Evaluation of Immune-Modulating Therapies For Parkinson's Disease

Katherine E. Olson
University of Nebraska Medical Center

Follow this and additional works at: https://digitalcommons.unmc.edu/etd

Part of the Nervous System Diseases Commons

Recommended Citation
https://digitalcommons.unmc.edu/etd/235

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@UNMC. It has been accepted for inclusion in Theses & Dissertations by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.
Evaluation of Immune-Modulating Therapies

For Parkinson’s Disease

By

Katherine E. Olson-Johnson

A DISSERTATION

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

Department of Pharmacology and Experimental Neuroscience

Under the Supervision of Professor Howard E. Gendelman

University of Nebraska Medical Center

Omaha, Nebraska

December, 2017
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my Ph.D. advisor, Dr. Howard E. Gendelman. This work would not have been possible without your support and guidance. You have been supportive of my ideas and research goals and have worked actively to provide me with multiple opportunities throughout my graduate career. With this support and guidance, you have helped me become a better student and scientist. You taught me how to think critically and how to “hold my own.” Also, thank you for creating a lab environment that supports collaboration. Not only did I receive constant interaction with fellow scientists, I made some wonderful companions along the way. For this, I am forever thankful.

Secondly, I am exceedingly grateful for Dr. R Lee Mosley. Thank you for always pushing me to come up with the answer on my own and asking “what is your question?” It may have been frustrating at times, but it allowed me to think independently and for that, I am a better scientist. Not only have you helped shape my scientific view, you have made me a better person. Your daily presence in lab is something I look forward to. You help lighten the burden of graduate school and are a joy to be around. Thank you for always being there and taking an interest in my life. Future students will be lucky to have you.

To my committee members, Dr. Joyce Solheim, Dr. Myron Toews, and Dr. Pamela Santamaria, you have been extremely helpful the past 5 years. Thank you for taking the time to shape my scientific career, and thank you for providing
me with constructive criticisms. This support was invaluable. Without your expertise and knowledge, I would not be where I am today.

To my past and present lab members, Annie, Kristi, Lisa, Charles, Rebecca, and Krista, thank you for teaching me the ropes. Thank you for taking the time to help me feel involved and needed. I could not have done this work without your help, and I would not have made it through graduate school without you. You make the lab environment fun and memorable, making these experiences something I will never forget.

To my parents, Dan and Teri, thank you for passing on your love of medicine and science. I may not be delivering babies or teaching high school science, but I am helping shape the scientific and medical world in my own way. I would not have embarked on this journey without your continued support. Dad, I want to especially thank you for always making me laugh and helping make a joke out of anything. This attitude towards life helps keep me afloat during the tough times. Mom, thank you for always being one phone call away. Thank you for keeping me grounded and for helping me talk through anything and everything. You understand how I am, and for that, I am extraordinarily thankful. Again, thank you for everything you have done and will continue to do.

Finally, to my husband, Matt, and daughter, Alyssa, words cannot express how much gratitude I have for you. You are my people. I could not have done this without your constant love and support. Matthew, thank you for dealing with my grad school craziness. Thank you for your patience, and thank you for spending hours in the lab with me over the past few years. You are my rock. Alyssa Rae,
thank you for always bringing a smile to my face on tough days, and thank you for reminding me that, sometimes, work can wait. You bring a light to my life every day and for that, I am forever grateful.
Parkinson’s disease (PD) is the second most common neurodegenerative disorder, second only to Alzheimer’s disease (AD). It is characterized by a progressive loss of dopaminergic neurons along the nigrostriatal axis and the formation of proteinaceous inclusions of alpha-synuclein (α-syn). Secondary to the loss of dopaminergic neurons is a progression in motor and non-motor symptoms. Motor symptoms are characterized by slowness in movement, stiffness and tremor. Non-motor symptoms include depression, constipation, sleep abnormalities and loss of sense of smell. The cause of disease remains incompletely understood. However, age, genetics, environment, viral infection, and interplay between the innate and adaptive immune system can contribute to disease onset and progression. Currently, treatments are palliative, and no known intervention to halt disease progression exists. Drug therapiest employ dopamine or a dopamine precursor that affect neurotransmitter signaling while showing no effect on the neurodegenerative process. Nonetheless, the available
therapies improve walking, movement and tremor debilities. Therefore, it remains essential that therapies are developed to combat PD itself rather than simply alleviating symptoms.

Neuroinflammation and immunity can speed nigrostriatal degeneration in PD. The neuroinflammatory cascade begins with aggregation of misfolded or post-translationally modified alpha synuclein (α-syn) resulting in the occurrence of neuronal cell death and the presence of chronically activated glia. Such changes in the glial phenotypes can affect the central nervous system (CNS) microenvironment by producing pro-inflammatory factors that speed nigrostriatal degeneration.

To halt or slow disease progression, a change in the microenvironment of the brain may be necessary. One potential mechanism to achieve this goal is through the induction and/or enhancement of immune-modulating cells such as regulatory T cells (Tregs). Tregs maintain immune homeostasis by suppressing pro-inflammatory immune responses, such as those associated with neuroinflammation and PD. Furthermore, Tregs taken from PD patients compared to control subjects lack the capacity to suppress proliferation of other immune cells, suggesting a dysfunctional Treg response associated with disease. Previously, our laboratory utilized granulocyte-macrophage colony-stimulating factor (GM-CSF) to restore Treg numbers and function in animal models and a clinical trial. This cytokine induced a neuroprotective phenotype when assessed in animal models; however, in the clinical setting, some mild to moderately severe adverse events were identified. Thus, it remains important to
identify different means for drug delivery or other compounds with Treg-inducing activity and without potential untoward side effects for easy translation to human use.

With this goal in mind, our lab evaluated the efficacy of vasoactive intestinal peptide (VIP) analogs in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Our data indicate that treatment with a stable VIP analog results in a decrease in pro-inflammatory cytokine production, decrease in microglial reactivity, increase in neuron survival, and increase in suppressive immune phenotypes. Taken together, these findings support the use of VIP as a potential immunotherapy for the treatment of PD.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS

ABSTRACT

TABLE OF CONTENTS

LIST OF TABLES and FIGURES

LIST OF ABBREVIATIONS

CHAPTER ONE: Introduction

1.1 Immunity and t cell subsets
   Innate and adaptive immunity
   Effector T cells
   Regulatory T cells

1.2 Parkinson’s disease and neuroinflammation
   Clinical features, diagnosis, and treatments
   Disease-associated immune dysfunction
   Influence of viral infection on Parkinson’s disease

1.3 Current immunotherapies in Parkinson’s disease

1.4 Summary Figures

CHAPTER TWO: Selective VIP receptor agonists facilitate nigrostriatal neuroprotection

2.1 Abstract

2.2 Introduction

2.3 Materials and methods
<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4 Results</td>
<td>47-60</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>60-66</td>
</tr>
<tr>
<td>2.6 Tables and figures</td>
<td>67-101</td>
</tr>
<tr>
<td>CHAPTER THREE: Utilization of bioimaging to evaluate therapeutic potential following VIPR treatment in vivo</td>
<td>102-139</td>
</tr>
<tr>
<td>3.1 Abstract</td>
<td>102</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>103-105</td>
</tr>
<tr>
<td>3.3 Materials and methods</td>
<td>105-109</td>
</tr>
<tr>
<td>3.4 Results</td>
<td>109-114</td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>114-121</td>
</tr>
<tr>
<td>3.6 Figures</td>
<td>122-139</td>
</tr>
<tr>
<td>CHAPTER FOUR: Interplay between viral infection and Parkinson's disease</td>
<td>140-182</td>
</tr>
<tr>
<td>4.1 Abstract</td>
<td>140</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>140-142</td>
</tr>
<tr>
<td>4.3 Materials and methods</td>
<td>142-145</td>
</tr>
<tr>
<td>4.4 Results</td>
<td>146-152</td>
</tr>
<tr>
<td>4.5 Discussion</td>
<td>152-159</td>
</tr>
<tr>
<td>4.6 Figures</td>
<td>160-181</td>
</tr>
<tr>
<td>CHAPTER FIVE: Discussion</td>
<td>182-194</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>195-236</td>
</tr>
</tbody>
</table>
List of Tables and Figures

CHAPTER ONE:

Figure 1. Neuroinflammation in Parkinson’s disease. 29
Figure 2. Modulating the neuroinflammatory response. 31

CHAPTER TWO:

Table 1. Amino acid sequences for the peptides evaluated in these studies. 67
Figure 1. LBT-3393 and LBT-3627 peptides are VIPR1 and VIPR2 agonists. 68
Figure 2. LBT-3627 is resistant to protease activities. 70
Figure 3. LBT-3627 pretreatment is neuroprotective. 72
Figure 4. Administration of a VIPR antagonist blocks the neuroprotective effects of LBT-3627. 74
Figure 5. The neuroprotective effect of LBT-3627 is dose-dependent. 76
Figure 6. Adoptive transfer of splenocytes from mice treated with VIPR agonists is neuroprotective. 78
Figure 7. Pretreatment with VIPR agonists decreases reactive microglia levels during MPTP-induced inflammation. 80
Figure 8. Adoptive transfer of splenocytes from VIPR agonist treated mice diminishes microgliosis during MPTP-intoxication. 82
Figure 9. VIPR agonist pretreatment does not affect CD4+ and CD4+CD25+ T cell frequencies. 84
Figure 10. LBT-3627 treatment elicits changes in Treg function. 86
Figure 11. LBT-3627 treatment decreases pro-inflammatory cytokine release in CD4+ T cell subsets. 88
Figure 12. VIPR2 agonism induces dysregulation of genes associated with inflammatory responses and cell-to-cell signaling.

Figure 13. VIPR2 agonism elicits changes in immune pathways.

Figure 14. Targeting VIPR2 with LBT-3627 changes inflammation-associated gene expression in microglial populations.

Figure 15. Targeting VIPR2 with LBT-3627 prior to MPTP intoxication elicits changes in the inflammatory response pathway.

Figure 16. MPTP metabolism in the midbrain and striatum is not diminished by pretreatment with VIPR agonists.

Figure 17. LBT-3627 treatment following MPTP intoxication does not attenuate neurodegeneration.

CHAPTER THREE:

Figure 1. Comparison of MEMRI signal enhancement between controls and MPTP-intoxicated mice at day 2 post-MPTP.

Figure 2. Comparison of MEMRI enhancement between controls and MPTP-intoxicated mice at day 7.

Figure 3. Assessment of neuronal survival at day 2 following MPTP intoxication and LBT-3627 pretreatment.

Figure 4. Quantification of dopaminergic neuronal survival following pretreatment with multiple different doses LBT-3627.

Figure 5. Assessment of microglial reactivity after 2 days following MPTP intoxication and LBT-3627 pretreatment.

Figure 6. Assessment of microglial reactivity after 7 days post MPTP intoxication and LBT-3627 pretreatment.

Figure 7. GFAP reactivity at 2 days post MPTP intoxication and LBT-3627 pretreatment.
Figure 8. GFAP reactivity is increased after MPTP-intoxication.

Figure 9. Time comparison of immunohistology and signal enhancement.

CHAPTER FOUR:

Figure 1. Co-administration of HIV and MPTP intoxication does not affect neuronal degeneration in humanized mice.

Figure 2. HIV infection does not increase microglial reactivity after MPTP intoxication in humanized mice.

Figure 3. EcoHIV co-infection exacerbates MPTP-induced neurodegeneration.

Figure 4. EcoHIV infection leads to increased microglial reactivity.

Figure 5. EcoHIV infection does not affect peripheral T cell populations.

Figure 6. EcoHIV infection alone leads to changes in the inflammatory.

Figure 7. EcoHIV co-infection results in gene changes in the inflammatory response pathway.

Figure 8. EcoHIV and MPTP co-administration does not exacerbate neuronal degeneration in non-humanized NSG mice.

Figure 9. Microglial reactivity following EcoHIV and MPTP co-administration is not enhanced in non-humanized NSG mice.

Figure 10. Immunodeficient scid mice are not susceptible to MPTP intoxication.

Figure 11. MPTP intoxication does not lead to an inflammatory response in scid mice.
List of Abbreviations

6-OHDA  6-hydroxydopamine
α-syn  alpha-synuclein
Aβ  amyloid beta
AAV  adeno-associated virus
AD  Alzheimer’s disease
ADNF  activity-dependent neurotrophic factor
ADNP  activity-dependent neuroprotective protein
ADP  adenosine diphosphate
AIDS  acquired immunodeficiency syndrome
ALS  amyotrophic lateral sclerosis
AMP  adenosine monophosphate
APC  antigen presenting cell
ATP  adenosine triphosphate
BDNF  brain-derived neurotrophic factor
BBB  blood-brain barrier
Ca  calcium
cAMP  cyclic adenosine monophosphate
CBA  cytometric bead array
CFSE  carboxyfluorescein succinimidyl ester
CNS  central nervous system
Cop-1  copolymer 1
COX  cyclooxygenase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA4</td>
<td>cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DBS</td>
<td>deep brain stimulation</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>FasL</td>
<td>fas ligand</td>
</tr>
<tr>
<td>FoxP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPA</td>
<td>ingenuity pathway analysis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IPEX</td>
<td>immunodysregulation polyendocrinopathy enteropathy X-linked</td>
</tr>
<tr>
<td>iTreg</td>
<td>induce regulatory T cell</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>levodopa</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy body</td>
</tr>
<tr>
<td>LN</td>
<td>Lewy neurite</td>
</tr>
<tr>
<td>Mac-1</td>
<td>macrophage-1 antigen</td>
</tr>
<tr>
<td>MAMP</td>
<td>microbe-associated molecular pattern</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MDSC</td>
<td>myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MEMRI</td>
<td>manganese-enhanced magnetic resonance imaging</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP-1 α</td>
<td>macrophage inflammatory protein – 1 alpha</td>
</tr>
<tr>
<td>Mn</td>
<td>manganese</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>N-α-syn</td>
<td>nitrated alpha-synuclein</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD SCID gamma</td>
</tr>
<tr>
<td>nTreg</td>
<td>natural regulatory T cell</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reverse phase-high performance liquid chromatography</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STR</td>
<td>striatum</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Teff</td>
<td>effector T cell</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>Tresp</td>
<td>responder T cell</td>
</tr>
<tr>
<td>UPDRS</td>
<td>unified Parkinson's disease rating scale</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
</tr>
</tbody>
</table>
VIPR vasoactive intestinal peptide receptor
CHAPTER ONE

1.1 immunity and T cell subsets

Innate and Adaptive Immunity

Both innate and adaptive immune responses are important for mounting the body’s defense against a pathogen or foreign microorganism (Getz, 2005). The innate response is the first line of defense, which is relatively rapid, recognizes a broad spectrum of antigen patterns, does not require immune memory, and is characterized by phagocytic activity mediated by resident mononuclear phagocytes such as macrophages, dendritic cells (DC), and microglia (Turvey and Broide, 2010). The adaptive immune response requires substantially greater time to develop, produce and utilize immunological memory, and affords narrow specificity for antigens based on lymphoid receptors for antigen expressed by T cells and B cells (Dempsey et al., 2003). The main function of the adaptive immune system is to recognize and destroy foreign pathogens and relieve pathogen-associated toxicities. However, to initially mount an immune response, the innate arm of the immune system must first be activated by recognition via broadly-specific pattern recognition receptors (PRRs) which respond to microorganism-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) (Turvey and Broide, 2010). In an innate response, antigens recognized by PRRs of mononuclear phagocytes are engulfed,
digested, and processed to bind with molecules of the major histocompatibility complex (MHC). Upon maturation, mononuclear phagocytes acquire an antigen presenting capability whereby processed and MHC-bound antigen is expressed on the cell surface in a configuration necessary for T cells to recognize via the T cell receptor (TCR). Unlike immunoglobulins (Ig) on B cells, which recognize cellular- or non-cellular-bound antigen, the TCR only recognizes antigen presented by MHC molecules on antigen presenting cells (APCs) (Cohn, 2017). APCs also express co-stimulatory molecules such as CD80, CD86, CD70, CD40, and CD200 that are necessary to generate an effective, robust, and specific immune response (Stephen et al., 2005). As APCs, mononuclear phagocytes bridge the innate and adaptive arms of the immune system by providing the antigen and co-stimulation necessary for naïve T cells to become activated. Additionally, APCs also deliver cytokine signals, undergo polarized differentiation, transforming them into activated T cells with specific effector functions, which will be described in the next section (Effector T cells) (Turvey and Broide, 2010). Once activated, T cells proliferate and undergo clonal expansion to increase their cell number and potential to challenge invading pathogens (Dempsey et al., 2003). One mechanism by which effector T cells expand in the presence of antigen is to secrete pro-growth cytokines to the surrounding environment. For instance, activated T cells produce and secrete IL-2 cytokine that binds their own IL-2R to enhance proliferation in an autocrine fashion, as well as enhance proliferation of surrounding T cells in a paracrine fashion (Ozaki et al., 2000). In addition, to achieve an efficient effector function,
activated T cells migrate to areas of infection and inflammation to interact with other immune cells, such as macrophages or microglia, ultimately, bestowing collaborative effector functions to rid the host of a foreign assault (Campbell, 2015). Thus, T cells are important for the general cellular-mediated response of the adaptive immune arm.

**Effector T cells**

T cells represent the arm of the adaptive immune system responsible for mounting a response against foreign pathogens. T cells are derived from bone marrow lymphocyte progenitors that mature and are immunologically educated within the thymus (Romagnani, 2000). From there, they migrate to the periphery and reside throughout the tissues of the body, but are located mainly in spleen, lymph nodes, and peripheral circulation (Romagnani, 1999). T cells, acting as effector cells, provide the stimulus and signals for directing the cellular and antibody responses necessary to clear foreign pathogens and antigens, but also, when acting as regulatory T cells (Tregs), endow immunological tolerance to the individual and actively restrain the immune system from recognizing itself as foreign (Corthay, 2009).

Generally, T cells are characterized by expression of the TCR-CD3 complex on the cell surface (Romagnani, 2000). While the TCR recognizes presented antigen, it does not possess the cytosolic machinery necessary for successful signal transduction. Therefore, the CD3 complex serves as the signaling mechanism that bridges the antigen recognition and conveyance of
signal for effector function (Dempsey et al., 2003). T cells are subdivided into two major lineages based on the expression of either CD4 or CD8 on the cell surface. CD4+ T cells are considered helper T (Th) cells. With the TCR recognizing the antigen, the CD4 molecule acts as a co-receptor and binds the MHC II molecule that presents the antigen from the APC (Getz, 2005). Depending on the cytokines generated by the APC, activated CD4+ T cells develop into specific effector T cell (Teff) subsets that include type-1, -2, -17, -22, or -9 Th cells designated as Th1, Th2, Th17, Th22, or Th9 cells, respectively (Romagnani, 2000). Th subsets secrete a variety of cytokines that act as either pro- or anti-inflammatory mediators.

Classically, Th1 cells are important for mounting immune responses against intracellular pathogens and are characterized by secretion of predominately IFN-γ, TNF-α, and IL-2 (Romagnani, 2000). IFN-γ from Th1 cells enhances macrophage activation necessary for immunity to pathogens. In addition, Th1 cells produce IL-2 and IL-21 to promote and maintain antigen-specific CD8+ cytotoxic T lymphocytes (CTLs) that lyse virally-infected cells (Hiroishi et al., 2010). CTLs are primarily responsible for ridding the host of intracellular pathogens and virus-infected cells, by recognizing antigen presented by MHC class I, which is expressed in all nucleated cells. When activated and expanded, CTL effectors produce perforins and granzymes to induce cell-mediated cytolysis in infected cells (Fan and Zhang, 2005). On the other hand, Th2 cells preferentially assist antibody-mediated responses through the secretion of IL-4, IL-10, IL-13, and IL-5, which act on B cells for antibody production.
Because the prototypical cytokines from Th1 and Th2 cells regulate the expression of each other’s master-controlling transcription factors, this preferentially selects T cells to differentiate into either Th1 or Th2 effector cell types in response to pathogens (Romagnani, 1999).

Th17 cells are a more recently discovered subset of Teffs. They predominantly secrete IL-17, as well as TNF-α, and are thought to be important for protection against extracellular infections (Korn et al., 2009). In addition to the Th17 effectors, another subset expresses IL-9 and is thought also to be important in resolution of extracellular infections (Li and Rostami, 2010). The Th9 subset has been implicated in promoting the migration of Th17 cells to the central nervous system (CNS) (Zhou et al., 2011) and potentiation of Th17 effectors via the ability to increase Th17-produced IL-17. Moreover, recent evidence indicates that other T cell types such as Tregs, Th1, and Th17 can also secrete IL-9 with pleiotropic effects that may ultimately alter the predominant proinflammatory response (Li and Rostami, 2010). Along the same lines, Th22 cells are a separate lineage of CD4+ Teffs that primarily secrete proinflammatory cytokines such as IL-22, IL-13 and TNF-α, and express the skin homing-associated chemokine receptors CCR4, CCR6 and CCR10 (Zhang et al., 2011). Th22 cells differentiate from naïve T cells in the presence of IL-6 and TNF-α under control of the transcription factors aryl hydrocarbon receptor and GATA3 (Azizi et al., 2015). These Teffs are recruited to the skin and thought to be involved in microbial immunity and tissue repair and remodeling. Skin disorders such as psoriasis, eczema, and contact dermatitis may be due to dysregulation of Th22
migration or function.

Each of these T cell subsets plays crucial, yet independent roles in mounting a robust and effective adaptive immune response. As these effector cells provide potent weapons toward immunity to foreign invaders, they also serve equally as potential liabilities due to increased pro-inflammatory cytokines, antibody-mediated cytotoxicity to cells, or hyperactivation of innate immune cells. Thus, regulation of the Teffs is necessary to prevent untoward pathological sequelae. CD4+CD25+ regulatory T cells (Tregs) provide that regulation, seemingly via expression and control of a master regulatory transcription factor, forkhead box 3 (FoxP3).

Regulatory T cells

Tregs are a subset of T cells that regulate and suppress the activities of Teffs and myeloid lineage cells such as DCs, microglia, and macrophages that comprise innate immunity. Tregs can be further subdivided into natural Tregs (nTregs) that are derived from the thymus and induced Tregs (iTregs) that arise from naïve T cells in the periphery (Corthay, 2009). Moreover, a primary function of Tregs is the maintenance of immunological tolerance to self and thus, inhibition of initial autoimmune responses and suppression of auto-reactive T cells that may arise in peripheral tissues. As such, individuals that do not produce functional nTregs due to mutated FOXP3, develop immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, a systemic, multi-organ autoimmune disorder (Wildin et al., 2002).
Treg-mediated regulatory functions are achieved at many levels. First, Tregs inhibit initiation of immune responses by diminishing antigen processing and antigen presentation by MHC molecules as well as regulating 2nd and 3rd signals from APCs (Sakaguchi et al., 2008). This is achieved through interaction with cytotoxic T-lymphocyte-associated protein 4 (CTLA4) on the Treg cell surface and CD80 or CD86 on the APC cell surface. This interaction acts as an “off” switch for the APC. Second, Tregs secrete anti-inflammatory cytokines such as IL-10, IL-35, and TGF-β that suppress activated mononuclear phagocytes and Teffs (Dasgupta and Saxena, 2012). Third, Tregs have the capacity to elicit metabolic disruption in effector cell types through cytokine deprivation or cell-mediated apoptosis. To maintain this function, Tregs express CD39 and the IL-2R, also known as CD25. CD39 is an ATPase, which phosphohydrolyzes ADP/ATP to AMP, and CD25 binds extracellular IL-2 (Antonioli et al., 2013). Upregulation of these receptors results in a decrease in surrounding IL-2, a cytokine needed for cellular proliferation, and a decrease in available ATP, which is needed for cellular energy. Finally, Tregs also possess the ability to mediate cytolysis through the secretion of granzymes and perforins, ultimately leading to apoptosis of the target cell (Dasgupta and Saxena, 2012). However, unlike the Th2 subset, which also elicits anti-inflammatory and protective effects, Tregs do not traditionally interact with B cells to elicit a response. Therefore, Tregs generally regulate the immune response independently rather than via the activation of other cell types.

The interplay between the innate and adaptive arms of the immune
system is essential to the relationship between neuroinflammation, 
neuroprotection, and neurodegeneration. While neuroinflammation and 
neurodegeneration are associated with the pathobiology of neurodegenerative 
diseases, they are also responsible for the overall neuroprotective homeostasis 
of the host CNS in infectious disease surveillance. Similarities between multiple 
neurological disorders have provided common mechanisms of immune 
interactions that lead to protective or destructive effects within the CNS and 
peripheral nervous system (PNS). Although neuroinflammation and T cell 
interactions play a prominent role in disease progression, it should be noted that 
the immune response can vary from a very prominent primary T cell response, as 
in multiple sclerosis (MS), to seemingly less intense T cell responses as in the 
cases of amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD) and 
Parkinson’s disease (PD) (Town et al., 2005, Holmoy, 2008, Fletcher et al., 2010, 
Mosley and Gendelman, 2017). Recent findings in human neurodegenerative 
disorders, specifically in PD and in corresponding animal models, have shown 
the involvement and putative mechanisms of T cells and subsequent secondary 
responses in disease initiation and progression, which will be discussed in the 
following section.

1.2 Parkinson’s disease and neuroinflammation

Clinical features, diagnosis, and treatment

PD is the second most common neurodegenerative disorder, but it is the most 
prevalent neurodegenerative movement disorder (Kalia and Lang, 2015). The
first clinical description of PD was originally described as a “shaking palsy” by James Parkinson in 1817 (Donaldson, 2015). It is a neurological disease that is characterized by two disease hallmarks, the progressive loss of dopaminergic neurons that originate within the substantia nigra (SN) and innervate into the striatum and the presence of proteinaceous inclusions called Lewy bodies (LB) and Lewy neurites (LN). LB and LN are intracellular inclusions consisting of modified and misfolded α-syn accompanied by ubiquitin (Carrithers et al., 2000, Scanlon et al., 2008). Dopaminergic neurons are the main producers of dopamine, the key neurotransmitter needed for proper movement. Thus, the loss of this neuron population causes the majority of the motor symptoms associated with PD (Maiti et al., 2017).

The classical motor symptoms of PD are recognized as prominent features for disease diagnosis, but only appear after ~50-60% neuronal loss (Olanow et al., 2009). These motor symptoms include bradykinesia, rigidity, resting tremor, postural instability, and gait dysfunctions. However, the severity and degree of these motor features remains heterogeneous within the diseased population. Apart from prevalent motor dysfunction, patients with PD display many non-motor symptoms as well including olfactory deficits, sleep disturbances, cognitive impairments, pain, fatigue, constipation, and some psychiatric disorders (Titova et al., 2017). Frequently, these non-motor features arise well before the onset of motor dysfunction, indicating that the pathogenic cause of the disease occurs years prior to noticeable disease onset. One hypothesis for the onset of non-motor dysfunctions prior to motor dysfunction lies
with Braak and colleagues (Rietdijk et al., 2017). They hypothesize that symptoms start in the periphery before entering the CNS via a nasal or gastric route, eventually contributing to neuronal loss. Thus, continued evaluation of biomarkers for early diagnosis remains essential for combating the disease. To date, a variety of biomarkers are being evaluated using clinical, pathological, biochemical, genetic, and bioimaging techniques (Kalia and Lang, 2015). These include, but are not limited to, detection of olfactory impairments, neuronal imaging using PET/SPECT, skin biopsies, changes in biochemical makeup of the CSF, saliva, or blood, and genetic sequencing.

Without easily defined and detectable diagnostics, clinicians focus on treating patients after diagnosis based on motor symptoms. Initially, motor dysfunctions are managed symptomatically. Currently, the readily available therapies for PD are palliative; however, there is no known cure or intervention to slow or halt disease progression (Olanow et al., 2009). In general, drug therapies employ dopamine agonists or the dopamine precursor, L-DOPA, to assist with normal dopaminergic neurotransmission (Kalia and Lang, 2015). A second therapeutic avenue is through deep brain stimulation (DBS), a surgical approach aimed at stimulating either the subthalamic nucleus or globus pallidus internus to counteract the motor disturbances (Follett and Torres-Russotto, 2012, Hamani et al., 2017). However, each of these therapies only targets the symptoms and shows no effect on the progressive nature of the disease. Also, as the disease progresses, it is not as easily managed, and is characterized by the worsening of motor disturbances, resulting in freezing, falls, dysphagia, and slurred speech
(Kalia and Lang, 2015). Thus, it remains essential that therapies are developed for combating PD progression rather than simply alleviating symptoms.

These disease hallmarks and symptoms can present themselves in both sporadic and familial cases of PD. Familial PD is genetically-linked and accounts for approximately 10% of all PD cases. Likewise, several genes have been identified in patients with a family history of disease, including SNCA, LRRK2, PINK1, DJ-1, and Parkin (Olanow et al., 2009). Apart from genetics, other risk factors include increased age, male gender, Hispanic ethnicity, and exposure to environmental toxins (Kalia and Lang, 2015). In accordance with known genetic links and risk factors, both sporadic and familial cases are linked to dysfunctions in common cellular networks. These include dysregulation of protein homeostasis, such as abnormalities in protein aggregation, intracellular protein trafficking, and ubiquitin-based protein disposal, and dysfunctional mitochondrial processes (Good et al., 1998, Moon and Paek, 2015).

The importance of the mitochondrial function for parkinsonism was verified by Dr. Langston in “The Case of the Frozen Addicts (Langston et al., 1983).” Men and women had ingested heroin containing an impurity called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). After ingestion, they suddenly became immobile, as if they had instantly acquired PD. Some continued to experience effects years after the fact (Langston et al., 1999). It was discovered that the impurity resulted in motor dysfunction and is now utilized as a model for inducing PD-like symptoms and hallmarks in rodent and primate models (Przedburski et al., 2001). When MPTP is administered peripherally, it rapidly crosses the blood-
brain barrier (BBB) where it is metabolized into its toxic form, MPP+. This toxin accumulates within dopaminergic neurons via uptake by the dopamine transporter (DAT), due to the similar structure to dopamine itself. Once inside the neuron, MPP+ inhibits complex I of the electron transport chain within the mitochondria, ultimately inhibiting ATP production and generating the formation of superoxide radicals. Superoxide radicals react with nitric oxide (NO) and produce peroxynitrite, causing cellular damage. In turn, tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine production, is oxidized and nitrated causing it to become inactive. This inactivation leads to decreased dopamine production, resulting in Parkinson-like motor symptoms (Przedborski et al., 2000).

However, even with the known risk factors and known associations with cellular dysfunction, the underlying driving force behind the progression of PD is still unknown. It is hypothesized that the overall cause and pathogenesis of disease is multi-factorial and results in an elaborate interplay between multiple insults. For instance, genetics, environment, age and interplay between innate and adaptive immune systems contribute to disease onset and progression. Recently, neuroinflammation and immune dysfunction have been shown to be a characteristic feature of PD, but whether or not neuroinflammation is a cause or effect of the disease is not yet known. However, while the pathobiology of PD remains in study, neuroinflammation and immunity are thought to speed nigrostriatal degeneration (Mosley et al., 2012, Anderson et al., 2014). The neuroinflammatory cascade associated with PD begins with the aggregation of
misfolded or post-translationally modified α-syn. Such aggregation results in neuronal cell death and the presence of chronically activated microglia and astrocytes, leading to the production of tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), IL-6, reactive oxygen species (ROS), and enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Liu and Hong, 2003). These changes in the microglial phenotype can affect the CNS microenvironment by producing a pro-inflammatory milieu that speeds PD pathogenesis (Figure 1). Compelling evidence in both animal and human studies helps to support the idea of an overactive inflammatory state and lack of immune regulation within the PD population.

*Disease-associated immune dysfunction*

Initial studies of peripheral lymphocyte populations from PD patients show decreased frequencies and total numbers of CD4+ T lymphocytes compared to controls (Bas et al., 2001, Baba et al., 2005, Saunders et al., 2012, Stevens et al., 2012). However, due to conflicting reports on T cell phenotypes, a firm consensus of other T cell subset changes in PD patients has proven difficult. For instance, the diminution of CD4+ T cell numbers in PD patients was found chiefly from decreased numbers of CD4+CD45RA+ naïve T cells and to a lesser extent from CD4+CD29+ memory subsets (Saunders et al., 2012). On the other hand, Stevens and colleagues reported decreased levels of CD4+CD45R0+ memory T cells (Stevens et al., 2012). A recent study by Saunders and colleagues showed slight, yet significant increases in frequencies of CD4+CD45R0+ memory/effector
T cells with a parallel diminution of CD4+CD45RA+ resting/naïve T cell levels (Saunders et al., 2012). Additionally, frequencies of peripheral CD4+ T cells with effector-associated phenotypes expressing FAS were increased in patients, whereas those expressing α4β7 integrins and CD31 were diminished. Notably, these changes in CD4+ T cell phenotypes were correlated with severity of motor function as quantified by the Unified Parkinson’s Disease Rating Scale, part III (UPDRS III) (Saunders et al., 2012). Differences in modulation of T cell immunology profiles may be the consequence of disease heterogeneity and severity, but clearly require further investigation.

Post-mortem studies of PD patient brain tissues show both CD4+ and CD8+ T cells in close proximity to dopaminergic neurons within the SN at levels exceeding 10-fold those found in controls (Brochard et al., 2009). Moreover, these levels of T cells were not detected in non-lesioned brain regions. Microarray analysis of peripheral blood leukocytes and SN brain tissue showed many genes expressed were in common with those expressed by Th17-mediated immune reactions and suggest that idiopathic parkinsonism is a Th17 dominant autoimmune disease (Qian et al., 2010). However, whether T cell infiltration is primary or secondary to PD progression is unknown at this time. Similarly, conflicting reports of Tregs in PD also produced variances in levels detected ranging from increased frequencies in PD patients to little or no differences among groups (Fiszer et al., 1994a, Baba et al., 2005, Saunders et al., 2012, Stevens et al., 2012). However, one study demonstrated the diminished capacity of Tregs from PD patients compared to those of controls to inhibit the
proliferation of responder T cells from healthy donors (Saunders et al., 2012). This suggests that a dysfunction in Tregs leads to a hyper-activated immune state and increased disease progression. The notion that hyper-activated immune responses support increased dopaminergic loss is provided by use of animal studies.

Multiple studies in animal models demonstrate the involvement of the adaptive immune system in dopaminergic neurodegeneration using both active and passive transfer of immunity (Kurkowska-Jastrzebska et al., 1999b, Brochard et al., 2009, Kosloski et al., 2013). In the MPTP mouse model, numbers of T cells in the SN are increased after intoxication, and interestingly, numbers of CD8+ T cells predominate those of CD4+ T cells (Brochard et al., 2009). While in agreement of the relative proportions of T cell subsets within the SN of MPTP-treated mice, the total numbers of CD4+ T cells vary widely between studies. The importance of T cells for mediating MPTP-induced neurodegeneration was found initially in adoptive transfer and reconstitution studies of functional T cells to immunodeficient mice (Benner et al., 2008, Brochard et al., 2009). While both studies confirm that immune deficient mice were not susceptible to MPTP intoxication, reconstitution of those mice with functional naïve lymphocytes partly restored MPTP susceptibility (Benner et al., 2008) and CD4+ T cells were found to be chiefly responsible for MPTP susceptibility (Brochard et al., 2009). These studies point to a deleterious role of CD4+ T cells in PD. The phenotypes of CD4+ populations required for increased degeneration were determined to be Th1 and Th17 cell lineages. Similarly, T cells isolated from mice immunized with
a modified self-antigen, nitrated α-synuclein (N-α-syn) recognized only N-α-syn, but not unmodified α-syn in in vitro challenge assays. Moreover, N-α-syn specific Teffs exacerbated neuroinflammation and increased neuronal injury and subsequent neurodegeneration of dopaminergic neurons within the SN of MPTP mice (Benner et al., 2008). These findings indicate that N-α-syn, a modified self-protein, either evades or breaks immunological tolerance to self α-syn and induces N-α-syn specific T cells. Likewise, another second study showed that Th17 Teffs possess a significantly greater capacity to exacerbate dopaminergic neurodegeneration than the same number of Th1 Teffs (Reynolds et al., 2010). Together, these data indicate that CD4+ T cells play an important role in the neuroinflammation and subsequent neurodegeneration in models of PD. It also indicates that Th17 Teffs are more potent at direct killing of neurons or alternatively, enhancing neurotoxic microglia than Th1 Teffs. These data also support the idea that increased peripherally circulating Teffs, as found in PD patients, are capable of migrating to the sites of neuroinflammation and can exacerbate and accelerate PD disease progression.

Activated microglia and Teffs are thought to be key mediators of neuroinflammatory processes in PD progression. If left uncontrolled, these mediators support an inflammatory cascade that affects the tempo of the disease (Reynolds et al., 2007a). Initially, studies directed to harness the inflammatory cascade using an anti-inflammatory agent demonstrated that adoptive transfer of CD4+ T cells from copolymer-1 (Cop-1) immunized donor mice protected dopaminergic neurons and striatal termini in MPTP-treated mice (Laurie et al.,
2007). These observations supported the hypothesis that subpopulations of T cells potentiate neurodegeneration in the MPTP animal model of PD. These findings are consistent with known mechanisms by which Cop-1 regulates proliferative and inflammatory responses by preferentially inducing Th2, Th3, and Tregs that secrete anti-inflammatory cytokines (Benner et al., 2004, Laurie et al., 2007, Reynolds et al., 2007b, Lalive et al., 2011). In a separate line of study, researchers found that CD4+CD25+ Tregs were most capable of suppressing neuroinflammation and neurodegeneration in the MPTP model with as few as 3.5 × 10^6 Tregs being sufficient to provide virtually complete neuroprotection to dopaminergic neurons along the nigrostriatal axis (Reynolds et al., 2007b). Moreover, the degree of protection afforded by Tregs seems to increase with increasing inflammatory responses as evidenced by increased neuroprotection with Treg co-transfer with N-α-syn specific Th17 cells in the MPTP model (Reynolds et al., 2010). Also, use of VIP, a known inducer of Treg activity (Gonzalez-Rey and Delgado, 2007), increased the neuroprotective capability of Tregs from VIP-treated donors in the MPTP model (Reynolds et al., 2010). Along those same lines, studies using GM-CSF showed that pretreatment with GM-CSF prior to MPTP-intoxication increased Treg activity in a dose-dependent fashion and diminished the neuroinflammatory response, affording significant dopaminergic neuroprotection (Kosloski et al., 2013). Based on these findings, our lab carried out an investigator-initiated phase I clinical trial to target the dysregulated Treg population in PD using sargramostim, also known as GM-CSF. In theory, treatment would upregulate Treg numbers and function to
suppress the neuroinflammatory cascade in the PD population, with the hopes that treatment would afford a neuroprotective outcome to inhibit or slow disease progression (Gendelman et al., 2017). Prolonged treatment with GM-CSF in PD patients resulted in decreased motor severity, as determined by the UPDRS III, and these changes were linked to increases in Treg function. Treatment was also associated with increased adverse events, such as injection site reactions, eosinophilia, increased white cell counts, bone pain, urticarial reaction, vasculitis, and stroke. However, some of these adverse events are known to be associated with the drug due to its ability to rapidly reconstitute all parts of the immune system (Vial and Descotes, 1995a). Thus, we now aim to utilize an analog of another potent immune modulator, VIP, in the hopes that it would result in a neuroprotective phenotype with a lower likelihood of the development of untoward side effects.

Influence of viral infection on Parkinson’s disease

Apart from neuroinflammation and immune dysfunction, there has also been a potential association between viral infection and PD. Besides the overlapping symptomology and generation of movement disorders, a number of viruses have already been associated with parkinsonism development. These include, but are not limited to, influenza viruses, herpes simplex viruses, Epstein-barr virus, cytomegalovirus, polio virus, human immunodeficiency virus (HIV), West Nile virus, and Japanese encephalitis B virus (Jang et al., 2009). Findings suggest that viruses, such as these, possess neurotropism, have the ability to enter the
brain, cause cell death, and elevate pro-inflammatory cytokine production responses, resulting in a neurodegenerative response (Karim et al., 2014).

These findings help support the idea that viral infection could participate in the development of neurologic disease such as PD. However, other studies have generated opposing findings, suggesting that some viral infections such as hepatitis or HIV show no significant associations with development of PD (Boyd et al., 2016, Pakpoor et al., 2017). Thus, past epidemiologic studies have yielded conflicting reports, and there is a lack of experimental findings utilizing animal models to determine the resulting effects of viral infection on neurologic deficits and degeneration. Therefore, amidst testing immunotherapies in PD models, we also aim to identify a potential relationship between viral infection and a Parkinson-like state.

1.3 Current Immunotherapies in Parkinson’s Disease

While immunosuppression is associated with nigrostriatal neuroprotection for PD, the direct cause and effect relationships have not yet been realized. Thus, modulating the immune system for therapeutic gain has been openly debated. Currently, researchers are revealing how innate and adaptive immunity can affect neurodegenerative disease pathology, and similarly, how the immune response can be harnessed for successful treatments. The overarching idea is to employ immune modulating agents as a neuroprotective strategy for disease treatment. To date, many disease-modifying treatments have been or are currently being developed as neuroprotective strategies for PD in both experimental animal
models and for human disease translation, as discussed below. Overall, the long-
term goal of this research is to effectively harness the immune system to slow or
prevent PD pathobiology.

Upon observational evaluation of large cohorts of individuals, those who
use non-steroidal anti-inflammatory drugs (NSAIDs), specifically ibuprofen,
exhibit a lower risk of developing PD, and treatment with NSAIDs is protective in
both 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP) and 6-
hydroxydopamine (6-OHDA)- induced neuronal lesions (Rees et al., 2011).
Ultimately, this suggests that there is an association with anti-inflammatory use
and a decrease in the potential of developing PD. One of the first anti-
inflammatory treatments to be investigated was minocycline. Minocycline
possesses potent neuroprotective and anti-inflammatory activity in various
inflammatory rodent models of PD, and has been carried into clinical trials as
well. Its mechanisms of action are mediated by blocking activation of NADPH
oxidase, decreasing microglial activation, and decreasing the production of iNOS,
IL-1β, and TNFα (Du et al., 2001, He et al., 2001, Tikka et al., 2001, Kim and
Suh, 2009, Lu et al., 2010). In both the MPTP and 6-OHDA models, minocycline
reduced the numbers of reactive microglia and spared nigral dopaminergic
neurons along with their projections into the striatum in a dose-dependent
manner. However, in the clinical setting, neuroimaging analysis of PD patients
following minocycline treatment suggests that microglial activation is perturbed,
but motor assessments failed to show any clinical benefit (2006, 2008, Dodel et
al., 2010). In conclusion, anti-inflammatory agents, while having been extensively
studied, have failed to provide improved clinical outcomes.

Many natural and/or endogenous compounds have recently been shown to be effective in rodent models of PD as well, such as resveratrol (Lin et al., 2014), tanshinone (Ren et al., 2015), silymarin (Borah et al., 2013), resolvins (Tian et al., 2015), daidzein (Chinta et al., 2013), and apocynin derivatives (t Hart et al., 2014). The majority of these compounds act as both anti-inflammatory agents and anti-oxidants. Collectively, they mediate their actions by down-regulating glial activation, decreasing pro-inflammatory cytokine production, suppressing M1 microglial phenotypes, and reducing NF-κB activation. Specifically, resveratrol and tanshinone treatment both show decreases in IL-1β, IL-6, and TNFα (Zhang et al., 2010, Lofrumento et al., 2014, Wang et al., 2015). Also, resveratrol causes an upregulation of suppressor of cytokine signaling (SOCS-1), and tanshinone treatment inhibited nitric oxide (NO) production, as well as NADPH and iNOS expression. In the MPTP model, silymarin treatment stabilizes mitochondrial membrane potential, decreases levels of TNFα, iNOS, and IL-1β, and ultimately, decreases striatal levels of caspase-3 and NF-κB indicating an anti-apoptotic and anti-inflammatory function (Geed et al., 2014, Jung et al., 2014, Lee et al., 2015). Taken together, these findings help to justify the potential beneficial use of natural anti-inflammatory agents for the treatment of PD. However, these agents have yet to be translated into clinical investigation for efficacy in PD patients.

To date, there have also been many recent investigations into the role of peroxisome proliferator-activated receptor (PPAR) agonists and their
neuroprotective mechanisms within the CNS. PPAR is a receptor super family of transcription factors that is activated by small molecules such as hormones and steroids, and its activation plays an important role in mediating the inflammatory response. PPAR agonists, such as pioglitazone and rosiglitazone, are shown to possess neuroprotective and anti-inflammatory activity both in vitro and in vivo by selectively targeting neurotoxic factors within reactive microglia (Carta and Pisanu, 2013, Pisanu et al., 2014). Their use in the MPTP model of PD led to decreases in microglial activation, iNOS production, NF-κB activation, and ROS production (Garrido-Gil et al., 2012, Pisanu et al., 2014). Along with a decrease in pro-inflammatory mediators, it is associated with increases in anti-inflammatory mediators such as IL-10 and transforming growth factor-β (TGF-β), as well as restored levels of CD206, a marker for anti-inflammatory M2 microglial responses (Pisanu et al., 2014). These findings suggest a shift from a pro-inflammatory response to an anti-inflammatory one, perhaps resulting in a more protective and neurotrophic environment. To further confirm neuroprotective effects and the ability to modulate the inflammatory response, pioglitazone was tested in parkinsonian monkeys. Oral administration led to higher stereological counts of tyrosine hydroxylase-positive neurons and modulated CD68+ inflammatory cells in a dose-dependent manner (Swanson et al., 2011). To this end, these findings prompted translation of PPAR agonists, specifically pioglitazone, into a multi-center, double-blind phase 2 clinical trial (2015). This trial also attempted to find peripheral biomarkers for PD that included leukocyte PGC-1α, IL-6, and urine 8-hydroxydeoxyguanosine (Simon et al., 2015). There were no changes in
peripheral biomarkers observed. Additionally, the agonist failed to show benefit as indicated by UPDRS III scores.

Explanations for why PPAR agonists failed in human PD studies likely reflect the fact that the neurotoxin-inducing neuroinflammatory models incompletely reflect human disease. In addition, multiple inflammatory response targets are likely needed, along with secondary drug-induced neuroprotective activities. The UPDRS III scores may show other limitations. Indeed, there is the potential that using motor tests as an singular end point may not preclude other disease events that could be influenced by PD-modifying treatments. The span of time for the assessment was also relatively short. This would not allow evaluation as to whether the treatment had long-term beneficial effects. Potentially, the anti-inflammatory activities of PPAR agonists may slow the rate of progression in the long term. However, the lack of observed efficacy in treating motor dysfunction should prompt researchers to continue to explore other therapeutic alternatives.

Due to the lack of clinical efficacy using anti-inflammatory agents, potential therapies aimed at directly modulating immune aberrancies are actively being studied and investigated in the clinical setting. Currently, two main therapeutic avenues are being explored and have shown promise in both rodent models of disease and PD itself. These two methods are based on the specific targeting of aberrantly misfolded proteins and peripheral immune modulation to elicit immune transformation into a protective phenotype.

The therapeutic strategy of targeting alpha-synuclein is aimed at ameliorating PD-associated neuroinflammation and disease progression via
targeting misfolded α-syn. In recent years, it has been shown that toxic versions of α-syn can accumulate within neurons and be secreted into the extracellular environment where the misfolded, oligomeric protein can cause a neurotoxic, inflammatory immune response (Benner et al., 2008). Thus, immunotherapeutic approaches are being developed to target and enhance clearing of misfolded or post-translationally modified α-syn. There is potential benefit in targeting both the N-terminal and/or C-terminal region of the protein. A recent study using an AAV-α-syn rat model of PD indicates that anti-human α-syn N-terminal peptide antibodies can protect against dopaminergic neuron loss and decrease microglial activation to some extent (Shahaduzzaman et al., 2015). Treatment with the vaccine also lead to altered IgG production, enhanced MHCII expression, and increased CD4+ T cell infiltration into the CNS. Recently, it has also been shown that use of monoclonal antibodies against the C-terminal region of α-syn or misfolded α-syn reduces levels of protein propagation and improves PD-like pathologies, ameliorates dopaminergic neuronal cell loss, and attenuates motor deficits in mouse models of the disease (Fagerqvist et al., 2013, Games et al., 2014, Lindstrom et al., 2014, Tran et al., 2014). Based on these findings, in 2013, Roche and Prothena further developed and commercialized PRX002 to specifically target α-syn. A phase I clinical trial was initiated with the finding that the vaccine was safe and well-tolerated, prompting a second phase I trial to assess dose response, tolerability, pharmacokinetics, exploratory biomarkers, and immunogenicity (NCT02095171 and NCT02157714).

With a similar directive in mind, a current study using a vaccine against α-
syn demonstrated that vaccination prevented Parkinson-like inclusions, as well as increased Treg recruitment, increased glial cell-derived neurotrophic factor (GDNF) production, and increased antibody formation (Sanchez-Guajardo et al., 2013). Excitingly, another vaccine that has been developed to produce antibodies that are specific for α-syn while sparing neuroprotective beta-synuclein, AFFITOPE PD01A, has entered clinical trials (Schneeberger et al., 2012, Mandler et al., 2014). PD01 targets the phosphorylated form of α-syn that is responsible for induction of a neurotoxic proinflammatory cascade through microglial activation. The first phase is completed and has indicated that the vaccine is safe and tolerable in two doses, 15 and 75 µg (NCT01568099). Half of the patients developed α-syn antibodies suggesting that the vaccine is effective as well. However, further investigations are underway to test PDO1A’s clinical benefit in PD, as well as other parkinsonian-associated diseases (Mandler et al., 2015) (NCT02216188 and NCT02270489).

Collectively, these studies show that α-syn-targeted immunotherapies may have the potential to attenuate neuroinflammatory-associated neurotoxicities and as such, delay dopaminergic neurodegeneration associated with PD. Alternatively, targeting α-syn may have some potential pitfalls. Vaccination and antibody-mediated therapies are associated with antigen-specific T cell responses that may contribute to an inflammatory state operative during PD. This concern was realized in studies of immunotherapies for AD. Here, extensive T cell reactivity and an autoimmune meningoencephalitis followed immune-stimulating strategies for amyloid beta (Aβ) clearance (Orgogozo et al., 2003).
This, coupled with the presence of intracellular misfolded proteins, makes it imperative to generate vaccination designs that are long-lasting and highly specific for α-syn. The robustness of the immune response generated needs to be evaluated together with any potential cross-reactivity of common nonpathogenic forms of α-syn or members of its protein families.

Lastly, since there has been little success in anti-inflammatory therapy translation into the clinic, therapeutic strategies aimed at modulating the aberrant immune response during disease progression may be more appropriate. The majority of immunotherapies are designed to protect the host from something foreign; however, in neurodegenerative diseases, the target is a self-protein, making the type and duration of the peripheral immune response elicited extremely important. In the acute MPTP model of PD, nitrated α-syn enhances the neurotoxic element of microglia activation, ultimately leading to neuronal cell loss (Reynolds et al., 2008). In PD, our data supports that with inflammation and chronic immune stimulation in the brain, there is an increase in effector and memory T cell populations that can secrete pro-inflammatory and neurotoxic cytokines (Mosley et al., 2012, Anderson et al., 2014). This can drive microglial reactivity, producing even greater levels of pro-inflammatory mediators (Figure 1). Thus, inhibiting Teff subsets, along with microglial activation may be of clinical benefit (Benner et al., 2004). One such strategy utilizes the immune suppressing capabilities of Tregs to target neuroinflammation (Figure 2). In the MPTP mouse model, our lab has shown that Tregs can attenuate microgliosis, protect against dopaminergic neuronal death, and spare striatal termini (Kosloski et al., 2013).
Tregs can elicit a down-regulation of pro-inflammatory mediators such as iNOS, TNFα, IL-1β, and IFN-γ, as well as decrease levels of ROS production and NF-κB activation (Lan et al., 2012, Lowther and Hafler, 2012). Through these mechanisms, they may also have the ability to mediate their protective actions by switching microglial responses from a neurotoxic M1 response to a neurotrophic M2 response (Reynolds et al., 2007b). However, it should be noted that even resting microglia can exist in a spectrum of phenotypic states rather than solely existing in an M1 and M2 condition that are exclusively pro- or anti-inflammatory (Cherry et al., 2014). Rather, microglia may simply be shifted into a more or less anti-inflammatory state. Nonetheless, these findings indicate a phenotypic transition during Teff or Treg interactions with microglia or peripheral monocyte-macrophages. Furthermore, as discussed previously, Tregs can act directly on activated Teff populations and antigen presenting cells by cytolysis, metabolic disruption, inhibiting maturation, and/or secreting suppressive cytokines (Dasgupta and Saxena, 2012). However, in the overall PD population, there is a Treg deficit and an increase in Teff populations that are associated with increased movement dysfunction and disease severity (Saunders et al., 2012), indicating a need for a therapy that may induce or enhance Treg functions. One appropriate therapeutic avenue would be to enhance Treg numbers or function through the use of potent immune modulating agents such as VIP, pituitary adenylate cyclase-activating polypeptide (PACAP), and GM-CSF, or through the use of vaccine strategies that induce Treg populations. Notably, the use of PACAP, VIP, GM-CSF, and a Bacillus Calmette-Guerin vaccination have shown
promising results in PD models. Such therapeutic interventions have also shown effectiveness in the treatments of other chronic inflammatory conditions and as such, support their ability to restore immune homeostasis and repair tissue injuries (Schabitz et al., 2008, Reynolds et al., 2010, Delgado and Ganea, 2013, Kosloski et al., 2013, Lacan et al., 2013, Waschek, 2013, Shivers et al., 2014, Fraccaroli et al., 2015, Kelso et al., 2015).

For instance, adoptive transfer of VIP- or GM-CSF-induced Tregs following MPTP intoxication leads to enhanced neuronal survival of nearly 100%, and a decrease in microglia activation (Reynolds et al., 2010, Kosloski et al., 2013). The potential use of this therapeutic strategy, along with clearance of aberrant protein, could possibly lead to induction of Tregs that will induce a phenotypic shift in effector subsets and microglia, ultimately ameliorating disease. Although, there remains the possibility that the neuroinflammatory response that is occurring with progressed PD will not be ameliorated simply through the induction of Tregs. Tregs may not have the ability to suppress the detrimental immune response to a point in which protection is observed. However, pre-clinical studies have already shown potential promise and the ability of effector to regulatory cell transformation. Thus, here, we report the therapeutic potential of a stable VIP analog in the MPTP mouse model of PD.
1.4 Summary Figures

Figure 1. *Neuroinflammation in Parkinson’s disease.*
Figure 1. *Neuroinflammation in Parkinson’s disease*. The pathogenesis of PD is affected by modified α-syn released extracellularly when dopaminergic neurons become damaged or die. The modified aggregated protein elicits microglial immune responses leading to a neurotoxic cascade within the substantia nigra pars compacta. Reactive microglia secrete neurotoxic factors that include proinflammatory mediators further accelerating neuronal cell death. Drainage of modified α-syn across the blood-brain barrier and into secondary lymphoid tissues causes an aberrant activation of the adaptive immune system. In response to α-syn, infiltrating effector T cells exacerbate neurodegeneration by contributing to the inflammatory neurotoxic cascade through the secretion of factors that include TNF-α and IFN-γ.
Figure 2. Modulating the neuroinflammatory response.
Figure 2. *Modulating the neuroinflammatory response.* The use of agents that induce Treg promote neurotrophic support and ultimately, neuronal cell survival by suppressing effector T cell activation, proliferation, secretion of neurotoxic mediators, and infiltration into the brain. These events modulate microglial reactivity by secreting immune suppressive factors such as IL-10, IL-4, and FASL (Fas ligand). The immune events temper neurotoxic innate and adaptive immune responses associated with disease progression, ultimately leading to a neuroprotective phenotype within the affected nervous system.
CHAPTER TWO

SELECTIVE VIP RECEPTOR AGONISTS FACILITATE NIGROSTRIATAL NEUROPROTECTION

2.1 Abstract

Vasoactive intestinal peptide (VIP) mediates a broad range of biological responses by activating two related receptors, VIP receptors 1 and 2 (VIPR1 and VIPR2). Although use of native VIP facilitates neuroprotection, clinical application of the hormone is limited due to VIP’s rapid metabolism and inability to distinguish between VIPR1 and VIPR2 receptors. In addition, activation of both receptors by therapeutics may increase adverse secondary toxicities. Thus, we developed two metabolically stable and receptor-selective agonists, one for VIPR1 and one for VIPR2 in order to improve the pharmacokinetic and pharmacodynamic therapeutic endpoints. These two selective agonists were investigated for their abilities to protect mice against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nigrostriatal degeneration used to model Parkinson’s disease (PD). Survival of tyrosine hydroxylase (TH⁺) neurons in the substantia nigra was quantified by stereological tests after MPTP intoxication in mice pretreated with VIPR1 or VIPR2 agonist, or following adoptive transfer of splenic cell populations from agonist-treated mice administered into MPTP-intoxicated animals. Treatment with VIPR2 agonist or splenocytes from those

---

mice resulted in increased neuronal sparing when compared to native VIP or VIPR1 agonist. Immunohistochemical tests showed that agonist-treated mice displayed reductions in microglial responses, with the most pronounced effects in VIPR2 agonist-treated, MPTP-intoxicated mice. In parallel studies, we observed changes in pro-inflammatory cytokine release that included decreases in IL-17A, IL-6, and IFN-γ and increases in GM-CSF transcripts in CD4+ T cells recovered from VIPR1 or VIPR2 agonist-treated animals. Moreover, a phenotypic shift of effector to regulatory T cells was observed. These results support the use of VIPR2-selective agonists for PD treatment.

2.2 Introduction

Aberrant innate and adaptive immune responses are known disease initiators for PD (Ha et al., 2012, Mosley et al., 2012, Kosloski et al., 2013). These result in chronic immune activation perpetuated by the extracellular accumulation of aggregated and post-translationally modified α-syn (Benner et al., 2008). Prion-like aggregated α-syn induces an inflammatory neurotoxic cascade affecting nigrostriatal degeneration (Anderson et al., 2014). Infiltrating CD4+ and CD8+ T cells, microglial activation, and dopaminergic cell loss are linked to human disease and are observed in PD mouse models and post-mortem human brain tissues (McGeer et al., 1988, Fiszer et al., 1994b, Kurkowska-Jastrzebska et al., 1999a, Bas et al., 2001, McLaughlin et al., 2006, Brochard et al., 2009). PD patients present higher frequencies of Teff phenotypes with reduced Treg function, and T cell changes relative to controls without disease correlate with
increased PD-associated movement disorder scores (Saunders et al., 2012). Other clinical studies have shown altered frequencies in peripheral CD4+ T cells and phenotype changes within the PD population, substantiating the possible role changes in T cells in PD progression (Romero-Ramos et al., 2014).

Work performed in animal models demonstrates that adoptive transfer of Teff exacerbates neurodegeneration (Benner et al., 2008, Kroenke et al., 2008), whereas transfer of Tregs elicits neuroprotective responses (Reynolds et al., 2007b, Huang et al., 2009, Kosloski et al., 2013). Treg-mediated protection from dopaminergic cell death is complemented by decreased microglial reactivity. Our work and the work of others have shown that GM-CSF, which modulates Teff immunity, is neuroprotective in MPTP-treated mice and in traumatic brain injury models (Schabitz et al., 2008, Kosloski et al., 2013, Kelso et al., 2015). GM-CSF induces an immune transformation from a neurotoxic response to a protective regulatory response (Kosloski et al., 2013). Nonetheless, extended use of GM-CSF is associated with secondary toxicities including bone pain, fatigue and nausea (Vial and Descotes, 1995a). Therefore, a search for alternative immune-modulating treatment strategies that restore regulatory capacity to a system imbalanced by disease pathobiology or means for the achievement of improved drug delivery is warranted.

One agent that could improve PD outcomes is vasoactive intestinal peptide (VIP). VIP is the natural 28-residue agonist of the G protein-coupled receptors, VIPR1 and VIPR2 (Reubi and Waser, 2003). It acts as a cytokine and neuropeptide by positively affecting immune responses (Delgado and Ganea,
The immunomodulatory capabilities of VIP support its potential to transform T cell phenotypes, leading to protection in models of inflammatory and autoimmune conditions (Delgado et al., 2000, Delgado and Ganea, 2001, Abad et al., 2003, Abad et al., 2005, Chen et al., 2008, Abad et al., 2010, Deng et al., 2010). These conditions include rheumatoid arthritis, inflammatory bowel disease, endotoxic shock, multiple sclerosis, and Crohn's disease. Thus, we reasoned that VIP could also transform adaptive immune responses in PD. Indeed, prior works show that VIP protects against neurotoxicity by attenuating microglial activation and degradation of neuronal cell bodies and termini in both MPTP- and 6-OHDA-induced injuries (Offen et al., 2000, Delgado and Ganea, 2003b, Reynolds et al., 2010, Korkmaz et al., 2012, Tuncel et al., 2012). The protective mechanism arises from altering the cytokine response into an anti-inflammatory profile (Vial and Descotes, 1995b, Delgado et al., 1999, Chen et al., 2008, Reynolds et al., 2010).

Based on these findings, we hypothesize that VIP-induced neuroprotective responses can be harnessed for clinical benefit if the rapid proteolytic degradation and lack of VIPR selectivity of the native hormone itself can be overcome (Domschke et al., 1978). To this end, we developed backbone-modified analogues of VIP that resist protease degradation and display selective agonism of VIPR1 or VIPR2. Both VIPR1 and VIPR2 agonists caused reductions in the release of pro-inflammatory cytokines, IL-17A, IL-6, and IFN-γ from stimulated CD4+ T cells. Transformation of Teff responses into an anti-
inflammatory T cell phenotype was observed. Likewise, VIPR2-selective agonist elicited a neuroprotective response, which provides evidence for its potential to ameliorate PD.

2.3 Materials and Methods

*Peptide synthesis and purification.*

Protected α-amino acids, resins, and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphatate (HBTU) were purchased from GL Biochem. Protected β-homoamino acids were purchased from PepTech. ACPC was acquired from Chemimpex and Polypeptide and APC was purchased from the organic chemistry facility at Fox Chase Cancer Center (Lee et al., 2001). Native VIP and [D-p-Cl-Phe⁶, Leu¹⁷]-VIP were purchased from Genway Biotech and Tocris Bioscience, respectively. All other reagents and solvents were purchased from Airgas, ChemImpex, Fisher Scientific, PharmcoAaper or Sigma Aldrich and used as received. Reverse-phase high performance liquid chromatography (RP-HPLC) was carried out on Supelco and Phenomenex analytical or preparative scale C18 columns using gradients between 0.1% trifluoracetic acid (TFA) in water or in acetonitrile using Agilent 1260 and Varian Prostar systems.

Peptides were synthesized by standard Fmoc-solid phase synthesis on Rink amide 4-methylbenzhydrylamine hydrochloride (MBHA) resin with norleucine. Microwave irradiation was used as previously described for peptide syntheses (Korendovych et al., 2010, Shandler et al., 2011). Briefly, protected
amino acids were activated with HBTU and N-hydroxybenzotriazole (HOBr) in the presence of N,N-diisopropylethylamine (DIEA) in N-methyl-2-pyrrolidone (NMP) for coupling reactions. Deprotection was achieved with 20% piperidine in dimethylformamide (DMF). After the final deprotection, peptides were capped using acetic anhydride/DIEA in DMF. After synthesis was complete, peptides were cleaved from the resin using a solution of 95% TFA, 2.5% H_2O, and 2.5% triisopropylsilane. Excess TFA was removed under a stream of nitrogen, and crude peptide was precipitated by addition of cold ether. Crude peptide solutions were purified using reverse-phase HPLC on preparative scale using C18 columns. The identity and purity of peptides were confirmed by mass spectrometry. After lyophilization, peptides were dissolved in TFE. An Agilent diode array, model 8453, was used to determine concentrations, with an extinction coefficient of 2980 M^{-1} cm^{-1} was applied. Aliquots for assays were dried under vacuum, resuspended in 20% acetonitrile, 0.1% HCl and lyophilized to exchange the counter ion. Dried peptide was resuspended in either DMSO or 1% DMSO in PBS.

**Cell-based assays.**

EC_{50} values for both VIPR1 and VIPR2 were determined in triplicate for each time point by monitoring intracellular cAMP concentrations of DiscoveRX’s PathHunter CHO-K1 VIPR1 β-arrestin or CHO-K1 VIPR2 β-arrestin cell lines, respectively. Both cell lines were used between 2 and 5 passages. To initiate assays, 2000 cells per well were plated into a 384-well plate and incubated 16
hours in F-12K medium supplemented with 10% FBS. The following day, each
reconstituted compound was diluted by twelve 3-fold serial dilutions and added to
the plated cells. The final concentrations for each compound in the assay wells
ranged between 5.6 pM and 1 µM in 1% DMSO/PBS. After a 90-minute
incubation period the medium and compound were removed and intracellular
cAMP concentrations were determined using the cAMP HiRange Kit available
from Cisbio. All kit components were used at concentrations according to the
manufactures recommendation. In brief, this kit functions by lysing the activated
cells to release the intracellular cAMP, which is then available to disrupt a FRET
pair. Homogenous time-resolved fluorescence signals were monitored using a
Tecan M1000 Pro in TR-FRET mode. All TR-FRET parameters including
excitation, emission, delay, and integration times were set according to the kits
recommendations and 100 flashes were used per well. The fluorescence
intensity of each well at both 620 nm and 665 nm was measured and the ratio of
these values was used to determine delta F%, which is defined as 
\[
\frac{\text{standard or sample ratio} - \text{ratio of the negative control}}{\text{ratio of the negative control}} \times 100.
\]
The resulting data were calibrated against a negative control, baseline corrected
then normalized using the average VIP response for each cell line as boundaries.
This normalized log inhibitor response curve was fit to a four-parameter
sigmoidal dose-response curve using least squares fitting in the GraphPad Prism
software package with a boundary constraint that each EC\textsubscript{50} fit was less than 1e^-6M.
Proteinase and pepsin assays.

The degradation rate of the peptide agonists was measured in the context of multiple aggressive proteases. All data were analyzed using analytical HPLC in triplicate for each time point. Pepsin Assay proteinase stock was prepared fresh using 185 nM pepsin, 34 mM NaCl and 84 mM HCl in deionized water. At time zero either 2.8 nmol of VIP or 7.0 nmol of Hybridtide was rapidly diluted into 350 µL of proteinase stock. Hybridtides were run at slightly higher concentrations to account for HPLC line broadening during detection. At specified times, 50 µL aliquots were removed and quenched with 10 µL of 1 M Tris pH 9.5. Quenched samples were evaluated by HPLC. Peak integrals were determined using Chemstation software and normalized to the average integral value for the first time-point for each dataset independently. These data were then fitted to a single exponential decay using least squares fitting through GraphPad Prism. Chymotrypsin and proteinase K assays were performed similarly to the pepsin assay with the following exceptions: all solutions were made in phosphate buffered saline, chymotrypsin was used at 40 nM, Proteinase K was used at 35 nM, and all time points were quenched with 10 µL of 99% acetonitrile/1% TFA.

Pharmacokinetic Analysis

Doses of LBT-3627 at 250 nmol/kg in 50 µL of 1% DMSO in PBS was prepared and dosed subcutaneously into the neck scruff of six 25-week-old C57BL/6 mice across two cohorts, which were used to minimize the amount of blood taken from each mouse according to IACUC approved protocols. Group 1 was sampled at 0,
30, 75 and 180 minutes, and Group 2 was sampled at 15, 45, 120 and 300 minutes. At each time point, 20 µL of blood was sampled from the tail vein using heparin-coated glass capillaries. Collected whole blood was mixed with broad-spectrum protease inhibitors (ThermoScientific) and sodium heparin (Sagent Pharmaceuticals) at 1X and 1 IU/mL, respectively. Whole blood samples were centrifuged for 10 min at 5000 x g to isolate plasma, which was flash-frozen in liquid nitrogen and stored at -20°C until further processing. To prepare samples for LC-MS/MS analysis an acetonitrile precipitation was performed by mixing 5 µL of thawed blood plasma with 15 µL of acetonitrile containing 1% formic acid and 13.3 nM LBT-3393 which was used as an internal standard. This mixture was vortexed at room temperature for 5 minutes at maximum speed on a Vortex Genie 2 and then centrifuged at room temperature for 5 minutes to remove the precipitated proteins. Supernatant was removed and transferred to an HPLC autosampler vial, and 23 µL of H₂O containing 5% formic acid was added to dilute the supernate prior to analysis.

For LC-MS/MS analysis, samples were transferred to an Agilent 1260 autosampler and held at 25°C until injection. Thirteen microletters of the sample was injected onto a Phenomenex Kinetex 2.6 µm C18 (50 x 3.0 mm) column heated to 50°C. An acetonitrile gradient of 20-35% over 2.5 minutes at 400µL/min was used to isolate LBT-3627 along with the internal standard. Compound elution was detected with an AB Sciex 4000 mass spectrometer equipped with a Turbo V ion source. Standard ionization parameters, as defined by the manufacturer, were used for drying and ionization. The two most intense
MS/MS transitions were collected, summed for quantification, and subsequently integrated with Analyst version 1.6 using default parameters. Integral intensities were normalized to the internal standard and compared to the linear range of a 10-point standard curve to determine plasma drug concentrations. The plasma exposure data were fitted to a single-compartment model for absorption and clearance using nonlinear regression within GraphPad Prism version 6.0f.

**Animals, drug treatment and MPTP intoxication**

Male C57BL/6 mice, six to eight weeks old (The Jackson Laboratory) were used as donor and recipient mice in all studies. Donor and pretreated mice were administered either VIP (VIP, human ovine porcine rat, Genway Biotech), LBT-3393, LBT-3627, or scrambled peptide, all of which, were reconstituted using Dulbecco’s PBS (DPBS) and given at a dosage of 15 µg, i.p., daily for 5 days prior to MPTP intoxication. For antagonist treatment, mice were administered with [D-p-Cl-Phe6,Leu17]-VIP at 8 µg i.p. daily for 5 days. Recipient mice received 4 s.c. injections of vehicle (DPBS, 10 ml/kg body weight) or MPTP-HCl (Sigma-Aldrich) at 16 mg MPTP (free base)/kg body weight in DPBS; each injection was given at 2-hour intervals. Twelve hours after MPTP intoxication, splenocytes were harvested from donors and adoptively transferred to MPTP-intoxicated recipient mice (n = 5-8 mice per group per time point). On days 2 and 7 following administration of MPTP, mice were sacrificed, and brains were harvested and processed for analysis. MPTP safety precautions were followed in accordance with the determined safety and handling protocols (Jackson-Lewis...
and all animal procedures were in agreement with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Isolation and adoptive transfer of CD4+ T cells

After 5 days of peptide administration, donor mice were sacrificed and single-cell suspensions were obtained from spleen and lymph nodes (brachial, axillary, cervical, and inguinal), and resuspended to 50 x 10^6 cells/0.25 ml for adoptive transfer. Recipient mice received 0.25 ml of cell suspension i.v. via the tail vein within 12 hours of the final MPTP injection. CD4+ T cells and CD4+CD25+ T cells were negatively selected using CD4+ T cell isolation kit II for mouse as per manufacturer’s instructions (Miltenyi Biotech) or CD4+CD25+ Treg isolation kit for mouse, with purity ranging from 65% to 90% depending on assay. Isolated CD4+ T cell populations were used for RNA isolation, genomic analysis, and cytometric bead assays (CBA). Freshly isolated CD4+CD25+ Treg were used for carboxyfluorescein succinimidyl ester (CFSE) inhibition assays (Quah and Parish, 2010).

CD11b+ microglia isolation

Following either MPTP-intoxication or 5 days of pretreatment with peptides, mice (n=8) were sacrificed and perfused with cold PBS to remove circulating immune cells. Ventral midbrains were collected from each mouse and dissociated into single-cell suspensions using the Neural Tissue Dissociation kit, as per
manufacturer’s instructions (Miltenyi Biotech). Myelin was removed using a 30% Percoll in DPBS solution. CD11b+ cells were magnetically isolated using PE-conjugated anti-CD11b antibodies followed by a magnetic bead-conjugated secondary antibody. The labeled cells were passed through a magnetic MS column (Miltenyi Biotech) according to protocol and as previously described (Nikodemova and Watters, 2012).

Perfusions and immunohistochemistry
Under terminal anesthesia (Fatal Plus, pentobarbital), mice were transcardially perfused with DPBS followed by 4% paraformaldehyde/DPBS (Sigma-Aldrich). Whole brains were harvested post-perfusion in order to assess dopaminergic neurons in the substantia nigra (SN) and termini in the striatum. Frozen midbrain sections (30 µm) were immunostained for tyrosine hydroxylase (TH) (anti-TH, 1:2000, EMD Millipore) and counterstained for Nissl substance (Benner et al., 2004). To assess microglial reactivity, midbrain sections (30 µm) were immunostained for Mac-1 (anti-CD11b, 1:1000, AbD Serotech). To assess dopaminergic termini, striatal sections (30 µm) were labeled with anti-TH (1:1000, EMD Millipore). To visualize antibody-labeled tissues, sections were incubated in streptavidin-HRP solution (ABC Elite Vector Kit, Vector Laboratories) and color was developed using an H₂O₂ generation system and diaminobenzidine (DAB) chromogen (Sigma-Aldrich). Within the SN, total numbers of Mac-1+ cells, TH+Nissl+ (dopaminergic neurons), and TH-Nissl+ (non-dopaminergic neurons) were quantified by stereological analysis using Stereo Investigator software with
the optical fractionator module (MBF Bioscience). Density of dopaminergic neuronal termini in the striatum was determined by digital densitometry using Image J software (National Institutes of Health), as previously described (Kosloski et al., 2013).

MPTP metabolism and reverse phase-high performance liquid chromatography (RP-HPLC)

Mice (n=5) were intoxicated with MPTP alone or pretreated for 5 days with either VIP, LBT-3393, or LBT-3627 followed by MPTP intoxication. Within 90 minutes post-MPTP, striatum and ventral midbrain were isolated and processed for MPTP and MPP+ levels (Jackson-Lewis and Przedborski, 2007). MPTP and MPP+ levels were determined using RP-HPLC analysis, using UV illumination and detection at 245 and 295 nm, respectively.

RNA isolations and polymerase chain reaction

Mice were pretreated with PBS, VIP, LBT-3393, or LBT-3627. CD4+ T cells or CD11b+ microglia were harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen). All procedures were performed under RNAse-free conditions. cDNA was generated from RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) and pre-amplification was performed using the appropriate primer mixes for RT² PCR arrays for Mouse T Helper Cell Differentiation or Mouse Proinflammatory Response and Autoimmunity (Qiagen). Quantitative RT-PCR was performed on an Eppendorf Mastercycler Realplex Ep
as per the manufacturer’s instructions (Eppendorf). Data analysis was performed using RT² Profiler PCR Array web-based data analysis software, version 3.5 (Qiagen). Gene networks were generated using Ingenuity Pathway Analysis (IPA, Qiagen) and were designed using the Pathway Designer Tool.

**Flow cytometric tests and cytokine assessments**

Cell fractions were isolated from total splenocytes following peptide treatment. Samples were permeabilized using the FoxP3 staining buffer set kit (eBioscience) and fluorescently labeled with monoclonal antibodies to CD4, CD25, CD8, and FoxP3 (eBioscience) to assess T cell frequencies within the total population and analyzed using a FACSCalibur flow cytometer. To assess cytokine production, CD4+ T cells were isolated, stimulated using anti-CD3/CD28 beads, and cell supernatant was collected at 12 hours. Supernatants were assessed for cytokine levels using a multianalyte Th1/Th2/Th17 cytometric bead array (BD Biosciences) and with data acquired using a BD FACSArray bioanalyzer (Reynolds et al., 2010).

**Statistical Analyses**

All values are expressed as means ± SEM. Differences in between-group means were analyzed using ANOVA followed by Fisher’s least significant difference (LSD) *post hoc* test (GraphPad Software, Inc.). Comparisons of slope and elevation for CFSE inhibition assays were evaluated using linear regression. Slopes for all lines were significantly non-zero (P < 0.0005), and no line deviated
from linearity as determined by runs test ($P \geq 0.6667$).

### 2.4 Results

*Peptide Stability.*

VIP exerts strong neuroimmunomodulatory effects (Delgado and Ganea, 2003a, Reynolds et al., 2010, Waschek, 2013). However, two features of VIP are potentially problematic for its pharmaceutical development. *First*, as is the case for all peptide hormones, VIP has a short half-life in vivo because of rapid proteolysis (Domschke et al., 1978). *Second*, VIP activates two widely-distributed receptors, VIPR1 and VIPR2, both members of the B-family of GPCRs (Reubi and Waser, 2003). Currently, it is not clear which of the two receptors is most relevant to its neuroprotective capabilities. Therefore, we developed analogs of VIP to resist protease degradation and display selectivity for either VIPR1 or VIPR2.

Our design strategy centers on replacing some of the original $\alpha$-amino acid residues with $\beta$-amino acid residues (Horne et al., 2008, Boersma et al., 2012, Cheloha et al., 2014, Johnson et al., 2014). These $\alpha \rightarrow \beta$ substitutions alter the covalent spacing between amide groups along the peptidic backbone, which disrupts protease recognition, while largely retaining native side chains and $\alpha$-helix propensity (Johnson and Gellman, 2013). Recent studies show that periodic $\alpha \rightarrow \beta$ replacements in the C-terminal portions of two other peptide hormones that act on B-family GPCRs, glucagon-like peptide-1 (GLP-1) (7-37) and parathyroid hormone (PTH) (1-34), yield potent agonists that display
prolonged activity in vivo relative to the native hormones (Boersma et al., 2012, Cheloha et al., 2014).

These precedents prompted us to explore the impact of periodic $\alpha \rightarrow \beta$ substitution in the C-terminal portion of VIP. These efforts led to LBT-3393 (Table 1), an analog that contains five $\alpha \rightarrow \beta$ replacements, at residues Thr11, Lys15, Val19, Leu23 and Leu27. At each replacement site, the $\beta$ residue bears the native side chain. In addition, LBT-3393 contains 2 $\alpha$ residue changes that are known to be well-tolerated in VIP, Met17$\rightarrow$Leu and Ser25$\rightarrow$Ala (Nicole et al., 2000, Igarashi et al., 2002a, Igarashi et al., 2002b). LBT-3393 is a full agonist of VIPR1, although ~10-fold less potent than VIP itself. LBT-3393 is highly selective for VIPR1 relative to VIPR2 (Figure 1), exhibiting only minimal VIPR2 activity at the highest concentrations tested.

Exploration of $\alpha \rightarrow \beta$ substitutions based on the sequence of VIP did not deliver a VIPR2-selective agonist; therefore, the highly VIPR2-selective peptide Ro 25-1553 (Table 1) was utilized as a starting point (O'Donnell et al., 1994a, O'Donnell et al., 1994b). Analogue LBT-3627 contains nine $\alpha \rightarrow \beta$ substitutions (Table 1), some bearing the native side chains (Tyr10, Gln16, Lys28 and Gly30) and others having a cyclic structure. Dose response assays showed that LBT-3627 is a potent agonist of VIPR2 and highly selective for VIPR2 vs. VIPR1 (Figure 1). LBT-3627 is superior to LBT-3393 in terms of resistance to proteinase activities (Figure 2A-C) which translates to an improved in vivo pharmacokinetic profile (t½ = 24.33min, Figure 2D) of the unformulated peptide when compared to previous human studies at a value of t½ <1min (Domschke et al., 1978).
VIPR agonists induce neuroprotective responses in MPTP-intoxication.

Our prior work demonstrated immune modulatory and neuroprotective activities for VIP (Reynolds et al., 2010). Indeed, previous work showed VIP induction of Treg with concomitant anti-inflammatory and neuroprotective responses in MPTP-intoxicated mice as well as a broad number of animal models of inflammatory and neurodegenerative diseases (Delgado et al., 2002, Delgado and Ganea, 2003b, Chen et al., 2008, Reynolds et al., 2010). To extend such observations, with an eye towards clinical translation, we assessed the abilities of the designed VIPR agonists to promote dopaminergic neuronal survival in our mouse model of PD. Mice were treated for 5 days with VIP, VIPR1 agonist (LBT-3393), or VIPR2 agonist (LBT-3627) and then intoxicated with MPTP. Following MPTP treatment, total numbers of surviving dopaminergic neurons (TH+Nissl+) were observed in the SN (Figure 3A). Numbers of dopaminergic neurons were determined by investigator-blinded stereological analysis. Immunohistochemical observations of dopaminergic neurons showed a significant increase in surviving neuron numbers associated with LBT-3627 treatment compared to PBS control (Figure 3B). Following MPTP intoxication, dopaminergic neuron numbers decreased from 9789 ± 1061 to 4411 ± 1228 without or with MPTP intoxication, respectively (Figure 3B). No differences in numbers of non-dopaminergic neurons (TH-Nissl+) were observed because this neuronal type is not susceptible to MPTP intoxication (Otto and Unsicker, 1993, Jackson-Lewis and Przedborski, 2007, Choi et al., 2008). Following VIPR agonist pretreatments, MPTP-induced
TH+ neuronal loss was attenuated for all treatment arms. Compared to MPTP alone, VIP, LBT-3393 and LBT-3627 pretreatment increased dopaminergic neuronal numbers to 5264 ± 1441, 4600 ± 945, and 7339 ± 2115, respectively. However, only LBT-3627 pretreatment showed significant protective effects. In contrast, striatal termini were not significantly spared by the peptide pretreatments (Figure 3C). To determine whether the observed neuroprotective effects are linked to specific peptide-receptor interactions, a scrambled peptide (LBT-SCR) as a negative peptide control and a specific VIPR antagonist ([D-p-Cl-Phe6,Leu17]-VIP) were employed. These experiments were designed to competitively inhibit binding of VIP, LBT-3393 or LBT-3627 to VIP receptors (Figure 4A). Co-administration of the VIP receptor antagonist, [D-p-Cl-Phe6,Leu17]-VIP, with VIP, LBT-3393, or LBT-3627 decreased previously observed VIP- or VIPR2 agonist-mediated neuroprotection. Under these conditions, numbers of TH+ neurons were not significantly different compared to those from mice treated with MPTP alone, or with MPTP and scrambled peptide. Additionally, striatal termini in MPTP-treated mice were not spared by any treatment (Figure 4B). To determine the neuroprotective efficacy with the current dose, we next addressed treatment using escalating doses of LBT-3627 (1.5 µg, 5 µg, 15 µg, 45 µg, 90 µg) (Fig 5A). When compared to MPTP alone, the lowest dose did not elicit a protective response, but the other four higher doses were effective. Linear regression analysis suggests that there is a dose-dependent effect in TH+ neuronal sparing with treatment ($R^2 = 0.4614, P = 0.001$) (Fig 5B). The lack of increased protection observed at the 90 µg dose and the high
variation observed at 45 µg may indicate that these doses are approaching a non-efficacious and/or toxic effect. Thus, the 15-µg dose seemed to be adequate for eliciting a strong neuroprotective response without generating any toxicity.

In a next series of experiments to assess the VIP-induced cellular response, donor mice were treated with VIP, LBT-3393 or LBT-3627 for 5 days, splenocytes were isolated from donor mice and adoptively transferred into recipient mice 12 hours after MPTP-intoxication. Dopaminergic neurons and striatal termini density were assessed 7 days after MPTP intoxication by immunohistochemical assessment of TH+ neurons in the midbrain and termini in the striatum (Figure 6A). MPTP diminished TH+Nissl+ counts from 13011 ± 1025 to 3414 ± 382 (Figure 6B). MPTP-induced neuronal loss was attenuated by adoptive transfer of splenocytes from non-intoxicated donor mice treated with any of the VIPR agonists showing significantly increased numbers of surviving dopaminergic neurons of 6896 ± 707 and 11094 ± 1233 TH+Nissl+ neurons in mice receiving spleen cells from donors treated with LBT-3393 or LBT-3627, respectively, compared to those treated with MPTP alone. Transfer of splenocytes from VIP-treated donors increased dopaminergic neuronal survival by 14%. Numbers of non-dopaminergic TH-Nissl+ neurons were not significantly different in any group treated with MPTP regardless of adoptive transfer. Compared to treatment with VIPR agonists prior to MPTP (Figure 3), striatal density analysis showed similar, non-significant effects for striatal sparing following adoptive transfer of splenocytes from mice treated with VIP or LBT-3393; however, adoptive transfer of splenocytes from LBT-3627-treated donors
showed significant sparing of striatal termini (Figure 6C).

**Microglial reactivity is decreased with VIPR2 agonism.**

As previously discussed, microglial activation is associated with neuronal cell death in human PD and in mouse models of disease (Kurkowska-Jastrzebska et al., 1999a, McLaughlin et al., 2006). Whether the neuroprotective effects upon either adoptive transfer from agonist-treated mice or agonist pretreatment of mice prior to MPTP intoxication were associated with changes in microglial morphology and pro-inflammatory responses were investigated. As before, MPTP intoxication was performed directly in mice receiving peptide pretreatment (Figure 7), or alternatively, donor animals were treated for 5 days prior to splenic cell isolation and adoptive transfer into recipient MPTP-intoxicated mice (Figure 8). Microglial responses were evaluated 2 days following MPTP-intoxication, at the peak of the MPTP-induced neuroinflammatory response (Kurkowska-Jastrzebska et al., 1999a). The SN was immunostained with macrophage antigen complex-1 (Mac-1) antibody, and reactive microglia were quantified. Both Mac-1 staining magnitude and microglial morphological changes, consisting of intensely-stained, amoeboid shaped microglia and reduced stained, ramified structures, were observed; the former as activated microglia, the latter as quiescent microglia (Figure 7). Stereological analysis showed numbers of activated microglia increased from 2.8 ± 1.1 cells/mm$^2$ in PBS-treated mice to 44.5 ± 6.9 cells/mm$^2$ following MPTP intoxication (Figure 7). VIP pretreatment decreased microglial reactivity to 25.1 ± 2.2 cells/mm$^2$ and VIPR1 agonism (LBT-
3393) showed similar numbers of activated microglia, 28.9 ± 3.3 cells/mm². The largest reduction in MPTP-induced microglial reactivity was seen following pretreatment with the VIPR2 agonist (LBT-3627) which significantly decreased the activated cell numbers to 18.1 ± 7.4 cells/mm². Moreover, treatment with LBT-3627 diminished numbers of activated microglia not only from mice treated with MPTP alone, but also from those treated with VIP or LBT-3393 and MPTP.

Adoptive transfer of splenic cells from agonist-treated donors was performed twelve hours following MPTP intoxication of recipient animals. Replicate data sets revealed reduced microglial reactivity in the SN after transfer of splenocytes from donors treated with VIP or either VIPR agonist (Figure 8). Compared to PBS-treated animals, MPTP intoxication increased the number of reactive microglia from 2.2 ± 0.5 cells/mm² to 29.6 ± 6.9 cells/mm² (Figure 8). Adoptive transfer of cells from animals treated with VIP significantly reduced the numbers of activated microglia to 18.7 ± 7.6 cells/mm². Comparable transfers from animals treated with either LBT-3393 or LBT-3627 scored at 23.9 ± 5.7 cells/mm², and 18.3 ± 2.9 cells/mm². However, only VIP or LBT-3627 treatment elicited significant decreases in activated microglial numbers when compared to MPTP alone. Together, these findings indicate that VIPR agonism can reduce reactive microgliosis associated with MPTP intoxication in each of two different treatment schemes. These favorable effects are likely to be associated with reduced inflammatory responses induced by VIP and VIPR agonists that in turn result in neuroprotection.
Neuroprotective mechanisms for VIPR agonists.

We showed that VIP treatment expands Treg numbers and elicits anti-inflammatory and immune-suppressive responses by modulating immune cell profiles and phenotypes (Reynolds et al., 2010). Thus, to understand the immune-modulating potential of VIPR agonism, we assessed the ability of VIPR agonists to affect levels of CD4+ T cell populations, the function CD4+CD25+ Treg, and/or modulate cytokine production. Flow cytometric analysis of total lymphocyte populations recovered from animals after five days of peptide treatment revealed no significant changes in either CD4+ (Figure 9A) or CD4+CD25+ T cell frequencies within the total lymphocyte population (Figure 9B). Next, Treg function was evaluated after peptide treatment as the capacity to inhibit CD3/CD28-stimulated proliferation of CD4+ T responder cells (Tresp) using a CFSE proliferation assay (Quah and Parish, 2010, Saunders et al., 2012). Tregs isolated from animals treated with LBT-3627 showed an increased functional capacity when compared to Tregs isolated from animals treated with PBS, VIP, or LBT-3393 (Figure 10). LBT-3627 treatment resulted in Tregs that caused a 74% inhibition of proliferation at a 1:1 Tresp:Treg ratio while the inhibition was 29% for Tregs from animals treated with PBS, 41.5% for Tregs from animals treated with VIP, and 47.5% for Tregs from animals treated with LBT-3393. The inhibitory capacity of isolated Tregs decreased in a dose dependent manner ($R^2 > 0.88$, $P < 0.0005$, for all treatments); however, LBT-3627 Tregs maintained enhanced suppressive capabilities even at the lowest dose when compared to all other treatment arms. Linear regression analyses of
Treg-mediated inhibition indicated that Treg dose responses from LBT-3627-treated mice were significantly enhanced (P < 0.03) over those from mice treated with PBS, VIP, or LBT-3393. Dose responses of Tregs from mice treated with LBT-3393 were significantly larger than those from PBS-treated controls (P = 0.016), whereas Treg responses from VIP-treated mice compared to PBS controls did not reach significance (P = 0.0788). Collectively, these data suggest that Treg frequencies are not affected by treatment with VIPR agonists, but functional properties of Tregs are enhanced upon these treatments, with the most pronounced enhancement resulting from the VIPR2-selective agonist.

Next, to determine mechanism(s) by which VIPR agonists could enhance Tresp suppression and diminish inflammation with neuroprotective effects, the effects of VIPR agonists on cytokine production were investigated after T cell stimulation. For these studies, mice were treated with PBS, VIP, LBT-3393, or LBT-3627 for 5 days. CD4+ spleen cells from each treatment arm were isolated, stimulated with anti-CD3/CD28, cultured and assessed for cytokine production by cytokine bead array. Relative to cytokine levels of stimulated CD4+ T cells from PBS-treated controls, LBT-3393 and LBT-3627 treatment significantly suppressed production of the proinflammatory cytokines IL-17A, IFN-γ, and IL-6, but not tumor necrosis factor-α (TNF-α) (Figure 11). Interestingly, VIP administration resulted in an opposite effect, with a significant upregulation of pro-inflammatory cytokines. This may be due to the fact that VIP is about 10-fold more potent than the selective VIPR agonists, suggesting that increased potency may yield an undesirable effect on pro-inflammatory cytokine production. Taken
together, these data demonstrated that selective agonism of either VIPR1 or VIPR2 induces downregulation of pro-inflammatory T cell phenotypes, and results in the enhanced immunosuppressive properties observed by Tregs. Observation of such T cell shifts in phenotype within 12 hours of stimulation also suggested that this VIPR agonist-mediated shift may occur prior to stimulation via CD3/CD28.

Because Treg numbers or cytokine production alone could not readily explain differences in the preferential effects on Treg function and neuroprotection mediated by VIPR2 versus VIPR1 agonism, we next examined potential phenotypic shifts elicited by VIPR agonists. We investigated T helper differentiation gene expression changes within the total CD4+ T cell population. CD4+ T cells were isolated from animals treated with 5 days with PBS, VIP, LBT-3393, or LBT-3627, RNA was isolated, and gene expression evaluated by RT-PCR array for genes associated with T helper cell differentiation. Gene expression levels of T cells from mice treated with VIP, LBT-3393, or LBT-3627 were compared to those from animals treated with PBS alone. Significant fold changes in mRNA expression were observed for each pretreatment (Figure 12); each of the three VIPR agonists produces a distinct profile change. For VIP pretreatment, transmembrane emp24 protein transport domain containing 1 (Tmed1) was significantly upregulated, whereas Fas ligand (FasL), IL-18 receptor accessory protein (Il-18rap), IL-21, interferon regulatory factor 4 (Irf-4), Rel, and suppressor of cytokine signaling 5 (Socs5) were significantly down-regulated. VIPR1 agonist pretreatment yielded a down-regulation in Irf-4, Rel,
and Tmed1. VIPR2 agonist pretreatment elicited a robust and significant increase in Gm-csf, as well as parallel increases in IL-17 receptor E (Il-17re), IL-18 receptor 1 (Il-18r1), RAR-related orphan receptor C (Rorc), Toll-like receptor 4 (Tlr4), and Tmed1. Il-21, inhibitor of DNA 2 (Id2) and Socs5 were increased as well, but these increases did not reach statistical significance. VIPR2 agonism did not lead to significant downregulation of any genes associated with T cell differentiation.

Because the majority of the detected neuroprotective and anti-inflammatory responses were associated with VIPR2 agonism, we focused on relationships among genes for which mRNA expression increased upon pretreatment with the VIPR2-selective agonist, LBT-3627. Mapping the relationships by Ingenuity Pathway Analysis (IPA) among genes for which mRNA increased suggests that changes in two overlapping networks are induced by VIPR2 agonism (Figure 13). IPA-mapped changes implicate immunological disease and inflammatory response networks and cell-to-cell signaling and interaction networks. Changes in Gm-csf (CSF2) are central to these networks. Changes linked to both anti- and pro-inflammatory genes as well as innate and adaptive immunity were observed, along with changes in T cell transcription factors associated with T cell differentiation.

Furthermore, since T cells readily interact with microglial populations to elicit an immune response, we next sought to examine the effect of VIPR2 agonism on CD11b+ cell populations isolated from the ventral midbrain of MPTP intoxicated mice. To do so, we investigated the inflammatory response mediated
by CD11b+ populations following both pre-treatment with LBT-3627 alone (Figure 14) or in combination with MPTP (Figure 15). The results suggest a down-regulation in multiple innate and adaptive immune mediators with LBT-3627 treatment. Specifically, with LBT-3627 treatment alone, mRNA transcripts for genes associated with a pro-inflammatory and/or oxidative immune response such as *Il-1β*, *Il-23a*, *Ifn-γ*, *Ptgs2* (prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase), *Ltb* (lymphotoxin B also known as TNF-C), *Tnf*, and *Il-7* were profoundly decreased with a fold decrease of more than 10 when compared to the inflammatory response associated with MPTP treatment alone (Figure 14). Also, in combination with MPTP intoxication, LBT-3627 pretreatment yielded similar decreases in the pro-inflammatory response, showing moderate decreases in *Ptgs2* and *Ltb* with at least a 2 fold decrease, as well as greater decreases in *Tnf*, *Il-7*, and *NFκB* expression at more than 10 fold decreases when compared to MPTP intoxication alone. Thus, the genomic analyses revealed that VIPR agonists, especially the agonist specific for VIPR2, can positively affect both innate and adaptive immune responses through modulation of gene expression in CD4+ T cells and CD11b+ microglial populations, with a coincident down-regulation of pro-inflammatory cytokine production *in vitro*.

**VIPR agonists do not affect MPTP metabolism.**

VIP can cross the BBB (Dogrukol-Ak et al., 2003), and possibly inhibit metabolism of the MPTP protoxin to the 1-methyl-4-phenylpyridinium (MPP+) toxin with putative neuroprotection due to diminished intoxication. To rule out that
possibility, we analyzed levels of MPTP and MPP+ by RP-HPLC within the midbrain and striatum of intoxicated mice and compared those to levels in intoxicated mice that received VIP, LBT-3393, or LBT-3627 before MPTP. Treatment with VIP or either VIPR agonist did not reduce the levels of MPP+ intoxicant in either the midbrain (Figure 16A) or the striatum (Figure 16B). Thus, conversion from MPTP into MPP+ was achieved in all treatment arms and, in fact, MPP+ levels were greater in mice treated with either VIPR agonist prior to MPTP. Thus, these data and the adoptive transfer data support the idea that VIPR2 agonist-mediated neuroprotective responses do not arise from effects on MPTP metabolism, which is consistent with a more direct immunomodulatory mechanism of action for these agonists.

_Treatment with VIPR agonist following MPTP intoxication is not neuroprotective._

Because PD is a chronic and progressive disease, we wanted to evaluate the treatment efficacy of LBT-3627 after administration of MPTP. This treatment scheme would, in theory, be more similar to human disease onset and resulting treatment. To evaluate this testing paradigm, mice were treated with MPTP, and after 12 hours, five days of consecutive treatment with LBT-3627 was started. After 7 days, mice were sacrificed and surviving dopaminergic cell bodies and striatal termini were quantified (Figure 17). With both MPTP intoxication alone and MPTP intoxication followed by LBT-3627 treatment, there was a significant decrease in both surviving cell bodies (Figure 17A) and termini (Figure 17B). Treatment with LBT-3627 did not lead to the neuroprotective response that was
observed in adoptive transfer and pretreatment studies. The lack of protective response may be attributed to the rapid inflammatory cascade that ensues following MPTP intoxication and the slow induction of protective T cell phenotypes following initial LBT-3627 treatment (Kurkowska-Jastrzebska et al., 1999a). Perhaps this treatment scheme would be more effective in a more chronic and less inflammatory model, such as the chronic MPTP model or an α-syn overexpressing model.

2.5 Discussion

VIP has potential for the treatment of neuroinflammatory conditions based on its ability to transform T cells (Gonzalez-Rey et al., 2007). One obstacle to the clinical use of VIP is the hormone's lack of specificity for VIPR1 and VIPR2 (Usdin et al., 1994, Reubi and Waser, 2003). A second obstacle is the rapid proteolytic degradation of VIP in vivo. Our work aimed to better define therapeutic potential in this arena by developing protease-resistant VIPR agonists that target each receptor independently. We hypothesized that targeting specific VIP receptors individually with physiologically stable agonists would elicit a robust neuroprotective response connected to changes in innate and adaptive immunity. It was demonstrated that the metabolically stable and VIPR2-specific agonist LBT-3627 is an effective immunomodulatory agent in the mouse model of PD. Treatment of MPTP-intoxicated mice with LBT-3627 significantly spared dopaminergic neuronal cell bodies within the nigrostriatal lesion, decreased the amount of reactive microgliosis associated with MPTP insult, decreased levels
pro-inflammatory gene expression associated with the inflammatory response in 
CD11b+ microglia populations, down-regulated pro-inflammatory cytokine 
production, and modulated T cell phenotypes with treatment. In contrast, 
treatment with the stable, VIPR1-selective agonist LBT-3393 yielded lesser 
neuroprotective responses. Thus, in this model of PD, VIPR2 agonism elicits a 
link between neuroprotection and modulation of the immune response with 
systemic treatment. Interestingly, significant sparing of striatal termini was only 
observed following adoptive transfer not during pretreatment. It's suggested that 
the lack of termini survival may be due to the increased MPP+ levels associated 
with VIPR pretreatment, whereas the increased MPTP conversion would not 
have been observed with adoptive transfer because no drug interaction would 
have been possible.

Similar anti-inflammatory and protective responses associated with the 
VIPR2 have been studied previously. For instance, specific targeting of VIPR2 
has been shown to mediate anti-inflammatory and therapeutic effects in 
rheumatoid arthritis (Juarranz et al., 2008) and models of spinal muscular 
atrophy (Hadwen et al., 2014), ultimately leading to enhanced production of Th2-
type transcription factors like c-Maf and JunB, anti-inflammatory cytokines such 
as IL-4 and IL-5 (Voice et al., 2004) and IL-10 (Larocca et al., 2007), and 
decreased macrophage-derived pro-inflammatory cytokine production (Gomariz 
et al., 2000). Also, the loss of VIPR2 receptor leads to enhanced and 
exacerbated disease states in experimental autoimmune encephalitis (EAE) and 
experimental colitis, suggesting that VIPR2 activity is important for mediating
disease processes (Yadav et al., 2011, Tan et al., 2015). This loss was associated with increased Th1/Th17 responses and decreased Th2/Treg responses (Tan et al., 2015). These previous findings are consistent with the observation of enhanced neuroprotective activity of a degradation-resistant and selective VIPR2 agonist relative to a selective VIPR1 agonist or the non-selective native hormone VIP. Together, these studies highlight the potential of a VIPR2-selective agonist to modulate the adaptive immune response in therapeutically favorable ways.

Initially, it was hypothesized that VIPR2 agonism would yield an increase in CD4+CD25+ Treg population, resulting in increased neuroprotection. Even though prior work has shown VIP-mediated increases in frequencies of Tregs (Fernandez-Martin et al., 2006, Gonzalez-Rey et al., 2006b, Reynolds et al., 2010), we were unable to demonstrate this response at the dosages we used, so we began to assess other avenues of regulatory and anti-inflammatory function that promote dopaminergic neuron survival upon MPTP-intoxication. Thus, we assessed the ability of Tregs isolated from VIPR agonist-treated mice to suppress the proliferation of Tresp in vitro. It was found that co-culture of Tresp with Tregs from animals treated with the VIPR2-selective agonist, LBT-3627, elicited an enhanced suppressive effect when compared to Tregs from animals subjected to other treatments. This suppressive effect corresponded with down-regulation of pro-inflammatory cytokine production associated with Th1/Th17 T cell phenotypes. Thus, the regulatory and immune-suppressive roles of LBT-3627 are likely a result of T cell phenotype modulation that leads to a change in
the adaptive immune response associated with inflammation.

The impact of VIPR agonists on MPTP-intoxicated mice led us to employ gene expression analysis for further exploration of T cell differentiation and possible phenotypic shifts induced by agonist treatment. Observed increases in genes associated with both pro- and anti-inflammatory responses following LBT-3627 treatment were found. The most abundant change was a 45-fold upregulation of the Gm-csf transcript. Previous studies have demonstrated the potent and robust neuroprotective responses associated with GM-CSF treatment, such as increased neuronal survival, decreased microglial reactivity, induction of Tregs, and changes in innate and adaptive immune responses associated with inflammation (Schabitz et al., 2008, Kosloski et al., 2013). Although genes associated with multiple T cell subsets were altered upon LBT-3627 treatment, many were associated with anti-inflammatory subsets including Tregs and Th2 populations. For instance, Id2 was upregulated 10-fold with treatment. This gene has been associated with maintaining Treg populations in inflammatory disease in order to enhance suppressive capabilities (Miyazaki et al., 2014). These changes were not observed for VIP or LBT-3393 treatment. Likewise, Tmed1 was upregulated 3.3-fold when compared to PBS treatment. Tmed1 has been shown to be involved in IL-33 signaling (Connolly et al., 2013); IL-33 is an IL-1-like cytokine that induces Th2 type cytokines such as IL-4, IL-5, and IL-13 (Schmitz et al., 2005). While mRNA levels for these Th2-associated cytokines were upregulated >2-fold with LBT-3627, the modest changes were not significant for the current sample size. Small changes in cytokine levels can
ultimately affect T cell subsets depending on the microenvironment at the sites of inflammation. The expression level changes we observed may suggest that LBT-3627 induces a shift toward an anti-inflammatory response associated with regulatory or Th2 subsets rather than a pro-inflammatory Th1/Th17-mediated response. This possibility is supported by the down-regulation of Th1/Th17 cytokine production following treatment with LBT-3627. Upon cytometric bead analysis, we observed a significant down-regulation of IFN-γ, IL-6, and IL-17A with LBT-3627 treatment. The VIPR2-selective agonist also generated a >2-fold decrease in IFN-γ gene transcript, although, the effect did not reach significance. Collectively, alterations in the inflammatory disease response and cell-cell signaling pathways following VIPR2 agonism were demonstrated. Gm-csf is a central component for both networks, and its upregulation can elicit changes linked to both innate and adaptive immunity (Kelso et al., 2015, Gendelman et al., 2017). This ability to modulate the immune response was strengthened with the observed changes in inflammatory response transcripts in microglial populations isolated from the ventral midbrain. Treatment with LBT-3627 alone elicited profound down-regulation of mRNA levels of potent pro-inflammatory mediators such as IL-1β, TNF-α, TNF-C, COX, and IFN-γ. Given the enhanced inflammatory cascade that occurs during MPTP intoxication, it is important to note that pre-treatment with LBT-3627 followed by MPTP intoxication was also able to elicit changes in some of the same detrimental mediators, such as decreased COX, TNF-c, and TNF-a. Collectively, these findings could be indicative of cross-talk between CD4+ T cell populations and their interactions
with CD11b+ microglia populations, creating a shift in the inflammatory response, ultimately leading to enhanced anti-inflammatory function and neuroprotective capabilities.

VIP can cross the BBB (Dogrukol-Ak et al., 2003), which may allow direct interaction with resident immune cells and neurons within the CNS. Both glia cells and neurons express VIP receptors (Chneiweiss et al., 1985, Hosli et al., 1989), and both VIPR1 and VIPR2 are expressed within the SN, as well as in many other parts of the brain (Joo et al., 2004). Activation of VIPR1 and/or VIPR2 within the CNS could lead to many other protective effects that are not directly associated with the modulation of T cell responses and/or phenotypes. VIP binding to its receptors on microglia and other antigen-presenting cells has been associated with down-regulation of co-stimulatory molecules, possibly resulting in desensitization of an inflammatory response (Ganea et al., 2003), as well as inhibition of pro-inflammatory cytokine production by microglia (Kim et al., 2000). VIP interaction with astrocytes results in increased neurotrophin production such as activity-dependent neurotrophic factor (ADNF), leading to decreased cellular toxicities and increased neuronal survival (Gozes and Brenneman, 1996). VIP interaction with neurons has been documented as well, causing VIP to be widely accepted as a neuropeptide for neuronal signaling and regulation of reactive gliosis (Brenneman et al., 1987, Waschek, 2013).

It has not been determined whether LBT-3393 or LBT-3627 can cross the BBB. It is possible that these VIPR1- and VIPR2-specific agonists share the ability of VIP to cross the BBB and act directly on microglia, astrocytes, and
neurons. Further exploration of the effects of LBT-3393 and LBT-3627 on cell types within and outside the CNS will be necessary to elucidate the immunomodulatory and neuroprotective effects manifested by these compounds in the MPTP mouse model of PD. Such insight would enhance the prospects for clinical translation. Ultimately, the pleiotropic functions of VIP and this hormone's ability to act broadly within the immune system, and possibly the central nervous system, suggest that targeting VIPR1 and/or VIPR2 provides a good strategy for identifying therapeutic candidates. The results reported here show that systemic administration of a VIPR2-selective agonist elicited profound neuroprotective and anti-inflammatory responses, supporting further exploration from a clinical perspective. Our evaluation of LBT-3393 and LBT-3627 to date has been limited to an animal model, but based on the results, we hypothesize that such agents would elicit similar anti-inflammatory responses and T cell phenotypic shifts in humans. Thus, such agonists might counteract the imbalanced neuroinflammatory response associated with neurodegeneration in PD. By enhancing the suppressive function of Tregs and down-regulating pro-inflammatory cytokine production, a long-acting VIPR2-selective agonist might restore pro-inflammatory and anti-inflammatory responses to a homeostatic state within the substantia nigra, ultimately sparing dopaminergic neurons, along with the possibility of direct neuronal stimulation. Overall, our studies support the likelihood that VIPR2 agonism has the potential to slow the pathogenesis of PD through modulation of the aberrant adaptive immune response.
2.6 Tables and figures

Table 1. Amino acid sequences for the peptides evaluated in these studies.

<table>
<thead>
<tr>
<th></th>
<th>Amino Acid Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP</td>
<td>HSDAV FTDNY TRLRK QMAVK KYLNS ILN-NH$_2$</td>
</tr>
<tr>
<td>LBT-3393</td>
<td>$^{a}$Ac-HSDAV FTDY $^{t}$RLR$k$ QLAv$k$ KYlNa $^{I}$IN-NH$_2$</td>
</tr>
<tr>
<td>Ro 25-1553</td>
<td>$^{b}$,$^{c}$Ac-HSDAV FTENY TKLRK $^{Q}$LAAK K<code>YlND</code> LKKGG $^{T}$-NH$_2$</td>
</tr>
<tr>
<td>LBT-3627</td>
<td>$^{a}$,$^{c}$Ac-HXDAX FTE$^{x}$ TKLRK $^{q}$LAAZ KYXND L$k$Gg $^{T}$-NH$_2$</td>
</tr>
<tr>
<td>LBT-SCR</td>
<td>Ac-Ty$^{X}$TX EgLKX GFKTK RHZKA $^{q}$YLXL ANAD$k$D</td>
</tr>
</tbody>
</table>

$^a$β3-amino acids are in blue lowercase font, cyclic β-amino acids are indicated in red capital font.

$^b$A lactam bridge between K* and D* exists in Ro25-1553 along with a single norleucine, indicated by $^{n}$L.

$^c$Both Ro 25-1553 and LBT-3627 are alkanoylated at the N-terminus.
Figure 1. *LBT-3393 and LBT-3627 peptides are VIPR1 and VIPR2 agonists.*
Figure 1. *LBT-3393 and LBT-3627 peptides are VIPR1 and VIPR2 agonists.* cAMP dose response assays comparing native VIP to LBT-3393 and LBT-3627, top and bottom, respectively. Data were generated by activating engineered CHO cells that over express either VIPR1 or VIPR2 with increasing concentrations of peptide and detecting intracellular cAMP activation using a two-component HTRF pair. The data was reported as normalized ΔF% (a measure of cAMP HTRF ratios that is quenched at higher cAMP concentrations). Higher concentrations of VIP (>10⁻⁸M) were excluded from analysis as appropriate since they began to increase in relative value, likely a result of receptor internalization (data not shown).
Figure 2. *LBT-3627* is resistant to protease activities.
Figure 2. *LBT-3627 is resistant to protease activities.* **A-C.** Relative stabilities of three peptides, VIP (solid line & circles), LBT-3393 (dotted lines and open circle) and LBT-3627 (dotted lines and open triangles) after exposures up to 120 minutes to different aggressive protease environments of pepsin (A), proteinase K (B), and chymotrypsin (C). Percentages of non-degraded peptide concentrations remaining from time zero were determined by RP-HPLC. **D.** Pharmacokinetic (PK) profile of 250 nmol/kg unformulated LBT-3627 drug concentration using LC-MS/MS analysis, with $T_{1/2} = 24$ min.
Figure 3. LBT-3627 pretreatment is neuroprotective.
Figure 3. *LBT-3627 pretreatment is neuroprotective.* A. Photomicrographs of TH+Nissl+ neurons in the SN and TH+ striatal termini (STR) in mice treated with PBS, MPTP, or pretreated with VIP, LBT-3393, or LBT-3627 prior to MPTP intoxication (40x image, scale bar, 200 µm). Sections were immunostained with anti-TH and HRP-conjugated secondary antibody and visualized with DAB. SN sections were counterstained with thionin. B. Total numbers of surviving dopaminergic neurons (TH+Nissl+) and nondopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment alone or pretreatment with VIP, LBT-3393, or LBT-3627. Percentages of spared dopaminergic neurons are included for each treatment (10x image, scale bar, 1000 µm). C. Relative TH densitometry of striatal dopaminergic termini following pretreatment. A-C. Differences in means (± SEM, n=8) were determined where P<0.05 compared with groups treated with PBS (a), MPTP (b), VIP (c), or LBT-3393 (d).
Figure 4. Administration of a VIPR antagonist blocks the neuroprotective effects of LBT-3627.
Figure 4. Administration of a VIPR antagonist blocks the neuroprotective effects of LBT-3627. **A.** Total number of TH+Nissl+ and TH-Nissl+ neurons within the SN following MPTP intoxication alone or with pretreatment of [D-p-CI-Phe6,Leu17]-VIP (Antag), scrambled peptide (LBT-SCR), or co-administration of VIP, LBT-3393, or LBT-3627 with antagonist. **B.** Relative TH densitometry of striatal dopaminergic termini following pretreatment. **C.** Differences in means (± SEM, n=8) were determined where P<0.05 compared with groups treated with PBS (a) or MPTP (b).
Figure 5. *The neuroprotective effect of LBT-3627 is dose-dependent.*
Figure 5. *The neuroprotective effect of LBT-3627 is dose-dependent.* A. Dose response for LBT-3627 at varying pretreatment doses of 1.5 µg, 5 µg, 15 µg, 45 µg, and 90 µg followed by MPTP intoxication. B. Linear regression analysis of dose response, $R^2 = 0.4614 \ P = 0.001$. Differences in means ($\pm$ SEM, n=8) were determined where $P < 0.05$ compared with groups treated with PBS (a) or MPTP (b).
Figure 6. Adoptive transfer of splenocytes from mice treated with VIPR agonists is neuroprotective.
Figure 6. *Adoptive transfer of splenocytes from mice treated with VIPR agonists is neuroprotective.* **A.** Photomicrographs of TH+Nissl+ neurons in the SN and STR in mice treated with PBS, MPTP, or MPTP followed by adoptive transfer of spleen cells from mice treated with VIP, LBT-3393, or LBT-3627 (40x image, scale bar, 200 µm). Sections were immunostained with anti-TH and HRP-conjugated secondary antibody and visualized with DAB. SN sections were counterstained with thionin. **B.** Total numbers of surviving dopaminergic neurons (TH+Nissl+) and non-dopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment and adoptive transfer (10x image, scale bar, 1000 µm). **C.** Relative TH densitometry of striatal dopaminergic termini. **B and C.** Differences in means (± SEM, n=8) were determined where P<0.05 compared with groups treated with PBS (a), MPTP (b), VIP (c), LBT-3393 (d).
Figure 7. Pretreatment with VIPR agonists decreases reactive microglia levels during MPTP-induced inflammation.
Figure 7. *Pretreatment with VIPR agonists decreases reactive microglia levels during MPTP-induced inflammation.* A. Photomicrographs of Mac-1+ microglia in the SN of PBS or MPTP treated mice pretreated with VIP, LBT-3393, or LBT-3627 (scale bars: *top panel*, 40x image, 200 µm and *bottom panel*, 400x image, 20 µm). B. Quantification of reactive microglia taken from midbrains 2 days post MPTP treatment. Sections were stained for Mac-1+ microglia using an anti-Mac-1 antibody, HRP-conjugated secondary antibody, and DAB for color visualization. Numbers of reactive microglia (amoeboid Mac-1+) were determined by stereological analysis. Differences in means (± SEM, n = 6) were determined where P < 0.05 compared with groups treated with PBS (a), MPTP (b), or LBT-3393 (c).
Figure 8. Adoptive transfer of splenocytes from VIPR agonist-treated mice diminishes microgliosis during MPTP intoxication.
Figure 8. *Adoptive transfer of splenocytes from VIPR agonist-treated mice diminishes microgliosis during MPTP intoxication.* A. Representative photomicrographs of Mac-1+ microglia within the SN of mice treated with PBS alone, MPTP alone, or MPTP-treated mice receiving splenocytes from donors treated with VIP, LBT-3393, or LBT-3627 (scale bars: *top panel*, 40x image, 200 µm and *bottom panel*, 400x image, 20 µm). B. Quantification of reactive microglia within midbrains, visualized as described in Figure 6. Differences in means (± SEM, n = 5) were determined where P < 0.05 compared with groups treated with PBS (a), or MPTP alone (b).
Figure 9. VIPR agonist pretreatment does not affect CD4+ and CD4+CD25+ T cell frequencies.
Figure 9. VIPR agonist pretreatment does not affect CD4+ and CD4+CD25+ T cell frequencies. Mice were treated with PBS, VIP, LBT-3393, or LBT-3627 for 5 days, and splenocytes assessed using flow cytometric analysis for frequencies of CD4+ T cells (A) and CD4+CD25+ Tregs within total splenocytes (B).
Figure 10. *LBT-3627 treatment elicits changes in Treg function.*
Figure 10. LBT-3627 treatment elicits changes in Treg function. Assessment of Treg-mediated inhibition (± SEM) of CFSE-stained CD4+ Tresps stimulated for proliferation with anti-CD3/CD28. Tregs were isolated from PBS-, VIP-, LBT-3393-, or LBT-3627-treated mice.
Figure 11. *LBT-3627 treatment decreases proinflammatory cytokine release in CD4+ T cell subsets.*
Figure 11. *LBT-3627 treatment decreases proinflammatory cytokine release in CD4+ T cell subsets.* Mice were treated with PBS, VIP, LBT-3393, or LBT-3627 for 5 days. Relative concentration of pro-inflammatory cytokines in cell culture supernatants of anti-CD3/CD28 stimulated CD4+ T cells following pretreatment (±SEM, n=3). Cytokine concentrations were determined by cytokine bead array for pro- and anti-inflammatory cytokines. Differences in means were determined where P<0.05 compared with groups treated with PBS (a) or VIP (b).
Figure 12. VIPR2 agonism induces dysregulation of genes associated with inflammatory responses and cell-to-cell signaling.
Figure 12. VIPR2 agonism induces dysregulation of genes associated with inflammatory responses and cell-to-cell signaling. PBS controls received no treatment, whereas VIP, LBT-3393, and LBT-3627 treatment groups were pretreated for 5 days followed by negative selection of the CD4+ T cell subset. qRT-PCR data shows gene expression changes by CD4+ T cells of mice pretreated with VIPR agonists (n = 3). Fold changes and p values were determined using SABioscience RT² Profiler PCR Array Data Analysis software, version 3.5. Fold changes and p values for differentially regulated mRNA levels with VIP (A), LBT-3627 (B), or LBT-3393 (C) treatment, normalized to PBS controls. Differences in fold change (n=3) were determined where p < 0.05.
Figure 13. VIPR2 agonism elicits changes in immune pathways.
Figure 13. **VIPR2 agonism elicits changes in immune pathways.** Shown are direct and indirect pathways utilizing T cell gene modulations that were significantly dysregulated with LBT-3627 treatment compared to PBS treatment group. Resulting gene networks from each treatment group were analyzed using Qiagen Ingenuity Pathway Analysis. Pink coloration indicates a modest increase in expression as compared to PBS control, and red indicates a profound increase in expression. Nodes lacking color indicate a molecule involved in the pathway, but not identified in the PCR data set. The central node, CSF-2 or GM-CSF is indicated in yellow color.
### LBT-3627 Only

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3ar1</td>
<td>Complement component 3a receptor 1</td>
<td>59.714</td>
</tr>
<tr>
<td>Ripk2</td>
<td>Receptor-interacting serine-threonine kinase 2</td>
<td>36.504</td>
</tr>
<tr>
<td>Cd14</td>
<td>CD14 antigen</td>
<td>14.520</td>
</tr>
<tr>
<td>Tnfsf14</td>
<td>Tumor necrosis factor ligand superfamily</td>
<td>11.632</td>
</tr>
<tr>
<td>Trl7</td>
<td>Toll-like receptor 7</td>
<td>11.472</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>Chemokine (C-X-C) ligand 3</td>
<td>10.126</td>
</tr>
<tr>
<td>Ccl24</td>
<td>Chemokine (C-C) ligand 24</td>
<td>7.311</td>
</tr>
<tr>
<td>Ccl7</td>
<td>Chemokine (C-C) receptor 7</td>
<td>4.595</td>
</tr>
<tr>
<td>Ccl12</td>
<td>Chemokine (C-C) receptor 12</td>
<td>4.084</td>
</tr>
<tr>
<td>Selc</td>
<td>Selectin</td>
<td>3.945</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
<td>3.837</td>
</tr>
<tr>
<td>Ccl8</td>
<td>Chemokine (C-C) ligand 8</td>
<td>3.784</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>Chemokine (C-X-C) receptor 4</td>
<td>2.770</td>
</tr>
<tr>
<td>Trl1</td>
<td>Toll-like receptor 1</td>
<td>2.462</td>
</tr>
<tr>
<td>Ccl25</td>
<td>Chemokine (C-C) ligand 25</td>
<td>2.346</td>
</tr>
<tr>
<td>Ccr1</td>
<td>Chemokine (C-C) receptor 1</td>
<td>2.158</td>
</tr>
<tr>
<td>Trl5</td>
<td>Toll-like receptor 5</td>
<td>2.071</td>
</tr>
<tr>
<td>Ccl22</td>
<td>Chemokine (C-C) ligand 22</td>
<td>-1.815</td>
</tr>
<tr>
<td>Ccl17</td>
<td>Chemokine (C-C) ligand 17</td>
<td>-2.189</td>
</tr>
<tr>
<td>C4b</td>
<td>Complement component 4B</td>
<td>-2.297</td>
</tr>
<tr>
<td>Ccl1</td>
<td>Chemokine (C-C) ligand 1</td>
<td>-2.969</td>
</tr>
<tr>
<td>Cebp</td>
<td>CCAAT/enhancer binding protein</td>
<td>-3.095</td>
</tr>
<tr>
<td>Trl2</td>
<td>Toll-like receptor 2</td>
<td>-3.580</td>
</tr>
<tr>
<td>Ccl5</td>
<td>Chemokine (C-C) ligand 5</td>
<td>-3.605</td>
</tr>
<tr>
<td>Ccr7</td>
<td>Chemokine (C-C) ligand 7</td>
<td>-4.857</td>
</tr>
<tr>
<td>Cd40</td>
<td>CD40 antigen</td>
<td>-6.589</td>
</tr>
<tr>
<td>Ccl4</td>
<td>Chemokine (C-C) ligand 4</td>
<td>-8.515</td>
</tr>
<tr>
<td>I1b</td>
<td>Interleukin 1 beta</td>
<td>-18.920</td>
</tr>
<tr>
<td>I23a</td>
<td>Interleukin 23, subunit a</td>
<td>-21.556</td>
</tr>
<tr>
<td>Ifng</td>
<td>Interferon gamma</td>
<td>-23.103</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>Chemokine (C-X-C) ligand 2</td>
<td>-24.420</td>
</tr>
<tr>
<td>Trl9</td>
<td>Toll-like receptor 9</td>
<td>-29.041</td>
</tr>
<tr>
<td>Ly96</td>
<td>Lymphocyte antigen 96</td>
<td>-34.776</td>
</tr>
<tr>
<td>Ptgs2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td>-42.814</td>
</tr>
<tr>
<td>Ccl2</td>
<td>Chemokine (C-C) ligand 2</td>
<td>-43.411</td>
</tr>
<tr>
<td>Trl4</td>
<td>Toll-like receptor 4</td>
<td>-51.984</td>
</tr>
<tr>
<td>Nr3c1</td>
<td>Nuclear receptor subfamily 3, group C</td>
<td>-52.710</td>
</tr>
<tr>
<td>Ltb</td>
<td>Lymphotoxin B</td>
<td>-61.820</td>
</tr>
<tr>
<td>Tnf</td>
<td>Tumor necrosis factor</td>
<td>-83.333</td>
</tr>
<tr>
<td>Ii7</td>
<td>Interleukin 7</td>
<td>-116.970</td>
</tr>
</tbody>
</table>

Figure 14. Targeting VIPR2 with LBT-3627 changes inflammation-associated gene expression in microglial populations.
Figure 14. Targeting VIPR2 with LBT-3627 changes inflammation-associated gene expression in microglial populations. CD11b+ microglial populations from the ventral midbrain of 8 mice were collected following treatment with MPTP alone, LBT-3627 pretreatment + MPTP, or LBT-3627 treatment only. Gene expression changes within the CD11b-enriched population were assessed using qRT-PCR (Inflammatory Response and Autoimmunity PCR profiler array) in each experimental group. Fold changes were determined using MPTP alone as the control. Fold changes for differentially regulated mRNA levels in response to LBT-3627 treatment only are displayed in the above table. Dark red coloration indicates a profound increase compared to MPTP, pink indicates a modest increase, light green indicates a modest decrease, and dark green indicates a profound decrease in expression.
Figure 15. Targeting VIPR2 with LBT-3627 prior to MPTP intoxication elicits changes in the inflammatory response pathway.
Figure 15. *Targeting VIPR2 with LBT-3627 prior to MPTP intoxication elicits changes in the inflammatory response pathway.* CD11b+ microglial populations from the ventral midbrain of 8 mice were collected following treatment with MPTP alone, LBT-3627 pretreatment + MPTP, or LBT-3627 treatment only. Gene expression changes within the CD11b+ enriched population were assessed using qRT-PCR (Inflammatory Response and Autoimmunity PCR profiler array) in each experimental group. Fold changes were determined using MPTP alone as the control. Fold changes for mRNA levels in response to LBT-3627 pretreatment followed by MPTP intoxication, normalized to MPTP alone. Dark red coloration indicates a profound increase compared to MPTP, pink indicates a modest increase, light green indicates a modest decrease, and dark green indicates a profound decrease in expression.
Figure 16. *MPTP* metabolism in the midbrain and striatum is not inhibited by pretreatment with VIPR agonists.
Figure 16. *MPTP metabolism in the midbrain and striatum is not inhibited by pretreatment with VIPR agonists.* Quantification of total MPTP and MPP+ levels in µg/g tissue in the midbrain (A) and striatum (B) of mice pretreated with VIP, LBT-3393, or LBT-3627 followed by MPTP intoxication. Tissues were collected at 90 minutes after the final MPTP injection. Differences in means (± SEM, n=5) were determined where P < 0.05 compared with groups treated with MPTP only (a) or VIP (b).
Figure 17. LBT-3627 treatment following MPTP intoxication does not attenuate neurodegeneration.
Figure 17. *LBT-3627 treatment following MPTP intoxication does not attenuate neurodegeneration.* Mice were intoxicated by MPTP and, after 12 hours, mice were then treated with LBT-3627 for 5 consecutive days. **A.** Total numbers of surviving dopaminergic neurons (TH+Nissl+) and non-dopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment and post-MPTP intoxication treatment of LBT-3627. **B.** Relative TH densitometry of striatal dopaminergic termini following MPTP intoxication or MPTP intoxication followed by LBT-3627 treatment. **A and B.** Differences in means (± SEM, n = 5) were determined where P < 0.05 compared with groups treated with PBS (a).
CHAPTER THREE

3 UTILIZATION OF BIOIMAGING TO EVALUATE THERAPEUTIC POTENTIAL FOLLOWING VIPR TREATMENT IN VIVO

3.1 Abstract

Neuroprotective immunity is defined by transformation of T cell polarity for therapeutic gain. For neurodegenerative disorders and specifically for PD, GM-CSF or VIPR2-agonists elicit robust anti-inflammatory microglial responses leading to neuronal sparing in MPTP-intoxicated mice. While the neurotherapeutic potential of immune modulation was demonstrated for PD, there remain inherent limitations in translating these inventions from the laboratory to patients. One obstacle in translating such novel neurotherapeutics is limited to the availability of suitable noninvasive methods to track disease progression and therapeutic efficacy. To this end, manganese-enhanced magnetic resonance imaging (MEMRI) assays were developed as a way to track a linkage between glial activation and VIPR2 agonist (LBT-3627)-induced neuroprotective immunity for MPTP-induced nigrostriatal degeneration. Notably, LBT-3627-treated, MPTP-intoxicated mice showed reduced MEMRI brain signal intensities. These changes were associated with reduced astrogliosis and sparing of nigral tyrosine hydroxylase neurons. Most importantly, the data suggest that MEMRI can be developed as a biomarker tool to monitor neurotherapeutic responses following treatment.

3.2 Introduction
Progressive nigrostriatal degeneration and its associated neuroinflammation contribute to the pathogenesis of PD. Although the cause of PD remains unknown, aberrant innate and adaptive immune responses direct the time course of nigral neuronal loss (Kosloski et al., 2010, Ha et al., 2012). Associated immune activation is propagated through the accumulation and extracellular release of α-syn, a self-protein (Benner et al., 2008). Extracellular release of aggregated and post-translationally modified α-syn results in glial activation that, in turn, affects the breakdown of immune tolerance. These pathological events underlie progressive dopaminergic neuronal loss and microglial activation. Indeed, neuronal loss is accompanied by robust microgliosis, astrocytosis and release of pro-inflammatory neurotoxic mediators (Mosley et al., 2012). Recent work from our own laboratories has served to highlight the role of Teff and Treg in attenuating such neuroinflammatory response cascades (Reynolds et al., 2010, Kosloski et al., 2013). The balance between Teff and Treg activities leads to degenerative or protective effects in the brain, respectively, depending on defined T cell polarity and cell phenotype (Kosloski et al., 2010). Immune profiles analyzed in PD patients support a functional balance between the cells because circulating immunocyte profiles demonstrate increased pro-inflammatory profiles and decreased Treg function (Saunders et al., 2012). Such aberrations serve to change the brain’s microenvironment such that a pro-inflammatory dominance ensues. These notions are further supported by pre-clinical studies performed in
an MPTP model of PD where intervention by immune modulation serves to protect dopaminergic neurons within the nigrostriatal pathway (Schabitz et al., 2008, Kosloski et al., 2013).

Mobilizing specific Treg adaptive immune responses affects microglial reactivity and protects against nigrostriatal degeneration (Reynolds et al., 2010, Kosloski et al., 2013, Olson et al., 2015). The use of immunomodulatory compounds shifts immune phenotypes and functions from neurotoxic to neuroprotective regulatory responses (Reynolds et al., 2010, Kosloski et al., 2013, Olson et al., 2015). Specifically targeting VIP receptor 2 (VIPR2) with a modified VIP analog (referred to as LBT-3627) showed robust neuroprotective activities in MPTP-intoxicated mice (Olson et al., 2015). LBT-3627 attenuates microglial activation and induces endogenous GM-CSF mRNA transcripts leading to increased Treg functional activities and potentiation of dopaminergic neuronal sparing. This has clinical relevance as recently shown in a randomized phase I clinical trial of Sargramostim where improvements in motor skills were observed as a consequence of immune modulation (Gendelman et al., 2017). LBT-3627 is a receptor-selective and metabolically stable agonist and a potent inducer of Treg. Moreover, it should not be associated with untoward adverse events, such as those observed with GM-CSF (Vial and Descotes, 1995a, Gendelman et al., 2017). Thus, further assessment of the neurotherapeutic capacities of LBT-3627 in a preclinically relevant model using bioimaging techniques was utilized. Manganese (Mn)-enhanced magnetic resonance imaging (MEMRI) was used as a noninvasive imaging biomarker assay to follow
disease progression and to assess the abilities of LBT-3627 therapies to slow neurodegenerative activities. However, even with promising results, a potential downside of its use rests in the fact that Mn exposure can be linked parkinsonism in humans (Olanow, 2004, Kwakye et al., 2015). Thus, owing to the toxicity of Mn, MEMRI has only been utilized in animal studies. The feasibility of using MEMRI in humans is still under investigation. Nonetheless, we now show that MEMRI is an effective noninvasive tool that can be developed as a means to follow immune-modulatory neurotherapies in experimental models for preclinical testing.

3.3 Materials and methods

Animals, Drug treatment, and MPTP Intoxications

Male C57BL/6J mice, 6–8 weeks old (The Jackson Laboratory, Bar Harbor, ME, USA) were used in all studies. Mice were pretreated with LBT-3627 (Longevity Biotech, Inc., Philadelphia, PA, USA), a VIPR2 agonist, at intraperitoneal dosages of 15 µg or 45 µg daily for 5 days prior to MPTP intoxication. On the sixth day, mice received 4 subcutaneous injections of vehicle [Dulbecco's phosphate-buffered saline (DPBS), 10 ml/kg body weight] or MPTP –HCl (SigmaAldrich, St. Louis, MO, USA) at 16 mg MPTP (free base)/ kg body weight in DPBS, with each injection given every 2h. MPTP safety precautions were followed according to published safety and handling protocol (Przedborski et al., 2000, Jackson-Lewis and Przedborski, 2007). All animal procedures used in this report were approved by the Institutional Animal Care and Use Committee of the
University of Nebraska Medical Center and were in agreement with the guidelines set by the National Institutes of Health.

Manganese-enhanced MRI (MEMRI)

A 50 mM solution of MnCl2 ·4H 2O (Sigma-Aldrich) in 0.9 % w/v NaCl (Hospira, Lake Forest, IL, USA) was used for MEMRI. For MEMRI examinations, 60mg(0.3mmol)/kg MnCl2 solution was administered intraperitoneally at 48 and 24 h prior to initiation of examination. The fractioned MnCl2 administration scheme (1 i.p. dose per day for 2 days) were used to control uneven Mn\(^{2+}\) distribution caused by a variety reasons, including differences in extracellular and intracellular Mn\(^{2+}\) concentrations, anterograde transport through neurons, and injection variability. This design allows Mn\(^{2+}\) to reach a stable distribution as previously published by others and our laboratory (Grunecker et al., 2010, Grunecker et al., 2013). The period of stable distribution allows for reproducible and even distribution of signal during MRI (Silva et al., 2004, Silva and Bock, 2008, Grunecker et al., 2010, Massaad and Pautler, 2011, Grunecker et al., 2013). For studies performed 2 days post-MPTP treatment, the first MnCl2 was injected 4 h after MPTP/DPBS administration. After lesion formation (day 5), MEMRI examinations were performed on a staggered basis for days 7–16 post MPTP/DPBS treatment and were binned together (hereafter designated as day-7 examinations). As only 4 MEMRI examinations could be completed in 1 day, staggering examinations using 1 subject from each of 4 treatment groups (4 total/day) were necessary to minimize between group variability. For both day-2
and day-7 examinations, mice were scanned in a 7T/21 cm MRI scanner (Bruker, Billerica, MA, USA). Animals were anesthetized by inhalation of isoflurane in 100 % oxygen and maintained at 40–80 breaths/minute. Anesthetized mice were scanned using a T1 mapping sequence (fast spin echo with variable repetition time from 0.4 s to 10 s, 12 slices, slice thickness 0.5 mm, in-plane resolution 0.1 × 0.1 mm²) and T1-weighted (T1-wt) MRI (FLASH, repetition time 20 ms, flip angle 20°, 3-dimensional isotropic resolution 0.1 × 0.1 × 0.1 mm³). After data acquisition, the mice were euthanized and tissues removed for immunohistological studies. T1 and T1-wt scans were performed prior to MPTP treatment, and the acquired image served as baseline data for the calculation of Mn-mediated signal enhancement.

**MRI Data Processing**

Brain volumes in the T1-wt images were first extracted using an in-house MATLAB program based on the level sets method. The brain images were then registered to the MRI-based mouse brain atlas (Laboratory of Neuro Imaging, University of California Los Angeles) using affine transformation first, and then nonlinear transformation (DiffeoMap; John Hopkins University, Baltimore, MD, USA). The baseline and postMnCl₂ injection, T1-wt images were calibrated using T1 values to minimize the MRI system variations between the baselines and post-MnCl₂ injection scans as described (Bock et al., 2008). Signal enhancement maps were calculated as follows: \( \frac{S_{Mn} - S_{bl}}{S_{bl}} \), where \( S_{Mn} \) and \( S_{bl} \) are T1-wt signal intensity of post- and pre-MnCl₂ injection, respectively. The MEMRI
enhancement maps were segmented automatically into 16 brain regions/subregions using the atlas. The enhancements in the regions were measured, and 1-way analysis of variance (ANOVA) performed to examine the significance of enhancement change among groups. A pixel-by-pixel comparison was also performed among groups using one-way ANOVA. A pixel on a brain image was compared with corresponding pixels on the same locus on other brain images.

**Tissue Acquisitions and Immunohistochemistry**

Following MEMRI scanning, mice were terminally anesthetized and transcardially perfused with DPBS followed by 4 % paraformaldehyde/DPBS (Sigma-Aldrich) at day 2 or days 7– 16 post-MPTP intoxication (referred to as day 7). Whole brains were harvested, processed, and flash-frozen to assess dopaminergic neuronal survival and glial reactivity in the substantia nigra (SN) as previously described [36]. Frozen midbrain sections (30 µm) were immunostained for either tyrosine hydroxylase (TH) (anti-TH, 1:2000; EMD Millipore, Billerica, MA, USA), Mac-1 (anti-CD11b, 1:1000; AbD Serotech, Raleigh, NC, USA), or glial fibrillary acidic protein (anti-GFAP, 1:1000; Dako, Carpinteria, CA, USA). Within the SN, numbers of GFAP+ cells/mm², Mac1+ cells/mm², total numbers of TH+Nissl+ (dopaminergic neurons), and total numbers of TH-Nissl+ (nondopaminergic neurons) were estimated using stereological analysis via Stereo Investigator software with the optical fractionator module (MBF Bioscience, Williston, VT, USA), as previously described (Benner et al., 2008).
**Statistical Analysis**

All immunohistochemical values are expressed as mean ± SEM. Differences in between-group means were analyzed using ANOVA followed by Fisher’s least significant difference post hoc test (GraphPad Software, Inc., La Jolla, CA, USA).

**3.4 Results**

*Detection of LBT-3627 therapy using MEMRI signal enhancement.*

Peak neuroinflammation and rate of neuronal death as measured by reactive microglia occurs 2 days post-MPTP intoxication (Kurkowska-Jastrzebska et al., 1999b, a). We chose to examine the ability of MEMRI to detect changes between MPTP and DPBS controls during this time. MEMRI provides information relevant to assessments of neural anatomy, function, and connectivity (Eschenko et al., 2010, Inoue et al., 2011). Mn\(^{2+}\) enters neurons through voltage-gated calcium Ca\(^{2+}\) channels, owing to its similarities with Ca\(^{2+}\), linking Mn\(^{2+}\) accumulation to MEMRI signal intensity (Massaad and Pautler, 2011). Additionally, in prior studies, we demonstrated that MEMRI signals are increased during neuroinflammation due to elevated neuronal Mn\(^{2+}\) uptake and accumulation through astrocyte activation (Bade et al., 2013, Bade et al., 2016). Thus, comparisons of pretreatment scans and day-2 or day-7 DPBS post-treatment scans demonstrated that MnCl\(_2\) administration yielded enhanced signal intensities (hereafter designated as MEMRI signal) (Figure 1 and 2). Previous reports of Mn enhanced T1-wt signaling confirm these results (Bade et al., 2013,
To minimize between-group by day variability, one subject from each of 4 treatment groups were assessed every day on days 7–16; a time after MPTP-induced lesion forms (Jackson-Lewis et al., 1995). Linear regression of all dependent experimental parameters for an effect of day showed no significant effects on any dependent variable except for MEMRI signal intensities and changes in intensities for mice treated with MPTP and 15 µg LBT-3627 (r² =0.64, p=0.0176). However, comparison of signal levels obtained on days 7–10 with those obtained on days 11–14 showed no significant differences by t test (p =0.1091). Thus, data acquired on days 7–16 were binned as 1 time point and are hereafter designated as day-7 data. Using baseline T1-wt images, we prepared mean MEMRI signal enhancement maps for day 2 and day 7 acquisitions (Figure 1A and 2A). Following bioimaging, results indicate that on day 2 following MPTP intoxication (Figure 1A), the MEMRI signal in the SN was diminished (column 3, row 4), while that in the striatum was increased (column 3, rows 1 and 2). Pixels with significant signal changes (p<0.05) were superimposed on anatomical structures to form a p-value map (Figure 1B). Significantly decreased MEMRI signals were found primarily within the SN (row 4). Striatum contained the most pixels of significantly increased signals (rows 1 and 2). A region-by-region comparison facilitated by the MRI-based atlas indicated the same findings (data not shown). A number of pixels on cerebral cortex and hippocampus also showed significant enhancement increases. However, the comparison did not find enhancement differences between the 2 groups. Comparison of subregions of hippocampus and cortex was not performed because the MRI-based mouse
brain atlas employed for enhancement map segmentation does not identify these subregions.

Next, we assessed MEMRI signal after lesion formation in mice pretreated with LBT-3627. By day 7, the major inflammatory condition associated with reactive microglia in MPTP is essentially resolved, leaving only astrogliosis and the nigrostriatal lesion to be observed (Jackson-Lewis et al., 1995). In contrast to day 2, by day 7 MEMRI signals for DPBS controls and MPTP intoxicated mice were similar in most brain regions, including SN (column 3, rows 3 and 4). Signal intensity within the SN was significantly decreased in low-dose LBT-3627-treated mice compared with MPTP-intoxicated mice (columns 4 and 5, rows 3 and 4). The pixel-wise p-value map also showed the most pixels on 15 µg-treated with lower signal compared with MPTP-treated mice (Figure 2B). Mice treated with the higher dose of LBT-3627 also had lower MEMRI signal when compared with MPTP; however, it did not reach significance. The lack of significance is potentially due to the high variation of signal intensity and low number of animals. In accordance with our previous dose–response findings, a 45-µg dose appears to elicit an extremely variable response, indicating that we may be approaching a therapeutic threshold (Olson et al., 2015). In addition, both cerebral cortex and hippocampus included pixels that had significantly lower signal (Figure 2B). However, the brain atlas-based regional comparison did not find a significant difference between 15-µg-treated and MPTP mice in these 2 regions.
Changes in neuron numbers and glial reactivity following treatment with LBT-3627.

In order to assess the ability of MEMRI to detect neuronal loss and/or inflammation during MPTP, we carried out immunohistological evaluations to determine dopaminergic neuron numbers, microglial reactivity, and astrogliosis. As expected, TH+Nissl+ dopaminergic neurons were lost following MPTP intoxication (Figure 3A), whereas TH-Nissl+ neurons remained unchanged. At day 2, neuron numbers significantly dropped from 8020 ± 379 to 3938 ± 317, equivalent to a 51% loss (Figure 3B). At day 7, MPTP intoxication markedly reduced neuron levels to 42 %, with an increased loss compared with day 2 (Figure 4A and 4B). Both the low and high dose of LBT-3627 significantly spared dopaminergic neurons, resulting in 62% and 64% neuron survival compared with DPBS alone. At 2 and 7 days post MPTP, reactive microglial populations were also assessed (Figure 5 and 6). When compared with day 2 DPBS controls, MPTP significantly increased reactive microglial cell numbers from 2.8 ± 0.3 cells/mm² to 46 ± 5 cells/mm², indicating an inflammatory response associated with MPTP intoxication (Figure 5). By day 7, there was a significant decrease in observed microglial reactivity, when compared with MPTP-treated mice (Figure 6). The number of reactive microglia decreased by 48% from day 2 to day 7, indicating a resolution of inflammation. LBT-3627 treatment also led to a small decrease in reactive microglial numbers, standing at 15.18 ± 3 cells/mm² and 14.96 ± 2.7 cells/mm² with both a low and high dose, respectively. However, the magnitude of changes in microglial activation at day 7 is small, which is expected
in this model. With the acute MPTP model of PD, the reactive microglial response generally subsides by day 7; thus, the minute differences observed between groups is in accordance with what has been previously demonstrated in the literature (Kohutnicka et al., 1998, Kurkowska-Jastrzebska et al., 1999a).

In addition, GFAP-immunoreactive cells were assessed following MPTP intoxication at both time points, as well as following low- and high-dose LBT-3627 pretreatment (Figure 7 and Figure 8). At day 2, MPTP intoxication led to an unremarkable increase in astroglial response (Figure 7). However, by day 7, the increase was highly significant (Figure 8A). Compared with DPBS, MPTP intoxication noticeably increased GFAP reactivity by nearly 6-fold from 12 ± 2 cells/mm² to 61 ± 8 cells/mm² (Figure 8B). Both doses of LBT-3627 pretreatment attenuated astroglial response by significantly decreasing GFAP reactivity by ~50% when compared with MPTP treatment alone (Figure 8B). In developing MEMRI as a potential therapeutic biomarker, we sought to correlate signal intensity change with the immunohistological findings (Figure 9). When comparing dopaminergic neuron (Th+Nissl+) number and signal intensity, data was found to be associated in all treatment arms except for MPTP-treated mice at day 7 that did not receive LBT-3627 treatment (Figure 9A). Microglial reactivity seemed to be inversely related to signal enhancement, indicating that microglial response over time does not contribute to the MEMRI signal strength (Figure 9B). As expected, correlation analysis of combined day 2 and day 7 data points revealed a positive relationship between signal intensity and TH+ neuronal number (r=0.36, p=0.0208), and a negative relationship between signal intensity
and Mac-1+ microglia cell numbers ($r = -0.42, p=0.0066$), indicating that increases in signal intensity correlate with increased neuron number and decreased microglial response. Interestingly, for GFAP+ staining, there was not a significant correlation observed when including all data points. Conversely, when comparing data on day 2 alone, changes in GFAP+ staining was negatively related to changes in signal intensity in MPTP-treated groups ($r = -0.74, p=0.0092$), as shown in Figure 9C. At day 2, when MEMRI signal intensity was significantly decreased from DPBS control levels, GFAP+ astrocyte numbers were also low, with no significant change observed between DPBS and MPTP treatment. However, by day 7, MEMRI signal intensity corresponded with significantly higher GFAP+ reactivity, indicating that the increased signal may be due to an activation of astrocytes, which outweighed the expected decreased signal due to the significant neuronal loss seen 7 days after the formation of an MPTP-mediated lesion. Likewise, in the mice pretreated with LBT-3627, the decrease in MEMRI signal was likely due to the loss of neurons from MPTP. Also, there was an insufficient astroglial response to restore the MEMRI signal to that of the DPBS controls.

3.5 Discussion

Progressive neuronal degeneration underlies the pathobiology of a broad range of neurodegenerative diseases that include PD (Kalia and Lang, 2015). While the causes of most neurodegenerative diseases are not known, the relationship between neurodegeneration and immune system activation is clear. Therapies
are constantly being developed and tested to modulate or transform the immune system with the intention to clear misfolded, aggregated, and nitrosylated proteins, as well as to restore the damaged central nervous system microenvironment (Olanow et al., 2009). Amongst these therapies, there are agents that can change a dominant effector phenotype to one that promotes immune tolerance and is regulatory, anti-inflammatory, and neuroprotective. One such candidate is LBT-3627. This drug has shown robust neuroprotective potential in MPTP-intoxicated animals to attenuate neuroinflammatory responses while affecting Treg function (Olson et al., 2015). Despite such effects, broad obstacles still lie ahead in bringing the treatments to human use. With this in mind, our laboratory has pioneered preclinical development of these agents and entered into randomized clinical evaluations (Gendelman et al., 2017). All such efforts have shown promise but with known limitations. One such limitation rests in uncovering suitable biomarkers to track disease and therapeutic effectiveness. While immune monitoring of circulating T cell numbers and functions have proven helpful, these tests are cumbersome, time consuming, and require multiple technical arms for evaluation and confirmatory assessments. Thus, the need for suitable biomarkers to track disease and therapeutic efficacy are real and timely.

Here, we report that MEMRI can be used to assess inflammatory activities related to neuronal loss and astrocyte activation. With a neurotherapeutic directive in mind, our imaging results suggest the ability of MEMRI to detect immune modulation following MPTP treatment and LBT-3627 pretreatment. In
this case, inflammation reduction seemed to outweigh the neuronal protection effect, possibly leading to lower MEMRI enhancement in LBT-3627-treated mice compared with MPTP mice. Mn\textsuperscript{2+} serves as a surrogate measure of calcium influx linking MEMRI signal to the accumulation of the ion in neurons in an activity-dependent manner. As such, MEMRI signal is directly related to neural viability and activity (Takeda et al., 1998, Pautler and Koretsky, 2002). To date, some studies have utilized MEMRI for studying pathobiology of neurodegenerative diseases, such as PD (Kondoh et al., 2005, Pelled et al., 2007, Soria et al., 2011, Weng et al., 2016). However, neurodegeneration is a dynamic process rather than a static event. Immune responses, neuronal impairment, and other pathological events are involved in neurodegenerative diseases, which can ultimately affect MEMRI signal intensity output. Delineation of the cellular basis of MEMRI is critical if it is harnessed as an imaging biomarker to gauge disease progression in experimental models.

Previously, MEMRI signal change following a neuroinflammatory lesion was explored in a prior study from our laboratory (Bade et al., 2013). MEMRI's ability to detect astrocyte, microglia and neuronal activation were measured following treatment with lipopolysaccharide or proinflammatory cytokines, both in vitro and in vivo. We were able to distinguish differences in MEMRI signal from either activated glia or neuronal injury. As such, we used the agent as a marker for neural activity in inflammatory and degenerative disease states. We also found axonal loss detected by traditional neurofilament staining coupled with MEMRI signal loss (Bade et al., 2016). These findings support the notion that
MEMRI reflects neuronal excitotoxicity and impairment that can occur through a range of insults including neuroinflammation. We concluded that the MEMRI signal enhancement is induced by inflammation stimulating neuronal Mn$^{2+}$ uptake. As previously stated, Mn$^{2+}$ ions enter neurons through Ca$^{2+}$ channels, and brain function depends on complex metabolic interactions amongst only a few different cell types, with astrocytes providing critical support for neuron function and survival. Astrocyte functions include buffering toxins in its extracellular environment, providing support to neurons, modulating glutamate and glutamine levels, and affecting neuronal synaptic transmission (Kimelberg and Nedergaard, 2010, Khakh and Sofroniew, 2015). Neurons undergo extremely oxidative metabolism making them highly susceptible to injury, whereas astrocytes are affected by glycolytic metabolism and are much less susceptible to oxygen deprivation. These differences in metabolism provide therapeutic opportunities to address Mn$^{2+}$ sensitivity. Interactions between both cell types changes the metabolism of neurons through a range of mechanisms, including active glycolysis, lactate extrusion, and glutamate uptake by dysregulation of transporters (Araque, 2006, Ricci et al., 2009). Thus, activated astrocytes have a profound influence on neuronal metabolic susceptibilities and are changed by environment, disease, and therapeutic interventions that can alter function and lead to enhanced proliferation and cell surface receptor expression. In turn, the astrocyte phenotypic shift affects neuronal function. In support of this idea, in our prior study, we observed elevated Mn$^{2+}$ uptake by neurons caused by astrogliosis (Bade et al., 2013). Thus, we also posit that
reactive astrocytes release cytokines stimulating neuronal Ca$^{2+}$ channel activity and, ultimately, Mn$^{2+}$ uptake.

We demonstrated that MEMRI signals are increased during neuroinflammation owing to elevated neuronal Mn$^{2+}$ uptake and accumulation through astrocyte activation through calcium channels (Bade et al., 2013, Bade et al., 2016). Likewise, as Mn$^{2+}$ is rapidly taken up into both neurons and astrocytes, neuronal death leads to the release of Mn$^{2+}$, and leads to decreased MEMRI signal (Massaad and Pautler, 2011). Similarly, in a model of traumatic brain injury, regions of MEMRI hyper-intensity are seen at lesion sites and are associated with increased accumulation and infiltration of astrocytes to the lesion (Talley Watts et al., 2015). Similar effects can be observed in the current study at 2 days following MPTP intoxication, a time of peak neuronal loss and peak microglial response within the SN. Neuronal injury often leads to proliferation and migration of activated microglia to assist with cellular debris cleanup (Neumann et al., 2009). It could be hypothesized that activated microglia are ingesting released Mn$^{2+}$ during this period, potentially leading to an increase in signal. However, we have previously found that macrophage uptake of Mn$^{2+}$ using inductively coupled plasma mass spectrometry is not operative in these conditions (unpublished data). In accordance with this previous finding, increased early microgliosis appears to have a minimal effect on MEMRI enhancement at day 2, even with peak reactivity. Yet, there is the possibility that enhanced microglial reactivity could potentially be inhibiting neuronal uptake of Mn$^{2+}$ due to elevated inflammatory states and neuronal degeneration, adding to a decrease in
MEMRI signal. Interestingly, there was a significant signal enhancement found in the striatum of mice treated with MPTP rather than a decrease like that observed in the SN. This finding was not confirmed by immunohistology, but may be owing to the fact that striatal termini begin to degenerate prior to the loss of dopaminergic cell bodies (Burke and O'Malley, 2013). Early degeneration of termini may induce astroglial reactivity more quickly than is seen in the SN, resulting in an increased signal enhancement.

By 7 days after initial MPTP intoxication, TH+ neuronal death is dominant, with limited microgliosis and a robust astroglial response. Treatment with LBT-3627 significantly attenuates the astroglial response, paralleled by a coincident decrease in signal enhancement. The results suggest that MEMRI signal enhancements can reflect both neuronal loss and reduced astrogliosis seen following therapeutic activities. Nonetheless, at day 7, MEMRI signal shows no differences between saline-treated controls and MPTP-intoxicated mice. This seemingly discordant result can be explained by the astrocyte signal. As shown previously, astrocyte activation elicits MEMRI signal enhancement (Bade et al., 2013). Moreover, astrogliosis and neurodegeneration can occur at the same time but have opposite effects on the MEMRI signal. That is, neuronal death elicits a signal decrease, while astrocytic activation enhances the signal. Interestingly, certain areas within the hippocampus and cerebral cortex were found to be significantly increased in MEMRI enhancement in MPTP-treated mice on day 2, and lower enhancement in 15-µg LBT-3627-treated mice compared with MPTP-treated mice on day 7. In future studies, we will identify these areas and
investigate the pathology underlying the MEMRI signal changes. Relationships between MEMRI signal enhancement and glial interactions has been shown in a range of neurological disease models (Haapanen et al., 2007, Immonen et al., 2008, Soria et al., 2011, Talley Watts et al., 2015). However, results have been conflicting. This has suggested that only astrocyte responses affect signal enhancement. Others have found that both activated microglia and astrocytes elicit signal change (Haapanen et al., 2007, Wideroe et al., 2009, Kawai et al., 2010), or that astrocyte activation may play no role at all (Immonen et al., 2008). Thus, we chose to utilize an inflammatory model of disease that would allow us to evaluate longitudinally and independently microgliosis and astrocytic engagement in the MEMRI responses (Kohutnicka et al., 1998).

By assessing early events (2 days after MPTP intoxication), a time of peak inflammation and microglial reactivity coincident with a peak rate of neuronal death, we could assess signal decreases primarily linked to neuronal loss. Here, astrocyte involvement was not observed. However, after 7 days, when microglial activation is diminished and nervous tissue repair is operative, coincident with astrocyte activation and facilitated Mn$^{2+}$ uptake, MEMRI signal enhancement appears to be associated with changes in GFAP$^+$ reactivity. Such events are observed during treatment of MPTP intoxication. Indeed, we demonstrate that inflammation-induced astrocyte activation can stimulate Mn$^{2+}$ uptake, potentially leading to MEMRI signal enhancement. However, the sensitivity of the MEMRI signal changes in relation to the changes in immunohistology is relatively low. The moderately low spatial resolution of MEMRI (100 µm$^3$ pixel size) compared
with histological slices is one potential cause of the low sensitivity observed. Another reason can be attributed to the calculated T1wt signal enhancement as the measure of tissue Mn$^{2+}$ concentration. For accurate calculation of MEMRI enhancement, scanner system variations among imaging sessions must be corrected, as described in a previous study (Bade et al., 2016). Currently, we are developing 3-dimensional high-resolution (<100 µm3 pixel size) T1 mapping methods for future use, potentially providing improved assessment of underlying histopathology. Nonetheless, MEMRI is a valuable tool as it provides in vivo imaging examination with minimal invasiveness.
3.6 Figures

Figure 1. Comparison of MEMRI signal enhancement between controls and MPTP-intoxicated mice at day 2 post-MPTP.
Figure 1. *Comparison of MEMRI signal enhancement between controls and MPTP-intoxicated mice at day 2 post-MPTP.* **A** MEMRI enhancement maps. The first column (from left) shows coronal slices of the averaged MEMRI of control mice (n=5) as an anatomical reference. The sagittal slice (upper left) shows respective coronal slices (red lines from rostral to caudal) that correspond to images in rows 1-4 showing sections with striatum (rows 1-2) or midbrain (rows 3-4). The second column shows the average enhancement in control mice on the coronal slices. The third column represents the average enhancement of MPTP-intoxicated mice (n=6). The color bar for the enhancement maps is at the top of the figure. *Dark blue* color (0%) means no change in enhancement from Mn$^{2+}$ compared to pre-injection signal intensity. *Dark red* color represents 90% signal increase compared to pre-injection signal intensity. **B** Statistical comparison of MEMRI enhancement between control and MPTP-intoxicated mice. The left column shows the pixels (red color) with significant decrease ($p < 0.05$) in signal intensity overlaid onto the averaged brain image. The right column shows the pixels (red color) with significant increase ($p < 0.05$) in signal intensity overlaid onto the averaged brain image.
Figure 2. Comparison of MEMRI enhancement between controls and MPTP-intoxicated mice at day 7.
Figure 2. *Comparison of MEMRI enhancement between controls and MPTP-intoxicated mice at day 7*. **A** MEMRI enhancement maps. The *first column* (from *left*) shows coronal slices of the averaged MEMRI of control mice (*n*=5) as an anatomical reference. The sagittal slice (*upper left*) shows respective coronal positions (*red lines*). The *second column* shows the average enhancement in control mice on the coronal slices. The *third column* represents the average enhancement of MPTP-intoxicated mice (*n*=6). The *forth column* represents the average enhancement of MPTP-intoxicated mice treated with 15 µg LBT-3627. The *forth column* represents the average enhancement of MPTP-intoxicated mice treated with 45 µg LBT-3627. The color bar for the enhancement maps is at the top of the figure. *Dark blue color* (0 %) means no change in enhancement from Mn\(^{2+}\) compared to pre-injection signal intensity. *Dark red color* represents 90 % signal increase compared to pre-injection signal intensity. **B** Statistical comparison of MEMRI enhancement between MPTP-intoxicated mice and MPTP-intoxicated mice treated with 15 µg LBT-3627. It shows the pixels (red color) with significant decrease (*p* < 0.05) in signal intensity overlaid onto the averaged brain image.
Figure 3. Assessment of neuronal survival at day 2 following MPTP intoxication and LBT-3627 pretreatment.
Figure 3. *Assessment of neuronal survival at day 2 following MPTP intoxication and LBT-3627 pretreatment.* **A.** Photomicrographs of TH+Nissl+ neurons in the substantia nigra (4x) in mice treated with PBS or MPTP, followed by quantification of TH+Nissl+ and TH-Nissl+ neuronal counts. **B.** Quantification of surviving TH+ dopaminergic neurons within the substantia nigra at 2 days post MPTP Intoxication. All sections were immunostained with anti-TH, color was generated with DAB, and sections were counterstained with thionin. Differences in means (± SEM, n=5-8) were determined ANOVA followed by Fisher’s least significant difference (LSD) *post hoc* test where P<0.05 compared with PBS (a).
Figure 4. Quantification of dopaminergic neuronal survival following pretreatment with 2 different doses of LBT-3627.
Figure 4. **Quantification of dopaminergic neuronal survival following pretreatment with 2 different doses of LBT-3627.** **A.** Photomicrographs of surviving TH+Nissl+ neurons after 7 days within the SN following treatment with MPTP or LBT-3627 pretreatment of 15 μg or 45 μg doses. **B.** Quantification of TH+Nissl+ and TH-Nissl+ neurons following treatment. Sections were stained as described in Figure 3. Differences in means (± SEM, n=5-8) were determined ANOVA followed by Fisher’s least significant difference (LSD) *post hoc* test where P<0.05 compared with PBS (a) or MPTP (b).
Figure 5. Assessment of microglial reactivity after 2 days following MPTP intoxication and LBT-3627 pretreatment.
Figure 5. Assessment of microglial reactivity after 2 days following MPTP intoxication and LBT-3627 pretreatment. Representative photomicrographs of Mac-1+ microglia within the SN of mice treated with PBS alone or at day 2 after MPTP intoxication (4x with 40x inset). The right panel depicts number of reactive microglia taken from midbrains 2 days following MPTP treatment. Differences in means (± SEM, n=5-8) were determined by ANOVA followed by Fisher’s least significant difference (LSD) post hoc test where P<0.05 compared with groups treated with PBS (a).
Figure 6. Assessment of microglial reactivity after 7 days post MPTP intoxication and LBT-3627 pretreatment.
Figure 6. Assessment of microglial reactivity after 7 days post MPTP intoxication and LBT-3627 pretreatment. Nigral sections were immunostained for Mac-1+ microglia using an anti-Mac-1 antibody, HRP-conjugated secondary antibody, and DAB. Numbers of reactive microglia were assessed by stereological analysis. A. Photomicrographs of reactive microglia at day 7 after MPTP intoxication, followed by quantification of Mac-1+ cells/mm² (B). Differences in means (± SEM, n = 5-8) were determined by ANOVA followed by Fisher’s least significant difference (LSD) post hoc test where P<0.05 compared with groups treated with PBS (a).
Figure 7. GFAP reactivity at 2 days post MPTP intoxication and LBT-3627 pretreatment.
Figure 7. GFAP reactivity at 2 days post MPTP intoxication and LBT-3627 pretreatment. Representative photomicrographs of GFAP immunoreactive cells 2 days post MPTP intoxication within the SN of mice (10x with 40x inset) treated with PBS or MPTP are displayed. Quantification was assessed using stereological analysis and was reported as GFAP+ cells/mm², as indicated in the bar graph. No significant differences were observed.
Figure 8. GFAP reactivity is increased after MPTP-intoxication.
Figure 8. *GFAP reactivity is increased after MPTP-intoxication.*  
A. Photomicrographs of GFAP+ cells following MPTP intoxication and LBT-3627 pretreatment at day 7. B. Quantification of day 7 reactive astrocytes. Serial sections were stained with a primary antibody against GFAP, followed by a biotinylated secondary antibody, and DAB color development. Sections were counterstained with thionin, and quantification was assessed using stereological analysis. Differences in means (± SEM, n=5-8) were determined by ANOVA followed by Fisher’s least significant difference (LSD) *post hoc* test where P<0.05 compared with groups treated with PBS (a) and MPTP (b).
Figure 9. *Time comparison of immunohistology and signal enhancement.*
Figure 9. *Time comparison of immunohistology and signal enhancement.* Change in signal intensity ± SEM was determined from PBS controls and was compared to total number of TH+ neuron (A), numbers of Mac-1+ microglia/mm² (B), and numbers of GFAP+ astrocytes/mm² (C) at days 2 and 7. Change in signal intensity is labeled in blue and cells numbers in red. Differences in means (± SEM, n=5-8) were determined by ANOVA followed by Fisher’s least significant difference (LSD) *post hoc* test where P<0.05 compared with groups treated with PBS (a) and MPTP (b).
4.1 Abstract

Recent advances in human immunodeficiency virus (HIV) treatment have dramatically prolonged the life expectancy of individuals living with the disease. Increased aging is associated with the likelihood of developing age-related disease, such as Parkinson’s disease. Interestingly, it has been shown that viruses can enter the central nervous system and potentially lead to neuropathologies themselves. HIV infection is associated with motor dysfunction and postural and gait abnormalities similar to the late stages of Parkinson’s disease, and it has also been associated with other disease hallmarks such as dopaminergic cell loss and neuroinflammation. Thus, we address whether viral infection itself can induce Parkinson-like neurodegeneration or whether it can speed or exacerbate MPTP-induced neurodegeneration using two models of viral infection, HIV and EcoHIV. Findings suggest that there is a synergistic mechanism of immune dysfunction during chronic periods of infection that causes increased dopaminergic cell loss. Likewise, EcoHIV appears to play a direct role in dopaminergic cell death, indicating that the virus itself is neurotoxic, even in the absence of an inflammatory stimulus.

4.2 Introduction

Since the advent of highly active anti-retroviral therapies for the treatment of HIV infection, persons living with the disease are aging at a rate similar to the normal
population. Infected individuals are living longer and the number of patients over the age of 50 is increasing (Wing, 2016). Consequently, these patients are now prone to suffer from age-related neurodegenerative diseases such as PD, a disease characterized by motor dysfunction due to the loss of dopaminergic neurons along the nigrostriatal system and the formation of Lewy bodies resulting from the accumulation of misfolded or modified α-syn (Olanow et al., 2009, Kalia and Lang, 2015). Past studies indicate that the CNS is highly susceptible to HIV infection, with increased susceptibility in dopamine-rich areas, such as those affected by PD itself (Ellis et al., 2009, Kumar et al., 2009). Along the same lines, post-mortem brain tissues isolated from individuals living with HIV display increased α-syn within the substantia nigra (Khanlou et al., 2009). Patients also suffer from other overlapping disease characteristics such as neuroinflammation, lymphocyte infiltration into the CNS, impaired mitochondrial function, increased peripheral pro-inflammatory cytokine levels, and increased oxidative stress (Jones et al., 1998, Hong and Banks, 2015, Moulignier et al., 2015).

Interestingly, HIV patients also display many key motor and non-motor features observed in advanced PD such as bradykinesia, altered posture, impaired dexterity, altered gait, and cognitive impairments (Navia et al., 1986, Sheppard et al., 2015). Parkinsonism and Parkinson-like symptoms are also present in those suffering from acquired immunodeficiency syndrome (AIDS) (Rosso et al., 2009). Importantly, a non-invasive MEG study to monitor motor control demonstrated that HIV-infected patients have reduced beta responses indicative of a deficit in movement planning (Wilson et al., 2013). These motor
dysfunctions are most likely the result of the loss of neurons within dopamine-rich regions such as the basal ganglia, substantia nigra, and striatum (Berger and Arendt, 2000). Loss of dopaminergic neurons results in decreased availability of dopamine, a neurotransmitter needed for movement-related signaling (Groger et al., 2014). For example, HIV-infected patients display up to a 53% decrease in dopamine availability within affected brain regions and when treated with anti-retrovirals, they still experience a 45% decrease in dopamine levels (Kumar et al., 2011). Similarly, they also display decreases in the dopamine metabolite, homovanillic acid (HVA) (Kumar et al., 2009, Kumar et al., 2011). Thus, their movement and gait dysfunctions can be attributed to decreases in dopamine and dopamine metabolite availability.

Due to the overlap of disease hallmarks and characteristics between viral infection and Parkinson's-like symptoms, a direct association between the two is hypothesized. Likewise, several other viruses have already been shown to be associated with potential parkinsonism and Parkinson's-like symptoms due to the neurotrophic character of viruses (Jang et al., 2009). To extend these findings, we tested whether viral infection impacts nigrostriatal degeneration by employing two mouse models of viral infection in parallel with MPTP intoxication.

4.3 Materials and methods

Animals, infections, and MPTP intoxication

For HIV infection in humanized mouse studies, new-born NOD.Cg-Prkdcscid
dII2rgtm1Wjl/SzJ (NOD/scid-IL-2Rγcnull or NSG) mice (stock 005557, Jackson
Laboratories, Bar Harbor, ME) were transplanted with human CD34+ stem cells obtained from blood taken from the human umbilical cord, as previously described (Knibbe-Hollinger et al., 2015). Balb/cJ mice (stock 000651, Jackson Laboratories) served as non-humanized controls. After 15 weeks of reconstitution, humanized NSG mice were selected for study use based on human CD45+ cells numbers within peripheral blood. The selected animals were infected with HIV-1\textsubscript{ADA} via intraperitoneal injection at $10^4$ tissue culture infective dose 50 (TCID\textsubscript{50})/mouse. After four weeks of infection, mice received four s.c. injections of vehicle (DPBS, 10 ml/kg body weight) or MPTP-HCl (Sigma-Aldrich) at 14 mg MPTP (free base)/kg body weight in DPBS; each dose given at two hour intervals. Seven days post-MPTP intoxication, mice were sacrificed, brains harvested, and tissues processed for analysis. MPTP safety precautions were followed in accordance with determined safety and handling protocol (Jackson-Lewis and Przedborski, 2007). All animal procedures were also in agreement with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Six- to eight-week old, male C57BL/6J mice (stock 000664) or B6.CB17-Prkdc\textsuperscript{scid}/SzJ (C57BL/6 scid) mice (stock 001913) (The Jackson Laboratory) were used in all EcoHIV studies. In order to achieve infection, mice were inoculated intraperitoneally using EcoHIV, kindly provided by the laboratory of Dr. David Volsky (Columbia University, New York), at a dose of $3\times10^6$ pg p24/mouse. EcoHIV virus stock was prepared as previously described (Potash et al., 2005).
After 4 weeks of infection, animals were intoxicated with MPTP in the same manner as the HIV studies.

*Plasma collection, flow cytometric assessment and T cell Profiling*

During the course of infection, peripheral blood was isolated using a maxillary bleed. Plasma was collected for analysis of viral load, and whole blood was stained with fluorescently-conjugated monoclonal antibodies to human CD45, CD3, CD4, CD25, CD8, and FoxP3 in order to quantify T cell frequencies. For intracellular staining, samples were permeabilized using the FoxP3 staining buffer set kit (eBioscience). Fluorescent staining was analyzed using a FACSCalibur flow cytometer.

*Perfusions and immunohistochemistry*

Following administration of terminal anesthesia (Fatal Plus, pentobarbital), mice were transcardially perfused with DPBS followed by 4% paraformaldehyde/DPBS (Sigma-Aldrich). On day 7, to assess dopaminergic neurons in the substantia nigra (SN) and termini in the striatum, whole brains were isolated and cryosectioned. Frozen midbrain sections (30 µm) were immunostained for tyrosine hydroxylase (TH) (anti-TH, 1:2000, EMD Millipore) and counterstained for Nissl substance, as previously described (Benner et al., 2008). To determine the microglial reactivity, midbrain sections (30 µm) were immunostained for Mac-1 (anti-CD11b, 1:1000, AbD Serotech), as previously described (Kosloski et al., 2013). Within the SN, Stereo Investigator software (MBF Bioscience) was utilized to determine, by stereological analysis, the total numbers of Mac-1+
cells/mm², TH+Nissl+ (dopaminergic neurons), and TH-Nissl+ (non-dopaminergic neurons) within the substantia nigra. Density of dopaminergic neuronal termini in the striatum was calculated by digital densitometry using Image J software (National Institutes of Health).

**RNA isolations and polymerase chain reaction**

Following 4 weeks of infection and MPTP intoxication, fresh ventral midbrain tissue was isolated, and total RNA was harvested using an RNeasy Mini Kit (Qiagen), using RNase-free conditions. cDNA was generated from RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) and pre-amplification was performed using the appropriate primer mixes for RT² PCR arrays for the mouse Inflammatory Response array (Qiagen). Quantitative RT-PCR was performed on an Eppendorf Mastercycler realplex ep as per the manufacturer’s instructions (Eppendorf). Data analysis was performed using RT² Profiler PCR Array web-based data analysis software, version 3.5 (Qiagen), and gene networks were generated using Ingenuity Pathway Analysis (IPA, Qiagen). The resulting pathway was designed using the Pathway Designer Tool.

**Statistical Analysis**

All values are expressed as means ± SEM. Differences in between-group means were analyzed using ANOVA followed by Fisher’s least significant difference (LSD) post hoc test (GraphPad Software, Inc.).
4.4 Results

*HIV infection does not exacerbate neurodegeneration in MPTP-intoxicated humanized mice.*

To determine the interplay between HIV infection and development of Parkinson-like symptoms, we utilized humanized NSG mice that were either infected with HIV or uninfected, followed by MPTP intoxication. Following infection, mice were sacrificed and stereological analysis was used to quantify surviving neuron numbers. TH+Nissl+ dopaminergic neuron counts were decreased with MPTP intoxication, as expected from 9012 ± 679 to 6596 ± 580 (Figure 1A and 1B). However, co-infection with HIV did not increase the degree of degeneration observed, suggesting no effect on speed or amount of neurodegeneration associated with MPTP intoxication. Similarly, with MPTP intoxication alone or HIV infection + MPTP intoxication, TH density within the striatal termini decreased 49%, respectively compared to PBS controls (Figure 1C). There were no significant differences in microglial reactivity between treatments. Mac-1+ counts remained similar between groups (Figure 2A and 2B), indicating that the neuroinflammatory response had subsided by day 7. HIV infection did not enhance microglial reactivity above that observed with MPTP intoxication alone. Taken together, these results indicate that chronic and active HIV infection, as determined by viral loads in plasma, did not lead to enhanced nigrostriatal degeneration in the inflammatory mouse model of PD. These findings may either show either the lack of a synergistic effect causing neuronal loss or a lack of interaction between reconstituted human lymphocytes and murine microglia.
within the humanized system. The small loss of dopaminergic neurons is most likely due to the initial neuronal toxicity of the toxic MPTP metabolite, MPP⁺, rather than the ensuing inflammatory cascade associated with immune activation. Thus, based on these confounds, we began to explore the use of EcoHIV as a model of HIV infection in order to utilize a less complicated and non-humanized system. The EcoHIV virus has the ability to infect murine cells and exhibits neuroinvasiveness, allowing us to utilize non-humanized mouse models to test its effects on neurodegenerative disease (Potash et al., 2005).

*EcoHIV infection enhances MPTP-induced nigrostriatal degeneration.*

To determine a time course evaluation of EcoHIV infection in normal mice, male, C57BL/6 mice were inoculated with EcoHIV for 2, 3, or 4 weeks, followed by MPTP intoxication. At the time of sacrifice, brains were collected to assess neuronal survival and microglial reactivity following dual insult. Surviving neuron numbers were decreased with all treatments when compared to the control group (Figure 3A-C). EcoHIV infection alone resulted in a significant decrease at all time points decreasing neuron counts from 9556 ± 326 for PBS to 7450 ± 350 at 2 weeks of infection, 7359 ± 364 at 3 weeks of infection, and 6836 ± 999 after 4 weeks of infection (Figure 3C). Addition of MPTP intoxication lead to a further decrease in neuron numbers at all time points as well, resulting in 5404 ± 671 at 2 weeks of infection + MPTP, 6238 ± 743 at 3 weeks of infection, and 4103 ± 103 at 4 weeks of infection + MPTP. Striatal termini were also affected in all treatment groups (Figure 3D). At 4 weeks of infection and MPTP intoxication, numbers of
surviving dopaminergic neurons were decreased within the substantia nigra, when compared to mice treated with PBS, MPTP, or 3 weeks EcoHIV infection + MPTP, indicating a synergistic effect with longer periods of viral infection.

Based on these findings, we sought to elucidate a mechanism for the increased neuronal degeneration observed at 4 weeks of infection followed by a second insult with MPTP. It was shown that microglial activation and increased reactivity are associated with neuronal cell death in the MPTP mouse model of PD, so we also quantified numbers of Mac-1+ reactive microglia within the ventral midbrain of mice treated with PBS, MPTP, EcoHIV, and EcoHIV + MPTP at varying time points (Figure 4A) (Kurkowska-Jastrzebska et al., 1999a). The numbers of reactive microglia observed were significantly increased compared to PBS controls in MPTP without infection and at 4 weeks of infection followed by MPTP intoxication (Figure 4B). The other experimental groups were similar to controls. Interestingly, 4 weeks of infection followed by MPTP intoxication increased reactive microglial counts nearly 10 fold, increasing counts from 3 ± 0.3 cells/mm² in PBS-treated mice to 29 ± 4.1 cells/mm². These findings support the notion that EcoHIV infection exacerbates neuronal degeneration and enhances the neuroinflammatory response.

Furthermore, T cells interact with microglial populations to elicit active immune responses (Brochard et al., 2009). Therefore, mice were bled and peripheral blood was collected for analysis of changes in lymphocyte populations following EcoHIV infection (Figure 5). Flow cytometric analysis indicates that CD3, CD4 (Figure 5A and 5D), CD8 (Figure 5B and 5E), and CD25 (Figure 5C
and 5F) populations remained relatively unchanged over the course of infection and remained stable after MPTP intoxication, revealing the lack of a shift in number or frequency of T cell populations, suggesting that EcoHIV does not result in a diminution of CD4+ T cells as has been noted with HIV infection itself (Hong and Banks, 2015). Also, these results indicate that sufficient levels of CD4+ T cells exist to support necessary immune reactions necessary for successful neurodegeneration following MPTP intoxication. Moreover, these data support the continued use of the EcoHIV infection model rather than HIV infection in a humanized model.

Next, we investigated possible mechanisms for the increased neuronal loss and microglial reactivity observed at 4 weeks co-insult. We isolated the ventral midbrain of mice following infection at 4 weeks EcoHIV infection alone or 4 weeks EcoHIV infection followed by MPTP intoxication and isolated total RNA to assess changes in inflammatory response gene expression (Figure 6 and Figure 7). Although these results are not significant, they suggest a change in the inflammatory response pathway upon co-insult. When compared to EcoHIV alone, co-insult resulted in increased gene expression levels of the genes encoding for CCR7, CCL2, IFNγ, IL-10, IL-9, and NFκB and decreased levels of TLR4 and IL-5 (Figure 6 and 7B). Similarly, when compared to MPTP alone, co-insult led to an upregulation of, CCL2, IFNγ, IL-10, IL-9, NFκB, TLR4, and IL-5 (Figure 7A and 7B). These results suggest an interaction between two insults causing increased dysregulation in the inflammatory response pathway within the ventral midbrain.
EcoHIV infection does not affect MPTP-induced neuronal degeneration in immunodeficient mice.

Next, in order to evaluate the role of T cell-mediated neurodegeneration in EcoHIV infection, we utilized immunodeficient, non-humanized NSG mice and C57BL/6 scid mice. Each set of mice was infected with EcoHIV for 4 weeks, followed by MPTP-intoxication. On day 7 post-MPTP, animals were sacrificed and brains were harvested. Neuronal survival and microglial reactivity was assessed and quantified using stereological analysis. In non-humanized NSG mice, when compared to PBS controls, MPTP intoxication lead to a 13% and 6% loss in TH+ dopaminergic neurons, indicating that this strain may not be readily susceptible to MPTP intoxication (Figure 8A and 8B). This is most likely due to the lack of lymphocytes needed for MPTP susceptibility (Benner et al., 2008, Brochard et al., 2009). However, EcoHIV infection alone significantly decreased neuronal numbers by 26% compared to PBS, suggesting that EcoHIV may have a direct effect on dopamine–producing neurons, which is similar to the results observed in the immune competent, C57BL/6 strain of mice (Figure 3). Likewise, in humanized NSG mice, dopaminergic neuron numbers diminished after infection with EcoHIV alone (Figure 8B), further supporting the idea that EcoHIV may be directly neurotoxic. However, even though the neuronal cell bodies remained intact following MPTP intoxication, striatal termini were not spared (Figure 8C). MPTP intoxication significantly deceased the relative TH+ density within the striatum compared to PBS controls, whereas EcoHIV infection alone did not result in a significant loss of termini. These findings support the idea that
striatal termini are more susceptible to MPTP-intoxication and degeneration may occur in cell termini prior to the degeneration of neuronal bodies (Burke and O'Malley, 2013). To assess the presence of an inflammatory response, microglial populations were also stained and quantified in the same tissues, as previously performed. Reactive microglial numbers were significantly increased with MPTP intoxication when compared to PBS controls (Figure 9A and 9B); however, levels remained far below those observed in co-infection in C57BL/6, as shown in Figure 4.

In studies using immunodeficient, C57BL/6 scid mice, the results observed were similar to non-humanized NSG mice. TH+ neuron counts indicate no significant decrease in cell number after MPTP intoxication (Figure 10A and 10C). However, MPTP intoxication still resulted in an 8% and a 24% loss of dopaminergic neurons within the substantia nigra (Figure 10C). EcoHIV infection alone also led to a 7% loss in cell number, further supporting the concept that the virus causes dopaminergic cell death. Similarly, striatal termini in all treatment groups were significantly diminished (Fig 10B and 10D). MPTP intoxication alone resulted in a 39% loss of striatal termini, and EcoHIV infection plus MPTP-intoxication displayed a 52% loss, indicating increased susceptibility of striatal termini (Figure 10D). EcoHIV infection alone also resulted in a significant termini loss of 20%. The loss in both dopaminergic cell bodies and striatal termini were a result of direct MPP+ toxicity and viral neurotoxicity, rather than a resulting adaptive immune response generally associated with MPTP intoxication. Likewise, microglial populations remained unreactive (Figure 11A). There were
no significant changes in reactive microglia numbers among treatment groups (Figure 11B). In fact, virtually no inflammatory microglial response could be detected. This is most likely due to the lack of functional T cell populations in scid mice. In order for the neuroinflammatory cascade to occur in MPTP, an adaptive immune response is necessary (Benner et al., 2008, Brochard et al., 2009). Collectively, the data using two immunocompromised models help justify that an adaptive immune response is needed to achieve significant nigrostriatal degeneration in the MPTP model, and that EcoHIV infection alone exhibits a neurodegenerative signature along the nigrostriatal axis in an independent manner.

4.5 Discussion

Viral infection, specifically HIV infection, affects the CNS and causes Parkinson's-like symptoms (Navia et al., 1986). Post-mortem studies of HIV patients also indicate the presence of increased alpha-synuclein and decreased dopamine bioavailability, similar to that observed in PD (Kalia and Lang, 2015). However, in spite of these similarities, a paucity of scientific literature exists investigating the interplay of viral infection and development and/or progression of neurodegenerative disease using animal models. Thus, we investigated the potential effects of viral infection on MPTP-induced neurodegeneration utilizing two different mouse models of infection, HIV and EcoHIV. Infection of humanized mice with HIV-1 prior to MPTP intoxication did not result in enhanced nigrostriatal degeneration. The speed or magnitude of degeneration was unaffected, as was
the inflammatory response associated with MPTP. These results suggest either the lack of a synergistic effect of viral infection with an MPTP-induced lesion or that resident mouse microglia and neurons do not successfully interact with the humanized immune system. In order to initiate the neurodegenerative cascade in MPTP intoxication, MPTP is converted into its neurotoxic form, MPP⁺. The resulting neurotoxicity from MPP⁺ uptake and binding to complex I of the electron transport chain accounts for approximately 10% of the observed neuronal cell death (Przedborski et al., 2001). Further death results from the induced pro-inflammatory microenvironment induced by dying cells and subsequent activated microglial responses (Kurkowska-Jastrzebska et al., 1999a). For this response to occur, CD4⁺ cells must be present. Studies from Brochard et al indicate that mice lacking CD4⁺ cells are not susceptible to MPTP intoxication (Brochard et al., 2009). Therefore, we hypothesize that the lack of an accelerated neurodegenerative response in these studies is due to the lack of human CD4⁺ cell interaction with mouse microglial cells. The complexity and compounding effects of interspecies immune interaction make our findings difficult to interpret definitively. Our studies using immunodeficient models also help justify the importance of an adaptive immune response in the MPTP model of PD (Benner et al., 2008). In mice lacking a functional adaptive immune system, MPTP intoxication resulted in a minor loss in dopaminergic neuron number. This is presumably due to the effect of MPTP being metabolized into its toxic form, MPP⁺. MPP⁺ inhibits complex 1 of the electron transport chain causing cell death (Jackson-Lewis and Przedborski, 2007). This accounts for the initial drop
in cell viability. The secondary inflammatory reaction due to reactive microglia that ensues following initial neuronal loss continues the neurotoxic cascade. Without CD4+ T cells, this cascade does not occur (Benner et al., 2004, Brochard et al., 2009). Likewise, if CD4+ T cells are not functional, as in NSG and scid mice, a successful secondary reaction is not engaged. This also was observed in the humanized system. The lack of interaction between mouse microglia and human lymphocytes may account for the lack of MPTP susceptibility. Thus, taking these findings into account, we chose to utilize a less complex and species-specific model using EcoHIV infection in an immunocompetent, non-humanized system to address this question.

EcoHIV/NDK is a chimeric virus on the backbone of HIV-1 clade D, NDK, containing the gp80 envelope protein of murine leukemia virus instead of gp120 (Potash et al., 2005). It has been shown to infect murine cells, replicate in murine lymphocytes and macrophages, induce antiviral immune responses, invade the CNS, and increase inflammatory mediators in the brain. Use of EcoHIV inoculation to mimic HIV infection in humans is efficient and reproducible, and offers a suitable testing paradigm to study the neurodegenerative effects of infection (Potash et al., 2005, Hadas et al., 2007). Potash et al. showed that EcoHIV enters and establishes an infection in the mouse brain as early as 3 weeks after inoculation. The infected brains also express inflammatory factors including C3, IL-1B, STAT-1, and IL-6 indicating an active infection and anti-viral response within the brain (Hadas et al., 2007). The mechanism of the observed neuroinvasiveness of EcoHIV lies in its ability to increase BBB permeability after
2 weeks of infection (Jones et al., 2016). EcoHIV infection results in decreased expression of tight junction proteins, ZO-1 and claudin-5, within the BBB, allowing virus to actively enter the brain. Similarly, increased BBB permeability through the weakening of tight junctions is a mechanism used by HIV, further supporting the use of EcoHIV infection to mimic HIV infection (Dallasta et al., 1999). In our findings, we did not observe a significant decrease in neuron loss until 4 weeks of EcoHIV infection. This finding is most likely due to the time it takes for virus to enter the brain, travel to vulnerable sites, and successfully replicate. It also appears that with co-insult, microglial reactivity is increased at this time point as well. Microglia have the capacity to be infected causing them to become reactive (He et al., 1997, Bissel and Wiley, 2004). Likewise, EcoHIV may also have the capacity to infect microglia or microglia may be engulfing viral particles and proteins such as p24 or tat. A second possibility would be the effect of direct neuronal death, causing resident microglia to become activated as they sample the microenvironment (Kreutzberg, 1996). A third possibility would be the infiltration of infected peripheral macrophages across the leaky BBB to areas of degeneration (McArthur et al., 2005). Increased numbers of infiltrating cells would yield higher numbers of reactive Mac-1+ cells, as we observed. Further studies should be performed utilizing co-staining for Mac-1+ and viral proteins such as p24 and/or isolation by laser capture microdissection to determine whether microglial populations are productively infected.

With the significant increase in both neuronal death and microglial reactivity at 4 weeks EcoHIV infection, followed by MPTP intoxication, we
assessed potential changes in the inflammatory response cascade. Although the changes observed did not reach statistical significance, small changes in gene expression can lead to large fluctuations in protein content, thus giving rise to a more profound response. Therefore, these changes may be important in its mechanism of action. We observed changes in gene expression of both pro- and anti-inflammatory mediators with EcoHIV infection followed by MPTP-intoxication, indicating a general dysregulation of the inflammatory response. Increases in pro-inflammatory mediators including CCL2, IFNγ, NFκB, TLR4, and IL-5 were observed, but increases in anti-inflammatory mediators, IL-9 and IL-10, were also detected. Previously, HIV-1 infection was linked to increases in the pro-inflammatory cytokines, IL-5 and IFNγ (Navikas et al., 1994, Resino et al., 2000). The observed increase in CCL2 here may be linked to BBB permeability. Studies show that CCL2 mediates the transmigration of HIV-infected leukocytes across the BBB, resulting in viral neuroinvasiveness (Eugenin et al., 2006). Likewise, this may indicate a potential mechanism of CNS invasion with EcoHIV infection. HIV infection also activates NFκB and TLR4, which correlates with increased expression observed in our EcoHIV study (Fiume et al., 2012, Hernandez et al., 2012). Increased expression and activation of the NFκB pathway would ultimately lead to increases in overall pro-inflammatory gene levels (Fiume et al., 2012). Similarly, changes in TLR4 levels could affect the pro-inflammatory response by promoting viral replication and disease progression (Hernandez et al., 2012). Overall, changes in protein levels based on these gene changes may give rise to enhanced microglial reactivity observed after 4 weeks
co-infection, as well as led to an enhanced inflammatory microenvironment, thus, further exacerbating neurodegeneration. On the other hand, the upregulation of anti-apoptotic and anti-inflammatory interleukins may result from counteracting the neuroinflammatory response associated with infection in order to maintain a homeostatic balance within the brain. For instance, IL-9 has been linked to protecting cortical neurons and thymic lymphoma cells against apoptosis (Renauld et al., 1995, Fontaine et al., 2008). The upregulation of this interleukin would serve to decrease neuronal cell death associated with viral infection and MPTP intoxication. Similarly, IL-10 is needed for an anti-inflammatory response and is often secreted to suppress an overactive pro-inflammatory response, like that observed in MPTP intoxication or viral CNS infection (Kwon and Kaufmann, 2010).

Interestingly, EcoHIV viral infection alone yielded neuronal death in both immunocompetent and immunodeficient mice, indicating a direct effect of EcoHIV on the dopaminergic system that does not require cell-mediated immune responses. Until recently, in vivo and in vitro studies of neuronal infection have not yet yielded few compelling results. Some studies have shown that various neuronal subsets display the appropriate co-receptors for viral infection, such as CXCR4, CCR5, and CCR3 (Lavi et al., 1997, Rottman et al., 1997, Sanders et al., 1998). CD4 has also been detected on some neuron types (Funke et al., 1987). Also, multiple studies have shown that neuronal exposure to gp120 directly induces neuronal apoptosis (Brenneman et al., 1988, Xu et al., 2004). However, it should be noted that EcoHIV does express gp120, as it has been
replaced by gp80, and the effect that gp80 on neuronal survival has not yet been tested. To our knowledge, no study has explored the effect of HIV or EcoHIV infection on neurons within the substantia nigra or ventral midbrain specifically. This brain region exhibits increased susceptibility to neuronal cell death itself, so the presence of virus or viral particles may lead to increased neuronal cell death that may not be presently observed in other regions of the brain (Naoi and Maruyama, 1999, Sulzer and Surmeier, 2013, Haddad and Nakamura, 2015). This neuronal susceptibility to viral infection could, in turn, lead to the likelihood of developing PD or Parkinson-like symptoms later.

A second possibility for the neuronal death detected in this group is the presence of activated astrocytes within the lesioned region. Results utilizing in vitro models of astrocyte infection using cell lines indicate that they can be infected (Cheng-Mayer et al., 1987). In vivo findings show that cells with astrocyte morphology stain positive for p25, nef, and viral particles, indicating that they may be infected with HIV (Pumarola-Sune et al., 1987, Anderson et al., 2003). Cells immunostained with GFAP also tested positive for HIV gag DNA, furthering supporting the notion that astrocytes may have the potential to be actively infected (Trillo-Pazos et al., 2003). In these studies, we did not examine the astrocyte population, but this cell type may be important in the observed neuronal cell death. Astrocytes are important generators of neuroprotective mediators, such as nerve growth factor (NGF) and glial-derived neurotropic factor (GDNF), and infected and/or dying astrocytes could result in surrounding neurodegeneration (Belanger and Magistretti, 2009).
As stated previously, MPTP intoxication helps to generate a neuroinflammatory response, similar to that observed in human PD (Kurkowska-Jastrzebska et al., 1999a, Hirsch et al., 2012, Kalia and Lang, 2015). It also mimics dopaminergic neuronal loss associated with PD progression (Przedborski et al., 2001). However, it does not mimic all aspects of PD. For instance, this model of MPTP intoxication is acute and does not simulate the chronic and progressive nature of human disease (Kurkowska-Jastrzebska et al., 1999a, Przedborski et al., 2000, Przedborski et al., 2001). Thus, it should be appreciated that the dual effect observed using this model may not be observed in other more chronic and progressive disease models, such as chronic MPTP intoxication or alpha-synuclein overexpression models (Mochizuki et al., 2006, Meredith et al., 2008). Nonetheless, these findings suggest a potential effect of HIV/EcoHIV infection on neuronal survival, especially those neurons within the dopaminergic system. This negative effect can be partially attributed to enhanced microglial reactivity and changes in the inflammatory response pathway. If viral infection can initiate neurodegeneration in areas primarily affected in PD, then it is likely that viral infection could potentially lead to formation of PD later in life or at least increase the probability of developing symptoms similar to those associated with PD.
Figure 1. Co-administration of HIV and MPTP intoxication does not affect neuronal degeneration in humanized mice.
Figure 1. Co-administration of HIV and MPTP intoxication does not affect neuronal degeneration in humanized mice. **A.** Representative images of the SN and STR of humanized NOD/scid-IL-2Rγc null mice treated with MPTP alone or inoculated with HIV for 3 weeks or 4 weeks followed by MPTP intoxication. **B.** Total numbers of surviving dopaminergic neurons (TH+Nissl+) and nondopaminergic neurons (TH-Nissl+) in the SN following MPTP intoxication, or HIV infection followed by MPTP intoxication. Percentages of spared dopaminergic neurons are displayed on the representative bar for each treatment. **C.** Relative TH+ density within striatal termini of dopaminergic neurons. Differences in means (± SEM, n = 3-6) were determined where P < 0.05 compared to PBS (a).
Figure 2. *HIV infection does not increase microglial reactivity after MPTP intoxication in humanized mice.*
Figure 2. *HIV infection does not increase microglial reactivity after MPTP intoxication in humanized mice.* **A.** Representative images Mac-1+ microglia within the ventral midbrain of humanized NOD/scid/IL-2Rγc null mice treated with MPTP alone or inoculated with HIV for 3 weeks or 4 weeks followed by MPTP intoxication. **B.** Quantification of reactive microglia cells/mm² located within the substantia nigra.
Figure 3. *EcoHIV* co-infection exacerbates MPTP-induced neurodegeneration.
Figure 3. *EcoHIV co-infection exacerbates MPTP-induced neurodegeneration.* A. Photomicrographs of TH+Nissl+ neurons in the substantia nigra (SN) in C57/Bl6 mice treated with PBS, MPTP, or EcoHIV infection at multiple time points prior to MPTP intoxication. Sections were immunostained with anti-TH and HRP-conjugated secondary antibody and visualized with DAB. B. Photomicrographs of TH+ striatal termini (STR) following PBS, MPTP or EcoHIV coinfection. C. Total numbers of surviving dopaminergic neurons (TH+Nissl+) and nondopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment alone, EcoHIV infection alone, or EcoHIV infection followed by MPTP intoxication. Percentages of spared dopaminergic neurons are displayed on the representative bar for each treatment. Differences in means (± SEM, n = 8) were determined where P < 0.05 compared to PBS (a), MPTP (b), 3 wk EcoHIV + MPTP (c), and 4 wk EcoHIV + MPTP (d). D. Relative TH density of striatal dopaminergic termini following infection. Differences in means (± SEM, n = 8) were determined where P < 0.05 compared to PBS (a), 2 wk EcoHIV (b), and 4 wk EcoHIV (c).
Figure 4. EcoHIV infection leads to increased microglial reactivity.
Figure 4. *EcoHIV infection leads to increased microglial reactivity.* A. Representative photomicrographs of Mac-1+ microglia with the SN of C57/Bl6 mice treated with PBS, MPTP, EcoHIV, or EcoHIV + MPTP over time (4x image, inset = 40x image). B. Quantification of reactive microglia within the SN 7 days post MPTP intoxication. Sections were stained using an anti-Mac-1 antibody, followed by an HRP-conjugated secondary antibody and DAB. Numbers of amoeboid microglia were assessed using stereological analysis. Differences in means (± SEM, n = 8) were determined where P < 0.05 compared with groups treated with PBS (a) and 4 wk EcoHIV + MPTP (b).
Figure 5. *EcoHIV infection does not affect peripheral T cell populations.*
Figure 5. *EcoHIV infection does not affect peripheral T cell populations.* C57/Bl6 mice were inoculated with EcoHIV for 2-4 weeks and then intoxicated with MPTP. Mice were assessed for frequencies of CD3+CD4+ (A), CD3+CD8+ (B), and CD4+CD25+FoxP3+ (C). Differences in means (± SEM, n = 8) were determined where P < 0.05 compared with groups treated with PBS (a). D-F. Changes in T cell populations over time following EcoHIV infection.
Figure 6. *EcoHIV infection alone leads to changes in the inflammatory response.*
Figure 6. EcoHIV infection alone leads to changes in the inflammatory response. RT-PCR data shows gene expression changes in the ventral midbrains of C57BL/6 mice inoculated with EcoHIV followed by MPTP intoxication (n = 3-4). Fold changes were determined using SABioscience RT² Profiler PCR Array Data Analysis software, version 3.5. Changes in the inflammatory response pathway associated with EcoHIV infection alone vs EcoHIV + MPTP co-treatment. Resulting gene networks from each treatment group were analyzed using Qiagen Ingenuity Pathway Analysis. Pink coloration indicates a modest increase in expression and red indicates a profound increase in expression. Green coloration indicates a decrease in expression. Nodes lacking color indicate a molecule involved in the pathway, but not identified in the PCR data set.
Figure 7. EcoHIV co-infection results in gene changes in the inflammatory response pathway.
Figure 7. *EcoHIV* co-infection results in gene changes in the inflammatory response pathway. Fold changes were determined using SABioscience RT² Profiler PCR Array Data Analysis software, version 3.5. **A.** Changes in the inflammatory response pathway associated with MPTP intoxication alone vs *EcoHIV* + MPTP co-treatment are shown in the above pathway. Resulting gene networks from each treatment group were analyzed using Qiagen Ingenuity Pathway Analysis. Pink coloration indicates a modest increase in expression and red indicates a profound increase in expression. Green coloration indicates a decrease in expression. Nodes lacking color indicate a molecule involved in the pathway, but not identified in the PCR data set. **B.** Fold changes for differentially regulated mRNA levels with *EcoHIV* + MPTP treatment normalized to either *EcoHIV* treatment alone or MPTP intoxication alone.
Figure 8. EcoHIV and MPTP co-administration does not exacerbate neuronal degeneration in non-humanized NSG mice.
Figure 8. *EcoHIV and MPTP co-administration does not exacerbate neuronal degeneration in non-humanized NSG mice.* **A.** Photomicrographs of TH+Nissl+ neurons in the substantia nigra (SN) and striatum (STR) in non-humanized or humanized NOD/scid-IL-2Rγc null mice treated with PBS, MPTP, EcoHIV infection, or EcoHIV + MPTP co-administration. Animals were infected for 3 weeks prior to MPTP intoxication. **B.** Total numbers of surviving dopaminergic neurons (TH+Nissl+) and nondopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment alone, EcoHIV infection alone, or EcoHIV infection followed by MPTP intoxication. Percentages of spared dopaminergic neurons are displayed on the representative bar for each treatment. Differences in means (± SEM, n=3-4) were determined where P<0.05 compared to PBS (a). **C.** Relative TH density of striatal dopaminergic termini following infection and MPTP intoxication. Differences in means (± SEM, n = 3-4) were determined where P < 0.05 compared to MPTP (a) and EcoHIV + MPTP (b).
Figure 9. Microglial reactivity following EcoHIV and MPTP co-administration is not enhanced in non-humanized NSG mice.
Figure 9. *Microglial reactivity following EcoHIV and MPTP co-administration is not enhanced in non-humanized NSG mice.* **A.** Representative photomicrographs of Mac-1+ microglia within the midbrain following EcoHIV infection, MPTP intoxication, or EcoHIV infection and MPTP intoxication (4x image, inset = 40x image). **B.** Quantification of reactive microglia within the SN 7 days post MPTP intoxication. Differences in means (± SEM, n = 3-4) were determined where P < 0.05 compared with groups treated with PBS (a).
Figure 10. *Immunodeficient C57BL/6 scid mice are not susceptible to MPTP intoxication.*
Figure 10. *Immunodeficient C57BL/6 scid mice are not susceptible to MPTP intoxication.* A. Photomicrographs of TH+Nissl+ neurons in the substantia nigra (SN) and striatum (STR) (B) in C57BL/6 scid mice treated with PBS, MPTP, EcoHIV infection, or EcoHIV + MPTP co-administration. Animals were infected for 3 weeks prior to MPTP intoxication. C. Total numbers of surviving dopaminergic neurons (TH+Nissl+) and nondopaminergic neurons (TH-Nissl+) in the SN following MPTP treatment alone, EcoHIV infection alone, or EcoHIV infection followed by MPTP intoxication. D. Relative TH density of striatal dopaminergic termini following infection and MPTP intoxication. Differences in means (± SEM, n = 5) were determined where P < 0.05 compared to PBS (a) or EcoHIV alone (b).
Figure 11. *MPTP* intoxication does not lead to an inflammatory response in *C57BL/6 scid* mice.
Figure 11. *MPTP intoxication does not lead to an inflammatory response in C57BL/6 scid mice.* A. Representative midbrain images stained with Mac-1 following PBS, MPTP, EcoHIV + MPTP, or EcoHIV treatment. B. Quantification of Mac-1+ microglial cells/mm² within the substantia nigra. No significant differences were observed.
CHAPTER FIVE
DISCUSSION

Collectively, the data presented in this dissertation delineate a link between viral infection and neurodegenerative disease progression, a potential therapeutic agent for combating disease progression, a mechanism to track immune-modulating therapies in vivo using bioimaging techniques, and a shift in the current treatment paradigms for PD. First, PD has been associated with many risk factors, including male gender, aging, exposure to pesticides, and genetics (Kalia and Lang, 2015). It has also been suggested that viral infections may be linked to disease onset and progression (Jang et al., 2009, Moulignier et al., 2015). However, this has not been extensively studied or definitively linked. Thus, the studies presented here indicate that viral infection can, in fact, act directly on susceptible brain regions involved in the development of PD. Our data show the idea that viral invasion into the CNS results in direct neurotoxicity of dopaminergic cell bodies along with their striatal termini following infection with a chimeric murine virus that simulates HIV infection in humans. This also indicates that active viral infection potentiates and/or accelerates neurodegeneration, as observed in dual insult studies. One possibility for these neurotoxic findings would be the direct infection of dopaminergic neurons themselves, as discussed previously. Other possibilities include viral effects on surrounding astrocytes and microglia, the BBB, or peripheral immune populations.

Astrocytes are a glial subset that is normally involved in the maintenance of brain homeostasis and can also potentiate or mitigate neuroinflammatory
circuits. In the presence of hyper-activated microglia, astrocytes can directly participate in inflammatory reactions to secrete pro-inflammatory mediators such as IL-6 and TNFα, upregulate expression of FasL, and diminish trophic activities that eventually lead to the acquisition of a neurotoxic phenotype that exacerbates neurodegeneration (Dong and Benveniste, 2001). Similarly, while microglia that exist in an alternatively-activated (M2) state are generally considered neuroprotective and release anti-inflammatory cytokines as well as neurotrophic factors, under neuroinflammatory conditions, microglia can readily switch to an M1 cytotoxic phenotype. The phenotypic switch is accompanied with upregulation of oxidative stress-generating enzymes such as iNOS and NADPH oxidase; increased levels of ROS and RNS; and secretion of neurotoxic levels of proinflammatory cytokines including IL-1β, TNFα, and IFN-γ (Liao et al., 2012) that lead to secondary neuronal damage (Walsh et al., 2014). Similarly, apart from reactive glia, viral infection also has the capacity to increase BBB permeability, allowing both cellular and non-cellular immune mediators to enter the normally immune privileged site (Dallasta et al., 1999). Chronic viral infection causes upregulation of cytotoxic mediators as an anti-viral response, and progressive HIV disease is also associated with systemic and chronic immune activation, leading to B-cell activation, increased T cell turnover, increased frequencies of activated T cell phenotypes, and increased levels of proinflammatory cytokines and chemokines in peripheral blood and serum (Beltran et al., 2015). With a compromised BBB, these cytotoxic and neurotoxic mediators, such as CD8+ CTLs, activated CD4+ cells, activated macrophages, or
proinflammatory cytokines, may enter the brain, ultimately contributing to the neuroinflammatory microenvironment associated with both CNS viral infection and PD.

The presence of T cells within the brain of PD patients is higher than those typically found in the CNS which suggests a role of T cells beyond normal surveillance (Stone et al., 2009). For instance, during viral infections and neuroinflammation, MHC II is upregulated by microglia, whereas MHC I is constitutively expressed by most cells including oligodendrocytes, neurons, microglia, and endothelia (Redwine et al., 2001). Therefore, neurons in a pro-inflammatory environment, such as that associated with PD or other neurodegenerative diseases and viral infection, could serve as targets for CD8+ T cells with direct killing of neurons through antigen-specific interactions mediated by cytotoxic granzyme release. For instance, in animal models of MS, studies demonstrated that CD8+ CTLs form stable adhesions with neurites in an MHC I peptide-dependent fashion. Furthermore, MBP-specific CD8+ T cells induce direct tissue damage when injected into immunocompromised recipient mice (Neumann et al., 2002). Likewise, increased numbers of CD8+ T cells are found in close proximity to activated microglia in post mortem studies of PD patients (Brochard et al., 2009). However, their role in PD and PD animal models remains unknown. Based on past studies, the probability exists that, apart from CD4+ T cells, CD8+ Teffs may also play a role in inflammation-associated neurodegenerative disorders. However, in our studies, we did not focus on the potential detrimental roles of CD8+ CTLs in CNS viral infection and PD
progression. This cell types, along with chronically activated glia, may be potentiating some of the neuronal loss observed in the dual infection studies. Nonetheless, based on our findings, there is a synergistic effect of infection and MPTP-induced neuronal loss. The exact mechanism and/or mechanisms have not yet been deciphered, but our studies suggest a role for T cell-mediated neurodegeneration and a dysregulation in the neuroinflammatory response. Thus, in patients living with HIV and Parkinson's-like symptoms, therapies aimed at suppressing the neuroinflammatory condition and over-activation of immune mediators may be of clinical benefit.

Second, the studies discussed here point to a potential therapeutic agent that merits further study for both the treatment of PD alone and PD patients living with HIV comorbidities or vice versa. VIP is an endogenous peptide that is widely expressed and secreted by cells in the immune system, endocrine system, central nervous system, and peripheral nervous system (Delgado and Ganea, 2003a, Gonzalez-Rey et al., 2007). It acts as a neuropeptide, exerting a wide variety of biological effects, including neurotransmission and neuroprotection (Gonzalez-Rey et al., 2007). However, it is difficult to harness its effects for clinical use due to its rapid degradation and short half-life in vivo (Domschke et al., 1978). Thus, we synthesized an analog of VIP, LBT-3627, with increased receptor selectivity and improved half-life. When utilized in an inflammatory model of PD, LBT-3627 resulted in significant dopaminergic neuron sparing, decreased microglial reactivity, decreased astrocyte reactivity, decreased pro-inflammatory cytokine production, and a shift in inflammatory T cell phenotypes.
to suppressive subsets. Each of these findings helps to strengthen its potential clinical utility for the treatment of neuroinflammatory and neurodegenerative disorders. Previously, our lab utilized another known immune modulator, GM-CSF, in preclinical models, and observed similar effects (Kosloski et al., 2013, Kelso et al., 2015). Based on its pre-clinical efficacy and neuroprotective profile, a phase I clinical trial for its use in patients with PD was initiated (Gendelman et al., 2017). GM-CSF treatment yielded suggested improved motor function and cortical motor electrical activities, as well as modulated immune functions and changes in serum metabolites. GM-CSF treatment also corrected known PD-associated Treg dysfunction, potentially contributing to the observed improved motor outputs. However, these promising therapeutic results were paralleled by the development of multiple adverse events, including injection site reactions, increased white blood cell counts, upper torso and extremity bone pain, feelings of “chest tightening”, stroke, and vasculitis. Many of these reactions anticipated with extended use of GM-CSF, from studies in which GM-CSF is utilized to reconstitute the immune system following chemotherapy (Arellano and Lonial, 2008). From a clinical perspective, it would be beneficial to develop a treatment that utilizes similar immune-mediated mechanisms, but does not have these associated untoward side effects and does not require reconstitution of the entire immune system to achieve its neuroprotective effects. LBT-3627 meets these standards. Along with its neuroprotective potential, treatment with LBT-3627 resulted in an increase in GM-CSF gene transcript within the entire CD4+ T cell population, yet it did not modulate the number of T cells observed. This suggests
that LBT-3627 has the potential to mediate the neuroprotective activities that may be associated with GM-CSF treatment, without having to treat with GM-CSF protein itself for an extended amount of time. Clinically, this may benefit PD patients and may result in decreased adverse events associated with current immunotherapy treatments.

Previous studies utilized native VIP for neuroprotection from HIV toxicity (Brenneman et al., 1988, Brenneman et al., 1999, Brenneman et al., 2000, Zusev and Gozes, 2004). Various studies have shown that VIP treatment prevents HIV-1-induced neuronal death (Brenneman et al., 1988, Brenneman et al., 1999). This protection is partially mediated through VIP-associated signaling within astrocytes. When astrocyte and cortical neuron cultures were treated with VIP, there was an increase in macrophage inflammatory protein-1α (MIP-1α), beta-chemokine, and RANTES (Brenneman et al., 1999, Brenneman et al., 2000). This chemokine upregulation blocks receptor interactions that are needed for viral entry and toxicity, resulting in neuronal survival. Likewise, when VIP binds to the VIPR2 on astrocytes, it induces changes in activity-dependent neuroprotective protein (ADNP), which is associated with cell survival and development, further supporting the neuroprotective effects of VIP that could be achieved by targeting VIPR2, similar to our data with LBT-3627 treatment (Zusev and Gozes, 2004). Interestingly, activated CD4+ T cells in HIV infection also display elevated levels of VIPR2, thus, targeting this receptor may help to limit immune activation in the CD4+ T cell subset in a selective manner (Ipp et al., 2014). VIP-mediated signaling through VIPR2, rather than VIPR1, results in an
immunosuppressive profile and shift in T cell phenotypes, as observed in our studies. Thus, use of LBT-3627 in our dual insult studies would be expected to counteract the enhanced neurodegeneration observed. LBT-3627 would likely lead to a neuroprotective phenotype in this case, making its clinical utilization more appealing.

Along the same vein, VIP has been used in many other inflammatory and neurodegenerative conditions, including rheumatoid arthritis, inflammatory bowel disease, AD, MS, Huntington’s disease, endotoxin shock syndrome, and Crohn’s disease. Previous works show that VIP protects against neurotoxicity by decreasing microglial activation and degradation of neuronal cell bodies and termini in both MPTP- and 6-OHDA-induced injuries (Offen et al., 2000, Delgado and Ganea, 2003b, Reynolds et al., 2010, Korkmaz et al., 2012, Tuncel et al., 2012). The protective mechanism arises from altering the cytokine response into an anti-inflammatory profile by downregulation of proinflammatory mediators such as TNFα, IL-6, and IL-12 (Vial and Descotes, 1995b, Delgado et al., 1999, Chen et al., 2008, Reynolds et al., 2010), and by protecting neuronal tissue against oxidative stress and apoptosis by reducing lipid peroxidation and DNA fragmentation (Tuncel et al., 2012). Likewise, as stated previously, it also mediates protective effects via modulation of astrocyte activity and neurotrophin secretion and through the induction of immunosuppressive Treg populations (Zusev and Gozes, 2004, Fraccaroli et al., 2015).

Here, we propose the idea that VIP-induced transformation of T cell populations and enhanced Treg-mediated suppression are one of the main
mechanisms of its neuroprotective capacity. However, contrary evidence suggests that Tregs are detrimental to cell survival and can enhance neurodegeneration. Notably, this was shown during the acute phase of experimental stroke in which Treg expansion exacerbated brain injuries (Schuhmann et al., 2015). However, it should be noted that the acute phase is a relatively short span of time and is during a period consisting of an intense initial inflammatory response. Throughout this period, such an innate response in affected brain tissue is required to enhance phagocytosis of cellular debris, which may explain the coincident increase in inflammation associated with Treg infiltration. In contrast, another study demonstrated that IL-10-producing Tregs elicited a reduction in infarct size and attenuated the inflammatory response associated with ischemic stroke (Na et al., 2015). Likewise, another group showed that brain-derived neurotrophic factor (BDNF)-positive Tregs are increased in stroke patients, and this increase is associated with improved clinical outcomes (Chan et al., 2015). Further support of Tregs as neuroprotective cells is observed in tests using an experimental model of ALS and in human disease. Tregs were shown to modulate disease progression and to actively contribute to a neuroprotective cascade through their interactions with microglial populations (Beers et al., 2011, Zhao et al., 2012, Henkel et al., 2013). Similarly, a study was carried out using bee venom phospholipase A2 to protect dopaminergic neurons through the modulation of the inflammatory response associated with MPTP intoxication that occurred following Treg treatment transfer. When endogenous Tregs were blocked with anti-CD25 antibody, the
protective phenotype was no longer observed, indicating their specific involvement in neuroprotection (Chung et al., 2015). Collectively, these findings, along with our data, further support a direct neuroprotective role of Tregs in neurodegenerative disorders.

However, it should be appreciated that induction of Treg subsets may simply be a primary endpoint in a cascade of beneficial immunological changes. Tregs are not the only suppressive cell type within the immune system. Other types of immunosuppressive agents include CD8+ regulatory T cells, regulatory B cells, myeloid-derived suppressor cells (MDSC), and tolerogenic DCs (McHugh and Shevach, 2002, Gabrilovich and Nagaraj, 2009, Wang and Alexander, 2009, Rosser and Mauri, 2015). VIP induces and mediates its actions via these cell types as well as through the induction of Tregs (Chorny et al., 2005, Chorny et al., 2006, Gonzalez-Rey et al., 2006a, Li et al., 2015).

DCs are a heterogeneous population of APCs that contribute to innate immunity and initiate the adaptive immune response associated with inflammation and autoimmunity. However, apart from this, DCs also play an important role in maintaining immune homeostasis and immune tolerance (Mildner and Jung, 2014). Unlike classical DC function, tolerogenic DCs should not stimulate T cell proliferation or inflammatory cytokine production. Instead, they should act by suppressing the immune response and the effector populations required for the response. In support of this, VIP treatment regulates DC differentiation by causing an upregulation of CD86 in immature DCs and a downregulation of CD80 and CD86 in LPS-stimulated DCs (Chorny et al., 2006).
Also, induced CD4+ T cells by VIP-treated immature DCs exhibit an anti-inflammatory Th2 phenotype. Another study found that VIP induces tolerogenic DCs that cause surrounding CD4+ T cells to release anti-inflammatory cytokines such as IL-10 and TGFβ, indicating the formation of a regulatory subset rather than an effector population (Chorny et al., 2005). Likewise, in human studies, VIP treatment generated tolerogenic DCs that induced both CD4+ Tregs and CD8+ Tregs, further supporting the idea that signaling via VIPRs is involved in the generation of multiple immunosuppressive subsets (Gonzalez-Rey et al., 2006a).

Additionally, VIP also has the capacity to induce and generate myeloid-derived suppressor cells (MDSCs) (Li et al., 2015). MDSCs are an important population of regulatory cells within the innate immune system (Zhao et al., 2016). They have been linked to cancer pathogenesis due to their immunosuppressive function (Hoechst et al., 2008, Fujimura et al., 2012). These functions are mainly mediated through increased production of arginase, TGFβ, IL-10, and IDO (Zhao et al., 2016), ultimately leading to the crosstalk between MDSCs and Tregs. Therefore, we suggest that the observed regulatory T cell induction and shift in inflammatory state following LBT-3627 treatment may also be linked to changes in MDSC and tolerogenic DC populations, as well as through a direct interaction with T cells. For that reason, it would be beneficial to evaluate other regulatory cell populations following treatment to obtain a better understanding of shifts in the immune cascade in response to VIPR agonism. Such studies could yield additional potential clinical targets for the treatment of PD and other neuroinflammatory conditions.
Third, clinical diagnosis of PD is mainly limited to the associated motor dysfunction linked to neuronal loss along the nigrostriatal axis (Olanow et al., 2009). However, this aberrant motor read out does not present until 50-70% of the neurons are lost. The slowly progressive nature of the disease makes early detection difficult, and currently, diagnostic tests or clinical biomarkers for early recognition of the disease are lacking (Kalia and Lang, 2015). Therefore, the studies in Chapter 3 focused on utilizing bioimaging to track not only neuroinflammation associated with disease progression over time, but to evaluate treatment efficacy using LBT-3627 in a non-invasive manner. Collectively, our results confirm the ability of MEMRI to detect neurodegeneration in areas of brain inflammation, as well as to monitor the success of an anti-inflammatory and immune-modulating therapy in vivo. MEMRI can be used to identify brain subregions where inflammation is active and may also be useful for longitudinal measurement of inflammation in rodent models of neuroinflammatory disease, providing measurement tools to evaluate putative neurotherapeutics. However, Mn$^{2+}$ has been linked to neurotoxicity itself, potentially making it unsafe for use in humans (Olanow, 2004). Thus, further examination into bioimaging techniques suitable for human use needs to be carried out. Nonetheless, the use of MEMRI in animal models allows non-invasive tracking of glial and neuronal populations in response to treatment and neuroinflammatory insults, supporting the idea that bioimaging, such as MRI, PET/SPECT, or MEG, could potentially be used to diagnose disease, follow disease progression, or track treatment efficacy in the clinical setting.
Lastly, progressive neurodegenerative disorders, such as PD, present a major challenge for developing treatments because onset of the disease may be unknown, and the exact mechanism of disease initiation is poorly understood. However, neuroinflammation associated with activated microglial responses and adaptive immune aberrancies are widely considered to participate in the pathogenesis of PD, making them potential suitable targets. The pro-inflammatory cytokines, chemokines, ROS, and RNS secreted by reactive microglia trigger neurotoxic cascades that have detrimental effects within the CNS by exacerbating neuronal lesions. Thus, therapies targeting neuroinflammation either directly or indirectly should be explored. Many anti-inflammatory agents and immune modulating compounds discussed in the introduction share this commonality and have shown promising results in experimental models of the disease. However, even with their success in animal models, clinical advances using these agents are limited. This may be due to the fact that no animal model of PD encompasses all aspects of PD. Although some therapies have shown the potential to be translated into a clinical setting, they may not be as successful in treating patients with progressing PD. In acute, inflammatory models of PD, targeting inflammation specifically using anti-inflammatory agents has been shown to be neuroprotective, but with the multifactorial and slowly progressing nature of PD in humans, simply targeting one aspect may not be effective. Perhaps a combinatorial approach that targets many aspects of the disease and modifies disease microenvironment may be better suited for PD therapy.
One potential way of achieving this is by modulating the innate and adaptive immune system, as well as shifting peripheral T cell populations from pro-inflammatory, activated Teffs to anti-inflammatory phenotypes by utilizing agents that enhance Treg-mediated suppression of the immune response, such as LBT-3627. The data presented here, using a Treg-mediated therapy, document considerable progress toward improving disease outcomes in models of nigrostriatal degeneration. Some of the mechanisms by which the immune system can be used to target and control neuroinflammation are now realized. The therapeutic potential of immunomodulating therapies is an exciting advance and raises real possibilities for successfully combating disease and changing the course of PD pathogenesis. Thus, further pre-clinical exploration into the development of LBT-3627 as a neuroprotective agent should be carried out, with a clinical directive in mind.
BIBLIOGRAPHY


Cherry JD, Olschowka JA, O'Banion MK (2014) Are "resting" microglia more "m2"? Front Immunol 5:594.


Cohn M (2017) An observation that illustrates most T cell receptor structure-function relationships. Immunol Res.


Fujimura T, Kambayashi Y, Aiba S (2012) Crosstalk between regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) during melanoma growth. Oncoimmunology 1:1433-1434.


Kurkowska-Jastrzebska I, Wrońska A, Kohutnicka M, Członkowski A, Czlonkowska A (1999b) MHC class II positive microglia and lymphocytic infiltration are


1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. Ann Neurol 46:598-605.


receptor (VPAC2) for vasoactive intestinal peptide. J Immunol 172:7289-7296.


