THE ROLE OF TUMOR NECROSIS FACTOR (TNF) IN MICROGLIAL ACTIVATION AND PROGRESSIVE DEGENERATION OF DOPAMINERGIC NEURONS

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DEDICATION

To my family and friends for their unwavering love and support.

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THE ROLE OF TUMOR NECROSIS FACTOR (TNF) IN MICROGLIAL ACTIVATION AND PROGRESSIVE DEGENERATION OF DOPAMINERGIC NEURONS

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THE ROLE OF TUMOR NECROSIS FACTOR (TNF) IN MICROGLIAL ACTIVATION AND PROGRESSIVE DEGENERATION OF DOPAMINERGIC NEURONS

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Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by a loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). A number of studies have implicated chronic inflammation in the pathophysiology of PD; however, it is unclear which inflammatory mechanisms directly contribute to neuronal loss in PD. A number of cytokines, including TNF, are elevated in post-mortem brain and cerebrospinal fluid of patients with PD. Previous studies from our group have shown that blocking solTNF signaling at the time of a unilateral 6-OHDA striatal lesion

attenuated behavioral deficits and the acute loss of dopaminergic neuron loss by 50%. However, a critical question of clinical relevance is whether delayed solTNF signaling inhibition can prevent the progressive loss of DA neurons that occurs after a CNS insult. I report here that a single intranigral injection of a lentivirus encoding a dominant negative TNF inhibitor delivered 2 weeks after an intrastriatal 6-OHDA lesion attenuated microgliosis in SNpc and halted the progressive loss of nigral DA neurons and the associated locomotor deficits. Given the potential contribution of microglial activation to PD and the suggestion that anti-TNF therapies in the CNS may exert neuroprotective effects on vulnerable dopaminergic neuron populations, I also investigated the role of TNF in regulating microglia effector functions to gauge the potential detrimental effects of anti-TNF therapies on the microglia functional response. I found that microglia from TNF-null mice produced reduced protein levels of cytokines and chemokines in response to LPS stimulation and they displayed no cytotoxic effects on dopaminergic neuroblastoma MN9D cells when activated *in vitro*. I also demonstrated that microglia isolated from TNF-null mice failed to display the expected morphological changes in response to LPS stimulation, including enhanced perinuclear expression of the activation marker CD45. My results suggest that TNF plays a critical role in microglia activation, regulation of several microglia effector functions and is the primary microglia-derived inflammatory factor that compromises survival of dopaminergic neurons. Furthermore, these studies suggest that TNF-dependent neuroinflammation directly contributes to the delayed and progressive degeneration of nigral DA neurons after neurotoxic injury and further validates solTNF as a potential therapeutic target in PD.

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6-OHDA – 6-hydroxydopamine

AADC – L-aromatic amino acid decarboxylase, dopa decarboxylase

AAV – adeno-associated virus

ANOVA – analysis of variance

ASK-1 – apoptosis signal regulating kinase 1

ATP – adenosine triphosphate

BBB – blood brain barrier

CNS – central nervous system

COR – c terminal of ROC

COX – cyclooxygenase

CSF – cerebrospinal fluid

DA – dopamine

DAT – dopamine transporter

DBS – deep brain stimulation

DN-TNF – dominant negative TNF

ETAN – etanercept

GABA – gamma aminobutyric acid

GAD – glutamic acid decarboxylase

GDNF – glial cell line derived neurotrophic factor

GFP – green fluorescent protein

GTPases – guanosine triphosphate phosphohydrolases

ICAM – intracellular cell adhesion molecule

IFNγ – interferon gamma

IL-1 – interleukin-1

IL-6 – interleukin-6

JEV – Japanese encephalitis virus

KO – knockout

L-DOPA – L-dopamine

LPS – lipopolysaccharide

LRRK2 – leucine rich repeat kinase 2

MAC1 – membrane attack complex 1

MAO-B – monoamine oxidase-B

MAPK – mitogen activated protein kinase

MCP-1 – monocyte chemoattractant protein-1

MHC – major histocompatibility complex

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NeuN – neuronal nuclei

NF-κB – nuclear factor kappa B

NO – nitric oxide

NRF2 – nuclear factor erythroid 2 related factor

NSAIDS – non-steroidal anti-inflammatory drugs

PD – Parkinson's disease

PINK1 – PTEN-induced kinase 1

SEM – standard error of the mean

SNpc – substantia nigra pars compacta

SNPs – single nucleotide polymorphisms

solTNF – soluble TNF

TACE – TNFα converting enzyme

TBI – traumatic brain injury

TH – tyrosine hydroxylase

TLR4 – toll-like receptor 4

tmTNF – transmembrane TNF

TNF – tumor necrosis factor

TNFR1 – TNF receptor 1

TNFR2 – TNF receptor 2

TRAF2 – TNF receptor associated factor 2

TRAP1 – TNF receptor associated protein 1

UPS – ubiquitin proteosome system

VCAM – vascular cell adhesion molecule

VTA – ventral tegmental area

WT – wild type

CHAPTER ONE
Introduction

PARKINSON'S DISEASE

Etiology, Pathology, and Clinical Symptoms of Parkinson's Disease

Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder that was first described by James Parkinson in his original manuscript "An Essay on the Shaking Palsy" published in 1817 in which he details the clinical manifestations of the disease (Parkinson 2002). Parkinson's disease is characterized by a slow protracted loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) that project to the caudate/putamen or dorsal striatum. The primary motor symptoms associated with this disease are an involuntary resting tremor (5-7Hz), rigidity, difficulty initiating movement (akinesia), slowed movement (bradykinesia), and postural instability (Whitton 2007). At the time of clinical manifestation fifty percent of the dopamine producing neurons in the substantia nigra pars compacta have already degenerated reducing striatal levels of dopamine by up to eighty percent (Lang and Lozano 1998; Deumens et al. 2002). Neuropathological analysis has revealed a histopathological hallmark of the disease: ubiquitin positive, intracytoplasmic inclusions of alpha-synuclein, the primary protein component of Lewy bodies (Whitton 2007). Although there are other pathological changes throughout the brain and body, it is believed that the loss of dopamine producing neurons in the SNpc is the primary cause of motor symptoms associated with the disease (Braak et al. 2004; Surmeier et al. 2010)..

Populations at risk for developing Parkinson's diease

Today, Parkinson's disease is the most common neurodegenerative movement disorder (Whitton 2007). Like most neurodegenerative diseases, the risk of developing Parkinson's disease is strongly associated with age. Approximately 3% of the population affected is between the ages of 65-70 and the risk increases to 4-5% in people 85 years of age or older (Whitton 2007). Studies have shown that the mean age of onset is 70 in sporadic Parkinson's disease and 50 for familial early-onset forms of Parkinson's disease.

Pathogenesis of Parkinson's Disease

Although the exact mechanism responsible for initiation and progression of PD is unknown, a prevalent hypothesis is that genetic susceptibility combined with environmental influence may be responsible for the selective degeneration of dopaminergic neurons in the SNpc. Currently research has focused on three main themes of cellular dysregulation that are associated with genetic and environmental components: ubiquitin proteasome dysfunction resulting in the formation of Lewy bodies, oxidative stress, and mitochondrial dysfunction resulting in apoptotic cell death.

Ubiquitin proteosome system

The ubiquitin proteasome system (UPS) is an essential protein processing and degradation mechanism utilized by cells to regulate levels of intracellular proteins. Within the cell, the UPS serves as a protective mechanism by allowing the cell to selectively target toxic or damaged proteins for degradation by the addition of a mono or

poly ubiquitin tag. In the context of neurodegenerative diseases such as PD, UPS dysfunction is indicated in the pathogenicity of the disease by the presence of Lewy bodies, intracytoplasmic inclusions of the aggregated protein alpha-synuclein. Under conditions of oxidative stress or toxic insults in neurodegenerative diseases such as PD, the UPS becomes impaired leading to the accumulation of toxic proteins (Betarbet et al. 2005). In sporadic cases of PD, UPS dysfunction is evident in postmortem brain tissue by reduced expression of 20S/26S proteasomal subunits and the presence of Lewy bodies (McNaught et al. 2003). In *in vitro* cell models, alpha-synuclein is normally targeted for degradation to the UPS (Bennett et al. 1999) and proteasomal inhibitors increase the number of alpha-synuclein immunoreactive inclusions (Rideout et al. 2001; McNaught et al. 2002). Furthermore, Parkin, an E3 ubiquitin ligase in which mutations are genetically linked to familial early onset PD, has been shown to ubiquitinate glycosylated forms of alpha-synuclein (Shimura et al. 2001) indicating UPS dysfunction underlies the vulnerability and degeneration of dopaminergic neurons in genetic and sporadic cases of PD.

Oxidative Stress

In dopaminergic neurons, the synthesis and oxidative metabolism of dopamine is a large source of hydrogen peroxide and other reactive oxygen/nitrogen species that cause oxidative stress and subsequent neurodegeneration. In addition to reactive oxygen/nitrogen species, it is current knowledge that decreased levels of antioxidants and increased levels of reactive iron can also impact oxidative stress (Olanow and Tatton 1999). While the cell maintains natural mechanisms to prevent or decrease sources of

oxidative stress, it has been shown in postmortem brain tissue that glutathione levels are lower, and iron deposition is higher than in age-matched controls, indicating oxidative stress as a key player in PD pathogenesis (Sofic et al. 1988; Hirsch et al. 1991; Sian et al. 1994). In support of oxidative stress in the pathogenesis of PD, studies utilizing natural plant based antioxidants such as flavonoids and polyphenols, have all shown neuroprotection in *in vitro* and *in vivo* models of PD (Zhao 2009). Dopamine synthesis and oxidative metabolism, decreased natural antioxidants, and high intracellular levels of reactive metals provide key insight into why dopaminergic neurons in the brain are specifically vulnerable to degeneration in disease such as PD.

Mitochondrial dysfunction

Within the cell, mitochondria are essential for cellular respiration and generation of energy in the form of adenosine triphosphate (ATP). It has been shown that PD patients display reduced mitochondrial complex 1 activity in the SNpc and systemically (Schapira 2008; Bueler 2009) implicating mitochondrial dysfunction in PD. Environmental toxins associated with PD susceptibility such as rotenone and MPTP are potent inhibitors of mitochondrial complex 1 (Di Monte et al. 2002; Bueler 2009). Defects or inhibition of mitochondrial complex 1 can lead to disruptions in the mitochondrial membrane potential resulting in decreased ATP production, release of apoptosis initiating factors and cytochrome c, and eventual apoptotic cell death (Olanow and Tatton 1999). Recent studies in rodents have shown that mitochondrial mass is lower in dopaminergic neurons in the SNpc than dopaminergic neurons in the ventral tegmental area (VTA) or any other

cell type in the ventral midbrain (Liang et al. 2007) suggesting dopaminergic neurons in the SNpc are more vulnerable to mitochondrial complex 1 inhibition and degeneration.

The Genetics of Parkinson's disease

Sporadic or idiopathic Parkinson's disease makes up about 90-95% of diagnosed cases and the remaining 5-10% are heritable forms of PD (Mizuno et al. 2001; Van Den Eeden et al. 2003; Tansey et al. 2007). In the heritable cases of PD, five main genes with multiple genetic mutations have been identified and associated with developing the 'Parkinsonian'-like symptoms (Hardy et al. 2006). (Table 1)

Dominantly inherited mutations

Alpha synuclein, a natively unstructured protein with a uncertain function is the main component of Lewy bodies and the first gene identified with mutations linked to familial early onset PD (Hardy et al. 2006). The first point mutation identified, A53T, has an autosomal dominant inheritance pattern and was identified in a family of Greek/Italian descent with the average age of onset at 55 years (Polymeropoulos et al. 1996; Polymeropoulos et al. 1997). Later, two additional dominant point mutations, A30P and E46K, in the alpha-synuclein gene were identified and linked to autosomal dominant, inherited forms of PD (Kruger et al. 1998; Zarranz et al. 2004). In addition to families with point mutations, early onset cases of familial PD have also been causally linked to duplication and triplication events in the alpha-synuclein gene with increased copy number lowering the average age of onset in these patients (Singleton et al. 2003;

Chartier-Harlin et al. 2004). Although the exact function of alpha-synuclein is not known, research has shown that it may play an important role in the central nervous system (CNS) by modulating vesicle trafficking, a critical process of synaptic transmission by binding to lipid membranes and stabilizing membrane curvature defects (Cabin et al. 2002; Fortin et al. 2004; Nuscher et al. 2004; Hardy et al. 2006). Immunohistochemical studies have shown that Lewy bodies, a histopathological hallmark of PD, intracytoplasmic inclusions of aggregated protein, are primarily composed of misfolded alpha-synuclein (Spillantini et al. 1997). It is currently hypothesized that increased expression of alpha-synuclein, the presence of Lewy bodies, and genetic mutations causally linked to the onset of PD, alpha-synuclein may be the link between sporadic and familial forms of the disease.

Leucine-rich repeat kinase 2 (LRRK2), a protein named for its leucine rich repeats and kinase domain, is another gene in which autosomal dominant mutations have been identified and linked to heritable forms of PD. In these patients, the age of onset and pathology are highly variable, with all cases presenting with nigral degeneration and some cases presenting with Lewy body and/or neurofibrillary tangle pathology (Wszolek et al. 2004; Zimprich et al. 2004). LRRK2 has been shown to be the most prevalent cause of familial PD and represents about 1.5% of all PD cases in the United States, with a higher prevalence in other populations (Hardy et al. 2006). LRRK2 is large protein member of the ROCO family of Guanosine Triphosphate Phosphohydrolases (GTPases) that has several domains including a Roc GTPase, an associated C-terminal of Roc (COR) domain, a WD40 repeat, an armadillo and anykrin repeat region, leucine-rich

repeats, and a kinase domain (Paisan-Ruiz et al. 2004; Zimprich et al. 2004). The large structure of the protein, variable pathology, and numerous domains indicate that LRRK2 may have functions other than kinase and GTPase activity associated with disease (West et al. 2005). Although there are many point mutations identified in the LRRK2 gene, two disease causing point mutations in the kinase domain (G2019S and I2020T) have shown an increase in kinase activity *in vitro* causing neurotoxicity and neurodegeneration (West et al. 2005; Gloeckner et al. 2006; Smith et al. 2006). While the downstream substrates for LRRK2 kinase activity are relatively unknown, recently, mitogen activated protein kinase kinases (MAPKK) and moesin have been identified as a substrates for LRRK2's kinase activity indicating cellular stress, actin dynamics, and vesicular transport involvement in LRRK2-associated neurodegeneration (Jaleel et al. 2007; Gloeckner et al. 2009). Due to the large structure of the protein and numerous domains, research pursuing LRRK2 interactions with other proteins will be key in identifying the mechanistic role of LRRK2 in PD pathology.

Recessively inherited mutations

Autosomal recessive mutations or large deletions in Parkin, a gene encoding for an E3 ubiquitin ligase, were first identified in a Japanese cohort of patients with juvenile onset PD (Kitada et al. 1998). Today, loss of function mutations in the Parkin gene account for nearly half of all the familial cases of early onset PD (Lucking et al. 2000). Parkin, a RING finger-containing protein with E3 ubiquitin ligase activity, participates in protein turnover by the addition of mono or polyubiquitin chains therefore targeting its substrates for degradation to the ubiquitin proteosome (Shimura et al. 2000; Lim et al. 2006). Due

to the loss-of-function mutations associated with PD, it is thought that a build up of ubiquitinated proteins may be the cause of toxicity; however, it is unclear if there is a specific substrate accumulation responsible for the dopaminergic neuron degeneration (von Coelln et al. 2004). Interestingly, Parkin deficient mice have dopamine dysregulation, but do not display nigrostriatal degeneration (Goldberg et al. 2003) indicating a potential role for environmental interactions to induce nigral degeneration, a hypothesis that is supported by the varying age of onset in patients and a study detailing that Parkin deficiency increases susceptibility to inflammation-induced nigrostriatal degeneration (Frank-Cannon et al. 2008). While the mechanism by which Parkin deficiency induces nigrostriatal neuron loss is currently unknown, studies focusing on genetic deficiency and environmental interplay may better explain the role of Parkin in PD.

Rare mutations in the autosomal recessive gene DJ-1, a protein involved in oxidative stress signaling, were first identified in a small group of families with early onset PD (Bonifati et al. 2003). While the function of DJ-1 in nigrostriatal degeneration is relatively unknown, studies have shown that it is an important player in the oxidative stress response and mitochondrial dysfunction, two mechanisms thought to play a role in dopaminergic system dysfunction in PD (Schapira 2008). DJ-1 is a dimeric protein present in a variety of tissues, but is primarily localized to the cytoplasm of the cell, inner membrane space, and matrix of the mitochondria (Zhang et al. 2005). It has shown that DJ-1 translocates to the outer membrane of the mitochondria during conditions of oxidative stress, a mechanism thought to protect neurons from oxidative

neurodegeneration (Canet-Aviles et al. 2004). DJ-1 has been shown to stabilize nuclear factor erythroid 2-related factor (NRF2) and is required for NRF2's induction of the oxidative response (Clements et al. 2006). Considering that loss-of-function mutations in DJ-1 are associated with early onset PD, it is thought that DJ-1 may confer a protective mechanism in dopaminergic neurons by promoting NRF2's induction of the oxidative stress response, although the exact mechanism is unknown. Consistent with this hypothesis, studies have shown that DJ-1 deficiency causes an increased sensitivity to oxidative toxins (Meulener et al. 2005) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neuron death (Kim et al. 2005). Considering the oxidative process involved in synthesizing dopamine in neurons, further research involving DJ-1's role in the oxidative stress response may be informative as to why DJ-1 deficiency leaves dopaminergic neurons susceptible in PD.

Mutations in PTEN-induced kinase 1 (PINK1), a serine/threonine kinase, account for a small percentage (1-4%) of autosomal recessive early onset PD cases (Valente et al. 2004). PINK1 is a protein kinase primarily localized to the mitochondria and is thought to play a protective role, suggesting loss-of-function mutations can cause increased vulnerability to disease (Deng et al. 2005; Petit et al. 2005). In support of this hypothesis, TNF Receptor Associated Protein 1 (TRAP1), a mitochondrial chaperone, has recently been identified as a kinase substrate of PINK1. Phosphorylation of TRAP1 by PINK1 has been shown to block cytochrome c release from the mitochondria protecting against oxidative stress-induced cell death (Pridgeon et al. 2007). Interestingly, PINK1 has been shown to be upstream in pathways involving Parkin. PINK1 deficiency in drosophila

produces a strong mitochondrial phenotype that can be rescued by Parkin expression (Greene et al. 2003; Clark et al. 2006). One caveat to these studies is the lack of reproduction of these results in mammalian systems. PINK1 deficient mice are viable have mitochondrial dysfunction but no nigrostriatal degeneration (Gispert et al. 2009). Further studies using mammalian systems and in vivo approaches directed at identifying further kinase substrates and pathways involving PD related genes will be useful in understanding the neuroprotective role of PINK1.

Environmental Factors Contributing to Parkinson's Disease

It has been reported that 90-95% of PD cases are considered sporadic. Studies with monozygotic and dizygotic twins have shown that in idiopathic PD, genetic factors do not play a role, indicating there must be an underlying mechanism in which environmental factors may contribute to the disease (Tanner et al. 1999). It has previously been reported that certain groups of individuals with common environmental influences share a similar risk for developing idiopathic PD. For example, individuals with common exposure to toxic compounds such as MPTP (Langston et al. 1983), pesticides such as paraquat and/or maneb, organochlorides, and heavy metals such as iron, copper, and manganese all have increased risk for developing Parkinson's disease (Rybicki et al. 1993; Meco et al. 1994; Seidler et al. 1996; Liou et al. 1997; Bhatt et al. 1999; Corrigan et al. 2000; Lai et al. 2002). Lifestyle has also been under investigation for risk factor assessment in idiopathic PD. Rural lifestyles, specifically agricultural, plantation, and mining professions are all associated with an increased risk for developing PD (Rybicki et al.

1993; Meco et al. 1994; Tuchsen and Jensen 2000; Lai et al. 2002; Petrovitch et al. 2002; Baldi et al. 2003). In addition to lifestyles and professions with exposure to toxins, medical conditions such as viral encephalitis, gastrointestinal infections, autoimmunity, and traumatic brain injury have all been associated with increased risk for developing disease (Tansey et al. 2007). While none of these environmental factors have been proven to be causative in PD, research is underway to elucidate the mechanisms involved in the pathogenesis.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure

MPTP was first discovered as a selective catecholaminergic neurotoxin after a group of individuals intravenously injected a MPTP-contaminated synthetic opiod mixture causing rapid development of Parkinsonian-like symptoms that could be reversed with levodopa treatment (Langston et al. 1983). MPTP injected systemically readily crosses the blood brain barrier (BBB) and is converted to its active form, MPP+, by monoamine oxidase B in glial cells and then released extracellularly. MPP+ is taken up into the neurons by the dopamine transporter (DAT) and acts on mitochondrial complex 1, where it is a selective inhibitor of the electron transport chain (Przedborski et al. 2003). In complex 1, MPP+ disrupts the flow of electrons through the electron transport chain therefore inhibiting production of ATP and increases the generation of reactive oxygen species (Rossetti et al. 1988; Chan et al. 1991). Due to the selectivity and systemic administration, MPTP has become a well characterized PD model in rodents and non-human primates.

Pesticides

In epidemiological studies, one strong association between environment and disease susceptibility is exposure to toxic chemicals such as pesticides involved in agriculture (Di Monte et al. 2002). Pesticide exposure was originally investigated as a potential risk for disease when the structure of the selective neurotoxin MPP+ proved to be strikingly similar to the herbicide paraquat (Snyder and D'Amato 1985). Rotenone, a widely used pesticide in agricultural professions, was shown to bind at the same site in complex 1 of the electron transport chain as MPP+, therefore identifying it as a potent mitochondrial complex 1 inhibitor (Nicklas et al. 1985). Since then, epidemiological and animal studies have yielded conflicting results as to whether paraquat and rotenone exposure can be linked to the development of PD (Di Monte et al. 2002). In support of pesticide involvement in disease pathogenesis, direct injection of rotenone into the rodent striatum mimics the effects of MPP+ (Heikkila et al. 1985) and high systemic doses of rotenone can cause striatal damage and hypokinetic behavior in rats (Ferrante et al. 1997; Betarbet et al. 2000; Thiffault et al. 2000). Paraquat, another potent complex 1 inhibitor, crosses the BBB by a selective carrier, enters the nigrostriatal system causing a moderate loss (10-20%) of dopaminergic neurons in the SNpc (Shimizu et al. 2001; McCormack et al. 2002). Furthermore, paraquat coupled with the fungicide maneb can cause pathological damage to dopaminergic nerve terminals and tyrosine hydroxylase (TH) immunoreactive cell bodies leading to reduced motor activity in a mouse model of PD (Thiruchelvam et al. 2000). Even though the epidemiological data may not be as convincing, these rodent pesticide models give researchers the potential to investigate new mechanisms of environmental involvement in the pathogenesis and susceptibility for PD.

Heavy Metals

It has been reported that prolonged or repeated exposure to heavy metals such as iron, manganese, and copper are associated with increased risk of PD (Rybicki et al. 1993; Lai et al. 2002). Several studies have shown that iron deposition in the brain, particularly in large quantities in areas such as the basal ganglia and SNpc, increases with age (Sofic et al. 1988; Gerlach et al. 1994; Zecca et al. 1994) indicating a long latency for toxicity (Gorell et al. 1997). In addition, increases in dietary iron have also been associated with increased susceptibility for developing disease (Powers et al. 2003). Intracellular iron is normally bound to ferritin, a protein that binds iron in an unreactive state, and has decreased expression in PD patients (Dexter et al. 1990). It is thought that accumulation of iron in an unbound state may lead to generation of hydroxyl radicals causing oxidative stress and subsequent neurodegeneration particularly in the basal ganglia (Di Monte et al. 2002). Other studies have shown that manganese toxicity, primarily in the globus pallidus, can cause Parkinsonian-like symptoms that cannot be reversed with levodopa treatment (Pal et al. 1999). More recently, multiple metal exposures has been associated with a higher risk for PD, indicating a mechanism of synergistic degeneration of nigrostriatal neurons (Di Monte et al. 2002).

Viral encephalitis

Viral encephalitis associated with the H5N1 avian influenza pandemic of 1918 and the Japanese encephalitis virus (JEV) have been associated with a phenomenon known as post-encephalitic parkinsonism, a form of parkinsonism that presents with pathological and motor symptoms similar to idiopathic PD (Shoji et al. 1993; Dale et al. 2004; Tansey

et al. 2008). Furthermore, rodent models infected with JEV show hypokinetic behaviors and catecholamine depletion (Hamaue et al. 2006) and rodents infected with H5N1 show significant nigral neuron loss, active microgliosis indicative of inflammation, and alpha-synuclein aggregation (Jang et al. 2009). It is thought that chronic inflammation and immune dysregulation in the CNS induced by encephalitis may selectively target vulnerable dopaminergic neuron populations causing significant degeneration and PD like symptoms.

Gastrointestinal infection and autoimmune disease

In addition to motor symptoms of PD, patients have reported a variety of non-motor symptoms including reduced colon motility or constipation prior to motor disease onset (Abbott et al. 2001). In line with sporadic PD, alpha-synuclein positive Lewy body inclusions are also found in peripheral neurons involved in gastrointestinal function(Braak et al. 2003). It is a current hypothesis among researchers that the triggering event initiating sporadic PD cases may come from the periphery, suggesting that gastrointestinal infections and autoimmune diseases may play a role in increasing one's susceptibility for PD (Weller et al. 2005). In support of this theory, studies have shown individuals with sporadic PD are more likely than control individuals to be seropositive for *Heliobacter pylori*, the bacterial species implicated in peptic ulcers (Dobbs et al. 2005). It has also been shown that individuals with polymorphisms in the nucleotide-binding oligomerization domain 2 (NOD2) gene, a gene known to be with associated with the autoimmune disorder Crohn's disease, are highly represented in populations with sporadic PD (Hugot et al. 2001; Ogura et al. 2001; Bialecka et al. 2007).

How chronic gastrointestinal infection or autoimmune disorders may be the trigger initiating autonomic dysfunction is not well understood, but it is suspected that inflammation may play a contributing role.

Traumatic brain injury

Recent studies have indicated that mild to moderate head injury, particularly injuries resulting in loss of consciousness or amnesia, can increase susceptibility for idiopathic PD (Goldman et al. 2006). The mechanism by which TBI is associated with increased risk is currently unknown, but rodent models have shown that TBI induces nitration and redistribution of alpha-synuclein in aged mice (Uryu et al. 2003). Although the mechanism is not known, nitrative stress has been shown to initiate alpha-synuclein aggregation and stabilize aggregated forms linking TBI to alpha-synuclein initiated neurotoxicity (Souza et al. 2000; Paxinou et al. 2001; Norris et al. 2003).

Genetic and environmental interactions

While genetics and environmental exposure have been individually studied and assessed as risk factors for the development of PD, there is currently little or no evidence for one definitive factor in the initiation of idiopathic PD. Research strongly implicates genetics; however, genetic mutations represent a small percentage of the total number of PD cases and the age of disease onset is variable indicating there may be other environmental influences needed to initiate PD progression (Ross and Smith 2007). In support of this theory, research is now focusing on genetic susceptibility and environmental exposure in the development of PD research models. For example, mutations in the parkin gene

alone do not cause nigrostriatal neuron loss (Goldberg et al. 2003), but parkin deficiency coupled with chronic systemic inflammation does initiate persistent neuroinflammation leading to a selective loss of dopaminergic neurons in the SNpc (Frank-Cannon et al. 2008). Furthermore, systemic paraquat administration in mice upregulates alpha-synuclein protein and initiates its aggregation (Manning-Bog et al. 2002). These studies implicate the importance of synergy between genetics and environmental influences such as inflammation and oxidative stress in the pathogenesis of PD.

Inflammation in Parkinson's Disease

In addition to the mechanisms such as UPS and mitochondrial dysfunction and oxidative stress, inflammation has recently been investigated for its function in PD pathogenesis. Currently, inflammation in the form of microgliosis, leukocyte infiltration, BBB breakdown and expression of pro-inflammatory cytokines is tightly linked in some way to all mechanisms of disease pathogenesis indicating a central role in dopaminergic neuron degeneration (Whitton 2007). This is further confirmed by clinical studies showing that regular users of non-steroidal anti-inflammatory drugs (NSAIDs) reduced the risk of developing sporadic Parkinson's disease by up to 46% (Chen et al. 2003; Chen et al. 2005), implicating inflammation as a potential risk factor for developing idiopathic PD.

Inflammation in Parkinson's disease patients

The involvement of inflammation in PD pathology was first identified in patients displaying significant up regulation of major histocompatibility complex (MHC) molecules, activated microglia (McGeer et al. 1988), elevated levels of antibodies to dopamine oxidation products (Rowe et al. 1998), and CD4+ lymphocyte infiltration (Brochard et al. 2009). Researchers have also reported elevated levels of the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukins 1 beta (IL-1b) and 6 (IL-6) in the striatum (Mogi et al. 1994; Blum-Degen et al. 1995) and activated glial cells in the SNpc producing high levels of TNF, IL-1b, interferon gamma (IFNγ), and nitric oxide (NO) (Hunot et al. 1996; Hirsch et al. 1998). It has also been reported that PD patients have elevated levels of pro-inflammatory cytokines TNF, IL-1, and IL-6 in the brain and cerebrospinal fluid (Mogi et al. 1994; Mogi et al. 1996; Nagatsu et al. 2000). In addition to inflammation's role in idiopathic PD, researchers found activated microglia present in postmortem brain tissue 16 years following exposure to MPTP, alluding to inflammation's participation in toxin-induced death of dopaminergic neurons (Langston et al. 1999). Prolonged expression of inflammatory factors following a toxic insult in combination with those released from degenerating or dying dopaminergic neurons may be enough to amplify and sustain the ongoing inflammatory process risking destruction of additional dopaminergic neurons in the SNpc.

Microglia

Within the CNS, microglia represent approximately 15% of the total cell population and are the monocyte-derived, immunocompetent phagocytic cells responsible for activating the innate immune system. In the event of an immune challenge, microglia act as

scavenger cells mediating the earliest response to infection, inflammation, trauma, ischemia, and neurodegeneration by secreting a battery of cytokines and chemokines (Carson et al. 2006; Whitton 2007; Tansey et al. 2008). CNS resident microglia are indistinguishable from monocyte-derived macrophages and express common markers such as Iba-1, the membrane glycoprotein F4/80, and membrane attack complex 1 (MAC1: CD11b/CD18). However, CNS resident microglia express lower levels of the protein tyrosine phosphatase CD45 than peripheral macrophages (Carson et al. 2006). In a resting state microglia exhibit a ramified morphology distinguishable by visible arms or processes and lower expression of the aforementioned markers (Iba-1, MAC1, CD45) (Lawson et al. 1990; Kreutzberg 1996). Following a proinflammatory stimulus such as lipopolysaccharide, a gram-negative bacterial endotoxin known to activate microglial cells (Lieberman et al. 1989; Kim et al. 2000), the inner cytoskeleton changes allowing the cell body to become enlarged, displaying an amoeboid or macrophage-like appearance and proliferation (Lawson et al. 1990; Raivich et al. 1999). In addition, activating stimuli promote microglial cells to up-regulated expression of adhesion molecules such as intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 allowing the cells to adhere to neurons by the direction of neuron secreted monocyte chemoattractant protein-1 (MCP-1) and IFNγ (Aloisi et al. 2000; Aloisi 2001; Orr et al. 2002). It has been reported that when the activating stimulus disappears microglia can return to the resting state, but in the context of neurodegenerative diseases such as PD, this is not the case as evidenced by the presence of activated microglial cells clustered around dopaminergic neurons years following the initial toxic insult (Bronstein et al. 1995; Langston et al. 1999).

Lipopolysaccharide and the expression of pro-inflammatory cytokines

Pro-inflammatory cytokines have an important function in defending the host from foreign pathogens and are essential for innate and acquired immunity (Feldmann and Saklatvala 2000). The innate immune system is important for a pre-programmed reaction to microorganisms and recognition of bacteria and parasites by phagocytic cells. One of the key phagocytic cells in the CNS is the microgial cell. Microglia and macrophages display receptors that recognize, bind and signal the presence of microbial pathogens (Feldmann and Saklatvala 2000). Toll-like receptor 4 (TLR-4) in the central nervous system, is the important receptor responsible for recognizing the bacterial endotoxin LPS, an important cell wall component in gram negative bacteria and potent activator of microglial cells (Lieberman et al. 1989; Kim et al. 2000). TLR-4 recognizes and binds LPS with LPS binding protein, therefore activating the microglial cell increasing its phagocytic capabilities. In addition to increased phagocytic properties, the activated microglia increases the production and secretion of cytokines and chemokines (Feldmann and Saklatvala 2000).

Activated microglial cells secrete an important inflammatory mediator: IL-1. IL-1 proteins are the products of two genes: IL-1α and IL-1β (Feldmann and Saklatvala 2000). IL-1β has been implicated in Parkinson's disease patients by elevated levels in postmortem brains and cerebrospinal fluid (Blum-Degen et al. 1995; Mogi et al. 1996) and in animal models of PD. One study utilizing an intranigral LPS injection found a specific IL-1 receptor antagonist reduced nigral protein levels of TNF and IFNγ and

attenuated dopaminergic neuron loss (Koprich et al. 2008). IL-1β signals through the IL-1 receptor, which is widely expressed on all immune cells and throughout the brain (Feldmann and Saklatvala 2000). The main pro-inflammatory function of IL-1 is to signal the up regulation of adhesion molecules on endothelial cells, stimulate the release of chemokines to attract monocytes, and stimulate the production of the small inflammatory mediator cyclooxygenase-2 (COX-2). COX-2 is an important enzyme involved in the synthesis of prostaglandin $E_{2,}$ which is important for vasodilatation and the perception of pain (Feldmann and Saklatvala 2000). COX-2 has been shown to be up regulated in activated microglial cells present in the SNpc of postmortem brain tissues of PD patients (Knott et al. 2000) and selective COX-2 inhibition attenuated dopaminergic neuron loss in 6-Hydroxydopamine rat model of PD (Sanchez-Pernaute et al. 2004) implicating its role in idiopathic and toxin-induced PD. IL-1 is also known to stimulate the release of IL-6, a cytokine known for its pro and anti-inflammatory effects. The most important anti-inflammatory feature of IL-6 is its ability to down-regulate the production of TNF, a major player in the inflammatory cascade with strong implications in PD (Feldmann and Saklatvala 2000).

Tumor Necrosis Factor Signaling and Parkinson's Disease

TNF structure and function

TNF is an important pro-inflammatory cytokine produced by activated microglia and macrophages. TNF is involved in protection from bacterial infections, cell growth, viral replication, immune system regulation, and is a major player in many autoimmune and

neurodegenerative diseases (Aggarwal 2000). TNF has two distinct forms: soluble and transmembrane. Transmembrane TNF is a type II transmembrane protein 233 amino acids in length and a molecular weight of 26 kiloDaltons (kDa) (Aggarwal 2000). Transmembrane TNF is converted to the soluble form by proteolytic cleavage by the TNFα –converting enzyme (TACE). TACE is a membrane bound matrix metalloproteinase that cleaves the C-terminal end of transmembrane TNF and releases a mature soluble TNF of 17 kDa. Soluble TNF is a homotrimer with a molecular mass of 50 kDa and is produced by many cell types in the mammalian body (Aggarwal 2000). TNF is produced by activated macrophages, microglia, lymphocytes, natural killer cells, dendritic cells, endothelial cells, leukocytes, astrocytes and mast cells and has many important signaling roles modulating growth, differentiation, inflammation, and tumorigenesis (Aggarwal 2000).

TNF Signaling

TNF signaling occurs through two different cell surface receptors with divergent functions. TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) are type II transmembrane proteins with respective molecular masses of 55-60 kDa and 75-80 kDa respectively (Aggarwal 2000). TNF receptors are generally abundant on cell surfaces and have a density between 1000-5000 sites per cell depending on the cell type. Both receptors contain 3 distinct domains: the extracellular, transmembrane, and intracellular domains and have affinity for soluble TNF. Both receptors contain four cystine rich domains with 6 cystine residues per repeat (Aggarwal 2000). The main difference between the two receptors is in the amino acid sequences of the intracellular domain.

These unique intracellular sequences allow each receptor to activate different signaling pathways. TNFR1 is expressed by most cell types and modulates the majority of TNF pro-inflammatory activities including activation of apoptosis and nuclear transcription factor-κB. TNFR1 is also responsible for TNF-mediated inflammation, viral infection, and protection against bacterial and fungal growth (Aggarwal 2000). TNFR2 function still remains unclear; however, TNFR2 is believed to primarily mediate cytotoxicity and T cell proliferation. TNFR2 is expressed by a smaller population of cells including immune cells, hematopoietic, endothelial, and prostate cells (Aggarwal 2000). TNF receptors have been implicated in many signaling cascades based upon their distinct intracellular domains. TNFR1 contains an 80 amino acid death domain involved in TNF-mediated cell death (Aggarwal 2000). The intracellular death domain binds and recruits TNF receptor-associated death domain protein (TRADD), a protein that interacts with TNF receptor-associated factor 2 (TRAF2). TRAF2 recruits NF-κB inducing kinase (NIK), a serine/threonine kinase that binds IKKα and IKKβ. IKKα and IKKβ promote the phosphorylation and degradation of IκBα in a ubiquitin/proteosome dependent manner (Aggarwal 2000). Degradation of IκBα releases the transcription factor NF-κB allowing it to translocate to the nucleus and promote transcription of additional pro-inflammatory cytokines. In addition to TRAF2, the TRADD protein can also bind FADD which interacts with FLICE, a known activator of caspases leading to apoptotic cell death. Apoptosis signal-regulating kinase 1 (ASK1) can also interact with TRAF2 and activate JNK and p38 MAPK signaling cascades (Aggarwal 2000). In contrast to TNFR1, TNFR2 does not have an intracellular death domain. However, studies have shown that it can also activate apoptosis, NF-κB, and JNK. The TNFR2 intracellular

domain can directly bind TRAF2 which can activate the signaling cascade similar to TNFR1 but the mechanism is not fully understood (Aggarwal 2000).

TNF actions in the CNS

As mentioned previously, TNF is primarily produced by activated macrophages and microglia in response to an inflammatory stimulus (Aggarwal 2000). TNF is produced by many other cell types, but TNF function beyond inflammation is not well understood. TNF knockout mice are viable, suggesting that TNF does not have a major role in normal mouse development, however, histopathological analysis has revealed that their splenic architecture is severely disrupted (Pasparakis et al. 1996). TNF knockout mice fail to develop monocyte-derived follicular dendritic cell clusters and germinal centers compromising their ability to fight bacterial infection (Aggarwal 2000) and one study has shown that TNF knockout mice display a reduced monocyte-derived microglial population size (Lambertsen et al. 2009). Studies have shown that TNF knockout mice are more resistant to TNF and LPS challenge (Scherbel et al. 1999) indicating they may be more resistant to inflammation induced neuronal degeneration. In addition, studies have implicated the detrimental role of TNF by showing memory loss and motor deficits were less severe in TNF knockout mice following a traumatic brain injury (Scherbel et al. 1999). From these studies it is evident that TNF plays a deleterious role in the acute response to brain injury, however, it is also necessary for recovery from these injuries since wild type mice were able to overcome their motor deficits four weeks post injury (Scherbel et al. 1999).

TNF in Parkinson's disease

TNF is believed to participate in tissue injury and inflammation. TNF message and protein levels in postmortem brain and cerebrospinal fluid appear to be elevated in many central nervous system disorders including Parkinson's disease (Boka et al. 1994; Mogi et al. 1994). At this time, evidence supporting the role of TNF in the etiology of Parkinson's disease is limited; however, recent studies have shown that TNF may be playing a role in the acceleration of disease onset. One study found single nucleotide genetic polymorphisms (SNPs) in the TNF promoter that increase transcriptional activity and TNF production are associated with earlier onset of disease in patients with idiopathic PD (Nishimura et al. 2001). In support of the role of TNF in PD, studies have shown that blocking soluble TNF signaling using a dominant negative inhibitor specific for soluble TNF rescued up to fifty percent of dopaminergic neurons following a single striatal injection of the neurotoxin 6-hydroxydopamine in rats. In addition, these studies showed that co-infusion of the dominant negative inhibitor with a low dose of bacterial LPS into the substantia nigra also attenuated dopaminergic neuron loss (McCoy et al. 2006; McCoy et al. 2008) indicating a role for TNF participation in the progression dopaminergic neuron loss in animal models of PD.

Rodent Models of Parkinson's Disease

Given that PD is a progressive disorder involving genetic and environmental factors in the initiation and progression of disease, rodent models are generally utilized to study the mechanisms underlying the pathophysiology. PD in humans is a complex disorder with

variable pathology that is difficult to replicate in rodents, sparking the debate on which rodent models best represent the progressive nature of the disease. While it is controversial as to which rodent models best reproduce the pathological hallmarks of the disease, a common and indispensable feature of each model is the selective loss of dopaminergic neurons in the SNpc (Meredith et al. 2008). Given that most murine genetic knockouts such as Parkin, PINK1 and DJ-1 display no nigrostriatal loss, the most widely used rodent models are those that employ selective neurotoxins such as 6-OHDA, MPTP, and pesticides or inflammogens such as LPS to induce nigral loss to mimic the pathophysiology of sporadic PD

6-Hydroxydopamine

6-OHDA, a structural analog of dopamine and norepinephrine, is taken up into the dopaminergic neuron via the dopamine transporter present on the plasma membrane (Breese and Traylor 1971). Once inside the cell, 6-OHDA is metabolized into the reactive species hydrogen peroxide and paraquinone, both of which are highly toxic to the cell, initiating degeneration (Saner and Thoenen 1971). 6-OHDA does not readily cross the BBB, so it is generally administered unilaterally into the SNpc, medial forebrain bundle (MFB) or striatum via a stereotaxic procedure. Delivery of 6-OHDA into the ventral midbrain or striatum results in direct toxic damage to the dopaminergic neuron terminals and axons surrounding the injection site, initiating a critical loss of dopaminergic neurons in the ipsilateral SNpc (Meredith et al. 2008). Following the direct toxic insult, retrograde cell loss occurs in two distinct phases: an acute or rapid phase of cell death generally lasting a few weeks, and a slow or progressive phase of

dopaminergic cell loss lasting several weeks to months after the lesion (Kirik et al. 1998). In this model, the degree of dopaminergic neuron loss and locomotor deficits are completely dependent on the dose and subregion of the striatum lesioned (Kirik et al. 1998). Lesions in the dorsomedial striatum have pronounced effects on locomotion and drug induced rotational behaviors whereas lesions in the ventrolateral striatum have noticeable effects on movement initiation, sensorimotor and skilled motor behaviors (Kirik et al. 1998). 6-OHDA lesions induce nigrostriatal loss making them a useful model to study dopaminergic neuron degeneration; however, these lesions are generally not progressive and lack extra-nigral and Lewy body pathology, the pathological hallmark in PD diagnosis in human patients (Meredith et al. 2008).

MPTP and MPP+

MPP+, the toxic metabolite of MPTP, is a potent inhibitor of mitochondrial complex 1 taken up into dopaminergic neurons via the dopamine transporter (Przedborski and Vila 2003). MPTP is delivered systemically in large doses via an intraperitoneal or subcutaneous injection paradigm, causing acute nigrostriatal loss in mice (Meredith et al. 2008). MPTP administered acutely (4 injections given in 1 day) or chronically (once daily for 5-8 days) causes a rapid loss of dopaminergic neurons in the SNpc and a downregulation of TH (Xu et al. 2005; Meredith et al. 2008). MPP+ does not cross the BBB due to its charge, but can be administered unilaterally by direct infusion into the lateral cerebral ventricle via an osmotic mini pump for 28 days initiating a progressive loss of dopaminergic neurons in the SNpc (Yazdani et al. 2006). MPTP and MPP+ rodent models are valuable for studying molecular mechanisms for mitochondrial

dysfunction in dopaminergic neuron loss; however, motor deficits are difficult to detect and do not correlate with neuronal loss and there is no Lewy body pathology.

Pesticides (Rotenone, Paraquat and Maneb)

Rotenone, a naturally occurring pesticide and potent mitochondrial complex 1 inhibitor (Nicklas et al. 1985), has recently been used to generate a chronic rodent model of PD (Betarbet et al. 2000; Di Monte et al. 2002; Meredith et al. 2008). Rotenone when administered intravenously, subcutaneously, and intraperitoneally induces a loss of dopaminergic nerve terminals followed by a progressive loss of cell bodies in the SNpc. In addition to progressive loss of dopaminergic neurons in the SNpc and locomotor deficits, microgliosis, and iron accumulation, these degenerating neurons have also shown intracytoplasmic inclusions of alpha-synuclein and ubiquitin reminiscent of Lewy body pathology in postmortem brain tissues (Betarbet et al. 2000; Fleming et al. 2004). In addition, rotenone administered intraperitoneally to rats delivers less variability in terms of dopaminergic neuron loss and locomotor deficits and recapitulates some of the non-motor symptoms of PD by inducing a loss of enteric neurons (Alam and Schmidt 2002; Cannon et al. 2009). With a newer intraperitoneal dosing paradigm to reduce variability, the rotenone model is an attractive model for therapeutic testing in both the early and later stages of PD.

Paraquat, a widely used herbicide that can cross the BBB, disrupts mitochondrial activity by producing reactive oxygen species through interactions with mitochondrial complex 1. When administered in rodents, paraquat has been shown to initiate a small

loss of dopaminergic neurons in the SNpc and initiate the up-regulation and aggregation of alpha-synuclein (Manning-Bog et al. 2002; Meredith et al. 2008). While the nigrostriatal loss and locomotor deficits are highly variable or non existent in paraquat models, paraquat co-administered with maneb, a fungicide that disrupts glutamate transport and dopamine uptake and release, has been shown to produce progressive dopaminergic neuron loss (Thiruchelvam et al. 2000). Co-administration of paraquat and maneb has been shown to recapitulate the different stages of clinical PD including a progressive loss of dopaminergic neurons, microgliosis, and locomotor deficits, however, this model does not display intranigral Lewy body pathology and can induce lung toxicity in older rodents (Saint-Pierre et al. 2006).

Lipopolysaccharide

LPS, a gram-negative bacterial endotoxin, initiates an inflammatory response in the brain characterized by activated microglia and elevated levels of pro-inflammatory cytokines that is subsequently followed by dopaminergic neuron degeneration in the SNpc. LPS (5-10 ug) administered directly into the cortex, hippocampus, striatum, or SNpc initiates a specific, non-progressive loss of nigrostriatal neurons that is accompanied by locomotor deficits and microglial activation. When administered chronically (5 ng/hour, 2 weeks) into the midbrain area above the SNpc by a cannula connected to an osmotic mini pump, a progressive loss of dopaminergic neurons is observed from 6 to 10 weeks post LPS exposure (Gao et al. 2002). It has also been reported that a high dose single systemic injection in mice can also initiate a progressive loss of dopaminergic neurons seen in a delayed fashion (7-10 months post injection) (Qin et al. 2007). Furthermore, prenatal

exposure to LPS from a single injection into pregnant female rats disrupts dopaminergic neuron development resulting in a significant loss of nigrostriatal neurons in offspring implicating prenatal infections as a potential risk factor for PD (Ling et al. 2002). Taken together, these LPS models display significant dopaminergic neuron loss and microgliosis, however, it is not currently known if locomotor deficits and intraneuronal alpha-synuclein inclusions accompany the nigrostriatal loss observed in these models.

Genetic Models

Rodent genetic models of PD currently studied fall into three main categories. First, are the mouse models involving the genetic knockdown or knockout of genes important for dopaminergic neuron development and maintenance such as the homeobox transcription factors Pitx3 (Nunes et al. 2003) or Engrailed 1 (Sonnier et al. 2007). Second are the rodent models involving genetic manipulations (knockout or transgenic) of genes implicated in familial early onset PD such as Parkin (Goldberg et al. 2003), PINK1 (Gispert et al. 2009), DJ-1 (Chen et al. 2005) and LRRK2 (Li et al. 2009). Last, genetic models have been created that utilize virally delivered genes implicated in PD pathogenesis such as alpha-synuclein to induce dopaminergic system dysfunction (Reviewed in Meredith et al. 2007). While all of these models involve some form of genetic manipulation relevant to PD pathogenesis, many models lack the progressive loss of dopaminergic neurons in the SNpc, detectable locomotor deficits, and/or Lewy body pathology.

Current Therapeutic Strategies for Parkinson's Disease

Parkinson's disease is a chronic and progressive disorder with a wide variety of motor and non-motor symptoms associated with the subsequent loss of nigrostriatal neurons. While there is no cure, current therapies such as pharmacological and surgical intervention are designed and focused to alleviate the symptoms, not to slow the progression of the disease (Olanow et al. 2009).

Dopamine replacement therapy

Currently the most widely used and effective pharmacological treatment to alleviate debilitating motor symptoms associated with PD is levodopa (L-dopa). L-dopa, a chemical precursor to the naturally occurring dopamine in the brain, readily crosses the BBB and is taken up by dopaminergic neurons where it is converted to dopamine by the enzyme L-aromatic amino acid decarboxylase (dopa-decarboxylase, AADC). Since L-dopa is administered orally and is taken up into the bloodstream via the gut, L-dopa is often administered with the AADC inhibitor, carbidopa to prevent metabolism before entering the brain. Although highly effective to alleviate motor symptoms associated with the loss of striatal dopamine, resistance to L-dopa increases over time requiring the administered dose to be increased for continued effectiveness. Increases in L-dopa administered orally can lead to decreased responsiveness, additional side effects, and the development of dyskinesias (Olanow et al. 2009). While effective at temporarily relieving motor symptoms, L-dopa treatment does not slow or modify disease progression.

Dopamine agonists

An alternative or replacement treatment to L-dopa is the use of dopamine agonists that enter the brain and act to directly stimulate striatal D_1 and D_2 dopamine receptors independent of degenerating dopaminergic neurons. When used in conjunction or as a replacement to L-dopa therapy, dopamine agonists can reduce the dsykinesias associated with high levels of L-dopa therapy thereby prolonging the effectiveness of L-dopa treatment (Olanow et al. 2009). Currently, dopamine agonists are used for treatment in the early symptomatic stages of PD to reduce the risk of additional motor symptom complications initiated by L-dopa therapy. In addition, dopamine agonists have been an attractive therapy for study due to their neuroprotective and anti-apoptotic effects in PD models (Olanow et al. 1998; Schapira 2002; Iravani et al. 2006). While effective at relieving L-dopa-induced dyskinesias and potential for neuroprotection, prolonged treatment with dopamine agonists can lead to receptor insensitivity resulting in the increase or reappearance of dyskinesias.

Monoamine oxidase B inhibitors

Monoamine oxidase B (MAO-B) is a key enzyme required for the oxidation and subsequent breakdown of dopamine in the synapse. Inhibitors targeting MAO-B block the oxidation of dopamine thereby increasing the bioavailability of dopamine in the synapse. MAO-B inhibitors are generally administered in conjunction with L-dopa therapy therefore increasing the effectiveness of the therapy and prolonging onset of dyskinesias. Studies have highlighted a potential neuroprotective role for MAO-B inhibitors by blocking the metabolism of MPTP to MPP+ in animal models of PD

(Heikkila et al. 1984). Currently research studies are targeted to elucidate the potential neuroprotective and disease modifying role of MAO-B inhibitors (Olanow et al. 2009).

Deep brain stimulation

Deep brain stimulation (DBS) to the globus pallidus, thalamus and subthalamic nucleus via electrodes surgically implanted deep into the brain is an FDA-approved, non-pharmacological alternative therapy utilized to treat tremor, rigidity and akinesia in PD patients. DBS to the thalamus can potentially alleviate symptoms of tremor while DBS to the subthalamic nucleus and globus pallidus are reported to alleviate severe symptoms of tremor, rigidity, and L-dopa induced dyskinesias. Although it is not as widely used and has varying results in patients, DBS is the most successful surgical option for the treatment of severe motor symptoms associated with PD progression (Olanow et al. 2009).

Exercise therapy

Recently moderate rehabilitative exercise, dance and social therapy have emerged as viable, low cost treatments for patients with PD. In conjunction with pharmacological therapy, studies have found that exercise regimens or rehabilitation programs improved activities of daily living and self assessed quality of life in patients with PD (Yousefi et al. 2009) implicating exercise science as a potential target therapy to combat motor symptoms of PD.

Gene Therapy in Parkinson's Disease

Recently interest in gene therapy to slow the progression of PD has undergone intense research and is now the subject of several phase I/II clinical trials. Utilizing viral technology to integrate DNA into the host genome of specific brain regions allows for continuous production of desired proteins and an alternative treatment for the symptoms and cell loss observed in PD patients. Currently, gene therapy has focused on the delivery of additional neurotrophic factors to help slow disease progression, and promote terminal sprouting and enzymes important for neurotransmitter synthesis and stability.

Viral technology

Currently viral technology is utilized for specific delivery and expression of desired proteins into the diseased host brain. Viral technology is generally safe to use in human and animal models and can be manipulated to infect and transduce in specific cell types based on the viral subtype and promoter used to drive expression. In clinical trials, adeno-associated virus (AAV) is often selected as the viral delivery method due to its high titer (infections particles/milliliter), ability to infect and transduce post-mitotic neurons, long term expression of protein, and its low immune response in host brain regions. Lentiviral technology has also gained interest and is commonly utilized in gene therapy studies in PD animal models and recently in a clinical trial. Lentiviruses are a subtype of retroviruses capable of integrating DNA in both mitotic cells such as glia and post-mitotic cells, making them an attractive technology for the expression of desired proteins in the brain (Lim et al. 2010).

Neurotrophic factor gene therapy

Neurturin, a glial cell line-derived neurotrophic factor (GDNF) homolog, has shown neuroprotective effects on dopaminergic neurons *in vitro* and in animal models of PD (Tseng et al. 1998; Hoane et al. 1999; Rosenblad et al. 1999; Oiwa et al. 2002; Hurelbrink and Barker 2004). These neuroprotective effects led the biotech company Ceregene to undergo phase I and II clinical trials with AAV delivered neurturin (CERE-120) into the putamen of patients with advanced stages of PD. While the results in phase I were positive showing significant clinical and radiographical improvement, a double blind, randomized phase II clinical trial failed to show a significant improvement over placebo controls (Kaplitt 2009). Contrary to clinical data collected from the clinical trials, CERE-120 has shown significant protection and behavioral improvement in 6-OHDA lesioned rat and MPTP treated non-human primate models of PD (Kordower et al. 2006; Gasmi et al. 2007).

Glutamic acid decarboxylase gene therapy

Glutamic acid decarboxylase (GAD) is the key enzyme in the synthesis of gabaminobutyric acid (GABA), a key inhibitory neurotransmitter produced by inhibitory neurons in the brain. Neurologix Inc. has recently sponsored a successful phase I clinical trial in which AAV-GAD was delivered to the subthalamic nucleus in a small cohort of advanced PD patients (Kaplitt et al. 2007). Patients receiving GAD replacement therapy showed a significant improvement of motor function and radiological scores 3 months post GAD replacement and lasted up to 12 months post surgery (Kaplitt et al. 2007). GAD delivered to the subthalamic nucleus can increase production of GABA therefore

increasing the amount of inhibitory neuron firing and reducing motor symptoms associated with PD (Olanow et al. 2009). Currently phase II clinical trials are underway.

AADC gene therapy

AAV administered AADC into the putamen of PD patients is currently being studied in a phase II clinical trial sponsored by Neurologix Inc. Phase I clinical trials offered significant (about 30%) improvement of clinical scores in advanced PD patients (Christine et al. 2009). AADC expression in the putamen can increase synthesized dopamine from the precursor L-dopa administered orally, therefore increasing synaptic levels of dopamine and subsequently enhancing the efficacy of L-dopa therapy in patients (Christine et al. 2009; Olanow et al. 2009). AADC expression in MPTP lesioned non-human primate models of PD improved clinical scores, reduced L-dopa requirements and dyskinetic side effects (Bankiewicz et al. 2006).

Cell Replacement Therapy in Parkinson's Disease

Within the spectrum of the CNS, PD was the first neurodegenerative disorder targeted for cell replacement based therapies. The theory behind cell replacement therapy in PD is based on the subsequent replacement of degenerating dopaminergic neurons with enriched populations of fetal midbrain and terminally differentiated human embryonic stem cells. After implantation, these cells could potentially engraft into the patient brain and ameliorate symptoms associated with PD with little or no immune response

(Goldman and Windrem 2006). Highly enriched dopaminergic neuron progenitors derived from embryonic midbrain tissue when transplanted into animal models of PD has shown promising results (Studer et al. 1998; Sawamoto et al. 2001), but transplantation in to adult PD brains has yielded inconclusive results (Kordower et al. 1998; Hauser et al. 1999; Freed et al. 2001; Hagell et al. 2002). In order to overcome ethical issues and lower yield of dopaminergic progenitors, investigators have now focused on terminally differentiated human embryonic stem cells. Human embryonic stem cells when subjected to a standardized differentiation protocol can yield a larger quantity of dopaminergic neurons *in vitro* (Lee et al. 2000; Studer et al. 2000) and *in vivo* (Ye et al. 1998). Transfer of these dopaminergic neuron enriched progenitors derived from human embryonic stem cells in to rodent and non-human primates has yielded positive results restoring striatal levels of dopamine and improving behavioral outcomes (Kim et al. 2002; Takagi et al. 2005). While the results have been promising in animal models, there are several technical challenges associated with the use and differentiation of human embryonic stem cells. For example, pure enrichment of dopaminergic progenitors is difficult to achieve leaving other differentiated cell types available for transfer into the adult brain to interact with other cells in the CNS. In addition, studies have demonstrated issues with long term survival of engrafted cells (Park et al. 2004; Takagi et al. 2005) and cells that are not fully differentiated could lead to tumorigenesis after implantation (Bjorklund and Isacson 2002; Bjorklund et al. 2002).

Table 1.1 Genes Linked to PD

Gene, Protein (locus)	Function	Inheritance	Pathological Features
SNCA, α-synuclein (PARK1/4)	Unclear: vesicle trafficking?	Dominant	Always Lewy bodies: some cases have dementia as presenting feature
LRRK2, dardarin (PARK8)	Cytosolic kinase	Dominant	Variable: usually with Lewy bodies, some tau lesions and some neuron-loss only cases
PRKN, parkin (PARK2)	E3 ligase	Recessive; rare "pseudo-dominant" cases reported	Variable: usually benign and without Lewy body pathology
PINK1 (PARK6)	Mitochondrial kinase	Recessive	Unknown
DJ-1 (PARK7)	Oxidative stress signaling molecule	Recessive	Unknown

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CHAPTER TWO

DELAYED DOMINANT NEGATIVE TNF GENE THERAPY HALTS PROGRESSIVE LOSS OF NIGRAL DOPAMINERGIC NEURONS AND BEHAVIORAL DEFICITS IN A RAT MODEL OF PARKINSON'S DISEASE

ABSTRACT

Parkinson's disease is a progressive neurodegenerative disorder characterized by a loss of dopaminergic (DA) neurons in the substantia nigra pars compacta. A number of studies have implicated chronic inflammation in the pathophysiology of PD; however, it is unclear which inflammatory mechanisms directly contribute to neuronal loss in PD. A number of cytokines, including Tumor Necrosis Factor, are elevated in post-mortem brain and cerebrospinal fluid of patients with PD. Previous studies from our group have shown that blocking soluble TNF (solTNF) signaling at the time of a unilateral 6-hydroxydopamine (6-OHDA) striatal lesion attenuated behavioral deficits and the acute loss of dopaminergic neuron loss by 50%. However, a critical question of clinical relevance to potential therapeutic intervention is whether delayed solTNF signaling inhibition can prevent the progressive loss of DA neurons that occurs after a CNS insult. We report here that a single intranigral injection of a lentivirus encoding a dominant negative TNF inhibitor delivered 2 weeks after an intrastriatal 6-OHDA lesion attenuated microgliosis in SNpc and halted the progressive loss of nigral DA neurons and the associated locomotor deficits. Our findings suggest that TNF-dependent neuroinflammation directly contributes to the delayed and progressive degeneration of

nigral DA neurons after neurotoxic injury and further validate solTNF as a potential therapeutic target in PD.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by a loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). A number of studies have highlighted chronic inflammation as a key player in the pathophysiology of PD (McGeer and McGeer, 2008; Frank-Cannon et al., 2009). Therefore, identification and selective targeting of inflammatory mediators that contribute to the progressive loss of nigral DA neurons may possibly delay disease progression if administered in a timely manner to PD patients in the early stages of the disease.

Tumor Necrosis Factor (TNF) is a cytokine produced by activated immune cells in all areas of the body including the central nervous system (Aggarwal, 2000). In patients with PD, TNF mRNA and protein levels are elevated in post-mortem brain and cerebrospinal fluid (Boka et al., 1994; Mogi et al., 1994). In addition, single nucleotide genetic polymorphisms (SNPs) in the TNF promoter that increase transcriptional activity and TNF production are associated with earlier onset of disease in patients with idiopathic PD (Nishimura et al., 2001). Lastly, the majority of studies involving use of the mitochondrial complex I inhibitor 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) to induce nigral degeneration in rodents suggest mice functionally deficient in

TNF signaling display reduced sensitivity to the neurotoxin (Sriram et al., 2002; Ferger et al., 2004; Sriram et al., 2006). Together, these studies strongly implicate TNF as a critical mediator in degeneration of nigral DA neurons.

Using TNF inhibitors selective for the soluble form of the ligand, previous studies from our group have directly demonstrated that solTNF is the ligand species mediating the cytotoxic effects on DA neurons. Specifically, chronic infusion of dominant negative TNF inhibitor protein (DN-TNF) (McCoy et al., 2006) or a single intranigral injection of a lentivirus encoding DN-TNF (lenti-DN-TNF) administered at the time of the neurotoxic insult (McCoy et al., 2008) attenuated locomotor deficits and nigral degeneration in rats induced by a unilateral intrastriatal 6-hydroxydopamine (6-OHDA) striatal lesion by nearly 50%. However, because the neuroprotective effects obtained in these studies were achieved by co-administration of the inhibitors at the time of the 6-OHDA lesion, it was difficult to ascertain whether solTNF-dependent toxicity occurred both in the acute stage of cell death induced by the neurotoxin or during the progressive stage of degeneration, a period known to be characterized by persistent neuroinflammation (Sauer and Oertel, 1994). Therefore, the objective of this study was to determine the extent to which delayed inhibition of solTNF by a single intranigral injection of lenti-DN-TNF afforded neuroprotection and attenuated locomotor deficits in the late and progressive phase of neurotoxin-induced cell death.

Our results demonstrate that selective inhibition of solTNF beginning in the late or progressive phases of nigral DA neuron degeneration is sufficient to halt further loss of

DA neurons and the associated locomotor deficits. These unexpected findings have clear implications for therapeutic intervention and suggest that even delayed delivery of anti-TNF gene therapy may halt the progressive loss of nigral DA neurons and prevent the appearance of disabling motor symptoms in patients with PD.

MATERIALS AND METHODS

Cloning and Preparation of Lentiviral Stocks

The human full length dominant negative-TNF (TNF variant A145R/I97T) DNA sequence or Green Fluorescent Protein (GFP) sequence were subcloned into a constitutive self-inactivating lentiviral vector and validated *in vitro* and *in vivo* according to previously published reports (McCoy et al. 2008; McAlpine et al. 2009). The GFP-expressing lentivirus has been previously described and validated (Pfeifer et al. 2002; Taylor et al. 2006). Both DN-TNF and GFP lentiviral stocks were produced and purified according to previously published protocols (Taylor et al. 2006). The final titer was 102 ug/mL p24 and 1 x 10exp8 IU/mL for the lenti-GFP control and 84 ug/mL and 2 x 10exp8 IU/mL for lenti-DN-TNF. All viruses were diluted in Hanks Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA).

Animal Studies

Young adult Sprague Dawley SASCO rats (200-250g) were purchased from Charles River Laboratories (Wilmington, MA). All rats were housed in a pathogen-free, climate controlled facility at The University of Texas Southwestern Medical Center. All studies

and animal protocols were approved and guided by the Institutional Animal Care and Use Committee at UT Southwestern.

Surgical Procedures

Immediately before surgical procedures 6-OHDA (Sigma) was dissolved at 5mg/mL with sterile saline. Lentivirus stocks were diluted 1:2 in sterile HBSS) and kept on ice protected from light throughout the entire procedure. Striatal 6-OHDA lesions were performed as described previously (Sauer and Oertel 1994; McCoy et al. 2006; McCoy et al. 2008). Young adult female Sprague-Dawley rats (200-250g) were anesthetized by a single intraperitoneal injection of a ketamine/xylazine cocktail (100mg/kg ketamine, 20mg/kg xylazine, 10mg/kg acepromazine) and placed into a stereotaxic frame. Burr holes were drilled to allow a single unilateral injection of 20 µg 6-OHDA (Sigma) or sterile saline at the following stereotaxic coordinates (AP): 1.0mm from bregma, (ML): 3.0mm, (DV): -4.5mm below surface of the dura. 4 µL of 6-OHDA or saline were infused into the right striatum at the rate of 0.5 µL/min. For the co-inhibition groups, a second burr hole was drilled to allow a single unilateral injection of 2 µL of lenti-DN-TNF or lenti-GFP using a 28-gauge needle at a rate of 0.5uL/min into the right substantia nigra pars compacta at the following coordinates (AP): -5.3mm from bregma, (ML): -2.3mm, (DV): -7.3mm below surface of dura. For the delayed inhibition groups, this procedure was performed 2 weeks after the 6-OHDA lesion. Following all intracranial injections, a 5 minute waiting period and slow needle retraction were employed to allow proper diffusion of 6-OHDA and/or virus. Post-operatively and for the following 3 days

animals received subcutaneous injections of the buprenomorphine HCL (0.05 mg/kg) and were monitored closely for signs of pain or discomfort.

Amphetamine-Induced Rotational Behavior

Two weeks after 6-OHDA lesion, animals were tested for amphetamine-induced rotational behavior according to a previously published protocol (McCoy et al. 2006; McCoy et al. 2008) as an indirect measure of striatal dopamine depletion. Animals received a single intraperitoneal injection of D-amphetamine (2.5 mg/kg) (Sigma) and rotational asymmetry was scored for 20 minutes during the hour following the i.p. injection. Full ipsilateral rotations were scored and plotted as rotations/minute.

Vibrissae-Evoked Forelimb Placement

Prior to surgery and at weeks 1, 3 and 5 post-lesion, animals were tested for vibrissae-evoked forelimb placement to assess deficits in sensorimotor integration according to previously published protocols (Woodlee et al. 2005). Forelimb placement was scored as the number of successful paw reaches out of 10 independent trials for all combinations of vibrissae stimulation and forelimb placement. The percent of contralateral forelimb reaching is plotted at the times tested.

Forelimb Asymmetry Test

Prior to surgery and at weeks 1, 3 and 5 post-lesion, animals were tested for forelimb asymmetry according to previously published protocols (Woodlee et al. 2005) with the following modifications. Animals were allowed to explore in an unfamiliar glass cylinder

(20 cm in diameter) and videotaped for 3-5 minutes undisturbed. The first 50 paw contacts with the side of the cylinder were recorded and used for analysis. The following equation was used to calculate the percent contralateral forelimb usage: (# left paw touches + # both paw touches)/(# right paw touches + # both paw touches + # left paw touches) x 100.

Tissue Processing

At 5 weeks post lesion, animals were deeply anesthetized with Euthasol (Butler, Dublin, OH) (100 mg in 500 µL sterile saline) and transcardially perfused with 250 mL of heparinized (1mL/L) phosphate buffered saline (PBS) pH 7.4 followed by 500 mL of 4 % paraformaldehyde (PFA) in PBS pH 7.4. Brains were post-fixed for 24 hours in 4 % PFA and then dissected out and placed into a 20 % sucrose solution in PBS for 26-28 hours. Brains were cryosectioned coronally on a Leica1650 cryostat (cut thickness: 30 µM); sections were collected serially thoroughout the striatum and substantia nigra pars compacta into tissue collection solution (50 % 0.1M phosphate buffer, 25 % glycerol, 30 % ethylene glycol) and stored at -20 for further analysis.

Brightfield Immunohistochemistry

Free floating immunohistochemistry on SNpc sections was performed using previously published DAB protocols (Taylor et al. 2006). Briefly, sections were labeled overnight at 4 degrees with primary antibodies: Tyrosine hydroxylase (TH) at 1:5000 (Millipore) followed by Neuronal nuclei (NeuN) at 1:2000 (Millipore). Sections were then labeled with the appropriate biotinylated secondary antibody diluted at 1:200 (Vector

Laboratories Burlingame, CA) for 2 hours at room temperature and then incubated in neutravidin-horse radish peroxidase conjugated antibody (Thermo Scientific) at 1:5000 for one hour at room temperature. The enzymatic reaction for TH was allowed to proceed for 4-6 minutes in a 0.05 % 3,3'-diaminobenzidine, 0.01 % hydrogen peroxide, and 0.04 % nickel chloride solution. The reaction for NeuN was allowed to proceed for 4-6 minutes in a 0.05 % 3,3'-diaminobenzidine, 0.01% hydrogen peroxide solution. Immunolabeled sections were mounted on glass slides (SuperFrost Plus, Fisher) and dehydrated/coverslipped as previously described (Frank-Cannon 2008).

Stereological Estimate of Neuron Number

Unbiased stereological estimates of dopaminergic (TH-positive cell) and total neuron (NeuN-positive cell) number were performed using StereoInvestigator analysis software (MicroBrightField Inc., Williston, VT) and the optical fractionator method (West et al. 1991) according to previously published reports (McCoy et al. 2008). Boundaries in the SNpc were defined according to previously defined anatomical analysis in the rat (German and Manaye 1993) and cells were counted under a 40X oil-immersion objective on a Nikon 80i microscope. Counting frames were designated as previously described (McCoy et al. 2008) with the following modifications: average mounted thickness, 20 μM; optical dissector, 16 μm and upper and lower guard zones, 2 μm.

Fluorescence Immunocytochemistry

Free floating immunohistochemical analyses of SNpc and striatal sections was performed as previously described (Taylor et al. 2006). Sections were labeled with GFAP antibody

diluted 1:1000 (Dakocytomation), IBA-1 antibody diluted 1:600 (AbCam), GFP antibody diluted 1:1000 (Rockland), or huTNF antibody diluted 1:1000(R&D systems) overnight at 4 degrees. Appropriate Alexa-conjugated secondary antibodies diluted 1:1000 (Invitrogen, Carlsbad, CA) were used at room temperature for 2.5 hours. Sections were mounted onto glass slides and coverslipped using BioMeda Gel-Mount. Images were captured with a Photometrics CoolSnap CCD ES monochromatic digital camera and analyzed with MetaMorph software (Universal Imaging Systems, West Chester, PA).

Quantification of Microglia

Images of midbrain sections double-labeled with IBA-1 and TH were captures under 4x objective lens on a Nikon 90i fluorescence microscope and quantified by thresholding analysis using Nikon Elements software.

Statistical Analysis

Statistical analysis was performed using Graph Pad software for student's t-test, one way ANOVA with Tukey's post-hoc tests, and two way ANOVA with Bonferroni's post hoc tests for significance. A p value of 0.05 or less was considered significant. Values shown are means $\pm$ S.E.M.

RESULTS

Co- and Delayed TNF Inhibition attenuates 6-OHDA-induced TH/NeuN positive neuron loss in the SNpc

We have previously shown that blocking solTNF signaling with a single intranigral injection of lentiviral DN-TNF at the time of an acute 6-OHDA pre-terminal lesion (Kirik et al., 1998) attenuated nigral DA neuron loss by approximately 46% (McCoy et al., 2008). However, those studies did not directly address the role of solTNF signaling in the progressive phase of nigral DA neuron degeneration, a critical question relevant to disease progression and development of anti-TNF therapeutics in PD models. To determine what role solTNF plays in the progressive phase of 6-OHDA-induced nigral DA neuron death, we chose a 6-OHDA model that consisted of an acute phase followed by a progressive phase of nigral DA neuron death (Sauer and Oertel, 1994) and subjected adult Sprague Dawley rats to experimental paradigm shown in Figure 2.1. First, using unbiased stereology to estimate the number of DA neurons (tyrosine hydroxylase, TH+ and neuronal antigen N, NeuN+ double-labeled cells) in the substantia nigra pars compacta (SNpc) SNpc, we confirmed that this 6-OHDA lesion resulted in nigral DA neuron death that progressed between week 2 and week 5. Specifically, we observed that at 2 weeks post-lesion the number of nigral DA neurons on the ipsilateral side was 60% of the number of nigral DA neurons on the contralateral side and at 5 weeks post-lesion it was 35% of the contralateral side (Figure 2.2A).

Next, we either co-administered lesions and virus injections or we delayed the virus injection by 2 weeks as shown schematically in Figure 2.1. Animals were divided into two groups: a Co-Inhibition group that received a striatal 6-OHDA (or saline) lesion at the same time as an intranigral injection of lenti-GFP (negative control) or lenti-DN-TNF virus and a Delayed Inhibition group that received intranigral lenti-GFP or lenti-

DN-TNF two weeks after the striatal 6-OHDA lesion. Five weeks after the initial lesion, animals were sacrificed for immunohistological analysis. Representative brightfield immunohistochemical sections double-labeled with antibodies against TH and NeuN used for stereological analyses are included in Supplemental Figure 2.1. Unbiased stereological analysis of sections throughout the SNpc double-labeled for TH and NeuN revealed an overall loss of 70% of nigral DA neurons by week 5 on the lesioned side in animals that received lenti-GFP at the time of the 6-OHDA lesion (Figure 2.2D) or 2 weeks after the lesion (Figure 2.2C) consistent with data from animals that received no virus injections (Fig. 2.2A). However, in animals that received lenti-DN-TNF at the time of the 6-OHDA lesion (Figure 2.2D) or 2 weeks after the lesion (Figure 2.2C) nigral DA neuron loss was attenuated by 50%. A concomitant reduction in the numbers of NeuN-positive cells was also observed (Figure 2.2C, D), indicating the reduction of TH-positive cells was due to frank neuronal loss and not mere down-regulation of TH expression. Remarkably, comparison of the nigral DA neuron number in 6-OHDA lesioned animals at the beginning of the progressive phase (week 2) with that at week 5 in animals that received delayed lenti-DN-TNF indicated that delayed inhibition of solTNF halted further loss of nigral DA neurons observed between week 2 and week 5 in animals that received lenti-GFP (Figure 2.2B). These results strongly suggest solTNF is mediating the progressive loss of nigral DA neurons elicited by the 6-OHDA neurotoxin.

Co- and Delayed DN-TNF Inhibition attenuates behavioral deficits in 6-OHDA lesioned rats

To determine if the rescue of nigral DA neurons was accompanied by an improvement in locomotor deficits, we performed two different behavior tests. To ensure all animals were appropriately lesioned prior to the delayed intranigral lentiviral injection at week 2, we performed amphetamine-induced rotational behavior at the end of the acute phase (i.e. week 2). We found that the 6-OHDA lesioned group had a statistically significant increase in the number of rotations per minute (Supplemental Figure 2.2) when compared to the saline group confirming detectable locomotor impairment prior to the second intervention. Forelimb asymmetry (Schallert and Jones, 1993) was tested prior to the 6-OHDA lesion and at weeks 1, 3, and 5 weeks post lesion. Our results indicate there was significant rescue of forelimb asymmetry at all weeks tested (weeks 1 through 5) for the co-inhibition DN-TNF group (Figure 2.3A) and at week 5 for the delayed inhibition DN-TNF group (Figure 2.3B).

Immunofluorescent localization of GFP-positive cells and DN-TNF protein expression after single lenti-DN-TNF injection

In order to localize lenti-DN-TNF-IRES-GFP transduced cells and determine the area of SNpc where DN-TNF protein was being expressed, we performed immunofluorescence analysis in brain sections from 6-OHDA/lenti-DN-TNF injected rats. An antibody specific for GFP revealed number of GFP-positive cells with glial morphology in the SNpc (Figure 2.4A, B) many of which were co-expressing DN-TNF protein detected with an antibody specific for the human DN-TNF sequence Detectable expression of DN-TNF protein in non-GFP-labeled regions marked the presence of

secreted DN-TNF protein in the region surrounding SNpc ((Figure 2.4C, D). Previous *in vivo* studies demonstrated that a single intranigral injection of this same lenti-DN-TNF virus injected resulted in variable expression of DN-TNF protein levels ranging from 0.6 to 2 ng/per midbrain as measured by human TNF ELISA (McCoy et al., 2008).

Transduction of SNpc with Lenti-DN-TNF attenuated gliosis in SNpc, but not in striatum

In previous *in vitro* studies, transduction of primary microglia with lenti-DN-TNF efficiently blocked solTNF-induced microglia activation (McCoy et al., 2008; McAlpine et al., 2009). In order to determine the extent to which transduction with lenti-DN-TNF blocked 6-OHDA-induced astrogliosis and microgliosis *in vivo*, we immunolabeled brain sections from the striatum and SNpc with antibodies specific for the microglial marker Iba-1 and the astrocyte marker glial fibrillary acidic protein (GFAP). As expected, transduction of cells with lenti-DN-TNF in the SNpc did not attenuate astrogliosis at the site of the 6-OHDA lesion in the striatum compared to lenti-GFP (Figure 2.5A). In contrast, lenti-DN-TNF significantly attenuated microgliosis in the SNpc (Figure 2.5B), as evidenced by a decrease in the microglial burden in the ipsilateral side relative to the contralateral or unlesioned side (36% for 6-OHDA/lenti-DN-TNF versus 53% for 6-OHDA/lenti-GFP). Similar inhibition of astrogliosis in SNpc was also observed (data not shown).

DISCUSSION

Using a rat model of parkinsonism consisting of an acute and a delayed,progressive phase of cell death, we demonstrate here that DN-TNF gene therapy in the SNpc results in transduction of glial cells and has robust neuroprotective effects regardless of whether it is administered at the same time as a neurotoxic lesion or with a two week delay. Specifically, single nigral injection of lenti-DN-TNF co-administered at the time of the 6-OHDA intrastriatal lesion afforded as much protection as a single nigral injection delivered at two weeks following the 6-OHDA lesion (Figure 2.2). Remarkably, nigral DA neuron number in the 6-OHDA/lenti-GFP group displayed a progressive decline from 60% to 35% of contralateral side between week 2 and week 5 but the 6-OHDA/lenti-DN-TNF group remained stable at approximately 50% of contralateral, leading us to conclude that administration of lenti-DN-TNF at week 2 during the progressive phase of 6-OHDA-induced nigral DA neuron death successfully halted this progression and stablilized nigral DA neuron number (Figure 2.2C). Consistent with these findings, co-inhibition with lenti-DN-TNF prevented the progressive forelimb asymmetry induced by 6-OHDA, while delayed inhibition with lenti-DN-TNF prevented further decline of forelimb usage between week 3 and week 5 (Figure 2.3). These results strongly support a role for solTNF signaling in the progressive phase of DA neuron loss induced by the oxidative neurotoxin 6-OHDA, suggesting it may have similar neuroprotective effects against progressive nigral DA neuron loss in early stages of PD.

In recent years, approaches targeting pro- and anti-inflammatory cytokines and cyclo-oxygenase-2 (COX-2) have emerged and undergone rigorous testing in pre-clinical

6-OHDA rat models (Sanchez-Pernaute et al., 2004; Koprich et al., 2008).While most manipulations attenuated nigral DA neuron loss when administered simultaneously with the lesion, few have shown efficacy specifically in the progressive phase of neuron loss (Sanchez-Pernaute et al., 2004). Although the 6-OHDA model used in our studies is considered to be modeling 'pre-clinical' stages of PD (Kirik et al., 1998), the instrastriatal 6-OHDA lesion achieved significant loss of nigral DA neurons (50%) and a behavioral correlate prior to DN-TNF transduction (Figures 2.2-2.3). Consistent with this, prior to DN-TNF transduction in the SNpc, all animals in the study showed a positive number of amphetamine-induced rotations, corresponding to a 40-50% striatal DA depletion and approximately 30-50% loss of nigral DA neuron cell bodies (Lee et al., 1996). Based on our analysis of microglial burden, the mechanism by which lenti-DN-TNF achieved rescue is likely to be a combination of neutralization of solTNF bioactivity (as expected based on the mode of action of this TNF inhibitor) as well as attenuation of microglia activation in the SNpc (Figure 2.5) which would be expected to result in reduced levels of other microglial-derived mediators with potential toxic effects on nigral DA neurons (including reactive oxygen and nitrogen species).

At this time, pharmacological and surgical therapies target and alleviate the non-motor and motor symptoms of PD, however, there are no therapies that can slow or halt the progression of PD (Olanow et al., 2009). Currently anti-TNF biologics are FDA approved for the treatment of patients with peripheral autoimmune disorders which are characterized by chronic systemic inflammation; however, these anti-TNF biologics can inhibit both soluble and transmembrane TNF (tmTNF) signaling therefore increasing the

patient's risk for opportunistic infections (van Oosten et al., 1996; Robinson et al., 2001; Tweedie et al., 2007). In contrast, the DN-TNF inhibitor used in our studies has been shown to preserve transmembrane TNF function thereby protecting against endotoxin-mediated hepatotoxicity and preserving immunity against *Mycobacterium bovis* and *Mycobacterium tuberculosis* infections in mice (Steed et al., 2003; Olleros et al., 2005). Therefore, if soluble TNF is the species promoting inflammation in the nigra as suggested by this and previous work from our group (McCoy et al., 2006; McCoy et al., 2008), selective targeting of solTNF with DN-TNF inhibitors in the CNS may prove to be the safer approach to avoid suppression of neuroimmune responses mediated by tmTNF.

Clinical trials are currently underway to investigate the therapeutic relevance of viral delivery of proteins designed to target many pathways in PD (Reviewed in (Olanow et al., 2009; Kaplitt, 2010; Lim et al., 2010). Our studies support the feasibility and efficacy of viral delivery mechanisms to slow the progression of PD and alleviate locomotor symptoms. Viral technology is generally safe to use in human and animal models and can be manipulated to infect and integrate DNA into the host genome of specific brain regions, allowing for continuous expression of desired proteins and a perhaps in the near future may become a viable alternative therapeutic approach for PD patients. For example, glutamic acid decarboxylase (GAD) is the key enzyme in the synthesis of gabaminobutyric acid (GABA) when delivered to the subthalamic nucleus in PD patients and non-human primate models with adeno-associated virus (AAV) can increase production of GABA therefore increasing the amount of inhibitory neuron firing and reducing motor symptoms associated with PD (Reviewed in (Olanow et al., 2009).

While GAD gene therapy may be effective in reducing motor symptoms, unlike DN-TNF it would not be expected to slow down the disease progression.

In summary, in a progressive rat model of PD, anti-inflammatory DN-TNF gene therapy reduces microgliosis in the SNpc, halts the progressive loss of nigral DA neurons and attenuates neurotoxin-induced behavioral deficits. While the exact mechanisms for inflammation-induced nigrostriatal degeneration of neurons have not been delineated, we provide neurohistological and behavioral evidence that virally delivered anti-TNF therapy selectively targeted to the nigral region of the midbrain halts the progressive loss of DA neurons. These promising findings should provide compelling reasons to investigate the efficacy of DN-TNF gene therapy in non-human primate models of PD with the long-term goal of delivering it during the pre-clinical stages of PD once early diagnosis of this age-related neurodegenerative disease is available.

FIGURE 2.1

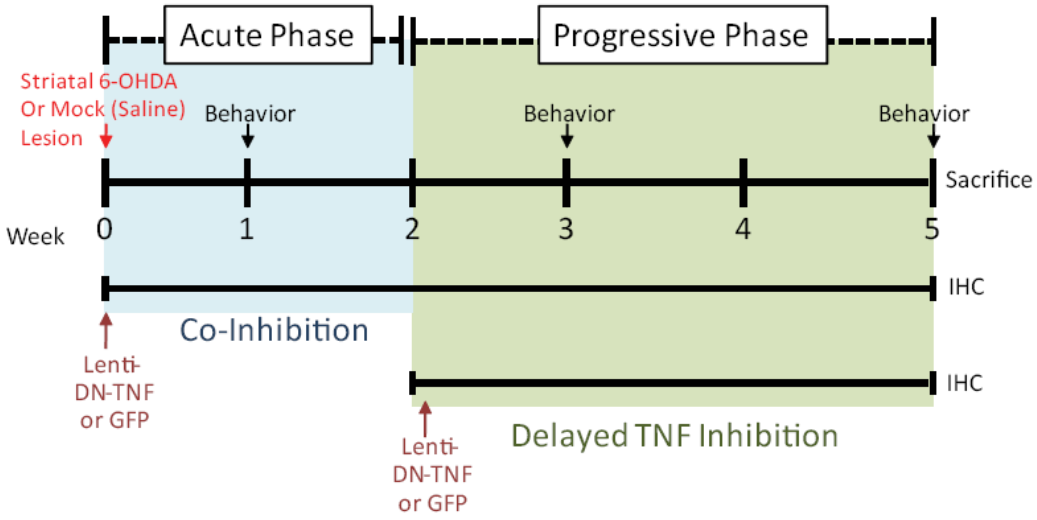

Acute Phase
Progressive Phase
Striatal 6-OHDA
Or Mock (Saline)
Lesion
Behavior
Behavior
Behavior
Sacrifice
Week
0
1
2
3
4
5
IHC
Co-Inhibition
Lenti-
DN-TNF
or GFP
IHC
Delayed TNF Inhibition
Lenti-
DN-TNF
or GFP

Figure 2.1: Schematic of experimental design and outcome measures.

Animals in the study were subjected to the surgical and testing paradigms as indicated (A). The number of animals utilized per group: co-TNFinhibition 5-7, delayed inhibition 7-9.

FIGURE 2.2

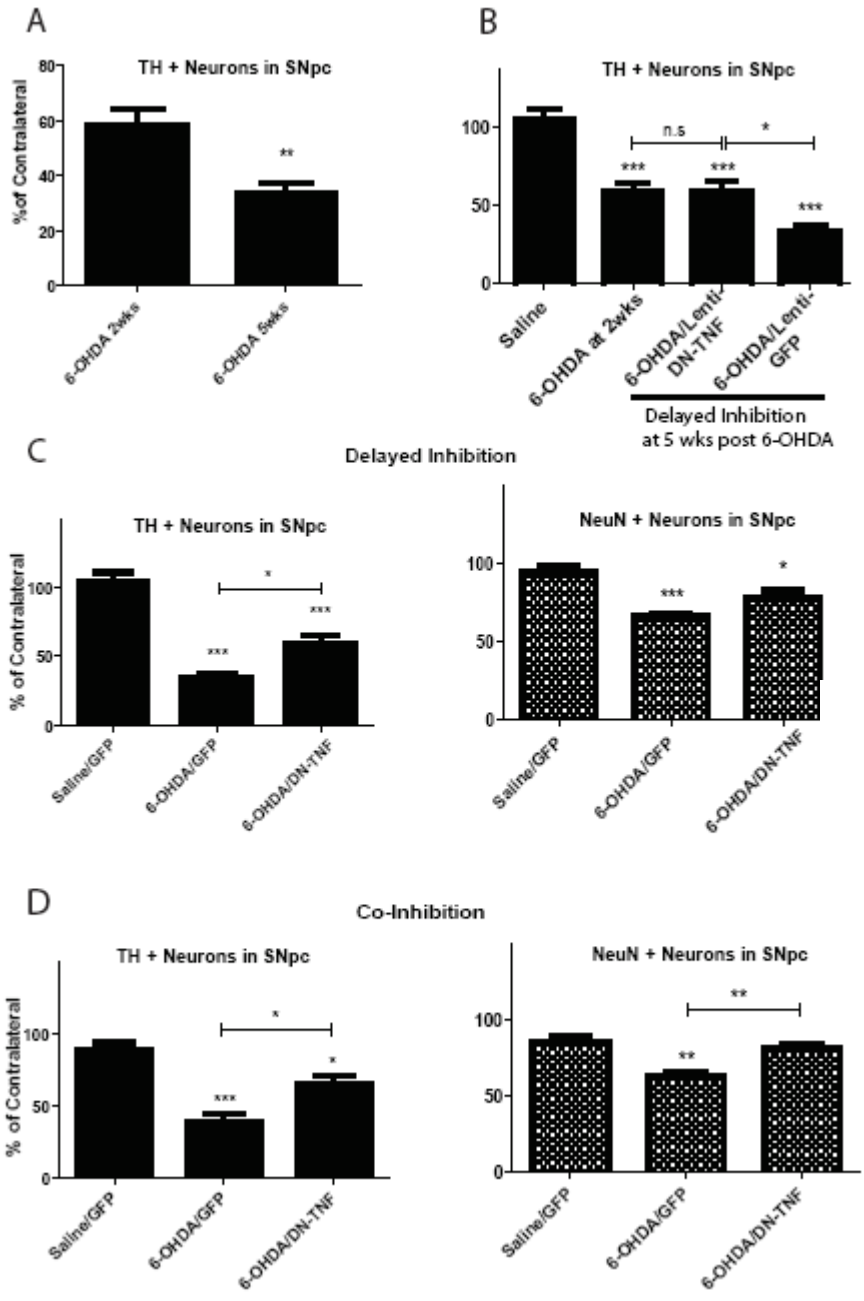

A
TH + Neurons in SNpc
%of Contralateral
80
60
40
20
0
**
6-OHDA 2wks
6-OHDA 5wks
B
TH + Neurons in SNpc
100
50
0
n.s
*
***
***
***
Saline
6-OHDA at 2wks
6-OHDA/Lenti-DN-TNF
6-OHDA/Lenti-GFP
Delayed Inhibition
at 5 wks post 6-OHDA
C
Delayed Inhibition
TH + Neurons in SNpc
% of Contralateral
100
50
0
*
***
***
Saline/GFP
6-OHDA/GFP
6-OHDA/DN-TNF
NeuN + Neurons in SNpc
100
50
0
***
*
Saline/GFP
6-OHDA/GFP
6-OHDA/DN-TNF
D
Co-Inhibition
TH + Neurons in SNpc
% of Contralateral
100
50
0
*
***
*
Saline/GFP
6-OHDA/GFP
6-OHDA/DN-TNF
NeuN + Neurons in SNpc
100
50
0
**
**
Saline/GFP
6-OHDA/GFP
6-OHDA/DN-TNF

Figure 2.2: Co- and Delayed TNF Inhibition attenuated 6-OHDA-induced loss of TH and NeuN double-positive neurons in the SNpc.

Estimates of dopaminergic (DA) neuron number (TH and NeuN double-positive cells) by unbiased stereology in the SNpc of lesioned animals sacrificed at 2 weeks and 5 weeks post- 6-OHDA lesion revealed progressive neuronal loss in the absence of treatment (A). Comparison of the nigral DA neuron number in 6-OHDA lesioned animals analyzed at 2 weeks post-lesion with animals lesioned and delayed injected with lenti-DN-TNF and analyzed at week 5 post-lesion revealed no difference while lesioned animals delayed injected with lenti-GFP developed further nigral DA neuron loss, suggesting that DN-TNF treatment halted the progression of the lesion between weeks 2 and 5 (B). DN-TNF treatment attenuated 6-OHDA-induced loss of TH and NeuN positive neurons in the SNpc in animals injected with lenti-DN-TNF at the same time (Co-inhibition, D) or 2 weeks following the 6-OHDA lesion (Delayed Inhibition, C). Frank neuronal loss was confirmed by the loss of NeuN-positive cells (C, D) Figures A, C, D, F One-way ANOVA with Tukey's post hoc test. *$p<0.05$, **$p<0.01$, *$p<0.001$. Figure B, Student's t-test **$p<0.003$. The number of animals utilized per group: Co- Inhibition n=5-7, Delayed Inhibition n=7-9.

FIGURE 2.3

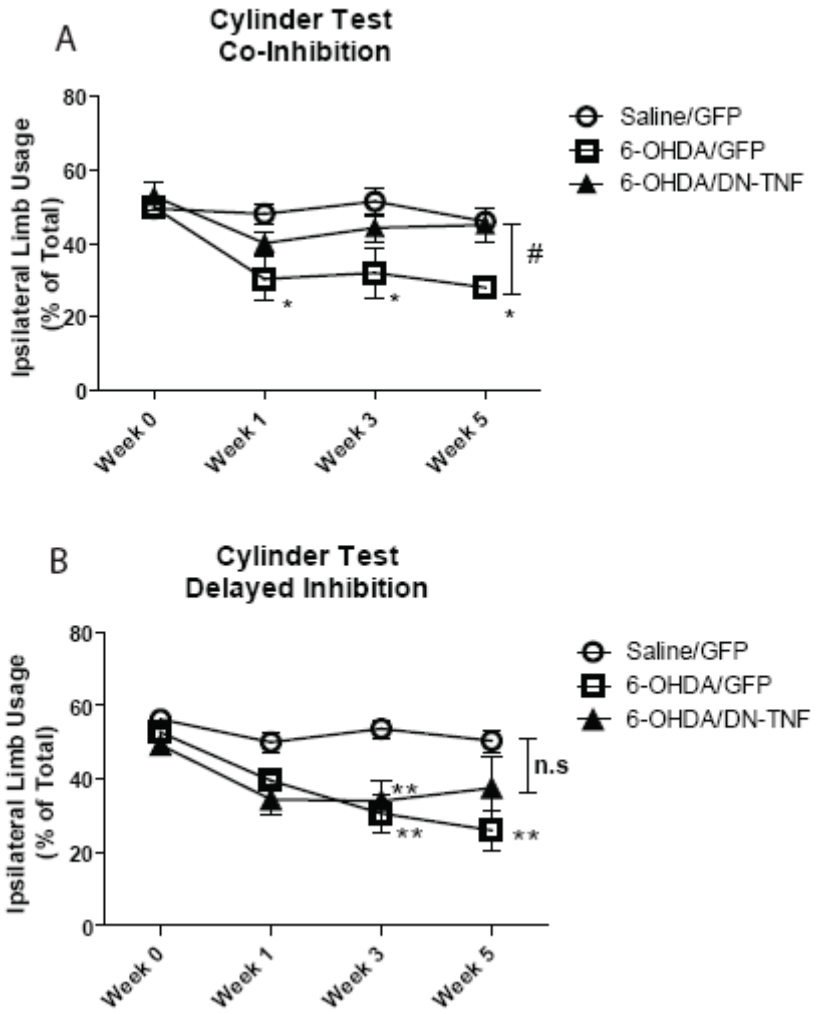

A
Cylinder Test
Co-Inhibition
Ipsilateral Limb Usage
(% of Total)
80
60
40
20
0
Week 0
Week 1
Week 3
Week 5
Saline/GFP
6-OHDA/GFP
6-OHDA/DN-TNF
#
*
*
*
B
Cylinder Test
Delayed Inhibition
Ipsilateral Limb Usage
(% of Total)
80
60
40
20
0
Week 0
Week 1
Week 3
Week 5
Saline/GFP
6-OHDA/GFP
6-OHDA/DN-TNF
n.s
**
**
**

Figure 2.3: Co- and Delayed DN-TNF Inhibition attenuates behavioral deficits in 6-OHDA lesioned rats.

At Weeks 0, 1, 3 and 5, 6-OHDA-lesioned animals were tested for forelimb asymmetry (cylinder test). Co-inhibition with lenti-DN-TNF attenuated 6-OHDA-induced deficits in forelimb asymmetry at weeks 1-5 (A; two way ANOVA with Bonferroni's post hoc test *$p<0.05$, #$p<0.05$). Delayed lenti-DN-TNF administration attenuated 6-OHDA-induced deficits in forelimb asymmetry at week 5 (B) Two way ANOVA with Bonferroni's post hoc test **$p<0.01$, n.s. not significant. The number of animals utilized per group: co-TNF Inhibition n=5-7, delayed inhibition n=7-9.

FIGURE 2.4

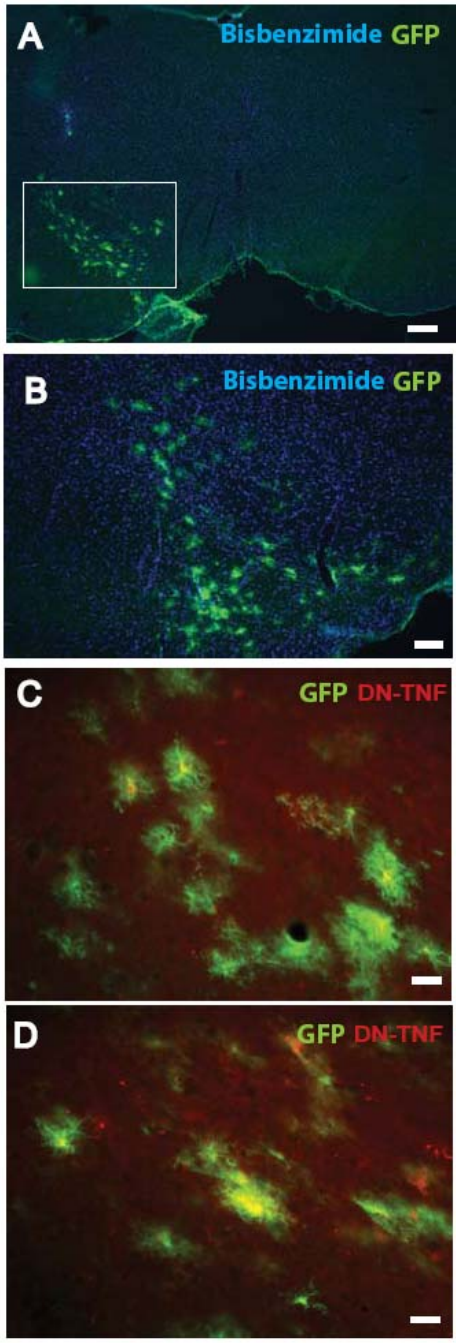

A
Bisbenzimide GFP
B
Bisbenzimide GFP
C
GFP DN-TNF
D
GFP DN-TNF

Figure 2.4: Immunofluorescent localization of GFP-positive cells and DN-TNF protein expression in cells of glial morphology after single lenti-DN-TNF injection. Anti-GFP immunofluorescence staining (green) (A, B) revealed cells of glial morphology transduced in the area of SNpc (white box) and hDN-TNF protein (red) expression in the surrounding areas (C, D). Scale bar in A, 400 μm; B, 200 μm; C and D, 50 μm.

FIGURE 2.5

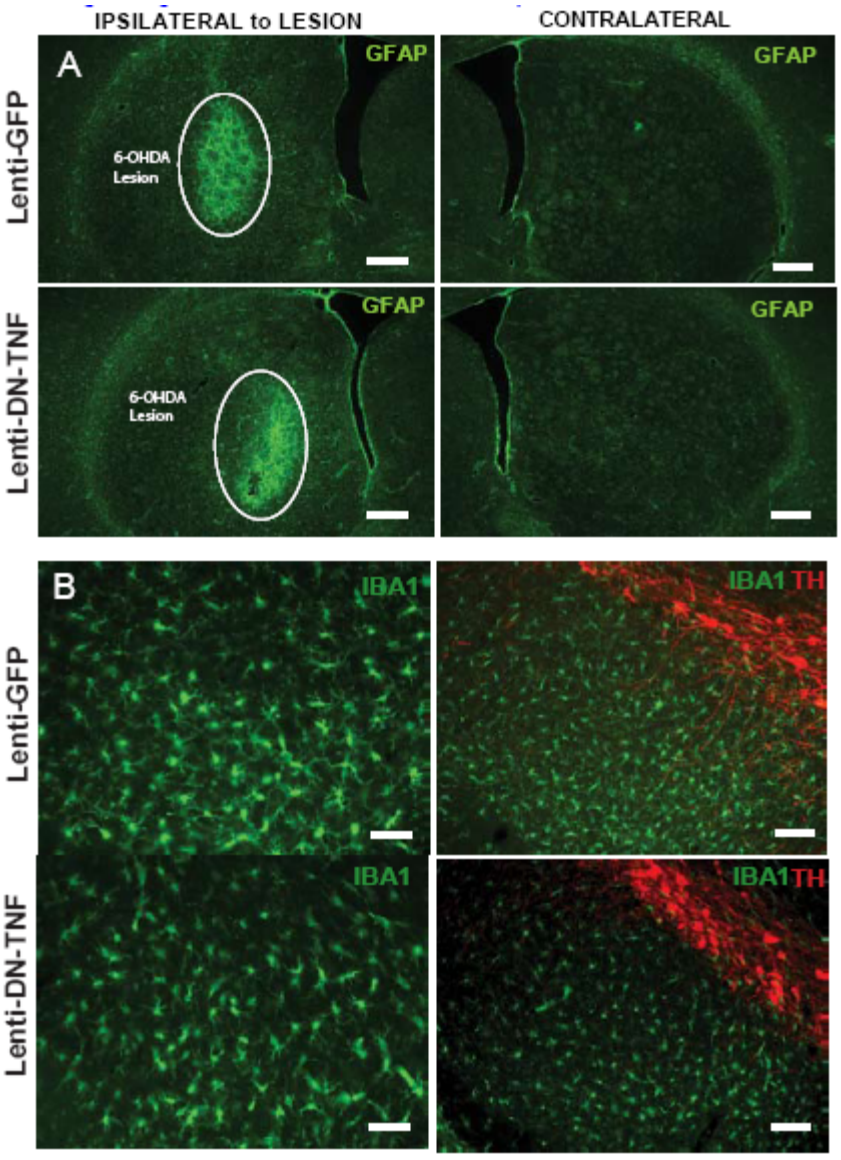
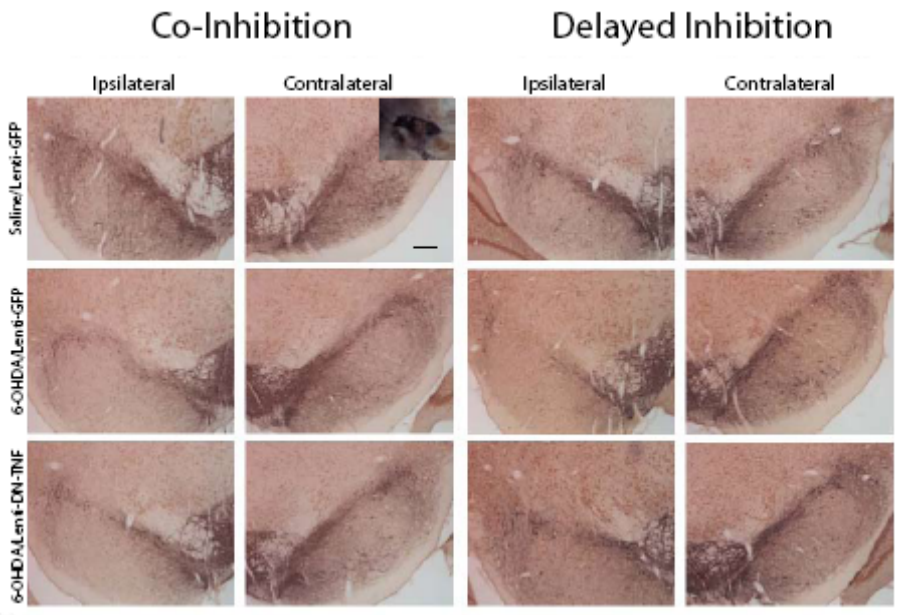

IPSILATERAL to LESION
CONTRALATERAL
A
GFAP
GFAP
6-OHDA Lesion
Lenti-GFP
GFAP
GFAP
6-OHDA Lesion
Lenti-DN-TNF
B
IBA1
IBA1 TH
Lenti-GFP
IBA1
IBA1TH
Lenti-DN-TNF

Figure 2.5. Transduction of SNpc with Lenti-DN-TNF attenuated microgliosis in SNpc, but not astrocytosis in striatum.

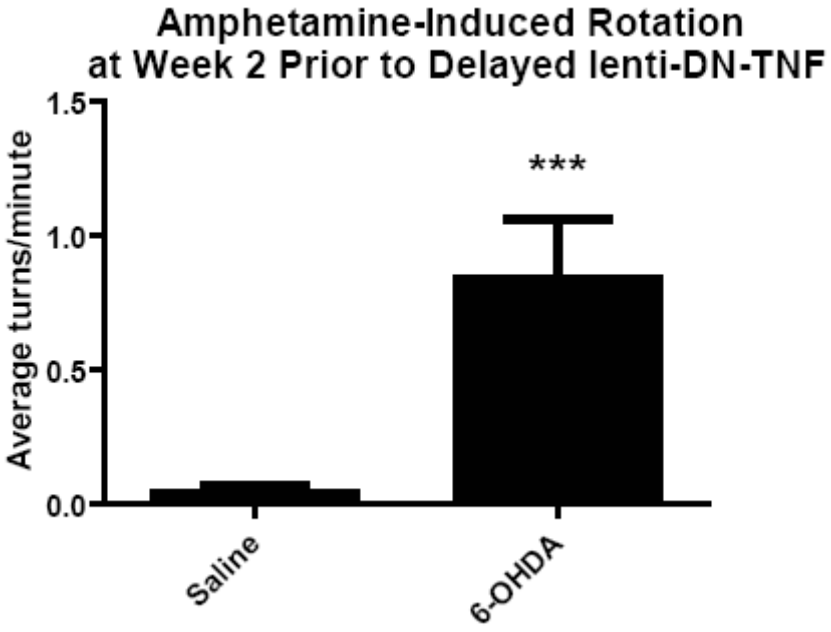
Activated astrocytes were identified by GFAP immunostaining (green) in the striatum (A). The area lesioned by the 6-OHDA neurotoxic is highlighted in white circle. Microglia were identified by IBA-1 immunostaining (green) in the SNpc area identified by TH immunostaining (red) (B). Scale bar in A = 400 μm; scale bar in B left panels, 100 μm; B right panels 200 μm.

SUPPLEMENTAL FIGURE 2.1



Supplemental Figure 2.1. Coronal brain sections double-labeled for NeuN and TH. Representative immunostained sections used for stereological estimate of total neuron number (NeuN, brown) and nigral DA neuron (TH, black) number. Inset shows higher magnification image of DA neuron (TH+/NeuN+) and non-DA neurons (NeuN+ only). Scale bar, 400 μm.

SUPPLEMENTAL FIGURE 2.2



Supplemental Figure 2.2. The 6-OHDA-lesioned rats destined for delayed solTNF inhibition demonstrated the expected behavioral deficits prior to the second surgery.

At week 2, 6-OHDA lesions were assessed for amphetamine-induced rotational behavior prior to second surgery in which the nigral injections were performed to deliver lenti-GFP or lenti-DN-TNF (B) Student's t-test ***$p<0.0001$.

ACKNOWLEDGEMENTS

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CHAPTER THREE

REGULATION OF MICROGLIA EFFECTOR FUNCTIONS BY TUMOR NECROSIS FACTOR (TNF): IMPLICATIONS FOR ANTI-TNF THERAPY IN THE CNS

ABSTRACT

Tumor Necrosis Factor (TNF) is a Th-1 class cytokine produced by activated microglia and macrophages involved in regulation of the immune system but whose function in the central nervous system (CNS) is not well understood. Neuroinflammation, and in particular TNF signaling, has been implicated in the pathophysiology of a number of neurodegenerative diseases; therefore anti-TNF drugs are currently under investigation as potential neuroprotective agents which could slow down the progressive loss of vulnerable neuronal populations. Given that microglia are monocyte-derived cells in charge of immune surveillance in the brain, we investigated the role of TNF in regulating microglia effector functions. By flow cytometry, we found no detectable differences in the basal activation state of brain microglia harvested from TNF-null mice compared to wild type mice and no differences in their LPS-induced phagocytic responses. However, microglia from TNF-null mice produced reduced protein levels of cytokines (IL-10, IL-6, IL-1β, and IL-12) and one chemokine (CXCL1) in response to LPS stimulation compared to wild type microglia and they displayed no cytotoxic effects on differentiated dopaminergic neuroblastoma MN9D cells derived from ventral mesencephalon when activated *in vitro*. To investigate whether reduced secretion of TNF or another cytokine accounted for the reduced microglial cytotoxicity, we treated MN9D cells with IL-6 or

IL-1β and found that only solTNF compromised MN9D viability. To distinguish between the developmental effects of TNF deficiency and pharmacological inhibition of TNF on cytokine secretion by microglia, we pre-treated wild type microglia with anti-TNF biologics prior to LPS stimulation. Multiplexed immunoassays revealed reduced levels of solTNF in the conditioned media but no change in levels of other cytokines. These data indicate that genetic ablation of TNF in microglia results in abnormal production of cytokines upon activation but prolonged pharmacological inhibition of solTNF signaling does not interfere with secretion of cytokines by wild type microglia. Although the baseline pattern of inflammatory gene expression measured by real time PCR in the brains of adult TNF-null mice was similar to that of wild type mice, microglia isolated from TNF-null mice failed to display the expected morphological changes in response to LPS stimulation, including enhanced perinuclear expression of the activation marker CD45. On the basis of these results we conclude that TNF-null microglia display aberrant effector functions as a result of developmental changes but pharmacological TNF inhibition does not impair the normal cytokine response upon activation but does protect dopaminergic cells from TNF cytotoxicity. One direct implication of our findings is that delivery of anti-TNF therapeutics to the CNS is not likely to have adverse effects on microglia function but may be an effective way to afford neuroprotection to dopaminergic neurons in the early stages of Parkinson's disease.

INTRODUCTION

Tumor necrosis factor (TNF) is a Th1-class cytokine produced by activated microglia and macrophages. TNF is involved in regulation of the immune system, protection from bacterial infections, cell growth, and viral replication and is a major player in many autoimmune and neurodegenerative diseases (Aggarwal 2000). TNF knockout mice are viable and fertile, but histological analysis revealed that their spleenic architecture is severely disrupted (Pasparakis et al. 1996). In addition, TNF knockout mice fail to develop monocyte-derived follicular dendritic cell clusters and germinal centers which compromises their immune system and ability to fight systemic *Listeria monocytogenes* bacterial infections (Aggarwal 2000). These findings prompted us to hypothesize that TNF is also important for development and function of brain microglia, the monocyte-derived macrophage population responsible for the innate immune response in the brain. Microglia make up about 10-20% of the total glial cell population (Carson et al. 2006) and are recruited to sites of injury or infection where they phagocytose invading pathogens and extracellular debris (Ransohoff and Perry 2009). Microglia secrete a number of inflammatory cytokines including TNF, interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) in response to inflammatory stimuli.

Neuroinflammation and chronic microglial activation have been implicated in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (Whitton 2007; Frank-Cannon et al. 2009). However, conflicting results from genetic studies aimed at determining whether TNF was required for the death of dopaminergic neurons in the midbrain substantia nigra induced by 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine

(MPTP), the mitochondrial complex I inhibitor commonly used to induce experimental parkinsonism in rodents, were likely due to differences in dosing regimens (Rousselet et al. 2002; Sriram et al. 2002; Ferger et al. 2004; Sriram et al. 2006). Importantly, follow-up studies suggested differences in microglia activation between adult TNF knockout mice and wild type mice may have influenced the neuroinflammatory and neurodegenerative responses to MPTP (Sriram et al. 2006). In agreement with genetic studies demonstrating reduced sensitivity to MPTP toxicity in mice lacking functional TNF signaling (Sriram et al. 2002; Ferger et al. 2004; Sriram et al. 2006), chronic *in vivo* inhibition of soluble TNF signaling with dominant negative TNF (DN-TNF) inhibitor proteins significantly attenuated neurotoxin- or endotoxin-induced DA neuron death and microglial activation (McCoy et al. 2006; McCoy et al. 2008), directly implicating soluble TNF in neurodegeneration of nigral DA neurons. Given the potential contribution of microglial activation to neurodegenerative diseases and the suggestion that anti-TNF therapies in the CNS may exert neuroprotective effects on vulnerable neuronal populations, the overall goal of these studies is to determine the role of TNF in regulating microglia effector functions in the brain as a way to gauge the potential for collateral damage of such approaches on microglia function in the brain.

MATERIALS AND METHODS

Animals

TNF knockout (B6;129S6-Tnftm1Gkl/J; stock number 003008) mice on a C57Bl/6 background strain along with wild type mice as non-transgenic controls were obtained

from Jackson Labs (Bar Harbor, ME). Experimental procedures involving the use of animals or animal tissue were performed in accordance with the NIH Guidelines for Animal Care and Use and approved by the Institutional Animal Care and Use Committee at The University of Texas Southwestern Medical Center in Dallas. Animals were housed in a climate controlled facility with a twelve hour light/dark cycle.

Primary Microglia

For immunocytochemistry, phagocytosis, and target effector assays murine microglia were isolated from postnatal day 3-5 (P3-P5) TNF knockout or wild type C57/Bl6 mouse pups according to published protocols (Lee et al. 2008) with minor modifications. Briefly, whole brains were isolated, minced, and placed in ice-cold dissociation media containing sterile filtered DNase1 (1μL/mL, Invitrogen, Carlsbad, CA), Dispase II (1.2 U/mL, Roche, Indianapolis, IN), and Papain (1 mg/mL, Sigma-Aldrich, St. Louis, MO) dissolved in DMEM/F12 (Invitrogen). Cells were dissociated for 30 minutes at 37ºC with agitation every 10 minutes. Following mechanical and chemical dissociation, mixed glial cells were filtered through a 40 μm-pore filter (BD Falcon) and plated in DMEM/F12 supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 1% penicillin/streptomycin (Sigma), and 1% L-glutamine (Sigma). Mixed glial cultures were maintained *in vitro* at 37ºC and 5% CO_2 for 14-18 days and media was replenished every 3-4 days. Once cultures reached confluence, primary microglial cells were isolated from the glial cell bed by mechanical agitation on an orbital shaker (150 rpm) for 1 hour. Following isolation, cells were plated for all experiments in DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (Sigma) supplemented with 1%

penicillin/streptomycin, and 1% L-glutamine. For flow cytometry, primary microglia were isolated from postnatal day 5 (P5) pups by MACS® neural dissociation and OctoMACS® CD11b magnetic bead separation according to the manufacturer's protocol (Miltenyi Biotech). Isolated microglia cells were labeled with fluorescently conjugated anti-F4/80 and anti-CD68 antibodies, fixed with 4% paraformaldehyde in PBS, and sorted by flow cytometry according to published protocols (Lee et al. 2008).

Multiplexed Immunoassays

WT microglia treated overnight with XENP345 (200ng/mL) and etanercept (200ng/mL) and treated with LPS for 24 hours (n=3 for each condition). Media was collected and run on an MSD 7-spot pro-inflammatory immunoassay per the manufacturer's instructions (Meso Scale Discovery).

Target-Effector Assays

The dopaminergic neuron-like cell line derived from ventral mesencephalon MN9D (Choi et al. 1991) was maintained in DMEM supplemented with 10% Fetal Clone III (Hyclone) and 1% penicillin/streptomycin (Sigma). MN9D cells were plated at a density of 7,000 cells/well in 96-well plates and allowed to adhere overnight. Twenty four hours later, MN9D cells were terminally differentiated as published in (Lee et al. 2008) in serum-free DMEM supplemented with 5 mM valproic acid (Sigma) and N2 supplement (Invitrogen) for 72 hours. During this differentiation period, primary microglia were isolated and plated at a density of 50,000 cells/well in 24-well plates and treated with PBS, low LPS (10 ng/mL) or high LPS (1 ug/mL) for 24 hours. Following treatment,

microglia conditioned media were harvested and clarified of cell debris by centrifugation. Microglial cells were fixed in 4% paraformaldehyde in PBS (pH 7.4). For target-effector assays, MN9D cell media was removed and replaced with primary microglia conditioned media (CM) supplemented with 5 mM valproic acid (to maintain the MN9D differentiated state) for 48 hours. MN9D viability was assayed using the CellTiter 96 Aqueous Assay reagent (Promega, Madison, WI). MTS or formazan absorbance was measured at a wavelength of 492 nm on an absorbance plate reader (Thermo Lab Systems Multiskan Ascent) and the values plotted and analyzed with GraphPad software.

Flow Cytometry

Primary microglial cells were isolated from postnatal day 3 (P3) wild type or TNF-null mice using the MACS neural dissocation kit followed by CD11b magnetic bead separation as per the manufacturer's instructions (Miltenyi Biotech). Immediately following isolation, microglial cells were immunolabeled with anti-F4/80-TxRed and anti-CD68-FITC (Serotec) for 15 min on ice in the dark. Cells were washed 3 times with PBS containing 0.01 % NaN_3, 0.05 % BSA, and fixed with 4 % PFA for 10 min on ice in the dark. TxRed and FITC-conjugated monoclonal antibodies (mAbs) with irrelevant specificity were used as negative controls. Cells were resuspended in wash buffer for flow cytometric analysis within 48 hours after fixation. A total of 10^4 cells with light scatter characteristics of cells of each sample were analyzed using a FACSCalibur instrument (BD Biosciences).

Quantitative Polymerase Chain Reaction

Real-time quantatative polymerase chain reaction (qPCR) was performed according to previously published protocols (Kurrasch et al., 2004). Total RNA was isolated from freshly-dissected brain tissue, treated with DNAse I (Invitrogen, CA) ,and reverse transcribed into cDNA using Superscript II RNAse H-reverse transcriptase (Invitrogen, CA). qPCR was performed on an ABI Prism 7000 Detection System (Applied Biosystems). All reactions were performed in a 384-well format with 50 ng cDNA , 10 uL SYBR green PCR mastermix, and 150 nM each forward and reverse oligonucleotide primers. All reactions were performed in triplicate and mRNA levels normalized to the housekeeping gene cyclophilin. Oligonucleotide primers for Tumor Necrosis Factor Receptor 1 (TNFR1), TNF Receptor 2 (TNFR2), MAC1, Macrosialin (CD68), Leukocyte common antigen or protein tyrosine phosphatase (CD45) were obtained from Integrated DNA Technologies (Coralville, IA). Primer sequences are available on request.

Fluorescence Immunocytochemistry

Fluorescence immunocytochemistry was performed as previously described (McCoy et al. 2006). Anti-CD45 (Serotec) was used at 1:250 and the nuclear counterstain Hoechst 33258 (Invitrogen) at 1:20,000. Appropriate Alexa-conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:1000. All digital images were acquired on an Olympus BX61 fluorescence microscope with a CoolSnap CCD ES monochromatic camera and analyzed with Metamorph software.

Phagocytosis Assay

Primary microglia were isolated and plated at a density of 50,000 cells/well in 96-well plates and allowed to adhere for 8 hours. Upon adherence, cells were treated overnight with low LPS (10 ng/mL) or high LPS (1 μg/mL). Phagocytic activity of fluorescent *E. coli* bioparticles was measured using a Vibrant Phagocytosis Assay kit (Invitrogen).

Statistics

Statistical analysis was performed using Graph Pad software for one or two-way ANOVA with Bonferroni's post-hoc tests for significance. A p value of 0.05 or less was considered significant. Values shown are means $\pm$ SEM.

RESULTS

Microglia from TNF-null mice display deficits in LPS-evoked cytokine production

Given that TNF is a potent Th1-type cytokine believed to promote pro-inflammatory cascades, we first investigated whether production of other inflammatory factors by activated microglia was affected by the lack of TNF expression in microglia. Multiplexed immunoassay analyses of conditioned media from resting or LPS-stimulated primary microglia harvested from the brain of wild type or TNF-null mice revealed a statistically significant reduction in secretion of several inflammatory mediators including TNF, IL-1β, IL-6, IL-10, CXCL1, and IL-12 by TNF-null microglia compared to wild type microglia (Figure 3.1), suggesting that TNF regulates gene expression and/or secretion of these microglial-derived factors.

TNF-null microglia display reduced cytotoxicity on differentiated dopaminergic MN9D cells

Because pro-inflammatory factors, and in particular TNF, have been implicated in the death of dopaminergic neurons (McGeer and McGeer 2008; Tansey and Goldberg 2009), we investigated whether this reduced inflammatory factor production from TNF-null microglia would translate into reduced toxicity on DA neuron-like MN9D cells. To assess whether any soluble factors secreted by resting or activated microglia have effects on dopaminergic cell viability, we performed target-effector assays where conditioned media (CM) from resting or LPS-stimulated primary wild type or TNF-null microglia are transferred directly onto terminally differentiated dopaminergic MN9D cells which do not express the LPS receptor TLR4. In agreement with previous work (Lee et al. 2008; Tran et al. 2008), we found that CM of LPS-stimulated wild type microglia reduced viability of differentiated MN9D cells in a dose-dependent manner. In contrast, CM of LPS-stimulated TNF-null microglia did not reduce MN9D viability under any LPS concentration tested (Figure 3.2). However, given the fact that TNF-null microglia produced significantly reduced levels of several cytokines upon treatment with LPS, the reduced toxic effect could be the result of reduced production of several inflammatory cytokines and not just reduced TNF expression. To address this possibility, we next measured the direct effect of TNF, IL-6 and IL-1β on MN9D cell viability. We found there was a dose-dependent decrease in MN9D cell viability upon treatment with soluble TNF but not with either IL-6 or IL-1β stimulation (Figure 3.3). These findings are

consistent with previous findings from our group demonstrating that neutralization of soluble TNF in the CM of LPS-stimulated microglia with the soluble decoy receptor etanercept or the dominant-negative TNF inhibitor XENP345 blocks the cytotoxic effects of LPS-stimulated microglia on dopaminergic MN9D cells and primary DA neurons *in vitro* and *in vivo* (McCoy et al. 2006). Taken together, these findings demonstrate that TNF is not only critical for inflammatory factor expression in microglia but is also the primary microglial-derived factor with toxic effects on the MN9D dopaminergic cell line.

Primary microglia from wild type and TNF-null mice display similar cell-surface markers and reduced expression of Mac-1 and TNFR2

To investigate the extent to which global absence of TNF affects brain microglia number, we measured the percentage of CD68-positive microglia and F4/80-positive microglia by flow cytometry immediately following harvest from the postnatal brains of wild type or TNF-null mice. We found no significant differences in the mean fluorescence intensity of CD68+ or F4/80+ microglia between genotypes (Figure 3.4)**.** Similar results were obtained with primary peritoneal macrophages from young adult wild type or TNF-null mice (data not shown). To confirm and extend these findings, we measured the mRNA levels of microglia cell surface genes and TNF receptor (TNFR1 and TNFR2) genes by real-time quantitative PCR (QPCR) in brain tissue of wild type or TNF-null mice. In agreement with results from flow cytometry experiments, we found no genotype differences in the levels of CD68 or CD45 expression in isolated microglia (Figure 3.5). However, we found a statistically significant decrease in expression of TNFR2 and in

expression of MAC-1 (a key component of the Macrophage Attack Complex) in TNF-null mouse brains compared to wild type mouse brains, suggesting that TNF may be required for developmental expression of these and other genes of functional importance to microglia not measured in our studies.

Morphological analysis reveals differences between activated microglia and normal phagocytic responses

To investigate the extent to which TNF regulates microglia effector functions other than cytokine expression, we measured LPS-evoked morphological changes and phagocytic responses of microglia isolated from wild type or TNF-null newborn mice. We treated primary microglia for 24 hours with low (10 ng/mL) or high (1 μg/mL) lipopolysaccharide (LPS) then performed immunocytochemical analysis of the activation marker CD45, a tyrosine phosphatase. We found that TNF-null microglia displayed increased cytoplasmic vacuole-formation in response to LPS, the functional significance of which is unclear at this time. TNF-null microglia also displayed attenuated perinuclear localization of CD45 compared to wild type microglia (Figure 3.6), an effect which was more prominently detected after low LPS treatment. Surprisingly, phagocytic responses were not affected by TNF deficiency (Figure 3.7).

Previous work from our group demonstrated that LPS-induced microglial activation can be attenuated transiently by co-addition of TNF inhibitors *in vitro* (McCoy et al. 2006; McAlpine et al. 2009). Therefore, in order to determine if the blunted

cytokine secretion by TNF-null microglia exposed to LPS (Figure 3.1) was due to developmental changes in gene transcription as a result of TNF deficiency or due to the lack of TNF signaling during the LPS stimulus, we treated primary wild type microglia overnight with the anti-TNF biologics XENP345 and etanercept prior to the 24-hour LPS stimulus. We found that pre-treatment with the anti-TNF biologics only reduced the amount of soluble TNF secreted into the medium and did not affect secretion of all other cytokines (Figure 3.8)**.** Together, our data indicate that TNF deficiency during microglia development impairs the normal increase in gene expression and production of inflammatory mediators during the microglia activation response.

DISCUSSION

The role of TNF in the CNS has not been explored extensively; but TNF signaling has been shown to participate in a form of synaptic plasticity termed synaptic scaling, glutamatergic neurotransmission, regulation of blood-brain barrier (BBB), and injury-mediated microglial and astrocyte activation (reviewed in (McCoy and Tansey 2008). While genetic and pharmacological studies have strongly implicated TNF and microglial activation in dopaminergic neuron death and seem to provide compelling rationale for use of TNF inhibitors in the CNS as a neuroprotective strategy in PD, the role of TNF in microglial effector function must be elucidated to avoid potentially harmful bystander effects of such approaches on microglia function. In this study we provide detailed molecular, cell biological, and functional analyses of the effects of TNF deficiency on microglia populations and their effector functions.

Our results indicate that TNF is critical for the increased expression of several cytokines and chemokines produced by activated microglia *in vitro*. Specifically, following LPS stimulation, TNF-null microglia showed reduced protein expression of several cytokines (TNF, IL-1β, IL-6, IL-12 p70, IL-10) and chemokines (CXCL1) (Figure 3.1). One clear implication from these findings is that results involving use of TNF-null mice to investigate the role of TNF in a particular disease process should be interpreted with caution due to the fact that TNF-null mice display an altered cytokine profile in addition to being deficient in TNF production.

Brain microglia are responsible for innate immune responses in the CNS; but in recent years chronic microglial activation and overproduction of pro-inflammatory cytokines has been implicated in the pathophysiology of several neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS). In PD patients, TNF mRNA and protein levels are elevated and microglial activation is widespread, in particularly in the substantia nigra pars compacta (SNpc), the midbrain area most affected by degeneration in this disease (Duke et al. 2007). Using a murine dopaminergic neuron-like cell line derived from mouse ventral mesencephalon (Choi et al. 1991), here we demonstrate that TNF is the critical microglial-derived factor that compromises the viability of these cells whey they are exposed to conditioned media from activated microglia (Figures 3.2 and 3.3). Other studies have suggested that IL-1β may also compromise DA neuron survival (Godoy et al. 2008); however, while both IL-1β and IL-6 are produced by activated

microglia in our assays, neither have direct toxic effects on MN9D cell line viability (Figure 3.3). Nevertheless, it has been demonstrated that *in vivo* inhibition of IL-1β affords neuroprotective effects in part derived from the indirect effects of reduced TNF production (Koprich et al. 2008). On the basis of these findings and our data, we conclude that TNF is likely to be the critical microglial-derived inflammatory mediator that compromises DA neuron survival in the inflamed nigrostriatal pathway.

Based on the rationale that microglia are monocyte-derived and that monocyte-derived dendritic cell populations were found to be arrested in TNF-null mice (Pasparakis et al. 1996), we investigated whether TNF-null mice displayed similar developmental arrest in their microglia populations. We performed flow cytometry on microglia harvested from brain tissue of postnatal day three (P3) TNF-null or wild type mice and found that there was no difference in basal expression of CD68 (also known as macrosialin) and no difference in the percentage of F4/80-positive microglia (Figure 3.4). These findings were confirmed by gene expression analyses of CD68 and CD45 in brain tissue harvested from adult wild type or TNF-null mice (Figure 3.5).

We also found that in response to an inflammatory challenge, primary microglia from TNF-null mice did not undergo the typical morphological changes from a resting "ramified" morphology to a round amoeboid-like shape which also normally accompany perinuclear translocation of CD45 (Figure 3.6), in agreement with a recent study reporting that TNF-null mice have a reduced population size of CD45-positive resident microglia (Lambertsen et al. 2009). In contrast, TNF deficiency did not appear to affect

phagocytic responses. In light of published reports that CD68 is a lysosomal membrane-associated protein expressed in macrophages and microglia with an important role in phagocytosis (Holness et al. 1993), the normal phagocytic response of TNF-null microglia is consistent with their normal cell surface expression of CD68 (Figure 3.4 and 3.5).

Anti-TNF biologics are an FDA approved, safe treatment for a number of peripheral inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (Tansey and Szymkowski 2009) and our group has used them to investigate the role of TNF signaling in pre-clinical models of neurodegeneration (McCoy et al. 2006; McAlpine et al. 2009). However, the extent to which long-term use of such TNF inhibitors affects microglial function remains to be determined. The studies presented her provide direct evidence that DN-TNF and etanercept selectively inhibited microglial-derived TNF production without affecting production of other cytokines. The therapeutic implication of these findings is that anti-TNF biologics are likely to afford potent neutralization of TNF-dependent processes without pan-inhibition of microglia activation.

In summary, these findings establish the relative importance of TNF in several different microglia effector functions. TNF is not critical for development of microglia number, their basal activation state, or their phagocytic responses which are critically important against pathogen invasion and debris clearance. However, TNF is required for the typical morphological changes that occur in microglia upon activation by LPS and the

functional consequence of TNF deficiency is attenuated production of several pro-inflammatory cytokines and chemokines by activated microglia. Importantly, although activated microglia produce a number of pro-inflammatory cytokines that may affect neuronal survival, TNF seems to be the critical microglial-derived factor that compromises survival of dopaminergic neuron-like cells. Together with previous observations that chronic TNF signaling inhibition *in vivo* affords neuroprotection to nigral DA neurons (McCoy et al. 2006; McCoy et al. 2008), the studies presented here strengthen the notion that TNF inhibition in the CNS may be a viable therapeutic approach to attenuate the progressive loss of nigral DA neurons that occurs in PD without compromising critical microglia effector functions in the CNS such as phagocytosis.

FIGURE 3.1

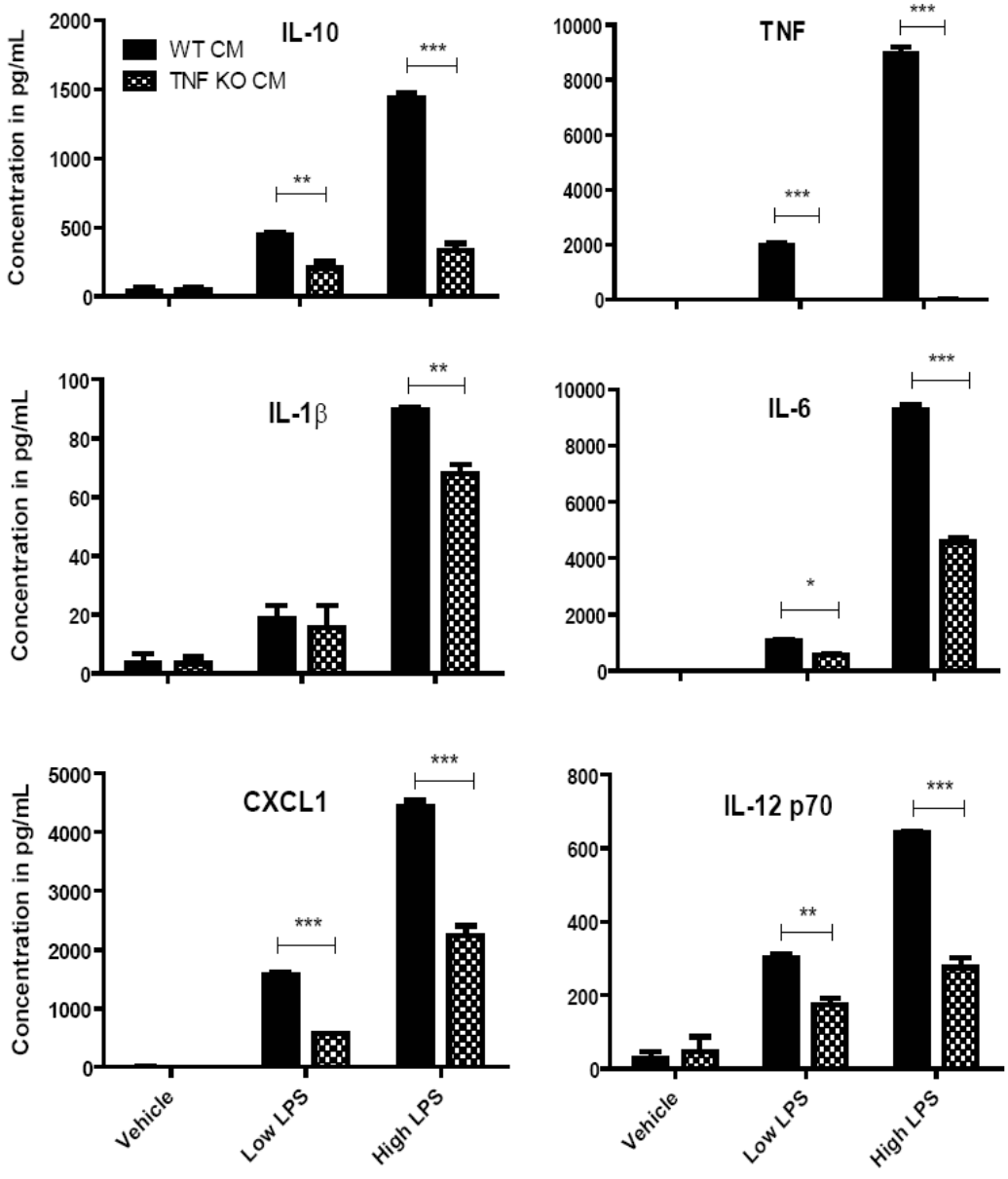
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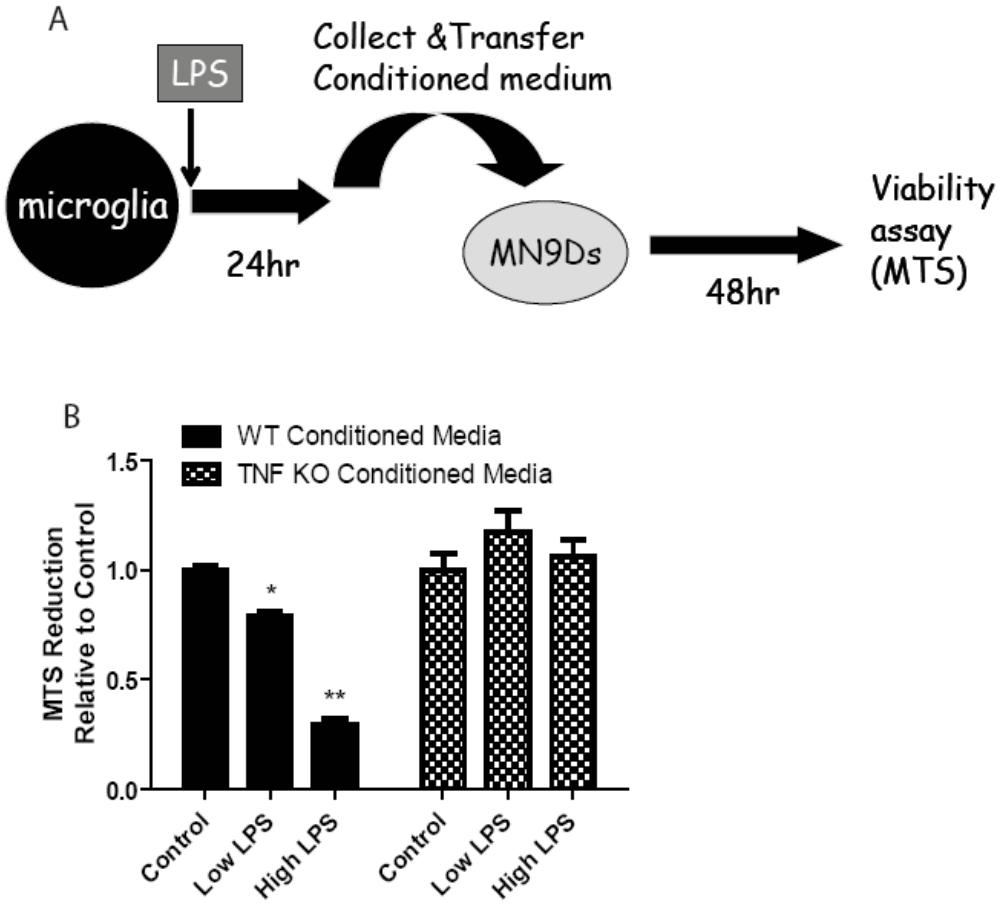
Figure 3.1. Microglia from TNF-null mice display deficits in LPS-evoked cytokine production.

Primary microglia cells isolated from wild type or TNF-null postnatal day 3 (P3) pups were plated (50,000 cells/well) and treated with low (10 ng/mL) or high (1 μg/mL) LPS for 24 hrs. Conditioned media was collected and analyzed for inflammatory factor production by multiplexed immunoassay (MesoScale). TNF-null microglia secrete significantly lower protein levels of all inflammatory mediators measured. Two-way ANOVA with Bonferroni's post hoc test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

FIGURE 3.2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Low LPS
High LPS

Figure 3.2. TNF-null microglia display reduced cytotoxicity on differentiated dopaminergic MN9D cells.

Schematic of the target-effector assay (A); Primary microglia isolated from wild type or TNF-null postnatal day 3 (P3) pups were treated for 24 hrs with low (10 ng/mL) or high (1 μg/mL) LPS. Conditioned media (CM) from resting or treated microglia were collected and transferred directly onto cultures of differentiated dopaminergic MN9D cells for 48 hrs. Cell viability of MN9D dopaminergic cell line was assayed using an MTS reduction assay (B). Two-way ANOVA with Bonferroni's post hoc test. $*p < 0.05$, $**p < 0.01$.

FIGURE 3.3

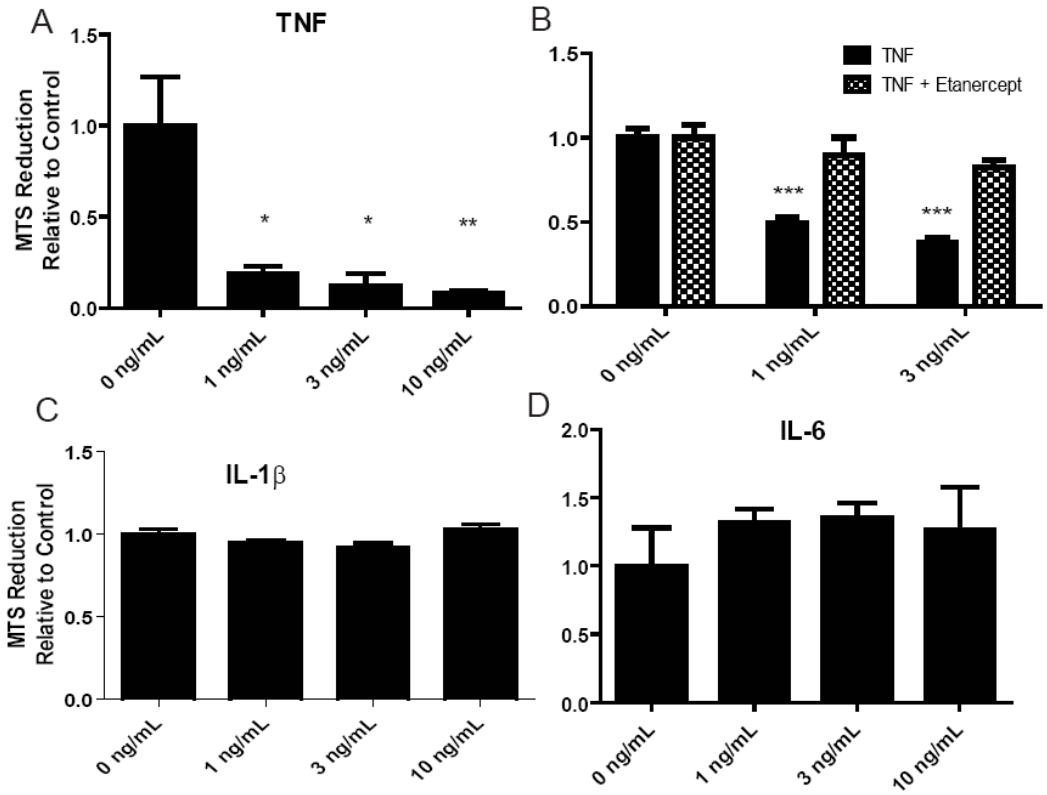
A
TNF
MTS Reduction Relative to Control
1.5
1.0
0.5
0.0
*
*
**
0 ng/mL
1 ng/mL
3 ng/mL
10 ng/mL
B
TNF
TNF + Etanercept
***
***
C
IL-1β
D
IL-6
2.0

Figure 3.3: TNF, not IL-6 or IL-1β, exerts cytotoxic effects on differentiated dopaminergic MN9D cells.

Differentiated dopaminergic MN9D cells were treated for 48 hours with TNF (A), TNF plus the decoy receptor etanercept (200 ng/mL) (B), IL-1β (C), or IL-6 (D). Cell viability of MN9D dopaminergic cell line was measured by MTS reduction assay. One way ANOVA.*$p<0.05$, **$p<0.01$, ***$p<0.001$.

FIGURE 3.4

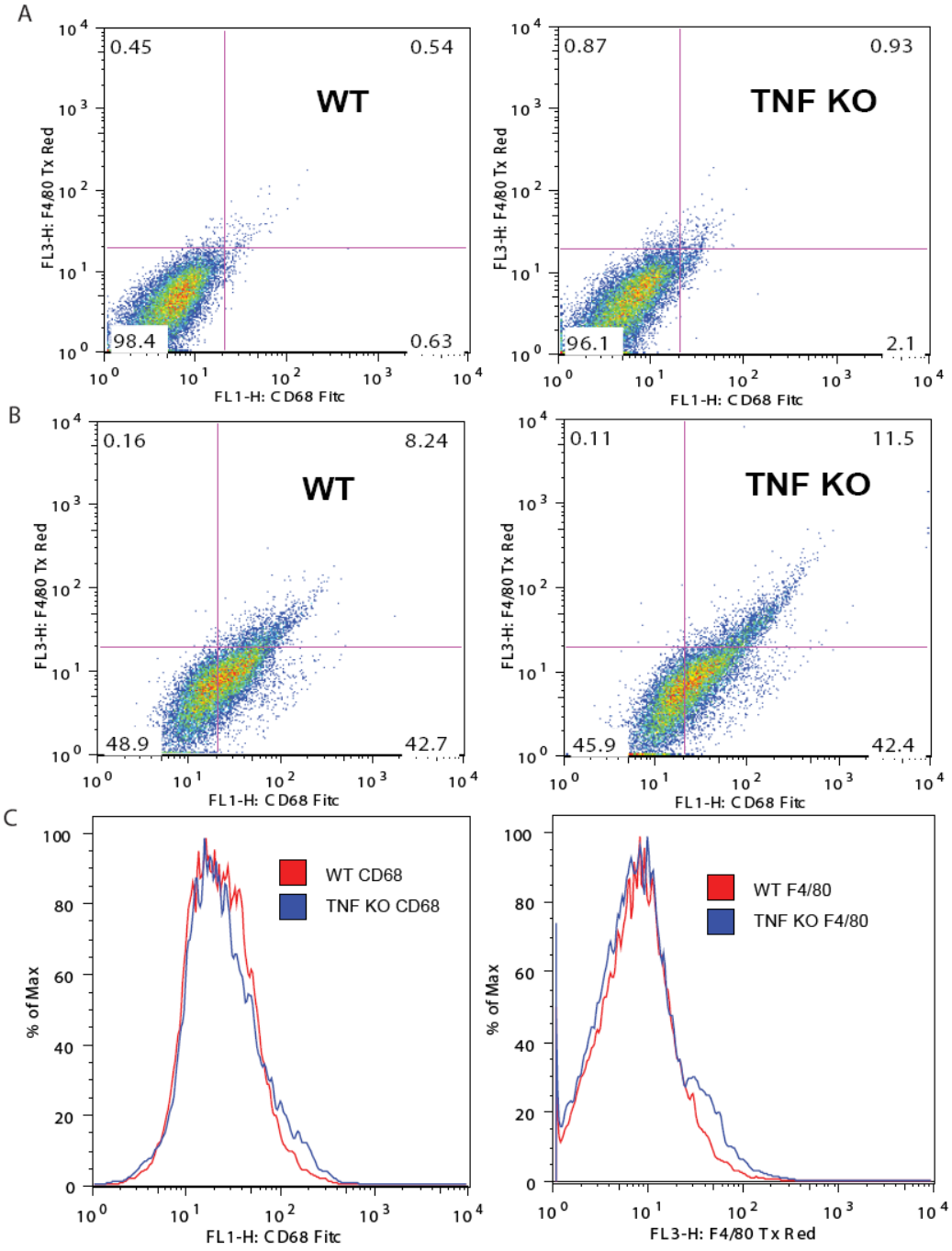
A
0.45
0.54
WT
98.4
0.63
FL3-H: F4/80 Tx Red
FL1-H: CD68 Fitc
0.87
0.93
TNF KO
96.1
2.1
B
0.16
8.24
WT
48.9
42.7
0.11
11.5
TNF KO
45.9
42.4
C
WT CD68
TNF KO CD68
% of Max
WT F4/80
TNF KO F4/80
FL3-H: F4/80 Tx Red

Figure 3.4. Primary microglia from wild type and TNF-null mice display similar cell-surface markers.

Primary microglia were isolated from postnatal day 5 (P5) pups by MACS® neural dissociation and positively selected using CD11b magnetic bead separation (Miltenyi Biotec). Isolated microglia cells were double-labeled with fluorescently conjugated F4/80 and CD68 antibodies, fixed and sorted by flow cytometry. Unstained (A), and Double-labeled (B) cells; Histogram plots for single-labeled populations (C). FACs analysis of the live cell population revealed no difference in the mean fluorescence intensity between genotypes.

FIGURE 3.5

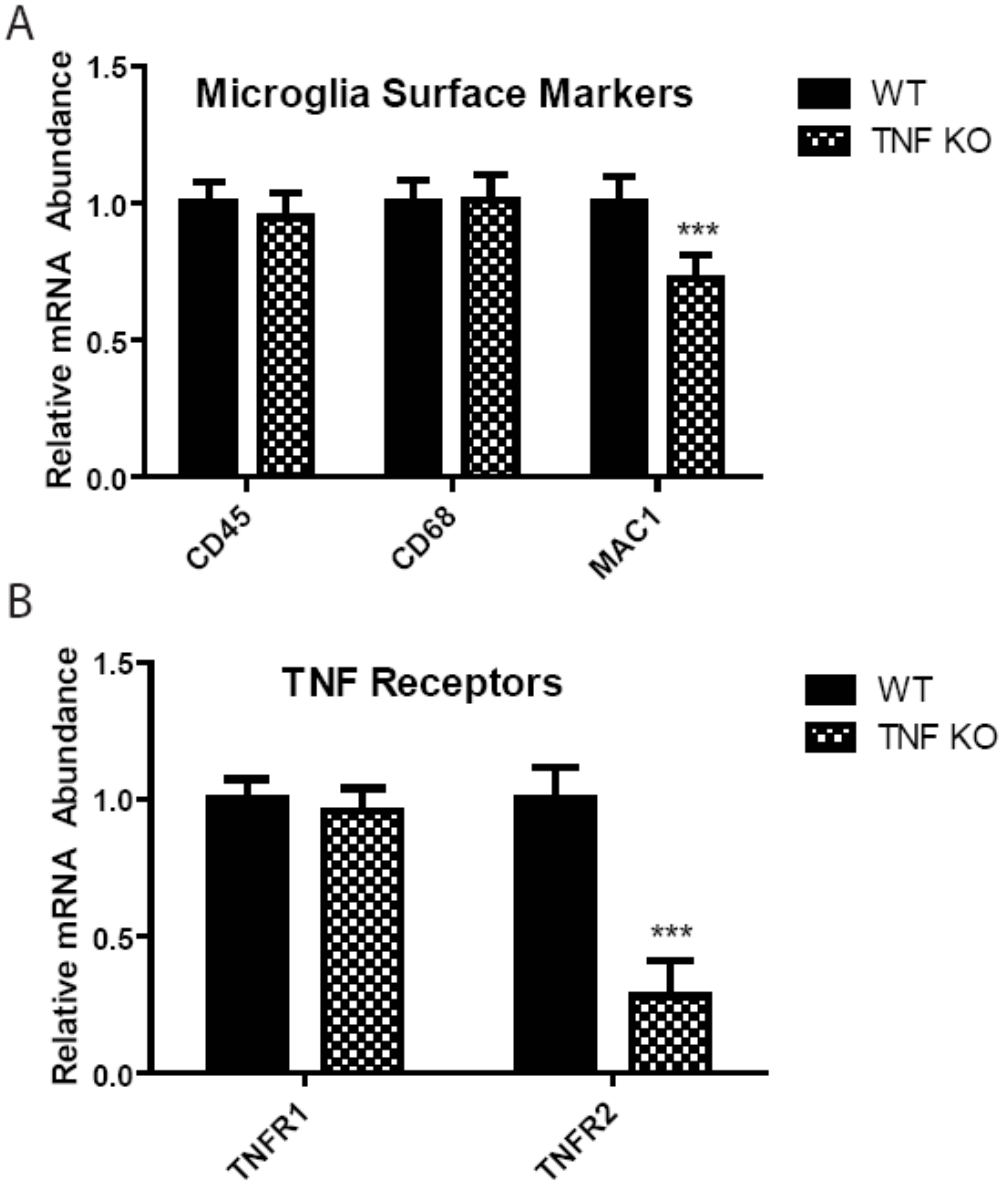
A
Microglia Surface Markers
Relative mRNA Abundance
1.5
1.0
0.5
0.0
***
CD45
CD68
MAC1
WT
TNF KO
B
TNF Receptors
Relative mRNA Abundance
1.5
1.0
0.5
0.0
***
TNFR1
TNFR2
WT
TNF KO

Figure 3.5. Inflammatory gene expression analysis reveals reduced expression of Mac-1 and TNFR2 in adult brains of TNF-null compared to wild type mice. Brains (n = 4 mice/genotype) were rapidly removed and microdissected. Total RNA was extracted and reverse transcribed into cDNA for real-time PCR analysis of microglial surface markers (A) or TNF receptors (B). Expression of CD45, CD68, and TNFR1 was not significantly different between genotypes but a significant reduction in expression of MAC1 and TNFR2 was found in brain of adult TNF-null mice. Unpaired t-test, $*p < 0.05$, $***p < 0.001$.

FIGURE 3.6

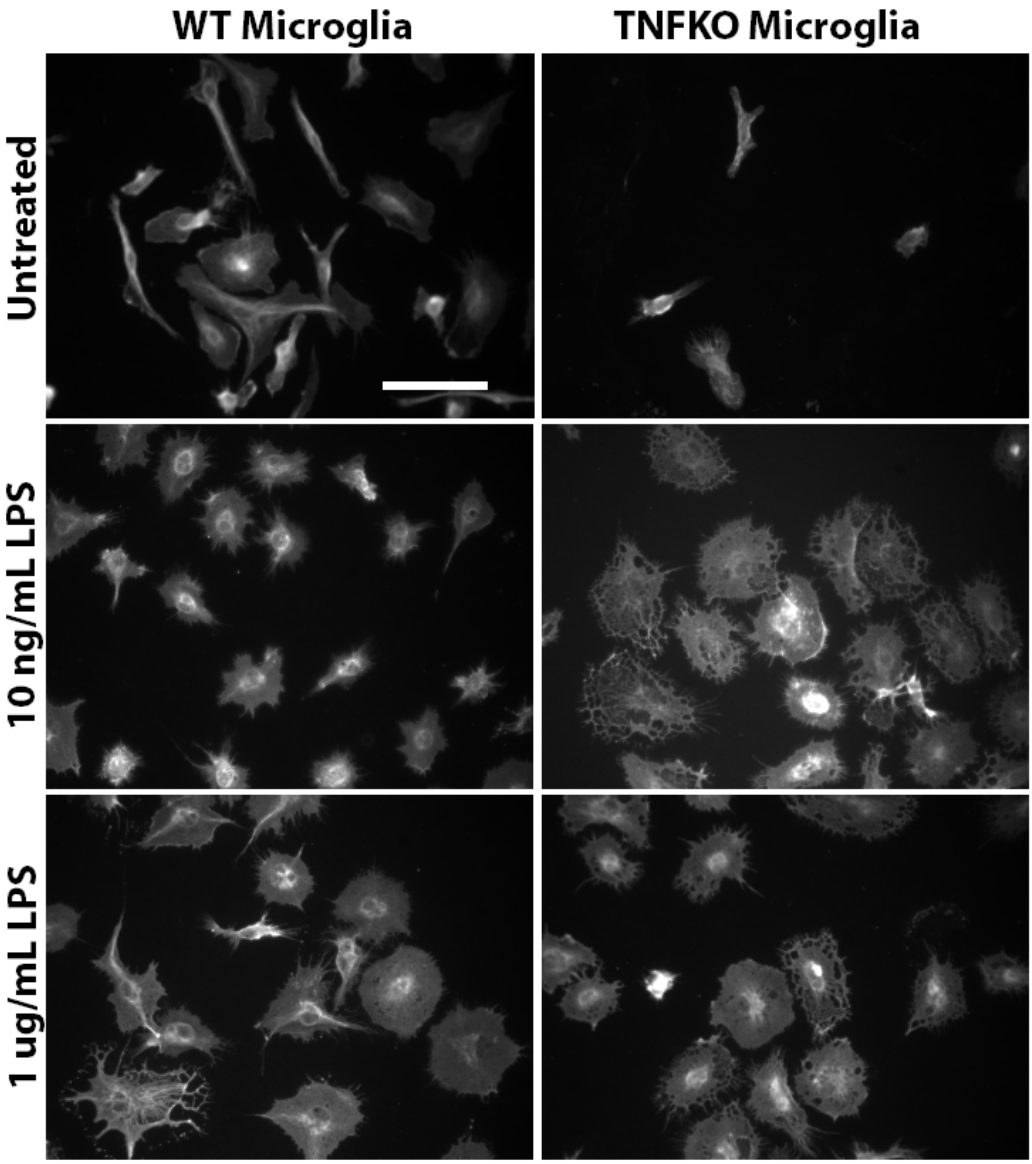
WT Microglia
TNFKO Microglia
Untreated
10 ng/mL LPS
1 ug/mL LPS

Figure 3.6. Morphological analysis reveals differences between activated microglia isolated from TNF-null and wild type mice.

Primary microglia were isolated from wild type or TNF-null postnatal day 3 (P3) pups and plated at a density of 50,000 cells/well. 24 hrs post-plating, microglia cells were treated with 10 ng/mL or 1 μg/mL lipopolysaccharide (LPS). Cells were fixed and stained with rat anti-mouse CD45 to assess microglia morphology. TNF-null microglia cells display vacuolation and decreased perinuclear CD45 immunoreactivity. Scale bar = 50 μm.

FIGURE 3.7

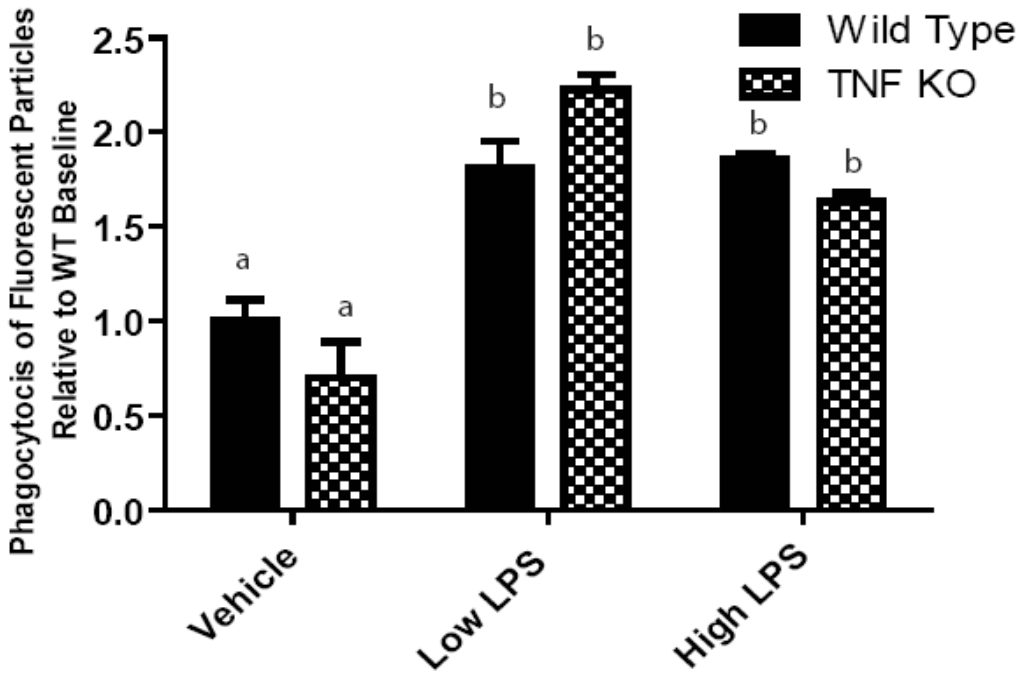
Phagocytocis of Fluorescent Particles
Relative to WT Baseline
2.5
2.0
1.5
1.0
0.5
0.0
Wild Type
TNF KO
a
a
b
b
b
b
Vehicle
Low LPS
High LPS

Figure 3.7. Microglia from TNF-null mice display normal phagocytic responses. Primary microglia cells were isolated from wild type or TNF-null postnatal day 3 (P3) pups were plated and treated overnight at a density of 50,000 cells/well with low (10 ng/mL) or high (1 ug/mL) LPS. Phagocytic activity measured using the Vibrant Phagocytosis Assay (Invitrogen). Two-way ANOVA and Bonferroni's post hoc test. Conditions with different letters are significantly different from each other at $p < 0.05$.

FIGURE 3.8

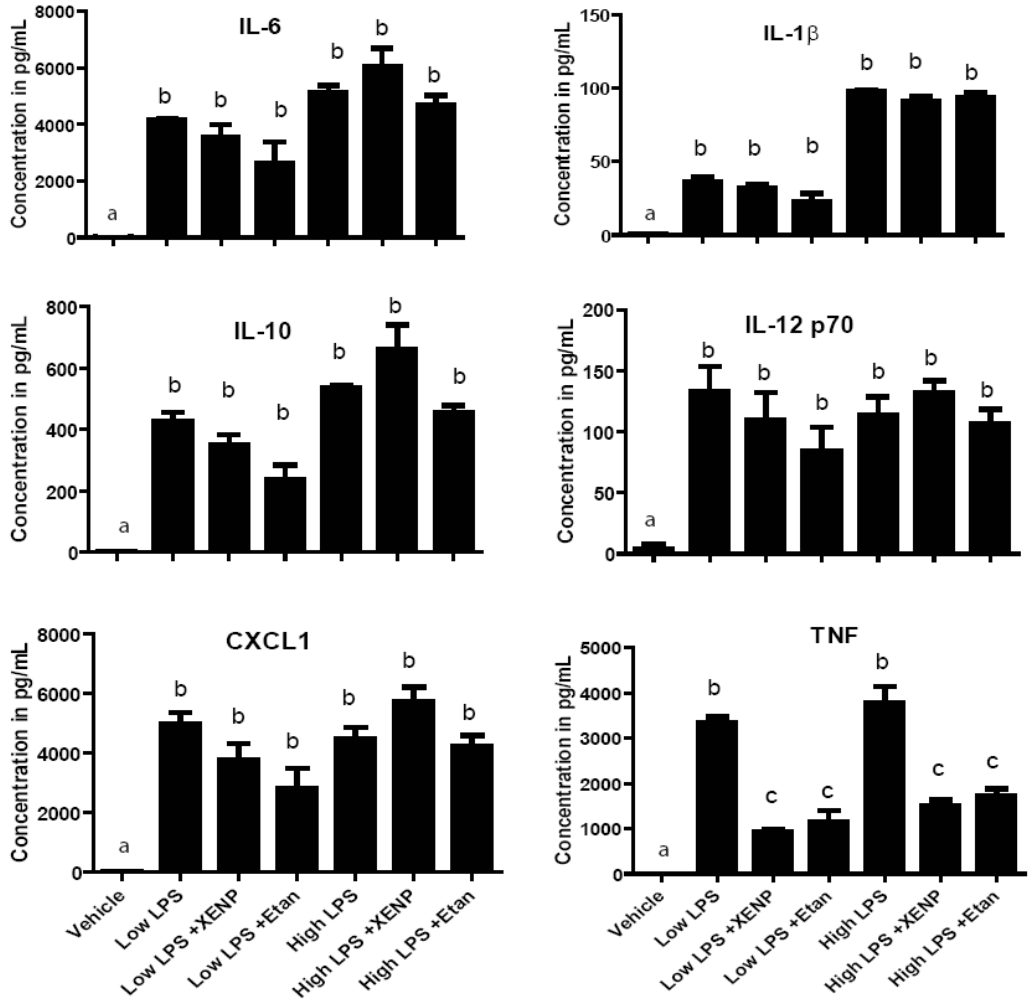
IL-6
Concentration in pg/mL
8000
6000
4000
2000
0
a
b
IL-1β
150
100
50
IL-10
800
600
400
200
IL-12 p70
CXCL1
TNF
5000
4000
3000
2000
1000
c
Vehicle
Low LPS
Low LPS +XENP
Low LPS +Etan
High LPS
High LPS +XENP
High LPS +Etan

Figure 3.8. Neutralization of solTNF with DN-TNF inhibitor XENP345 decreases amount of solTNF but not other cytokines.

WT microglia treated overnight with XENP345 (200ng/mL) and Etanercept (200ng/mL) and treated with LPS for 24 hours. Media was collected and run on an MSD 7-spot pro-inflammatory ELISA. n=3 for each condition. One-way ANOVA and Bonferroni's post hoc test. Conditions with different letters are significantly different from each other at $p<0.05$.

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CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Inflammatory mechanisms have been implicated in PD pathogenesis for quite some time. In PD patients and animal models of disease, TNF expression has been implicated in neuroinflammatory mechanisms of pathogenesis. TNF message and protein levels are elevated in post mortem brain tissue and cerebrospinal fluid (Mogi et al. 1994) and SNP's in the TNF promoter causing heavy production are associated with early onset PD (Nishimura et al. 2001). Based on the role of inflammation and microglial activation in the later stages of PD, I hypothesized that TNF is a critical mediator of inflammation-induced microglial activation and progressive dopaminergic neuron degeneration in animal models of PD. Furthermore, I hypothesized that specific anti-TNF therapy administered during the progressive phases of dopaminergic neuron death would provide neuroprotection and attenuate neurotoxin-induced microgliosis.

It has been shown that dopaminergic neurons are sensitive to TNF in a dose dependent manner *in vitro* (McGuire et al. 2001) and *in vivo* studies utilizing the mitochondrial complex 1 inhibitor MPTP have found that functional TNF signaling is critical in the death of dopaminergic neurons in the SNpc (Rousselet et al. 2002; Sriram et al. 2002; Ferger et al. 2004; Sriram et al. 2006). In animal models of PD, TNF message and protein levels are elevated following MPTP and 6-OHDA-induced

degeneration of nigrostriatal neurons, implicating TNF as an important inflammatory mediator in neurotoxin-induced cell death (Mogi et al. 1999). I have demonstrated that co and delayed inhibition of solTNF signaling in the form of a single intranigral injection of lentiviral delivered dominant negative TNF halted disease progression and attenuated locomotor deficits in a progressive 6-OHDA model of PD (Sauer and Oertel 1994) (Chapter 2). I also found that the same lentiviral delivered dominant negative TNF attenuated 6-OHDA-induced microgliosis in the SNpc *in vivo*, therefore effectively reducing the inflammatory response to the oxidative neurotoxin (Chapter 2). Previous studies have implicated TNF participation in dopaminergic neurodegeneration by utilizing non-specific TNF inhibitors such as minocycline (Du et al. 2001) and thalidomide (Ferger et al. 2004) as a form of anti-inflammatory therapy, but did not definitively implicate TNF-dependent toxicity in disease pathogenesis.

Previous studies using a solTNF specific dominant negative inhibitor attenuated dopaminergic neuron loss by 46%, strongly implicating TNF participation in the acute phases of neurotoxin and inflammation-induced nigrostriatal neuron death (McCoy et al. 2008) (Chapter 2). While these dominant negative TNF inhibitor studies provide strong evidence for TNF participation in acute phases of neurotoxin and inflammation induced cell death, they do not address the role of TNF in the progressive phases of nigrostriatal neuron loss, a critical phase of clinical relevance. The studies presented in this thesis have significantly advanced the understanding of the role of microglial-derived TNF in the progressive phases of dopaminergic neuron death in a neurotoxin based animal model

of PD as well as the specific role of TNF in regulating inflammation-induced microglial effector functions with relevance to PD.

Given the potential contribution of microglial activation to PD and the suggestion that anti-TNF therapies in the CNS may exert neuroprotective effects on vulnerable dopaminergic neuron populations, I also investigated the role of TNF in regulating microglia effector functions to gauge the potential detrimental effects of anti-TNF therapies on the microglia functional response. I have shown that TNF is a critical regulator of microglia-derived cytokine and chemokine expression and morphological phenotype switching in response to an inflammatory stimulant *in vitro.* Additionally I have also demonstrated that TNF is the critical microglia-derived inflammatory mediator compromising the viability of dopaminergic neuron-like cells (MN9Ds) *in vitro* therefore strongly implicating microglia-derived TNF as a critical mediator in inflammation-induced neurodegeneration *in vivo* (Chapter 3).

Based on the results presented in these studies, lentiviral delivered dominant negative TNF is an efficacious therapeutic strategy in both the early and progressive stages of neurotoxin-induced degeneration of dopaminergic neurons by inhibiting microgliosis and reducing inflammation-induced solTNF release. Furthermore, these results have implicated that TNF expression is critical for inflammation-induced cytokine release, chemokine release and morphological phenotype switching potentially offering further protection from microglia-derived cytotoxicity. Further research should be

focused on using this safe and novel anti-inflammatory approach in non-human primate models of PD and eventually in clinical trials.

Future Directions

Based on the results of anti-TNF therapy in 6-OHDA rat models of PD, it is important to investigate the role of TNF in a more progressive PD model involving multiple mechanisms of pathogenicity reminiscent of sporadic PD prior to clinical trials. I believe that it is important to investigate the efficacy of lentivirally delivered dominant negative TNF in a non-human primate MPTP model as well as a more pathologically relevant rodent model to further establish the role of TNF in dopaminergic neuron degeneration. Currently a rodent rotenone model of PD has been developed showing a progressive loss of dopaminergic neurons, enteric nervous system dysfunction, microgliosis and alpha-synuclein positive Lewy-body pathology (Cannon et al. 2009). I believe it is important to validate the results of the lentiviral dominant negative TNF studies in a model that bridges neurotoxin-induced dopaminergic neuron death with a more complete model of PD pathology. Utilizing this progressive rotenone model I could elucidate the role TNF in a model of PD that links genetics (alpha-synuclein pathology), environmental toxins (rotenone), and the inflammatory response (microgliosis). Studies have shown that alpha-synuclein can activate microglia promoting increased TNF expression, activation of the adaptive immune response, and contributing to inflammation-induced dopaminergic neuron degeneration (Zhang et al. 2005; Su et al. 2008). Based on the results from these studies, I hypothesize that lenti-DN-TNF can provide neuroprotection

in the progressive, more disease relevant stages of DA neuron loss while attenuating inflammation, alpha-synuclein pathology and microgliosis. If lentiviral delivered dominant negative TNF can attenuate microgliosis and inflammation-induced cytokine release, I predict that it can also provide neuroprotection from alpha-synuclein-induced microglial activation.

As a potential gene therapy, I have shown lentiviral dominant negative TNF transduces microglia and blocks activation *in vitro* (McCoy et al. 2008; McAlpine et al. 2009) and *in vivo* (Chapter 2). Considering microglial phenotype switching from a resting to activated state is important for CNS immune challenges, I believe it is important to fully investigate how DN-TNF expression affects microglia function *in vivo* and *in vitro*. I believe it is also important to investigate the neuroprotective role of DN-TNFs in response to additional neurotoxins like MPTP, rotenone, and paraqat *in vitro*.

Given that TNF signaling is important for sensitivity to MPTP-induced nigrostriatal degeneration (Sriram et al. 2002; Sriram et al. 2006) and TNF knockout microglia display reduced expression of pro-inflammatory cytokines following an inflammatory stimulus, I believe it is important pursue additional mechanisms involving TNF that allow for this neuroprotection. While it has been reported that differences in microglial activation between adult TNF knockout mice and wild type mice may have influenced the neuroinflammatory and neurodegenerative responses to MPTP (Sriram et al. 2006), a recent publication has identified Nurr1, a nuclear orphan receptor that is important for the development and/or maintenance of dopaminergic neurons and

transcriptional regulation of NF-κB dependent pro-inflammatory cytokine release (Saijo et al. 2009). Mutations resulting in decreased expression of Nurr1 are associated with familial PD and a prolonged inflammatory response that accelerates the loss of nigrostriatal neurons (Saijo et al. 2009). Given that TNF knockout microglia display reduced cytotoxicity on dopaminergic neuron-like cells and an attenuated inflammation-induced cytokine profile (Chapter 3), I hypothesize that TNF regulates Nurr1 expression in the context of inflammation-induced PD. Understanding how TNF affects Nurr1 expression may help identify novel therapeutic strategies for inflammation-induced dopaminergic neuron degeneration.

In the context of the CNS, dopaminergic neurons are a particular population of neurons that are vulnerable to inflammation-induced degeneration. Research elucidating the role of TNF in mediating neuroprotective and neurotoxic responses in the brain is currently underexplored. Due to the death receptor domain, TNFR1 has been shown to mediate neurotoxic effects while TNFR2 has been shown to mediate pro-survival effects in cortical neurons following excitotoxic stimuli (Marchetti et al. 2004). Currently, the role of cross-talk between the receptors in mediating dopaminergic neuronal survival remains unclear. Based upon the functional outcomes of TNFR signaling, I hypothesize that TNFR1 and TNFR2 signaling can antagonize one another to regulate dopaminergic neuron survival and that the TNFR1/TNFR2 expression ratio determines the outcome of TNF stimulus. Research focusing on the role of TNFR signaling may explain the differential vulnerability of dopaminergic neurons in the ventral midbrain further validating our anti-solTNF therapeutic approach.

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APPENDIX A

POSITIVE SELECTION OF POSTNATAL BRAIN MICROGLIA FOR PHENOTYPIC CHARACTERIZATION AT THE CELLULAR AND MOLECULAR LEVEL

BACKGROUND

Microglia are the monocyte-derived resident macrophages of the brain in charge of immune surveillance (Ransohoff and Perry 2009). Activated microglia secrete neurotrophic factors to limit tissue injury, protect vulnerable neuronal populations, and aid in brain repair processes. However, activated microglia can also overproduce prostaglandins, chemokines, cytokines, and reactive oxygen and nitrogen species which can have a deleterious effect on neuronal survival by enhancing oxidative stress and activating cell death pathways (reviewed in (McGeer and McGeer 2004), raising the interesting possibility that chronic microglia activation may contribute to the etiology and/or progression of neurodegenerative disease (reviewed in (Tansey et al. 2007). *In vitro* analyses of microglia phenotype and their effector functions is expected to significantly improve the ability to generate testable hypotheses about the *in vivo* role of microglia in normal and pathophysiological states in the brain. Yet conventional methods used to obtain homogeneous populations of microglia require maintainence of primary neuron-glia cultures for extended periods of time (from 1 to 2 weeks), increasing the likelihood for loss of morphological and functional microglial phenotype.

OBJECTIVE

To shorten the time between brain harvesting and microglia isolation, and characterization we utilized the MACS® neural dissociation kit followed by OctoMACS® CD11b magnetic bead isolation technique to positively select for brain microglia expressing the pan-microglial marker Cd11b, a key subunit of the Membrane Attack Complex (MAC). Primary microglia from C57Bl6 mice were plated for next day analyses of morphology and cellular markers by immunocytochemistry or for analysis of gene expression under resting or LPS-stimulated conditions. To compare the activation state of wild-type microglia with that of microglia isolated from TNF-null mice, CD11b positively selected microglia were immediately fixed and double labeled for Cd11b and the activation marker F4/80 and subjected to flow-cytometric analyses.

RESULTS

Immunocytochemical analyses of primary microglia positively selected with CD11b magnetic beads reveal morphological changes upon LPS stimulation

Methods: Primary microglia were isolated from eight postnatal day 5 (P5) C57Bl/6 wild-type pups using the MACS® neural dissociation it (Miltenyi Biotec). The total cell number was determined by trypan-blue exclusion and found to be 40 million. To enrich for primary microglia, OctoMACS® CD11b magnetic bead separation (Miltenyi Biotech) was performed according to the protocol provided by the manufacturer. The number of cells obtained post-magnetic CD11b bead isolation was 4 million or 10% of the total

number of cells in the single-cell suspension obtained with the MACS neural dissociation kit, which is in the expected range. After (1) day *in vitro* in DMEM/F12 with 10% FBS the cells were treated with lipopolysaccharide (LPS *E. coli* strain O111:B4, 10ng/mL or 1 ug/mL for 24 hours) then fixed in 4% paraformaldehyde in PBS and processed for fluorescence immunocytochemistry as previously described (McCoy et al. 2006). The antibody dilutions were as follows: CD45 (Serotec) 1:250, CD68 (Serotec) 1:250, and Hoechst 33258 (Invitrogen) 1:20,000. Appropriate Alexa conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:1000. All images were acquired using the CoolSnap CCD ES monochromatic camera and Metamorph software. (Figure A1) illustrates images from a representative experiment demonstrating that the number of cells found to be immunoreactive for CD45 was approximately (99%) and for CD68 was (98%), indicating that the purity of the isolated microglia population is high.

Gene expression analyses of microglia isolated using CD11b magnetic beads indicate responsiveness to inflammatory stimuli

Methods: Primary microglia were isolated from ten postnatal day 5 (P5) C57Bl/6 wild-type pups by MACS® neural dissociation followed by OctoMACS® Cd11b magnetic bead positive selection and then plated in 6-well plates at a density of 0.5 million/well. After 2 days in culture in DMEM/F-12 supplemented with 10% FBS, cells were stimulated with lipopolysaccharide (LPS, 1ug/mL) for 4 hrs at 37 degrees in a humidified CO2 incubator. Cells were harvested in RNA STAT60 (Tel-Test, Friendswood, TX) for isolation of total RNA and briefly treated with DNAse I (Invitrogen) then reverse

transcribed into cDNA using Superscript II RNAse H-reverse transcriptase (Invitrogen). Real-time quantatative polymerase chain reaction (qPCR) was performed as previously described (Kurrasch et al. 2004; Lee et al. 2008) using an ABI Prism 7000 Detection System (Applied Biosystems Inc.). All reactions took place in 384-well format with 50 ng cDNA , 10 uL SYBR green PCR mastermix, and 150 nM each forward and reverse primer. Oligonucleotide primers for TNF, IL-1β, iNOS, MIP-1α, CD45 were designed using Primer Express software and purchased from Integrated DNA Technologies (Coralville, IA). All reactions were performed in triplicate and levels of various mRNAs were normalized using cyclophilin as the housekeeping gene. Gene expression analyses in (Figure A2) revealed low level expression of mRNA for iNOS, MIP1α, IL-1β, TNF, CD45 which increased significantly upon stimulation with LPS.

Quantitative flow-cytometric analyses of primary microglia isolated from brains of wild-type or TNF-deficient neonatal mouse pups reveal similar levels of basal activation states

Methods: Primary microglial cells were isolated from eight postnatal day 5 TNF-deficient pups or wild-type pups by MACS® neural dissociation followed by OctoMACS® CD11b magnetic bead separation (Miltenyi Biotech). Cells were labeled with a fluorescently conjugated F4/80 and CD11b antibody, fixed (1% PFA) and sorted by flow cytometry as previously described (Lee et al. 2008). FACS analysis from the live cell population in (Figure A3) revealed TNF-deficient mice had similar numbers of F4/80-positive microglia relatively similar degree of activation compared to wild-type mice.

SUMMARY

Primary microglia harvested from neonatal mouse pups can be isolated using the MACS® neural dissociation kit followed by positive selection with OctoMACS® Cd11b magnetic beads. This protocol yields a viable and highly pure (> 95%) microglial population of approximately 500,000 cells per pup that is amenable for *in vitro* characterization within hours or days after being harvested from brain tissue. The ease of isolation enables investigators to perform molecular and cellular analyses without having to wait 1-2 weeks to isolate microglia by conventional methods involving mechanical agitation off astrocytes beds.

FIGURE A1

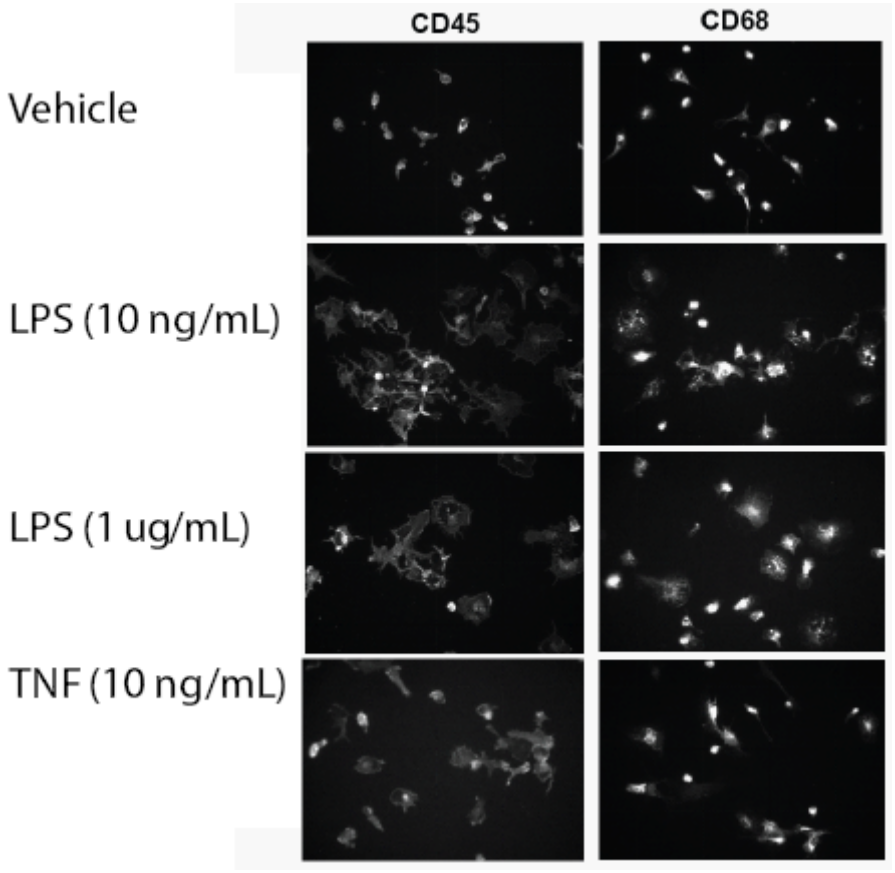

Figure A1. Primary microglia positively selected with CD11b magnetic beads reveal normal morphological changes upon LPS stimulation.

Primary microglial cells were isolated from postnatal day 5 (P5) C57Bl/6 pups using the MACS® neural dissociation kit followed by CD11b magnetic bead separation. Cells were plated and treated for 24 hours with LPS or TNF, fixed, and stained with anti-CD45 (Serotec) and anti-CD68 (Serotec). Immunocytochemical analysis revealed normal morphological changes and expression of microglial markers CD45 and CD68 after stimulation LPS or TNF.

FIGURE A2

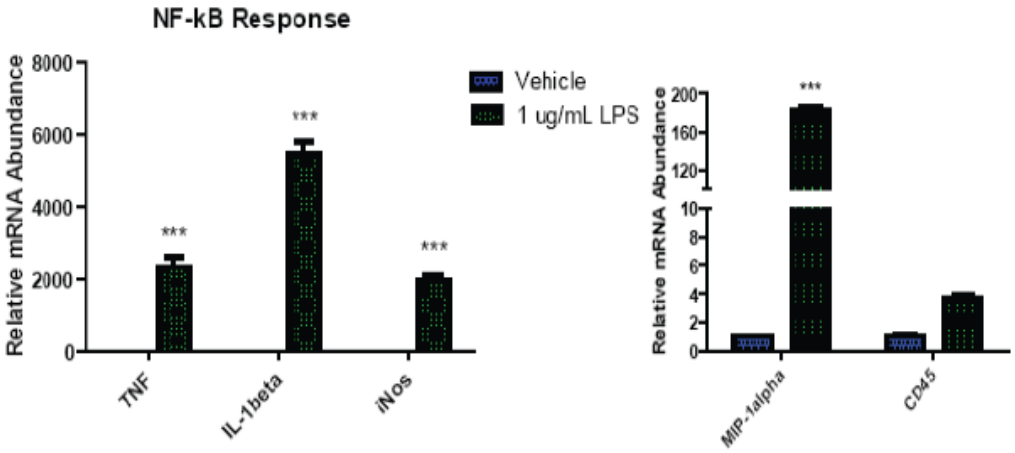
NF-kB Response
Relative mRNA Abundance
8000
6000
4000
2000
0
***
***
***
Vehicle
1 ug/mL LPS
TNF
IL-1beta
iNos
Relative mRNA Abundance
200
160
120
10
8
6
4
2
0
***
MIP-1alpha
CD45

Figure A2. Primary microglia positively selected with CD11b magnetic bead separation are responsive to inflammatory stimuli.

Primary microglia were isolated from ten postnatal day 5 (P5) C57Bl/6 pups by MACS® neural dissociation followed by CD11b magnetic separation and treated for 4 hours with 1ug/mL LPS. Total RNA was harvested using phenol/chloroform extraction and reverse transcribed into cDNA. Quantitative PCR analysis revealed low level expression of mRNA for iNOS, MIP1α, IL-1β, TNF, CD45 which increased significantly upon stimulation with LPS.

FIGURE A3

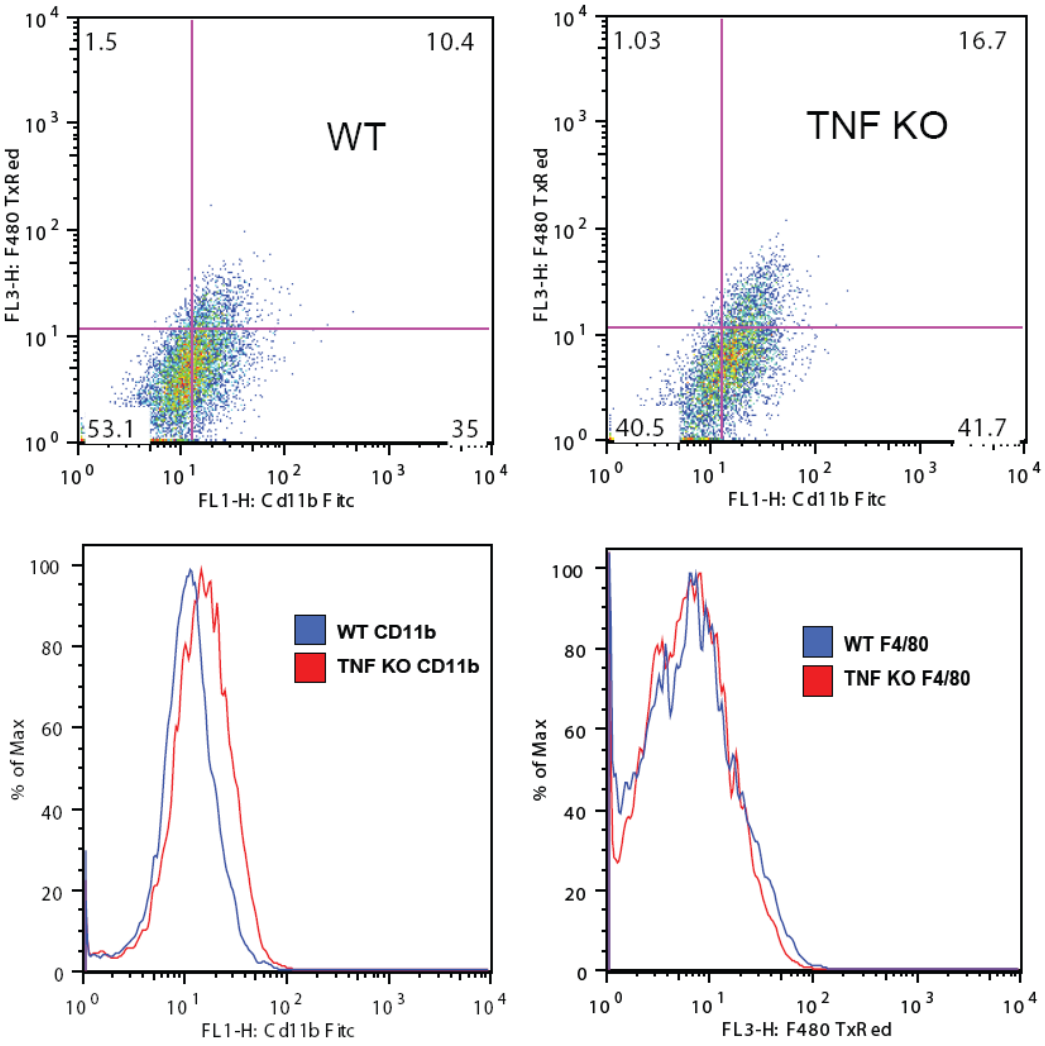
1.5
10.4
WT
53.1
35
FL3-H: F480 TxRed
FL1-H: Cd11b Fitc
1.03
16.7
TNF KO
40.5
41.7
WT CD11b
TNF KO CD11b
% of Max
WT F4/80
TNF KO F4/80

Figure A3. Primary microglia positively selected with CD11b magnetic bead separation display the expected microglial cell-surface markers.

Primary microglia were isolated from postnatal day 3 (P3) TNF-deficient (TNF KO) and wild type (WT) pups by MACS® neural dissociation and CD11b magnetic bead separation. Cells were double-labeled with an antibody against the activation marker F4/80 conjugated to the fluorophore Texas Red (Caltag) and an antibody against the microglial marker Cd11b conjugated to the fluorephore FITC (Miltenyi Biotec), fixed in 1% paraformaldehyde and subjected to flow cytometry. FACS analyses of the live cell population revealed no significant differences in the activation status of the microglia isolated from the two different genotypes.

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