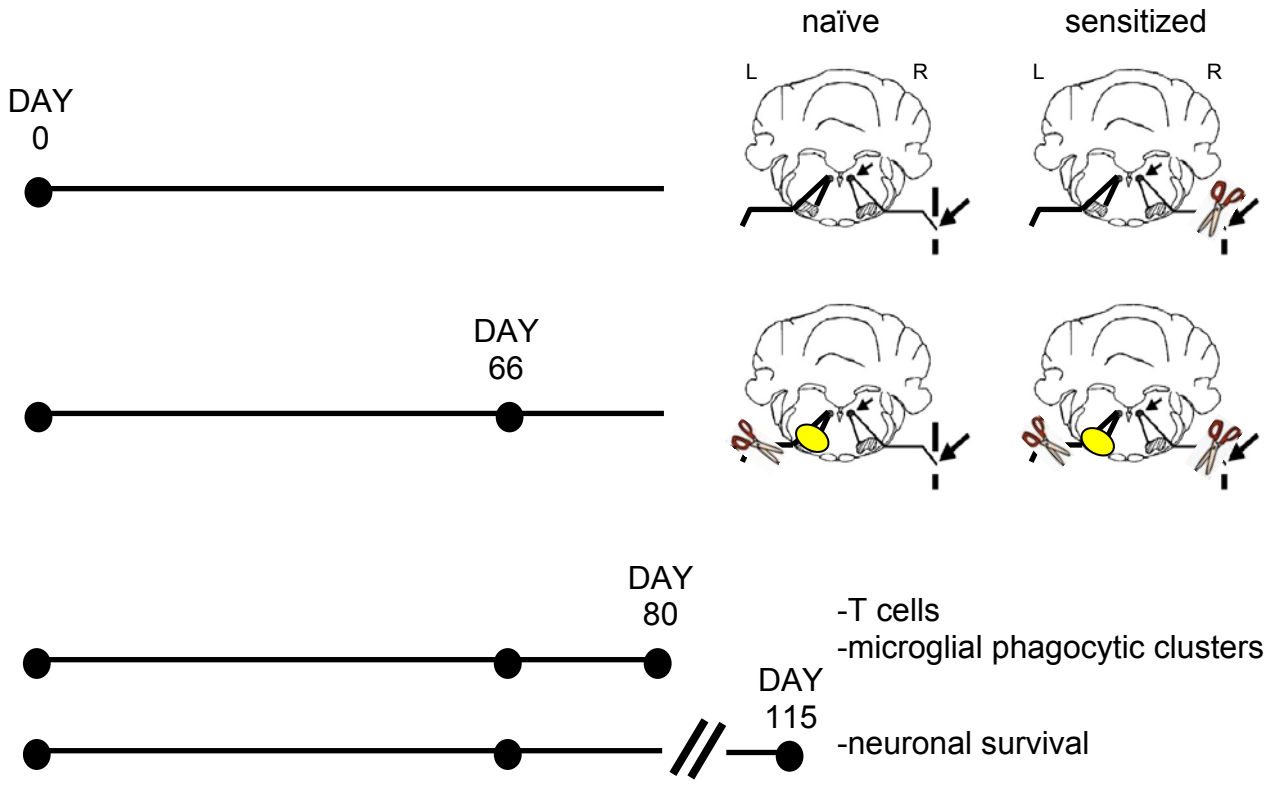

Figure 4-1. Schematic of the double injury paradigm.

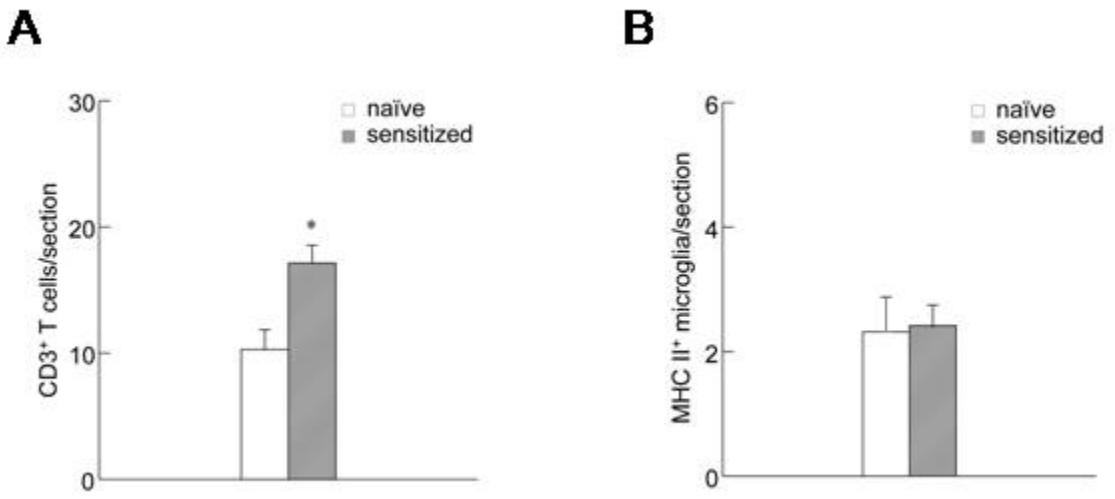

Figure 4-2. Quantification of CD3+ T cells (A) and MHC+ microglia (B) in the injured FMN of naïve and sensitized mice at 14 days post-axotomy. Each bar represents the mean S.E.M. of 15 (naïve) and 17 (sensitized) mice. *p<0.01

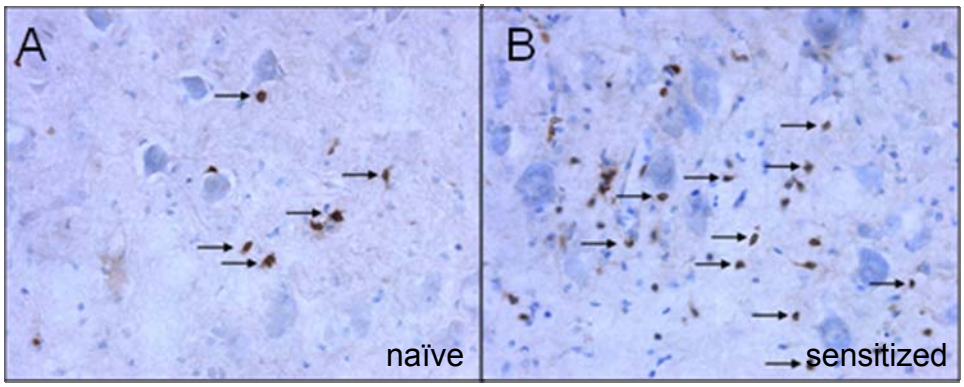

Figure 4-3. Immunohistochemistry for CD3+ T cells in the injured FMN of naïve (A) and sensitized (B) mice. Note the robust T cell response in the injured FMN of sensitized mice compared to naïve mice.

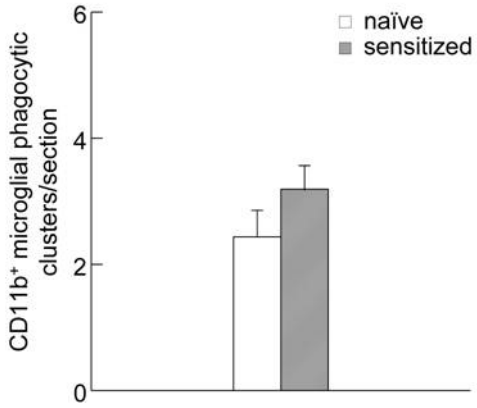

Figure 4-4 Quantification of CD11b+ microglial phagocytic clusters in naïve and sensitized mice at 14 days post-axotomy. Each bar represents the mean S.E.M. of 15 (naïve) and 16 (sensitized) mice.

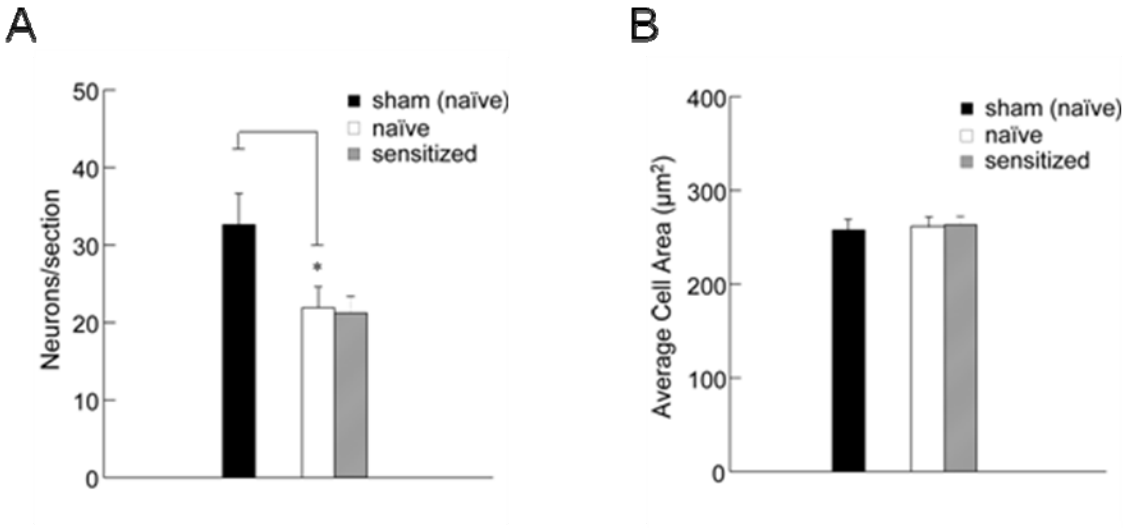

Figure 4-5. Quantitative cell counts (A) and average neuronal cell area (B) in the injured FMN of naïve and sensitized mice at 49 days post-axotomy. Each bar represents the mean S.E.M. of 7 (naïve) and 9 (sensitized) mice. *$p<0.05$

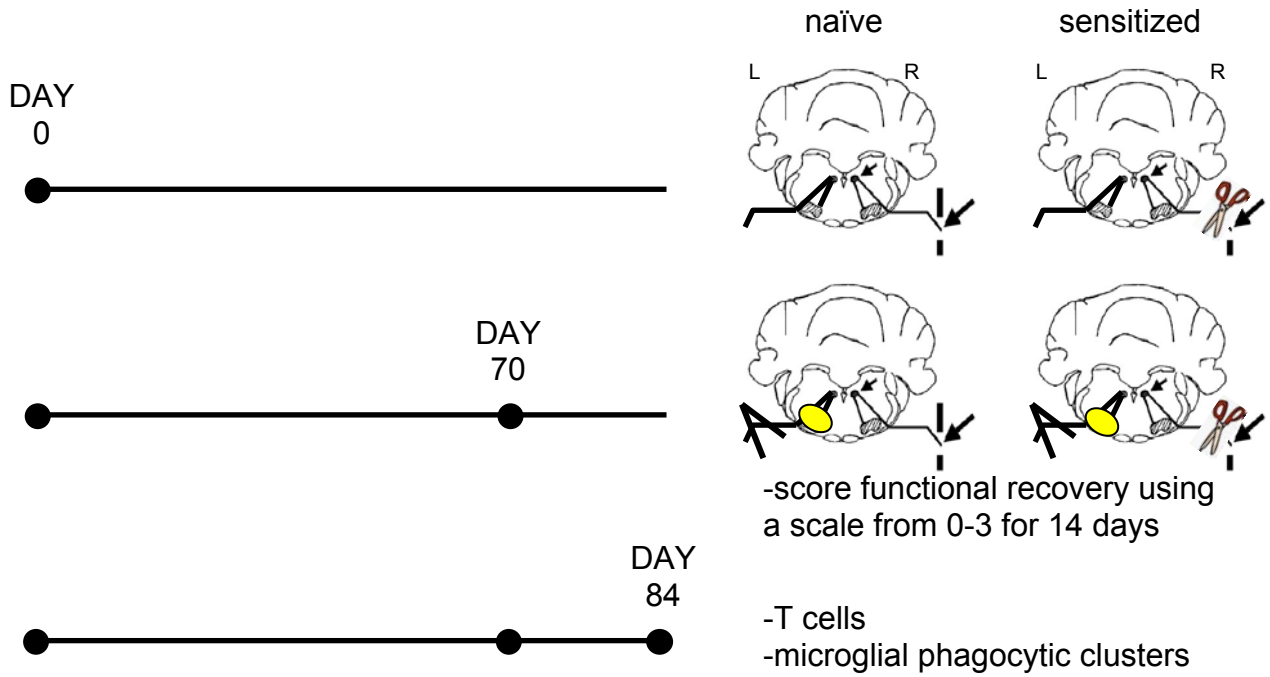

Figure 4-6. Schematic of the modified double injury paradigm.

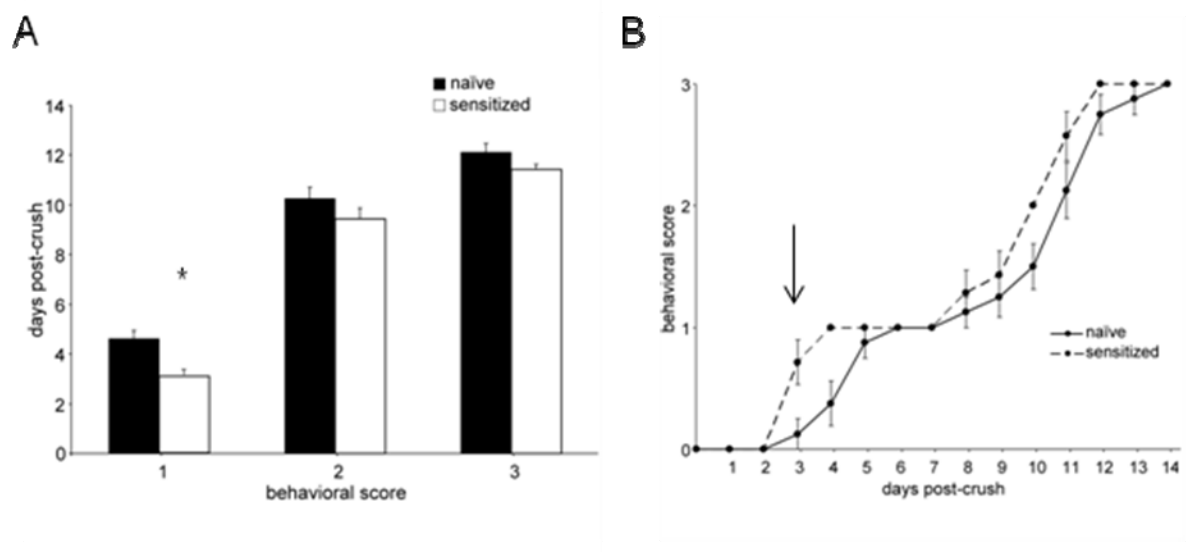

Figure 4-7. Comparison of functional recovery in naive and sensitized mice. The number of days required for each group to reach each behavioral score is depicted in Fig. 2A, where each bar represents the mean S.E.M. of 8 naïve and 7 sensitized mice. In 2B, the overall rates of recovery are shown for naïve (solid) and sensitized (dashed) mice, with differences between groups indicated by the arrow. *p<0.01

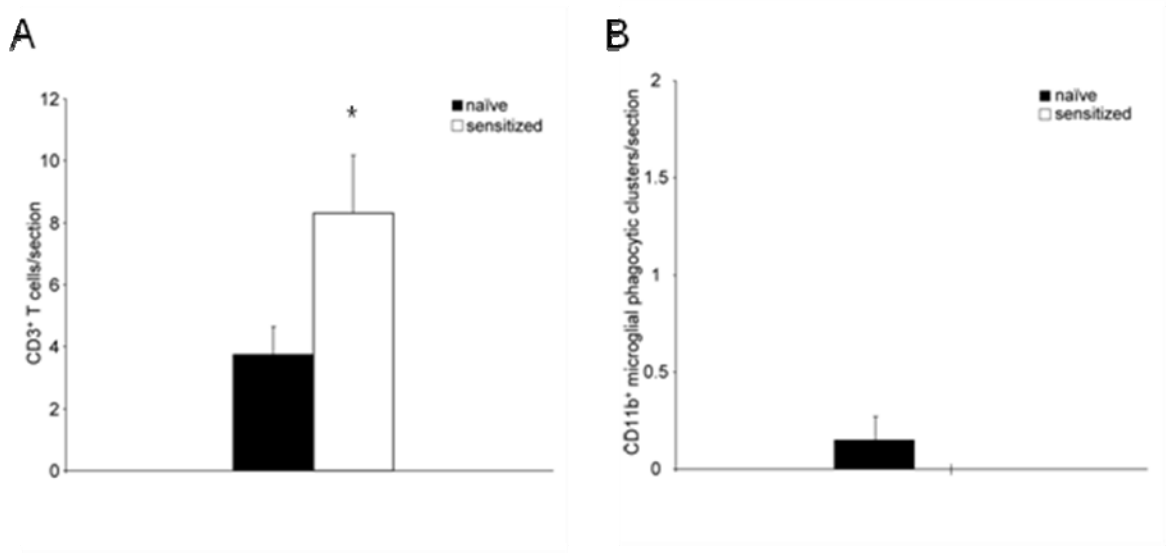

Figure 4-8. Quantification of $CD3^+$ T cells and $CD11b^+$ microglial phagocytic clusters in the injured FMN of naïve and sensitized mice at 14 days post-crush. For A, each bar represents the mean S.E.M. of 7 naïve and 8 sensitized mice. For B, each bar represents the mean S.E.M. of 5 naïve and 3 sensitized mice. *$p<0.05$

CHAPTER 5
EFFECT OF INJURY SEVERITY ON THE RATE AND MAGNITUDE OF THE T CELL AND NEURONAL DEATH RESPONSE FOLLOWING FACIAL NERVE AXOTOMY

Introduction

The effect of T cells in the central nervous system (CNS) appears to be context-dependent, as their presence has been shown to promote neuronal survival following certain types of injuries or, conversely, contribute to CNS pathology, such as in experimental autoimmune encephalomyelitis (EAE) and infection (Byram et al., 2004; Martino and Hartung, 1999; Nau and Bruck, 2002; Schwartz, 2001; Serpe et al., 1999). Peripheral transection of the facial nerve in adult mice induces retrograde neuronal cell loss in the facial motor nucleus (FMN) that is accompanied by a site-specific infiltration of T cells across an intact blood-brain-barrier to the injured motor neurons (Moran and Graeber, 2004; Raivich et al., 1998). Although the trafficking of T cells to the injured FMN has been assumed to depend on injury severity, findings from independent studies are conflicting. While the presence of T cells has been shown to be associated with substantial neuronal death in some studies, others have reported significant T cell trafficking following injury despite minimal neurodegeneration (Galiano et al., 2001; Ha et al., 2007; Ha et al., 2006; Raivich et al., 2003; Raivich et al., 2002). Differences in animal models and methodology used in these studies may contribute to the disparate findings. Understanding the significance of T cells in relation to neuronal death may thus be critical to gain further insight regarding the bi-directional effects of T cells in the injured CNS.

Although the time course of T cell accumulation in the FMN following facial nerve transection had been studied, it was unknown how the T cell response would be altered by other forms of facial nerve axotomy that vary in injury severity (Raivich et al., 1998). Thus, the present study was designed to address whether the degree of neuronal cell death induced by the severity of peripheral nerve axotomy influences the T cell response in the injured CNS. To

address these issues, we used two extreme variations of the facial nerve axotomy model, nerve crush and resection, to compare injuries that are of the same nature (i.e., mechanical lesion) but that differ in their severity. A comparison of the T cell response between these two injuries, which differ considerably in their capacity for nerve regeneration and in the extent of neuronal loss, enabled us to examine T cell trafficking in relation to disparate levels of neuronal degeneration. We hypothesized that the differences in the level of neuronal injury induced by facial nerve crush versus resection would be associated with changes in the rate of accumulation and magnitude of the T cell response in the FMN. Comparisons were made cross time from 1-49 days post-injury in the FMN of C57BL/6 mice given facial nerve crush or resection and the number of $CD3^+$ T cells and $CD11b^+$ microglial phagocytic clusters were quantified. In addition, we assessed the level of neuronal survival to determine whether differences in the level of neuronal death at the various time points reflected cumulative neuronal loss over time.

Results

A Comparison of the Rate and Magnitude of the T Cell and Neuronal Death Response to Facial Nerve Crush and Resection

The time course of the T cell and neuronal response to facial nerve crush and resection is shown in Figure 5-1. Neuronal death (dashed line) in the FMN for both treatment groups was not apparent at day 1 post-injury but increased to less than one microglial phagocytic cluster/section between days 3-7 post-injury. T cell infiltration (solid line) to the FMN in both treatment groups was minimal (<1 T cell/section) by day 1 post-injury and showed comparable increases of 3-4 T cells/section by day 7 post-injury. T cell infiltration in the contralateral, uninjured FMN across all time points was negligible, with an average of less than 0.1 cells per 15 μm section. Differences in the rate of accumulation and magnitude of the T cell response became apparent by 14 days post-injury, when the levels of neuronal death reached their peak in

both treatment groups. The number of $CD11b^+$ microglial phagocytic clusters reached a maximal 0.5/section following facial nerve crush (open circles, dashed line) and coincided with a peak number of 6.5±0.27 $CD3^+$ T cells/section (open circles, solid line). By contrast, facial nerve resection induced a peak number of 2.54±0.45 $CD11b^+$ microglial phagocytic clusters/section (closed circles, dashed line) by 14 days post-injury that was followed by a maximum number of 26.1±2.29 $CD3^+$ T cells/section (closed circles, solid line) one week later at 21 days. Interestingly, there was a second modest increase in the T cell response between 21 and 28 days post-crush, where the average number of T cells rose from 1.63±0.85 cells/section at 21 days to 4.10±1.57 cells/section at 28 days. By 49 days post-injury, the rate of neuronal death was decreased in both treatment groups and the number of T cells in the FMN declined to 0.19±0.06 cells/section (crush) and 2.50±0.78 cells/section (resection). In Figure 5-2A, Spearman's rank correlation coefficient (ρ) revealed that the number of $CD11b^+$ microglial phagocytic clusters was highly correlated with the number of $CD3^+$ T cells when mice from all time points and both treatment groups were combined ($\rho=0.68$, $p<0.01$). Similar significant ρ-values were obtained for nerve crush (Fig. 5-2B, $\rho=0.57$, $p<0.01$) and nerve resection (Fig. 5-2C, $\rho=0.74$, $p<0.01$) alone.

Neuronal Cell Loss Following Facial Nerve Crush and Resection

As seen in Figure 5-3 and as expected, neuronal cell loss at 49 days post-injury was significantly greater in mice that received facial nerve resection compared to nerve crush [$F(1,4)=20.853$, $p<0.05$].

Discussion

The findings in Chapter , demonstrated that the magnitude and rate of T cell accumulation in the injured FMN were influenced by differences in the level of neuronal death induced by facial nerve crush and resection, two forms of mechanical injury to the facial nerve

that result in mild and severe neuronal loss, respectively. Interestingly, T cell and neuronal death responses were comparable in magnitude and followed a similar time course between 1-7 days post-injury, regardless of injury severity. Consistent with our hypothesis, greater T cell trafficking to the injured FMN was associated with higher levels of neuronal death, as demonstrated by the accumulation of microglial phagocytic clusters, between 14-21 days following facial nerve resection. By contrast, facial nerve crush induced minimal neuronal death that was accompanied by fewer numbers of T cells. Although Raivich et al. (1998) found an elevated plateau in the T cell response between 2-4 days that was followed by a more prominent peak at 14 days following facial nerve transection, we were unable to detect an early plateau in the T cell response in either nerve crush or resection injuries, possibly due to the limited number of early time points assessed.

Greater T cell trafficking to the injured FMN was associated with increased neuronal death and cumulative neuronal loss in mice that received facial nerve resection. This finding is consistent with our data in Chapter 3, where greater T cell trafficking to the injured FMN was associated with the strain exhibiting greater neuronal death at 14 days post-injury (Ha et al., 2006)). That greater T cell infiltration to the injured FMN might exacerbate neuronal cell death is unlikely, as we showed in Chapter 4 and others have shown that differences in the magnitude of the T cell response in the injured FMN were not associated with altered levels of neuronal death or long-term neuronal loss (Galiano et al., 2001; Ha et al., 2007; Huang et al., 2007; Raivich et al., 2003; Raivich et al., 2002). Here, we found that the number of T cells correlated with the number of microglial phagocytic clusters, suggesting that the T cell response to neuronal death is a graded event. The mechanisms that underlie this site-specific trafficking of T cells to the injured brain appear to involve the time-dependent expression of cytokines and cell

adhesion molecules by injured neurons and microglial phagocytic clusters (Raivich et al., 1998). The increased presence of microglial phagocytic clusters following facial nerve resection may facilitate the robust T cell response in the injured FMN. However, the correlation between the levels of neuronal death and T cells is reduced by the second increase in the T cell response observed at 28 days post-crush, which occurs in the absence of notable neuronal cell death. Interestingly, a comparison of findings from the current study with that of Raivich et al. (1998) revealed that the number of T cells in the injured FMN following facial nerve transection in C57BL/6 mice, while higher than the response following nerve crush, was more comparable to levels induced by resection. Facial nerve transection results in nerve severing with a potential for reconnection to occur and produces neuronal loss that is considered intermediate to that induced by facial nerve crush and resection (Moran and Graeber, 2004; Raivich et al., 2004). While it would be expected that facial nerve transection would induce a T cell response that is also intermediate in magnitude to that induced by facial nerve crush and resection, it is plausible that the two injuries would result in T cell responses of similar magnitude but of different clearance kinetics, as nerve resection and transection involve mechanical shearing of the neural sheath that differ in the potential for the nerve to reconnect. Future comparisons which include graded degrees of injury may be useful to further characterize the T cell response to neuronal injury.

The differences in the timing of the peak T cell response in relation to the peak of neuronal death between facial nerve crush and resection suggest that parameters of the T cell response may be directed by factors in addition to the level of neuronal cell death. The maximal T cell response coincided with peak levels of neuronal death at 14 days post-crush and is consistent with the time course previously described following nerve transection (Raivich et al.,

1998). By contrast, for facial nerve resection, we found that the maximal T cell response occurred at 21 days post-injury, one week following the peak rate of neuronal death. Moreover, we observed significant T cell infiltration in the injured FMN following facial nerve crush despite the presence of few microglial phagocytic clusters, which is consistent with other findings where T cells have been found to traffic to the injured FMN despite the absence of neuronal death (Raivich et al., 2003). It is interesting to speculate that the time course of the T cell response in the injured FMN may partly be dependent on the ability of the nerve to reconnect. Previously, it was shown that the decreased expression of NeuN in the FMN following facial nerve crush was transient in mice, where the return of NeuN expression coincides with the time when mice generally show a return of whisker function (McPhail et al., 2004b). That this loss of neuronal phenotype failed to return in mice that received facial nerve resection was suggested to be related to the inability of the nerve to reconnect. In the current study, the decline in the T cell response in the injured FMN between 14-21 days post-crush appears to be associated with the time when mice gain functional recovery of the whisker response. By contrast, the persistence of T cells in the injured FMN, as observed in this study for resection and in the transection studies by Raivich et al. (Raivich et al., 2004; Raivich et al., 1998), may be related to the absence of recovery due to permanent nerve disconnection or a prolonged recovery period, as in the case with nerve transection.

We confirmed that the increased rate of neuronal death in resected animals, as demonstrated by the by performing cell counts of Nissl stained neuronal cell bodies at 49 days post-injury, the latest time point assessed. It is important to note that overall neuronal death (as assessed by the number of microglial phagocytic clusters across all time points) correlated with cumulative neuronal loss that occurred long-term, as more cell death observed following facial

nerve resection was associated with greater neuronal loss at 49 days post-resection, compared to nerve crush. Interestingly, the percentage of neurons taken up by phagocytic microglia did not account for the larger percentage of neurons lost at 49 days post-injury. With an average of 1700 neurons counted in the uninjured contralateral FMN of our mice, cumulative neuronal loss by phagocytic microglia was approximately 4% and 16% following nerve crush and resection, respectively, while neuronal loss quantified by Nissl stain at 49 days post-injury was approximately 8% and 58% following nerve crush and nerve resection, respectively. The disparity between the two measures of neuronal loss may be due to insufficient sampling of the number of microglial phagocytic clusters, as all post-injury time points were not accounted for. Alternatively, studies by McPhail et al. (2004a) suggest that following facial nerve resection, the majority of injured neurons may reside in a shrunken and atrophic state, rendering them undetectable by Nissl staining. Re-injuring the facial nerve resulted in a reversal of neuronal atrophy such that the number of countable neurons increased to 79% of the contralateral side. It is noteworthy that their finding translates to an actual loss of only 21%, which more closely matches the loss accounted for by phagocytic microglia in our study and provides further evidence that microglial phagocytic clusters may serve as an informative and conservative marker of neuronal death.

In conclusion, our data demonstrate that the rate of accumulation and magnitude of T cells in the injured FMN is related to the level of neuronal death. Further understanding of the mechanisms by which T cells respond to different forms of neuronal injury may be useful in elucidating their role in CNS degeneration and injury.

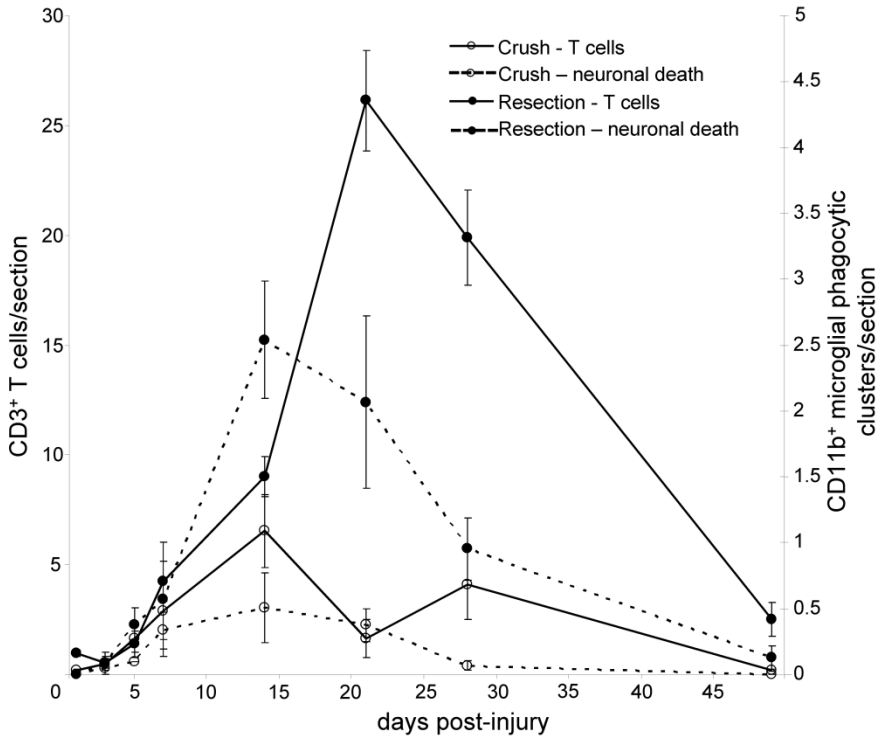

Figure 5-1. Temporal relationship between infiltrating CD3+ T cells (solid lines) and the number of CD11b+ microglial phagocytic clusters (dashed lines) in the FMN following facial nerve resection (●) and crush (○). For the T cell curve, each point represents the mean±S.E.M. of 4 mice/treatment group except for days 5, 7, and 21 in the resection group, where n=3/time point. For the neuronal death curve, each point represents the mean±S.E.M. of 4 mice/treatment group except for day 21 in the crush group (n=2) and days 1, 5, 7, and 21 in the resection group, where n=3/time point.

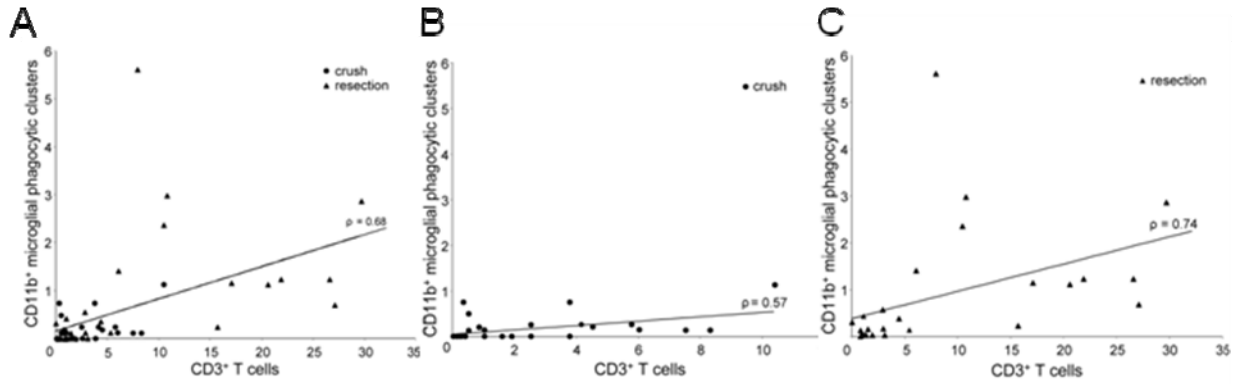

Figure 5-2. Spearman's rank correlation analysis between the number of CD3+ T cells and CD11b+ microglial phagocytic clusters for both treatment groups combined (A), crush only (B), and resection only (C). Each graph represents data pooled across all time points. Spearman's rank correlation coefficients (ρ) are indicated and are significant in all cases ($p<0.01$).

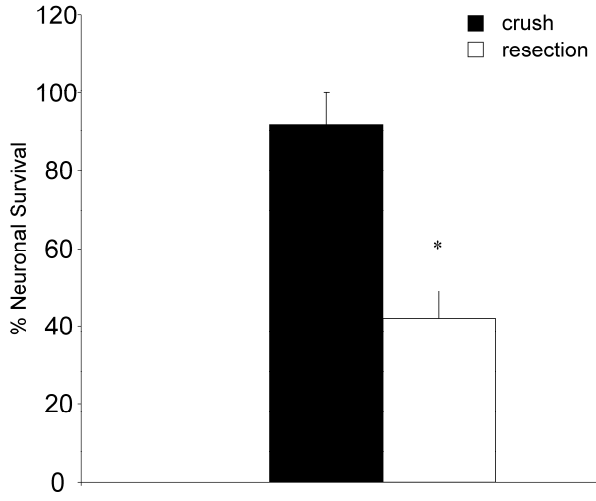

Figure 5-3. Comparison of neuronal survival in the FMN at 49 days following nerve crush and resection. Each bar represents the mean S.E.M. of 3 mice/group. *$p<0.05$

CHAPTER 6
IMMUNODEFICIENCY AND REVERSAL OF NEURONAL ATROPHY: RELATION TO T CELLS AND MICROGLIA

Introduction

Increasing evidence suggests that in some forms of neuronal injury, neurons may not actually die following nerve injury but reside in an atrophic state, characterized by extreme cell shrinkage and a decreased ability to take up Nissl stain (McPhail et al., 2004a). Recent studies by McPhail et al. demonstrated that a population of facial motor neurons undergo a protracted period of degeneration or atrophy following a resection of the facial nerve in adult mice. Re-injuring the facial nerve stimulated a reversal in the atrophic status of the injured neurons, causing an increase in both their size and number. Reversal of neuronal atrophy has also been demonstrated in several different models of CNS nerve injury (Hagg et al., 1989; Kwon et al., 2002). The mechanisms that mediate this regenerative response after a prolonged survival period remain unknown.

Under normal conditions, the CNS is subject to continuous immune surveillance by low numbers of circulating peripheral T lymphocytes (Cose et al., 2006; Hickey et al., 1991). In pathogenic states such as experimental autoimmune encephalomyelitis (EAE) and infection, the presence of T cells in the brain can have detrimental effects (Martino and Hartung, 1999; Nau and Bruck, 2002), while in other contexts, T cells have been shown to act in concert with glial cells to promote neuroregeneration (Byram et al., 2004; Martino and Hartung, 1999; Nau and Bruck, 2002; Raivich et al., 1998; Schwartz, 2003). Studies have demonstrated the effectiveness of T cells in preventing neuronal loss following injury (Armstrong et al., 2004; Jones et al., 2005a; Schwartz and Moalem, 2001). To date, research has focused on elucidating the role of T cells in preventing initial neuronal death or slowing the rate of neurodegeneration and gradual neuronal loss following facial nerve axotomy (Jones et al., 2005a; Serpe et al., 1999; Serpe et al.,

2000). Given that a substantial number of facial motor neurons undergo atrophy following axotomy and that the atrophied state of these neurons is reversible by re-injury**,** T cells may also be involved in mediating the reversal of neuronal atrophy following re-injury. In this study, we therefore used the re-injury model described previously by McPhail et al. (McPhail et al., 2004a; McPhail et al., 2005) to test the hypothesis that the reversal of motor neuron atrophy (i.e., increase in cell number and size) elicited by nerve re-injury would be impaired in immunodeficient recombinase activating gene-2 knockout (RAG-2 KO) mice, which lack mature T and B cells. Neuronal cell counts and mean cell size were compared in mice that received an initial resection of the facial nerve followed by a re-injury of the same nerve 10 weeks later versus mice that received only a single resection followed by a sham re-injury 10 weeks later. The re-injury paradigm is shown in Figure 6-1. In a landmark study, Raivich et al. (Raivich et al., 1998) demonstrated that T cells cross an intact blood-brain-barrier (BBB) and home to degenerating neuronal cell bodies following peripheral transection of the facial nerve, and established that the peak of this response occurs at 14 days post-axotomy. Measures of neuronal survival were therefore assessed at 14 days after the 2nd surgery in both groups, which also allowed us to assess the peak T cell response in the injured FMN. In Chapter 4, we showed that prior exposure to neuronal injury in the FMN early in adulthood induced a robust increase in T cell traffcking to the injured FMN, indicative of T cell memory, when the contralateral nucleus was injured later in adulthood (Ha et al., 2007). Interestingly, this enhanced T cell trafficking to the injured FMN was not correlated with improved neuronal survival. Interactions between T cells and microglia are important in the immune-mediated improvement of motor neuron survival (Byram et al., 2004). That we did not see an improvement in neuronal survival in the presence of a T cell memory response could be due to the possibility that the memory T cells

encountered microglia in the contralateral FMN that were naïve to injury and suggests that injury-experienced microglia may be needed to encode a type of memory that permits them to interact with memory T cells and mediate the improvement in neuronal survival. Thus, secondary hypotheses we sought to test were whether prior exposure to neuronal injury could elicit a T cell memory response when the same FMN is injured later in adulthood, and if the postulated increase in T cell homing to the FMN is correlated with the improvement in neuronal outcome measures in the current re-injury model. Studies documenting the presence of $CD4^+$ and $CD8^+$ T cells in the injured FMN are limited (Ankeny and Popovich, 2007; Bohatschek et al., 2004; Liu et al., 2005), and none have been performed using this resection or re-injury model. To address the aforementioned T cell memory hypothesis and to assess the distribution of $CD4^+$ and $CD8^+$ T cells in the injured FMN, we compared the number of $CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells in the FMN of 3 groups of WT mice. One group was assessed at 14 days post-resection but did not receive prior nerve injury and served as controls (acute resection). A second group received prior nerve injury and was assessed at 14 days after a re-injury of the same nerve (chronic resection + re-injury). The third group was assessed at 12 weeks after a single resection (chronic resection + sham). Finally, to determine whether prior injury alters the microglial response to re-injury, we also quantified the number of $MHC2^+$ microglia in these subject groups.

Results

Effect of Immunodeficiency on the Reversal of Neuronal Atrophy

We compared the effect of treatment (chronic resection + re-injury vs. chronic resection + sham re-injury) on motor neuron survival in RAG-2 KO and WT mice. As seen in Figure 6-2A and as expected from the literature, motor neuron survival was significantly increased in chronically resected WT mice that received nerve re-injury compared to those that received sham

re-injury [$F(1,10)=6.083$, $p<0.05$]. By contrast, the level of motor neuron survival did not differ between chronically resected RAG-2 KO mice that received re-injury or sham re-injury. We also compared the effect of treatment on the cross-sectional area of neurons measured in 3 representative sections throughout the FMN in RAG-2 KO and WT mice. As seen in Figure 6-2B, average cell size was significantly increased in chronically resected WT mice that received re-injury compared to those that received sham re-injury [$F(1,6)=18.981$, $p<0.01$]. By contrast, average cell size did not differ between chronically resected RAG-2 KO mice that received re-injury or sham re-injury. As seen in Figures 6-3A and 6-3B, binning of the neurons by cell size revealed a noticeable shift from smaller to larger cell sizes following nerve re-injury in WT mice. This shift in cell size following nerve re-injury was not apparent in the RAG-2 KO mice (Figures 6-3C and 6-3D). Figure 6 shows representative sections of the FMN from chronically resected WT and RAG-2 KO mice that received re-injury or sham re-injury. In Figures 6-4A and 6-4B, note the increase in the number and size of motor neurons in re-injured WT compared to sham re-injured WT mice. In Figure 6-4F, motor neurons remained shrunken or appeared to be lost to axotomy in RAG-2 KO mice that received re-injury.

Effect of Nerve Re-Injury on the T Cell Response in the FMN

To determine whether a T cell memory response is elicited following nerve re-injury, we compared the effect of treatment on the number of $CD3^+$ T cells in the FMN of WT mice. Mice that did not receive prior nerve injury were assessed for their T cell response in the FMN at 14 days post-resection (acute resection) and were used as controls. As depicted in Figure 5A, there was a significant decrease in the number of $CD3^+$ T cells in chronically resected mice that received re-injury [compared to acute resection $F(1,9)=7.943$, $p<0.05$]. There was also a significant decrease in the number of $CD3^+$ T cells in chronically resected mice that received sham re-injury compared to acute resection [$F(1,9)=11.577$, $p<0.01$]. The number of T cells in

the injured FMN was comparable between chronically resected mice that received re-injury or sham re-injury. By 12 weeks post-injury, there was an occasional T cell (<1 T cell/section) in the injured FMN of chronically resected mice that received sham re-injury.

In Figures 6-5B and 6-5C, we quantified the number of $CD4^+$ and $CD8^+$ T cells, respectively, that were recruited to the FMN in acutely resected WT mice to determine whether there is a predominance of either T cell subtype in the FMN. The number of $CD4^+$ and $CD8^+$ T cells was comparable in the FMN of acutely resected mice. We also compared the number of $CD4^+$ and $CD8^+$ T cells in the FMN of chronically resected mice that received re-injury or sham re-injury versus the acute resection group to determine whether re-injury alters their distribution in the FMN. ANOVA revealed a significant decrease in the number of $CD4^+$ T cells in chronically resected WT mice that received re-injury [$F(1,8)=5.366$, $p<0.05$] or sham re-injury [$F(1,9)=15.771$, $p<0.01$] compared to acute resection. The number of $CD4^+$ cells did not differ between the chronic axotomy groups that received re-injury or sham re-injury. Similarly, there was a significant decrease in the number of $CD8^+$ T cells in chronically resected mice that received re-injury [$F(1,9)=6.376$, $p<0.05$] or sham re-injury [$F(1,9)=7.621$, $p<0.05$] compared to acute resection. The number of $CD8^+$ T cells did not differ between the chronic axotomy groups that received re-injury or sham re-injury.

Effect of Nerve Re-Injury on the Microglial Response in the FMN

To determine whether nerve re-injury alters microglial reactivity in the FMN, we compared the number of $MHC2^+$ microglia between chronically resected WT mice that received re-injury and acutely resected WT mice. As shown in Figure 6-6, although there were fewer $MHC2^+$ microglia following re-injury in chronically resected mice that received re-injury compared to acute resection mice, the groups were not statistically significant. ANOVA revealed a significant decrease in the number of $MHC2^+$ microglia in chronically resected WT

mice that received sham re-injury when compared to those that received re-injury [$F(1,9)=16.771$, $p<0.01$] or acute resection [$F(1,8)=13.236$, $p<0.01$].

Discussion

We demonstrated that immunodeficiency prevented the re-injury-induced reversal of neuronal atrophy in RAG-2 KO mice. Although the number and size of injured motor neurons were not increased following nerve re-injury in RAG-2 KO mice, the expected increase in both measures was observed in re-injured wild-type mice. The degree of improvement in measures of neuronal survival, however, was not as robust as the improvement seen in the study by McPhail et al., (2004), likely because we performed our assessments at 14 days instead of 7 days following re-injury and suggests that the regenerative effect following re-injury is transient. It is possible that neuronal regeneration did occur in RAG-2 KO mice but that the survival of those neurons was unsustainable due to immunodeficiency. In a previous study, the role of T cells in delaying neuronal loss was found to be time-dependent, with a substantial reduction in neuroprotection seen between 4 and 10 weeks post-axotomy (Serpe et al., 2000). Interestingly, the level of neuronal survival between RAG-2 KO and wild-type mice at 12 weeks post-resection was comparable, suggesting that the eventual fate of injured neurons in immunodeficient and wild-type animals is the same.

Contrary to our initial hypothesis, we did not observe an increased number of T cells, indicative of T cell memory, in the re-injured FMN of wild-type mice. In fact, we failed to observe a notable T cell response in the re-injured FMN of these mice, despite a marked improvement in neuronal survival. This finding is in contrast to the double injury model described in Chapter 4 in which the second injury was performed on the contralateral nerve, where sensitized T cells were exposed to neurons that were naive to injury and undergoing degeneration. Although T cell memory is typically characterized by greater T cell responses

following re-exposure to antigen, there may have been a functional enhancement of the few T cells present in the FMN, which allowed them to mediate the improvement in survival that was observed in the wild-type mice. Alternatively, T cells may have entered the FMN earlier than 14 days following re-injury to provide the necessary molecules (cytokines, chemokines, neurotrophic factors) to mediate neuroregeneration.

That few T cells were observed in the FMN following re-injury does not preclude the possibility that T cells might exert their effects peripherally at the site of the nerve injury. T cells can accumulate at the nerve stump following certain forms of peripheral nerve injury (Kleinschnitz et al., 2006; Moalem et al., 2004). Moreover, facial motor neurons have been shown to increased their expression of the anti-apoptotic gene bcl-2 following induction of an inflammatory response in the facial muscles of the rat, suggesting that facial motor neurons are able to respond to peripheral immune signals (Mariotti et al., 2001). Although unlikely, T cell tolerance induced in this re-injury model could have accounted for the lack of a prominent T cell response.

Given that additional T cells did not appear to infiltrate the FMN following re-injury, it is plausible that T cells are not involved in neuroregenerative processes (i.e., reversal of atrophy) and that their neuroprotective benefit could be attributable to their actions following the initial injury where they may sustain the viability of injured neurons by maintaining them in an atrophied state. Current work underway in the lab will address this working hypothesis by using adoptive transfer strategies to determine the importance of the timing of the T cell response in this re-injury model. Briefly, RAG-2 KO mice were immune reconstituted by adoptive transfer at two distinct time points, prior to the first or second injuries, and were subjected to the re-injury paradigm described in Figure 6-1. It is predicted that RAG-2 KO mice immune reconstituted

prior to the re-injury will be impaired in their regenerative capacity due to the lack of T cell-associated support at the time of the initial injury. An additional study nearing completion will attempt to detect the presence of atrophied neurons by using the retrograde tracer True Blue, which was applied to the nerve stump at the time of facial nerve resection. It is predicted that wild-type mice will show greater True Blue-positive neuronal labeling in the injured FMN than RAG-2 KO mice.

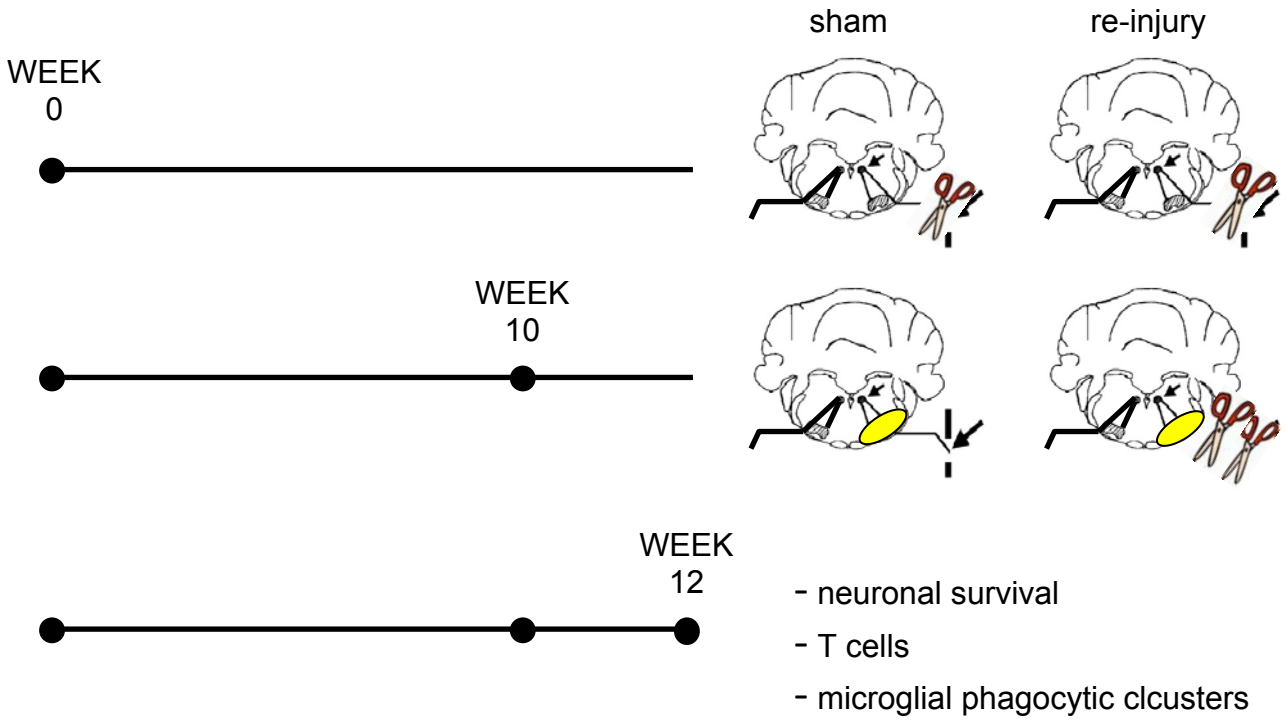

Figure 6-1. Schematic of the re-injury paradigm.

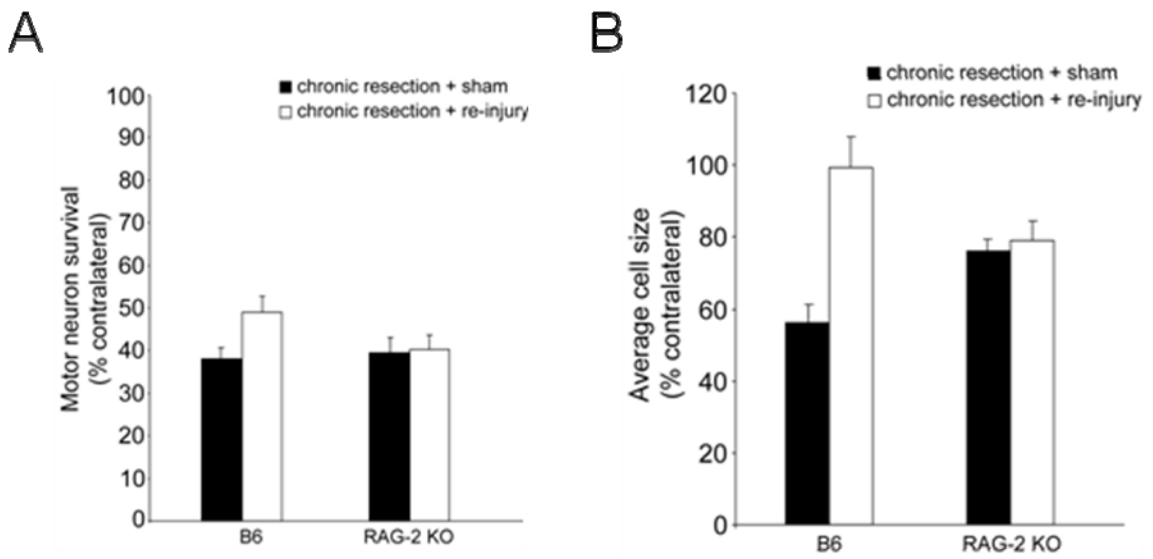

Figure 6-2. Quantification of mean cell counts (A) and mean cell size (B) in chronically resected WT and RAG-2 KO mice that received nerve re-injury or sham re-injury. Each bar in A represents the S.E.M. of 6 WT mice/treatment and the S.E.M. of 6 (chronic resection + sham) and 5 (chronic resection + re-injury) RAG-2 KO mice. Each bar in B represents the S.E.M. of 4 WT mice/treatment and the S.E.M. of 3 RAG-2 KO mice/treatment. *$p<0.05$, **$p<0.01$

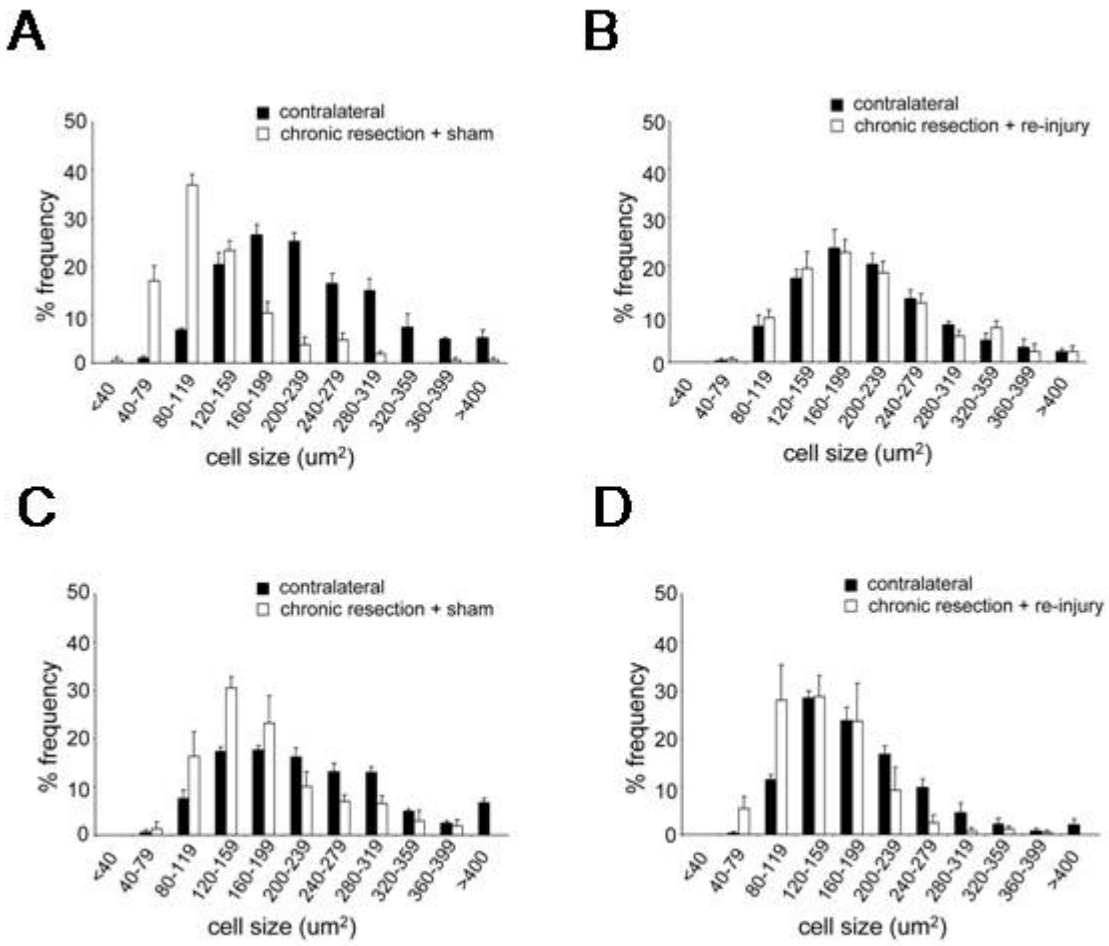

Figure 6-3. Facial motor neurons binned according to cell size following sham re-injury and re-injury in WT (A-B) and RAG-2 KO (C-D) mice. Note that the distribution of neurons in the injured FMN shifts from small to large cell sizes and is normalized to the contralateral uninjured side following re-injury in B6 but not in RAG-2 KO mice.

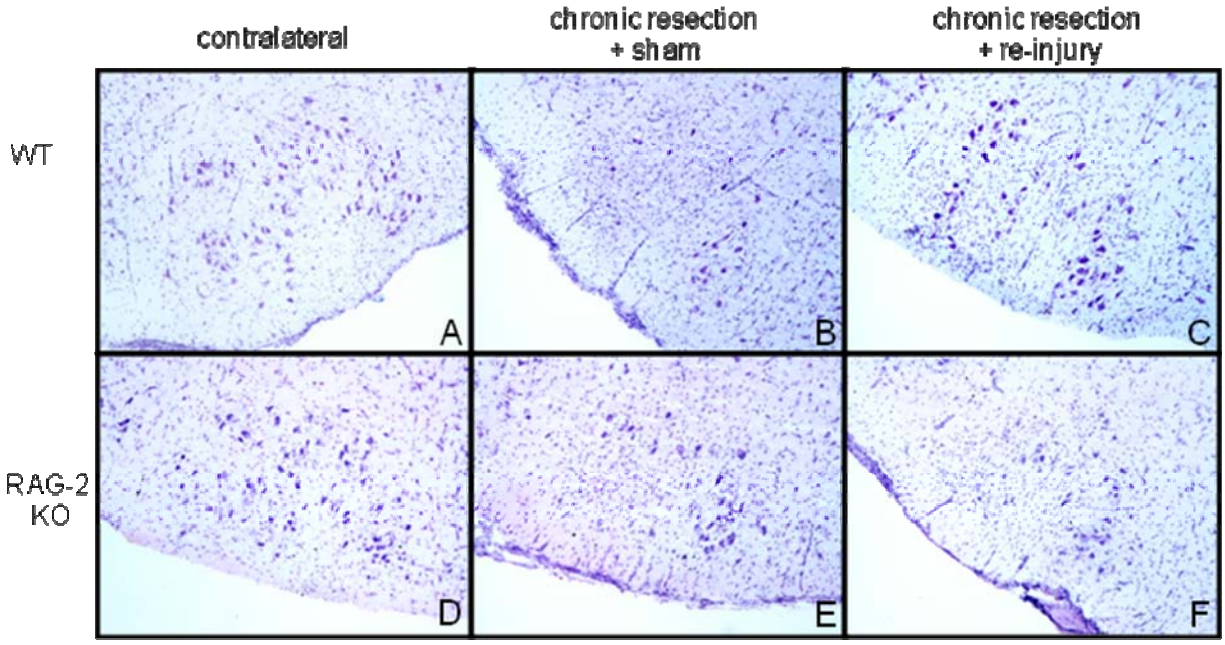

Figure 6-4. Photomicrographs of Nissl stained facial motor neurons in WT and RAG-2 KO mice. The facial motor nucleus is oriented so that the medial sub-nucleus is located on the right. There was a significant loss and shrinkage of neurons in WT and RAG-2 KO mice that received a chronic resection + sham injury (12 weeks after initial resection; B & E) compared to their respective contralateral controls (A & D). Following nerve re-injury, the number and size of injured motor neurons were markedly increased in WT (C) but not in RAG-2 KO mice (F).

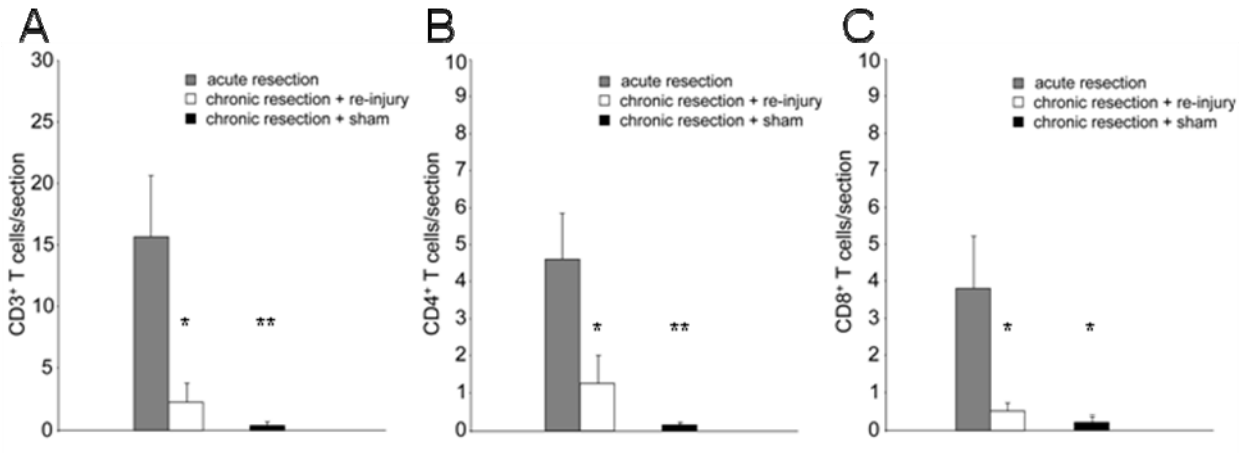

Figure 6-5. Quantification of CD3+, CD4+, and CD8+ T cells in the FMN of WT mice. For A and C, each bar represents the S.E.M. of 5 (acute resection) and 6 (chronic resection + re-injury, chronic resection + sham) mice. For B, each bar represents that S.E.M. of 5 (acute resection, chronic resection + re-injury) and 6 (chronic resection + sham) mice. *p<0.05, **p<0.01 (compared to acute resection)

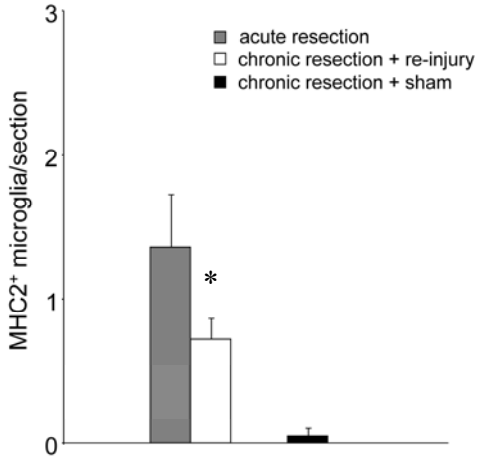

Figure 6-6. Quantification of MHC2+ microglia in the FMN of WT mice. Each bar represents the S.E.M. of 5 (acute resection, chronic resection + sham) and 6 (chronic resection + re-injury) mice. *p<0.01 (compared to acute resection and chronic resection resection + re-injury)

CHAPTER 7
PERSPECTIVES

Future Directions

Although the facial nerve axotomy model has been useful for extending our knowledge of the role of T cells in one form of CNS injury, it will be important to determine whether T cells operate in a similar manner to other forms of injury in different regions of the CNS. One model of interest is the fimbria fornix transection model, which results in a retrograde degeneration of neurons and site-specific microglial activation in the medial septum (Hollerbach et al., 1998). A feature of the fimbria fornix transection paradigm that distinguishes it from the facial nerve axotomy model is that the injury is initiated within the CNS. Thus, T cells would be operating under conditions where the integrity of the BBB is impacted. Moreover, the findings in Chapter 6 suggest that T cells may prevent the death of a particular population of neurons by promoting their atrophic status following injury. Given that medial septal neurons have been shown to atrophy following fimbria fornix transection, it would be interesting to examine whether immunodeficiency impairs the atrophy and subsequent regeneration of injured medial septal neurons. Insight gained from these studies could have significant relevance to neurodegenerative conditions that involve deficits in learning and memory processes.

The findings in Chapter 4 demonstrated that the presence of T cell memory in the injured FMN did not affect levels of neuronal survival. The methods used to quantify the number of surviving neurons in the studies of Chapter 4 failed to account for a population of neurons that have been shown to shrink and undergo atrophy following facial nerve axotomy. In light of the findings in Chapter 6 that suggest that T cells promote the atrophy of injured neurons, the presence of T cell memory in the injured FMN may increase the number of neurons that atrophy and their subsequent ability to regenerate. By combining the double injury model used in

Chapter 4 with the re-injury model used in Chapter 6, the regenerative response (reversal of atrophy) can be compared in mice that are naive and sensitized to neuronal injury. It is expected that the presence of T cell memory in sensitized mice will increase the number of neurons that atrophy such that re-injury elicits a greater regenerative response (i.e., more atrophied neurons available to regenerate). The proposed method of detecting neuronal atrophy by retrograde labeling with True Blue may also provide additional insight regarding T cell memory and its effect on atrophied neurons.

Concluding Remarks

The use of T cells in developing immune-based strategies to treat neurodegenerative disease or injury holds much promise. Vaccines designed to boost the T cell response in the CNS are being proposed to aid in the treatment of various types of CNS injury and neuropsychiatric and neurodegenerative disease (Schwartz, 2000; Schwartz and Hauben, 2002). By exploiting the intrinsic ability of T cells to seek out regions of neurodegeneration and damage, it would also be possible to engineer T cells to deliver neuroprotective or regeneration-related genes vital to the survival of the neuron. Indeed, current treatment for certain types of cancer has taken advantage of the ability of T cells to recognize and destroy tumor cells. With regards to the delicate nature of the CNS, however, it is critical to approach these promising strategies with caution. As discussed in Chapter 1, T cells have the potential to exert detrimental or beneficial effects in the CNS, depending on context. Before moving forward with such strategies, it was important to address some of the fundamental questions in this work regarding the role of endogenous T cell responses in the injured CNS.

Based on our findings, we propose that T cells respond to the neuronal death induced by injury and aid in promoting the long-term survival of the surrounding neurons by maintaining them in an atrophied state where they can be prompted to regenerate. As the interactions

between T cells and microglia have been shown to promote neuronal survival following injury, it will be important to understand the potential mechanisms underlying this process. It has been shown that supernatant collected from cells from the cervical lymph nodes of axotomized wild-type mice and re-activated by anti-CD3 was able to rescue injured facial motor neurons when administered to RAG-2 KO mice (Serpe et al., 2005). The neuroprotective effect was diminished when the supernatant was incubated with anti-BDNF, providing indirect evidence that BDNF produced by T cells aid in promoting survival. In relation to our work, it is possible that interactions between T cells and microglia promote the atrophy of neurons by mediating growth factor production in the injured FMN. In addition, the molecular underpinnings of these interactions must be addressed. Recent studies suggest that T cells may influence the expression of pituatary adenylate cyclase activating polypeptide (PACAP) in injured motor neurons following facial nerve axotomy (Armstrong et al., 2004; Zhou et al., 1999). It will be interesting to determine whether the expression of PACAP and other related genes in injured neurons aid in promoting their atrophied phenotype and whether increases in the level of endogenous T cells (i.e., T cell memory) augment such gene expression.

It is noteworthy that our studies used immunologically intact mice, where the interactions between T cells, microglia, and injured neurons were examined under physiologically relevant conditions. Prior to this work, the information regarding the role of T cells in the facial nerve axotomy model was gained from studies that used adoptive transfer strategies to restore the peripheral immune system of immunodeficient mice. While these strategies have served as useful tools for the intitial identification of T cell-mediated neuroprotection in the facial nerve axotomy model, potential confounds are introduced with adoptive transfer methods where T cells have been shown to undergo significant proliferation and exhibit an activated, memory

phenotype long after transfer. Thus, it is important to consider whether these findings hold in immunologically intact mice. In our studies of intact mice, enhanced T cell responses did not result in the profound improvements in neuronal survival that were reported by Jones and colleagues. Our findings did, however, shed light on an elusive population of neurons that appear to be impacted by the actions of T cells. Using immunodepletion strategies, it will be important in future studies to further elucidate the different populations of T cells that mediate neuroprotection.

In the studies of Chapter 4, T cell memory was elicited using a double injury model in which T cells were exposed and primed *in vivo* to endogenous CNS antigen. It is noteworthy that the presence of T cell memory in the injured FMN was associated with a modest recovery of function and did not worsen measures of neuronal survival and death. By contrast, several studies have used immunization protocols in which T cells were primed to myelin basic protein (MBP) prior to injury, in an effort to boost T cell responses to injury (Hauben et al., 2000; Jones et al., 2004; Jones et al., 2002; Jones et al., 2005b). The identification of the specific CNS antigen to which T cells respond following neuronal injury is currently unknown. The finding that immunization with T cells primed to CNS antigen results in the marked improvement of functional recovery and neuropathology following spinal cord injury has not been replicated in independent studies. In fact, a study that followed the same immunization and injury protocol showed that aspects of recovery and neuronal outcome were exacerbated. These findings raise questions regarding potential intrinsic differences between T cell responses derived from experimental vs. endogenous sources of antigen. These and other relevant questions regarding antigen specificity should be the focus of future studies.

Generally, the profound presence of inflammatory infiltrates in the CNS is thought to be predictive of severe neuropathology. As demonstrated by the findings in Chapter 5, the magnitude of the T cell response was related to the severity of peripheral nerve injury and resulting neuronal loss. It would be hasty to assume, however, that greater T cell responses in the CNS result in exacerbated pathology. In Chapter 6, we demonstrated that T cells may affect the long-term survival of atrophied neurons, suggesting that actions of T cells in the injured CNS may be more enduring than previously thought (Jones et al., 2005a). Notably, injured rubrospinal neurons have been shown to survive in an atrophied state for up to one year (Kwon et al., 2002). Subsequent treatment with BDNF applied at the site of the neuronal cell bodies reversed the atrophy and promoted the extension of the chronically injured axons into peripheral nerve grafts. Thus, treatment strategies that suppress overall immune responses following certain forms of CNS injury may be disadvantageous by preventing a normal and necessary response to tissue damage which could have the potential to provide long-term neuroprotection. A more fine-tuned approach in the treatment and management of these injuries would be to inhibit aspects of the immune response known to cause damage while permitting the beneficial aspects of the response.

In conclusion, our studies provide the impetus for further investigation into the role of endogenous T cell responses in the compromised CNS. Understanding the physiological role of T cells in the CNS under different conditions will provide information regarding the importance of context in driving the beneficial vs. detrimental aspects of the T cell response. Insight gained from these studies can be used to guide the development of immune-based treatment strategies that promote the repair and regeneration of damaged CNS tissue.

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BIOGRAPHICAL SKETCH

A New Orleanian by birth, Grace was transplanted to Tampa, FL where she spent most of her childhood. The Florida sun was not enough, however, to keep her from venturing to the chilly northeast where she attended Boston University. Although she initially dabbled in the field of biomedical engineering, Grace soon realized that neuroscience was her calling. After four years in Boston, Grace decided to return home where she completed her graduate studies at the University of Florida in the laboratory of Dr. John Petitto. With her husband Abe, Grace relishes the company of their family, friends, two dogs, Lyle and Fletcher, and cat, Nala. In her free time, Grace can be found enjoying a variety of activities, among which running, reading, and cooking are her favorite pastimes.