Immunotherapy for the Treatment of Parkinson’s Disease

By
Matthew A. Follett
Department of Integrative Physiology, University of Colorado at Boulder

Research Conducted at the University of Nebraska Medical Center,
Department of Pharmacology and Experimental Neuroscience

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Thesis Advisor:
Dr. Teresa Foley, Department of Integrative Physiology

Defense Committee:
Dr. Teresa Foley, Department of Integrative Physiology
Dr. David E. Sherwood, Department of Integrative Physiology
Dr. Daniel C. L. Jones, College of Arts & Sciences Honors Program
Abstract

Parkinson’s disease is the most common neurodegenerative movement disorder, affecting an estimated 5 million people worldwide (Olanow, Stern, & Sethi, 2009). The exact etiology of the disease is unknown, but current treatments are able to control symptoms in many patients. However, no treatments currently exist that can slow or stop the progression of the disease. Research has indicated that the immune system likely plays a role in the etiology of Parkinson’s disease, so immunotherapy strategies have been proposed as a potential way to better combat the disease. This study examines the effect of granulocyte macrophage-colony stimulating factor on dopaminergic neuron damage seen in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson’s disease. Significant results were found that support the role of regulatory T cells in the protection of neurons from MPTP-induced damage, supporting the possibility of immunotherapy to treat Parkinson’s disease.
Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that was first described by James Parkinson in 1817 in “An Essay on the Shaking Palsy” (Smeyne & Jackson-Lewis, 2005). The disease is estimated to affect 5 million people worldwide and is seen mainly in people age 60 and older. Approximately 1 to 2% of individuals in that age group have the disease (Olanow, Stern, & Sethi, 2009), making it the most common neurodegenerative movement disorder (Saunders et al., 2012). While the exact etiology of the disease is unknown, it is suspected that environmental and genetic factors are involved in causing PD (Amor, Puentes, Baker, & van der Valk, 2010; Smeyne & Jackson-Lewis, 2005). Many PD features are known including that dopaminergic neurons in the brain, specifically in the substantia nigra pars compacta (SNpc) and striatum, are damaged and killed (Smeyne & Jackson-Lewis, 2005) and that deposits composed mainly of the nitrated form of protein α-synuclein, known as Lewy bodies, are found in affected neurons and microglia (Reynolds et al., 2010).

Parkinson’s disease is known mainly as a movement disorder. The motor symptoms, which begin to appear when 60 percent of the neurons in the SNpc are lost (Smeyne & Jackson-Lewis, 2005), are most often characterized by resting tremor, rigidity, bradykinesia, gait disturbances or postural instability, and freezing (Olanow et al., 2009). These symptoms, in addition to nonmotor symptoms including autonomic dysfunction, pain, mood disorders, and dementia (Olanow et al., 2009), can make completing basic daily activities difficult and become a source of disability in the lives of PD patients. Current treatments for PD are only palliative, not curative (Kosloski et al., 2010).
Based on the observed loss of dopamine in the brain following the death of dopaminergic neurons, dopamine replacement therapies have been developed and introduced, including levodopa (L-DOPA). Levodopa, a precursor of dopamine first offered on the market in the 1960s, is the current gold-standard PD treatment due to its effectiveness in reducing the motor symptoms in a large percentage of affected individuals and improving their quality of life (Olanow et al., 2009). Levodopa is often given with monoamine oxidase-B (MAO-B) inhibitors or catechol-O-methyl-transferase (COMT) inhibitors, which slow the degradation of dopamine and prolong the drug’s effects (Duty & Jenner, 2011). However, this treatment is not without its drawbacks as L-DOPA is not curative, it fails to control all PD symptoms, and it induces side effects with chronic administration.

Levodopa is often effective in controlling the classic motor symptoms of PD, but it does not affect other aspects of the disease, including mood disorders, freezing, and dementia. In addition, chronic L-DOPA usage can induce motor-related side effects in up to 90 percent of individuals who use the drug for greater than five to ten years, including motor fluctuations and dyskinesias (Olanow et al., 2009). Motor fluctuations occur when a drug dose wears off, unexpectedly stops working, or when it takes longer than expected to take effect. These create periods where the drug satisfactorily controls PD symptoms (known as “on” periods) alternating with periods when the drug does not satisfactorily control symptoms (known as “off” periods). As individuals with PD continue to take L-DOPA, these fluctuations between “on” and “off” periods can worsen and become debilitating. Over time, the drug may become less effective so the dosage has to be increased, worsening drug side effects. Levodopa can also induce involuntary movements throughout the body, known as dyskinesias. These often occur as L-DOPA begins to take effect (entering the “on” period) or when it begins to lose effect (entering
the “off” period). As the dosage is increased to reduce motor fluctuations or to counter reduced drug effectiveness, dyskinesias tend to become worse and can also become debilitating (Olanow et al., 2009). Administering L-DOPA with peripheral decarboxylase inhibitors does reduce some systemic side effects through preventing the peripheral conversion of the drug and allowing more to enter the brain (Duty & Jenner, 2011), which allows for lower doses, but L-DOPA-induced symptoms can still occur, especially after an individual has been using the drug for an extended time (Olanow et al., 2009). Additionally there is currently debate over whether L-DOPA may increase the rate of disease progression by exacerbating neuronal damage (Olanow et al., 2009; Smeyne & Jackson-Lewis, 2005).

Additional treatments exist, but like L-DOPA they are only palliative. A number of dopamine agonists, which are similar to L-DOPA in function but do not need to be first converted to dopamine within the body before working, have been tested and used in the treatment of Parkinson’s disease. Two early dopamine agonists, apomorphine and ergot derivatives, were shown to be effective but had worrisome side effects that caused them to consequently fall into disuse (Duty & Jenner, 2011). As a result, non-ergot derivatives are now the dopamine agonists of choice and are currently used to reduce the prominence of “off” periods or as an early treatment option to delay the use of L-DOPA. These dopamine agonists are not without their problems either, as side effects including nausea, vomiting, hallucinations, and impulse control disorders can occur, and, like L-DOPA, they do not treat the nonmotor symptoms of PD. Anticholinergic drugs can also be administered, but are only effective in treating resting tremor and are not frequently used (Olanow et al., 2009).

Surgical procedures offer benefits in the treatment of PD as well. One surgical intervention creates lesions in targeted overactive brain regions, including the ventral
intermediate nucleus of the thalamus to treat Parkinsonian tremor or the globus pallidus pars interna, which can produce improvement in tremor, rigidity, and dyskinesias. However, this procedure includes risks of damaging neighboring structures and, as a destructive treatment, can prevent the future use of more effective therapies that might become available. More recently, deep brain stimulation has been introduced to treat PD. Deep brain stimulation includes the implantation of one or more electrical leads into certain brain regions, including the ventral intermediate nucleus of the thalamus, globus pallidus pars interna, and subthalamic nucleus, connected to a subcutaneous pulse generator implanted under the skin of the chest. The pulse generator creates high frequency stimulation in the chosen region of the brain and is thought to create a pattern of interference to reduce abnormal or overactive neuronal signals, which can provide significant improvement of PD motor symptoms including motor fluctuations and dyskinesias. Advantages of deep brain stimulation over creating lesions include that the pulse generator can be reprogrammed as needed to reduce emerging PD symptoms, the surgery is not destructive, and it does not prevent the use of future therapies in targeted brain regions (Olanow et al., 2009). However, these surgical approaches, like the pharmacological treatments, are only able to target certain Parkinsonian symptoms, are not without their own side effects, and are not curative.

No interdictive treatment for PD exists, so it is clear that there is a need for further research into neuroprotective or restorative therapies that could stop the disease before it starts, or stop or reverse disease progression once the disease has began. A number of attempts have been made in the past to do so, including the use of antioxidants, neurotrophic factors, and anti-inflammatory approaches, which have yet to produce an effective treatment (Duty & Jenner,
2011). However, looking more in depth at the proposed pathogenesis of PD, it is clear that there are a number of targets for a neuroprotective therapy.

**Proposed Inflammatory Pathogenesis of Parkinson’s Disease**

The etiology of PD is unknown, but it is suspected that an interaction of genetic and environmental factors could trigger the disease, or that there could be a genetic cause for being sensitive to environmental toxins (Amor et al., 2010; Smeyne & Jackson-Lewis, 2005). Some known genetic mutations exist which are thought to be the cause of familial PD, although those mutations only account for a small number of PD cases, leaving most cases with an unknown etiology. Some of the genes that have been identified to cause PD when mutated include SNCA, LRRK2, Parkin, PINK1, and DJ-1.

SNCA is the gene that codes for α-synuclein. Mutations or multiplications of the SNCA gene are autosomal dominant and can lead to mutations in the α-synuclein protein (Lubbe & Morris, 2013), giving rise to Lewy bodies, one of the characteristic pathological features of PD. Parkinsonian Lewy bodies are found in the SNpc and are known to be composed mainly of nitrated-α-synuclein and ubiquitin (Smeyne & Jackson-Lewis, 2005), a protein marker for cellular degradation of the marked item. The fact that Lewy bodies contain ubiquitinated α-synuclein proteins shows that the human body tried to clear the misfolded or mutated proteins but was unable, seeming to indicate that the presence of Lewy bodies is the result of a protective measure taken by the body (Jellinger, 2010). LRRK2 is another autosomal dominant gene that can mutate and cause PD, also giving rise to Lewy bodies. Parkin and PINK1 are two genes that are autosomal recessive, and together regulate mitochondrial biogenesis and mitochondrial
quality control. Mitochondrial dysfunction is known to play a role in the pathogenesis of PD. Finally, DJ-1 is a gene that rarely mutates but can be a cause of autosomal recessive PD (Lubbe & Morris, 2013).

Beyond possible genetic influences on PD, the immune system seems to play an important role as well. Normally, microglia are the resident innate immune cells of the brain that present antigens to adaptive immune cells, clear debris, and survey the environment to look for foreign antigens (Sanchez-Guajardo, Annibali, Jensen, Sci, & Romero-Ramos, 2013). They can be either pro- or anti-inflammatory (Kosloski et al., 2010), but in a healthy state microglia should help maintain an anti-inflammatory environment (Amor et al., 2010) and therefore should not cause any damage to neurons. T cells are part of the adaptive immune system and are responsible for helping innate cells fight off infections as well as for maintaining an immunological memory to prevent repeat infections. When an antigen-presenting cell (e.g., microglia, macrophage, dendritic cell) finds a foreign antigen (or what the antigen-presenting cell perceives as a foreign antigen), it processes the antigen and presents it to naïve T cells (Kosloski et al., 2010). The activated T cell becomes a CD8+ cytotoxic T cell, a CD4+ helper T cell (Th), or CD4+ regulatory T cell (Treg), depending on the signals it receives from the antigen presenting cell, and can then enter the immune-privileged environment of the brain (Amor et al., 2010). In PD, CD4+ cells (both T helper and Treg) seem to play an important role. Helper T cells are generally pro-inflammatory and Tregs are generally anti-inflammatory cells. In a healthy state, Tregs help maintain an anti-inflammatory environment in the central nervous system, as neurons present proteins and molecules that favor activation of Tregs. However, in PD these roles can change and as neurons are damaged the T cells are less able to maintain an anti-inflammatory environment (Amor et al., 2010).
The chronic inflammation model of PD (see Figure 1) proposes that the expression of nitrated-α-synuclein is one possible cause of the neuroinflammatory environment associated with PD. Microglia bind α-synuclein, causing their activation and inducing the pro-inflammatory NF-κB signaling cascade to produce pro-inflammatory mediators (Cao, Standaert, & Harms, 2012; Reynolds, Stone, Mosley, & Gendelman, 2009). The microglia phagocytize the nitrated-α-synuclein into Lewy bodies, and produce inflammation- and oxidative stress-causing cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (Niranjan, 2013), which together cause the upregulation of inducible nitric oxide synthase. The increased levels of inducible nitric oxide synthase produced in the SNpc lead to the production of nitric oxide, which then damages neurons through oxidation (Smeyne & Jackson-Lewis, 2005). This damage is exacerbated by the brain’s relatively low levels of antioxidants (Niranjan, 2013). Microglia are also able to produce reactive oxygen species, including hydrogen peroxide (Reynolds, Banerjee, Liu, Gendelman, & Mosley, 2007), which can damage neurons via oxidation as well.

The oxidative damage that neurons experience in this inflammatory environment can damage intracellular organelles, most importantly mitochondria. Mitochondria play a key role in cellular survival, as they aid in protein production and are the source of adenosine triphosphate (ATP) energy. Mitochondria can also play a role in the death of cells when directed to do so by the immune system or when damaged. Mitochondria can be damaged in a number of ways. First, reactive oxygen species and nitric oxide produced by microglia damage mitochondria via oxidation to impair complex I of the electron transport chain. Second, mutations of PINK1 or Parkin can damage mitochondria by impairing oxidative phosphorylation (Jellinger, 2010), of which the electron transport chain is a component, or prevent regulation of mitochondria. Third, mutations in the α-synuclein gene can impair complex I of the electron transport chain (Schapira
Fourth, the α-synuclein protein may directly interact with mitochondrial proteins and contribute to mitochondrial dysfunction (Jellinger, 2010). Finally, mutations in the mitochondrial DNA may cause mitochondrial dysfunction, although mitochondrial DNA mutations themselves rarely cause parkinsonism (Schapira & Gegg, 2011). Damaged and dysfunctional mitochondria then produce reactive oxidants, including reactive oxygen species and superoxide radicals (Smeyne & Jackson-Lewis, 2005), potentially as a consequence of electrons escaping the electron transport chain and reacting with oxygen (Jellinger, 2010), further increasing the concentration of reactive oxygen species and worsening the oxidative damage that occurs to neurons. In addition, the superoxide radicals produced by mitochondria can react with nitric oxide to form peroxynitrite, which is a particularly destructive oxidizing molecule (Smeyne & Jackson-Lewis, 2005) that damages proteins, lipids, and fatty acids. Oxidative damage to mitochondria not only produces more oxidative molecules, but it also reduces ATP production in the cell, impairs calcium buffering, and causes cell death (Jellinger, 2010). Contributing to further reactive oxygen species production, as mitochondria are damaged and ATP availability is reduced, dopamine is released from intracellular stores and is oxidized to create more superoxide radicals. The culmination of the oxidative conditions within neurons that exist as a result of a large number of reactive oxygen species, nitric oxide, and peroxynitrite sources cause the nitration of α-synuclein protein, and as the neurons die the nitrated-α-synuclein is released into the extracellular environment (Kosloski et al., 2010) along with chemoattractants that draw in more microglia. Occurring in parallel to these intracellular processes, dopaminergic neurons continue to activate microglia through presentation of nitrated-α-synuclein, which continues to push microglia into an activated and pro-inflammatory state (Niranjan, 2013), as
evidenced by the increasing numbers of activated microglia that occur with PD progression (Reynolds et al., 2009).

Next, the nitrated-α-synuclein protein could either further activate more microglia and start the cycle over again, or it could cross the blood-brain barrier and drain to lymph nodes outside the central nervous system, where it is phagocytized by antigen presenting cells and presented to naïve T cells via major histocompatibility complex (MHC) II molecules. The T cells that are activated to the nitrated-α-synuclein in the lymph nodes differentiate into pro-inflammatory CD4+CD25- Th17 cells (Th17 refers to a subset of helper T cells) and can cross back over the blood brain barrier into the brain (Kosloski et al., 2010) where they further activate microglia to cause them to produce higher concentrations of pro-inflammatory cytokines, including reactive oxygen species. That T cells play a role in the process is supported by the finding of CD4+ T cells in the SNpc in PD (Amor et al., 2010). The increasing microglial activation and infiltration of Th cells worsens neuronal damage, specifically to dopaminergic neurons in the SNpc and striatum, leading to a chronically-activated inflammatory environment (Kosloski et al., 2010). This likely occurs for years, eventually causing symptoms when 60 to 70% of the SNpc dopaminergic neurons are destroyed (Smeyne & Jackson-Lewis, 2005), and then continues to worsen in the absence of an interdictive treatment.

Normally, CD4+CD25+Foxp3+ regulatory T cells (Tregs, where CD4+CD25+Foxp3+ specifically denotes the regulatory T cell) should quiet the immune responses of Th cells to self-antigens in order to maintain an immune tolerance to self (Corthay, 2009; Kosloski et al., 2010). However, in PD Tregs seem to be functionally deficient. The Tregs are able to proliferate as they should, indicating that the problem does not lie in the activation of the cells, but they are not as functionally competent in PD as they should be and therefore cannot suppress Th cell responses
(Kosloski et al., 2010; Saunders et al., 2012). In other words, in PD Tregs are unable to antagonize Th cells, so Th cells are almost unopposed and are able to maintain a pro-inflammatory environment (Kosloski et al., 2010). Normally, Tregs would be able to antagonize Th cell activity and reduce microglial-induced inflammation through the release of IL-10 and TGF-β (Kosloski et al., 2010) or through Fas-Fas ligand (Fas-FasL) interactions that cause the microglia to undergo apoptosis (Kosloski et al., 2010; Reynolds et al., 2009). However, in PD Treg are unable to fulfill this responsibility.

Parkinson’s Disease Model: MPTP

To better understand the pathogenesis of PD, various animal models have been developed. One of the most common is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin model, which was accidentally discovered first in humans when California heroin users presented in emergency rooms with parkinsonian symptoms after using the drug. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine can be used in mice and primates to produce symptoms similar to those seen in humans (Smeyne & Jackson-Lewis, 2005), with the mouse model being more widely used. In this model, MPTP is injected systemically into the mouse, where it is converted to toxic forms in the periphery or in the central nervous system, although only central nervous system MPTP damage causes parkinsonism. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine is a protoxin that is converted by MAO-B within glial cells (Smeyne & Jackson-Lewis, 2005) to 1-methyl-4-phenyl-2,3-dihydropyridium (MPDP+) which is then oxidized to the toxic form of the drug, 1-methyl-4-phenylpyridinium (MPP+). 1-methyl-4-phenylpyridinium is released into the extracellular environment and can then be taken up into dopaminergic neurons by the dopamine transporter. Within the dopaminergic neurons, MPP+
targets mitochondria to produce reactive oxygen species through inhibition of complex I of the electron transport chain, as seen in PD, giving this model high construct validity (i.e., a pathogenesis similar to that of PD). As a result of damaged mitochondria, cellular ATP availability decreases, which, when combined with high levels of reactive oxygen species such as hydroxyl radicals, causes neurons to die via apoptosis (Duty & Jenner, 2011; Niranjan, 2013; Smeyne & Jackson-Lewis, 2005). As in PD, the glial cells that processed the MPTP become activated and pro-inflammatory, and interact with naïve T cells. T cells are then activated, and CD4+ T cells become toxic to dopaminergic neurons and exacerbate damage with Fas-FasL interactions (Jellinger, 2010).
Immunotherapy Strategy

Current treatments for PD are only palliative and some may exacerbate the disease (Olanow et al., 2009; Smeyne & Jackson-Lewis, 2005), so there is a clear need for further research into more effective treatments. A number of researchers have identified places within the proposed inflammatory PD pathogenesis model for interventions that could halt or reverse disease progression, but so far no methods have translated from animal models to show neuroprotective features in humans, in part due to the inherent difficulty in creating treatments for a disease with an unknown etiology (Duty & Jenner, 2011). However, recent studies on inflammation in PD have found that in the MPTP model the induction of Tregs with certain cytokines can beneficially attenuate the Th17-mediated damage to dopaminergic neurons (Parajuli et al., 2012; Reynolds et al., 2010). The cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) is produced by dendritic cells, natural killer cells, and activated CD4+ T cells and is suspected to cause Treg proliferation (Kared et al., 2008; Shi et al., 2006). GM-CSF also has a clinical use, as it is currently given to humans to increase white blood cell counts when those individuals have decreased white blood cell counts that can occur with chemotherapy. Using this finding, I hypothesize that Tregs induced by GM-CSF will decrease damage to dopaminergic neurons in the MPTP mouse model of PD.
Materials and Methods

Mouse Groups

The mice used in this experiment were 5 week old male C57BL6/J mice from The Jackson Laboratory (Bar Harbor, ME). One control group, plus four experimental groups of mice were included in the analysis:

- Group 1, control, Phosphate Buffered Saline (PBS) injections only: This group allows for comparisons to treated mice in the other four experimental groups (n=7)
- Group 2, MPTP injections only: This group shows the effect of MPTP treatment alone on dopaminergic cells and allows for a comparison to the other groups (n=8)
- Group 3, GM-CSF pretreatment: GM-CSF was given to these mice before they received MPTP injections in order to examine the neuroprotective effects of this cytokine alone. This group shows whether intrinsically generated increases in Treg cell populations (i.e., Treg proliferation induced with GM-CSF) can modify MPTP-induced damage to dopaminergic neurons (n=7)
- Group 4, MPTP injection followed by adoptive transfer of all CD4+ T cells from donor animals treated with GM-CSF to induce Treg proliferation: This group will show whether CD4+ T cells transferred from other animals (adoptive transfer) can reduce MPTP-induced damage to dopaminergic neurons (i.e., whether a neuroprotective effect is related specifically to CD4+ T cells) (n=8)
- Group 5, MPTP injection followed by adoptive transfer of only Treg from donor animals treated with GM-CSF to induce Treg proliferation: This group will show whether Tregs
transferred from other animals can reduce MPTP-induced damage to dopaminergic neurons (i.e., whether a neuroprotective effect is related specifically to Treg cells) (n=7)

- One additional group of animals was used as donors of the GM-CSF-induced CD4+ T cells and Tregs.

The control and MPTP groups (groups 1 and 2) allow the three treatment groups (groups 3, 4, and 5) to be compared to baselines to determine the effect of those treatments on reducing dopaminergic neuron death caused by MPTP intoxication. All animal experiments were conducted in accordance with guidelines published by the National Institutes of Health and the experiments were approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center.

**Pretreatment of Donor Mice, Extraction and Purification of T Cells**

Recombinant mouse GM-CSF in sterile Dulbecco’s PBS (DPBS) was injected intraperitoneally (i.p.) into the donor mice daily for 5 days at 50 µg/kg body weight. On day 6, the donor mice were sacrificed and single cell suspensions of T cells were obtained from brachial, axillary, and inguinal lymph nodes by pressing lymph tissues from those locations through a 70µm cell strainer (Fisher Scientific). CD4+ T cells were then isolated by using a CD4+ T cell isolation kit II, mouse, per the manufacturer’s instructions (Miltenyi Biotech, Auburn, CA). Similarly, Treg were also isolated with a CD4+CD25+Regulatory T cell isolation kit (mouse), per manufacturer instructions (Miltenyi Biotech, Auburn, CA). The population of CD4+ T cells was resuspended in DPBS, as was the Treg population.
**MPTP-Intoxication and Adoptive Transfer to Recipient Mice**

The recipient mice in this study (groups 4 and 5) were injected with MPTP (Sigma-Aldrich, St. Louis, MO) subcutaneously 4 times at 2 hour intervals with 16 mg MPTP/kg body weight in DPBS. (Control mice were injected subcutaneously with DPBS alone at the same intervals and volume). Twelve hours after the final MPTP injection, 0.25ml of the isolated and resuspended CD4+ T cells from donor animals was injected into designated MPTP-intoxicated mice (group 4), and 0.25ml of the isolated and resuspended Treg cells from donor animals was injected into designated MPTP-intoxicated mice (group 5) via intravenous (i.v.) tail injections. The mice were sacrificed 5 days after MPTP intoxication.

**Transcardial Perfusion and Tissue Fixing and Freezing**

Mice received i.p. injections of 0.11-0.14cc of pentobarbital (Nembutal, Sodium Solution, Abbott Laboratories, North Chicago, IL, 50mg/ml, 20ml vial, NDC 0074-3778–04), followed by terminal anesthesia. Once the mouse was fully anesthetized (when it did not respond to a forcep pinch to the feet), the sternum was cut away to expose the heart. Dulbecco’s PBS was perfused into the left ventricle of the heart first for 3 minutes, followed by 4% paraformaldehyde (PFA; Sigma-Aldrich) in DPBS for 8 minutes. Next, the mouse was decapitated just above the shoulders and the skull carefully removed to expose the brain. The brain was removed and placed in PFA at 4°C for 24h on a rotating shaker to ensure that the tissue was fixed, and then was placed in 30% sucrose in PBS at 4°C for 24h on a rotating shaker. Once the tissue was fixed, the brains were snap frozen (30 seconds each) in 2-methylbutane in a beaker pre-cooled on a bed of dry ice for 20 minutes. After being immersed for 30 seconds, the brain was removed and
wrapped in foil (before being stored at -80°C as needed). In preparation to be sectioned, the brains were then embedded in Optimal Cutting Temperature Compound (OCT) and were attached to a Cryostat (cryomicrotome) specimen chuck with the OCT at the same time. This OCT helps to hold the tissue in place while it is being sectioned and helps to keep the tissue at the temperature needed for sectioning.

*Tissue Sectioning, Mounting, and Staining*

In preparation to be sectioned, the tissue was warmed to -20°C. Fifty sections through the SNpc centered at bregma -3.64mm, each 30µm thick, and 50 sections through the striatum centered at bregma 0.62mm, each 30µm thick, were then taken using a Cryostat. Individual tissue slices were placed into wells filled with sodium azide to remove the OCT and preserve the tissue. The tissue sections were then immunostained using the anti-tyrosine hydroxylase (TH) free float immunostaining method with a 1:2000 dilution for the SNpc, 1:1000 dilution for the striatum of anti-TH antibody in tris buffered saline (Calbiochem/EMD Millipore, San Diego, CA). The anti-TH stain is a marker of dopaminergic neurons in the brain. Tissue was mounted on slides and allowed to dry overnight before it was counterstained for Nissl substance with thionin staining (Benner et al., 2008; Benner et al., 2004). The Nissl stain is used as a marker of the endoplasmic reticulum and nucleus that allows visualization all neurons, both dopaminergic and non-dopaminergic.
**Tissue Densitometry**

The total numbers of dopaminergic neurons (TH+Nissl+) and non-dopaminergic neurons (TH-Nissl+) in the SNpc were estimated using a stereological analysis with Stereo Investigator optical fractionator module software (MBF Bioscience, Williston, VT). The density of dopaminergic neurons in the striatum was estimated using digital densitometry with ImageJ software (National Institutes of Health, Bethesda, MD) to analyze scanned images of stained striatal sections.

**Statistical Analyses**

Statistical analyses were completed to determine the significance of the four treatments compared to each other and compared to the control group. One-way ANOVA tests, followed by the Bonferroni correction post-hoc test for multiple comparisons were completed with GraphPad Software (GraphPad Software, Inc., La Jolla, CA). Values here are expressed as the mean ± the standard deviation. P-values <0.05 were considered statistically significant.
Results

Neuroprotective Effects of GM-CSF in the Substantia Nigra

Stained sections from each of the five groups were analyzed to determine the total numbers of dopaminergic neurons (TH+Nissl+) surviving in the SNpc in each sample. The mean total number of TH+ neurons in each group was:

- PBS only: 6442 TH+Nissl+ neurons ± 2157 TH+Nissl+ neurons
- MPTP only: 3434 TH+Nissl+ neurons ± 1742 TH+Nissl+ neurons
- GM-CSF pretreatment: 6291 TH+Nissl+ neurons ± 1992 TH+Nissl+ neurons
- CD4+ T cell adoptive transfer: 7077 TH+Nissl+ neurons ± 1365 TH+Nissl+ neurons
- Treg adoptive transfer: 6996 TH+Nissl+ neurons ± 1918 TH+Nissl+ neurons

As shown in Figure 2, there was a significant loss of dopaminergic neurons in the MPTP group compared to the PBS control group (P < 0.05). Figure 3 shows representative examples of photomicrographs of stained SNpc sections from each group, which show stained TH+Nissl+ neurons. TH+ cell counts in the three treatment groups (GM-CSF pretreatment, CD4+ T cell adoptive transfer, Treg adoptive transfer) were not significantly different from the control group or compared to each other (P > 0.05), but were significantly greater than TH+ cell counts in the MPTP intoxication-only group (MPTP vs. GM-CSF pretreatment: P < 0.05; MPTP vs. CD4+ T cell adoptive transfer: P < 0.01; MPTP vs. Treg adoptive transfer: P < 0.05). As also seen in Figure 2, there were no significant differences between any of the five groups in the mean total number of TH- (non-dopaminergic) neurons.
Neuroprotective Effects of GM-CSF in the Striatum

Stained striatal sections were analyzed to determine the relative density of TH+Nissl+ neurons compared to the PBS control in each group. The mean relative densities of TH+Nissl+ neurons in each group (normalized to the PBS-only control group) were:

- PBS only: 1.000 ± 0.156
- MPTP only: 0.398 ± 0.095
- GM-CSF pretreatment: 0.396 ± 0.057
- CD4+ T cell adoptive transfer: 0.409 ± 0.044
- Treg adoptive transfer: 0.551 ± 0.095

As shown in Figure 4, there was a significant loss of TH+Nissl+ neurons in all of the treatment groups compared to the PBS control (P < 0.001 for all comparisons). Figure 5 shows representative examples of photomicrographs of stained striatal sections from each group, which show stained TH+Nissl+ neurons. Of the three treatment groups, only the Treg adoptive transfer group (group 5) showed significant improvement from the MPTP intoxication only group (P < 0.05). All other comparisons were insignificant (P > 0.05).
Discussion

Parkinson’s disease is a debilitating neurodegenerative disorder that can present with both motor and nonmotor symptoms, creating a source of disability in the lives of affected individuals. While the precise etiology of PD is unknown, some potential causes have been identified, including genetic mutations and exposure to environmental toxins such as herbicides, giving rise to an increased prevalence of PD in rural areas (Olanow et al., 2009). These factors may trigger an initial event that sets up a sustained pro-inflammatory environment in the brain, causing slow but steady neuronal death by oxidative damage (Jellinger, 2010). Despite the unknown etiology, treatments have been developed to combat PD. Unfortunately, current treatments are only able to treat the symptoms of the disease (Kosloski et al., 2010) and may actually increase its tempo (Olanow et al., 2009; Smeyne & Jackson-Lewis, 2005). However, the growing understanding of the role of inflammation in the pathophysiology of PD offers potential targets for treatments that could slow or stop the progression of the disease, including targets involving quieting the pro-inflammatory Th cells in the brain.

Previous research on the immune response in PD has shown evidence that reducing inflammation in the brain can reduce or stop neuronal loss in the MPTP mouse model (Reynolds et al., 2007; Reynolds et al., 2010). Building on this work, I hypothesized that increasing the Treg populations in the brain would decrease damage to dopaminergic neurons caused by MPTP intoxication. In this study, the number of Tregs was increased in one of two ways: 1) by administering GM-CSF to induce Treg proliferation directly in the experimental animals, or 2) by using GM-CSF in donor animals to cause Treg proliferation, then transferring CD4+ cells or Tregs from those animals into the experimental animals. Using the MPTP mouse model of PD as a platform, five experimental groups of mice were studied including a PBS injection-only
control, MPTP injection-only, GM-CSF injection followed by MPTP injection, MPTP injection followed by CD4+ T cell adoptive transfer, and MPTP injection followed by Treg adoptive transfer.

Significant losses in the number of dopaminergic neurons were observed with MPTP injections alone compared to the PBS control, confirming that MPTP caused the expected result in our test animals. In comparison to the PBS control and MPTP injections alone, the three treatment groups (GM-CSF pretreatment, CD4+ T cell adoptive transfer, and Treg adoptive transfer) all offered significant protection against the effects of MPTP-induced neuronal damage to dopaminergic neurons in the SNpc. No significant effect was seen in the SNpc in the numbers of non-dopaminergic neurons between any of the five groups, indicating that the changes in dopaminergic cell counts in the MPTP-only and in the three treatment groups were due to changes in dopaminergic neuron counts and not due to changes in the larger population of dopaminergic and non-dopaminergic neurons. This observation is consistent with the known dopaminergic neuron-specific effect of MPTP in the animal model (and consistent with the human condition of PD in which dopaminergic neurons are lost). This result is also consistent with the conclusion that the experimental treatments (GM-CSF and adoptive transfer of CD4+ and Treg cells) were also specific to dopaminergic cell populations.

In the striatum, there was a significant loss of dopaminergic neurons within all treatment groups compared to the PBS control. Only the Treg adoptive transfer group yielded a significant improvement in the number of surviving dopaminergic neurons compared to the MPTP injection-only group, but even here there was still a significant loss of dopaminergic neurons compared to the PBS control. Of the three treatments studied here, the adoptive transfer of Tregs produced overall the most significant neuroprotective effect as demonstrated by elimination of
MPTP-induced damage of dopaminergic neurons in the SNpc and greater protection of dopaminergic neurons in the striatum compared to the other two treatment groups.

Earlier research has shown that GM-CSF does not interfere with the conversion of MPTP to MPP+ (Kosloski-Bilek, 2013) and does not interfere with the MPTP model, but instead promotes neuroprotection through another mechanism. The observation that GM-CSF induces Treg proliferation (Kared et al., 2008) in combination with the new data presented here that pretreatment with GM-CSF or transferring CD4+ and Tregs from other animals reduces MPTP-induced dopaminergic cell damage supports the conclusion that GM-CSF creates neuroprotection against the inflammatory environment created by MPTP by inducing Tregs. Whether GM-CSF also increases the effectiveness of Tregs is unknown. The neuroprotection created by GM-CSF pretreatment or the adoptive transfer of CD4+ T cells or Tregs might result from restoration of the balance between the pro-inflammatory Th cells and anti-inflammatory Tregs.

It is unclear why the treatment groups had less of an effect in protecting dopaminergic neurons in the striatum compared to the SNpc. The damage in the striatum could possibly be caused by non-immune factors that are more influential in the striatum than the inflammatory processes are in the SNpc. The greater protection of striatal dopaminergic neurons against MPTP damage afforded by Treg adoptive transfer (group 5 animals) compared to the other two treatments (GM-CSF and CD4+ adoptive transfer) might be related to the relatively greater number of Tregs added to the animals’ immune systems in group 5 animals. The transfer of immune cells from donor to recipient animals used a fixed volume of solution in groups 4 and 5. In the CD4+ donor solution (group 4), only a portion of the transferred cells were Tregs (there were fewer total number of Tregs in the volume of solution given to these recipient animals). In contrast, the Treg donor solution was entirely Tregs (there was a greater number of Tregs in the
same volume of solution). Treg adoptive transfer likely provides the largest number of new Tregs in the immune system and provides the best neuroprotection in the striatum, which supports the importance of Tregs in producing neuroprotection against MPTP damage. Whether GM-CSF treatment (group 3) produces fewer Tregs than are provided by the Treg transfer from donor animals is unknown, but the data presented here indicate that GM-CSF alone results in fewer or less effective Tregs compared to donor Treg with respect to protecting striatum from MPTP damage. If the damage in the striatum is only partially due to inflammation, adoptively transferring purified Tregs (group 5, with the largest number of Tregs transferred) would have the best possible chance to restore the Th-Treg balance, whereas transferring all CD4+ T cells adds cells that may not be as effective in reducing inflammation. However, even with the transfer of purified, anti-inflammatory Tregs, if there were damaging non-immune inputs in the striatum, the Tregs would not reduce or eliminate all the MPTP damage.

These results, even in striatum, support some role of inflammation in the etiology of PD as it relates to the MPTP mouse model, based on the finding that the induction or addition of anti-inflammatory immune cells significantly reduced or eliminated dopaminergic cell loss in the SNpc and had a small effect in the striatum. Further, this supports the involvement of T cells in neuroprotection in the MPTP mouse model due to the significant protection afforded by the induction or addition of anti-inflammatory T cells.

While these results are encouraging, GM-CSF treatment has yet to be translated to human studies of PD. Whether the neuroprotective effects observed here in the MPTP model, which might result from restoration of the Th-Treg balance to reduce inflammation, might occur in humans is not known. GM-CSF is currently in clinical use (Sargramostim; Leukine™, Sanofi-Aventis) to increase neutrophil counts (e.g., in people with low blood counts after
chemotherapy). It has been studied as a possible immune-modulator to treat inflammatory diseases in humans (e.g., Crohn’s disease, an inflammatory bowel disease) with some success (Korzenik, Dieckgraefe, Valentine, Hausman, & Gilbert, 2005). It is possible that GM-CSF treatment of humans with PD will produce meaningful neuroprotection to slow or stop the progression of the disease, but research will first need to be conducted to assess the safety of GM-CSF in humans with PD to ensure that no serious adverse events occur from the treatment. A pilot study is being organized for this purpose (H. Gendelman and L. Kosloski-Bilek, personal communication, December 2013). Our growing understanding of the role of inflammation in PD offers exciting new opportunities to develop restorative or regenerative therapies for this debilitating disease. Immune modulation with cytokines such as GM-CSF might prove to be one such approach.
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Appendix A: Abbreviations Used

α-syn: alpha-synuclein
CD4+CD25+Foxp3+: regulatory T cell
COMT inhibitor: catechol-O-methyl-transferase inhibitor
IL-1β: interleukin-1β
IL-6: interleukin-6
i.p.: intraperitoneal
i.v.: intravenous
L-DOPA: levodopa
MAO-B inhibitor: monoamine-oxidase-B inhibitor
MHC: major histocompatibility complex
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NO: nitric oxide
N-α-syn: nitrated-alpha-synuclein
PD: Parkinson’s disease
ROS: reactive oxygen species
SNpc: substantia nigra pars compacta
TH+Nissl+ neurons: dopaminergic neurons
TH-Nissl+ neurons: non-dopaminergic neurons
TNF-α: tumor necrosis factor-α
Figure 1: The proposed inflammatory pathogenesis and chronic inflammation model of Parkinson's disease.
Figure 2: Neuroprotective Effects of GM-CSF in the Substantia Nigra

The mean numbers of surviving dopaminergic (TH+ Nissl+) and non-dopaminergic (TH- Nissl+) neurons in the substantia nigra pars compacta is shown in each group. There was a significant loss of dopaminergic neurons with MPTP injections (n=8) compared to the PBS control (n=7; p < 0.05). Each of the three treatment groups [GM-CSF Pretreatment (n=7), CD4+ adoptive transfer (n=8), and Treg adoptive transfer (n=7)] showed significant neuroprotection against MPTP-induced neuronal loss (MPTP vs. GM-CSF Pretreatment: p < 0.05; MPTP vs. CD4+: p < 0.01; MPTP vs. Treg: p < 0.05). There were no significant changes in the numbers of non-dopaminergic neurons across any of the groups. Values represent group mean ± standard deviation. Percentages indicate surviving dopaminergic neurons compared to the PBS control. Letters placed above each group indicate significance between groups, where groups are labeled a, b, c, d, and e from left to right (i.e. PBS is group a, MPTP is group b, GM-CSF pretreatment is group c, CD4+ adoptive transfer is group d, and Treg adoptive transfer is group e).
Figure 3: Representative Substantia Nigra Photomicrographs

Representative photomicrographs showing immunohistochemistry staining of TH+ neurons within the substantia nigra pars compacta (SNpc) of each of the five groups are shown. Note the dark color of the stained SNpc in the PBS control, indicating relatively large numbers of dopaminergic neurons, and the significant loss in those neurons with MPTP injections. GM-CSF pretreatment, CD4+ adoptive transfer, and Treg adoptive transfer protected SNpc neurons significantly compared to MPTP injections alone.
Figure 4: Neuroprotective Effects of GM-CSF in the Striatum

Relative densities of striatal dopaminergic (TH+Nissl+) neurons within the striatum of each of the five groups are shown. The MPTP (n=8), GM-CSF Pre-treatment (n=7), CD4+ (n=8), and Treg (n=7) groups are compared to the PBS control group (n=7), which is assigned a relative density value of 1.0. All groups showed significant losses in neuronal density compared to the PBS control (p < 0.001). Only the Treg group showed a significant increase TH+ density compared to the MPTP group (p < 0.05). Values represent group mean ± standard deviation. Letters placed above each group indicate significance between groups, where groups are labeled a, b, c, d, and e from left to right (i.e. PBS is group a, MPTP is group b, GM-CSF pretreatment is group c, CD4+ adoptive transfer is group d, and Treg adoptive transfer is group e).
Figure 5: Representative Striatum Photomicrographs

Representative photomicrographs showing TH+Nissl+ staining within the striatum of each of the five groups are shown. Note the dark color of the striatum (the dark central sections) in the PBS control group, indicating a high density of dopaminergic neurons (TH+Nissl+) relative to the lighter colored, less dense TH+Nissl+ neurons in the other four groups. Neither the GM-CSF pretreatment, CD4+ adoptive transfer, nor the Treg adoptive transfer fully protected the TH+Nissl+ neurons in the striatum. Only the Treg adoptive transfer group had a significantly higher density of TH+Nissl+ neurons in the striatum compared to the MPTP injection group.
References


