Forced-Exercise Dependent Changes in Cellular Immunity: Effects on Experimental Autoimmune Neuritis

Michael W. Calik

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LOYOLA UNIVERSITY CHICAGO

FORCED-EXERCISE DEPENDENT CHANGES IN CELLULAR IMMUNITY:
EFFECTS ON EXPERIMENTAL AUTOIMMUNE NEURITIS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY
MICHAEL W. CALIK
CHICAGO, IL
MAY 2011
ACKNOWLEDGMENTS

First, I would like to acknowledge and gratefully thank Dr. Evan B. Stubbs, Jr., who has been my dissertation advisor and mentor. He has taught me how to be, in essence, a critically thoughtful scientist. He has taught me patience, but also resilience, when conducting scientific research. For that, I am extremely grateful. I also would like to thank my dissertation committee, Dr. Edward J. Neafsey, Dr. John McNulty, Dr. John Clancy, and Dr. Morris Fisher, for their insightful critiques and guidance throughout my research.

I acknowledge the Veteran Affairs Department of Rehabilitation Services for financial assistance and for awarding me the VA Pre-Doctoral Associated Health Rehabilitation Research Fellowship.

I would like to thank my colleagues and labmates, Dr. Cynthia Von Zee and Kelly Clinkenbeard, for advice, motivation, and good times during my tenure in the lab.

I thank my mother, Barbara, and my father, Henryk, for emotional support throughout my doctoral career. I thank my twin brother, Martin, for his own humorous way of motivating me to finish my graduate studies. Finally, I graciously thank my wife, Marilyn, my son, Tristan, and my daughter, Lyra, who have been the foundation of my life.
For my lovely wife, Marilyn, my handsome son, Tristan, and my beautiful daughter, Lyra
Without you, this would have not been possible
Doubt is not a pleasant condition, but certainty is absurd.

Voltaire
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<td>AIDP</td>
<td>acute inflammatory demyelinating polyneuropathy</td>
</tr>
<tr>
<td>AMAN</td>
<td>acute motor axonal neuropathy</td>
</tr>
<tr>
<td>AMSAN</td>
<td>acute motor sensory axonal neuropathy</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BNB</td>
<td>blood–nerve barrier</td>
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<tr>
<td>CBG</td>
<td>corticosteroid-binding globulin</td>
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<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CIA</td>
<td>collagen-induced arthritis</td>
</tr>
<tr>
<td>CMAP</td>
<td>compound muscle action potential</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCC</td>
<td>dextran-coated charcoal</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EAN</td>
<td>experimental autoimmune neuritis</td>
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<tr>
<td>GBS</td>
<td>Guillain-Barré Syndrome</td>
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<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin-4</td>
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<td>Abbreviation</td>
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<td>IL-6</td>
<td>interleukin-6</td>
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<td>IL-10</td>
<td>interleukin-10</td>
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<td>IL-12p70</td>
<td>interleukin-12 subunit p70</td>
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<td>IFN-γ</td>
<td>interferon-γ</td>
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<tr>
<td>IVIg</td>
<td>intravenous immunoglobulin</td>
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<tr>
<td>MFS</td>
<td>Miller-Fisher Syndrome</td>
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<td>MHC II</td>
<td>major histocompatibility complex class II</td>
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<td>MNCV</td>
<td>motor nerve conduction velocity</td>
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<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PES</td>
<td>plasmapheresis</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>Tc</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>Th1</td>
<td>type 1 helper T cell</td>
</tr>
<tr>
<td>Th2</td>
<td>type 2 helper T cell</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17 cell</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>Tr1</td>
<td>type 1 regulatory T cell</td>
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Guillain-Barré Syndrome (GBS) is a debilitating inflammatory autoimmune disease of the peripheral nervous system that is characterized by rapid-onset paraparesis with areflexia progressing, in some patients, to neuromuscular paralysis. GBS occurs with an incidence of 2-4 cases per 100,000, similar to that reported for multiple sclerosis. Recognized as a heterogeneous disorder, the most common (85-90%) form of GBS observed in North America and Europe is acute inflammatory demyelinating polyneuropathy (AIDP). Enhanced infiltration of pro-inflammatory type 1 helper T (T\(_{\text{h}1}\)) cells into peripheral nerves of AIDP patients is strongly suggestive of an immune-mediated pathogenic process. Cellular immunity directed against specific constituents of the peripheral nerve myelin is considered causal in AIDP, leading to segmental demyelination and secondary axonal loss. The treatment of AIDP is currently palliative and utilizes non-specific immune-modulating therapies.

Increasing evidence now supports the application of exercise as a safe and effective non-pharmacological rehabilitative intervention strategy. Exercise attenuates the onset and progression of some autoimmune diseases and improves some measures of peripheral nerve function in diabetic neuropathy. In type 1 diabetes mellitus, exercise diminishes disease severity by promoting a type 2 helper T (T\(_{\text{h}2}\)) protective immune response. In patients with GBS, exercise has been shown to increase muscle strength recovery and aerobic exercise capacity, decrease fatigue, and improve quality of life.
Whether exercise alters the progression of GBS remains unclear.

In this study, we determined the effects of forced-exercise on development and progression of experimental autoimmune neuritis (EAN), an established animal model of AIDP. We report that a moderate regimen of forced-exercise significantly attenuates the severity of actively induced EAN in Lewis rats. We also investigated the mechanism by which forced-exercise alters the development of EAN. Adult male Lewis rats were randomized to three groups: sedentary adjuvant control, sedentary EAN, and forced-exercise EAN. Rats assigned to the forced-exercise group were trained on a motorized treadmill (6 km per week) for three weeks. Sedentary rats were allowed to explore the treadmill for the same duration of time without exercise. After three weeks, rats received a single injection of complete Freund’s adjuvant (CFA controls) or were actively immunized with an emulsified mixture of complete Freund’s adjuvant containing P2 peptide fragment (53-78, EAN). CFA control-injected rats did not develop EAN. By comparison, sedentary rats immunized with CFA containing P2 peptide developed a monophasic course of EAN. In contrast, rats subjected to forced-exercise and injected with CFA containing P2 peptide exhibited a significantly attenuated course of EAN. In addition, during severe EAN, forced-exercise rats lost less weight than sedentary rats. This attenuation was most likely not due to stress hormone levels since levels were similar in sedentary and forced-exercise EAN rats. Near peak of disease, evoked compound muscle action potential amplitudes were significantly reduced in sedentary rats compared to forced-exercise rats with mild EAN. In contrast, forced-exercise did not protect against deficits in evoked compound muscle action potential amplitudes, or conduction velocities, and in sciatic nerve pathology in sedentary and forced-exercise rats.
with severe EAN. Lymphocytes recovered from the popliteal lymph nodes of forced-exercise EAN rats exhibited increased proliferative response to P2 peptide compared to sedentary EAN controls. Moreover, the supernatant obtained from the lymphocyte proliferation assays of forced-exercise EAN rats near onset and peak of disease show a marked increase in the anti-inflammatory cytokine interleukin-10. When analyzed by flow cytometry, the percentage of T_{h}1 lymphocytes present in lymph nodes, spleen, and blood of forced-exercise EAN rats were reduced compared with sedentary EAN controls. Collectively, these data support a protective effect of forced-exercise against the development of EAN, in part, by selectively enhancing anti-inflammatory cytokine release and decreasing percent distribution of pro-inflammatory autoreactive T_{h}1 lymphocytes.
CHAPTER I
INTRODUCTION

Statement of the Problem

Human peripheral neuropathies affect approximately 2.4% of the population, causing a burden on the healthcare system (White et al., 2004). Peripheral neuropathies are a disparate group of diseases that are heterogeneous in etiology, diverse in pathology, and varied in severity (Martyn and Hughes, 1997). Current interventions for treating peripheral neuropathies are largely limited to management of discomfort and pain (Martyn and Hughes, 1997; White et al., 2004).

Guillain-Barré Syndrome (GBS) is an acute acquired autoimmune mediated human peripheral neuropathy with an incidence of about four cases per 100,000 people. Males are more commonly affected than females (Alter, 1990; Hughes and Rees, 1997; Cosi and Versino, 2006; Vucic et al., 2009). GBS encompasses a spectrum of clinical disorders characterized by acute and sub-acute progressive weakness of at least two limbs, usually beginning in the legs, for up to four weeks (Asbury et al., 1978; Hughes and Rees, 1997; Vucic et al., 2009). GBS affects motor and sensory nerves of the arms and legs and may involve the respiratory muscles and the facial, bulbar, and ocular motor nerves (Hughes and Rees, 1997). Autonomic nerve involvement can occur causing urine retention, ileus, sinus tachycardia hypertension, cardiac arrhythmia, and postural hypotension. Respiratory failure occurs in 25% of patients with GBS, with general
supportive therapy needed (Hughes and Cornblath, 2005; Vucic et al., 2009).

In North America and Europe, GBS typically presents in the form of acute inflammatory demyelinating polyneuropathy (AIDP) (Hadden et al., 1998; Hughes and Cornblath, 2005; Vucic et al., 2009). This common variant of GBS involves multifocal mononuclear cell infiltration of the peripheral nervous system (Asbury et al., 1969; Hughes and Cornblath, 2005; Vucic et al., 2009). Autoreactive pro-inflammatory lymphocytes and macrophages invade the myelin, thus demyelinating and damaging the axon, leading to paraparesis, paralysis, and areflexia of the limbs. Similar disease pathology has been shown to occur in experimental autoimmune neuritis (EAN), an established animal model of GBS (Hughes and Cornblath, 2005; Vucic et al., 2009).

The treatment of AIDP/GBS is currently palliative and utilizes non-specific immune-modulating therapies. Plasmapheresis or intravenous immunoglobulin therapies currently benefit the GBS patient by reducing the need for supportive care and expediting recovery. However, these benefits are generally observed only if treatment is administered early in the course of the disease (Hughes and Cornblath, 2005; Vucic et al., 2009). While most patients recover from GBS, there is a mortality rate of about 10% in population-based studies, and at least 10% of survivors are left with severe disability (Hughes and Rees, 1997; Plasma Exchange/Sandoglobulin Guillain-Barre Syndrome Trial Group, 1997; Vucic et al., 2009). Advancement of care for these affected patients waits on the development of new selective immune-modulating agents or the novel application of existing therapeutic strategies.

Increasing evidence now supports the application of exercise as a safe and
effective non-pharmacological rehabilitative intervention. Exercise decreases inflammatory diseases by increasing anti-inflammatory and decreasing pro-inflammatory cytokines, and by reducing pro-inflammatory toll-like receptor signaling on innate immune cells (Gleeson et al., 2006; Gleeson, 2007; Rosa Neto et al., 2010). Exercise attenuates the onset and progression of autoimmune diseases and peripheral neuropathies (Le Page et al., 1994; Le Page et al., 1996; White et al., 2004; Lowder et al., 2010; Navarro et al., 2010). In multiple sclerosis, exercise improves the quality of life, in part, by attenuating pro-inflammatory cytokine production (Schulz et al., 2004; White et al., 2006a; White et al., 2006b). Exercise also decreases peripheral neuropathy in type 2 diabetes mellitus by decreasing pro-inflammatory cytokines and leukocyte-endothelial interactions and adhesion (Fisher et al., 2003; Balducci et al., 2006; Gleeson et al., 2006; Roberts et al., 2006; Fisher et al., 2007; Hopps et al., 2011; Teixeira-Lemos et al., 2011a). In patients with GBS, exercise has been shown to increase muscle strength recovery and aerobic exercise capacity, decrease fatigue, and improve the quality of life (Garssen et al., 2004; El Mhandi et al., 2007). However, the effects of exercise on immune parameters of GBS were not studied. In this dissertation research study, we determined the mechanism by which exercise attenuates the onset and progression of experimental autoimmune neuritis.

**Hypothesis**

Forced-exercise protects against the onset and progression of experimental autoimmune neuritis, in part, by suppressing pro-inflammatory type 1 helper T (Th1) cell-mediated autoimmunity.
Specific Aims

The hypothesis was tested with the following two specific aims.

Specific Aim 1

We determined whether forced-exercise protects against the onset and progression of EAN in adult male Lewis rats. This was accomplished with the following sub-aims:

1A. Effects of forced-exercise on the clinical onset and progression of EAN, compared with sedentary controls, were quantified using established blinded scoring criteria.

1B. Effects of forced-exercise on EAN-induced changes in peripheral nerve function, compared with sedentary controls, were quantified by evoked-response electrophysiology.

1C. Effects of forced-exercise on EAN-induced changes in peripheral nerve pathology, compared with sedentary controls, were semi-quantified by light microscopy.

Specific Aim 2

We determined whether forced-exercise protects against the onset and progression of EAN in adult male Lewis rats by a mechanism that alters pro-inflammatory T<sub>h</sub>1 and anti-inflammatory type 2 helper T (T<sub>h</sub>2) cell activation. This was accomplished with the following sub-aims:

2A. Effects of forced-exercise on EAN-induced changes in P2 peptide or lectin-stimulated nodal and splenic lymphocyte proliferation, compared with sedentary controls, were quantified by [³H]thymidine uptake.

2B. Effects of forced-exercise on EAN-induced changes of T<sub>h</sub>1 and T<sub>h</sub>2 cell populations in the spleen, popliteal lymph nodes, or blood, compared with
sedentary controls, were quantified by flow cytometric analyses.

2C. Effects of forced-exercise on EAN-induced changes in P2 peptide or lectin-stimulated nodal and splenic lymphocyte cytokine production, compared with sedentary controls, were quantified by multiplex immunoassay.

2D. Effects of forced-exercise on corticosterone and corticosteroid-binding globulin, compared with sedentary controls, were quantified by enzyme-linked immunosorbent assay and radioimmunoassay, respectively.

A major finding of this dissertation is that a pre-induction three-week regimen of moderate forced-exercise attenuates the clinical severity, weight loss, and peripheral nerve damage of P2 peptide induced EAN in Lewis rats, an animal model of GBS. Our results show that in forced-exercise EAN rats, there is increase retention of adaptive immune cells in the popliteal lymph nodes and spleens. Lymphocytes obtained from popliteal lymph nodes of forced-exercise EAN rats have increased capacity to proliferate near peak of disease when stimulated with P2 peptide. Moreover, the supernatant obtained from the lymphocyte proliferation assays of forced-exercise EAN rats show a marked increase in the anti-inflammatory cytokine interleukin-10. Lymphocytes obtained from spleens of forced-exercise EAN rats have an increased capacity to proliferate when stimulated with ConA. Flow cytometric analysis of adaptive immune cells obtained from popliteal lymph nodes, spleens, and blood of forced-exercise EAN rats show a clear decrease in pro-inflammatory \( T_{h1} \) cells. These results illustrate that forced-exercise has anti-inflammatory effects that can attenuate EAN in rats. To our knowledge, this is the first study to show attenuation of the clinical severity of EAN by moderate intensity
forced-exercise.
CHAPTER II

LITERATURE REVIEW

Human Peripheral Neuropathies

Peripheral neuropathies affect approximately 2.4% of the population, causing a burden on the national healthcare systems (Martyn and Hughes, 1997; White et al., 2004). Peripheral neuropathies are a heterogeneous group of genetic or acquired disorders where one or all parts of the peripheral nervous system are damaged. Patients with peripheral neuropathies develop symptoms of numbness or altered sensation, usually starting at the extremities and progressing proximally, and muscle weakness (White et al., 2004). *Peripheral neuropathies are disabling, and improved interventions to alleviate the symptoms of peripheral neuropathies are critically needed.*

Autoimmunity

Autoimmune diseases are a family of more than 80 chronic, and often debilitating, diseases affecting more than 14.7 million people in the United States (The Autoimmune Diseases Coordinating Committee, 2005). The prevalence of these diseases is rising. *A better understanding of autoimmunity is crucial for future treatments.*

In a normal immune response, a foreign antigen is recognized and endocytosed by antigen presenting cells (APCs). These foreign antigens are recognized by toll-like receptors (TLRs) expressed on APCs, which are pattern recognition receptors that recognize pathogenic antigens (Toubi and Shoenfeld, 2004; Waldner, 2009; Torres-
Aguilar et al., 2010). APCs, such as dendritic cells, macrophages, and B cells, migrate to secondary lymphoid organs where these APCs present the foreign antigen in the context of major histocompatibility complex class II (MHC II) and co-stimulatory signals, CD80 and CD86, to naïve T cells with specific T cell receptors to that foreign antigen (Romagnani, 2006; Mackay, 2008). These T cells clonally expand. After clonal expansion, naïve T cells differentiate into a specific subset of helper T cells that participate in cell-mediated or humoral-mediated immune responses (Zhu and Paul, 2008; Veldhoen, 2009). The differentiated helper T cells then egress from the lymph nodes into circulation to survey and attack the foreign antigen (Mackay, 2008). Eventually, these activated and differentiated immune cells will encounter the foreign antigen at the affected target organ and will initiate a cascade of events, including release of cytokines and activation of macrophages and B cells to dispose of the foreign antigen (Romagnani, 2006; Mackay, 2008; Veldhoen, 2009; Torres-Aguilar et al., 2010). When this process involves a self-antigen, then the immune response is targeted at self-tissue and/or organs. This is defined as autoimmunity (Toubi and Shoenfeld, 2004; Romagnani, 2006; Mackay, 2008; Veldhoen, 2009; Waldner, 2009; Torres-Aguilar et al., 2010).

The immune system has in place a set of checkpoints to prevent the development of an immune response against a self-antigen (Goodnow et al., 2005; Parish and Heath, 2008). In the first checkpoint against autoimmunity, thymic T cells undergo central tolerance, a process of selecting T cells that strongly recognize self-antigen. Thymic T cells that strongly recognize self-antigen either undergo further rearrangement of antigen-receptor genes to avoid reactivity to self-antigen or face deletion by apoptosis.
(Fathman and Lineberry, 2007; von Boehmer and Melchers, 2010). However, central tolerance is only 60 to 70 percent efficient, with many of the autoreactive T cells escaping to the secondary lymphoid organs (Parish and Heath, 2008).

Autoreactive T cells that escape central tolerance are naïve (ignorant) but can be activated by APCs that have processed and presented a self-antigen to the T cells in the context of MHC II (Parish and Heath, 2008). In the second checkpoint against autoimmunity, autoreactive T cells can undergo peripheral tolerance, involving anergy, deletion by apoptosis, or suppression when presented with a self-antigen by an APC (Goodnow et al., 2005; Romagnani, 2006; Parish and Heath, 2008). Through anergy, APCs present to the T cells the self-antigen in the context of MHC II without the necessary co-stimulatory receptors CD80 and CD86, thus making the T cells functionally inactive to future interactions with an APCs (Romagnani, 2006). Deletion through apoptosis (activation-induced cell death) occurs when T cells encounter high antigen concentrations or when T cells are heavily activated, and is mediated by high expression of Fas and its ligand Fas ligand (Romagnani, 2006). Finally, peripheral tolerance can occur through suppression. Autoreactive T cells can be suppressed by type 1 regulatory T cells (Tr1), a subset of helper T cells known to control immune response to antigens by secreting the anti-inflammatory cytokine IL-10 (Romagnani, 2006).

Peripheral tolerance can be overcome by bystander activation or molecular mimicry (Romagnani, 2006). In bystander activation, pathogenic microbes induce inflammation and release of sequestered self-antigens from apoptotic cells. These self-antigens are then processed by APCs and presented to autoreactive T cells causing
autoimmunity (Romagnani, 2006). In molecular mimicry, foreign antigens from pathogenic microbes can strongly resemble self-antigens. When APCs process and present these foreign antigens to autoreactive T cells, the resulting immune response will not be able to distinguish between the foreign and self-antigens, and self-tissues and/or organs will be compromised (Rose and Mackay, 2000; Romagnani, 2006; Ryan et al., 2007). Both bystander activation and molecular mimicry are dependent on the self-antigens being recognized by TLRs on the APCs. The levels of TLRs on APCs differ depending on the inflammatory milieu encountered by APCs. For example, inflammation caused by an infection of pathogenic microbes can increase surface expression of TLRs causing robust processing of the self-antigen by the APCs (Toubi and Shoenfeld, 2004; Waldner, 2009; Torres-Aguilar et al., 2010).

The final checkpoint to autoimmunity is whether activated T cells to self-antigens differentiate into helper T cells that participate in pro-inflammatory cell-mediated or anti-inflammatory humoral-mediated immunity (Figure 1). The pro-inflammatory cell-mediated immunity is represented by type 1 helper T (T_h1) and T helper 17 (T_h17) cells, and is responsible for the pathogenesis of autoimmune disorders (Zhu and Paul, 2008). The up regulation of T-bet transcription factor differentiates naïve T cells to become a T_h1 cells, which are characterized by secretion of pro-inflammatory cytokine interferon-γ (IFN-γ), and the proliferative cytokine interleukin-2 (IL-2) (Zhu and Paul, 2008). The up regulation of ROR-γ transcription factor differentiates naïve T cells to become T_h17 cells, which are characterized by secretion of pro-inflammatory cytokine interleukin-17 (IL-17) and the cytokine
Figure 1. Naïve T cell fates. Adapted from (La Cava, 2008; Zhu and Paul, 2008).
interleukin-6 (IL-6) (Zhu and Paul, 2008). The anti-inflammatory arm of cellular immunity is represented by type 2 helper T (T\textsubscript{h}2) and regulatory T (Treg) cells, and is responsible for allergic diseases (La Cava, 2008; Zhu and Paul, 2008; Veldhoen, 2009; Steward-Tharp et al., 2010). The up regulation of GATA-3 transcription factor differentiates naïve T cells to become T\textsubscript{h}2 cells, which is characterized by secretion of anti-inflammatory cytokines interleukin-4 (IL-4) and IL-10 (Zhu and Paul, 2008). The up regulation of Foxp3 transcription factor differentiates naïve T cells to become Treg cells, which are characterized by secretion of anti-inflammatory cytokine IL-10 and the cytokine transforming growth factor-β (TGF-β) (Zhu and Paul, 2008).

Differentiation of pro-inflammatory or anti-inflammatory autoreactive T cells during self-antigen presentation by APCs is determined by the activation status of APCs and cytokine milieu. For example, immature and mature dendritic cells can present the self-antigen to autoreactive naïve T cells. However, the immature and semi-mature dendritic cells express low levels of MHC II and the co-stimulatory molecules CD80 and CD86 and secrete less pro-inflammatory cytokines. This leads to decreases in differentiation of pro-inflammatory T cells, or increases in differentiation of autoreactive naïve T cells into Tregs that suppress pro-inflammatory cell-mediated immune responses. In contrast, mature dendritic cells expressing high levels of MHC II and the co-stimulatory molecules CD80 and CD86 differentiate autoreactive naïve T cells into T\textsubscript{h}1 and T\textsubscript{h}17 cells (Fehervari and Sakaguchi, 2004; Toubi and Shoenfeld, 2004; Romagnani, 2008; Torres-Aguilar et al., 2010). Moreover, the cytokine milieu derived from dendritic cells, macrophages, and contracting muscles (Nielsen and Pedersen, 2008), can
differentiate autoreactive naïve T cells into specific subsets (La Cava, 2008; Zhu and Paul, 2008; Torres-Aguilar et al., 2010). For example, during APC presentation of self-antigen, IFN-γ, interleukin-12 (IL-12), and interleukin-18 (IL-18) can differentiate autoreactive naïve T cells into T\textsubscript{h}1 cells, while IL-4 and IL-2 can differentiate autoreactive naïve T cells into T\textsubscript{h}2 cells. Moreover, TGF-β and IL-2 can differentiate autoreactive naïve T cells into Treg cells, while TGF-β, IL-6, interleukin-21, and interleukin-23 differentiate autoreactive naïve T cells into T\textsubscript{h}17 cells (Zhu and Paul, 2008).

**Guillain-Barré Syndrome**

The autoimmune disease Guillain-Barré Syndrome (GBS) encompasses a heterogeneous set of clinical syndromes in which idiopathic autoimmune peripheral neuropathy causes acute or sub-acute weakness of at least two limbs, usually starting in the legs, which progresses for up to four weeks and then reaches plateau (Hughes and Rees, 1997; Hughes and Cornblath, 2005; Vucic et al., 2009). GBS symptoms also include: parasthesia/dysaesthesia, backache/root pain, meningism, and muscle, joint, or visceral pain, though most cases of pain recover spontaneously at varying intervals after the onset of disease (Pentland and Donald, 1994). GBS can be divided into several patterns of pathology (Figure 2) (Ho et al., 1998).

Two patterns of mainly axonal involvement can be categorized in GBS: acute motor axonal neuropathy (AMAN) and acute motor sensory axonal neuropathy (AMSAN). AMAN is due to axonal degeneration without a prior demyelination of the peripheral motor nerves (Ho et al., 1998; Hughes and Cornblath, 2005; Vucic et al.,
2009). Generally, AMAN is characterized by weakness or paralysis without sensory loss. AMAN is diagnosed when electrophysiological studies of patients with GBS show a reduction in compound muscle action potential (CMAP) amplitudes without prominent conduction slowing. AMAN most commonly occurs in children in Asian countries, with antecedent *Campylobacter jejuni* infection, which suggests molecular mimicry (Ho et al., 1998; Kuwabara, 2004; Hughes and Cornblath, 2005; Kuwabara, 2007; Vucic et al., 2009). In fact, antigens on the *C. jejuni* are similar to those located at the nodes of Ranvier, signifying that AMAN is most likely a humoral-mediated immune response against the axon. Autoantibodies to gangliosides, such as GM1, GM1b, or GD1a, bind at the nodes of Ranvier leading to complement-mediated destruction and macrophage invasion, resulting in Wallerian-like degeneration (Li et al., 1996; Ho et al., 1998; Hughes and Cornblath, 2005; Vucic et al., 2009).

AMSAN is similar to AMAN in that it involves Wallerian-like degeneration and/or conduction block of peripheral motor nerves. Where AMSAN differs is that peripheral sensory nerves also undergo Wallerian-like degeneration and/or conduction block (Ho et al., 1998; Kuwabara, 2004, 2007). The incidence of AMSAN is very low, being less than 10 percent of AMAN (Kuwabara, 2004, 2007; Vucic et al., 2009).

Another variant of GBS is Miller-Fisher Syndrome (MFS), which is characterized by acute onset of unstable gait (ataxia), loss of reflexes (areflexia), and inability to move the eyes (ophthalmoplegia). Autoantibodies to the gangliosides GQ1b and GT1a are heavily distributed on oculomotor nerves and to a lesser extent on motor nerve terminals of somatic musculature, sensory neurons in the dorsal root ganglion, and a population of
Figure 2. An operational classification of GBS and related syndromes. Adapted from (Ho et al., 1998).
cerebellar neurons. Some cases of MFS are preceded by an antecedent infection of *C. jejuni*, which bear epitopes similar to GQ1b and GT1a, thus implicating molecular mimicry (Ho et al., 1998; Kuwabara, 2004; Hughes and Cornblath, 2005; Kuwabara, 2007; Vucic et al., 2009).

When GBS was first described, it was previously characterized only by lymphocytic infiltration/inflammation and peripheral nervous system (PNS) demyelination. With the realization that GBS encompasses many subtypes, the GBS form of lymphocytic infiltration/inflammation and PNS demyelination is now clinically defined as acute inflammatory demyelinating polyneuropathy (AIDP) (Ho et al., 1998). AIDP is the most common form of GBS in developed countries and is responsible for 85-90% of the patients with GBS (Kuwabara, 2004, 2007; Vucic et al., 2009).

AIDP is characterized by demyelination, and pro-inflammatory lymphocyte and macrophage infiltration. In some cases of AIDP, axonal degeneration may follow demyelination. Clinically, patients present with flaccid paralysis, areflexia, and some sensory loss (Hughes and Cornblath, 2005; Kuwabara, 2007; Vucic et al., 2009). Electrophysiological studies of the peripheral nerves usually reveal increases in distal latencies and absence of F waves or increases in F wave latencies, and reductions in nerve conduction velocities and CMAP amplitudes (Hughes and Cornblath, 2005; Vucic et al., 2009; Uncini et al., 2010). Also, early in AIDP, there are decreased proximal-to-distal CMAP amplitude ratios that, later in the disease, return to normal (Uncini et al., 2010). This signifies length-dependent CMAP amplitude reduction, and is thought to be due to demyelinating lesions scattered along the nerve followed by axonal degeneration.
Many markers of pro-inflammatory T cell activation, including soluble IL-2 receptor, IL-12, IL-18, and tumor necrosis factor-α (TNF-α), are found in the serum of AIDP patients (Ho et al., 1998; Lu and Zhu, 2010). Moreover, GBS patients have decreased percentages of anti-inflammatory regulatory T cells that may cause a dysregulation of pro-inflammatory T<sub>H</sub>1 cells (Pritchard et al., 2007; Vucic et al., 2009). An alternative, but not mutually exclusive, hypothesis is that the binding of autoantibodies to the Schwann cell is followed by complement-mediated destruction of peripheral myelin (antibody-dependent cell-mediated cytotoxicity) (Hughes and Cornblath, 2005; Vucic et al., 2009). Evidence for this theory comes from low incidence of antecedent infection of <i>C. jejuni</i>. It is thought that unknown antibodies against <i>C. jejuni</i> cross-reacts with self-antigens expressed on Schwann cells, thus implicating molecular mimicry (Ho et al., 1998; Hughes and Cornblath, 2005; Kuwabara, 2007; Vucic et al., 2009).

**Treatment for Guillain-Barré Syndrome**

A multidisciplinary approach is needed to prevent and manage potentially fatal complications of GBS. Respiratory failure occurs in about 25% of patients and early transfer to an intensive care unit for pre-induction intubation is required. Patients are also monitored for cardiac arrhythmia, autonomic failure, and vein thrombosis. Other complications requiring medical care are pain, urinary retention, and ileus (Hughes and Cornblath, 2005; Kuwabara, 2007; Vucic et al., 2009).

Immunotherapy is used as a treatment for GBS. These non-specific immune-modulating therapies are indicated for patients who are unable to walk, and they consist
of plasmapheresis (PES) or intravenous immunoglobulin (IVIg) infusion (Ho et al., 1998). Both treatments help to speed the recovery of the patient, but only if the treatments are implemented early (before 4 weeks for PES and before 2 weeks for IVIg) in the disease (Hughes and Cornblath, 2005; Vucic et al., 2009). IVIg and PES have similar outcomes and either should be offered to patients who are considered immobile (Cortese et al., 2011). The mechanism of IVIg therapy remains to be understood, but it is thought that IVIg modulates complement activation products, neutralizes idiotypic antibodies, saturates the Fc receptors on macrophages, and suppresses various pro-inflammatory mediators, such as cytokines, chemokines, and matrix metalloproteinases (Kuwabara, 2004, 2007). The combination of PES and IVIg does not add an extra benefit to the recovery of GBS (Hughes et al., 2007; Kaida and Kusunoki, 2009; Vucic et al., 2009).

Corticosteroids are widely used as a therapy in autoimmune disorders. However, in studies with GBS patients, corticosteroids failed to modulate the course of the disease (Kuwabara, 2004; Kaida and Kusunoki, 2009; Vucic et al., 2009). One possible explanation for the lack of benefit seen in corticosteroid treatment is that corticosteroids might adversely affect the recovery process by inhibiting macrophage clearance of myelin debris and hinder remyelination of the peripheral nerves (Hughes and Cornblath, 2005; Kaida and Kusunoki, 2009).

*Even though current therapies help to speed the recovery process, there is a pressing need to find more effective and specific immune modulating therapies for the treatment of GBS and related neuropathies.*
**Experimental Autoimmune Neuritis**

Experimental autoimmune neuritis (EAN) is an animal model that closely resembles AIDP. It is induced by immunization with peripheral nerve myelin or its proteins, P2, P0, or PMP22 (Hughes and Cornblath, 2005; Kaida and Kusunoki, 2009). It is also induced by adoptive transfer of autoreactive T cells reactive towards P2, P0, or PMP22 protein (Gold et al., 2000; Hughes and Cornblath, 2005). The main mechanism of EAN is a pro-inflammatory autoreactive T\(_h\)1 cell-mediated response against one or more of the myelin proteins (**Figure 3**). Due to activation of the adaptive immune response by innate immune cells, the activated pro-inflammatory autoreactive T\(_h\)1 cells, as part of normal immune surveillance, egress from secondary lymphoid organs into the circulation, cross the blood-nerve barrier (BNB), encountering a cross-reactive antigen in the endoneurium (Spies et al., 1995a; Maurer and Gold, 2002; Hughes and Cornblath, 2005; Schwab and Cyster, 2007). The activated autoreactive T\(_h\)1 cells release pro-inflammatory cytokines, which break down the BNB, and recruit and stimulate macrophages and autoreactive cytotoxic T cells. Macrophages are the effector cells invading the myelin sheaths and causing demyelination (Maurer and Gold, 2002; Hughes and Cornblath, 2005; Lu and Zhu, 2010). Autoreactive cytotoxic T cells play a role in EAN by migrating to peripheral nerves and releasing the cytotoxins perforin, granzymes, and granulysin (Constantinescu et al., 1998; Pelidou et al., 2000; Yun et al., 2007). In addition, autoantibodies against the myelin proteins produced by autoreactive T cell-activated B cells can enter the compromised BNB and cause conduction block of the peripheral nerves. The autoantibodies can also activate complement-mediated destruction of
peripheral nerve myelin (Hughes et al., 1981; Spies et al., 1995b; Maurer and Gold, 2002; Vucic et al., 2009).

EAN, similar to AIDP, is a monophasic disease (Gold et al., 2000). It is induced in Lewis rats. Starting as early as ten days after active immunization with purified P2 peptide fragment, animals begin to lose muscle tone in the tail. Progression of EAN is characterized by weakness (paraparesis) or paralysis in the hind limbs. In severe cases, paraparesis or paralysis of the fore limbs and respiratory muscles can occur, leading to death. Weight loss is common, and begins before the clinical symptoms of EAN appear. Tactile sensitivity testing of the hind paws reveals tactile hypersensitivity before onset of clinical symptoms (Moalem-Taylor et al., 2007; Luongo et al., 2008; Zhang et al., 2008b). Electrophysiological studies show a reduction in motor nerve conduction velocity, and CMAP amplitudes at peak EAN severity compared to pre-immunization responses. The neuropathological features of EAN include demyelination of the peripheral nerves and nerve roots, infiltration of lymphocytes and macrophages, and nerve edema. In severe EAN, axonal damage may follow demyelination (Harvey and Pollard, 1992; Sarkey et al., 2007). *Many of the immunopathological mechanisms of EAN are well understood, making it important tool for the development of specific therapies in autoimmune disease.*

**Exercise, Autoimmunity and Guillain-Barré Syndrome**

Exercise has potent anti-inflammatory effects on the innate and adaptive immune systems (*Figure 4*). Regarding innate immunity, exercise is known to alter innate
Figure 3. Pathogenesis of AIDP/EAN. m, macrophage; T (blue), autoreactive naïve T cell; T (red), autoreactive activated T cell; B, autoreactive activated B cell; C5-9, complement activation by autoantibody; NO, nitric oxide. Adapted from (Hughes and Cornblath, 2005; La Cava, 2008).
immune cell trafficking (Woods et al., 1999; Sugiura et al., 2000; Ho et al., 2001; Chiang et al., 2010; Suchanek et al., 2010), decrease TLRs, and alter APC function (Woods et al., 1999; Lancaster et al., 2005; Flynn and McFarlin, 2006; Gleeson et al., 2006; McFarlin et al., 2006; Lambert et al., 2008; Timmerman et al., 2008; Simpson et al., 2009; Oliveira and Gleeson, 2010).

Regarding adaptive immunity, exercise is known to increase (Sugiura et al., 2000; Rogers et al., 2008), or decrease (Moraska et al., 2000; Nielsen, 2003; Gleeson et al., 2006; Gleeson, 2007; Rosa Neto et al., 2010) lymphocyte proliferation, increase differentiation of anti-inflammatory T cells and secretion of anti-inflammatory cytokines while decreasing pro-inflammatory T cells and cytokines (Lancaster et al., 2004; Pastva et al., 2004; Yeh et al., 2006; Flynn et al., 2007; Gleeson, 2007; da Silva Krause and de Bittencourt, 2008; Donnikov et al., 2008; Haaland et al., 2008; Nielsen and Pedersen, 2008; Yeh et al., 2008; Hewitt et al., 2009b; Lowder et al., 2010; Rosa Neto et al., 2010), and impair the T cell-mediated induction phase of an in vivo immune response (Harper Smith et al., 2011). Moreover, exercise can increase catecholamines and glucocorticoids (Woods et al., 1999; Pastva et al., 2005; Ortega et al., 2007; Hewitt et al., 2009a) decreasing a pro-inflammatory immune response. Exercise can also alter immune cell trafficking by promoting lymphocyte egress into peripheral tissues (Chen et al., 2010; Turner et al., 2010; Adams et al., 2011). With many pleiotropic anti-inflammatory effects on the innate and adaptive immune systems, exercise can be a safe and effective non-pharmacological rehabilitative intervention for inflammatory diseases.

Exercise reduces the complications associated with type 2 diabetes. Exercise
increases the release of anti-inflammatory cytokines from muscles, and decreases the release of pro-inflammatory cytokines from adipose tissue (Nielsen and Pedersen, 2008; Hopps et al., 2011), thus decreasing inflammation. Diabetic men who exercised had significant reductions in pro-inflammatory cytokines, leukocyte-endothelial interactions and adhesion, and peripheral neuropathy (Fisher et al., 2003; Balducci et al., 2006; Roberts et al., 2006; Fisher et al., 2007). Similarly, in a rat model of type 2 diabetes mellitus, exercise was shown to decrease pro-inflammatory cytokines (Teixeira de Lemos et al., 2009; Teixeira-Lemos et al., 2011a).

Exercise is known to affect autoimmune and inflammatory diseases in humans. In asthma, an inflammatory disease of the airways, exercise decreases disease-related hospital admission of asthmatics (Lucas and Platts-Mills, 2005). In patients with rheumatoid arthritis (RA), an inflammatory cell-mediated autoimmune disease with signs and symptoms of joint and systemic inflammation, exercise increases strength and quality of life, and decreases pain, fatigue, and inflammation (Metsios et al., 2010; Hurley et al., 2011). In patients with multiple sclerosis (MS), an inflammatory cell-mediated autoimmune demyelinating disease of the central nervous system, exercise increases fitness parameters and quality of life, and decreases fatigue and pro-inflammatory cytokines (TNF-α, IFN-γ,) (Schulz et al., 2004; White et al., 2006a; White et al., 2006b; Castellano et al., 2008). Patients suffering from GBS also benefit from exercise. For these patients, exercise improves physical fitness and muscle strength and decreases recovery time, fatigue, anxiety, and depression (Garssen et al., 2004; El Mhandi et al., 2007).

However, the effects of exercise on immune parameters have not been investigated
Diagram 3. Possible mechanisms by which exercise increases susceptibility to infection but reduces inflammation and risk of developing chronic disease.

TLR, Toll-like receptor; TH1, T helper 1; IL-1ra, IL-1 receptor antagonist. The encircled minus sign represents an inhibitory action of IL-6 on TNF production.

Figure 4. Exercise reduces inflammation and risk of chronic disease. TLR, toll-like receptor; TH1, type 1 helper T cell; IL-1ra, interleukin-1 receptor antagonist; IL-10, interleukin-10. Adapted from (Gleeson, 2007).
in GBS patients.

In the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), forced-exercise has been shown to decrease the clinical severity and delay the onset of symptoms (Le Page et al., 1994; Le Page et al., 1996; Rossi et al., 2009). Three weeks of exercise in rats has been shown to increase blood-brain barrier function by decreasing expression of matrix metalloproteinases (Guo et al., 2008). In studies of EAE, the blood-brain barrier becomes permeable to lymphocytes (Engelhardt, 2006), and thus exercise might have a therapeutic role in MS. In an animal model of RA, collagen-induced arthritis (CIA), exercise has been shown to decrease severity of CIA by decreasing immune cell activation and increasing blood glucocorticoids (Navarro et al., 2010). In the mouse model of asthma, ovalbumin-induced asthma, exercise has been shown to decrease severity of asthma by increasing glucocorticoids, and increasing the percentage and suppressive activity of anti-inflammatory Tregs with a concomitant increase in anti-inflammatory cytokines, while decreasing pro-inflammatory cytokines and transcription factors (Pastva et al., 2004; Pastva et al., 2005; Hewitt et al., 2009b; Lowder et al., 2010).

There have been no studies on the effects of exercise on EAN. However, due the anti-inflammatory effects of exercise, EAN might be attenuated. Moreover, further beneficial effects of exercise in the PNS are increased capacity of axonal regeneration and sprouting after peripheral nerve injury and decreased age-related apoptosis of Schwann cells (Ilha et al., 2008; Sabatier et al., 2008; Shokouhi et al., 2008). Promoting regeneration or decreasing Schwann cell apoptosis in EAN or GBS would be ideal as a therapeutic intervention. To our knowledge, the effect of exercise on EAN has not been
investigated, and the beneficial effects of exercise may attenuate this autoimmune peripheral neuropathy.
CHAPTER III
MATERIAL AND METHODS

This study was conducted using protocols approved by the Edward Hines Jr. VA Institutional Animal Care and Use Committee in accordance with the principles of laboratory animal care (Institute for Laboratory Animal Research, 2010). Male Lewis rats (6-8 weeks of age; 200-220g) were housed two to a cage and allowed free access to Harlan 2018 rat chow and water *ad libitum*. Rats were maintained on a 12h/12h light/dark cycle. Tissue harvest and blood collection were performed between the hours of 0500 and 0800. For induction of experimental autoimmune neuritis (EAN) and for electrophysiological studies, rats were anesthetized with a combination of ketamine (90 mg/kg) and xylazine (7.5 mg/kg). 0.1 M phosphate-buffered saline (PBS) (pH 7.4) was used in this study.

**Forced-Exercise Experimental Design**

Rats were randomized to two groups: sedentary control or forced-exercise. Rats randomized to the forced-exercise group were acclimated to treadmill running using an Exer-3R motorized treadmill apparatus equipped with a motivational electric shock grid set at 20 µA (Columbus Instruments, USA). During acclimation, rats were subjected to treadmill training at 15 m/min x 20 minutes on the first day. Subsequently, rats were subjected to progressively faster and longer training sessions until the fifth day of training where the rats ran consistently at 20 m/min x 60 minutes. Rats acclimated to
treadmill training received an additional three weeks of forced-exercise at 20 m/min x 60 minutes, five days a week. Sedentary control rats were allowed to explore a similar “treadmill” environment for an identical duration of time but without receiving exercise. Both sedentary and forced-exercise rats were weighed daily throughout this study.

**Induction of Mild Experimental Autoimmune Neuritis**

At the completion of the three-week sedentary- or forced-exercise program, all rats were actively induced with experimental autoimmune neuritis (EAN), an established animal model of acquired inflammatory demyelinating neuropathy (AIDP), the predominant subtype of Guillain-Barré Syndrome (GBS) occurring in North America and Europe. EAN was induced (day 0) by injecting in the base of the tail (**Figure 5**) 100 µl of an emulsified 1:1 PBS:CFA (Sigma, USA) solution containing a final concentration of 5 mg/ml heat-inactivated H37RA *Mycobacteria tuberculosis* (Difco, USA) and 100 µg of commercially prepared P2 peptide (residues 53-78 of bovine P2 myelin protein; Dana-Farber Cancer Institute, Harvard University, USA). Forced-exercise rats immunized at the base of the tail were allowed to continue their training throughout the disease course.

All rats were closely observed for development of EAN. Clinical assessment of EAN severity was performed by investigators blinded to group assignment and was semi-quantified as follows: 0 = normal; 1 = limp tail; 2 = abnormal gait; 3 = mild paraparesis; 4 = severe paraparesis; 5 = hind limb paraplegia. Intermediate scores were assigned using 0.5 increments. Rats were sacrificed near peak (day 18) of EAN.

**Induction of Severe Experimental Autoimmune Neuritis**

At the completion of the three-week sedentary- or forced-exercise program, all
rats were actively induced with EAN, an aggressive animal model of AIDP. EAN was induced (day 0) by injecting in the hind left footpad (Figure 6) 100 μl of an emulsified 1:1 PBS:CFA (Sigma, USA) solution containing a final concentration of 5 mg/ml heat-inactivated H37RA *Mycobacteria tuberculosis* (Difco, USA) and 100 μg of commercially prepared P2 peptide (residues 53-78 of bovine P2 myelin protein; Dana-Farber Cancer Institute, Harvard University, USA). Forced-exercised rats immunized in the hind left footpad were not subsequently subjected to further training due to the local inflammatory response.

As an immunization antigen-free adjuvant (CFA) control, a subgroup of sedentary rats were injected in the hind left footpad with 100 μl of an emulsified 1:1 PBS:CFA solution containing 5 mg/ml of heat-inactivated H37RA *Mycobacteria Tuberculosis* in the absence of added antigen.

All rats were weighed daily and closely observed for development of EAN. Clinical assessment of EAN severity was performed by investigators blinded to group assignment and was semi-quantified as follows: 0 = normal; 1 = limp tail; 2 = abnormal gait; 3 = mild paraparesis; 4 = severe paraparesis; 5 = hind limb paraplegia. Intermediate scores were assigned using 0.5 increments. Rats were further subdivided into two disease groups, sacrificed either near onset (day 14) or near peak (day 18) of EAN.

**Peripheral Nerve Conduction Studies**

Evoked-response electrophysiological studies (Figure 7) were performed on rats prior to induction of (day 0) and near peak (day 18) of mild (Figure 5) and severe EAN (Figure 6). Evoked compound muscle action potential (CMAP) amplitudes and latencies
were recorded from the right leg of ketamine/xylazine sedated rats as previously described (Sarkey et al., 2007). Briefly, evoked responses were elicited from the ankle (tibial nerve, distal) or the sciatic notch (sciatic nerve, proximal) with unipolar needle electrodes using supramaximal stimuli (25 mA, 0.05 ms duration). Evoked compound muscle action potential (CMAP) amplitudes were recorded from the plantar muscles with needle electrodes. Body temperature of the sedated rat was maintained near 37° C with an activated thermal heating pad. A series of 25 individual responses were evoked at a frequency of 1 Hz. Evoked responses were amplified, recorded, and averaged using a Teca Synergy electromyograph system (CareFusion, USA). Each response series was repeated three times. Proximal/distal ratios (R ratios) were calculated using the following formula: proximal CMAP amplitude ∕ distal CMAP amplitude. Motor nerve conduction velocities (MNCVs) were calculated using the following formula: distance between ankle and sciatic notch placed electrodes (typically 55 mm) ∕ (sciatic notch latency recording – ankle latency recording).

**Neuropathology**

Near peak of severe EAN (day 18) (Figure 6), rats were sacrificed by CO₂ asphyxiation, and sciatic nerves were rapidly harvested and immediately post-fixed in ice-cold 2% paraformaldehyde (Sigma, USA) + 2.5% glutaraldehyde (EMS, USA) PBS (pH 7.4) solution for 48 hours at 4° C. Fixed nerves were washed in PBS, osmicated by brief immersion in 1% OsO₄, dehydrated by washing with ascending series of alcohol solutions (50-100%) and propylene oxide. Dehydrated nerves were embedded in Embed-812 (Electron Microscopy Sciences, USA). Serial transverse sections (0.5 µm thick) were
cut using a Reichert Ultracut S microtome and stained with 0.5% toluidine blue/borax solution. Stained sections were examined by light microscopy using a Leitz DMRB inverted phase-contrast microscope equipped with a Leica Wild MPS photographic system (W. Nuhsbaum, USA). Histopathological changes (inflammatory infiltrates, myelin ovoids, or damaged axons) in sciatic nerves were quantified by two investigators blinded to group assignment by using a four-point scoring system as follows: 0 = normal; 1 = mild demyelination, axonal damage, or cellular infiltrates; 2 = moderate demyelination, axonal damage, or cellular infiltrates; 3 = severe demyelination, axonal damage, or cellular infiltrates (Sarkey et al., 2007).

**Proliferation Assay**

Rats were sacrificed by CO₂ asphyxiation near onset (day 14) or near peak (day 18) of severe EAN, and spleens and popliteal lymph nodes were harvested (Figure 6). Splenic lymphocytes were cleared of contaminating red blood cells by brief exposure to ammonium chloride lysing buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA; pH 7.4). Lymphocytes were prepared as a single-cell suspension. Prepared splenic or nodal lymphocytes were cultured at an initial density of 2.0 x 10⁶ cells/ml in RPMI-1640 GlutaMAX-I media supplemented with 9% heat-inactivated fetal bovine serum (Invitrogen, USA), 0.05 μM 2-mercaptoethanol, 90 U/ml penicillin and 90 μg/ml streptomycin.

Cells were assayed for 96 hours in either the absence (antigen-free control) or presence of 10 μg/ml of P2 peptide or 5 μg/ml of the lectin concanavalin A (Sigma, USA). To determine proliferation indices, 0.5 μCi of [³H]thymidine (6.7 Ci/mmol; MP
Figure 5. Experimental design of forced-exercise and mild EAN. In this experimental model, rats were induced with mild EAN in the base of the tail. After induction, forced-exercise rats continued to run. EAN, experimental autoimmune neuritis; P.I., post-induction.
Figure 6. Experimental design of pre-induction forced-exercise and severe EAN. In this experimental model, rats were induced with severe EAN in the hind left footpad. CFA injection in the hind left footpad of sedentary rats was used as a non-disease control. After induction, forced-exercise rats ceased to run. CFA, complete Freund’s adjuvant; EAN, experimental autoimmune neuritis; P.I., post-induction.
Figure 7. Representative M wave tracings from the rat left plantar muscle stimulated at the ankle or sciatic notch. A recording electrode was placed in the plantar muscle of the rat, and M waves were recorded by stimulating distally at the ankle or proximally at the sciatic notch as described in Materials and Methods (top). Adapted from (Lawlor et al., 2001). Representative tracing from one rat are shown (bottom).
Biomedicals, USA) was added to each culture for the final 24 hours of control-, antigen-, or lectin-stimulation. Cells were briefly trypsinized and collected by centrifugation. DNA was precipitated with ice-cold 10% (w/v) trichloroacetic acid, solubilized with NaOH, neutralized with HCl, and an aliquot was added to 5 ml of Beckman Coulter Ready Safe liquid scintillation counting cocktail. Disintegrations per minute (dpm) of $[^{3}H]$thymidine incorporated into the splenic or nodal lymphocyte DNA was quantified using a Tri-Carb 2810 liquid scintillation analyzer (Perkin Elmer, USA). Stimulation indices were calculated as a ratio of dpm of DNA recovered in antigen- or lectin-stimulated cultures to dpm of DNA recovered in un-stimulated cultures (Sarkey et al., 2007).

**Cytokines**

Splenic or nodal leukocytes harvested near onset (day 14) or near peak (day 18) of severe EAN (Figure 6) were cultured for 96 hours at a density of $2.0 \times 10^6$ cells/ml in the absence or presence of 10 µg/ml of P2 peptide or 5 µg/ml of concanavalin A. Cell culture supernatants were collected and analyzed for the presence of interleukin-2, interleukin-4 interleukin-6, interleukin-10, interleukin-12p70, interferon-γ, and tumor necrosis factor-α pro- or anti-inflammatory cytokines. The content of cytokines present in culture supernatant were quantified simultaneously using multiplex technology with an available Bio-plex system (Bio-Rad Laboratories, USA) and a commercially available rat-specific cytokine Milliplex kit (Millipore, USA).

**Flow Cytometric Analysis**

Splenic, nodal, or blood lymphocytes were prepared from rats sacrificed near onset (day 14) or near peak (day 18) of severe EAN (Figure 6). Blood was collected in 5
mM EDTA tubes.

To determine the relative abundance of CD4$^+$ and CD8$^+$ T cells, splenic, nodal, or blood lymphocyte preparations were co-incubated at a density of 1 x $10^6$ cells for 30 min at 4° C in the presence of 1 µg each of FITC-conjugated mouse anti-rat CD4 (clone W3/25), and PE-conjugated mouse anti-rat CD8α (clone G28) monoclonal antibodies (BioLegend, USA).

To determine the relative abundance of type 1 helper T (T$_{h1}$, CD4$^+$) cells (Yamane et al., 2000; Ozenci et al., 2001; McQuaid et al., 2003; Kano et al., 2008) or activated cytotoxic T (T$_c$, CD8$^+$) cells (McQuaid et al., 2003; Bontkes et al., 2005), splenic, nodal, or blood lymphocyte preparations were co-incubated at a density of 1 x $10^6$ cells for 30 min at 4° C in the presence of 1 µg of rabbit anti-rat IL-12Rβ1 (clone C-20) monoclonal antibody (Santa Cruz Biotechnology) followed by incubation in the presence of 1 µg APC-conjugated F(ab')$_2$ fragment goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, USA) as a secondary antibody.

To determine the relative abundance of type 2 helper T (T$_{h2}$, CD4$^+$) cells (Rojo et al., 2008; Zhu and Paul, 2008; Simpson et al., 2010), prepared splenic, nodal, and blood lymphocyte preparations were co-incubated at a density of 1 x $10^6$ cells for 30 min at 4° C in the presence of 1 µg of FITC-conjugated mouse anti-rat CD4 (clone W3/25) and PE-conjugated hamster anti-rat CD278 (clone C398.4A) monoclonal antibodies (BioLegend, USA).

Co-immunostained cells from splenic or nodal lymphocyte preparations were washed twice with ice-cold serum (5% bovine calf serum)-supplemented PBS. Washed
cells were re-suspended in 1 ml of ice-cold 4% p-formaldehyde PBS (pH 7.4). After 10 min, fixed cells were washed twice and re-suspended in serum-supplemented PBS.

Co-immunostained cells from blood were incubated with CAL-LYSE red blood cell lysing solution (Invitrogen, USA) according to manufacturer’s protocol. After incubation, cells were washed twice and re-suspended in serum-supplemented PBS.

Cytometric data were collected using an available FACS Canto flow cytometer (BD Biosciences, USA) and analyzed with FlowJo software (Tree Star, Inc., USA). Lymphocytes were gated according to their forward and side scatter. In all cases, isotype controls were used to quantify and correct for background fluorescence.

A summary of differentiated T cells and their corresponding cell surface markers used in flow cytometric analyses are summarized in Table 1.

**Corticosterone and Corticosteroid-Binding Globulin**

To determine changes in corticosteroid levels in response to forced-exercise, blood was collected from sedentary control or forced-exercise rats gently sacrificed (CO₂ asphyxiation) near onset (day 14) or near peak (day 18) of severe EAN (Figure 6), plasma prepared and stored at -80º C until use. Rats were sacrificed in the morning between 0500 and 0800 hours. Plasma corticosterone levels were quantified using a commercially available competitive radioimmunoassay kit (Siemens Healthcare Diagnostics, USA).

Corticosteroid-binding globulin (CBG) levels in plasma were similarly quantified by competitive radioimmunoassay according to (Westphal, 1971; Brown et al., 2007). Briefly, plasma (20 µl) was pre-clarified with a dextran-coated charcoal (DCC) solution
Table 1. Summary of differentiated T cells and their corresponding cell surface markers used for flow cytometric analyses.

<table>
<thead>
<tr>
<th>Differentiated T cells</th>
<th>Cluster of Differentiation (CD)</th>
<th>Other Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve helper T (T&lt;sub&gt;h&lt;/sub&gt;) cell</td>
<td>CD4</td>
<td>-</td>
</tr>
<tr>
<td>Type 1 helper T (T&lt;sub&gt;h1&lt;/sub&gt;) cell</td>
<td>CD4</td>
<td>Interleukin-12 receptor β1 (IL-12Rβ1)</td>
</tr>
<tr>
<td></td>
<td>CD212</td>
<td></td>
</tr>
<tr>
<td>Type 2 helper T (T&lt;sub&gt;h2&lt;/sub&gt;) cell</td>
<td>CD4</td>
<td>Inducible T cell co-stimulator (ICOS)</td>
</tr>
<tr>
<td></td>
<td>CD278</td>
<td></td>
</tr>
<tr>
<td>Cytotoxic T (T&lt;sub&gt;c&lt;/sub&gt;) cell</td>
<td>CD8α</td>
<td>-</td>
</tr>
<tr>
<td>Activated cytotoxic T (activated T&lt;sub&gt;c&lt;/sub&gt;) cell</td>
<td>CD8α</td>
<td>Interleukin-12 receptor β1 (IL-12Rβ1)</td>
</tr>
<tr>
<td></td>
<td>CD212</td>
<td></td>
</tr>
</tbody>
</table>

Lymph node and spleen cells were prepared as described in *Materials and Methods*, and gated for lymphocytes according to forward and side scatter.
containing 0.5% activated charcoal (Sigma USA) and 0.05% dextran from *Leuconostoc mesenteroides* (Sigma, USA), centrifuged at 3000 g x 15 min, and the resultant clarified supernatant was diluted 1:2 (v/v) with 10 mM Tris buffer (pH 8.0) containing 1.0 mM EDTA, 10% glycerol (v/v), and 1.0 mM dithiothreitol. Clarified samples were aliquoted (100 µl) to four tubes, each containing 45 nM of [³H]corticosterone (70-78 Ci/mmol) (Perkin Elmer, USA). Unlabeled corticosterone (10 µM, Sigma, USA) was added to two tubes for determining non-specific binding. All tubes were incubated for 24 hours at 4°C. Ice-cold DCC (pH 7.4) was added to each tube and incubated for 10 min at 4°C. Samples were centrifuged at 3,000g at 4°C for 5 minutes to sediment DCC. An aliquot of the centrifuged sample was added to 5 ml of liquid scintillation Beckman Coulter Ready Safe counting cocktail. Disintegrations per minute of [³H]corticosterone were quantified using a Tri-Carb 2810 liquid scintillation analyzer (Perkin Elmer, USA). In all cases, non-specific binding was subtracted from the total binding to calculate specific CBG content.

**Statistical Analyses**

Sedentary and forced-exercise EAN data are expressed as mean ± SEM and sedentary adjuvant control data are expressed as mean, of (n) observations unless otherwise specified. Statistical significance between parametric multiple groups (between sedentary and forced-exercise EAN groups, and between post-induction day 14 and 18) was determined using two-way ANOVA (group × time) followed by a post hoc all pairwise multiple comparison Holm-Sidak test. This statistical post-hoc test was chosen because it is more powerful than the Bonferroni post hoc test and has stricter control of the familywise error rate than Tukey post hoc test (Salkind and Rasmussen, 2007). When
appropriate, two-way repeated-measures ANOVA (group × time) followed by a post hoc all pairwise multiple comparison Holm-Sidak test was used. Non-parametric clinical and neuropathological data were statistically analyzed using a Mann-Whitney non-parametric U test analysis and one-way ANOVA on ranks followed by a post hoc all pairwise multiple comparisons Dunn’s test, respectively. Sedentary adjuvant controls were not included in statistical analyses, but were included in figures as a reference for non-disease values. In all cases, $p < 0.05$ was considered significant.
CHAPTER IV

RESULTS

Forced-Exercise Attenuates Mild Experimental Autoimmune Neuritis

Increasing evidence supports the novel use of exercise as a safe adjunctive strategy for the management of some nervous system disorders, including immune-mediated peripheral neuropathies (Fisher et al., 2003; Garssen et al., 2004; Schulz et al., 2004; Balducci et al., 2006; Roberts et al., 2006; White et al., 2006a; White et al., 2006b; El Mhandi et al., 2007; Fisher et al., 2007; Castellano et al., 2008). First we sought to determine whether forced-exercise protects against autoimmune neuropathy by treadmill exercising adult male Lewis rats three weeks before and during the onset and progression of actively induced mild (base of the tail injection) experimental autoimmune neuritis (EAN) (Figure 5), an established animal model of Guillain-Barré syndrome (Hughes and Cornblath, 2005).

Prior to induction of mild EAN, Lewis rats were randomized to sedentary or forced-exercise training groups. Rats randomized to the forced-exercise group were subjected to daily 60 min sessions of running at a constant rate using a motorized treadmill as described within Methods and Materials. By comparison, rats randomized to the sedentary control group were allowed to explore a similar environment daily for 60 minutes without forced-exercise.

Following sedentary or forced-exercise training, rats were induced with mild
EAN by injecting into the base of the tail an emulsified solution containing 100 µg of a neuritogenic peptide fragment of bovine peripheral nerve P2 protein (residues 53-78) as described within Methods and Materials (Figure 5). Immunization at the base of the tail was chosen as an optimal injection site given the design of subjecting animals to forced-treadmill running. Within two-weeks of immunization, rats developed a monophasic course of paraparesis consistent with mild EAN (Figure 8; Table 1). Sedentary control rats immunized by injection at the base of the tail (Figure 8) developed clinical signs of mild EAN (defined here as a clinical score ≥ 0.5), beginning on post-induction day 12.33 ± 0.59 (n = 18; clinical score 0.61 ± 0.13) reaching peak severity at post-induction day 15.83 ± 0.35 (n = 18; clinical score 2.42 ± 0.33). By comparison, rats undergoing forced-exercise exhibited an attenuated monophasic course of mild EAN beginning on post-induction day 12.63 ± 0.53 (n = 16; clinical score 0.61 ± 0.17, n = 18) reaching peak severity on post-induction day 14.69 ± 0.73 (n = 16; clinical score 1.33 ± 0.27, n = 18). At post-induction days 13 to 18, forced-exercise EAN rats exhibited significantly lower (improved) clinical scores when compared to sedentary control EAN rats (Figure 8). The onset of mild EAN in sedentary rats, however, did not differ significantly from that of forced-exercise rats (p = 0.60) (Table 1). Whereas the clinical severity of mild EAN was significantly attenuated in rats undergoing forced-exercise, the day at which mild EAN peaked in these animals did not differ significantly (p = 0.25) compared with sedentary control EAN rats (Table 1). Two rats from the forced-exercise group failed to develop clinical signs of mild EAN.

Collectively, these data suggest that forced-exercise attenuates the development of
Figure 8. Forced-exercise attenuates mild EAN. Sedentary (open circles) or forced-exercise (closed circles) trained rats were immunized with P2 peptide fragment by injection in the base of the tail at day 0 and were clinically evaluated for signs of mild EAN. Data shown are the means ± SEM (n = 5-18). *p<0.05 vs. sedentary, Mann-Whitney non-parametric U-test.

Table 2. Effect of forced-exercise on development and clinical severity of mild EAN induced by injection in the base of the tail.

<table>
<thead>
<tr>
<th>Mild EAN</th>
<th>Sedentary (n = 18)</th>
<th>Forced-exercise (n = 16-18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical onset (post-induction day)</td>
<td>12.33 (12) ± 0.59</td>
<td>12.63 (12) ± 0.53</td>
</tr>
<tr>
<td>Clinical peak (post-induction day)</td>
<td>15.83 (16) ± 0.35</td>
<td>14.69 (16) ± 0.73</td>
</tr>
<tr>
<td>Peak clinical score</td>
<td>2.42 (2) ± 0.33</td>
<td>1.33 (1) ± 0.27*</td>
</tr>
</tbody>
</table>

Data shown are the means (median) ± SEM. Clinical onset is defined as the first day a minimum clinical score of 0.5 was reached. Clinical peak is defined as the first day a peak clinical score was reached. *p<0.05 vs. sedentary, Mann-Whitney non-parametric U-test. EAN, experimental autoimmune neuritis.
mild EAN. The mechanism by which this occurs was next addressed.

**Effect of Forced-Exercise on Mild EAN-Induced Peripheral Nerve Injury**

Previous studies demonstrate measurable deficits in peripheral nerve function following induction of severe EAN, supportive of an autoimmune-mediated inflammatory demyelinating nerve injury (Gabriel et al., 1997; Taylor and Pollard, 2001, 2003; Lin et al., 2007; Sarkey et al., 2007). Whereas forced-exercise attenuates the clinical severity of mild EAN (Figure 8), we next determined whether the moderate training program used in this study would alter mild EAN-induced changes in peripheral nerve large-fiber function in Lewis rats. The effect of forced-exercise on peripheral nerve function was determined by evoked-response electrophysiology as described within *Methods and Materials* (Figure 5).

As shown in Figure 8, rats immunized by injection in the base of the tail developed a mild course of EAN. Subjecting these animals to forced-exercise afforded a measure of protection against EAN-induced peripheral nerve injury (Figure 9). At post-induction day 18, sedentary control rats with mild EAN exhibited a significant reduction in both ankle- (4.65 ± 0.52 mV, n = 18) \[F_{1,34} = 5.68, p = 0.02\] and sciatic notch- (2.90 ± 0.39 mV, n = 18) \[F_{1,34} = 4.41, p = 0.04\] evoked compound muscle action potential (CMAP) amplitudes compared with pre-induction (day 0) responses (ankle 7.75 ± 0.46 mV, n = 18; sciatic notch 5.48 ± 0.49 mV, n = 18). In marked contrast, rats with mild EAN undergoing forced-exercise exhibited preserved ankle- (6.27 ± 0.57 mV, n = 18) and sciatic-notch- (4.26 ± 0.42 mV, n = 18) evoked CMAP amplitude responses that were statistically indistinguishable compared with their respective pre-induction (day 0)
Figure 9. Effect of forced-exercise on evoked peripheral nerve conduction amplitudes in rats with mild EAN. Relative changes in peripheral nerve CMAP amplitudes were determined by evoke-response electrophysiology prior to and 18 days following immunization with P2 peptide fragment by injection into the base of the tail as described within Methods and Materials. Data shown are the means ± SEM (n = 18) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training. *p<0.05 vs. respective day 0 responses, *p<0.05 vs. respective day 18 sedentary control responses demonstrating protection; two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test.
Figure 10. Effect of forced-exercise on R ratios in rats with mild EAN. Relative changes in peripheral nerve conduction ankle and notch amplitudes were determined by evoke-response electrophysiology, and R ratios calculated, prior to and 18 days following immunization with P2 peptide fragment by injection into the base of the tail as described within Methods and Materials. Data shown are the means ± SEM (n = 18) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training. These data were analyzed by two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Figure 11. Effect of forced-exercise on mild EAN-induced slowing of peripheral nerve conduction velocity. Peripheral nerve conduction velocities were calculated from the experimental data shown in Fig. 6. Data shown are the means ± SEM (n = 18) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training. *p<0.05 vs. respective day 0 responses; two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test.
responses (Figure 9). Absolute values of sciatic-notch evoked CMAP amplitudes were approximately 20% lower in magnitude compared with ankle-evoked responses in sedentary control and forced-exercise groups; there were no differences in R ratios between the groups \([F_{1, 34} = 0.23, p = 0.63]\) or between the post-induction days \([F_{1, 34} = 1.29, p = 0.26]\) (Figure 10). This could occur because of temporal dispersion or uniform demyelination (van der Meche et al., 1988; Harvey and Pollard, 1992; Lin et al., 2007; Daube and Rubin, 2009; Uncini et al., 2010).

Base-of-the-tail injected rats undergoing sedentary (60.15 ± 3.55 m/s, n = 18) or forced-exercise (59.59 ± 3.08 m/s, n = 18) training exhibited nearly identical rates \([F_{1, 34} = 2.74, p = 0.11]\) of motor nerve conduction velocities (MNCVs) prior to immunization (Figure 11, day 0). By comparison, sedentary rats with mild EAN exhibited significant slowing of MNCV (47.79 ± 1.60 m/s, n = 18) by post-induction day 18. Whereas forced-exercise rats with mild EAN also experienced significant slowing of MNCV (50.75 ± 2.29 m/s, n = 18), this occurred to a lesser degree compared with sedentary controls \([F_{1, 34} = 23.5, p < 0.001]\) (Figure 11).

Collectively, these data suggest that forced-exercise training program used in this study significantly attenuates the development of clinically mild EAN and its associated peripheral nerve electrophysiological deficits.

**Pre-Induction Forced-Exercise Attenuates Severe Experimental Autoimmune Neuritis**

We showed that forced-exercise before and during development and progression of mild EAN can attenuate the clinical and electrophysiological deficits of the disease. Next, we investigated whether three weeks of pre-induction exercise, in which Lewis rats
Figure 12. Effect of forced-exercise on weight gain in adult male Lewis rats. Rats undergoing forced-exercise on a motorized treadmill (solid circles) gained weight at a rate that was significantly less than sedentary controls (open circles). Data shown are the means ± SEM (n = 27). *p < 0.05 vs. sedentary control; two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test.
ceased running after induction, would similarly attenuate severe EAN induced by footpad P2 peptide injection. To determine the mechanism by which pre-induction exercise protects against autoimmune neuropathy, we challenged adult male Lewis rats to a moderate regimen of forced-exercise before induction and quantified clinically and immunologically the onset and progression of actively induced severe (hind left footpad injection) EAN (Figure 6).

Prior to induction of EAN, male Lewis rats were randomized to sedentary or forced-exercise training groups. Rats randomized to the forced-exercise group were subjected to daily 60 min sessions of running at a constant rate using a motorized treadmill as described within Methods and Materials (Figure 6). By comparison, rats randomized to the sedentary control group were allowed to explore a similar environment daily for 60 minutes without forced-exercise. As shown in Figure 12, both groups of rats steadily gained body weight throughout the three-week sedentary or forced-exercise training. During this period, sedentary control rats gained body weight at a rate of 2.7 ± 0.1 (n = 27) grams per day, consistent with previously published findings for male Lewis rats fed chow ad libitum (2.7 g/day, Harlan Lewis growth curve). In contrast, forced-exercise rats exhibited a significantly reduced rate (1.8 ± 0.1 g/day, n = 27) of body weight gain \( F_{19, 988} = 43.11, p < 0.001 \) (Figure 12). Post-hoc statistical analysis of these data revealed a significant difference in body weight gain between sedentary and forced-exercise rats between days 15 to 26. These findings suggest that the forced-exercise regimen used in this dissertation was of a moderate level of intensity. Importantly, at no time throughout this study did rats randomized to the forced-exercise group exhibit a
frank loss of body weight. While food consumption between sedentary and forced-exercise groups was not monitored in this study, rats undergoing forced-exercise gained body weight. These findings further suggest that secondary complications of confounding cachexia in response to forced-exercise were minimized.

To determine whether forced-exercise was effective at protecting against a clinically severe form of EAN, rats were immunized by injection in the hind left footpad as previously described (Sarkey et al., 2007). Injection into the footpad necessitated a change in experiment design of particular noteworthy significance. Given the injection site, rats undergoing forced-exercise were unable to sustain physical activity following footpad immunization. Thus, the experimental distinction between sedentary and forced-exercise footpad immunized rats was restricted to physical activity performed prior (pre-induction) to induction of EAN.

As previously reported (Sarkey et al., 2007), footpad-immunized sedentary control rats developed severe EAN (Figure 13; Table 2), beginning on post-induction day 12.33 ± 0.20 (n = 24; clinical score 0.75 ± 0.14) reaching peak severity at post-induction day 17.00 ± 0.33 (n = 12; clinical score 3.99 ± 0.20). Despite cessation of training prior to EAN induction, rats undergoing pre-induction forced-exercise similarly exhibited an attenuated course of severe EAN beginning on post-induction day 12.64 ±0.22 (n = 21; clinical score 0.54 ± 0.12, n = 23) reaching peak severity on post-induction day 15.70 ± 0.15 (n = 10; clinical score 2.74 ± 0.32, n=12 ). At post-induction days 13 to 18, rats undergoing pre-induction forced-exercise prior to EAN induction exhibited significantly lower clinical scores when compared to sedentary control EAN rats (Figure
suggesting a sustained effect of protection elicited by forced-exercise. The onset of EAN between sedentary and forced-exercise footpad immunized rats did not differ significantly ($p = 0.53$) (Table 2). A modest but significant difference in the day at which EAN peaked was observed between sedentary control and forced-exercise rats (Table 2). This difference was most likely due to lessening of disease development and severity by pre-induction forced-exercise. Two rats from the forced-exercise group failed to develop EAN.

Another reliable and reproducible clinical sign of EAN developmental onset and progression is body weight loss (Figure 14). A significant difference in the percentage of body weight between rats injected with complete Freund’s adjuvant (CFA) in the absence of P2 peptide (adjuvant immunization control) compared with sedentary control and forced-exercised rats receiving CFA + P2 peptide was observed [$F_{8, 264} = 4.67$, $p < 0.001$]. Post hoc statistical analyses revealed that at post-induction days 13 through 18, there was a significant attenuation of the percent body weight loss in CFA + P2 peptide injected forced-exercise rats compared with sedentary controls (Figure 14), consistent with exercise-facilitated clinical protection (Figure 13).

Collectively, these data suggest that (i) pre-induction forced-exercise attenuates the development of severe EAN and (ii) the protective effect of pre-induction forced-exercise appears sustainable for several weeks after cessation of treadmill running. The mechanism by which this occurs was next addressed.

**Effect of Pre-Induction Forced-Exercise and Severe EAN on Steady State Content of Corticosterone**

One mechanism by which pre-induction forced-exercise may attenuate
**Figure 13. Pre-induction forced-exercise attenuates severe EAN.** Sedentary (open circles) or forced-exercise (closed circles) trained rats were immunized with P2 peptide fragment by injection in the hind left footpad at day 0 and were clinically evaluated for signs of severe EAN. An adjuvant immunization control group (CFA control, open squares) is shown for comparison. Data shown are the means ± SEM (n = 5-24). *p<0.05 vs. sedentary, Mann-Whitney non-parametric U-test.

**Table 3. Effect of pre-induction forced-exercise on development and clinical severity of severe EAN induced by injection in the hind left footpad.**

<table>
<thead>
<tr>
<th>Severe EAN</th>
<th>Sedentary (n = 12-24)</th>
<th>Forced-exercise (n = 11-21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical onset (post-induction day)</td>
<td>12.33 (12) ± 0.20</td>
<td>12.64 (12.5) ± 0.22</td>
</tr>
<tr>
<td>Clinical peak (post-induction day)</td>
<td>17.00 (17.5) ± 0.33</td>
<td>15.70 (16) ± 0.15*</td>
</tr>
<tr>
<td>Peak clinical score</td>
<td>3.99 (4.25) ± 0.20</td>
<td>2.74 (2.83) ± 0.32*</td>
</tr>
</tbody>
</table>

Data shown are the means (median) ± SEM. Clinical onset is defined as the first day a minimum clinical score of 0.5 was reached. Clinical peak is defined as the first day a clinical peak score was reached. *p<0.05 vs. sedentary, Mann-Whitney non-parametric U-test. EAN, experimental autoimmune neuritis.
Figure 14. Pre-induction forced-exercise attenuates severe EAN-induced loss of body weight. Rats randomized to sedentary (open circles) or forced-exercise (closed circles) were immunized with P2 peptide fragment by injection in the left hind footpad at day 0 and were clinically evaluated for signs of EAN. An adjuvant immunization control group (CFA control, open squares) is shown for comparison. Rats randomized to the sedentary or forced-exercise groups both lost body weight beginning on post-induction day 10. Data shown are the means ± SEM (n = 5-24). *p<0.05 vs. sedentary control; two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test.
development and severity of severe EAN in Lewis rats may involve exercise-dependent increases in circulating levels of the stress hormone, corticosterone (Moraska et al., 2000; Weishaupt et al., 2001; Ploughman et al., 2005; Brown et al., 2007; Ploughman et al., 2007; Ke et al., 2011). The content of corticosterone and corticosterone-binding globulin (CBG) (Figure 15) in plasma prepared from blood were quantified as described within Material and Methods (Figure 6) between 0500 and 0800 hours.

Adjuvant control rats or sedentary or forced-exercise EAN rats were sacrificed by CO2 asphyxiation either at post-induction day 14 or at post-induction day 18. Resting levels of plasma corticosterone content in rats immunized with CFA without P2 peptide (CFA control) were 71 ± 14 ng/ml (n = 6; post-injection day 14) and 177 ± 68 ng/ml (n = 5; post-injection day 18), consistent with previously reported values for adult male Lewis rats at rest (Dhabhar et al., 1995; Martin et al., 2000; Duclos et al., 2001). By comparison, circulating corticosterone levels in sedentary EAN rats were not significantly elevated at post-induction day 14 (116 ± 51 ng/ml, n = 6) or day 18 (140 ± 62 ng/ml, n = 6) (Figure 15A). Interestingly, subjecting Lewis rats to pre-induction forced-exercise prior to severe EAN induction did not appear to alter circulating corticosterone content at post-induction day 14 (91 ± 29 ng/ml, n = 6) or day 18 (138 ± 57 ng/ml, n = 5) (Figure 15A). No statistical differences in the content of plasma corticosterone were observed between sedentary and forced-exercise EAN rats \( F_{1, 19} = 0.06, p = 0.80 \) and between post-induction days 14 and day 18 \( F_{1, 19} = 0.47, p = 0.50 \) for circulating corticosterone.

A limitation to quantifying circulating levels of corticosterone is its known diurnal variation (Fediuc et al., 2006) and its propensity to change rapidly in response to
Figure 15. Effect of pre-induction forced-exercise on circulating corticosterone and CBG content. Plasma corticosterone (A) and corticosterone-binding globulin (CBG) (B) content were quantified as described within Methods and Materials. Adjuvant (CFA control) immunization control (gray bars), sedentary (white bars) or forced-exercise (black bars) rats with severe EAN were sacrificed by CO\textsubscript{2} asphyxiation at post-induction day 14 or day 18. Data shown are the means ± SEM (n = 4-6). These data were analyzed by two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
environmental challenges such as exercise (Moraska et al., 2000; Ploughman et al., 2005; Brown et al., 2007; Ploughman et al., 2007; Ke et al., 2011). To determine if pre-induction forced-exercise training used in this study *chronically* alters steady state levels in corticosterone content, we quantified circulating levels of the carrier protein CBG (Westphal, 1971; Moraska et al., 2000; Brown et al., 2007). When circulating corticosterone is bound to CBG, the bound corticosterone cannot interact with its receptor. A reduction in plasma CBG content would therefore suggest a net increase in free circulating corticosterone that is able to interact with its receptor. As shown in **Figure 15B**, the content of CBG in plasma of adjuvant (CFA control) immunized rats at post-injection day 14 and day 18 were 162 ± 22 nM (n = 6) and 73 ± 13 nM (n = 5), respectively. This is consistent with previously published values for adult male Lewis rats at rest (Dhabhar et al., 1995). By comparison, circulating levels of CBG in sedentary EAN rats at post-induction day 14 (140 ± 41 nM, n = 6) and day 18 (144 ± 50 nM, n = 6) were similar to CFA controls (**Figure 15B**). Pre-induction forced-exercise did not alter CBG content in severe EAN rats at post-induction day 14 (138 ± 23 nM, n = 6) and day 18 (54 ± 4 nM, n = 4) (**Figure 15B**). Similar to plasma corticosterone, there were no differences in circulating CBG content between sedentary and forced-exercise EAN rats [$F_{1, 19} = 0.56, p = 0.47$], and between post-induction days 14 and 18 [$F_{1, 19} = 0.35, p = 0.56$].

Collectively, these findings suggest that pre-induction forced-exercise attenuates the development of severe EAN in adult male Lewis rats by a mechanism that occurs independent of changes in circulating corticosterone content.
Effect of Pre-Induction Forced-Exercise on Severe EAN-Induced Peripheral Nerve Injury

Previous studies demonstrate measurable deficits in peripheral nerve function following induction of severe EAN, supportive of an autoimmune-mediated inflammatory demyelinating nerve injury (Gabriel et al., 1997; Taylor and Pollard, 2001, 2003; Lin et al., 2007; Sarkey et al., 2007). Whereas pre-induction forced-exercise attenuates the clinical severity of severe EAN (Figure 13), we next determined whether the pre-induction moderate training program used in this study would alter severe EAN-induced changes in peripheral nerve large-fiber function in Lewis rats. The effect of pre-induction forced-exercise on peripheral nerve function was determined by evoked-response electrophysiology as described within Methods and Materials (Figure 6).

Sedentary control rats with severe EAN at post-induction day 18 (Figure 16) exhibited a marked reduction in both ankle- (2.60 ± 0.74 mV, n = 6) \([F_{1, 9} = 18.13, p = 0.002]\) and sciatic notch- (2.20 ± 0.64 mV, n = 6) \([F_{1, 9} = 13.95, p = 0.005]\) evoked CMAP amplitudes compared with pre-induction (day 0) responses (ankle 4.82 ± 0.38 mV, n = 6; notch 3.91 ± 0.39 mV, n = 6). Severe EAN rats undergoing pre-induction forced-exercise exhibited similarly marked deficits in ankle- (3.10 ± 0.27 mV, n = 5) and sciatic-notch- (1.99 ± 0.29 mV, n = 5) evoked CMAP amplitude responses compared with their respective pre-induction (day 0) responses (Figure 16). Moreover, severe EAN rats undergoing pre-induction forced-exercise exhibited significant decreases in R ratios (ratio 0.64 ± 0.03, n = 5) compared with their respective pre-induction (day 0) responses (ratio 0.84 ± 0.03, n = 5) and respective sedentary control responses (ratio 0.82 ± 0.11, n = 6) \([F_{1, 9} = 6.21, p = 0.03]\) consistent with injury to peripheral nerves that is not
uniformly distributed in forced-exercised EAN rats (Figure 17). This has been previously described in patients with GBS (van der Meche et al., 1988; Harvey and Pollard, 1992; Lin et al., 2007; Daube and Rubin, 2009; Uncini et al., 2010).

Additionally, severe EAN rats undergoing sedentary or pre-induction forced-exercise training exhibited nearly identical rates of MNCV (46.94 ± 5.58 m/s, n=6) [$F_{1, 9} = 0.02, p = 0.88$] prior to immunization (Figure 18 day 0). By comparison, sedentary rats with severe EAN exhibited marked slowing of MNCV (33.44 ± 1.63 m/s, n = 6) [$F_{1, 9} = 5.65, p = 0.01$] by post-induction day 18, consistent with previously published reports (Sarkey et al., 2007). Forced-exercise EAN rats also experienced a similar slowing of MNCV (35.00 ± 1.63 m/s, n = 6), though it did not reach significance.

Neuropathological changes in sciatic nerves from sedentary or forced-exercise rats with severe EAN were semi-quantified as described within Methods and Materials (Figure 6). Sciatic nerves harvested from sedentary and forced-exercise rats with severe EAN sacrificed at post-induction day 18 exhibited marked histopathological changes consistent with presence of inflammatory infiltrates, myelin ovoids, and axonal damage (Figures 19 and 20). Nerves harvested from the footpad-injected limb (2.24 ± 0.20, n = 5) of sedentary EAN rats exhibited more neuropathological changes compared with the non-injected limb (1.27 ± 0.38, n = 6) (Figure 19 top and Figure 20). The same neuropathological changes between footpad-injected (1.58 ± 0.41, n = 5) and non-injected limb (0.88 ± 0.20, n = 5) was seen in forced-exercise EAN rats (Figure 19 bottom and Figure 20). Sciatic nerves harvested from footpad-injected and non-injected limbs of EAN rats that underwent pre-induction forced-exercise training had lower scores
Figure 16. Effect of pre-induction forced-exercise on evoked peripheral nerve conduction amplitudes in rats with severe EAN. Relative changes in peripheral nerve CMAP amplitudes were determined by evoke-response electrophysiology prior to and 18 days following immunization with P2 peptide fragment by injection into the footpad as described within Methods and Materials. An adjuvant-immunization control group (CFA control, gray bars) is shown for comparison. Data shown are the means ± SEM (n = 3-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training. 
*p<0.05 vs. respective day 0 responses; two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test.
Figure 17. Effect of pre-induction forced-exercise on R ratios in rats with severe EAN. Relative changes in peripheral nerve conduction ankle and notch amplitudes were determined by evoke-response electrophysiology, and R ratios calculated, prior to and 18 days following immunization with P2 peptide fragment by injection into the base of the tail as described within Methods and Materials. An adjuvant-immunization control group (CFA control, gray bars) is shown for comparison. Data shown are the means ± SEM (n = 3-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training. *p<0.05 vs. respective day 18 sedentary control responses demonstrating length-dependent demyelination; two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test.
Figure 18. Effect of pre-induction forced-exercise on peripheral nerve conduction velocity in rats with severe EAN. Peripheral nerve conduction velocities were calculated from the experimental data shown in Fig. 8. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. Data shown are the means ± SEM (n = 3-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training. #p<0.05 vs. respective day 0 responses; two-way repeated measures ANOVA with post hoc Holm-Sidak multiple comparison test.
than sedentary EAN rats (Figure 19 and Figure 20); however, the neuropathological scores between the two groups did not differ significantly ($H_{3,17} = 6.76, p = 0.08$).

Collectively, these data suggest that while the pre-induction forced-exercise training program used in this study significantly attenuates the clinical severity of severe EAN; however, forced-exercise is most effective at protecting against mild EAN.

**Pre-Induction Forced-Exercise Alters Immune Cell Function in Rats with Severe EAN**

To determine whether pre-induction forced-exercise attenuates clinical severe EAN by altering cellular immunity, sedentary control or forced-exercise rats induced with severe EAN were sacrificed near disease onset (post-induction day 14) or near peak of disease (post-induction day 18), and splenic or nodal lymphocytes from spleen or popliteal lymph nodes, respectively, were harvested, purified, and analyzed as described within Material and Methods (Figure 6).

Pre-Induction Forced-Exercise Promotes Lymphocyte Retention

Significant differences in the total number of lymphocytes recovered from popliteal lymph nodes of sedentary or forced-exercise EAN rats were observed [$F_{1,27} = 6.82, p = 0.02$] (Figure 21). Near peak of severe EAN, the total number of nodal lymphocytes recovered from forced-exercise EAN rats was significantly greater (~2-fold) compared with sedentary EAN control rats (Figure 21), suggesting increased retention. Pre-induction forced-exercise increased retention of lymphocytes near onset of severe EAN; however, differences between sedentary and forced-exercise groups did not reach statistical significance. Lymphocyte retention is summarized in Table 4.
Figure 19. Effect of pre-induction forced-exercise on sciatic nerve neuropathology in rats with severe EAN. Sciatic nerves from P2 peptide injected (left) and un-injected (right) hind limbs were harvested from sedentary (top) and forced-exercise (bottom) EAN rats near peak (post-induction day 18) of disease, and histologically processed and imaged as described in Materials and Methods. Scale bar (red) = 25 μm.
Figure 20. Pre-induction forced-exercise does not protect against severe EAN-induced neuropathological changes in the sciatic nerves. Rats that were forced-exercised on a treadmill (black bars) or remained sedentary (white bars) were induced with severe EAN in the hind left footpad. Sciatic nerves were harvested at post-induction day 18 and prepared as described in Materials and Methods. Two blinded observers scored the embedded nerves; the scores were averaged. Data shown are the means ± SEM (n = 5-6).
Pre-Induction Forced-Exercise Promotes P2 Peptide-Stimulated Lymphocyte Proliferation

Lymphocytes prepared from harvested popliteal lymph nodes were assayed in vitro for non-specific (lectin-stimulated) and P2 peptide specific proliferation functional capacity as described within Methods and Material (Figure 6). Stimulation indices (SI) were obtained by dividing lectin- or P2-stimulated proliferation responses by media-stimulated control proliferation responses.

Significant differences in P2 peptide antigen stimulated proliferation of lymphocytes harvested from sedentary or forced-exercise EAN rats were observed \(F_{1,15} = 4.55, p = 0.05\). Lymphocytes harvested near onset of disease from sedentary EAN control rats exhibited a ~3-fold increase in P2 peptide-stimulated indices compared with CFA-immunization controls (Figure 22), consistent with disease onset. Forced-exercise EAN P2 peptide-stimulated proliferative responses were increased by ~2.5-fold compared to CFA-immunization; however, these proliferative responses were not significantly different from sedentary EAN control P2 peptide-stimulated proliferative responses. This slightly blunted proliferative response may indicate that forced-exercise alters the innate immune cell capacity to respond to P2 peptide and to initiate proliferation of lymphocytes as the EAN develops. In contrast, lymphocytes harvested from sedentary EAN control rats near peak of disease did not retain their ability to respond to P2 peptide-stimulation (Figure 22), consistent with lymphocyte anergy. Interestingly, lymphocytes harvested from forced-exercise EAN rats retained their ability to respond to P2 peptide-stimulation (Figure 22). These findings suggest that pre-induction forced-exercise retains autoreactive leukocytes to P2 peptide in the lymph
node.

To determine if the effect of pre-induction forced-exercise on lymphocyte proliferation is selective for P2 peptide-stimulation, parallel cultures of harvested and prepared lymphocytes were challenged *in vitro* with concanavalin A (ConA). This lectin potently non-selectively stimulates proliferation of leukocytes through toll-like receptors (TLRs) (Rostami et al., 1990; Rostami and Gregorian, 1991; Stenger and Modlin, 2002; Toubi and Shoenfeld, 2004; MacLeod and Wetzler, 2007; Yun et al., 2007; Unitt and Hornigold, 2011). Our results show that nodal lymphocytes harvested and prepared from CFA immunization control rats exhibited a robust (~40-fold) increase in stimulation indices near onset and peak, respectively (Figure 23). Nodal lymphocytes harvested from sedentary EAN control rats responded by exhibiting a ~13-fold and ~51-fold increase in proliferation to ConA-stimulation at near onset and peak, respectively. In contrast, both near onset (~5-fold) and near peak (~6-fold) of disease, lymphocytes harvested from forced-exercise rats responded only minimally to ConA-stimulation (Figure 23). Significant differences in ConA-stimulated lymphocyte proliferation were observed between sedentary EAN rats versus forced-exercise EAN rats \([F_{1, 16} = 5.24, p = 0.04]\). No differences, however, were observed between respective groups when comparing near onset and near peak, respectively \([F_{1, 16} = 2.95, p = 0.11]\). Post hoc tests confirmed significant decrease in ConA-stimulated proliferative responses of forced-exercise EAN lymphocytes compared to sedentary EAN lymphocytes near peak of disease. Non-selective lectin-mediated activation of lymphocytes appears compromised by pre-induction forced-exercise, possibly by influencing antigen-presenting cell (APC) TLR
Figure 21. Pre-induction forced-exercise promotes nodal lymphocyte cell retention in popliteal lymph nodes. Popliteal lymph nodes were harvested from each limb and quantified from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) as described within Methods and Materials. Data shown are the means ± SEM (n = 5-9) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. sedentary EAN controls; two-way ANOVA with post-hoc Holm-Sidak multiple comparison analysis.
Figure 22. Pre-induction forced-exercise promotes functional retention of P2 peptide-stimulated lymphocyte proliferation. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and P2 peptide-stimulated lymphocyte stimulation indices determined in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 5-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. sedentary EAN controls; two-way ANOVA with post-hoc Holm-Sidak multiple comparison analysis.
Figure 23. Pre-induction forced-exercise attenuates lectin-stimulated lymphocyte proliferation. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and concanavalin A (ConA)-stimulated lymphocyte stimulation indices determined in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 5-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. sedentary EAN controls; two-way ANOVA with post-hoc Holm-Sidak multiple comparison analysis.
dependence of lymphocyte proliferation (Flynn and McFarlin, 2006). Stimulated lymphocyte proliferation is summarized in Table 4.

Pre-Induction Forced-Exercise Promotes Splenic Lymphocyte Retention

Immune responses of localized nodal lymphocytes may differ from splenic lymphocytes. To determine if pre-induction forced-exercise alters splenic immune responses, spleens were harvested from treated rats and splenic lymphocytes prepared as described within Methods and Materials (Figure 6). When normalized to spleen wet weight, significant differences in total recovered splenic lymphocytes were observed between sedentary or forced-exercise EAN rats \([F_{1, 34} = 10.40, p = 0.003]\). The wet weights of spleens harvested between experimental groups were not statistically different \([F_{1, 33} = 0.19, p = 0.67]\). Near onset of disease, the total number of splenic lymphocytes recovered from spleens harvested from sedentary EAN rats was approximately 20% less compared with spleens from CFA immunization control or forced-exercised EAN rats (Figure 24). A similar trend in recovered splenic lymphocyte count was seen from spleens harvested near peak of disease. These data support the thesis that pre-induction forced-exercise promotes retention of lymphocytes in secondary lymphoid organs.

Splenic lymphocyte retention is summarized in Table 4.

Pre-Induction Forced-Exercise Preserves Lectin-Stimulated Splenic Lymphocyte Proliferation

Splenic lymphocytes prepared from harvested spleens were assayed in vitro for non-specific (lectin-stimulated) and P2 peptide specific proliferation functional capacity as described within Methods and Material (Figure 6). Stimulation indices (SI) were obtained by dividing lectin- or P2-stimulated proliferation responses by media-stimulated
control proliferation responses.

In marked contrast to nodal lymphocytes (Figure 22), splenic lymphocytes prepared from spleens harvested from CFA immunized control, sedentary or forced-exercise EAN rats were found to be largely un-responsive to P2 peptide-stimulation (stimulation index 1.11 ± 0.09, n = 48; combined from all groups), possibly due to inefficient antigen presentation by APCs (DeFranco et al., 2007).

By comparison, significant differences in ConA-stimulated proliferation of splenic lymphocytes harvested from sedentary or forced-exercise EAN rats were observed [$F_{1, 33} = 17.33, p < 0.001$]. Splenic lymphocytes harvested near onset or near peak of disease from sedentary EAN control rats exhibited a similar proliferative response to ConA compared with splenic lymphocytes from CFA-immunization controls (Figure 25). In contrast, splenic lymphocytes harvested from forced-exercise EAN rats near onset or near peak of disease exhibited a ~2-fold increase in stimulation indices compared with splenic lymphocytes from sedentary EAN control rats (Figure 25). In marked difference to nodal lymphocytes, these data suggest that pre-induction forced-exercise promotes retention (Figure 24) and preserves proliferative functional capacity (Figure 25) of splenic lymphocytes. Stimulated splenic lymphocyte proliferation is summarized in Table 4.

**Effect of Pre-Induction Forced-Exercise on Nodal Leukocyte Cytokine Production**

To further elucidate the influence of forced-exercise on immune cell function, we characterized the cytokine profile of nodal-derived leukocytes. We chose to evaluate representative pro-inflammatory (IL-2, IL-6, IFN-γ, TNF-α) and anti-inflammatory
Figure 24. Pre-induction forced-exercise promotes retention of splenic lymphocytes. Spleens were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18), weighed, and total recovered splenic lymphocytes quantified as described within Methods and Materials. Data shown are the means ± SEM (n = 5-19) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. sedentary EAN controls; two-way ANOVA with post-hoc Holm-Sidak multiple comparison analysis.
Figure 25. Pre-induction forced-exercise preserves lectin-stimulated splenic lymphocyte proliferation. Spleens were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and concanavalin A (ConA)-stimulated splenic lymphocyte stimulation indices determined in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 5-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. sedentary EAN controls; two-way ANOVA with post-hoc Holm-Sidak multiple comparison analysis.
Table 4. Summary of retention and proliferation of nodal and splenic lymphocytes from pre-induction forced-exercise rats compared to sedentary rats sacrificed near onset or near peak of severe EAN.

<table>
<thead>
<tr>
<th>Severe Experimental Autoimmune Neuritis</th>
<th>Popliteal Lymph Nodes</th>
<th>Spleen</th>
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<td>Retention</td>
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<tr>
<td>Stimulation</td>
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<td>P2 Peptide</td>
<td>Concanavalin A</td>
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<tr>
<td>Onset</td>
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<td>Peak</td>
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Forced-exercise EAN lymphocyte retention and proliferation was compared to respective sedentary EAN control lymphocyte and gauged as follows: ↑, increased retention or proliferation; ↓, decreased retention or proliferation; -, no change.
cytokines (IL-6, IL-10) released by P2 peptide-stimulated cells in vitro from leukocytes (Figures 26-30). Leukocytes were prepared from popliteal lymph nodes harvested from CFA immunized control, sedentary or forced-exercise EAN rats and cytokines simultaneously quantified using multiplex technology as described within Methods and Materials (Figure 6).

Pre-Induction Forced-Exercise Alters Nodal Leukocyte IL-2 Cytokine Production

IL-2 is a proliferative cytokine used in the differentiation of pro-inflammatory Th1 cells and anti-inflammatory Th2 and Treg cells (La Cava, 2008; Zhu and Paul, 2008). The content of IL-2 detected was significantly different in the culture media of un-stimulated (quiescent) leukocytes prepared from nodes harvested from sedentary or forced-exercise EAN rats near onset or peak of disease (Figure 26) \( [F_{1, 16} = 4.85, p = 0.04] \). Near disease peak, there was a significant decrease in IL-2 content from sedentary EAN leukocytes compared to forced-exercise EAN leukocytes.

Due to changes in proliferative functional capacity (Figure 22), P2 peptide-stimulated IL-2 production differed significantly from leukocytes prepared from nodes harvested from sedentary or forced-exercise EAN rats near onset or peak of disease (Figure 26) \( [F_{1, 16} = 22.54, p < 0.001] \). IL-2 production near disease onset was significantly decreased, and significantly increased near disease peak, in forced-exercise EAN leukocytes (38 ± 13 pg/ml and 98 ± 13 pg/ml, n = 5, respectively) compared to sedentary EAN leukocytes (87 ± 9 pg/ml and 31 ± 14 pg/ml, n = 5, respectively). The decrease of IL-2 production near onset corresponds with a blunted proliferative capacity of forced-exercise lymphocytes near disease onset. Moreover, the increase of IL-2
production near peak corresponds with increased proliferative capacity of forced-exercise lymphocytes near disease peak. Non-selective activation of nodal leukocytes with ConA elicited a marked 10-fold increase in IL-2 production that was unaffected by forced-exercise or disease course (Figure 26) \([F_{1, 16} = 0.37, p = 0.55; F_{1, 16} = 0.56, p = 0.47]\).

Pre-Induction Forced-Exercise Does Not Alter Nodal Leukocyte P2 Peptide-Stimulated IL-6 Cytokine Production

IL-6 is a cytokine released from leukocytes that can induce pro-inflammatory or anti-inflammatory differentiation of adaptive immune cells depending on the presence of other pro-inflammatory or anti-inflammatory cytokines (Nielsen and Pedersen, 2008; Zhu and Paul, 2008). IL-6 is increased in leukocyte proliferation assays from EAN rats (Bai et al., 1997; Zhang et al., 2008a; Zhang et al., 2009a). There were minimal un-stimulated (quiescent) and stimulated levels of IL-6 production from adjuvant control rats compared to severe EAN rats (Figure 27). In contrast to IL-2, resting levels of IL-6 released near onset or near peak of disease by nodal leukocytes prepared from nodes harvested from sedentary EAN rats were not different \([F_{1, 16} = 1.26, p = 0.28]\) compared to nodal leukocytes prepared from nodes harvested from forced-exercise EAN rats, and were unaffected by disease course\([F_{1, 16} = 0.72, p = 0.41]\) (Figure 27). Stimulating these prepared leukocytes with P2 peptide did not change the release of IL-6 between sedentary control and forced-exercise EAN rats \([F_{1, 16} = 0.60, p = 0.45]\), or between near disease onset or peak \([F_{1, 16} = 1.23, p = 0.20]\) (Figure 27). Changes in nodal leukocyte IL-6 release elicited by ConA were unaffected by forced-exercise or disease course \([F_{1, 16} = 0.00, p = 0.95; F_{1, 16} = 0.02, p = 0.50]\).
Pre-Induction Forced-Exercise Markedly Enhances Nodal Lymphocyte IL-10 Production

IL-10 is an anti-inflammatory cytokine released from Th2, regulatory T cells, and sometimes Th1 cells (Belkaid and Oldenhove, 2008; La Cava, 2008; Zhu and Paul, 2008). There were minimal levels of IL-10 production from un-stimulated (quiescent) and stimulated lymphocytes prepared from nodes harvested from adjuvant control rats compared to severe EAN rats (Figure 28), and the content of IL-10 detected in the culture media of un-stimulated (quiescent) lymphocytes were similar to previously published findings (Yun et al., 2007). The content of IL-10 detected in the culture media of un-stimulated (quiescent) lymphocytes prepared from nodes harvested from sedentary and forced-exercise EAN rats were indistinguishable [$F_{1,16} = 3.33, p = 0.09$], and unaffected by disease course [$F_{1,16} = 1.89, p = 0.19$]. The content of IL-10 detected in the culture media of P2 peptide-stimulated lymphocytes prepared from nodes harvested from sedentary and forced-exercise EAN rats were markedly different [$F_{1,14} = 4.61, p = 0.05$], and affected by disease course [$F_{1,14} = 7.34, p = 0.02$]. P2 peptide-stimulation increased levels of IL-10 released by nodal lymphocytes prepared from forced-exercise EAN rats near disease onset ($2904 \pm 949$ pg/ml, n = 5) by ~3-fold compared with sedentary EAN controls ($939 \pm 427$ pg/ml, n = 5) (Figure 28). However, only measurable levels of P2 peptide-stimulated IL-10 production from severe EAN rats were detected from lymphocytes harvested near peak of disease, consistent with antigen-stimulated cell anergy. A similar enhancement [$F_{1,15} = 18.30, p < 0.001$] of ConA-stimulated lymphocyte production of IL-10 by forced-exercise ($5304 \pm 306$ pg/ml, n = 4) was observed compared with sedentary controls ($2011 \pm 699$ pg/ml, n = 5) (Figure 28) that
was unaffected by disease course \([F_{1,15} = 1.81, p = 0.20]\).

Pre-Induction Forced-Exercise Prolongs Nodal Leukocyte P2 Peptide-Stimulated IFN-γ Production

IFN-γ is a cytokine released from leukocytes that induces differentiation of pro-inflammatory T_{h}1 cells (Zhu and Paul, 2008) and is increased in leukocyte proliferation assays from severe EAN rats (Bai et al., 1997; Zhang et al., 2008a; Zhang et al., 2009a). There were minimal levels of IFN-γ production from un-stimulated (quiescent) and stimulated lymphocytes prepared from nodes harvested from adjuvant control rats compared to severe EAN rats (Figure 29), and the content of IFN-γ detected in the culture media of un-stimulated (quiescent) lymphocytes were similar to previously published findings (Yun et al., 2007). Resting \([F_{1,16} = 0.38, p = 0.55]\) and P2 peptide-stimulated \([F_{1,15} = 3.52, p = 0.08]\) levels of IFN-γ from sedentary and forced-exercised leukocytes were unaffected by disease course (Figure 29). Resting levels of this pro-inflammatory cytokine in media of nodal leukocytes harvested from forced-exercise EAN rats was markedly elevated by ~8-fold (333 ± 95 pg/ml, n = 5) compared to sedentary EAN controls (42 ± 12 pg/ml, n = 5) \([F_{1,16} = 10.20, p = 0.01]\) near disease onset. Leukocytes from sedentary or forced-exercise EAN rats near disease onset exhibited a robust IFN-γ response (6828 ± 3161 pg/ml and 11298 ± 3943 pg/ml, n = 5, respectively) to P2 peptide antigen stimulation; however, there were no difference between these two groups \([F_{1,15} = 4.09, p = 0.06]\) (Figure 29). Near peak of disease, leukocytes from sedentary EAN control rats were unresponsive to subsequent P2 peptide-stimulation (68 ± 53 pg/ml, n = 4) compared to forced-exercise EAN rats (7247 ± 1942 pg/ml, n = 5); however, the difference does not reach significance \([F_{1,15} = 4.09, p = 0.06]\). This trend
can suggest that nodal leukocytes from forced-exercise EAN rats retained their ability to respond to P2 peptide-stimulation.

**Pre-Induction Forced-Exercise Does Not Alter Nodal Leukocyte TNF-α Cytokine Production**

TNF-α is a pro-inflammatory cytokine released from leukocytes (Leung et al., 2008) and is increased in leukocyte proliferation assays from EAN rats (Bai et al., 1997; Zhang et al., 2008a; Zhang et al., 2009a). The content TNF-α detected was not different in the culture media of un-stimulated (quiescent) leukocytes prepared from nodes harvested from sedentary or forced-exercise EAN rats near disease onset and was largely unaffected by disease course (Figure 30) [group: $F_{1,16} = 0.07, p = 0.80$; time: $F_{1,16} = 0.05, p = 0.83$]. TNF-α production elicited by nodal leukocytes from sedentary (24 ± 6 pg/ml, n = 5) or forced-exercise (34 ± 9 pg/ml, n = 5) EAN rats near disease onset stimulated by P2 peptide was not significantly different [$F_{1,16} = 3.00, p = 0.10$] (Figure 30). Leukocytes harvested near peak of disease from sedentary or forced-exercise EAN control rats exhibited reduced levels P2 peptide-stimulated TNF-α compared near onset [$F_{1,16} = 4.79, p = 0.04$]; however post hoc tests failed to reveal any significance. Non-selective activation of nodal leukocytes with ConA elicited no alterations in TNF-α production between EAN groups [$F_{1,15} = 1.10, p = 0.31$] that was also unaffected by disease course [$F_{1,15} = 1.80, p = 0.20$] (Figure 30).

Released cytokine changes from nodal leukocytes are summarized in Table 5.

**Effect of Pre-Induction Forced-Exercise on Splenic Leukocyte Cytokine Production**

To determine the influence of pre-induction forced-exercise on splenic immune cell function, we characterized the cytokine profile of spleen-derived leukocytes (Figures
Figure 26. Pre-induction forced-exercise alters nodal leukocyte resting or P2 peptide-stimulated IL-2 production. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A-stimulated leukocyte IL-2 cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 5) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. respective sedentary EAN controls, #p<0.05 vs. respective post-induction day 14 values, two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 27. Pre-induction forced-exercise does not alter nodal leukocyte IL-6 production. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A-stimulated leukocyte IL-6 cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 5) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Figure 28. Pre-induction forced-exercise enhances nodal lymphocyte antigen- and lectin-stimulated IL-10 production. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A-stimulated lymphocyte IL-10 cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 4-5) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. respective sedentary EAN controls, #p<0.05 vs. respective post-induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 29. Pre-induction forced-exercise prolongs nodal leukocyte P2 peptide-stimulated IFN-γ production. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A-stimulated leukocyte IFN-γ cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 4-5) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. respective sedentary EAN controls; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Pre-induction forced-exercise does not alter nodal leukocyte TNF-α production. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A-stimulated leukocyte TNF-α cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 4-5) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
31-35). We once again evaluated representative pro-inflammatory (IL-2, IL-6, IL-12p70, IFN-γ, TNF-α) and anti-inflammatory cytokines (IL-4, IL-6, IL-10) released by splenic lymphocytes in vitro. Splenic lymphocytes were prepared from spleens harvested from CFA immunized control, sedentary or forced-exercise EAN rats and cytokines simultaneously quantified using multiplex technology as described within Methods and Materials (Figure 6).

Resting Splenic Leukocyte Cytokine Levels

Despite measurable changes in lectin-stimulated cell proliferation (Figure 25), we were unable to detect resting or P2 peptide-stimulated changes in splenic lymphocyte production of IL-4 or IL-12p70 among spleens harvested from CFA immunization control, sedentary or forced-exercise EAN rats.

Culturing resting splenic lymphocytes harvested from sedentary or forced-exercise EAN rats near onset or near peak of disease did not significantly alter levels of IL-2 (Figure 31) [group: $F_{1,8} = 0.83$, $p = 0.39$; time: $F_{1,8} = 0.22$, $p = 0.65$], IL-10 (Figure 33) [group: $F_{1,20} = 2.51$, $p = 0.13$; time: $F_{1,20} = 0.11$, $p = 0.74$], or IFN-γ (Figure 34) [group: $F_{1,19} = 1.05$, $p = 0.32$; time: $F_{1,19} = 2.42$, $p = 0.14$], or TNF-α (Figure 35) [group: $F_{1,20} = 0.93$, $p = 0.35$; time: $F_{1,20} = 1.66$, $p = 0.21$].

A significant effect of group was observed for resting levels of IL-6 [$F_{1,20} = 5.47$, $p = 0.03$], but a post-hoc analysis suggested no significance between experimental groups (Figure 32). Disease course had no effect for resting levels of IL-6 [$F_{1,20} = 2.15$, $p = 0.16$]. These statistical findings suggest that changes in IL-6 most likely reflect experimental noise due to low resting levels of this cytokine.
P2 Peptide-Stimulated Splenic Leukocyte Cytokine Levels

Culturing P2 peptide-stimulated splenic lymphocytes harvested from sedentary or forced-exercise EAN rats near onset or near peak of disease with P2 peptide did not significantly alter levels of IL-2 (Figure 31) [group: $F_{1,8} = 3.20, p = 0.11$; time: $F_{1,8} = 0.47, p = 0.51$] or IL-10 (Figure 33) [group: $F_{1,20} = 0.52, p = 0.48$; time: $F_{1,20} = 0.79, p = 0.39$].

There were no alterations of IL-6 levels from P2 peptide-stimulation of splenic lymphocytes from sedentary and forced-exercise EAN rats near disease onset or peak [$F_{1,19} = 0.04, p = 0.84$] (Figure 32). Near disease peak, however, splenic lymphocytes from sedentary and forced-exercise EAN rats exhibited a decrease in P2 peptide-stimulated IL-6 production ($0 \pm 0$ pg/ml and $6 \pm 3$ pg/ml, $n = 6$, respectively) compared with splenic lymphocytes from disease onset ($76 \pm 27$ pg/ml and $62 \pm 24$ pg/ml, $n = 6$, respectively) [$F_{1,19} = 11.88, p = 0.003$], indicative of immune cell anergy (Figure 32).

P2 peptide-stimulation of splenic lymphocytes from sedentary and forced-exercise EAN rats harvested near disease onset failed to elicit pro-inflammatory TNF-α cytokine production (Figure 35). Near disease peak, however, splenic lymphocytes from forced-exercise EAN rats exhibited a measurable P2 peptide-stimulated increase in TNF-α production ($85 \pm 36$ pg/ml, $n = 6$) compared with splenic lymphocytes from sedentary EAN control rats ($2 \pm 0.4$ pg/ml, $n = 6$), and from splenic lymphocytes from forced-exercised EAN rats sacrificed at disease onset ($9 \pm 2$ pg/ml, $n = 6$) [$F_{1,20} = 5.33, p = 0.03$].

In contrast to TNF-α, splenic lymphocytes harvested from sedentary or forced-
exercise EAN rats near disease onset responded to P2 peptide-stimulation by increasing the release of pro-inflammatory cytokine IFN-γ (1965 ± 851 pg/ml and 1730 ± 822 pg/ml, n = 6, respectively) (Figure 34), though there were no differences between groups \[F_{1,20} = 0.01, p = 0.92\]. Near peak of disease, the IFN-γ response to P2 peptide was diminished in sedentary control rats (187 ± 95 pg/ml, n = 6) \[F_{1,20} = 7.29, p = 0.01\], consistent with anergy of autoreactive T-cells and the beginning of disease recovery.

There was a similar decrease near disease peak of P2-peptide stimulated splenic lymphocyte production of IFN-γ by forced-exercise leukocytes (298 ± 74 pg/ml, n = 6) compared to near onset levels (1730 ± 822 pg/ml, n = 6) \[F_{1,20} = 7.29, p = 0.01\], however, post hoc tests failed to reveal significance (Figure 35).

ConA-Stimulated Splenic Leukocyte Cytokine Levels

Splenic lymphocytes harvested from all experimental groups responded quite robustly to ConA-stimulation with some unique distinctions influenced by disease course (Figures 31-35). Compared with resting levels, ConA elicited a ~200-fold increase in IL-2 release from splenic lymphocytes harvested near disease onset from CFA immunization control rats (8468 ± 715 pg/ml, n = 4) (Figure 31). Pre-induction forced-exercise attenuated ConA-stimulated release of IL-2 (3928 ± 598 pg/ml, n = 3) compared to sedentary EAN control (6551 ± 376 pg/ml, n = 3) from splenic lymphocytes harvested near disease onset \[F_{1,11} = 10.20, p = 0.01\] (Figure 31). Compared with respective onset values, a decrease in IL-2 production was observed \[F_{1,11} = 10.20, p = 0.01\] near peak of disease for sedentary EAN control rats (4107 ± 605 pg/ml, n = 3) that was indistinguishable to peak forced-exercise EAN IL-2 levels, indicating anergy of sedentary
EAN immune cells.

With regard to IL-6, splenetic lymphocytes harvested from CFA immunization control rats responded to ConA-stimulation (194 ± 32 pg/ml, n = 4) by modestly increasing the production of this cytokine ~2-fold (Figure 32). By comparison, splenic lymphocytes from sedentary or forced-exercise EAN rats similarly responded to ConA-stimulation by enhancing IL-6 release ~9-fold (538 ± 115 pg/ml and 464 ± 145, n = 6, respectively). Near peak of disease, splenic lymphocytes from sedentary EAN rats (33 ± 15 pg/ml, n = 6) exhibited marked reduction in ConA-stimulated IL-6 release compared with respective onset values [F₁, 20 = 11.21, p = 0.003] (Figure 32).

Similar to IL-2, ConA elicited a marked ~15-fold release of TNF-α from splenic lymphocytes harvested near disease onset from all three experimental groups (Figure 35). In contrast, splenic lymphocytes from sedentary EAN control rats harvested near peak of disease responded only marginally (4 ± 1 pg/ml, n = 6) to ConA-stimulation compared with splenic lymphocytes harvested onset sedentary (191 ± 27 pg/ml, n = 6) or from peak forced-exercise (169 ± 19 pg/ml, n = 6) EAN rats (Figure 35) [F₁, 20 = 9.33, p = 0.01].

Pre-induction forced-exercise had little effect on ConA-stimulated release of IL-10 from splenic lymphocytes as shown in Figure 33. Splenic lymphocytes harvested near onset of disease from sedentary (1585 ± 246 pg/ml, n = 6) and forced-exercise (1838 ± 360 pg/ml, n = 6) EAN rats did not differ significantly [F₁, 20 = 1.61, p = 0.21]. This response was affected by disease course [F₁, 20 = 8.07, p = 0.01], with peak sedentary EAN IL-10 production (684 ± 327 pg/ml, n = 6) decreasing compared to onset sedentary
EAN. However, this is probably attributed to experimental noise.

Released cytokine changes from splenic lymphocytes are summarized in Table 5.

**Effect of Pre-Induction Forced-Exercise on severe EAN-Induced T Cell Bias**

Changes in nodal autoreactive lymphocyte cytokine profiling with pre-induction forced-exercise ([Figures 28 and 29]) suggest a possible localized shift from pro-inflammatory type 1 helper T (Th1) cellular immunity toward a more protective type 2 helper T (Th2) cellular immune response. To determine if pre-induction forced-exercise influences T cell bias, spleens and popliteal lymph nodes were harvested from severe EAN rats and prepared splenic and nodal lymphocytes, respectively, and lymphocytes obtained from blood collected from severe EAN rats, were fluorescently labeled as described within *Methods and Materials* ([Figure 6]). Using flow cytometry ([Figure 36]), relative changes in the percent distribution of helper T (Th), Th1, Th2, cytotoxic T (Tc), or activated Tc cells were quantified.

Pre-Induction Forced-Exercise Attenuates Severe EAN-Induced Nodal Th1 Cell Bias

The total percent distribution of helper T cells ([Figure 37]) recovered from popliteal lymph nodes harvested from sedentary or forced-exercise EAN rats was statistically indistinguishable \([F_{1, 28} = 3.27, p = 0.08]\) and unaffected by disease course \([F_{1, 28} = 0.05, p = 0.83]\), demonstrating that pre-induction forced-exercise does not by itself alter localized helper T cell distribution.

By comparison, the percent distribution of Th1 cells present in nodes harvested near disease onset from sedentary EAN control rats was found to be significantly \([F_{1, 28} = 6.57, p = 0.02]\) elevated \((5.10 \pm 0.87 \%, n = 8)\) compared with forced-exercise EAN
Figure 31. Pre-induction forced-exercise attenuates splenic lymphocyte lectin-stimulated IL-2 production. Splenic lymphocytes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A (ConA)-stimulated splenic lymphocyte IL-2 cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 3-4) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. respective sedentary EAN controls, #p<0.05 vs. respective post induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 32. Pre-induction forced-exercise alters splenic lymphocyte lectin-stimulated IL-6 production. Splenic lymphocytes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A (ConA)-stimulated splenic lymphocyte IL-6 cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 4-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. #p<0.05 vs. respective post induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 33. Pre-induction forced-exercise does not alter splenic lymphocyte IL-10 production. Splenic lymphocytes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A (ConA)-stimulated splenic lymphocyte IL-10 cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 4-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. respective post induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 34. Pre-induction forced-exercise does not alter splenic lymphocyte IFN-γ production. Splenic lymphocytes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated) or P2 peptide-stimulated splenic lymphocyte IFN-γ cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 4-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. respective post induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 35. Pre-induction forced-exercise enhances splenic lymphocyte P2 peptide- and lectin-stimulated TNF-α production. Splenic lymphocytes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and resting (un-stimulated), P2 peptide-, or concanavalin A (ConA)-stimulated splenic lymphocyte TNF-α cytokine production and/or release were quantified in vitro as described within Methods and Materials. Data shown are the means ± SEM (n = 4-6) of rats undergoing sedentary (white bars) or forced-exercise (black bars) training prior to severe EAN induction by footpad injection. An adjuvant immunization control group (CFA control, gray bars) is shown for comparison. *p<0.05 vs. respective sedentary EAN controls, #p<0.05 vs. respective post induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Table 5. Summary of cytokine release from cultured nodal and splenic leukocytes from pre-induction forced-exercise rats compared to sedentary rats sacrificed near onset or near peak of severe EAN.

<table>
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<th>Severe Experimental Autoimmune Neuritis</th>
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<tr>
<td></td>
<td>Popliteal Lymph Nodes</td>
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<td>IL-6</td>
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<td>IL-10</td>
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<td>Peak</td>
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<td>IFN-γ</td>
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<td>Peak</td>
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<td>TNF-α</td>
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<td>Onset</td>
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<td>Peak</td>
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Forced-exercise EAN leukocyte cytokine release was compared to respective sedentary EAN control leukocytes and gauged as follows: ↑, cytokine increase; ↓, cytokine decrease; -, no change.
rats (2.91 ± 0.55 %, n = 8) (Figure 38). Near disease peak, the percentage of T\(_h\)1 cells was still elevated in nodes harvested from sedentary EAN control rats (4.15 ± 0.70 %, n = 8) \([F_{1, 28} = 0.40, p = 0.53]\) (Figure 38).

Pre-Induction Forced-Exercise Does Not Affect Nodal T\(_h\)2 Cell Bias

In marked difference to T\(_h\)1 cell bias, no statistical distinctions were apparent between the percent distribution of T\(_h\)2 cells present in nodes harvested from sedentary and forced-exercise EAN groups \([F_{1, 19} = 2.60, p = 0.12]\); however, there was a statistical distinction in lymphocyte percentage between near onset and peak of disease in forced-exercise EAN rats \([F_{1, 19} = 5.78, p = 0.03]\) (Figure 39). This slight increase in T\(_i\)2 cells obtained from peak forced-exercise EAN rats was indistinguishable from peak sedentary EAN rats. These findings strongly suggest that pre-induction forced-exercise does not protect against the clinical development of severe EAN by a mechanism involving a localized Th1/Th2 shift in the cell bias of autoreactive lymphocytes, but instead decreasing the pro-inflammatory T\(_h\)1 response.

Pre-Induction Forced-Exercise Does Not Affect Nodal T\(_c\) Cells

The total percent distribution of T\(_c\) cells (Figure 40) recovered from popliteal lymph nodes harvested from sedentary or forced-exercise EAN rats was statistically indistinguishable \([F_{1, 16} = 0.17, p = 0.68]\) and unaffected by disease course \([F_{1, 16} = 1.32, p = 0.27]\), demonstrating that pre-induction forced-exercise does not alter T\(_c\) cell distribution.

Similarly, no effect of experimental group \([F_{1, 16} = 0.30, p = 0.59]\) or disease course \([F_{1, 16} = 0.33, p = 0.58]\) was apparent regarding the percent distribution of
Figure 36. Representative flow cytometric dot plots of nodal T cell bias from severe EAN rats. Popliteal lymph nodes were harvested from sedentary or forced-exercise EAN rats induced in the footpad near onset of disease. Lymphocytes were identified by gating against the forward and side scatter (A). The percentage (red box) of Th1 cells were further quantified by staining cells (B, top) with fluorescently tagged monoclonal antibodies to cell surface markers CD4 and CD212 compared with (B, bottom) corresponding isotype controls.
activated T<sub>c</sub> cells (Figure 40).

Pre-Induction Forced-Exercise Attenuates Severe EAN-Induced Splenic T<sub>h1</sub> Cell Bias

Whereas pre-induction forced-exercise prevented a localized bias in T<sub>h1</sub> cell distribution (Figure 38) consistent with a lessening of disease severity, we sought to determine if such a protective effect by pre-induction forced-exercise was similarly evident in the spleen.

The total percent distribution of T<sub>h</sub> cells recovered from spleens harvested from sedentary or forced-exercise EAN rats were statistically indistinguishable [F<sub>1,32</sub> = 1.14, p = 0.29], and unaffected by disease course [F<sub>1,29</sub> = 0.22, p = 0.65] (Figure 40). These findings agree well with localized changes in nodal T cell responses (Figure 37) and further support the thesis that pre-induction forced-exercise does not by itself alter helper T cell distribution.

Similar to that observed for nodal T cells (Figure 38), the percent distribution of T<sub>h1</sub> cells present in spleens harvested near disease onset from sedentary EAN control rats (11.41 ± 1.47 %, n = 6) was found to be elevated compared to forced-exercise EAN rats (8.63 ± 0.19 %, n = 6), though failed to reach statistical significance [F<sub>1,20</sub> = 6.21, p = 0.07] (Figure 43). Near disease peak, the percentage of T<sub>h1</sub> cells in spleens harvested from sedentary and forced-exercise EAN rats were significantly reduced [F<sub>1,20</sub> = 27.09, p < 0.001], consistent with the beginning of disease recovery (Figure 43).

Pre-Induction Forced-Exercise Does Not Affect Splenic T<sub>h2</sub> Cell Bias

No meaningful statistical distinctions were apparent between the percent distribution of T<sub>h2</sub> cells present in spleens harvested from sedentary and forced-exercise
EAN rats \([F_{1, 19} = 0.06, p = 0.81]\), and was unaffected by disease course \([F_{1, 19} = 0.80, p = 0.38]\) (Figure 44). These splenic findings strongly suggest that pre-induction forced-exercise does not protect against the clinical development of severe EAN by a mechanism involving a Th1/Th2 shift in the cell bias of autoreactive lymphocytes, but instead protects against a pro-inflammatory Th1 response.

Pre-Induction Forced-Exercise Affects the Percent Distribution of Splenic Activated T<sub>c</sub> cells

The total percent distribution of T<sub>c</sub> cells recovered from spleens harvested from sedentary or forced-exercise EAN rats (Figure 45) was statistically indistinguishable \([F_{1, 20} = 0.16, p = 0.69]\), but was affected by disease course \([F_{2, 20} = 5.49, p = 0.03]\). A reduction in the percent distribution of T<sub>c</sub> cells from spleens of peak forced-exercise EAN rats (17.33 ± 1.11 %, \(n = 6\)) compared to onset forced-exercise EAN rats (12.77 ± 0.91 %, \(n = 6\)) was noted, but this was most likely due to experimental noise. These findings suggest that pre-induction forced-exercise does not alter splenic cytotoxic T cell distribution.

The effect of pre-induction forced-exercise on the percent distribution of activated splenic T<sub>c</sub> cells is shown in Figure 46. Spleens harvested near disease onset from sedentary EAN control rats (6.07 ± 0.35 %, \(n = 6\)) exhibited an elevated percent distribution of activated T<sub>c</sub> cells compared to forced-exercise EAN rats (4.86 ± 0.19 %, \(n = 6\)) \([F_{1, 20} = 4.45, p = 0.05]\). Near peak of disease, spleens harvested from both groups exhibited a similar percent distribution of activated T<sub>c</sub> cells, and were significantly reduced compared to near onset of disease \([F_{1, 20} = 67.08, p < 0.001}\), consistent with
Figure 37. Pre-induction forced-exercise does not affect nodal helper T cell bias.
Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibody to cell surface marker CD4 or isotype control. Data shown are the means $\pm$ SEM (n = 5-8). These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Figure 38. Pre-induction forced-exercise attenuates severe EAN-induced nodal Th1 cell bias. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD4 and CD212, or isotype control. Data shown are the means ± SEM (n = 5-8). *p<0.05 vs. sedentary EAN control; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 39. Pre-induction forced-exercise modestly affects nodal T_h2 cell bias. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD4 and CD278, or isotype control. Data shown are the means ± SEM (n = 5-6). #p<0.05 vs. respective post induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 40. Pre-induction forced-exercise does not affect nodal T<sub>c</sub> cell bias. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibody to cell surface marker CD8 or isotype control. Data shown are the means ± SEM (n = 5-6). These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Pre-induction forced-exercise does not affect activated nodal \( T_c \) cell bias. Popliteal lymph nodes were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD8 and CD212, or isotype control. Data shown are the means \( \pm \) SEM (\( n = 5-6 \)). These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Figure 42. Pre-induction forced-exercise does not affect splenic helper T cell bias. Spleens were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibody to cell surface marker CD4 or isotype control. Data shown are the means ± SEM (n = 5-9). *p < 0.05 vs. CFA immunization control; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis. These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Figure 43. Pre-induction forced-exercise attenuates severe EAN-induced splenic Th1 cell bias. Spleens were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD4 and CD212, or isotype control. Data shown are the means ± SEM (n = 5-6). *p<0.05 vs. respective post-induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 44. Pre-induction forced-exercise does not affect splenic Th2 cell bias. Spleens were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD4 and CD278, or isotype control. Data shown are the means ± SEM (n = 5-6). These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Figure 45. Pre-induction forced-exercise does not affect splenic T<sub>c</sub> cell bias. Spleens were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibody to cell surface marker CD8 or isotype control. Data shown are the means ± SEM (n = 5-6). *p<0.05 vs. respective post-induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 46. Pre-induction forced-exercise does not affect activated splenic Tc cell bias. Spleens were harvested from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated T lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD8, CD212, or isotype control. Data shown are the means ± SEM (n = 5-6). *p<0.05 vs. sedentary EAN control; #p<0.05 vs. respective post-induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
disease recovery.

Pre-Induction Forced-Exercise Attenuates Severe EAN-Induced Circulating \( T_h \)1 Cell Bias

Whereas pre-induction forced-exercise prevented a localized and splenic bias in \( T_h \)1 cell distribution \( (\text{Figures 38 and 43}) \) consistent with a lessening of disease severity, we sought to determine if such a protective effect by pre-induction forced-exercise was similarly evident in circulation.

The total percent distribution of \( T_h \) cells in blood collected from sedentary or forced-exercise EAN rats near disease onset was statistically indistinguishable \( [F_{1, 18} = 2.21, p = 0.16] \) \( (\text{Figure 47}) \). By comparison, blood collected from sedentary control \( (55.97 \pm 0.98 \%, n = 6) \) and forced-exercise \( (57.50 \pm 2.42\%, n = 5) \) EAN rats near disease peak exhibited a modest increase \( [F_{1, 18} = 14.23, p = 0.001] \) in \( T_h \) cells compared to near disease onset \( (50.87 \pm 0.30 \% \) and \( 53.08 \pm 0.61\%, n = 5-6, \) respectively) that was most likely due to return of normal immune surveillance by \( T_h \) cells \( ] \) \( (\text{Figure 47}) \). These findings agree well with changes in nodal and splenic T cell responses \( (\text{Figures 35 and 40}) \) and further support the thesis that pre-induction forced-exercise does not by itself alter helper T cell distribution.

Similar to that observed for nodal and splenic T cells \( (\text{Figure 48}) \), the percent distribution of \( T_h \)1 cells present in blood collected from sedentary EAN control rats was found to be significantly \( [F_{1, 18} = 7.61, p = 0.01] \) elevated compared forced-exercise EAN rats \( (\text{Figure 48}) \). At disease onset, sedentary \( (7.01 \pm 0.94 \%, n = 6) \) and forced-exercise \( (5.04 \pm 0.32 \%, n = 5) \) EAN rats had similar percentages of blood \( T_h \)1 cells \( (\text{Figure 48}) \).
Near disease peak, the percentage of Th1 cells in blood collected from sedentary EAN rats (6.21 ± 0.95 %, n = 6) was sustained compared to sedentary onset levels, and significantly increased compared to forced-exercise EAN rats (3.58 ± 0.81 %, n = 5) signifying continued disease status. There was a modest reduction in Th1 cells in blood collected from forced-exercise EAN rats which consistent with the beginning of disease recovery (Figure 48).

Pre-Induction Forced-Exercise Does Not Affect Circulating Th2 Cell Bias

In marked difference to Th1 cell bias, no meaningful statistical distinctions were apparent between the percent distribution of Th2 cells present in blood collected from sedentary or forced-exercise EAN groups [$F_{1,16} = 0.59, p = 0.45$] or between near onset or peak of disease [$F_{1,16} = 0.19, p = 0.67$] (Figure 49). Similar to nodal and splenic findings (Figures 39 and 44), these circulatory findings strongly suggest that pre-induction forced-exercise does not protect against the clinical development of severe EAN by a mechanism involving a Th1/Th2 shift in the cell bias of autoreactive lymphocytes, but instead protects against a pro-inflammatory Th1 response.

Pre-Induction Forced-Exercise Does Not Affect Circulating Tc Cell Bias

The total percent distribution of Tc cells recovered from blood collected from sedentary or forced-exercise EAN rats (Figure 50) was statistically indistinguishable [$F_{1,18} = 1.38, p = 0.26$], but was affected by disease course [$F_{1,18} = 110.0, p < 0.001$]. Near disease peak, all groups had a ~35% decrease in Tc cell percentages compared to near disease onset, signifying the beginning of disease recovery.
Figure 47. Pre-induction forced-exercise does not affect circulating helper T cell bias. Blood was collected from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibody to cell surface marker CD4 or isotype control. Data shown are the means ± SEM (n = 3-6). *p<0.05 vs. respective post-induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 48. Pre-induction forced-exercise attenuates severe EAN-induced circulating Th1 cell bias. Blood was collected from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD4 and CD212, or isotype control. Data shown are the means ± SEM (n = 3-6). *p<0.05 vs. sedentary EAN control; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
Figure 49. Pre-induction forced-exercise does not affect circulating Th2 cell bias.

Blood was collected from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibodies to cell surface markers CD4 and CD278, or isotype control. Data shown are the means ± SEM (n = 3-6). These data were analyzed by two-way ANOVA with post hoc Holm-Sidak multiple comparison test and were found to be statistically indistinguishable.
Figure 50. Pre-induction forced-exercise does not affect circulating T<sub>c</sub> cell bias. Blood was collected from treated rats near onset (post-induction day 14) or near peak of disease (post-induction day 18) and gated lymphocytes were stained with fluorescently tagged monoclonal antibody to cell surface marker CD8 or isotype control. Data shown are the means ± SEM (n = 3-6). p<0.05 vs. respective post-induction day 14 values; two-way ANOVA with post hoc Holm-Sidak multiple comparison analysis.
CHAPTER V
DISCUSSION

Guillain-Barré Syndrome (GBS) is an acute acquired autoimmune mediated human peripheral neuropathy with an incidence of about four cases per 100,000 people. In North America and Europe, GBS typically presents in the form of acute inflammatory demyelinating polyneuropathy (AIDP), where autoreactive pro-inflammatory lymphocytes and macrophages invade the myelin, thus demyelinating and damaging the axon, leading to paraparesis, paralysis, and areflexia of the limbs. The treatment of AIDP/GBS is currently palliative and utilizes non-specific immune-modulating therapies. However, these benefits are generally observed only if treatment is administered early in the course of the disease (Hughes and Cornblath, 2005; Vucic et al., 2009). A better understanding of the autoimmune pathogenesis is crucial for development of better treatments for this peripheral neuropathy.

A major finding of this dissertation is that a three-week regimen of moderate forced-exercise attenuates the clinical severity, weight loss, and peripheral nerve damage of P2 peptide induced experimental autoimmune neuritis (EAN) in Lewis rats, an animal model of GBS. EAN is an inducible autoimmune disease that involves the activation, proliferation, and differentiation of the pro-inflammatory autoreactive adaptive immune cells in the secondary lymphoid organs. These autoreactive adaptive immune cells then egress from the secondary lymphoid organs (e.g. lymph nodes and spleen) to home and
demyelinate peripheral nerves leading to weakness and paralysis in the hind limbs. Exercise has been shown to have anti-inflammatory effects on innate and adaptive immune cells. Our results show for the first time that in forced-exercise EAN rats, there is increased retention of adaptive immune cells in the popliteal lymph nodes and spleens. Lymphocytes obtained from popliteal lymph nodes of forced-exercise EAN rats have an increased capacity to proliferate near peak of disease when stimulated with P2 peptide, and a decreased capacity to proliferate near peak of disease when stimulated with concanavalin A (ConA). Moreover, the supernatant obtained from the lymphocyte proliferation assays of forced-exercise EAN rats near onset and peak of disease show a marked increase in the anti-inflammatory cytokine interleukin-10 (IL-10). Splenic lymphocytes obtained from spleens of forced-exercise EAN rats have an increase capacity to proliferate when stimulated with ConA. Flow cytometric analysis of adaptive immune cells obtained from popliteal lymph nodes, spleen, and blood of forced-exercise EAN rats showed a clear decrease in pro-inflammatory type 1 helper T (T h1) cells. These results illustrate that forced-exercise has anti-inflammatory effects that can attenuate EAN in rats. To our knowledge, this is the first study to showed attenuation of the clinical severity of EAN by moderate forced-exercise.

Forced-exercise via treadmill allows for strict control of when, how long, and how far the rats run. A voluntary wheel-running paradigm was tried, but the male Lewis rats we used ran very little distances. Lewis rats that had access to wheels ran about 2 km per 24-hour period (data not shown). This was judged an unsatisfactory distance, so a 10% food restriction was implemented to increase the running distance of the Lewis rats.
After food restriction, half the rats ran 8 km per a 24-hour period, while the other half of rats ran about 4 km per 24-hour period (data not shown). This phenomenon of high-capacity and low-capacity runners is described in the literature, and most likely due to individual genetic differences between the rats (Britton and Koch, 2005). Due to the extra variability from the food restriction and voluntary wheel-running distances, it was decided that treadmill training would be best exercise model.

However, forced-exercise via treadmill has a negative aspect; rats run against their will, which can be stressful. Studies have shown that, acutely, treadmill training can increase the glucocorticoid corticosterone, and decrease corticosteroid-binding globulin (CBG), the carrier protein for corticosterone (Moraska et al., 2000; Ploughman et al., 2005; Brown et al., 2007; Ploughman et al., 2007; Ke et al., 2011). However, voluntary exercise can also lead to acute rise in corticosterone (Fediuc et al., 2006). In other studies, corticosterone and CBG normalize to physiological levels after eight weeks of chronic treadmill exercise (Leasure and Jones, 2008). In the present study, levels of corticosterone and CBG were not measured during exercise, and were only measured near onset and peak of EAN. Near onset and peak of EAN, there were no difference in stress hormone between sedentary and forced-exercise groups, or between non-EAN rats, but a glucocorticoid difference might have appeared if measurements were taken early in the exercise regimen. The levels of corticosterone and CBG in this study were similar to non-stressed Lewis rats (Dhabhar et al., 1995; Martin et al., 2000; Duclos et al., 2001).

EAN is an autoimmune disease mediated by pro-inflammatory autoreactive T_h,1
cells (Constantinescu et al., 1998; Gold et al., 2000; Maurer and Gold, 2002). However, 
EAN is not the only autoimmune disease mediated by $T_{h1}$ cells (Gold et al., 1997; 
Constantinescu et al., 1998; Elenkov et al., 2000; Gold et al., 2000), and there have been 
studies on how exercise affects these similar autoimmune diseases. For example, the 
effects of forced-exercise were studied in rats with experimental autoimmune 
encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). In these studies 
(Le Page et al., 1994; Le Page et al., 1996), forced-exercise was shown to decrease 
overall clinical severity and delay the onset, but did not decrease peak severity, of EAE in 
rats. However, these studies implemented a different exercise regimen. In these studies, 
the rats underwent acute severe exercise; the rats exercised either two days before or a 
few days after the induction of EAE. In another study (Rossi et al., 2009), mice with EAE 
had access to a voluntary wheel after induction. This acute voluntary exercise attenuated 
the course of EAE. In the present study, a chronic moderate forced-exercise regimen was 
used three weeks before induction of EAN. This pre-induction chronic exercise has been 
shown to have additional beneficial systemic effects, such as decreased inflammation, 
compared to acute exercise (Flynn et al., 2007; Gleeson, 2007; Haaland et al., 2008; 
Campbell et al., 2009; Lira et al., 2009; Rosa Neto et al., 2010; Teixeira-Lemos et al., 
2011b). In our study, chronic pre-induction exercise led to a decrease in clinical severity 
of EAN. Moreover, chronic exercise caused the forced-exercise rats to peak in clinical 
severity 1.3 days earlier than sedentary rats. This could be attributed to exercise 
attenuating the clinical severity of EAN and stunting EAN development. 

In our study, we used two methods of EAN induction in the rat: induction of
severe EAN using P2 peptide emulsified in complete Freund’s adjuvant (CFA) injected into the left footpad or induction of mild EAN using P2 peptide emulsified in CFA injected into the base of the tail. Injection of P2 peptide emulsified in CFA in either the footpad or base of the tail causes a local inflammatory response, edema, and tissue necrosis. We induced mild EAN in the base of the tail because our preliminary experimental design involved rats exercising throughout the course of EAN. Injecting in the footpad would have inhibited these rats from running after induction due to the local inflammatory response. After these preliminary experiments, we investigated whether pre-induction exercise alone could attenuate EAN. Our clinical data show that inducing EAN by footpad injection leads to a more severe disease state than inducing in the base of the tail. In fact, previous reports about the severity of EAN induced in the base of the tail (Zhang et al., 2008b) or in the footpad (Weishaupt et al., 1997; Stienekemeier et al., 1999; Stienekemeier et al., 2001; Ahn et al., 2010) correspond with our data. In other reports, base of the tail injections with twice the amount of antigenic P2 peptide was used to obtain clinical scores on par with footpad injections (Moalem-Taylor et al., 2007; Lonigro and Devaux, 2009).

It is known that the dosage of antigen used to induce EAN will change the clinical severity of EAN (Hahn et al., 1988). In this study, the same dose of P2 peptide was used in the footpad and base of the tail injection. The difference of clinical severity between the two injection sites could be explained by a difference in lymphatics at the injection site and the injection method. Regarding lymphatics, somatic areas subject to environmental hazards, such as the footpads, possess a diffuse regional drainage pattern,
while the base of the tail does not share this lymphatics feature. In addition, the footpad injections (popliteal lymph nodes) are closer to lymphoid organs than the base of the tail injections (gluteal lymph nodes) (Tilney, 1971). Moreover, there were two injection methods used: intradermal or subcutaneous injection. The tail involves an intradermal injection, which allows the immediate uptake of the injected material by the lymphatics. This shortens the time that antigen-presenting cells (APCs) have to process and present the antigen, which causes a less robust innate and adaptive immune response, leading to an attenuated disease course (Tilney, 1970; Romagnani, 2006; Waldner, 2009; Torres-Aguilar et al., 2010). In contrast, the footpad involves a subcutaneous injection, which allows for a far slower uptake of the injected material by the lymphatics and systemic circulation. The popliteal lymph nodes, as a result, receive a steady influx of the injected material (Tilney, 1971). The slow uptake of subcutaneous injection allows for ample time for the uptake, processing, and presentation of the injected material by APCs, which then migrate to the draining lymph to initiate the adaptive immune response (Romagnani, 2006; Waldner, 2009; Torres-Aguilar et al., 2010). Coupled with the diffuse lymphatics, the proximity of popliteal lymph nodes, and the subcutaneous injection, the footpad is optimal for an innate and adaptive immune response leading to robust EAN disease.

In the present study, there was a significant difference in weight gain between sedentary and forced-exercised rats. Both groups gained weight during the weeks before EAN induction, but the forced-exercise group gained significantly less weight. This has been reported in other studies, both in treadmill (Martin et al., 2000; Moraska et al., 2000; Leasure and Jones, 2008) and voluntary wheel-running (Fediuc et al., 2006; Droste et al.,
2007). However, we did not test whether the weight loss was due to changes in metabolic adaptations or food intake (Sherwin, 1998). Weight loss is a symptom of EAN, and the greater the clinical severity of EAN the greater the weight loss (Rostami et al., 1990; Gabriel et al., 1997; Kafri et al., 2005; Lin et al., 2007; Moalem-Taylor et al., 2007; Sarkey et al., 2007; Zhang et al., 2008b; Tran et al., 2010). Both forced-exercise and sedentary rats with severe EAN lost weight, but the forced-exercise rats loss significantly less weight. The attenuated weight loss is consistent with the attenuated clinical severity observed in forced-exercise EAN rats.

Peripheral nerve conduction studies were used to measure the EAN-induced changes of compound muscle action potential (CMAP) amplitudes and motor nerve conduction velocities (MNCVs) in the sciatic and tibial nerves (Gabriel et al., 1997; Taylor and Pollard, 2001, 2003; Lin et al., 2007; Sarkey et al., 2007). CMAP amplitudes and MNCV deficits are indicative of axonal damage and demyelination (Hughes and Cornblath, 2005; Daube and Rubin, 2009). The clinical severity of EAN is associated with deficits of CMAP amplitudes and MNCVs, with the more severe EAN corresponding with greater deficits (Taylor and Pollard, 2001, 2003; Lin et al., 2007; Sarkey et al., 2007). However, in one study, the clinical severity of EAN was not correlated with deficits of CMAP amplitudes and MNCVs; a greater clinical score did not lead to greater CMAP amplitudes deficits (Gabriel et al., 1997). These conflicting reports could be due to the size and body temperature of the rats, type of stimulating and recording electrodes used, and the clinical severity of EAN (Taylor and Pollard, 2001, 2003; Kurokawa et al., 2004). Small but significant changes in clinical severity of EAN
(Gabriel et al., 1997) might be indistinguishable, but large changes can be distinguished (Taylor and Pollard, 2001, 2003; Lin et al., 2007; Sarkey et al., 2007), by peripheral nerve conduction studies.

Exercise is also known to protect from CMAP and MNCV deficits in peripheral neuropathies (Fisher et al., 2003; Balducci et al., 2006; Fisher et al., 2007). Forced-exercise protected against CMAP amplitude deficits in rats induced with mild EAN in the base of the tail. However, pre-induction forced-exercise did not protect against CMAP amplitude deficits in rats induced with severe EAN in the footpad. Exercise also did not protect against MNCV deficits in either EAN induction method. The difference in CMAP amplitude protection between induction methods could be attributed to the more clinically severe EAN in footpad injection than base of the tail. A previous study showed that CMAP amplitude deficits were not observed until after the clinical score revealed paraparesis or paraplegia in the hind legs (Taylor and Pollard, 2001, 2003). EAN induction in the base of the tail leads to a less severe course of disease compared to footpad injection, and forced-exercise decreases clinical severity in both injection methods. Due to the combined effect of injection method and exercise, forced-exercise rats induced in the base of tail never developed paraparesis of the hind legs, a manifestation of severe clinical disease that has been associated with CMAP amplitude deficits. Even though pre-induction forced-exercise decreased clinical severity of severe EAN compared to sedentary rats, both groups developed paraparesis, which were revealed in the nerve conduction studies. A lack of change in CMAP amplitudes between the forced-exercise and sedentary rats with severe EAN injected in the footpad, and a
lack of change in MNCV between the forced-exercise and sedentary rats in both injection methods, could be due to the insensitivity of nerve conduction studies to distinguish small even if meaningful differences in clinical severity of EAN (Gabriel et al., 1997).

CMAP amplitudes stimulated from the rat hip (proximal) or ankle (distal), and recorded from the plantar muscle demonstrate physiological temporal dispersion, which is calculated as an R ratio (proximal to distal ratio, normal ratio > 0.7). A low R ratio (abnormal ratio < 0.7) can signify that abnormal temporal dispersion or conduction block is occurring, signifying segmental or multifocal demyelination (van der Meche et al., 1988; Harvey and Pollard, 1992; Lin et al., 2007; Daube and Rubin, 2009; Uncini et al., 2010). This decrease in R ratio has been described in patients with GBS, suggesting that demyelination of peripheral nerves is not uniformly distributed (van der Meche et al., 1988; Brown and Snow, 1991; Uncini et al., 2010). Specifically, in AIDP, this decrease of R ratios has been shown to occur early in the disease when the proximal CMAP amplitudes have decreased substantially while distal CMAP amplitudes have decreased minimally, while later in the disease both proximal and distal CMAP amplitudes have decreased substantially (Uncini et al., 2010). Even though both proximal and distal CMAP amplitudes were decreased in forced-exercise and sedentary rats with severe EAN there was a decrease of R ratios near peak of disease in forced-exercise EAN rats, but not in sedentary EAN rats. This could signify in forced-exercise EAN rats the distal tibial nerves were relatively spared in comparison to the proximal sciatic nerves while this was not true for sedentary EAN rats. The proximal ends of peripheral nerves are known to be more deficient in the blood-nerve barrier compared to other segments of the nerve, and
are more susceptible to cellular and humoral infiltrates of the immune system (Jacobs et al., 1976; Brown and Snow, 1991). Attenuation of the clinical severity of severe EAN due to pre-induction forced-exercise could be explained by an attenuation of the immunological injury of the distal tibial nerves. In contrast, there were no differences in R ratios between forced-exercise and sedentary rats with mild EAN injected in the base of the tail. This is probably due to decreased clinical severity of the base of tail injection.

Clinical severity of EAN has been correlated with neuropathological changes in sciatic nerves, with greater clinical severity corresponding to greater immune cell infiltrates, edema, demyelination, and axonal damage (Harvey and Pollard, 1992; Gregorian and Rostami, 1994; Spies et al., 1995b; Araga et al., 1999; Pelidou et al., 2000; Laura et al., 2006; Castro et al., 2007; Deng and Zhou, 2007; Sarkey et al., 2007; Yun et al., 2007; Zhang et al., 2008a; Tan et al., 2009; Zhang et al., 2009a; Zhang et al., 2009d; Zhang et al., 2009e; Tran et al., 2010). However, there is a conflicting report of attenuation of clinical severity of EAN without changes in neuropathological score. In this study, there were significant but small changes in EAN clinical severity (difference of about clinical score of 1 to 1.5) (Gabriel et al., 1997). In the studies that reported neuropathological score differences, the differences of EAN clinical severity were much larger (difference of a clinical score of > 2) (Gregorian and Rostami, 1994; Spies et al., 1995b; Araga et al., 1999; Pelidou et al., 2000; Laura et al., 2006; Castro et al., 2007; Deng and Zhou, 2007; Sarkey et al., 2007; Yun et al., 2007; Zhang et al., 2008a; Tan et al., 2009; Zhang et al., 2009a; Zhang et al., 2009d; Zhang et al., 2009e; Tran et al., 2010). The difference in attenuation of EAN could explain the discrepancy in neuropathological
score. In addition, neuropathological changes are also greater in the sciatic nerve of the leg that was injected compared to the sciatic nerve of the un-injected foot (Harvey and Pollard, 1992). In our study, we did not observe a difference in neuropathological score between forced-exercise and sedentary rats with severe EAN injected in the footpad, which correlates with the observed lack of change in CMAP amplitudes and MNCVs. As reported elsewhere (Harvey and Pollard, 1992), we did observe a difference in neuropathological score between injected (left) and un-injected (right) in sedentary and forced-exercise EAN rats, although these difference never reached significance.

EAN is an autoimmune disorder mediated by the pro-inflammatory autoreactive T\textsubscript{h}1 immune cells of the adaptive immune system (Rostami et al., 1990; Rostami and Gregorian, 1991; Takai et al., 1995; Fujioka et al., 2000; Gold et al., 2000; Maurer and Gold, 2002; Schmidt et al., 2003). When the P2 peptide and CFA emulsion is injected into the rat, the innate immune response is activated. APCs, through activation of toll-like receptors (TLRs), phagocytose the emulsion, process and present the P2 peptide in the context of major histocompatibility complex class (MHC) II with co-stimulatory signals CD80 and CD86, and migrate to secondary lymphoid tissue to activate autoreactive naïve T cells, which then proliferate and differentiate into pro-inflammatory autoreactive T\textsubscript{h}1 cells (Stenger and Modlin, 2002; Toubi and Shoenfeld, 2004; Deng and Zhou, 2007; Israeli et al., 2009; Waldner, 2009; Zhang et al., 2009c). These cells then egress from the lymphoid tissue to migrate to and cross the blood-nerve barrier to home in on the P2 protein on the myelin of the peripheral nerves, clonally expand again, release pro-inflammatory cytokines, activating macrophages and inducing Schwann cell apoptosis,
leading to demyelination and axonal damage (Hartung et al., 1995a, b; Fujioka et al., 2000).

Exercise increases circulating APCs (Ho et al., 2001; Chiang et al., 2010; Suchanek et al., 2010), decreases TLRs and alters APC function (Woods et al., 1999; Flynn and McFarlin, 2006; Gleeson et al., 2006; McFarlin et al., 2006; Lambert et al., 2008; Timmerman et al., 2008; Simpson et al., 2009; Oliveira and Gleeson, 2010), increase (Sugiura et al., 2000; Rogers et al., 2008) or decreases (Moraska et al., 2000; Nielsen, 2003; Gleeson et al., 2006; Gleeson, 2007; Rosa Neto et al., 2010) lymphocyte proliferation, increases differentiation of anti-inflammatory T cells and anti-inflammatory cytokines while decreasing pro-inflammatory T cells and cytokines (Lancaster et al., 2004; Pastva et al., 2004; Yeh et al., 2006; Flynn et al., 2007; Gleeson, 2007; da Silva Krause and de Bittencourt, 2008; Donnikov et al., 2008; Haaland et al., 2008; Yeh et al., 2008; Hewitt et al., 2009b; Lowder et al., 2010; Rosa Neto et al., 2010), impairs T cell-mediated induction phase of an in vivo immune response (Harper Smith et al., 2011), and promotes lymphocyte egress into peripheral tissues (Chen et al., 2010; Turner et al., 2010; Adams et al., 2011).

In this study, we quantified immune cells of two lymphoid organs: the popliteal lymph nodes, which are close to, and receive direct lymphatics from, the site of injection; and the spleen, which receives indirect lymphatics through the circulatory system (Cesta, 2006; Willard-Mack, 2006; DeFranco et al., 2007). We observed increases in lymphocyte number near disease peak and splenic lymphocyte concentration near disease onset in forced-exercise EAN rat popliteal lymph nodes and spleens, respectively.
An increase in splenic lymphocyte concentration due to exercise has been previously reported (Ferry et al., 1992). We did not observe an increase in lymphocyte number or splenic lymphocyte concentration in sedentary EAN rat popliteal lymph nodes and spleen, respectively, for the same time points. Both sedentary and forced-exercise EAN rats developed clinical signs, although the forced-exercise rats had attenuated clinical signs of EAN. A probable explanation of the decreased quantity of immune cells in lymph nodes and spleens, and thus a more severe clinical severity, of sedentary EAN rats is an increased egress of these immune cells from these lymphoid organs into the periphery where the cells can home in on peripheral nerve myelin (Fujioka et al., 2000). Exercise has been shown to affect egress and the migration process of immune cells (Chen et al., 2010; Turner et al., 2010; Adams et al., 2011), and our data might support decreased egress of immune cells from lymphoid organs from forced-exercise EAN rats. Sphingosine-1-phosphate is a major mediator in immune cell egress, but currently there are few reports on the effects exercise on sphingosine-1-phosphate which remain equivocal (Schwab and Cyster, 2007; Blachnio-Zabielska et al., 2008; Dube et al., 2011). Further research needs to be conducted to clarify the increased retention of immune cells in forced-exercise animals.

We normalized \((2.0 \times 10^6 \text{ cells/ml})\) and quantified the in vitro stimulated proliferative capacity and cytokine secretion of nodal and splenic lymphocytes from the popliteal lymph nodes and spleens, respectively, of rats with severe EAN. Proliferation assays serve to assess the functional capacity of immune cells (Nielsen, 2003). We also stained specific cell surface markers for lymphocytes from lymph nodes, spleens, and
blood, and performed flow cytometry to quantify T cell subsets involved in severe EAN (Hartung et al., 1995a, b) and altered in exercise (Lancaster et al., 2004; Flynn et al., 2007; Gleeson, 2007; da Silva Krause and de Bittencourt, 2008; Donnikov et al., 2008; Haaland et al., 2008; Lowder et al., 2010; Rosa Neto et al., 2010). Specifically, we looked at helper T (T_h) cells, which are CD4^+ (Zhu and Paul, 2008), and cytotoxic T (T_c) cells, which are CD8^+ (Andersen et al., 2006). From the T_h cells, we further divided this group into pro-inflammatory T_h1 cells, which are CD212^+ (Yamane et al., 2000; Ozenci et al., 2001; McQuaid et al., 2003; Kano et al., 2008), and anti-inflammatory T_h2 cells, which are CD278^+ (Rojo et al., 2008; Zhu and Paul, 2008; Simpson et al., 2010). We further divided T_c cells into activated T_c cells, which are CD212^+ (McQuaid et al., 2003; Bontkes et al., 2005).

Consistent with previous reports (Rostami and Gregorian, 1991; Yun et al., 2007), nodal and splenic lymphocytes from sedentary CFA control rats did not proliferate to P2 peptide and there were a small amount of pro-inflammatory T_h1 cells in the lymph nodes, spleen, and blood.

Sedentary EAN rats near onset of disease had increases in P2 peptide-stimulated lymphocyte proliferation and in pro-inflammatory T_h1 cytokine IFN-γ (Yun et al., 2007; Zhu and Paul, 2008; Bennett and Stuve, 2009). This proliferation data is expected since EAN sedentary rats have pro-inflammatory autoreactive T_h1 cells primed to P2 peptide and will proliferate when stimulated with the antigen (Hughes et al., 1981; Rostami et al., 1990; Rostami and Gregorian, 1991; Yun et al., 2007). We also showed that this increased proliferative capacity returns to baseline with a concomitant decrease of IFN-γ.
near peak of disease in sedentary EAN rats. Through flow cytometry, we showed an increase in $T_h1$ cells in lymph nodes near onset of disease consistent with EAN as $T_h1$-mediated autoimmune disease (Takai et al., 1995; Fujioka et al., 2000; Gold et al., 2000; Maurer and Gold, 2002; Schmidt et al., 2003), and that $T_h1$ cells return to normal levels near peak of disease. Near peak of disease, most effector T cells will undergo apoptosis or regulatory T cell-induced anergy, and lymphocytes will not proliferate or secrete cytokines when stimulated with P2 peptide antigen (Veldman et al., 2006; DeFranco et al., 2007; Vignali et al., 2008).

There have been reports of blunted proliferation (Moraska et al., 2000) or increased proliferation (Sugiura et al., 2000; Rogers et al., 2008) of immune cells, and increased differentiation of anti-inflammatory T cells and secretion of anti-inflammatory cytokines while decreasing pro-inflammatory T cells and cytokines (Jankord and Jemiolo, 2004; Lancaster et al., 2004; Goldhammer et al., 2005; Flynn et al., 2007; Gleeson, 2007; da Silva Krause and de Bittencourt, 2008; Donnikov et al., 2008; Haaland et al., 2008; Rosa Neto et al., 2010) due exercise training. However, there have been no reports on effects of exercise and autoimmune disease on immune cell proliferation. Forced-exercise slightly blunted the capacity of lymphocytes to proliferate to P2 peptide antigen and with a concomitant increase in IL-10 and IFN-γ and a decrease in IL-2 near onset of severe EAN. In contrast, we observed sustained P2 peptide-stimulated lymphocyte proliferation near peak of disease in forced-exercise EAN rats with only a concomitant increase in IFN-γ and IL-2. We also observed in forced-exercise rats decreased differentiation of $T_h1$ cells near disease onset in lymph nodes, and that $T_h1$
cells levels returned to normal near peak of disease.

We observed no differences near onset or peak of disease of T_h (Takai et al., 1995; Yun et al., 2007), T_c (Takai et al., 1995), and activated T_c cells in lymph nodes among the forced-exercised and sedentary EAN rats. We observed a modest increase near peak of disease in T_h2 cells in forced-exercise rats.

Exercise, by releasing IL-6 from contracting muscles, has the capacity to increase dendritic cells (DCs) in circulation and to decrease TLRs on APCs, therefore imposing an anti-inflammatory immature or semi-mature activation status on the innate immune system (Figure 51) (Ho et al., 2001; Flynn and McFarlin, 2006; Gleeson et al., 2006; McFarlin et al., 2006; Belkaid and Oldenhove, 2008; Lambert et al., 2008; Nielsen and Pedersen, 2008; Timmerman et al., 2008; Simpson et al., 2009; Chiang et al., 2010; Frick et al., 2010; Oliveira and Gleeson, 2010; Suchanek et al., 2010). This change in status of activation of APCs, which activate naïve T_h cells, induces a subset of T_h1 cells to secrete both the pro-inflammatory cytokine IFN-γ and the anti-inflammatory cytokine IL-10 (Trinchieri, 2001; Belkaid and Oldenhove, 2008). This unusual secretion of IL-10 is thought to occur by recently activated T_h1 cells. However, IL-10 secretion is transient, and T_h1 cells will then only secrete IFN-γ. This could be a regulatory mechanism to keep in check the pro-inflammatory immune response (Belkaid and Oldenhove, 2008), thus attenuating the clinical severity of EAN. Within the scope of our immune data, when stimulated with P2 peptide, lymphocytes from forced-exercise rats obtained near onset secrete both IL-10 and IFN-γ, and these lymphocytes obtained from popliteal lymph nodes are differentiated into T_h1 cells. These T_h1 cells limit their own proliferation and
differentiation by secreting the anti-inflammatory cytokine IL-10 (Zhu and Paul, 2008). Near peak of disease (Figure 52), these T\(_h\)1 cells lose their ability to secrete the large amounts of IL-10 observed near disease onset. These T\(_h\)1 cells continue to secrete IFN-\(\gamma\) leading to proliferation of lymphocytes. This transient secretion of IL-10 near onset of disease could account for the decreased amount of T\(_h\)1, and the slight increase in T\(_h\)2, cells in the lymph nodes.

Another possibility is that near onset of disease, there is an increase of both T\(_h\)1 cells and IL-10-producing type 1 T regulatory 1 (Tr1) cells, and these cells account for the increase in IFN-\(\gamma\) and IL-10, respectively (Figure 51). Tr1 cells are crucial in regulating pro-inflammatory immune response in autoimmune diseases (Fehervari and Sakaguchi, 2004; Veldman et al., 2006). Tr1 cells are differentiated and activated by immature DCs, and Tr1 cells blunt the proliferation of T\(_h\)1 cells by secreting the anti-inflammatory cytokine IL-10 (Fehervari and Sakaguchi, 2004; Veldman et al., 2006; Belkaid and Oldenhove, 2008). In fact, previous reports have shown a small quantity of regulatory T cells in EAN rats (Yun et al., 2007; Zhang et al., 2009b). Moreover, it has been shown that there are decreased percentages of circulating anti-inflammatory regulatory T cells in GBS patients insinuating dysregulation of pro-inflammatory T cells (Pritchard et al., 2007; Vucic et al., 2009). Exercise has been shown to increase immature DCs (Suchanek et al., 2010) which are implicated in inducing regulatory T cells (Fehervari and Sakaguchi, 2004; Veldman et al., 2006; Frick et al., 2010). Exercise has been shown to increase regulatory T cells (Lowder et al., 2010). It could be possible that after onset of disease the Tr1 cells have migrated into circulation and into the peripheral
nerves (Figure 52) (Gillen et al., 1998; Iellem et al., 2001), thus attenuating the clinical severity of the disease. When near peak of disease, Th1 lymphocytes no longer have regulation by Tr1 cells, thus Th1 cells continue to proliferate and release IFN-γ. Tr1 cell secretion of IL-10 near onset of disease could account for the decreased amount of Th1, and the slight increase in Th2, cells in the lymph nodes.

Both of these explanations also account for the decreased secretion of IL-2 in pre-induction forced-exercised. IL-10 producing Th1 or Tr1 cells induced from immature or semi-mature DCs decrease the pro-inflammatory Th1 cell response, and thus decrease IL-2 in our proliferation assays (Fehervari and Sakaguchi, 2004; Veldman et al., 2006; Zhu and Paul, 2008; Frick et al., 2010). An increase in IL-2 seen near disease peak can be due to a decrease in IL-10 production from Th1 cells, or the egress of IL-10-producing Tr1 cells from secondary lymphoid organs.

We also showed increased levels of TNF-α from severe EAN lymphocytes compared to non-EAN lymphocytes, but were no differences compared to forced-exercise and sedentary EAN lymphocytes. The most likely contributor of TNF-α are proliferating macrophages (Elenkov and Chrousos, 2002) or Tc cells (Johnson et al., 2010). Macrophages and Tc cells contribute to EAN pathogenesis (Constantinescu et al., 1998; Pelidou et al., 2000; Hughes and Cornblath, 2005; Yun et al., 2007).

Concerning splenic lymphocyte proliferation, we saw no increase in the capacity of immune cells to proliferate when stimulated with P2 peptide in EAN rats. Lymph nodes contain resident dendritic cells (DCs), APCs that are dedicated to presenting antigen to T cells. The resident DCs are naive until they encounter the immunogenic P2
peptide antigen, and these DCs contribute to stimulated lymphocyte proliferation. The spleen does not contain naïve DCs, but instead contains macrophages that are intended to encounter antigen from the blood and activated DCs that migrated to the spleen. Macrophages are APCs, but are not as efficient as DCs at processing and presenting the antigen to T cells (DeFranco et al., 2007). In some reports, there were increases in P2 peptide-stimulated splenic lymphocyte proliferation because irradiated thymocytes were used as APCs to activate the adaptive immune cells (Rostami et al., 1990; Rostami and Gregorian, 1991). Thymus has a large population of DCs used for positive and negative selection of autoreactive T cells (DeFranco et al., 2007), so irradiated thymocytes would contain naïve DCs. We believe that the macrophages in our splenic lymphocyte proliferation assays were incapable of efficiently presenting the P2 peptide to the T cells, and that we might have had increases in proliferation if we had used irradiated thymocytes. We would also note that although we did not see P2 peptide-stimulated splenic lymphocyte proliferation, we did observe P2 peptide-stimulated splenic lymphocyte production of TNF-α in the forced-exercise rats. This P2 peptide-stimulated exercise-induced production of TNF-α is probably secreted by CD8+ T cells that have migrated to the spleen (Sipos et al., 2008). TNF-α is also produced by macrophages since exercise is known to increase macrophage function (Elenkov and Chrousos, 2002; Chen et al., 2010).

Nodal and splenic lymphocytes were also stimulated with ConA, a plant lectin that increases T cell subset proliferation through activation of macrophages and immature dendritic cells through signaling of TLRs (Rostami et al., 1990; Rostami and Gregorian,
Our data show no difference between forced-exercise and sedentary EAN rats in ConA lymphocyte proliferation near onset, even though there were more T\textsubscript{h}1 cells in sedentary EAN lymph nodes. In contrast, our data show an increase in ConA lymphocyte proliferation in sedentary EAN rats near peak of disease, even though there were equal levels of T\textsubscript{h}1 cells between sedentary and forced-exercise EAN rats. The decreased lymphocyte proliferation in forced-exercised EAN rats correlates with an observed increase in the anti-inflammatory cytokine IL-10.

Activation of TLRs on APCs, depending on the activation status of APCs, is known to differentiate and activate naïve T cells to either a pro-inflammatory or an anti-inflammatory immune state (Kapsenberg, 2003; MacLeod and Wetzler, 2007). For example, exercise, by releasing heat shock proteins into the blood, decreases expression of TLR1, 2, and 4 on APCs (Flynn and McFarlin, 2006), and this increases the anti-inflammatory effects of immature DCs that leads to an anti-inflammatory immune state (IL-10 production) (Ozdemir et al., 2009). Hence, we see in our results decreased proliferation with corresponding production of IL-10 in forced-exercise EAN lymphocytes. However, we saw a decrease in proliferation in sedentary EAN lymphocytes near onset. A simple explanation of these data is that there are decreased amounts of immature DCs in the lymph nodes in which to react to ConA and to activate naïve T cells; mature DCs, in contrast, do not react to ConA due to a decrease in TLR signaling (MacLeod and Wetzler, 2007). Near peak of disease, immature DCs could reappear due to clearance of the P2 peptide, and when stimulated with ConA, DCs can
cause robust proliferation of lymphocytes.

In regards to the splenic lymphocytes, we observed an increase in ConA-stimulated proliferation and decreased quantity of T\(_h\)1 cells in forced-exercise rats near onset and peak. Sedentary EAN rats have an increased quantity of T\(_h\)1 cells. The spleen contains resident macrophages, and not naïve DCs, and ConA is a potent activator of macrophages and naïve DCs through TLRs (Toubi and Shoenfeld, 2004; DeFranco et al., 2007; MacLeod and Wetzler, 2007). Moreover, naïve T cells are unresponsive to TLR ligands, and TLRs are only up-regulated with T cell receptor activation (MacLeod and Wetzler, 2007). Since sedentary EAN rats have more T\(_h\)1 differentiation, it is feasible that macrophages have activated by T cells through the T cell receptor, and macrophages have egressed from the spleen and are not available to proliferate to ConA or to activate naïve T cells. In fact, exercise is known to suppress macrophage migration, and increase macrophage function and secretion of IL-6 and TNF-α (Sugiura et al., 2000; Chen et al., 2010; Teixeira-Lemos et al., 2011a), as has been observed in our experiments. The increased proliferation observed in forced-exercise EAN splenic lymphocytes could be attributed to an increase in basal levels of macrophages in the spleen, but also to increased capacity of the macrophages to proliferate and activate T cells. The increase in TNF-α and IL-6 in ConA-stimulated splenic lymphocytes from forced-exercise EAN rats could be due to production of TNF-α by macrophages or CD8\(^+\) cells (Elenkov and Chrousos, 2002; Sipos et al., 2008; Chen et al., 2010), and IL-6 by macrophages (Figure 53) (Teixeira-Lemos et al., 2011a).

We observed no differences near onset or peak of disease of T\(_h\) (Takai et al.,
1995), T\(_c\) (Takai et al., 1995), and T\(_{h2}\) cells in the spleen among the forced-exercised and sedentary EAN rats. We did, however, observed near onset an increase in activated T\(_c\) cells in the spleen in sedentary EAN rats. In patients with MS, and in animal models of MS, there is an increase in these effector CD8\(^{+}\) cells (Johnson et al., 2010), and an increase in activated T\(_c\) cells is correlated with an increase in T\(_{h1}\) cells (Joshi and Kaech, 2008). As observed in our study, the increase of T\(_{h1}\) cells corresponds with an increase in activated T\(_c\) cells.

Differentiated T cells in the lymph nodes and spleen must egress to the blood to home in on nerves (Hartung et al., 1995a, b; Fujioka et al., 2000). Exercise has been shown to increase egress of lymphocytes from the blood into peripheral tissues (Chen et al., 2010; Turner et al., 2010; Adams et al., 2011). We observed no differences near onset or peak of disease of T\(_h\) (Takai et al., 1995), T\(_c\) (Takai et al., 1995) and T\(_{h2}\) cells in the blood among the forced-exercised and sedentary EAN rats. We observed near onset and peak an increase in T\(_{h1}\) cells in the blood in sedentary EAN rats, similar to what we observed in the lymph nodes and spleen.

A weakness of this study is that innate immune cell quantity, type, TLR surface expression, and function were not measured. In our study, we used molecular mimicry to activate the innate immune response against the P2 peptide antigen, which then activated the adaptive immune system (Romagnani, 2006; Waldner, 2009; Torres-Aguilar et al., 2010). The innate immune system is crucial for the development of EAN. The innate immune system also undergoes changes to exercise (Ho et al., 2001; Flynn and McFarlin, 2006; Gleeson et al., 2006; McFarlin et al., 2006; Lambert et al., 2008; Timmerman et al.,
2008; Simpson et al., 2009; Chiang et al., 2010; Oliveira and Gleeson, 2010; Suchanek et al., 2010), which can lead to anti-inflammatory state of the adaptive immune system (Lancaster et al., 2004; Flynn et al., 2007; Gleeson, 2007; da Silva Krause and de Bittencourt, 2008; Donnikov et al., 2008; Haaland et al., 2008; Rosa Neto et al., 2010). The attenuation of EAN in forced-exercised rats is due to immunological changes occurring in these rats, but only the adaptive arm of the immune response was studied. The hypothesis that the anti-inflammatory effect of exercise is due to decreased TLR signaling on APCs that leads to secretion of IL-10 by either Th1 or Tr1 cells at the onset of disease needs to be further studied.

In summary, we show an attenuation of the clinical severity of EAN by forced-exercise. Lymphocytes obtained from popliteal lymph nodes of forced-exercise EAN rats are in an anti-inflammatory immune state demonstrated by their secretion of the anti-inflammatory cytokine IL-10. Flow cytometric analysis of adaptive immune cells obtained from popliteal lymph nodes, spleens, and blood of forced-exercise EAN rats showed a clear decrease in pro-inflammatory Th1 cells. These results illustrate that forced-exercise has anti-inflammatory effects that can attenuate EAN in rats.
Figure 51. Pre-induction forced-exercise attenuates severe EAN by decreasing nodal Th1 differentiation, and secretion of IL-2, while increasing IL-10 near disease onset. At onset of experimental autoimmune neuritis (EAN), mature dendritic cells (mDCs) have high expression of MHCII, CD80, and CD86, which leads to robust proliferation and differentiation of type 1 helper T (Th1) cells and secretion of interferon-γ (IFN-γ) and interleukin-2 (IL-2). Exercise, by decreasing toll-like receptors (TLRs), and increasing circulating interleukin-6 (IL-6), increases immature (iDCs) and semi-mature (smDCs) dendritic cells that induce transient interleukin-10 (tIL-10)-producing Th1 cells, and/or IL-10-producing type 1 regulatory T (Tr1) cells, which causes decreased differentiation of Th1 cells and secretion of IL-2, while secreting of IFN-γ and IL-10.
Figure 52. Pre-induction forced-exercise alters severe EAN-production of nodal lymphocyte IFN-γ near disease peak. Near peak of experimental autoimmune neuritis (EAN), type 1 helper T (Th1) cells are undergoing apoptosis or anergy, thus decreasing immune cells proliferation and interferon-γ (IFN-γ) secretion. Exercise, by decreasing toll-like receptors (TLRs), and increasing interleukin-6 (IL-6), induced transient interleukin-10 (tIL-10)-producing Th1 cells. At peak, these tIL-10 Th1 cells become Th1 cells that no longer secrete IL-10, but only secrete IFN-γ and interleukin-2 (IL-2), which increases immune cell proliferation. Another possibility is that IL-10-producing type 1 regulatory T cells have egressed from the lymph nodes, thus disinhibiting Th1 cells that secrete IFN-γ and interleukin-2 (IL-2), which increases immune cell proliferation.
Figure 53. Pre-induction forced-exercise alters severe EAN-induced splenic lymphocyte proliferation and cytokine secretion near disease onset and peak. Near onset or peak of experimental autoimmune neuritis (EAN), type 1 helper T (Th1) cells activate macrophages. Exercise decreases macrophage migration from the spleen, and increases cytokine secretions of IL-6 and TNF-α, which leads to further activation of T cells and proliferation near disease onset and peak.
REFERENCES


Balducci S, Iacobellis G, Parisi L, Di Biase N, Calandriello E, Leonetti F, Fallucca F


Hurley BF, Hanson ED, Sheaff AK (2011) Strength training as a countermeasure to aging


Kaida K, Kusunoki S (2009) Guillain-Barre syndrome: update on immunobiology and


training on type 1 and type 2 T lymphocytes. Exerc Immunol Rev 10:91-106.


Stienekemeier M, Herrmann T, Kruse N, Weishaupt A, Weilbach FX, Giegerich G,


Zhang Z, Zhang ZY, Schluesener HJ (2009a) Compound A, a plant origin ligand of
glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. J Immunol 183:3081-3091.


VITA

Michael W. Calik was born on December 1, 1982, in Oak Lawn, Illinois, along with his monozygotic twin, Martin O. Calik, and is the son of Polish immigrants, Henryk and Barbara Calik. He attended Loyola University Chicago in Illinois, where he earned a Bachelor of Science degree in Biology in 2005 and gained his interest in the interdisciplinary field of neuroscience.

Michael entered the Neuroscience Graduate Program at Loyola University Chicago in August of 2005. He joined the laboratory of Dr. Evan B. Stubbs, Jr. in 2006, where he began his work on the effects of forced-exercise on experimental autoimmune neuritis, a rat model of the human autoimmune disease Guillain-Barré Syndrome. During his time at Loyola, Michael has presented his research at the Society for Neuroscience conferences in Chicago and San Diego and at the American Society of Neurochemistry meeting in Santa Fe. He served as GSC co-president for the Graduate Student Council and in 2008 and 2009 received the VA Pre-Doctoral Associated Health Rehabilitation Research Fellowship to support his training.

After completing his doctorate, Michael will continue a research and academic career as a postdoctoral fellow with the ultimate goal of becoming principal investigator and professor at an academic institution. Michael married his wife, Marilyn, in 2007, and since then has fathered a son, Tristan, and a daughter, Lyra. They currently reside in Chicago, Illinois.
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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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