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Innate Immunity Mechanisms In Parkinson Disease

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INNATE IMMUNITY MECHANISMS IN PARKINSON DISEASE

by

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ABSTRACT

Parkinson disease (PD) is a late onset, progressive neurodegenerative movement disorder with cardinal symptoms of tremor at rest, bradykinesia, postural instability, and rigidity. These motor symptoms of PD are caused by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). However, despite PDs first modern observation in 1817, little is understood about the causes and molecular mechanisms behind dopaminergic neuron loss. The relatively weak understanding of pathological mechanisms has hindered the development of treatments to slow or halt the progression of PD. However, recently, mounting evidence from post-mortem, imaging, and retrospective studies suggest an important role for inflammation in the etiology of PD. Post-mortem analysis has implicated an elevated level of pro-inflammatory cells and cytokines in the brains and cerebrospinal fluid of patients with PD compared to control. Dopaminergic cells in the SNpc have been suggested to be particularly sensitive to these cells and cytokines found in post-mortem analysis, making the examination of inflammation as a pathological modifier or cause of PD of high therapeutic interest.

Familial studies of PD have yielded some clues to target inflammation in the study of PD. From these studies, mutations in *leucine rich repeat kinase 2* (*LRRK2*) have been shown to be the largest genetic cause of PD. Outside of PD, *LRRK2* is genetically and biochemically linked to inflammation. Genetically, *LRRK2* is associated with

inflammatory disorders such as leprosy and Crohn's Disease through genome wide association studies. At a biochemical level, expression studies show very high LRRK2 protein abundance in innate immune cells. Genetic or pharmacological manipulation of LRRK2 results in altered immune response, making LRRK2 an excellent mechanism to study inflammation in PD. Throughout this dissertation, we will utilize genetic manipulations of *LRRK2* to knockout or overexpress kinase active forms of LRRK2 coupled with robust models of inflammation both *in vitro* and *in vivo* to understand how LRRK2 influences immune cells and provide insight into inflammatory mechanisms in PD.

Keywords: PARK8, Macrophage, Chemotaxis, Thioglycollate, Innate Immunity

DEDICATIONS

This dissertation is dedicated to my family, friends, and mentors who have helped to foster my interest and abilities in science. Without your help, I would not be the person that I am today and would never have been able to reach this point.

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AD	Alzheimer Disease
ADP	Adenosine Di-Phosphate
ATP	Adenosine Tri-Phosphate
BAC	Bacterial Artificial Chromosome
CD	Cluster of Differentiation
DA	Dopamine
DAB	3,3'-Diaminobenzidine
EAE	Experimental Allergic Encephalitis
ELISA	Enzyme Linked Immunosorbant Assay
GS-LRRK2	G2019S LRRK2
GWAS	Genome Wide Association Study
HRP	Horseradish Peroxidase
i.p.	intra peritoneal
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IP	Immunoprecipitation
KO	Knockout
LPS	Lipopolysaccharide
LRRK2	Leucine Rich Repeat Kinase 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MS	Multiple Sclerosis
nTg	Non-transgenic
PBS	Phosphate Buffered Saline
PD	Parkinson disease
PET	Positron Emission Tomography
rAAV2	Recombinant Adeno Associated Virus 2
SNpc	Substantia Nigra pars compacta
TBS	Tris Buffered Saline
TEPM	Thioglycollate Elicited Macrophages
TH	Tyrosine Hydroxylase
TIP	Thioglycollate Induced Peritonitis
TNF	Tumor Necrosis Factor
WT	Wildtype
WT-LRRK2	Wildtype LRRK2

INTRODUCTION

CLINICAL PRESENTATION AND TREATMENT OF PARKINSON DISEASE

Parkinson disease (PD) is a progressive, chronic, neurodegenerative disorder that presents initially with the cardinal motor symptoms of tremor at rest, rigidity, postural instability, and slowness of movement (bradykinesia) (Fahn, 2003). These motor symptoms are associated with the loss of dopamine producing cells in the Substantia Nigra pars compact (SNpc). At first diagnosis, these motor symptoms are often asymmetric, effecting one limb more predominantly (Jankovic, 2008). However, as disease progresses, both sides of the body become affected and the motor symptoms can progress to where normal movement is impossible, and often results in severe morbidity. While the cardinal symptoms of PD are motor related, a number of non-motor symptoms exist that may even pre-date the motor symptoms of disease (Chaudhuri et al., 2006). Decreased gut motility, anosmia, dementia, and REM sleep disorder are all associated with non-motor symptoms of disease and increase the severe morbidity of PD (Langston, 2006, Caballol et al., 2007, Suzuki et al., 2015).

There are an estimated 1 million people in the United States currently diagnosed with PD, and another ~10 million patients worldwide, representing the second most common neurodegenerative disorder (Zhang et al., 2002, de Lau and Breteler, 2006). The costs of PD are estimated at over $25 billion per year in the United States alone, representing a large burden to care givers and the healthcare system. The largest risk factor for PD is age, with average age of onset of PD at 60 years old (Van Den Eeden et al., 2003, Jankovic, 2008). Both the population of patients with PD and burden caused by disease will increase as the United States population demographics shifts to an older

median age with the ageing of the Baby Boomer Generation. This shift in age demographics and large economic burden makes PD and other neurodegenerative disorders a major public health concern.

Symptomatic treatments of PD exist; however, there is no treatment that slows or alters the course of PD (West, 2014). Current symptomatic treatments rely on the modulation of dopamine through receptor antagonists, inhibition of break down, or through increasing dopamine synthesis (Rascol et al., Braun et al., 1987, Kanda et al., 2000). The most common, and oldest, pharmaceutical is L-DOPA (levodopa) (Marsden and Parkes, 1977). L-DOPA is the biochemical precursor to dopamine, and production of L-DOPA is the rate limiting step in dopamine synthesis. By administration of L-DOPA, dopamine synthesis is increased. L-DOPA and other dopamine based therapies produce reasonable symptomatic relief of PD motor symptoms, but their effectiveness decreases as death of SNpc neurons continues and tolerance develops. This necessitates higher and higher doses, but this leads to severe side effects, and eventually side effects outweigh the benefits of treatments (Melamed et al., 2007). Non-pharmacological therapies for PD exist, but they are only available to a small subset of PD patients. Deep brain stimulation can be very effective at eliminating or reducing the motor symptoms of PD, but requires serious surgery and represents a recovery period that is only afforded by a small subset of patients with PD. The large burden that PD represents, which will increase with the ageing of the Baby Boomer Generation, as well as the lack of disease modifying therapy, necessitates a better understanding of the underlying cause and molecular pathways behind PD.

PATHOLOGY AND ETIOLOGY OF PARKINSON DISEASE

PD is pathologically characterized by the loss of large, neuromelinated, dopamine producing neurons in the SNpc. Accompanying, and possibly preceding, the loss is the elimination of the SNpc dopaminergic neuron's synapses onto medium spiny neurons in the striatum. By the time of diagnosis, it is estimated that between 50 and 70% of SNpc neurons are lost (Kordower et al., 2013). This provides additional impetus for the development of disease modifying therapies in PD. If the death of SNpc neurons is slowed or stopped, it may prevent or greatly reduce PD associated morbidity.

In addition to loss of neuromelinated DA producing neurons in the SNpc, PD is characterized by the deposition of α-synuclein into proteinaceous inclusions in the cell bodies of remaining SNpc neurons deemed Lewy Bodies (Spillantini et al., 1997). These inclusions are the central pathological hallmark of disease. A confirmatory PD diagnosis on autopsy requires the pathological finding of accumulated α-synuclein into Lewy Bodies and Lewy Neurites (Braak et al., 2003). These accumulations, while of unknown cause and consequences with hypothesis suggesting they are protective are harmful, do correlate to the severity and stage of PD (Ross and Poirier, 2005).

The etiology and pathways of PD are largely unknown. Originally, PD was thought to be a purely environmental disease. This was supported by the discovery that the toxins MPTP, rotenone, and 6-OHDA could closely mimic the cell death and motor symptoms of PD in rodents and humans (Langston et al., 1984a, Langston et al., 1984b, Bove et al., 2005). However, this began to be challenged by the discovery of a kindred of patients that had an autosomal dominant inherited form of PD. This kindred eventually had their familial form of disease attributed to mutations in the *SNCA* gene, encoding α-synuclein (Polymeropoulos et al., 1996). A number of other loci following Mendelian

inheritance have since been attributed to causing genetic forms of PD including *LRRK2*, *Parkin*, *PINK1,* and *DJ1* (Farrer, 2006, Nalls et al., 2014). The discovery of these genes following Mendelian patterns, as well as genome wide association studies (GWAS) implicating a number of other genes in susceptibility to PD, challenged has challenged solely environmental causes of PD. Additionally, recent evidence suggests that upwards of 30% of PD cases are genetic in origin (Nalls et al., 2014). These genetic loci have provided some interesting clues in the etiology of PD, and may provide a molecular clue to dysfunctional pathways in PD which ultimately may lead to the design of the first disease modifying therapy for PD.

LEUCINE RICH REPEAT KINASE 2

The PARK8 locus, encoding LRRK2, was originally identified as a Mendelian inherited autosomal dominant locus in a Japanese kindred (Funayama et al., 2002). Since this discovery, mutations in *LRRK2* have been identified as the single largest cause of PD. *LRRK2* mutations account for 2 to 5% of PD cases in a typical "Western" population and upwards of 30% in some ethnic minorities such as Ashkenazi Jews and North African Arabs (Lesage et al., 2006, Ozelius et al., 2006, Healy et al., 2008, Bar-Shira et al., 2009). Mutations in *LRRK2* causes PD that is indistinguishable from idiopathic PD in pathology, age of onset, and symptoms (Healy et al., 2008). This extreme clinical overlap with idiopathic PD sets apart *LRRK2* with the other loci associated with PD, as almost all other PD linked genes cause an early onset form of Parkinsonism. Additionally, this clinical overlap with idiopathic PD suggests that LRRK2 activity may be involved in underlying pathology of all PD cases, making LRRK2 an exciting target for disease modifying PD therapy (West, 2014).

LRRK2 was originally discovered as part of an evolutionarily conserved family of GTPases in Disctostylium slime molds, making LRRK2 highly evolutionarily conserved (Bosgraaf et al., 2002). LRRK2 is a large, multi-domain ~280 kDa protein. At its N-terminus, there are several protein-protein interaction domains including leucine rich repeats, armadillo, and ankryin repeats with an additional protein-protein interacting WD40 domain at its C terminus. LRRK2 also has Roc (GTPase), COR, and serine threonine kinase domain. The presence of a GTPase and serine threonine kinase domain makes LRRK2 unique in the kinome, suggesting possible novel mechanisms of kinase regulation and activity. These three domains together encompass the "catalytic core" of the protein comprising LRRK2's different enzymatic activities.

Several mutations have been identified in *LRRK2*. These mutations cluster within the catalytic core of the protein (Cookson, 2010). The common theme among these mutations appears to be conferring increased kinase activity of the protein, although this is somewhat controversial (West et al., 2005, Sheng et al., 2012). The most common mutation in *LRRK2*, the G2019S mutation in the kinase domain, has been consistently shown to increase the kinase activity of the protein ~2 to 3 fold. Cellular consequences of this increased kinase activity are unclear, but have been shown to cause cellular toxicity in diverse model systems (MacLeod et al., 2006, Smith et al., 2006, Gillardon, 2009, Parisiadou et al., 2009, Sheng et al., 2012). A better understanding of LRRK2 signaling and what pathways it interacts with may lead to a better understanding of idiopathic PD as well as provide exciting potential for therapeutic targeting.

INFLAMMATION AND PARKINSON DISEASE

A recent hypothesis on the underlying pathology of PD is that inflammation may drive or exacerbate dopaminergic cell death (Hunot and Hirsch, 2003, Hirsch et al., 2012). Autopsy, PET imaging, and retrospective studies all indicate the inflammation plays a role in the etiology of PD. On autopsy, enhanced microglial activation, peripheral inflammatory cell recruitment, and immunoglobulin deposition are observed in and around the SNpc of PD patients compared to healthy control (McGeer et al., 1988). Additionally, pro-inflammatory cytokines TNF and IL-6 have been found in cerebrospinal fluid of patients with PD, and are positively correlated with increased disease severity (Muller et al., 1998, Nagatsu et al., 2000, Hofmann et al., 2009, Scalzo et al., 2010). PET imaging studies using ligands for activated inflammatory cells show 25 to 50% increase in binding compared to healthy, age matched controls in brain areas associated with PD (Gerhard et al., 2006, Bartels et al., 2010). Retrospective studies of PD have indicated that non-steroidal anti-inflammatory drugs reduce risk of PD moderately. Taken together, there is compelling evidence in humans to suggest an inflammatory component to PD.

Animal models of PD also suggest that inflammation may drive or exacerbate SNpc cell loss. Toxin based models of PD, such as the MPTP intoxication model, have a clear increase in inflammation in the SNpc (Bove et al., 2005, Pattarini et al., 2007). Additionally, pharmacological inhibition or genetic ablation of pro-inflammatory proteins provide moderate protection to dopaminergic neurons in the SNpc (Aubin et al., 1998, Wu et al., 2002, Sriram et al., 2006, Zhao et al., 2007, Morganti et al., 2012). Other models of PD, such as the rAAV2 based α-synuclein overexpression model both have dopaminergic cell death as well as inflammation (Kirik et al., 2003, St Martin et al.,

2007, Sanchez-Guajardo et al., 2010). Again, genetic ablation of pro-inflammatory proteins results in dopaminergic neuron protection (Cao et al., 2012, Harms et al., 2013). More evidence of inflammation in PD is discussed in chapter 1 of the thesis. Taken together, these results from a broad range of study types in both animals and humans implicate inflammation in the etiology of disease. However, mechanisms behind this inflammation and whether it exacerbates or initiates disease are still not understood.

LEUCINE RICH REPEAT KINASE 2 AND INFLAMMATION

In addition to *LRRK2's* genetic association to PD, GWAS implicates common variants of *LRRK2* in the susceptibility of Crohn's Disease and Leprosy, disorders with prominent inflammatory components (Van Limbergen et al., 2009, Zhang et al., 2009). When brain or innate immune cells are exposed to lipopolysaccharide (LPS) or interferon gamma, prototypical pro-inflammatory stimuli, LRRK2 increases in both expression and activity, implicating LRRK2 is responsive to inflammatory stimuli (Gardet et al., 2010, Dzamko et al., 2012, Moehle et al., 2012, Kuss et al., 2014). Examination of expression patterns for LRRK2 show that LRRK2 expression is particularly high in mature CD14+CD16+ cells (likely monocytes/macrophages) as well as CD19+ cells of the peripheral immune system (Thevenet et al., 2011). Both genetic and biochemical evidence points to a role for LRRK2 in the immune system. However, there is a lack of mechanistic insight into how LRRK2 and mutations in *LRRK2* affect the immune system.

With LRRK2's genetic and biochemical association to both PD and inflammatory disorders, LRRK2 may be particularly useful as a tool to understand how inflammation influences pathogenesis of PD. In this thesis, genetic manipulation of *LRRK2* either through transgenic mouse technology or through knockdown will be used in conjunction

with robust *in vitro* and *in vivo models* of inflammation to address the role of inflammation in PD. Additionally, these tools will allow a better understanding of pathways LRRK2 influences inside innate immune cells. These findings will also aid in the testing and design of LRRK2 inhibitors, which represent an exciting opportunity for novel therapeutic for PD.

M1 AND M2 IMMUNE ACTIVATION IN PARKINSON DISEASE: FOE AND ALLY?

by

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ABSTRACT

Parkinson Disease (PD) is a chronic and progressive neurodegenerative disorder of unknown etiology. Autopsy findings, genetics, retrospective studies, and molecular imaging all suggest a role for inflammation in the neurodegenerative process. However, relatively little is understood about the causes and implications of neuroinflammation in PD. Understanding how inflammation arises in PD, in particular the activation state of cells of the innate immune system, may provide an exciting opportunity for novel neuroprotective therapeutics. We analyze the evidence of immune system involvement in PD susceptibility, specifically in the context of M1 and M2 activation states. Tracking and modulating these activation states may provide new insights into both PD etiology and therapeutic strategies.

INTRODUCTION

Parkinson Disease (PD) is a chronic, progressive neurodegenerative disorder characterized by hallmark symptoms that include bradykinesia, ataxia, rigidity, and resting tremor. Pathologically, PD is characterized by the severe loss of melanated dopaminergic neurons in the substantia nigra pars compacta (SNpc), and deposition of α-synuclein into Lewy bodies and Lewy neurites in many remaining neurons (Spillantini et al., 1997, Spillantini et al., 1998). Markers of inflammatory responses have long been noted in and around the SNpc (Nagatsu et al., 2000, Hunot and Hirsch, 2003, Khandelwal et al., 2011). Initially, post-mortem examination using immunohistochemical techniques revealed a spectrum of different types of immune cells, as well as cytokines, in PD brain tissue (McGeer et al., 1988, Boka et al., 1994, Imamura et al., 2003). Later, ligands selective for activated immunological cells also demonstrated activation and inflammatory responses, both in early and late stages of disease (Gerhard et al., 2006, Bartels et al., 2010). Retrospective studies of anti-inflammatory therapeutics also implicates inflammation in some aspect of etiology (Gagne and Power, 2010). Several possibilities exist for understanding aspects of inflammation in PD: particular immunological responses are detrimental, benign, or beneficial. PD is not an acute disorder, so inflammatory responses may show temporal association with disease progression, where an initial response is beneficial and later becomes detrimental.

Therapeutic targeting of inflammation underlying disease pathogenesis represents an exciting approach for novel neuroprotective strategies. However, an incomplete understanding of the role of inflammation in PD will likely hinder successful implementation of rationally-derived therapeutics. The canonical role of microglia as

predominant resident immune cell in the brain has led to the hypothesis that these cells underlie the inflammatory processes noted in PD (Qian and Flood, 2008, Long-Smith et al., 2009). However, there is emerging evidence that peripheral immune cells may also be changed in PD (Hisanaga et al., 2001, Saunders et al., 2012, Funk et al., 2013). Understanding inflammation in the context of M1 and M2 activation paradigms may help clarify interpretation of these complex and dynamic processes.

In this review, we will discuss a context for M1 and M2 microglia and macrophage activation states. Emerging evidence for a critical role for these cells and activation states in PD will also be discussed, along with predictions about how modulating or blocking activation might be beneficial for the treatment of PD.

M1 ACTIVATION STATE

Macrophage activation states are understood within a continuum of activation paradigms that mirrors the responses of lymphocytes. The M1, or classical activation state, is associated with pro-inflammatory and pro-killing functions defined by macrophage responses to microbes. The M1 response was defined through studying the anti-microbial activity of macrophages towards Bacillus and Listeria after secondary exposure to other bacteria (Mackaness, 1962). This study highlighted an antigen dependent mechanism for macrophage activation, which has since been parsed into the prototypical M1 response.

The most common methods to track M1 responses include analysis of both secreted factors as well as cell surface and intracellular markers that increase in abundance. The M1 state causes the release of several pro-inflammatory cytokines including tumor necrosis factor (TNF), interleukin 6 (IL-6), IL-12, and IL-1β as well as several chemokines such as C-C motif ligand 2 (CCL2) and C-X-C motif ligand 10 (CXCL10). The production of these cytokines and chemokines are widely used as markers for the M1 state. Additional non-cytokine/chemokine markers of the M1 state include increased cell surface expression of major histocompatibility complex II (MHCII), increased cluster of differentiation marker 86 and 16/32 (CD86, CD16/32), and increased expression of inducible nitric oxide synthase (iNOS)(Nau et al., 2002, Martinez et al., 2006).

To induce a M1 state in macrophages *in vitro* and *in vivo,* more defined stimuli have been utilized to elucidate M1 responses in macrophages, including cytokine interferon-gamma (IFNγ) and lipopolysaccharide (LPS), an outer membrane component

of gram-negative bacteria. IFNγ signals through a dimer of the IFNγ receptor 1 and 2. Activated IFNγ receptors cause the recruitment of Janus kinase 1 and 2 (JAK1/2) which in turn phosphorylates and activates STAT1 and interferon regulatory factors (IRF), mainly IRF1 (Hu and Ivashkiv, 2009). The signal transduction cascade induces transcriptional changes that up-regulate the expression of cytokines, receptors, and hundreds of other genes associated with the M1 response (Dalton et al., 1993, Huang et al., 1993, Waddell et al., 2010).

The other prototypical M1 stimulus, LPS, signals through a different class of pattern recognition receptors known as toll-like receptors (TLR). LPS binds to TLR4 along with co-receptors MD2 and CD14. Other TLR4-independent LPS activation responses have also been described (Hagar et al., 2013, Kayagaki et al., 2013). TLR4 activation stimulates the transcription factors NFKβ, STAT5, AP1, and IRFs, through MyD88 and TRIF, which go on to cause a transcriptional up-regulation of a similar set of genes as IFNγ (Hu and Ivashkiv, 2009). Other TLRs show affinity for a variety of ligands. TLR2 binds a wide variety of microbial products including LTA. TLR3 binds dsDNA, TLR7 binds ssRNA, and TLR9 binds unmethylated CpG islands in DNA. These TLR activation cascades, through MyD88 or TRIF, skew macrophages towards the M1 state (Takeda and Akira, 2004, Yamamoto and Takeda, 2010, Casanova et al., 2011).

Granulocyte-modifying colony stimulating factor (GM-CSF) is another, more recently described stimulus to the M1 activation paradigm (Lacey et al., 2012, Bayer et al., 2013). However, as opposed to LPS, GM-CSF can induce pleomorphic activation states that can show elements of both M1 and M2 activation states (Weisser et al., 2013). GM-CSF binds to a large receptor that is comprised of a dodecamer of subunits (Hansen

et al., 2008). Intracellularly, GM-CSF utilizes many of the same effectors as that of the TLRs, but also utilizes ERK and AKT signal transduction pathways (Krausgruber et al., 2011). GM-CSF stimulation can produce similar cytokine responses to that of LPS, but to a much lesser extent as compared with other M1 stimuli (Lehtonen et al., 2007). GM-CSF function is understood through knockout studies in rodents as well as mutations in human populations, which highlight GM-CSF as a driver of hematopoietic (pre-cursors to myeloid lineage cells) cell differentiation and proliferation (Dranoff and Mulligan, 1994, Dirksen et al., 1997). The M1 activation state is graphically depicted in Figure 1, and listed in Table 1.

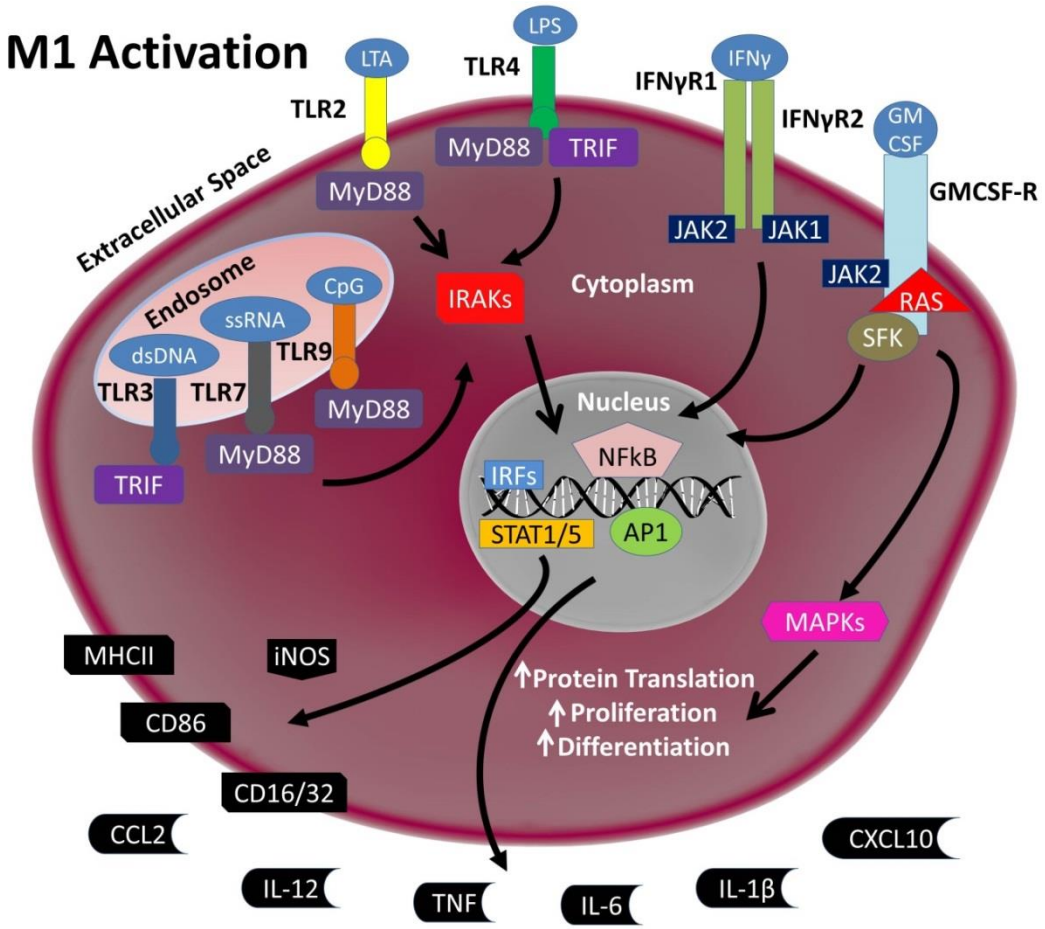

FIGURE 1. **Schematic of M1 Signaling.** A broad array of stimuli can induce an M1 pro-inflammatory response. TLR3 binds double stranded DNA (dsDNA). TLR7 binds single stranded RNA (ssRNA). TLR9 binds unmethylated CpG islands in stretches of DNA. Broadly, TLR3, 7, and 9 defend against viral infection. TLR2 binds lipoteichoich acid (LTA) and some other microbial products. TLR4, along with co-receptors MD2 and CD14 (not shown), binds lipopolysaccharide (LPS). TLRs signal through MyD88 and/or TRIF to activate the IRAK family of kinases. IRAKs then cause the translocation of several transcription factors to the nucleus, namely NFkβ, AP1, STAT5, and IRFs. Interferon-gamma (IFNγ) binds and activates a heterodimer of the IFNγ Receptor 1 and 2 (IFNγR1/2). Activation then leads to JAK1/2 activation which leads to STAT5 translocation to the nucleus. Granulocyte modifying- colony stimulating factor (GM-CSF) binds a dodecamer of subunits that forms the GM-CSF Receptor (GMCSF-R), which in turn activates JAK2, RAS and SFK. In addition to causing STAT5 translocation to the nucleus, GM-CSF alters the RAS pathway to increase protein translation, proliferation, and differentiation in innate immune cells. NFkβ, AP1, STAT1/5, and IRFs translocation to the nucleus leads to up-regulation of iNOS as well as the cell surface markers MHCII, CD86, and CD16/32. The production and release of cytokines TNF, IL-6, IL-1β, and IL-12 and chemokines CCL2 and CXCL10 are also up-regulated.

	M1	M2		
		M2a	M2b	M2c
Stimulus	LPS, IFNγ, LTA, GMCSF, dsDNA, ssRNA, unmethylated CpG Islands	Il-4	TLR and IgG	IL-10
Released Factors	TNF, IL-6, IL-12, IL-1β, CCL2, CCL10	IL-10, Polyamines	TNF, IL-6, IL-1β, IL-10	Matrix Proteins, TGFβ, IL-10
Cell Surface Markers	CD86, CD16/32, MHCII	CD206, SRs	CD86, MHCII	SLAM, CD206
Intracellular Markers	iNOS	Arg1*, YM1*, Fizz1*	NA	NA

TABLE 1. **Summary of M1 and M2 Stimuli, Markers and Released Factors**. Summary of the different stimuli, released factors, and markers for the M1 and M2 states. These markers and factors are the canonical markers for each state, and, as such is not an exhaustive list. *Denotes markers that only work in mice. YM1 and Fizz1 have no known human analogs. These markers should only be used for studies in mice.

M2 ACTIVATION STATES

The alternative M2 activation state encompasses a broad set of responses as compared to M1 responses. Generally, the M2 activation state is associated with healing and scavenging, opposing the pro-killing state of M1 activation states. The M2 state is further subdivided into M2a, M2b, and M2c. These three states have some biochemical overlap, but have distinct activation mechanisms as well as effector outputs.

The M2a category was the first alternative activation state described and was developed as a paradigm to understand host response to parasites, and, as such, is associated with encapsulation and killing of parasites as well as allergy. IL-4 is the prototypical M2a stimulus and can bind three different receptor pairs. Each receptor pair can activate JAK1 or JAK3 which activate STAT6 leading to transcriptional changes associated with the M2a state, including; CD206 (mannose receptor), scavenger receptors (SRs), and suppressor of cytokine release 1 (SOCS1) (Edwards et al., 2006, Martinez et al., 2013). M2a macrophages will secrete polyamines and IL-10, which will block pro-inflammatory (e.g., IFNγ, IL6, and TNF) cytokine production (Lu et al., 2013). With the exception of IL-10 secretion, which is released by all the M2 states (described below) to some degree, each of these biochemical changes indicates the M2a activation state.

M2b macrophages, also referred to as type II activated macrophages, are associated with a selective up-regulation of phagocytosis as well as regulation of inflammatory responses. To stimulate this response, TLR activation is required to fuse Fcγ Receptors, especially FcγRIIB, which can then bind IgG (released from B cells)(Anderson and Mosser, 2002). The M2b state is remarkably different than the M2a state in terms of secreted cytokines and associated changes in gene expression. M2b

macrophages will secret high amounts of IL10, as well as low to modest levels of typical pro-inflammatory cytokines, with CD86 highly expressed on the cell surface (Sanchez-Mejorada and Rosales, 1998, Takai, 2002, Edwards et al., 2006).

The last subcategory of M2 activation is M2c. M2c macrophages are associated with tissue repair, extracellular matrix repair, and de-activation of M1/Th1 immune responses (Fiorentino et al., 1989, Glocker et al., 2009). IL10 is the major stimulus for M2c. IL10 stimulates a dimer of the IL10 Receptor 1 and 2 subunits that causes JAK1 and subsequently STAT3 activation. This signaling results in the suppression of most M1 pro-inflammatory cytokines. IL10 also stimulates the release of CXCL13 as well as CXCL4. Several other gene products are also up-regulated including SLAM, which is a marker for this state (Park-Min et al., 2005). As compared with the M1 state, M2 stimuli are much broader and lead to a much larger array of possible responses. In summary, M2 activation states generally lead to healing and reparative responses as opposed to the pro-killing responses of the M1 state. The M2 states are graphically depicted in Figure 2, and listed in Table 1.

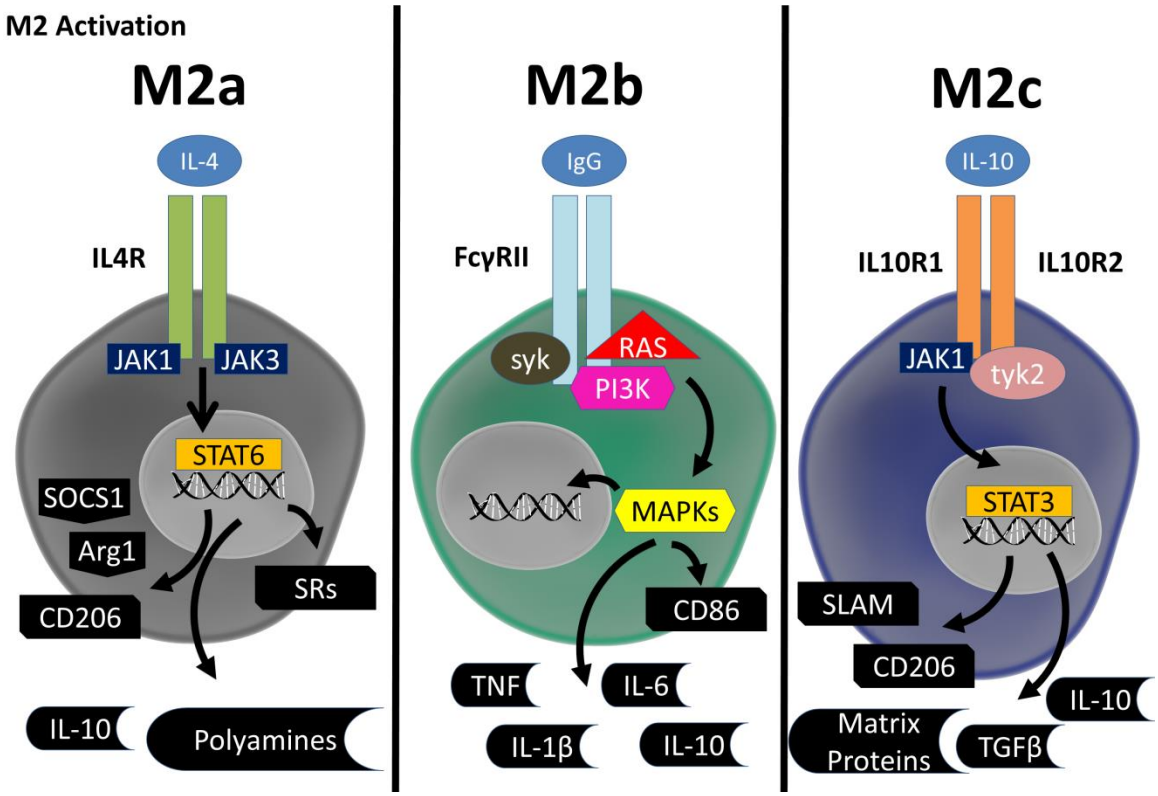

FIGURE 2. **Schematic of M2 Signaling**. The M2 activation state is further broken down into three sub-classes, dubbed the M2a, M2b, and M2c state, that have few overlapping characteristics. The M2a state is caused by IL-4 binding to one of three receptor pairs, which causes activation of JAK1/3. This in turn causes STAT6 translocation to the nucleus and upregulation of SOCS1, Arg1, CD206, scavenger receptors (SRs) and releases of IL-10 and polyamines. The M2b state has some characteristics of an M1 response. TLR activation is necessary to fuse the subunits of the Fcγ Receptor, which then binds IgG. Through a RAS, PI3K, and syk signaling cascade, there is increased release of typically pro-inflammatory cytokines such as TNF, IL-6 and IL-1β as well as typical M2 cytokine IL-10. Similarly to the M1 state, CD86 is up-regulated on the cell surface. The M2c state is caused by IL-10 binding to a heterodimer of IL10 Receptor subunits 1 and 2 which in turn causes activation of JAK1 and tyk. JAK1 and tyk then cause STAT3 translocation to nucleus and up-regulation of SLAM and CD206 as well as increased release of IL-10, TGFβ, and extracellular matrix proteins.

MICROGLIA AND MACROPHAGES IN PD

Microglia were originally thought to derive developmentally from origins similar to that of monocytes and macrophages (Rio-Hortega, 1939, Chan et al., 2007). However, recent studies have shown that microglia are not simply monocytes or macrophages from periphery that happen to reside in the brain. Instead, microglia arise from macrophages of the yolk sac blood islands that seed the developing brain early in development. In contrast, adult peripheral macrophages arise from the fetal liver (Ginhoux et al., 2010, Schulz et al., 2012, Kierdorf et al., 2013). Macrophages originating from the yolk sac cells remain the predominant population in the brain through life. In extreme cases such as inflammation and damage, peripheral cells will enter the brain in large numbers (de Groot et al., 1992, Ladeby et al., 2005, Mildner et al., 2007). Recently, the history and process of microglial origins has been eloquently reviewed (Ginhoux et al., 2013, Prinz and Priller, 2014)

Despite these distinct developmental origins, microglia and macrophages use remarkably similar transcription factors in development. For example, knockout of Pu.1 depletes both microglia and macrophage populations (McKercher et al., 1996, Beers et al., 2006). The cellular makeup of microglia and macrophages are very similar in terms of expressed proteins and cell surface markers, although new experimental paradigms tracking gene expression profiles are beginning to reveal differences between the two cells (Gautier et al., 2012, Butovsky et al., 2014). The M1 and M2 activation state paradigm has also been suggested to be similar in microglia and macrophages, although some differences have been noted in changes of cell surface markers such as CD206 and magnitude of responses to M2 stimuli (Durafourt et al., 2012).

This similarity between resident and peripheral cells in the brain has made the ability to distinguish resident cells from their peripheral counterparts extremely difficult. However, a few techniques have been suggested to differentiate central versus peripheral cells: CD45hi versus CD45low has been shown to mark macrophages and microglia respectively (Zhang et al., 2002, Prinz et al., 2011). Another recent study examining the gene transcription of adult microglia compared to peripheral cells has suggested that microglia lack CD169 and can be used as a staining antibody to distinguish the cell types (Butovsky et al., 2012).

In humans and animal models of PD, there is little understanding of macrophages and microglia as separate entities, even though different roles have been prescribed recently for each cell type in other disease states (Jung and Schwartz, 2012). In the context of AD, perivascular macrophages have been suggested to primarily clear protein aggregates from the brain, while microglia do not seem to significantly affect this process (Mildner et al., 2011). Recruited monocytes and macrophages increase disease severity in experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis (MS) (Ajami et al., 2011). Additionally, recruited cells may have distinct roles from microglia, e.g., demyelination, in disease etiology (Yamasaki et al., 2014).

Despite what is not known in PD, through these correlates there is reason to believe that peripheral macrophages may be involved in PD. For example, a recent study examined the expression and number of CCR2+ cells in blood and found that number of CCR2+ cells was decreased, but the expression of CCR2 was increased in PD patients (Funk et al., 2013). This is indicative of cells migrating into a tissue, and this CCR2 mechanism has been demonstrated to control migration of peripheral cells into the CNS

in EAE (Mahad and Ransohoff, 2003). These findings implicate peripheral cells being involved in PD and are reason to attempt to differentiate between macrophages and microglia in future studies.

EVIDENCE OF M1 ACTIVATION IN PD BRAIN

In PD, enhanced microglial activation, T and B cell infiltration, and immunoglobulin deposition can be found in the substantia nigra and other brain regions associated with α-synuclein aggregation (McGeer et al., 1988, Boka et al., 1994, Imamura et al., 2003). Increases in M1 associated cytokines such as TNF and IL6, possibly from TNF activation of astrocytes (Van Wagoner et al., 1999), have been reported in serum and cerebrospinal fluid from PD patients (Boka et al., 1994, Mogi et al., 1994, Muller et al., 1998, Mogi et al., 2000). Levels of these cytokines have correlated with increasing disability and poorer prognosis (Hofmann et al., 2009, Scalzo et al., 2010). Additionally, the increasing levels of α-synuclein deposition in post mortem PD brain correlates to an increasing number of MHCII positive cells, a marker of the M1 activation state (Croisier et al., 2005). However, these observations are correlative and skewed towards late-stages of disease, so understanding the role of M1 activation in PD becomes difficult.

Positron emission tomography (PET) ligands to peripheral benzodiazepine receptors (PBR) have provided further insight into immune cell activation in PD, particularly in earlier stages of disease compared with post-mortem studies. The PBR receptor increases in expression in the outer membrane of mitochondria of activated macrophages and microglia (Chen and Guilarte, 2008, Papadopoulos and Lecanu, 2009). PET studies have shown 25-50% increases in ligand biding in several areas of the brain associated with PD, such as structures in the basal ganglia, in patients with PD compared to healthy age matched controls (Ouchi et al., 2005, Gerhard et al., 2006, Bartels et al., 2010, Edison et al., 2013, Iannaccone et al., 2013). Unfortunately, it is unclear if PBR

ligands preferentially bind to M1 or M2 skewed microglia or macrophages, and interpretation must be limited to activation and not a specific state. Studies using PBR compounds indicate that PBR up-regulation may already be at maximum levels by the time of diagnosis, as patients followed for 2 years after the original PET scan did not show fluctuations in ligand binding. However, this plateau could also represent ceiling-effect technical limitation of the assay (Gerhard et al., 2006, Edison et al., 2013, Iannaccone et al., 2013). Another interpretation is that the plateau in PBR levels through disease is that PBR upregulation is important in initiation but not progression. Supporting this, PBR ligand binding did not correlate well to clinical severity of disease (Gerhard et al., 2006).

Retrospective epidemiological studies also highlight that inflammation may be important in the initiation or early progression of PD. Some studies show that non-steroidal anti-inflammatory drug (NSAID) use, especially ibuprofen, lowers PD susceptibility (Chen et al., 2003, Esposito et al., 2007, Wahner et al., 2007, Samii et al., 2009, Gagne and Power, 2010). While the preventative effect of NSAID use has failed to replicate in every study(Shaunak et al., 1995, Bornebroek et al., 2007, Becker et al., 2011), meta-analyses indicate that non-aspirin NSAID use is lowers risk for the development of PD (Samii et al., 2009, Gagne and Power, 2010, Noyce et al., 2012). Studies of NSAID use in AD highlight the complex relationship between neurodegeneration and anti-inflammatories. One study in AD prevention showed that naproxen use, a type of NSAID, was useful for prevention of AD in familial cases (Szekely et al., 2008). However, NSAID use did not modify susceptibility to AD in non-familial cases and actually was overall harmful in patients currently diagnosed with

idiopathic AD (Breitner et al., 2011, ADAPT, 2013). In AD, NSAID use for currently diagnosed patients could have attenuated M2 responses, which could explain the potential worsening observed in subjects. While NSAID usage is not indicated to prevent PD or AD due to the high frequency of adverse events associated with treatment, these retrospective studies provide proof-of-principle support for the hypothesis that inflammation is not a benign process in the development of PD.

Because it is not clear when NSAID usage may provide the most benefit for patients at risk or currently suffering from PD, there is a need to understand the activation state of macrophages and microglia throughout the brain during the disease process. Instead of PBR-binding compounds, PET ligands specific for surface receptors associated with an M1 or M2 states may be more useful. Typical approaches ubiquitous in immunological studies such as flow cytometry are not possible in PD since affected brain tissue is not available during the neurodegenerative process. Studies of peripheral cells, while providing interesting clues, may not accurately reflect local microglia and/or macrophage changes in the brain. Future PET approaches with refined ligands to M1 and M2 targets would allow for longitudinal studies to help understand the presumed cycling between M1 and M2 states. Such studies would provide temporal resolution of how inflammation initiates in the disease process.

PROTEIN AGGREGATES AS M1 STIMULI

Many neurodegenerative diseases, including PD, are defined pathologically by proteins that form insoluble aggregates in susceptible brain regions (Golde et al., 2013). How exactly macrophages or microglia become activated in PD are poorly understood. Protein aggregates comprised of α-synuclein can be generated in *vitro (Giasson et al., 2001, Volpicelli-Daley et al., 2011),* and these high molecular weight aggregates of α-synuclein have been shown to weakly induce an M1 response in *vitro (*Zhang et al., *2005b, Klegeris et al., 2008, Reynolds et al., 2008, Su et al., 2008, Freeman et al., 2013).* α-Synuclein aggregates have been shown to interact with a variety of receptors including CD36, TLR2, TLR4 and CD11b, largely depending on the constituency of the protein aggregates applied to cells (Zhang et al., 2007, Su et al., 2008, Lee et al., 2010). Mounting recent evidence suggests that these protein aggregates interact mainly with TLR4 or TLR2 receptors (Beraud et al., 2011, Fellner et al., 2013, Kim et al., 2013). Monomeric forms of α-synuclein do not seem to interact strongly with immunological receptors, nor do they elicit a M1 response. Protein aggregates in other neurodegenerative disorders, such as Aβ plaques in AD, have been shown almost exclusively to interact with TLR2 to induce an M1 response (Jana et al., 2008, Tukel et al., 2009, Liu et al., 2012). Interestingly, α-synuclein has unique affinity for lipids of a variety of compositions (Burke et al., 2013, Hellstrand et al., 2013). One explanation of α-synuclein interaction with TLR4 is that these bound-lipids serve as agonists that allow for TLR4 interaction.

Activation states elicited by neurodegeneration-linked protein aggregates are difficult to interpret in isolated primary microglial cells. For example, if aggregates of α-

synuclein are added to microglia and CD4+ T cells in co-culture, a much more robust M1/Th1 response is observed than either cell can mount on their own (Harms et al., 2013). Neurons in culture with those cells would presumably further alter immunological responses, for example through CX3CR1 signaling (Figure 3). Nevertheless, co-culture experiments point to a possible necessity for cells of the innate (e.g., macrophage or microglia) and adaptive (e.g., T-cells) immune system to work together.

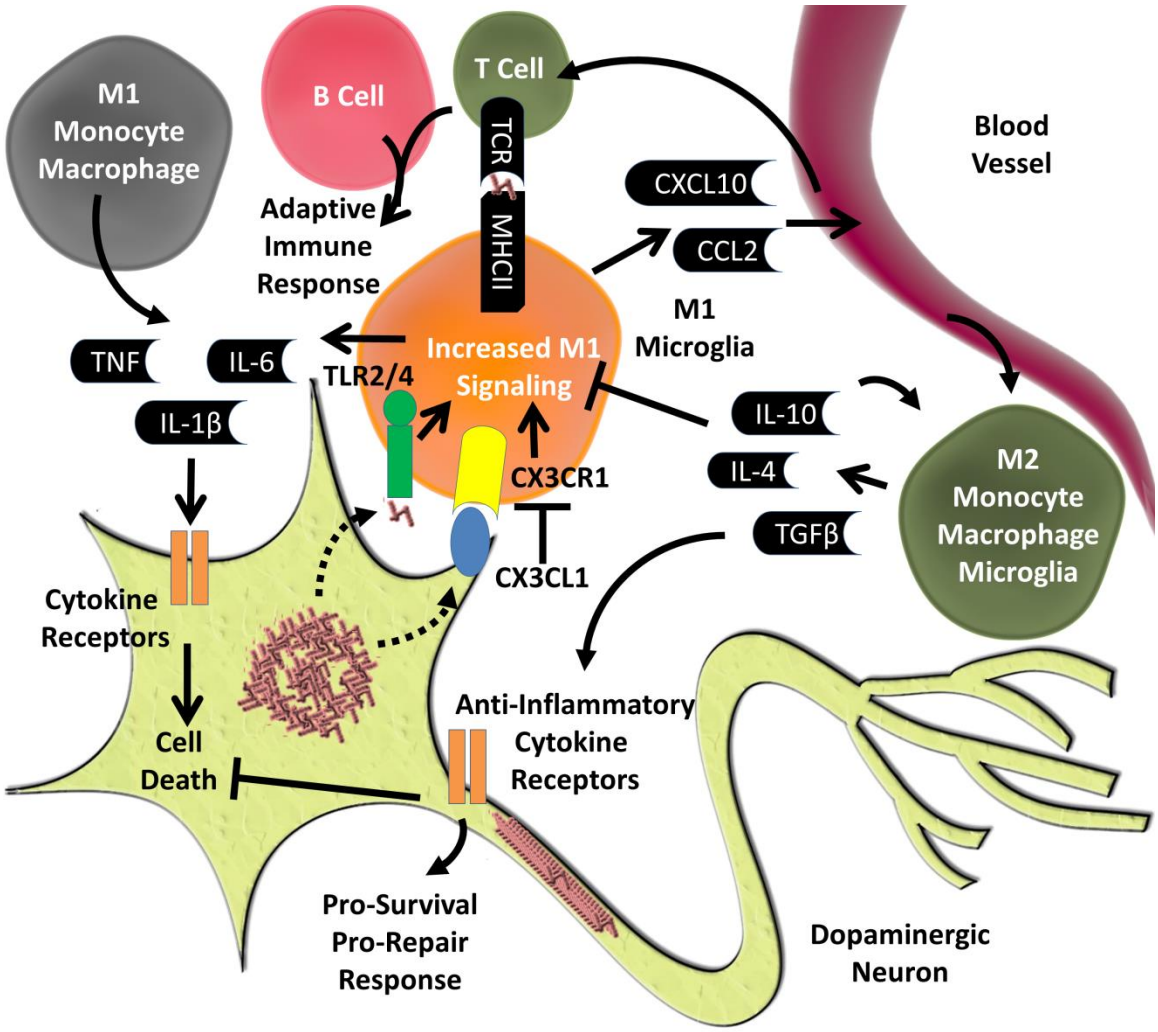

FIGURE 3. **Overview of Inflammatory Mechanisms in PD**. Inflammation is a common pathological hallmark in PD. One possible mechanism of how this arises is through direct activation of TLR2/4 by aggregated forms of α-synuclein. Another is through mechanisms by which neuronal health or dysfunction directly activates microglia. One mechanism is through CX3CR1, which is expressed on microglia, binding CX3CL1, which is expressed by neurons. Through injury, changes in health of neurons, or through α-synuclein CX3CL1 becomes down regulated which activates M1 signaling through CX3CR1 in microglia. With increased M1 signaling, microglia will release pro-inflammatory cytokines and chemokines. Chemokines will draw in innate immune cells from the peripheral immune system. These peripheral immune cells could lead to an adaptive immune response through T and B cells, or could lead to an increased M1 response through the recruitment of monocytes/macrophages and release of more pro-inflammatory cytokines and chemokines. These pro-inflammatory cytokines can act on a variety of cytokine receptors on dopaminergic neurons which could lead to cell death. Concurrently, or as a result of therapeutic intervention, M2 immune cells could release anti-inflammatory cytokines and chemokines that could decrease M1 activation and bind to anti-inflammatory cytokine receptors on neurons and promote survival and repair.

GENETICS OF PD RELEVANT TO THE M1 ACTIVATION STATE

Genome wide association studies (GWAS) have been useful to highlight genetic risk factors important for PD susceptibility (Sekiyama et al., 2014). Interestingly, the genes most strongly associated with PD *(α-synuclein and tau*) were already identified in neurodegeneration genetic linkage or genome wide association studies (Golbe et al., 1996, Polymeropoulos et al., 1996, Polymeropoulos et al., 1997, Martin et al., 2001, Zhang et al., 2005a). GWAS studies have identified the *human leukocyte antigen-DR* (*HLA-DR*) locus which points towards a role for inflammation in susceptibility to PD (Lampe et al., 2003, Hamza et al., 2010, Ahmed et al., 2012, Nalls et al., 2014). *HLA-DR*, encoding major histocompatibility complex 2 (MHCII), is expressed by a limited number of cells of the immune system, deemed antigen presenting cells (APCs). Microglia and macrophages are both APCs. The particular variant of *HLA-DR* associated with PD is believed to increase expression of MHCII (Wissemann et al., 2013). Of possible relevance, animal models of PD show that MHCII knockout reduces M1/Th1 inflammatory responses in response to α-synuclein overexpression, and MHCII knockout protects against dopaminergic neurodegeneration (Harms et al., 2013).

Mutations in the *leucine-rich repeat kinase 2 (LRRK2*) gene are the most common known genetic cause of familial PD (Paisan-Ruiz et al., 2004, Zimprich et al., 2004, Gilks et al., 2005, Healy et al., 2008, Kett and Dauer, 2012). LRRK2 shows high expression in myeloid cells (Thevenet et al., 2011) and knockdown, knockout, or pharmacological inhibition of LRRK2 decreases M1 inflammatory responses both *in vivo and in vitro (Moehle et al., 2012, Daher et al., 2014).* Additionally, *LRRK2* knockout rodents have decreased macrophage and microglia activation and dopaminergic cell death caused by

the prototypical M1-agonist LPS (Daher et al., 2014). These studies show that LRRK2 expression and activity are required for a full M1 response in model systems.

The genetic risk factors of PD implicate a role for inflammation in the etiology of disease. One recent study examined the expression of quantitative trait loci (eQTL), regions of the genome that regulate the expression of mRNA, in a wide range of inflammatory and neurodegenerative disorders in both lymphocytes and CD14+ Cd16- cells of the myeloid lineage that includes macrophages. These peripheral cells demonstrated an overrepresentation of monocyte specific eQTLs in PD. In fact, in contrast to other neurodegenerative disorders like MS, only one eQTL identified was not exclusively found in myeloid cells (Raj et al., 2014). These results suggest that myeloid cell changes predominate immunological responses in subjects with PD.

ANIMAL MODELS IMPLICATE M1 ACTIVATION IN DOPAMINERGIC NEURODEGENERATION

Innate immune activation, including M1 activation states, can drive dopaminergic cell loss in the SNpc in diverse models systems (Hirsch et al., 2012, Deleidi and Gasser, 2013).Through decades of work, it is thought that cells vulnerable to neurodegeneration in PD are particularly sensitive to secreted factors associated with M1 activation (Gonzalez-Hernandez et al., 2010). One hypothesis is that cells vulnerable in PD are found in brain regions enriched in cells capable of mounting M1 responses (Lawson et al., 1990). Direct injection of the canonical M1-agonist LPS into the SNpc produces robust loss of dopaminergic neurons, but not in other nearby brain regions like the ventral tegmental area that also harbor dopaminergic cells (Kim et al., 2000, Castano et al., 2002).

Another model that results in dopaminergic neurodegeneration involves rAAV mediated transduction of α-synuclein in the SNpc of rats and mice (Kirik et al., 2002, Kirik et al., 2003, St Martin et al., 2007). In this model, there is microglial activation, IgG deposition, as well as T and B cell infiltration, in addition to dopaminergic cell loss (Theodore et al., 2008, Sanchez-Guajardo et al., 2010, Barkholt et al., 2012). Interestingly, in this model, inflammation proceeds dopaminergic cell loss with pronounced inflammation 2 weeks to 3 months post injection and cell loss following at 4 to 6 months post injection (St Martin et al., 2007, Chung et al., 2009). This time course implicates that inflammation may be an initiating event in cell loss in this model. Genetically modifying the immune system in this model through *MHCII*, *CX3CR1*, or *FcγRIII* knockout blocks inflammation and protects from dopaminergic cell loss, but does not alter α-synuclein overexpression (Cao et al., 2010, Cao et al., 2012, Harms et al.,

2013). One possibility is that inflammation is secondary or a result of α-synuclein overexpression, but is critical for over cell loss. Some transgenic models of α -synuclein overexpression, such as Thy1- α-syn (line 61), MBP1-h α-syn, and A53T or A30P mutant α-synuclein overexpressing mice (driven by chicken β-actin promoter), also have pathological findings of inflammation in areas with high expression of α-synuclein, but in general these models have not been rigorously scrutinized for markers of M1 or M2 responses (Mendritzki et al., 2010, Chesselet et al., 2012, Valera et al., 2014).

The neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) can also be used to model dopaminergic cell death in model systems. MPTP was identified as the neurotoxin responsible for neurodegeneration in heroin addicts that injected contaminated preparations of drug. (Langston et al., 1984b). MPTP itself is not toxic and must be metabolized by the MAO-B enzyme to MPP+ to exert toxic effects (Langston et al., 1984a). Pathogenicity of MPTP comes, in part, from decoupling constituents of the electron transport chain in the mitochondria and increases reactive oxygen and nitrogen species (Bove et al., 2005). A common finding in MPTP models of dopaminergic cell death is robust inflammation associated with neurodegeneration (Pattarini et al., 2007, Ramsey and Tansey, 2014). The precise mechanism of the inflammation in MPTP intoxicated animals is not fully understood. However, CX3CR1 interaction with its ligand CX3CL1 has been shown to modulate MPTP, with loss of the interaction through *CX3R1* ablation worsening cell loss and increasing soluble CX3CL1 protecting from cell loss (Figure 3) (Cardona et al., 2006, Morganti et al., 2012).

Blocking M1 inflammation caused by MPTP offers some neuroprotection. Treatment with anti-inflammatory agents as well as genetic ablation of important pro-

inflammatory mediators, such as iNOS, protects from dopaminergic neurodegeneration (Wu et al., 2002, Watanabe et al., 2004, Zhao et al., 2007, Madathil et al., 2013, Thakur and Nehru, 2013). Interestingly, a recent study pre-treating MPTP intoxicated mice with GM-CSF, a typically weak M1 stimulus as described above, showed moderate protection from dopaminergic cell loss (Kosloski et al., 2013). This study indicates the possible complexity of the interplay between and pro and anti-inflammatory states in animal models of dopaminergic cell loss. Overall, neuroprotection studies in toxin models highlight the importance of inflammation to drive overt loss of neurons.

M2 ACTIVATION STATES IN PD AND NEURODEGENERATION

So far, the evidence presented for macrophage action in PD points to an M1 activation state contributing to susceptibility and/or progression of disease. However, it is important to note that, especially *in vivo*, macrophages or microglia are not necessarily only M1 or only M2, but can exist as continuums of M1 and M2 responses (Vogel et al., 2013, Martinez and Gordon, 2014). The M2 activation states in chronic disease have been the subject of intense recent interest and extensively reviewed in a series of recent publications (Shechter and Schwartz, 2013, Walker and Lue, 2013, Jiang et al., 2014, Miron and Franklin, 2014, Murray et al., 2014, Plemel et al., 2014).

Clear examples of the continuum of M1 and M2 inside neurodegenerative disease come from the prototypical neuroinflammatory disorder, multiple sclerosis, as well as acute CNS injury, which, unlike PD, have more defined periods for M1 and M2 activated cells. In contusion models of spinal cord injury in mice, there is a robust activation of resident microglia in the spinal column as well as invasion of macrophages. Despite the severity of the injury, there is a mixed M1 and M2 activation within the injury site (Shechter et al., 2009). A small and transient M2 activated cell population, defined as Arg1+ and CD206+ (M2a) localize to sites of injury (Kigerl et al., 2009). Furthermore, if this M2 population is down-regulated, lesion size and spinal cord motor neuron death are increased (Shechter et al., 2009). Conversely, supplanting the lesion site with macrophages exogenously manipulated to an M2 state, results in attenuated lesion size and spinal cord motor neuron death (Rapalino et al., 1998, Kigerl et al., 2009, Shechter et al., 2013). Similar mixed M1 and M2 populations have been identified within the cortex of brain in rodent models of traumatic brain injury (Zhang et al., 2012).

There is also very good evidence of mixed M1 and M2 populations in other chronic neuroinflammatory disorders. In MS, there is direct evidence of M2 macrophages and microglia. Arg1+CD163+ macrophages or microglia localize to both acute and chronic lesions of MS patients (M2a) (Boven et al., 2006, Zhang et al., 2011). Similar to lesion sites in acute injury, in MS models M2 cells are not the majority of innate immune cells within the lesion site. Animal models of MS, especially experimental autoimmune encephalitis (EAE), give further evidence that M1 and M2 activation states occur concurrently and can even predict some measures of disease (Mikita et al., 2011). Disease progression is dependent on M1 macrophages, since blocking M1 activation effectively blocks disease progression or initiation (King et al., 2009, Mildner et al., 2009, Moreno et al., 2014). The ratio of M1 to M2 cells has been shown to have some predictive value in determining relapses of EAE (Mikita et al., 2011). If the M1 state is dominant, a more progressive EAE is favored (King et al., 2009, Mildner et al., 2009). Conversely, if M2 states are favored through adoptive transfer or therapeutic intervention, a less aggressive and possibly regenerative state is achieved (Weber et al., 2007, Burger et al., 2009, Liu et al., 2013). Both EAE and human MS data point to roles for M1 and M2, and not just one activation state alone, in a chronic neuroinflammatory disease.

Similarly, a mixed M1 and M2 state could be occurring in PD, and could help to explain the heterogeneity of retrospective clinical data and observations made in model systems. Emergent data about α-synuclein's impact on M1 and M2 balance *in vitro* suggest sensitization of TLR based immunity and an intermediate M1/M2 phenotype of microglia (Roodveldt et al., 2013). Unfortunately, there is a relative paucity of information on M2 markers in PD or chronic animal models of PD based on α-synuclein,

so piecing together the whole puzzle of myeloid cell responses relevant to PD is not possible at this time.

THERAPEUTIC MANIPULATION OF M1 AND M2 RESPONSES IN PD

A question arises over where and how to target inflammation in neurodegenerative disease to achieve slowing or halting progression (Hirsch and Hunot, 2009). M1 and M2 activation states are embedded within the complexity of not only other immune cells, but also the interplay between neurons, glia, and interactions at the blood-brain barrier (Rock et al., 2004). NSAID studies in PD and AD illustrate that simply blocking inflammation with relatively non-specific targets is probably not going to have overall beneficial effects. Worse, neurodegenerative phenotypes may be exacerbated if NSAIDs are used at the wrong stage of disease (Breitner et al., 2011, ADAPT, 2013). A parallel may be drawn with minocycline usage in the MS model EAE where the anti-inflammatories can block beneficial effects of an M2 response (Li et al., 2005). Macrophage activation states in neurodegeneration may need to be treated more specifically by targeting and attenuating critical and specific M1 targets, and/or promoting M2 responses.

To accomplish M1 inhibition, products of M1 activation states could be blocked or signal transduction pathways underlying M1 activation could be directly attenuated. TNF provides a good target as the molecule itself can induce cell death in neurons, particularly dopaminergic substantia nigra neurons (Frankola et al., 2011). Blocking TNF from binding its receptor through neutralizing antibody therapy (adaluminab), decoy receptors (Etanercept), or through dominant negative TNF, are approaches already used in a number of human diseases and animal models with more established M1 activation states underlying pathogenesis (Peppel et al., 1991, Kempeni, 1999, Rau, 2002, Braun et al., 2007, McCoy et al., 2008, Harms et al., 2011). These therapeutics do not directly

affect the activation state of microglia or macrophages, but rather decrease the ability of TNF to act on its receptor in other cell types. These therapies all rely on large proteins (i.e., biologics), which typically do not cross the blood brain barrier. However, emerging technology to deliver biologics across the blood brain barrier using bispecific antibodies, polymers, or viruses may be on the horizon (Egleton and Davis, 2005, Xiao and Gan, 2013, Farrington et al., 2014).

Small molecules have a much better chance of crossing the blood-brain barrier to block M1 signaling in macrophages. Targeting JAK/STAT activation in signaling pathways that lead to M1 activation can effectively diminish downstream M1 responses (Mascarenhas et al., 2014). JAK/STAT inhibitors have promising efficacy in EAE models as they are able to decrease clinical score severity and associated M1 inflammatory responses (Liu et al., 2014). Whether through biological or small synthetic molecules, one important caveat with globally blocking components of the M1 response is that it could lead to decreased host responses to pathogens and greatly increase risk for infection (Kwon et al., 2014, Varley et al., 2014).

Instead, a therapy that promotes M2 responses could present a better therapeutic option in neurodegenerative disease that avoids caveats associated with blocking individual M1 responses. By polarizing microglia and macrophages into an M2 skewed phenotype, this would not only halt local M1 responses in a much more targeted and controlled way, but also promote healing and repair around the inflamed brain regions. Glimpses of benefit of an M2 targeting therapy have been seen in neurotoxin models. When IL10 was delivered virally into the midbrain or striatum of rodents undergoing MPTP or 6-OHDA intoxication, there was a robust amelioration of dopaminergic neuron

loss in the substantia nigra (Schwenkgrub et al., 2013, Joniec-Maciejak et al., 2014). In models of AD, activating CD200R, a membrane glycoprotein receptor induced by M2 cytokines, has been shown to decrease inflammation as well as decrease Aβ deposits (Lyons et al., 2007). However, the best evidence for M2 therapy comes from MS therapeutics. Glatiramer acetate and beta interferons are both currently approved therapies for MS. Their mechanism of action appears to be through altering the balance of M1/Th1 and M2/Th2 cells of the immune system (Weber et al., 2007, Burger et al., 2009, Kieseier, 2011). Glatiramer and interferon treatment are believed to skew macrophages to release M2 cytokines including IL10, as well as possibly releasing neuronal growth factors, decreasing chemotaxis of cells into the CNS, and decrease release of M1 cytokines, leading to a pro-M2 effect (Yong, 2002, Ziemssen et al., 2002, Ziemssen et al., 2005, Pul et al., 2011, Kurtuncu et al., 2012, Begum-Haque et al., 2013, Peelen et al., 2013). Treatment with these compounds is associated with decreased frequency of relapses in MS and possibly decreasing the progression of disability (Johnson et al., 1998, Buttinelli et al., 2007, Ford et al., 2010, Freedman, 2011).

By drawing on parallels between MS and PD, pro-M2 therapy may prove beneficial in PD, with particular utility in slowing progression. However, any therapy, whether pro-M2 or anti-M1, will likely require the additional development of biomarkers for inflammation within the CNS that are far more sensitive or specific than currently available options, such as currently available PET ligand options. Inflammation is widely postulated to start many years before the clinical onset of symptoms. Therapeutic targeting of inflammation could begin at this time point, but could only slow the progression of disease. If a sensitive biomarker of CNS inflammation could be found,

perhaps through PET, onset of clinical symptoms could be delayed by many years or possibly even prevent the clinical onset of PD.

CONCLUDING REMARKS

Better understanding M1 and M2 responses in PD presents opportunities for both enhanced clarity of pathogenic mechanisms underlying disease as well as potential therapeutic targets in neuroprotection approaches. Specifically, M1 activation may represent an insult that drives overt cell loss in the SNpc. Data from human genetic studies, pathological studies, and animal models suggest that M1 activation may have 2 possible relationships to dopaminergic cell death, ether as a secondary hit in response to α-synuclein aggregation or as primary, initiating event to inflammatory signals. Either way, in the relentless progression of neurodegenerative disease, somehow the underlying pathology subverts normal remediation of pro-inflammatory pathways or conversion to M2 pathways. Thus, therapeutic intervention to enact these responses seems well-justified. Ultimately, treating inflammation may present a unique opportunity for a disease modifying therapy in chronic neurodegeneration, but broad spectrum approaches to non-specifically attenuate immune cells seems likely to fail. Utilization of the mechanisms already in place in macrophages and microglia may represent a straightforward approach that delivers the specificity and efficacy necessary to deal with chronic neuroinflammation in PD.

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LRRK2 INHIBITION ATTENUATES MICRGOLIA INFLAMMATORY RESPONSES

by

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ABSTRACT

Missense mutations in leucine-rich repeat kinase 2 (LRRK2) cause late-onset Parkinson disease, and common genetic variation in LRRK2 modifies susceptibility to Crohn disease and leprosy. High levels of LRRK2 expression in peripheral monocytes and macrophages suggest a role for LRRK2 in these cells, yet little is known about LRRK2 expression and function in immune cells of the brain. Here, we demonstrate a role for LRRK2 in mediating microglial pro-inflammatory responses and morphology. In a murine model of neuroinflammation, we observe robust induction of LRRK2 in microglia. Experiments with TLR4-stimulated rat primary microglia show that inflammation increases LRRK2 activity and expression while inhibition of LRRK2 kinase activity or knockdown of protein attenuates TNFα secretion and iNOS induction. LRRK2 inhibition blocks TLR4 stimulated microglial process outgrowth and impairs ADP stimulated microglial chemotaxis. However, actin inhibitors that phenocopy inhibition of process outgrowth and chemotaxis fail to modify TLR4 stimulation of TNFα secretion and iNOS induction, suggesting LRRK2 acts upstream of cytoskeleton control as a stress-responsive kinase. These data demonstrate LRRK2 in regulating responses in immune cells of the brain and further implicate microglial involvement in late-onset PD.

INTRODUCTION

The leucine rich repeat kinase 2 (LRRK2) gene was discovered as part of an evolutionarily conserved family of proteins marked by GTPase domains usually encoded together with kinase domains (Bosgraaf and Van Haastert, 2003). Missense mutations in both the kinase and GTPase domain in LRRK2 cause late-onset Parkinson Disease (PD) with clinical and pathological phenotypes nearly indistinguishable from idiopathic disease, possibly through the up-regulation of LRRK2 kinase activity (Paisan-Ruiz et al., 2004; Zimprich et al., 2004; West et al., 2005). Disease penetrance of LRRK2 mutations in PD is incomplete as lifetime risk in clinical populations is estimated at ~22–32%, suggesting strong modifiers of LRRK2 disease (Goldwurm et al., 2007). A modifying role for the immune system in PD susceptibility is supported by the association of the HLA region with late-onset disease (Hamza et al., 2011), and pathological studies of PD brains demonstrate strong microglial and T-cell activation and infiltration in susceptible brain nuclei (McGeer et al., 1988). Genome-wide association studies also highlight LRRK2 in modification of susceptibility to the chronic autoimmune Crohn disease and Mycobacterium leprae infection (Zhang et al., 2009; Umeno et al., 2011), raising the possibility that mutations in LRRK2 may modify immunogenic responses in PD.

LRRK2 is expressed in many different cell types in mammals but the intracellular function of LRRK2 is not clear. In the brain, LRRK2 is expressed in diverse neuronal subtypes and localizes to cytoskeletal structures and a variety of vesicular and membranous organelles (Biskup et al., 2006). In neurons, LRRK2 has been described as a potent regulator of the cytoskeleton where knockdown of protein enhances neurite outgrowth and mutant (overactive) LRRK2 expression inhibits outgrowth (MacLeod et

al., 2006). LRRK2 may directly modify microtubule organization and the actin cytoskeleton through phosphorylation of substrates (Gillardon, 2009; Parisiadou et al., 2009). LRRK2 may also play additional kinase-dependent roles in the modification of synaptic vesicle storage and mobilization, in addition to kinase dependent roles in endocytosis, MAPK signaling, autophagy and apoptosis (Alegre-Abarrategui et al., 2009; Gloeckner et al., 2009; Piccoli et al., 2011).

Particularly high LRRK2 expression has been recently discovered in macrophage and monocytic cells, but not T cells, leading to speculation of a functional role for LRRK2 in the innate immune system (Thevenet et al., 2011). A number of powerful tools, including highly-specific rabbit monoclonal LRRK2 antibodies and potent and selective LRRK2 small molecule kinase inhibitors, have become available that allow for a careful dissection of LRRK2 function in cells of the immune system. Based on the expression of LRRK2 in monocytes, we hypothesized a role for LRRK2 in the immune cells of the brain. Our results show that LRRK2 is expressed in activated microglia and that LRRK2 modulates pro-inflammatory responses in these cells. Alterations in LRRK2 function may modify inflammatory responses in neurodegenerative and infectious diseases, potentially leading to disease initiation or modification of progression.

RESULTS

LRRK2 expression in TLR4-activated microglia

High LRRK2 expression in peripheral mouse monocytes and macrophages led us to examine whether LRRK2 may be expressed in brain resident macrophage cells (*i.e.*, microglia). We first applied recently characterized and highly specific rabbit monoclonal antibodies directed against LRRK2 to normal mouse brain tissue and failed to detect any cells positive for LRRK2 with morphology consistent with microglia, despite strong LRRK2 immunoreactivity in several neuronal populations. Since LRRK2 has been hypothesized as a stress-responsive kinase, we analyzed brain tissue from mice subjected to an intracranial injection of the potent toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS) for a period of 24 hours. Although we observed no loss of tyrosine hydroxylase (TH) positive cells at this time point, the intensity of TH expression was slightly diminished (Figure 1A–C). Whereas only blood vessels were labeled by isolectin B4 in the contralateral side to LPS injection (Figure 1D), numerous small strongly stained cells consistent with activated microglia were detected in the LPS treated SNpc (Figure 1E–F). LRRK2 staining in the LPS injected SNpc revealed a strong induction of LRRK2 immunoreactivity in small cells with a morphology and size consistent with the activated microglia identified by isolectin B4 (Figure 1H). This staining was abolished in LRRK2 knockout mice (Figure 1I). LRRK2 protein was undetectable in white matter tracts in normal brain tissue, although many LRRK2-positive small cells were found in the corpus callosum of mice after an intrastriatal LPS injection (Figure 1K). To rule out non-specific or cross-reactive labeling, non-immune rabbit IgG was also applied at comparable concentrations to LRRK2 antibody treated

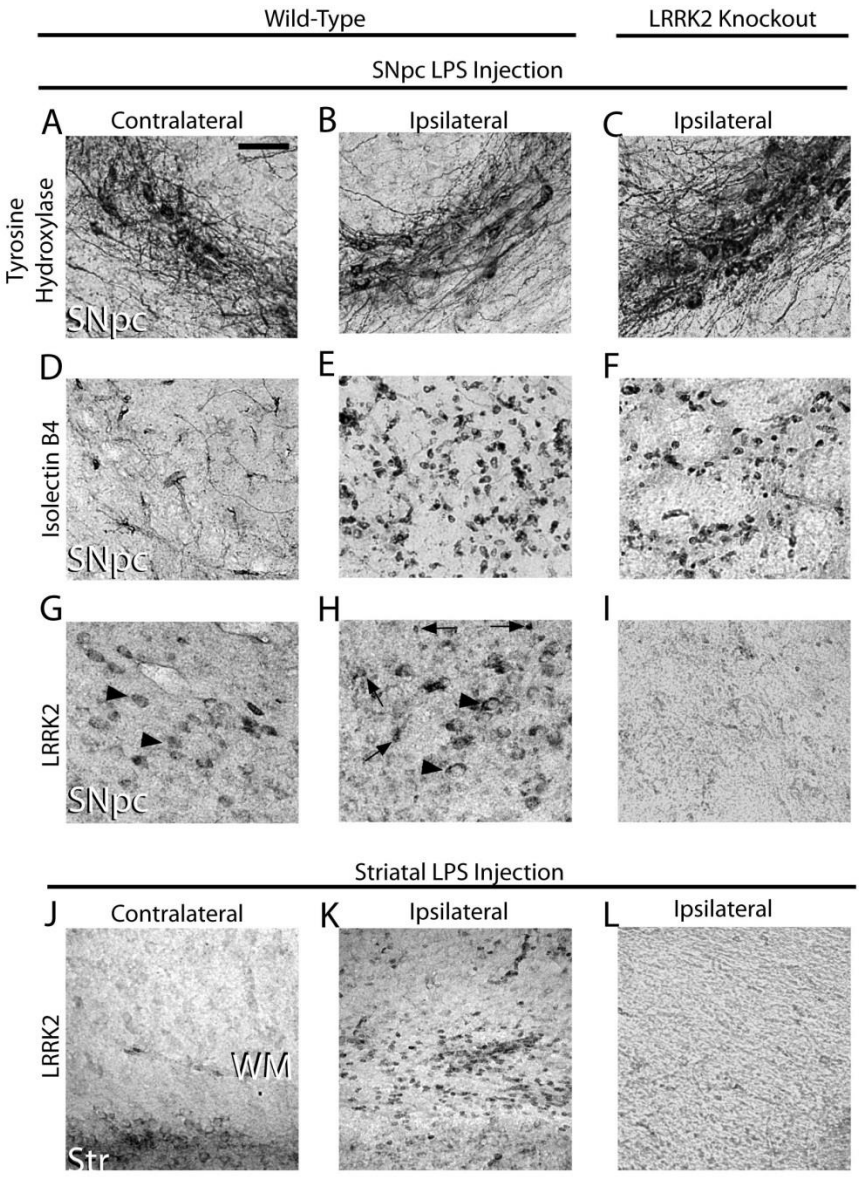


FIGURE1: **TLR4 stimulation triggers LRRK2 expression in microglia cells**. 5μg of LPS (E. coli 0111:B4) was unilaterally injected into the substantia nigra pars compacta (SNpc) or striatum of 12-week old male WT and LRRK2 KO C57BL6/J 12 mice. Immunohistochemistry for **(A–C)** tyrosine hydroxylase (TH), **(D–F)** isolectin B4 (marker for microglia and endothelial cells), or **(G–L)** LRRK2 was performed on serial coronal sections spanning the SNpc and striatum. Arrowheads indicate LRRK2 immunoreactivity on cells in the SNpc with the size and location of TH-positive neurons on both the contralateral and ipsilateral injection sides. Arrows indicate intense LRRK2 staining in numerous small cells observed exclusively on the ipsilateral side. WM is white matter, and Str is striatum. No specific cellular staining in these areas was observed when primary antibodies were replaced with species-matched whole IgG (data not shown). Indicated scale bar is 50 μm for all panels.

sections; negligible immunoreactivity was observed in these negative control sections and none reminiscent of microglia cells. Finally, staining in LRRK2 KO mice revealed the LRRK2 monoclonal antibody to be specific for LRRK2 (Figure 1I, L).

To co-localize LRRK2 with microglial markers in TLR4-activated microglia, a triple staining protocol that utilizes a single fluorescently labeled antibody was developed and applied to mice LPS-treated in either the SNpc or striatum (Figure 2). 24 hours post-injection, microglial cells rapidly accumulated and surrounded TH positive neurons (Figure 2A). Microglia could not be detected in the SNpc in animals that did not receive an LPS injection (Figure 2B). Similar to the SNpc, LRRK2 co-localized to microglial cells present in the white-matter tract post-striatal LPS injection (Figure 2C). Resident microglia with resting morphologies in non-injected animals failed to demonstrate immunoreactivity for LRRK2 (Figure 2D). As in Figure 1, whole-rabbit IgG control stained sections confirmed specificity of staining, and the LRRK2 antibody produces a single band of the correct size on western blot analysis (Figure 2E).

LRRK2 induction in TLR4-stimulated cells

TLR4 activation stimulates signal transduction pathways responsible for the up-regulation of pro-inflammatory factors. To further explore LRRK2 activity in response to TLR4 stimulation, mice transgenic for a FLAG-LRRK2 BAC insert were injected (bilateral) with LPS in the SNpc and the SNpc and immediately surrounding regions were removed after 24 hours. After LRRK2 immunoprecipitation from this tissue, LRRK2 was allowed to autophosphorylate for 15 min in the presence of 100 μM ATP, and protein eluted and transferred to membranes and probed with the recently described

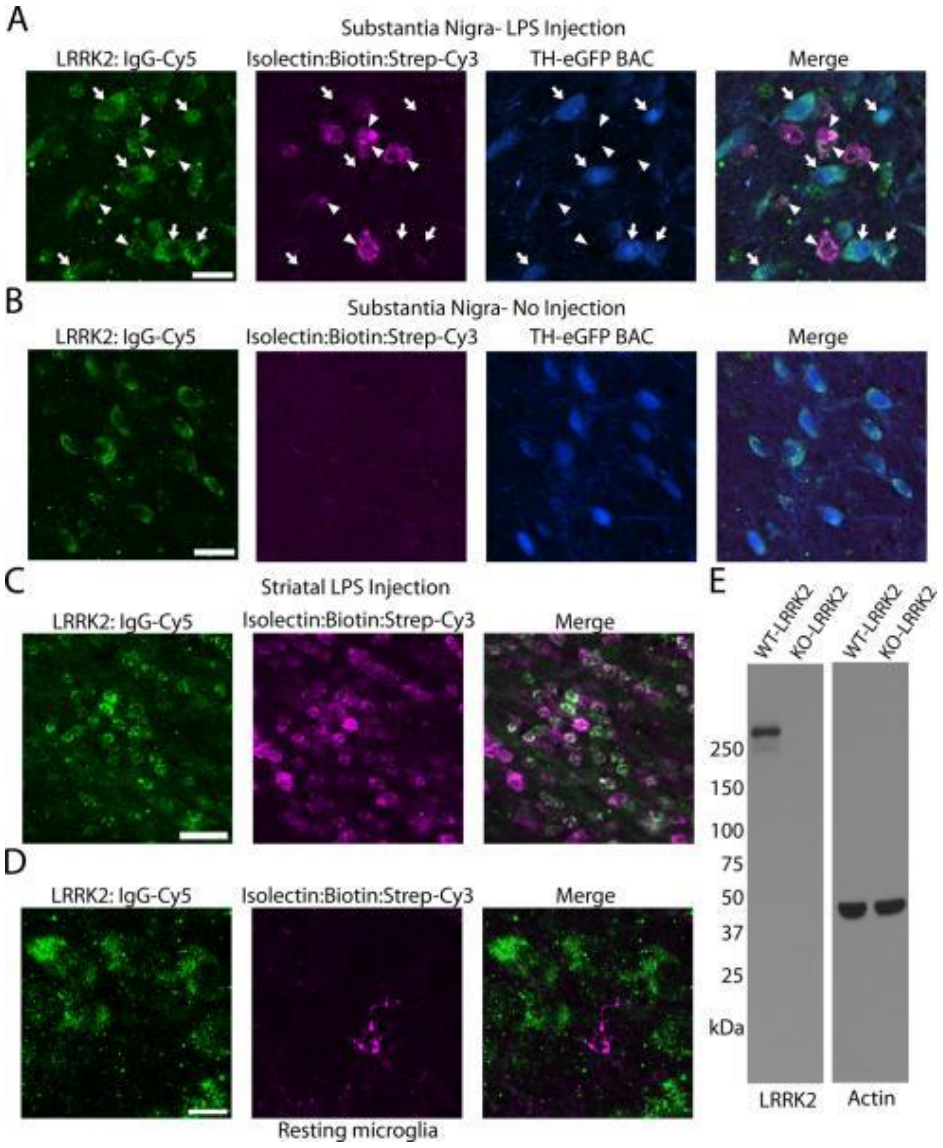


FIGURE 2: **LRRK2 co-localizes with TLR4 stimulated microglia**. TH-eGFP BAC mice were unilaterally injected with 5µg of LPS (E. coli 0111:B4) into the SNpc or striatum. Using a triple-staining protocol (anti-rabbit IgG-Cy5 to detect LRRK2 antibody, ExtraAvidin-Cy3 to detect Isolectin-B4:biotin positive cells, eGFP epifluorescence in TH positive cells), **(A)** LRRK2-labeled cells were observed in the SNpc as either large (arrows) or small (arrowheads) cells that co-localized with eGFP (arrows) or isolectin (arrowheads). Scale bar is 20 µm. **(B)** LRRK2 staining in the SNpc in control no-injection mice. Scale bar is 20 µm. **(C)** In striatal injected mice, LRRK2 was observed co-localized with most microglial cells in the white-matter projection tract. Scale bar is 30 µm. **(D)** LRRK2 staining could not be detected in microglia with resting morphology. Scale bar is 10 µm. Overlap of green (LRRK2) and magenta (microglia) is white, and overlap of blue (TH positive cells) and green (LRRK2) is cyan. **(E)** Western blot for LRRK2 with 20 µg total protein lysate loaded per well that was derived from LRRK2 KO or WT whole brain tissue.

autophosphorylation specific antibody pT1503 (Webber et al., 2011). LPS treatment significantly increased the proportion of LRRK2 in an activated state as revealed by an enhanced proportion of autophosphorylated LRRK2 (Figure 2B). To ensure the pT1503 antibody could not cross-react with non-autophosphorylated LRRK2 protein, recombinant protein harboring mutations in the 1503 autophosphorylation site or in the kinase domain (kinase dead, D1994A) was derived from transiently transfected cells and evaluated by western blot (Figure 2C). The pT1503 antibody could not detect signal in the kinase dead or the T1503A mutant LRRK2 protein, suggesting a high degree of specificity for this antibody.

To address whether LRRK2 expression also becomes up-regulated during TLR4 activation, primary microglia in culture were treated with increasing concentrations of LPS for 12 hours and LRRK2 protein levels determined by western blot (Figure 3E). 100 ng/mL of LPS was sufficient to increase levels of LRRK2 protein obtained in SDS-solubilized cell lysates, while higher concentrations of LPS failed to further increase LRRK2 induction. These primary microglia cultures were free from other cell types and >99% of cells in culture were CD-68 positive (data not shown). Quantification of LRRK2 protein levels across primary microglia, astrocytes, hippocampal neurons and oligodendrocytes, all derived from post-natal day 2 rats, unexpectedly revealed LRRK2 expression in primary astrocytes despite the lack of expression we could observe in these cells *in vivo*. LRRK2 expression in primary microglia cells is comparable to that of primary neurons in culture (Figure 3D).

We next determined whether LRRK2 expression is up-regulated at the mRNA level but failed to detect any significant differences after 12 hours of induction with

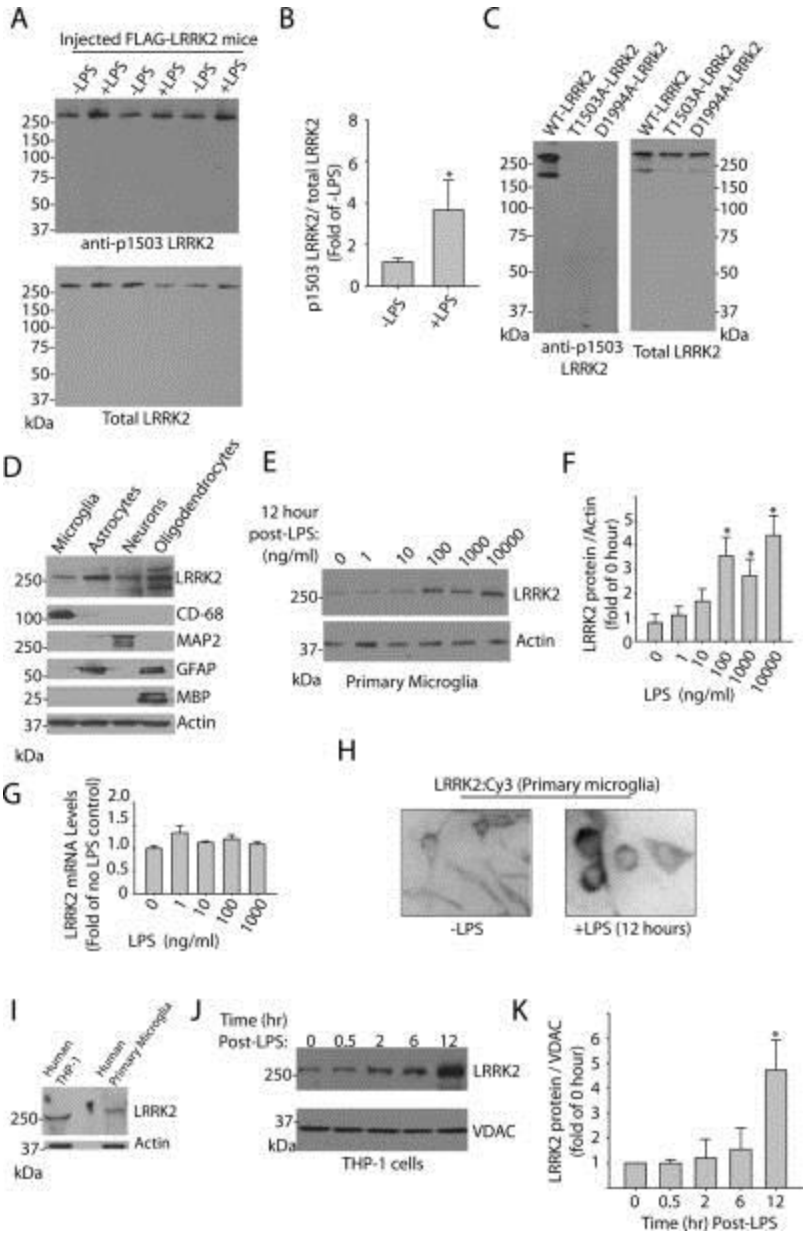


FIGURE 3: **LRRK2 induction by TLR4 stimulation (A)** 5μg of LPS or no LPS control was bi-laterally injected into the SNpc of FLAG-LRRK2 mice (Jackson strain #012466), the SNpc dissected after 24 hours and FLAG-LRRK2 immunoprecipitated and treated with ATP. Eluted protein was analyzed by western blot with either a total LRRK2 antibody or autophosphorylation specific pT1503 antibody. **(B)** Quantification of pT1503-autophosphorylated LRRK2 normalized to total LRRK2 from 4 LPS injected mice and 4 injection control mice. **(C)** Specificity of the pT1503 antibody is demonstrated by LRRK2 recombinant protein derived from transiently transfected HEK-293FT cells. **(D)** Primary cultures derived from post-natal 2 rats were analyzed by western blot for cell type specific markers and LRRK2 expression. Lysates were normalized to actin and approximately 20 μg of protein were loaded per lane. **(E)** Primary microglia treated with various concentrations of LPS for 12 hours and LRRK2 expression

evaluated by western blot, with **(F)** quantification normalized to actin for 3 independent experiments. **(G)** mRNA levels of LRRK2 were determined by relative quantification (ΔΔcT) normalized to TBP. **(H)** Representative immunofluorescence of LRRK2 staining in primary microglia cultures treated with LPS or control (−LPS) for 12 hours. **(I)** Human LRRK2 expression characterized by western blot in human primary microglia cultures in comparison to human macrophage/monocyte THP-1 cells. **(J)** THP-1 cells treated with 100 ng LPS for the indicated time and lysates analyzed by western blot, with **(K)** quantification of LRRK2 levels from three independent experiments. * is $p<0.01$ by two-tail unpaired t test for panel B and $p<0.01$ by one-way ANOVA with Tukey Kramer test for all other panels with respect to initial LRRK2 expression. Error bars are S.E.M.

various doses of LPS, suggesting important post-transcriptional regulation of LRRK2 in microglia (Figure 3G). LRRK2 distribution by immunofluorescence in primary microglia is consistent with that of previous reports with strong perinuclear staining and nuclear exclusion, both in non-LPS and LPS treated microglia (Figure 3H). LRRK2 expression in human derived cells of monocytic and microglia origin appears to be conserved (Figure 3I), with similar LRRK2 up-regulation in THP-1 cells treated with LPS. Quantitative PCR analysis for LRRK2 mRNA also revealed no significant changes in LRRK2 levels in these cells, despite strong up-regulation of LRRK2 protein levels (Figure 3K and data not shown).

Inhibition of LRRK2 kinase activity attenuates pro-inflammatory microglial signaling

A post-transcriptional induction of LRRK2 and enhanced autophosphorylation in purified protein suggests possible involvement of LRRK2 during a pro-inflammatory response. We first evaluated the inhibitory potential of the two most potent and specific LRRK2 inhibitors yet described, L2in1 and Sunitinib, under common conditions. We derived IC_{50} values of 40.3 and 126.4 nM under uniform conditions, respectively, demonstrating L2in1 as more potent against LRRK2 kinase activity *in vitro* (Figure 4A,B). Sunitinib inhibits with similar potency several receptor tyrosine kinases including the platelet-derived growth factor receptor (PDGF-R) and vascular endothelial growth factor receptor (VEGFR) in addition to LRRK2, and L2in1 is highly specific for LRRK2 but is predicted to inhibit several other kinases including MAPK7 and Aurora-A (Dzamko et al., 2011). However, neither small molecule inhibits any known kinases that obviously link to TLR4 receptor signaling when applied at nanomolar or low micromolar

concentrations. In addition, both inhibitors are structurally distinct from one another and there is no common off-targets known.

Secretion of the pro-inflammatory cytokine TNFα by microglia may represent a major modifier of neurotoxicity in models of Parkinson disease (Harms et al., 2011). Pre-treatment of microglia with nanomolar concentrations of either L2in1 or Sunitinib significantly attenuated the release and transcriptional induction of TNFα in primary cultured microglia (Figure 4D, E). iNOS and p38 are both critical targets of TNFα and effectors of continued TNFα release in autocrine signaling. In agreement with an attenuated inflammatory response to TLR4 stimulation, significantly reduced levels of iNOS induction and phosphorylated p38 were observed 6 hours post-LPS treatment (Figure 5F, G). To our knowledge, this is the first demonstration of anti-inflammatory activity for either small molecule LRRK2 inhibitor.

To determine whether removal of total LRRK2 protein produces effects comparable to acute kinase inhibition, we developed lentivirus capable of knocking down LRRK2 expression in primary microglia (Figure 5B). Lentivirus has previously been described as an effective method to genetically modify primary microglia without adverse effects on inflammatory responses (Balcaitis et al., 2005). To knockdown LRRK2 by RNAi in microglia, highly purified lentivirus was applied in increasing concentrations to determine the minimum amount of virus required to knockdown ~90% of LRRK2 protein. Control shRNA (non-coding, NC) lentivirus was also applied at identical concentrations and no adverse toxic or inflammatory effects on the microglia were observed. Knockdown of LRRK2 was sufficient to reduce TNFα secretion similar to LRRK2 kinase inhibition via small molecule inhibitor exposure (Figure 5C). LRRK2

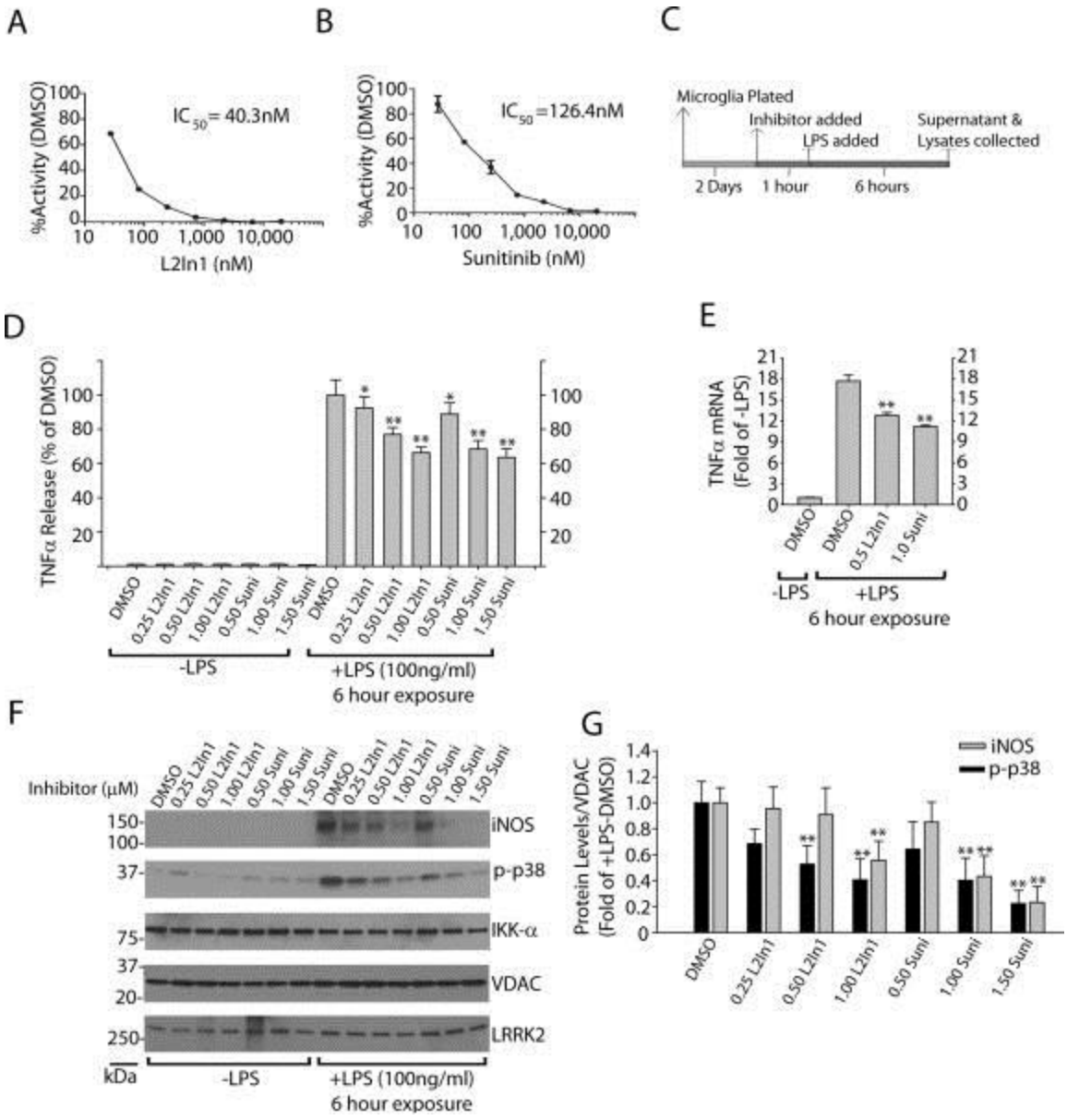


FIGURE 4: **LRRK2 kinase inhibition attenuates inflammatory signaling in microglia**. **(A)** Calculation of the inhibitory potential of the LRRK2 targeted compounds L2in1 and **(B)** sunitinib using standardized *in vitro* kinase assays consisting of 30 nM LRRK2 enzyme, 50 μM peptide and 100 μM ATP with reactions run for 30 minutes. IC_{50} values were calculated through non-linear regression with r^2 values of 0.960 and 0.968 for L2in1 and Sunitinib (respectively). **(C)** Graphical depiction of the experimental timeline used to generate lysates and serum from primary microglia analyzed in **(D–G)**. **(D)** Quantification of secreted TNFα by ELISA after a 6 hour exposure to LPS. "Suni" is Sunitinib. Drug concentrations are given in μm, and mean values are calculated from 3 independent experiments. **(E)** TNFα mRNA was measured by quantitative PCR (ΔΔcT) normalized to TBP, and mean values are calculated from 3 independent

experiments. **(F)** Representative western blot analysis of primary microglia lysates. (G) Quantification of 3 independent experiments with levels of iNOS and phospho-p38 levels normalized to VDAC expression. * represents $p<0.05$ and ** represents $p<0.01$ by one-way ANOVA with Tukey Kramer, with respect to DMSO (+LPS) conditions. Error bars are S.E.M.

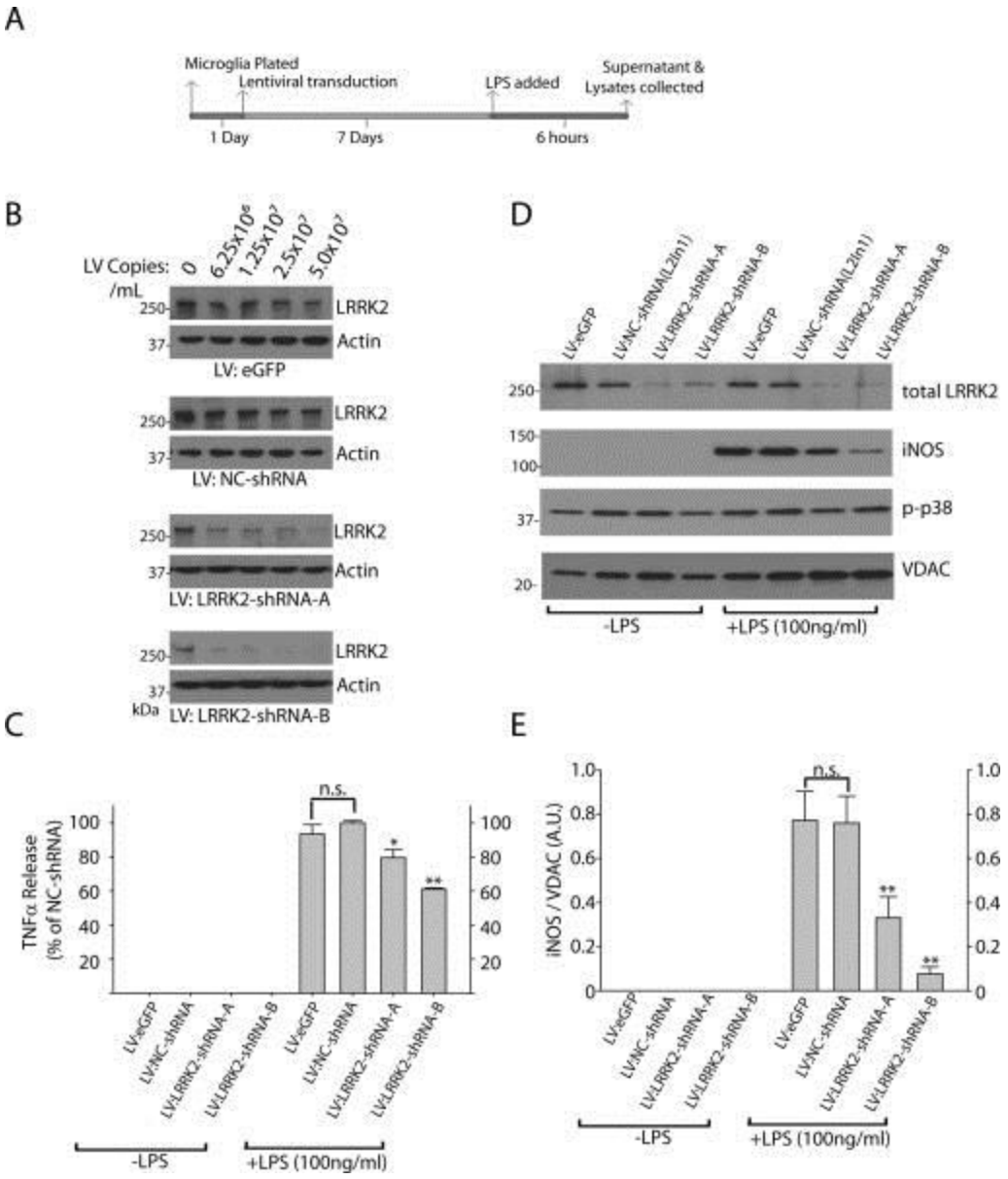


FIGURE 5: **LRRK2 knockdown attenuates inflammatory signaling in microglia**. (**A**) Graphical depiction of the experimental timeline used to generate lysates and serum from primary microglia cultures. (**B**) 5×10^5 primary microglia per condition were exposed to the indicated copies of purified lentivirus encoding either eGFP (no RNAi control), a non-coding control shRNA (NC-shRNA), or LRRK2 shRNAs A and B, in 0.5 mL in culture. (**C**) Serum from primary microglia treated with 2×10^7 LV copies/mL of the respective lentivirus for 7 days were analyzed for TNFα secretion 6 hours post-LPS (100 ng/mL) or control exposures. Results are calculated from three independent experiments. (**D**) Primary microglia exposed to LPS for 6 hours as indicated were lysed in SDS buffer and analyzed by western blot. 2µg of total protein was loaded per lane and analyzed with the indicated antibody. VDAC is used as a loading control. Blots shown are

representative of three independent experiments. (**E**) Quantification of iNOS levels normalized to VDAC expression. * represents $p<0.05$ and ** represents $p<0.01$ by one-way ANOVA with Tukey Kramer, with respect to LV-NC-shRNA (+LPS) conditions. Error bars are S.E.M.

knockdown also inhibited TLR4-mediated iNOS induction (Figure 6D, E). Phosphorylation of p38 was unchanged by LRRK2 RNAi, although basal levels of phospho-p38 rose to the levels of post-LPS phospho-p38 as a consequence of the extended *in vitro* culture period required for LRRK2 protein knockdown (Figure 6D). Collectively, these results suggest that LRRK2 kinase activity and expression are required for a full inflammatory response in microglia.

LRRK2 kinase activity is required for microglial morphological remodeling during activation

Microglia initially respond to LPS and other pro-inflammatory factors by extending processes into the environment (Sheng et al., 2011), with eventual (>12 hours) retraction into a classic activated amoeboid morphology as autocrine effects contribute to a positive feedback loop in pure microglial cultures. We noticed that in the presence of LRRK2 inhibitors or LRRK2 shRNA, LPS treatment failed to cause any significant morphological differences from non-LPS treated controls (Figure 6). This failure to morphologically respond to LPS was not due to an enhanced rate of cell death since Hoechst and propidium iodide staining revealed comparable results across conditions.

Microglial dynamic fine process extension have been suggested to play a critical role in mediating responses to local brain injury and stressors (Davalos et al., 2005). Because of migroglial dependence on LRRK2 for mediating a morphological response to LPS, we hypothesized that a chemotactic response to the potent microglial chemoattractant ADP may likewise be compromised in the absence of LRRK2 activity. Microglial cells immediately plated onto transwell permeable membranes after removal

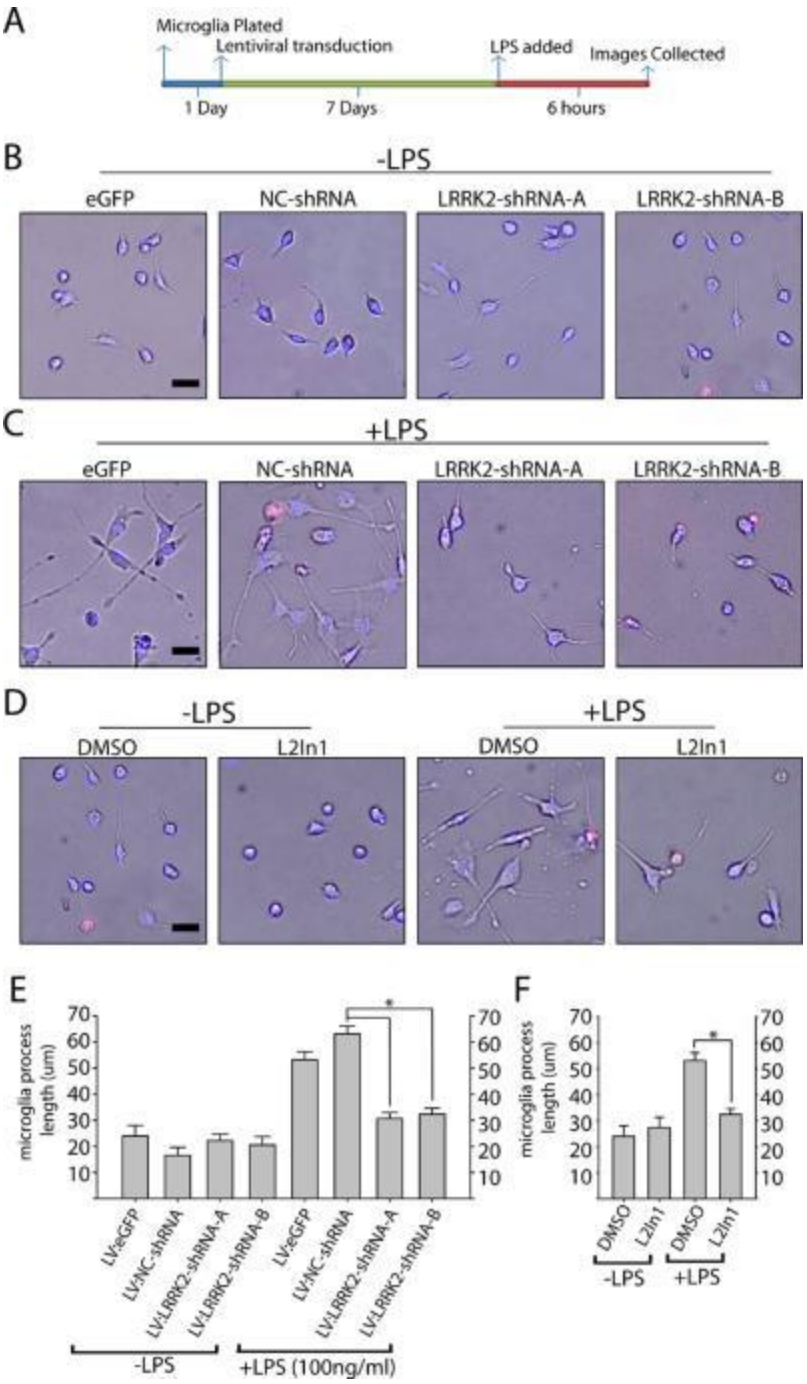


FIGURE 6: **LRRK2 controls TLR4-responsive process outgrowth**. (**A**) Graphical depiction of the experimental timeline. (**B**–**D**) Representative phase-contrast images with Hoechst stain (blue) and propidium iodide (red) stain overlay. Scale bars are 40μm. (**E**–**F**) Quantification of average microglia process length calculated from >150 microglia analyzed from three experiments per condition. L2in1 was used at 1 μM concentration with a 1 hour pretreatment before LPS addition. Round cells with healthy nuclear staining but no process extensions were counted as 0 for process length determination, and

propidium iodide positive cells or cells with abnormal Hoechst staining (less than 10% of cells in every condition) were excluded from analysis. * is $p<0.01$ as compared to LV:NC-shRNA/+LPS (panel E) or DMSO/+LPS (panel F) as determined by one-way ANOVA with Tukey Kramer test. Error bars are S.E.M.

from astrocyte cultures demonstrated reduced migration to the bottom-well in the presence of LRRK2 inhibitors (Figure 7). An actin inhibitor cytochalasin D nearly abolished migration of microglia through the membrane, demonstrating the importance of cytoskeleton architecture in this process.

Since inhibition of LRRK2 has been shown to modify intracellular cytoskeleton architecture and process outgrowth in neurons (MacLeod et al., 2006), we reasoned that LRRK2 inhibition of process outgrowth and chemotaxis may link LRRK2 action, albeit indirectly, to anti-inflammatory activities. To test this hypothesis, we applied the actin inhibitor cytochalasin D to repress LPS-induced morphological remodeling. A 2 μM concentration of cytochalasin D was sufficient to phenocopy LRRK2 in preventing LPS induced process extension, although as opposed to LRRK2 inhibition, cytochalasin D also reduced normal process morphology in non-LPS treated microglia (Figure 8B). Surprisingly, cytochalasin D failed to demonstrate any anti-inflammatory activity; rather, TNFα secretion was positively regulated although we were not able to detect an overall change in iNOS induction (Figure 8C–D). We conclude that LRRK2 dependent morphological changes are a result of upstream LRRK2-dependent anti-inflammatory signaling. Disruption of LRRK2 activity thus prevents a full inflammatory response in these cells that is reflected both morphologically, by TNFα secretion, and by iNOS induction.

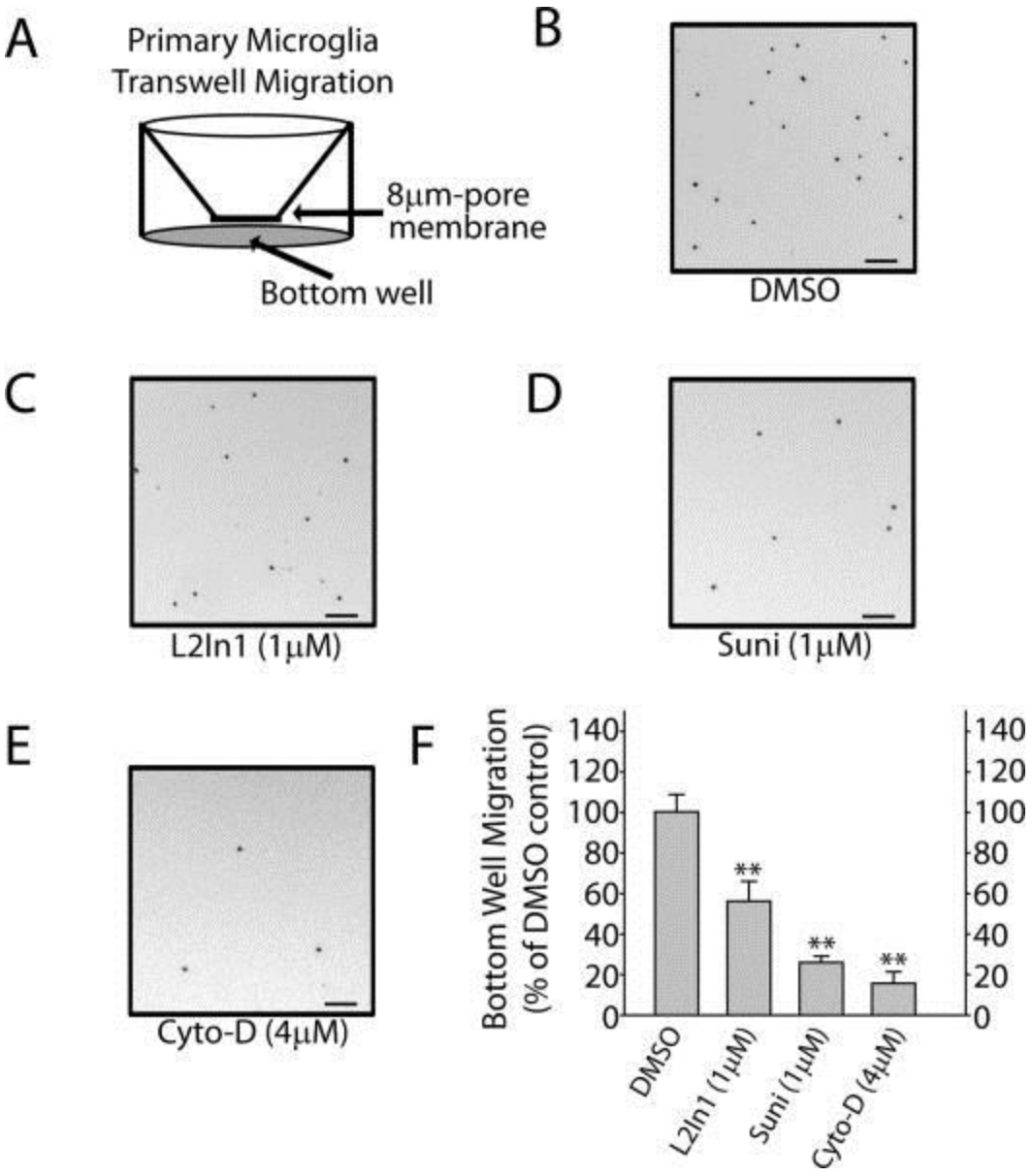
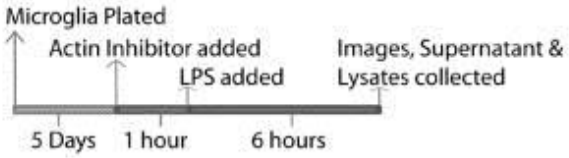


FIGURE 7: **LRRK2 inhibition impairs microglial chemotaxis**. (**A**) Graphical depiction of experimental approach. The bottom chamber is supplemented with 100 μM ADP and microglia are allowed to migrate through the 8 μm pore membrane over a 30 hour period of time to the bottom chamber. (**B**–**E**) Representative depiction of a 0.5 mm^2area of the bottom chamber with cells stained with Hoechst dye. "Suni" is sunitinib, and "Cyto-D" is the actin inhibitor cytochalasin D. 9**F**) Relative quantification of the number of microglia migrating to the bottom chamber, calculated from three independent experiments. ** is$p<0.01$ as determined by one-way ANOVA with Tukey Kramer test. Error bars are S.E.M.

A


Microglia Plated
Actin Inhibitor added
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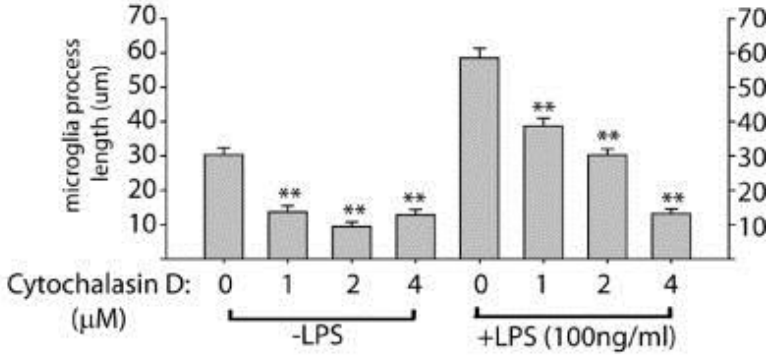
B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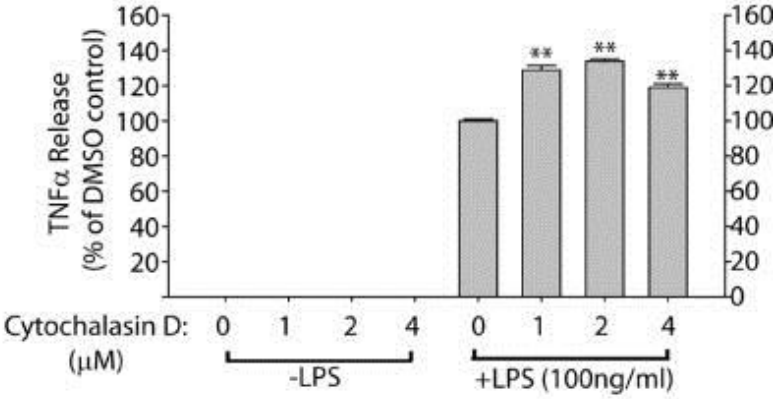
TNFα Release
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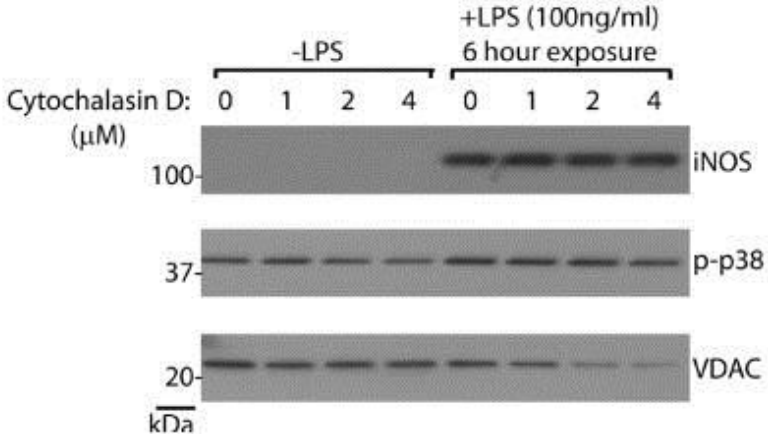
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p-p38
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VDAC
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FIGURE 8: **Inhibition of microglial process outgrowth does not affect pro-inflammatory signaling**. (**A**) Graphical depiction of experimental approach. (**B**) Quantification of average microglia process length in response to the indicated concentration of cytochalasin D. Mean lengths were calculated from >150 microglia analyzed from three independent experiments per condition. Round cells with healthy nuclear staining but no process extensions were counted as 0 for process length determination, and propidium iodide positive cells or cells with abnormal Hoechst staining (less than 10% of cells in every condition) were excluded from analysis. (**C**) Quantification of secreted TNFα by ELISA after a 6 hour exposure to LPS in the presence of the indicated concentration of cytochalsin D. Mean values were calculated from 3 independent experiments. (**D**) Representative western blots demonstrating levels of iNOS and phosphop38 levels in response to LPS addition in the presence of cytochalsin D or control. No significant differences in iNOS or phospho-p38 levels could be detected over three independent experiments. ** is $p<0.01$ as determined by one-way ANOVA with Tukey Kramer test. Error bars are S.E.M.

DISCUSSION

Genetic studies unambiguously tie LRRK2 to several human diseases. Most notably, high frequencies of causative pathological mutations have been identified in late onset PD. Whole genome association studies pinpoint LRRK2 as one of a few genes where common genetic variability underlies susceptibility to leprosy and Crohn's disease, highlighting a potential immunologic function for LRRK2. Other evidence also suggests that immune function is involved in the pathogenesis of PD, since genetic variation in the HLA region associates with PD (Hamza et al., 2011), and numerous pathologic studies describe microglia activation in PD. LRRK2 appears to be expressed in cells of the innate but not adaptive immune system. A recent study demonstrated that the expression of LRRK2 in cultured bone marrow- derived macrophages from mice is up regulated in response to LPS (Hakimi et al., 2011). Another recent study demonstrated that LRRK2 expression is also stimulated by the IFN-γ response in peripheral blood mononuclear cells (Gardet et al., 2010). Thus, it has been hypothesized that LRRK2 may mediate some aspect of signaling or differentiation in innate immune cells.

The recent development of highly specific and sensitive rabbit monoclonal LRRK2 antibodies and LRRK2 knockout mice enables immunohistochemistry studies with a higher degree of confidence. Our results show that in brain tissue challenged with the potent TLR4 agonist LPS, LRRK2 expression is induced in activated microglia. Immunoprecipitation of LRRK2 protein from brains treated with LPS revealed enhanced levels autophosphorylation, which implies greater LRRK2 activity. Direct demonstration of enhanced LRRK2 enzyme activity in neuroinflammation awaits the identification of

bone fide LRRK2 kinase substrates. Interestingly, we found that the accumulation of LRRK2 protein which occurs during inflammatory signaling in primary microglia is not accompanied by significant changes in mRNA levels, suggesting important post-transcriptional regulation.

We find that inhibition of LRRK2, either by small molecule kinase inhibitors or RNAi knockdown, attenuates pro-inflammatory signaling in response to TLR4 activation. LRRK2 is hierarchically clustered in the tyrosine-kinase like superfamily nearby kinases important for inflammatory signaling in immune activation, such as the Interleukin-1 receptor associated kinase (IRAK) family and the mixed lineage kinase (MLK) family (West et al., 2007). Thus, we hypothesized that LRRK2 may function as a stress response kinase during a neuroinflammatory stimulus in the brain by facilitating signal transduction pathways in affected cells. We utilized two approaches to dissect the role of LRRK2 in TLR4 mediated inflammatory responses in primary microglia: small molecule inhibition and RNAi knockdown of total protein. While a single inhibitory molecule may be confounded by off target effects, we find agreement with multiple effective molecules that are unlikely to have over lapping off targets. In cultured rat microglia, peak TNFα release was observed 6 hours post LPS exposure and reduced by 12 hours, and in the context of LRRK2 inhibition, peak induction was reduced by greater than 20% by either LRRK2 small molecule inhibition or RNAi. Likewise, reduced levels of the TNFα target iNOS were detected by western blot. Although this effect is modest in the overall pro-inflammatory response, a cumulative effect over time may link this modifying effect with susceptibility to neurodegeneration since both TNFα and iNOS have been implicated as critical determinants of neurotoxicity.

We also observed an effect of LRRK2 inhibition on the morphological response of microglia to LPS. Exposure of microglia to LPS in the context of inhibited LRRK2 prevents the normal morphological response of fine process extension and cytoskeleton remodeling. The functional role for microglial fine process extension during initial phases of inflammatory signaling are not clear, but may involve physical sequestration of infiltrating pathogens *in vivo* in damaged areas by providing a network blockage (Davalos et al., 2005). We hypothesized that inhibition of fine process extension during LPS stimulation may itself have an anti-inflammatory effect due to negative effects on receptor clustering and lipid raft shuttling. Although actin inhibition nearly abolished fine process extension, there were no anti-inflammatory effects observed. We therefore conclude that a full pro-inflammatory response mediates process extension, but process extension itself does not mediate pro-inflammatory signaling. Thus, LRRK2 inhibition may prevent a full inflammatory response required for fine-process extension, placing LRRK2 as an upstream stress-responsive kinase to TLR4 activation.

Although the current data would suggest an overall pro-inflammatory role for LRRK2, given the complexity of neuroinflammation and the limitations of interpreting microglial action in purified cultures *in vitro*, it is possible an overall anti-inflammatory role for LRRK2 may be likewise envisaged. Indeed, we find that LRRK2 inhibitors quell a chemotaxic response to the potent microglial chemoattractant ADP. However, we interpret these results with some caution since we were not able to perform comparable experiments under conditions of LRRK2 RNAi. In these experiments, it was not possible to remove microglia already transduced with lentiviral LRRK2 shRNA molecules, incubated for the time required to knockdown LRRK2 protein, without killing the

microglia cells. Besides microglia, LRRK2 may also be expressed in anti-inflammatory IL-10 producing macrophages in the brain, and inhibition of LRRK2 in these cells may mitigate anti-inflammatory signaling. Transgenic and LRRK2 knockout mice can be utilized in future studies to clarify overall effects of LRRK2 in neuroinflammation.

In this study, we contribute to a growing body of evidence that suggests a possible modifying role for the immune system and inflammation in PD by demonstrating LRRK2 activation in microglia and critical function in pro-inflammatory responses. It seems hypothetically possible that activating mutations in LRRK2, such as the G2019S missense mutation in the kinase activation loop, may serve to exaggerate neuroinflammatory responses that predispose to neurodegeneration susceptibility in PD. Further studies with*in vivo* models of neuroinflammation and associated neurodegeneration are warranted and will be critical to address the pathophysiological function of LRRK2.

MATERIALS AND METHODS

Immunohistochemistry and Immunofluorescence

Male 8–12 week old WT or LRRK2 KO C57BL6/J mice (provided by Heather Melrose) or Tg(TH-eGFP)DJ76Gsat were perfused with room temperature (RT) phosphate buffered saline solution (PBS, pH 7.4), then 4% paraformaldehyde (PFA) in PBS, and brains removed and post-fixed in 4% PFA in PBS at 4°C for 12 hours with agitation, then embedded in 30% sucrose/PBS for 24 hours at 4°C, then frozen in isopentane and sectioned at 40 μm width on a freezing microtome. Freshly cut sections were rinsed and immediately treated with 0.3% H_20_2 in methanol for 30 min at RT with mild agitation, rinsed and treated with 10 mM Na-Citrate, pH 6.0, 0.05% tween, for 30 min at 37°C. Sections were rinsed and blocked first in 3% non-fat milk in PBS with 0.3% triton x-100 for 1 hour RT and then in 10% normal goat serum in PBS with 0.3% triton x-100 for one hour. LRRK2 antibody solution (containing 0.2 μg/mL for DAB or 1 μg/mL for immunofluorescence rabbit monoclonal C-41, Epitomics, 5% goat serum, 0.1% triton x-100, and 0.01% sodium azide) was applied to sections for 24 hours at 4°C with mild agitation. Sections were rinsed and Goat Anti-Rabbit:biotin (Vector labs), Goat Anti-Rabbit DyLight 649 (Jackson Laboratories), Isolectin-B4:FITC or Isolectin-B4:Biotin (Sigma), was added (as indicated) for 24 hours at 4°C. Sections for immunofluorescence were mounted with ProLong Gold (Invitrogen) onto coverslips. Sections with biotinylated markers were developed with the Vectastain Elite ABC kit and Impact DAB (Vector labs) according to manufacturer's recommendations.

LPS injections

5 μg of LPS (15k endotoxin units, Sigma) was stereotactically injected in 1 μL volume with a flow rate of 0.2 μL/min using a NanoMite pump (Harvard Apparatus) fitted with a 32 gauge fully beveled needle and gas-tight syringe (Hamilton), with a 5 min wait for needle withdrawal, in mice anesthetized with isoflurane. Coordinates were −3.4 AP, −1.1 ML, and −3.9 DV for SNpc and +0.4 AP, −1.5 ML, and −2.5 DV for striatum, with respect to Bregma. For LRRK2 activity assays, FLAG-LRRK2 BAC mice (Jackson strain #012466) were utilized, and FLAG-M2 resin (Sigma) was utilized according to manufacturer's recommendations to immunoprecipitate LRRK2 protein. Animal usage was institutionally approved.

Western blotting, ELISA, and chemicals

Antibodies to LRRK2 (Epitomics, clone C-41), β-actin, GFAP, MBP (Sigma), CD-68 (Serotec), MAP2 (Millipore), GAPDH, IRF-1, VDAC (Santa Cruz), iNOS, Phospho-p38, Ikk-α (Cell Signaling) were used according to manufacturer's suggestions. Antibodies to pT1503 were previously described, and were combined with de-phosphopeptide at a concentration of 10 μg/ml during antibody incubations (Webber et al., 2011). Rat TNFα ELISA assays were from eBioscience. Sunitinib (LC Labs), L2In1 (provided by Dario Alessi), and cytochalasin D (Sigma) were dissolved at a concentration of 10 mM in DMSO. DMSO controls represent DMSO concentrations present at the highest amount in the experiment, and did not exceed 0.04% in any experiment.

Quantitative RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and first strand cDNA generated with Superscript III (Invitrogen). qPCR was performed using Taqman assays

Rn01455646_m1 TBP and Rn00562055_m1 TNFα primer sets (Invitrogen) and iQ Powermix (Bio-rad). Thermocycling was performed on a Bio-rad CFX96 machine.

LRRK2 kinase assays

Kinase assays were performed as previously described (Sen et al., 2009). Recombinant purified human LRRK2 (Invitrogen) was combined into kinase buffer with LRRKtide substrate (Enzo Bioscience) and activity measured by scintillation counting of P-81 Whatman phosphocellulose paper.

Lentiviral Purification

Lentivirus preparation was performed as previously described (Tomlinson, 2008). HEK293-FT cells were transfected with pLP1, pLP2, pVSV-G and lentiviral expression vectors pLKO.1_LRRK2 (Plasmid # TRCN0000022655, shRNA-A, and #TRCN0000022658 , shRNA-B, Open Biosystems), or pLKO.1_Non-coding (NC) shRNA (Addgene plasmid 1864, courtesy of David Sabatini), or cFUGW (no RNAi) control. For determination of titer, RNA was extracted and cDNA was synthesized using the SuperScript VILO cDNA synthesis kit (Invitrogen). Real time PCR reaction was performed using primers that target the RRE element (F-GCA GCA GGA AGC ACT ATG; R-CGC CTC AAT AGC CCT CAG C). Ct values obtained from the virus were compared to the plasmid standard curve in order to determine the number of copies of virus/ μL. eGFP epifluorescence was used to verify that >90% of cells were transduced for the entirety of the experiment.

Primary microglia cell cultures

Primary mixed glial cultures were isolated from the forebrains of 2-day old Swiss Webster rats of either sex using a differential detachment method. Forebrains were digested with Hank's Balanced Salt Solution (Gibco) containing 0.01% trypsin and 10 μg/ml DNase and triturated with DMEM (Invitrogen) containing 20% heat-inactivated fetal bovine serum (FBS, Hyclone) and 1% penicillin–streptomycin. The dissociated cell suspension was plated onto poly-D-lysine-coated flasks. Media changes with DMEM containing 20% fetal bovine serum and 1% penicillin-streptomycin were performed every other day for 7 days. Microglia were separated by shaking the flasks for 1 hour at 200 RPM. The resulting microglial cell suspension was removed and plated at a density of ~1×10^5 cells/cm^2 in DMEM supplemented with 10% FBS and 10 ng/mL GM-CSF (Peprotech). The purity of microglia was verified by anti-rat CD-68 (Serotec) immunolabeling and western blot.

Morphological assessment

Randomized captured phase contrast images were derived from live cultures in a 37°C humidified chamber at 5% CO_2 on a Carl Ziess Cell Observer using Axiovision 4.7 Mark and Find controller. Prior to image collection, cells were incubated with 2.5 μM propidium iodide and 10 μM Hoechst 33342 for 10 min. Resultant images were analyzed by an observer blinded to experimental identity using ImageJ software to calculate process length in microglia cells.

Chemotaxis assay

90,000 primary microglia cells were added to transwell plates (8 μm pore, 24-well inserts, Corning) immediately after microglial removal from astrocyte beds, and allowed to adhere to the upper chamber for 6 hours. The lower chamber was then supplemented

with 100 μM ADP (Sigma) to encourage migration through the membrane to the lower chamber, and experimental drug or DMSO was added to both the upper and lower chamber. After 30 additional hours, media was removed and the total number of cells counted, after an incubation with 10 μM Hoechst 33342 for 10 min, on a Carl Zeiss Cell Observer using Mark and Find software.

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THE G2019S LRRK2 MUTATION INCREASES MYELOID CELL CHEMOTACTIC RESPONSES AND ENHANCES LRRK2 BINDING TO ACTIIN-REGULATORY PROTEINS

by

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ABSTRACT

The Leucine rich repeat kinase 2 (LRRK2) gene is genetically and biochemically linked to several diseases that involve innate immunity including Parkinson disease. LRRK2 protein is highly expressed in phagocytic cells of the innate immune system, most notably differentiated myeloid cells capable of presenting antigen and mounting pro-inflammatory responses. Knockdown of LRRK2 protein in these cells broadly reduces pro-inflammatory responses. However, the effect of LRRK2 pathogenic mutations on myeloid cell function, particularly with respect to pro-inflammatory function, is not clear but could provide insight into LRRK2-linked disease. Here we find that rats expressing G2019S-LRRK2 have exaggerated pro-inflammatory responses and subsequent neurodegeneration after lipopolysaccharide injections in the brain, with a marked increase in recruitment of CD68 cells to the site of injection. Myeloid cells expressing G2019S-LRRK2 show enhanced chemotaxis both in vitro in two-chamber assays as well as in vivo in response to thioglycollate injections in the peritoneum. While G2019S-LRRK2 expression increases the chemotactic response of myeloid cells in these models, we did not detect any biochemical differences in un-stimulated myeloid cells that over-express wild-type or G2019S-LRRK2, or differences in immunological homeostasis in transgenic mice with G2019S-LRRK2 expression. Immunoprecipitation of LRRK2-positive protein complexes from M1-polarized myeloid cells shows a preferential interaction between G2019S-LRRK2 and actin-regulatory proteins that control chemotaxis, as compared to wild-type LRRK2. These experiments suggest that the primary mechanism of G2019S-LRRK2 with respect to myeloid cell function in disease

may be related to exaggerated chemotactic responses through possible up-regulation of actin-responses that mediate myeloid cell mobility.

INTRODUCTION

The *leucine rich repeat kinase 2* (*LRRK2*) gene was initially characterized as part of a newly defined Ras-of-Complex family member and encodes a large ~286 kDa protein with a number of protein-protein interaction domains (Bosgraaf and Van Haastert, 2003). The LRRK2 protein harbors two enzymatically active domains, a GTPase domain and a tyrosine-like serine/threonine kinase domain, separated by a linking domain known as COR (C-terminal of Ras domain). The *LRRK2* gene has been linked to disease susceptibility in several inflammatory disorders. Familial and genome wide association studies have implicated LRRK2 prominently in the susceptibility to Parkinson disease (PD), Crohn's disease (CD), and leprosy (Paisan-Ruiz et al., 2004, Zimprich et al., 2004, Satake et al., 2009, Van Limbergen et al., 2009, Zhang et al., 2009, Nalls et al., 2014). While many of the *LRRK2* genetic variants that are associated with disease are of unknown function, PD-linked familial mutations in *LRRK2* have yielded some insight into how mutations lead to disease. PD-linked mutations in *LRRK2* cluster within the "catalytic core" of the protein in the kinase, GTPase, and COR domains (West, 2014). The most common is the G2019S mutation in the kinase domain, and is believed to confer increased kinase activity of the protein (West et al., 2005, West, 2014). This increased kinase activity has been linked to cellular toxicity and dysfunction in diverse model systems, although the substrates and pathways through which LRRK2 acts remain unclear (Smith et al., 2005, Greggio et al., 2006, MacLeod et al., 2006, Smith et al., 2006, Nichols et al., 2010, Sheng et al., 2012, Stafa et al., 2012, MacLeod et al., 2013).

Following the genetic implication of *LRRK2* in inflammatory disease, the expression level of LRRK2 protein has been found to be highest in myeloid cells of the

innate immune system (Gardet et al., 2010, Hakimi et al., 2011, Thevenet et al., 2011, Moehle et al., 2012, Daher et al., 2014). Myeloid cells are a diverse class of cells that arise from hematopoietic stem cells of the bone marrow that spawn common myeloid progenitors. The progenitors then can differentiate into a wide variety of blood cells including erythrocytes, megakaryocytes, and innate immune cells including monocytes, macrophages, neutrophils, dendritic cells, and eosinophils (Alvarez-Errico et al., 2014, Crook and Liu, 2014, Chistiakov et al., 2015). *LRRK2* expression is particularly high or exclusively expressed in a subclass of myeloid cells that are CD14+ (part of the lipopolysaccharide [LPS] receptor complex) and CD16+ (binds Fc regions of antibodies and is associated with mature cell phenotypes) (Thevenet et al., 2011). Myeloid cells known to express *LRRK2* have diverse functions in the innate immune system such as secreting cytokines and chemokines, presenting antigen to adaptive immune cells, phagocytizing debris, pathogens and dying cells, and recognizing and moving to sites of danger/pathogen-associated molecular patterns through chemotaxis.

Knockdown or knockout of *LRRK2* expression and/or activity through genetic or pharmacological approaches has implicated *LRRK2* in many myeloid cell effector functions. RNAi knockdown of expression or pharmacological inhibition of LRRK2 has been shown to decrease the release of secreted cytokines such as TNF after pro-inflammatory stimuli in response to a number of pro-inflammatory agonists (Kim et al., 2012, Moehle et al., 2012, Daher et al., 2014). LRRK2 kinase inhibition has also been shown to decrease phagocytosis of pathogenic particles (Marker et al., 2012). Pharmacological inhibition of LRRK2 also decreases cell chemotaxis in cultured microglia cells and fibroblasts (Moehle et al., 2012, Caesar et al., 2013). The impairment

of chemotaxis due to loss of LRRK2 can also be supported through studies of knockout of LRRK2 homologs GbpC and ROCO4 (Bosgraaf et al., 2005, Cha et al., 2010, Kicka et al., 2011). However, mice and rats lacking LRRK2 also have systemic changes in immune cell homeostasis (Herzig et al., 2011, Baptista et al., 2013), notably including deficits in white blood cells. In addition, widely used LRRK2 kinase inhibitors have significant off-target effects (Liu et al., 2014), making it difficult to be able to understand the role of LRRK2 in myeloid cells using these models and tools.

While previous studies have focused on how loss of LRRK2 expression or activity influences cells of innate immunity, only a few studies have evaluated the effects of pathogenic missense LRRK2 mutations. Using mice that express R1441G pathogenic mutation, increased production of proinflammatory cytokines were detected in stimulated primary microglial cells (Gillardon et al., 2012). Several receptors, including toll like receptors (TLRs), scavenger receptors, and various chemokine receptors underlie these pro-inflammatory processes and can be utilized to determine cell type and activation state (Martinez and Gordon, 2014, Moehle and West, 2014). LPS is a canonical pro-inflammatory stimulus that elicits several of the effector functions of myeloid cells by binding to TLR4/CD14 complexes present in CD14+ cells known to express high LRRK2 levels (Thevenet et al., 2011). A direct LPS injection to the brain elicits inflammatory responses with respect to myeloid cell recruitment and activation, and subsequent dopaminergic neurodegeneration (Castano et al., 1998, 2002). LRRK2 knockout rats are protected from the effects of LPS-induced neurodegeneration (Daher et al., 2014), but the effects of pathogenic LRRK2 mutations on LPS-induced neurodegeneration and myeloid cell activation are not known.

In this study, we use transgenic rats and mice that over-express G2019S-LRRK2 or wild-type (WT) LRRK2 to explore myeloid cell responses altered by mutant LRRK2 expression. Through a combination of *in vitro* approaches using isolated primary cultured cells as well as several *in vivo* models, we find that G2019S-LRRK2 expression enhances chemotactic responses to a number of stimuli, but fails to alter other components of myeloid cell function affected by the loss of LRRK2 expression. Our findings revealed that in activated myeloid cells, the G2019S mutation robustly increases the association of LRRK2 with the actin-regulatory network that mediates chemotactic responses in myeloid cells. The role of LRRK2 in disease pathogenesis may be explained in part by its role in regulating myeloid cell chemotactic responses.

RESULTS

G2019S-LRRK2 Expression Enhances LPS-induced Brain Myeloid Cell Responses

Genetic knockout or knockdown of *LRRK2* both *in vivo* and *in vitro* produces an attenuated pro-inflammatory response to LPS (Moehle et al., 2012, Daher et al., 2014). The effect of pathogenic LRRK2 mutations on these myeloid cell-driven responses has not previously been studied. Recently, a line of Sprague Dawley rats that carry a human-derived bacterial artificial chromosome (hBAC) encoding the *LRRK2* gene recombineered with a G2019S-pathogenic mutation were developed by CJ Li and distributed in partnership with Cornell University and the Michael J. Fox Foundation. We verified strong transgenic expression of G2019S-LRRK2 in thioglycollate elicited primary macrophages (TEPM) cultured from these animals (Fig S1 A). The G2019S-LRRK2 protein is also expressed in dopaminergic substantia nigra pars compacta (SNpc) neurons and other brain nuclei susceptible to neurodegeneration from LPS exposure (West et al., 2014).

To examine myeloid cell responses in the LRRK2-G2019S rats compared to non-transgenic littermate controls (WT), we injected LPS or saline control (PBS) directly in the right SNpc to induce a robust and selective loss of dopaminergic neurons (Castano et al., 1998, Kim et al., 2000, Castano et al., 2002). After intracranial LPS injection, a panel of antibodies including IBA-1 (microglial morphology), CD68 (mature myeloid lineage phagocytic cells), and tyrosine-hydroxylase (dopaminergic neurons), were used to evaluate myeloid cell responses and their effects on dopaminergic neuron survival, respectively. Confocal analysis showed an increase in the intensity and proportion of CD68+/IBA1+ cells in G2019S-LRRK2 rats compared to non-transgenic littermate

controls (Fig. 1A). Commensurate with the increase in CD68+ cells in the ipsilateral SNpc were reduced numbers of TH+ cells (Fig. 1 B and C). These observations are consistent with an increased pro-inflammatory (M1) response in the G2019S-LRRK2 animals in response to LPS stimulation.

Previously we found that LRRK2 KO rats were resistant to LPS-induced dopaminergic neurodegeneration compared with WT rats (Daher et al., 2014). Stereological counts to TH+ cells in the SNpc revealed that the G2019S-LRRK2 rats have exacerbated dopaminergic neurodegeneration in response to LPS exposure (Fig. 1D). The G2019S-LRRK2 rats had a ~60% increase in TH neuron degeneration as compared to WT controls. Stereological counts of CD68+ cells in adjacent serial sections demonstrated that there were more CD68+ cells recruited to the SNpc of G2019S-LRRK2 rats compared to that of WT animals (Fig 1 E), and this correlated well with the extent of neurodegeneration occurring in the animal (Fig. 1 G).

To determine whether there were baseline differences between WT and G2019S-LRRK2 rats unrelated to LPS exposure, we analyzed by confocal analysis and stereology for TH and CD68 cells a separate cohort of animals injected with only saline (PBS, Fig S1 B and S2 A and B). We did not observe differences in the number of TH+ cells between G2019S-LRRK2 animals and WT controls in either the injected or un-injected side (Fig S 2). There was no significant CD68 reactivity in either the injected or un-injected sides in these animals. Overall, these data suggest an increase in pro-inflammatory responses in the G2019S-LRRK2 animals when challenged with LPS, but that the G2019S-LRRK2 rats have normal baselines of TH cell counts, consistent with

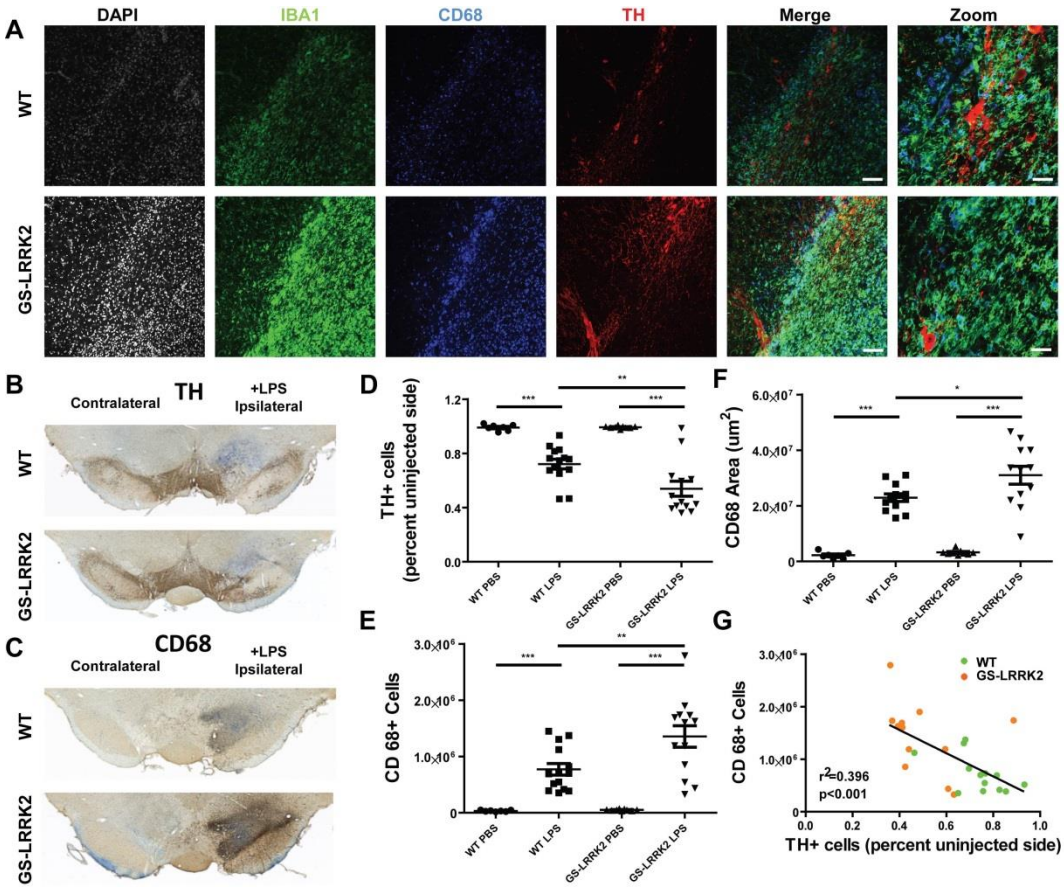

FIGURE 1**. Enhanced Neuroinflammation in G2019S-LRRK2 transgenic rats**. 10-12 week old non-transgenic littermate controls (WT, n=10) and G2019S-LRRK2 (GS-LRRK2, n=13) positive rats were unilaterally injected with 5 µg ultra-pure lipopolysaccharide (LPS) into the right substantia nigra pars compacta (SNpc). Animals were sacrificed 2-weeks post-injection. **A)** Representative confocal images of the SNpc stained for ionized calcium-binding adapter molecule 1 (IBA1) and cluster of differentiation protein 68 (CD68), both markers of myeloid cells, along with tyrosine hydroxylase (TH) expressed in nigral dopaminergic neurons. Scale bars for images are 10 µm and 100 µm for "Zoom" panels. **B)** Representative bright-field images of DAB-stained coronal brain sections from WT and G2019S-LRRK2 rats after LPS injection. TH staining and **C)** CD68 staining is shown with Nissl staining for contrast. **D)** Unbiased stereological quantification of TH+ neurons and **E)** CD68+ cells in the SNpc in WT and G2019S-LRRK2 LPS-injected rats. **F)** Volume calculation of the tissue area encompassing CD68 cell immunoreactivity. **G)** Correlation of CD68+cell counts to percent of TH+ cells remaining in the SNpc (calculated from panels D and E). Significance was calculated with one way ANOVA with Tukey's post-hoc comparisons, bars represent group means and error bars are S.E.M. * represents $p<0.05$, ** represents $p<0.01$, *** represents $p<0.001$.

other reports using independently derived human BAC G2019S-LRRK2 rat transgenic strains (Walker et al., 2014, Lee et al., 2015).

G2019S-LRRK2 Expression Does Not Affect Secretion of Pro-Inflammatory Factors or Phagocytosis

To determine the effects of G2019S-LRRK2 expression on critical aspects of myeloid cell function, we isolated TEPM cells from transgenic and non-transgenic littermate controls. WT-LRRK2 BAC mice that express LRRK2 protein at levels comparable to that in G2019S-LRRK2 BAC mice were used to control for the effects of over-expression of LRRK2 protein, compared to TEPM cells from non-transgenic littermate mice. WT-LRRK2 and G2019S-LRRK2 over-expression is closely matched in TEPM cells (Fig 2 A).

To test the role of G2019S and WT-LRRK2 over-expression on cytokines and chemokines secreted by TEPMs, cells were derived from non-transgenic littermate controls (WT, nTg), G2019S-LRRK2 mice and WT-LRRK2 mice. Cells were counted and plated at equivalent densities into tissue culture wells. Supernatants were collected from LPS-exposed or saline-treated wells and analyzed for a variety of cytokines and chemokines (Fig 2 B, Fig. S3). With all secreted factors measured, no significant differences could be observed between groups, showing that the transgenic over-expression of WT-LRRK2 or expression of G2019S-LRRK2 does not affect this aspect of myeloid cell inflammation.

Pathogenic and WT LRRK2 function are implicated in phagocytosis, endocytosis, lysosomal function, and vesicle recycling (Biskup et al., 2006, Alegre-Abarrategui et al., 2009, Higashi et al., 2009, Piccoli et al., 2011, Marker et al., 2012, Matta et al., 2012, MacLeod et al., 2013, Manzoni et al., 2013, Orenstein et al., 2013, Stafa et al., 2014). To

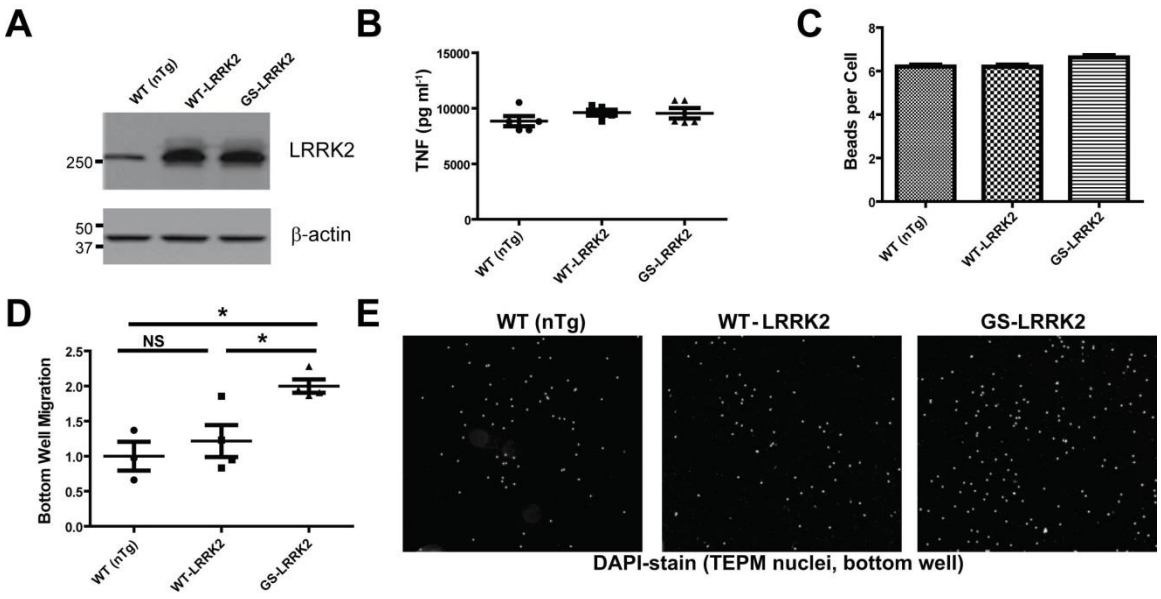

FIGURE 2**. Pathogenic G2019S-LRRK2 enhances chemotaxis but not phagocytosis or secretion of inflammatory cytokines and chemokines.** Thioglycollate-elicited peritoneal macrophages (TEPM) cells from adult male non-transgenic littermate controls (WT), WT-LRRK2 BAC and G2019S(GS)-LRRK2 BAC mice were placed in culture (at least 3 animals per strain per experiment were used). **A)** Representative western blots showing the magnitude of LRRK2 overexpression in TEPMs from GS-LRRK2 and WT-LRRK2 animals compared with non-transgenic (nTg) littermate controls. **B)** TEPMs were allowed to rest overnight after plating, and then stimulated with LPS (100 ng ml^{-1}) for 6 hours. No differences in TNF secretion could be detected as measured by ELISA, or with 32 other cytokines and chemokines measured by multiplex bead arrays (One Way ANOVAs, $p>0.5$ see Fig S 3 B-G for other representative secreted factors measured). **C)** Fluorescent Zymosan beads were added to TEPM cultures and the number of beads internalized per TEPM were determined by fluorescent microscopy (see Fig S 4 A-B for related histograms and images). No differences in the number of beads phagocytized per TEPM could be observed between groups (Kruskal-Wallis ANOVA, $p>0.5$). **D)** TEPM cells were plated into the top well of Boyden chambers in the presence of 100 μM ADP in the bottom chamber and the number of cells migrating to the bottom chamber over 36 hours was measured. Significantly more G2019S-LRRK2 TEPM cells migrated to the bottom well (one Way ANOVA, Tukey's post-hoc test, $*p<0.05$). **E)** Representative images of TEPMs visualized with DAPI stain in the bottom chamber of the chemotaxis assay. All bars represent group mean and error is shown as S.E.M.

determine if LPS-stimulated G2019S-LRRK2 expressing TEPM cells had increased phagocytosis compared to WT-LRRK2 expressing or TEPM cells from non-transgenic mice, zymosan beads were applied to cultured cells plated at equivalent density, and uptake of the beads determined by microscopy. A similar number of beads were phagocytized between the three groups of TEPM cells (Fig 2 C), indicating this aspect of myeloid cell function is unaffected by G2019S-LRRK2 expression. Histograms reveal comparable distributions and numbers of TEPM cells that have high phagocytic activity compared to cells with low phagocytic activity (Figure S4).

G2019S-LRRK2 Increases Myeloid Cell Chemotaxis

Myeloid cell chemotactic response is critical in a diversity of diseases and model systems that involve both innate and adaptive immune responses (Luster et al., 2005). The chemotactic ability of TEPM cells cultured from WT (nTg), WT-LRRK2 BAC, and G2019S-LRRK2 BAC mice were tested in Boyden chambers for differential migration through an 8.0 μm pore membrane. With addition of 100 μM ADP, a robust stimulant of chemotaxis used previously (Honda et al., 2001, Moehle et al., 2012), added into the bottom well media, the G2019S-LRRK2 expressing cells had an approximate 2-fold increase in bottom-well migration (Fig 2 D,E). The over-expression of WT-LRRK2 had no effect compared to cells from non-transgenic littermate controls.

In light of the finding that increased numbers of CD68+ cells were recruited to the brain in response to LPS injection (Figure 1A, C, E), we next sought to measure chemotactic responses *in vivo* in a second model of myeloid cell chemotaxis with a stimulant other than LPS. Thioglycollate induced peritonitis (TIP) is a robust model of inflammation used to measure acute chemotactic responses of immune cells into the

peritoneal cavity (Barski et al., 1955, Gallily et al., 1964). At sixteen hours post thioglycollate exposure, the majority of cells (>80%) recruited to the peritoneal cavity are monocytes, macrophages and neutrophils (Vodovotz et al., 1993, Schleicher et al., 2005, Ghosn et al., 2010). Flow cytometry revealed that G2019S-LRRK2 BAC mice have ~ 2 fold more cells recruited to the peritoneum than non-transgenic littermates (WT) and WT-LRRK2 BAC mice (Fig. 3 A). No difference was observed between non-transgenic mice and animals over-expressing WT-LRRK2. Classification of the TIP cells showed that there was a ~2 fold increase in both recruited monocytes/macrophages (CD11b+ Ly6G(A18)+) cells as well as neutrophils (CD11b+ Ly6G(A18)-). Gating strategies for flow cytometry are given in Fig. S5. These results indicate that the G2019S mutation in LRRK2 is associated with a substantial increase in chemotaxis responses of innate immune cells with a variety of stimulants including LPS, ADP, and thioglycollate.

LRRK2 KO animals have previously been shown to have several alterations in blood chemistry, including a decrease in white blood cells under basal conditions (Herzig et al., 2011, Baptista et al., 2013, Ness et al., 2013). In order to test whether baseline differences in immunological homeostasis exist in G2019S-LRRK2 mice that could account for the observed increase in the accumulation of myeloid cells in the peritoneum (Fig 3), a complete blood chemistry panel was performed (Table 1). However, as opposed to *LRRK2* KO rodents, no differences were observed in any tested element of blood chemistry and increases in white-blood cells were not detected in the G2019S-LRRK2 mice (Table 1).

Although the number of circulating white blood cells is comparable between the LRRK2 mouse strains used in this study, the basal activation (i.e., polarization) state of

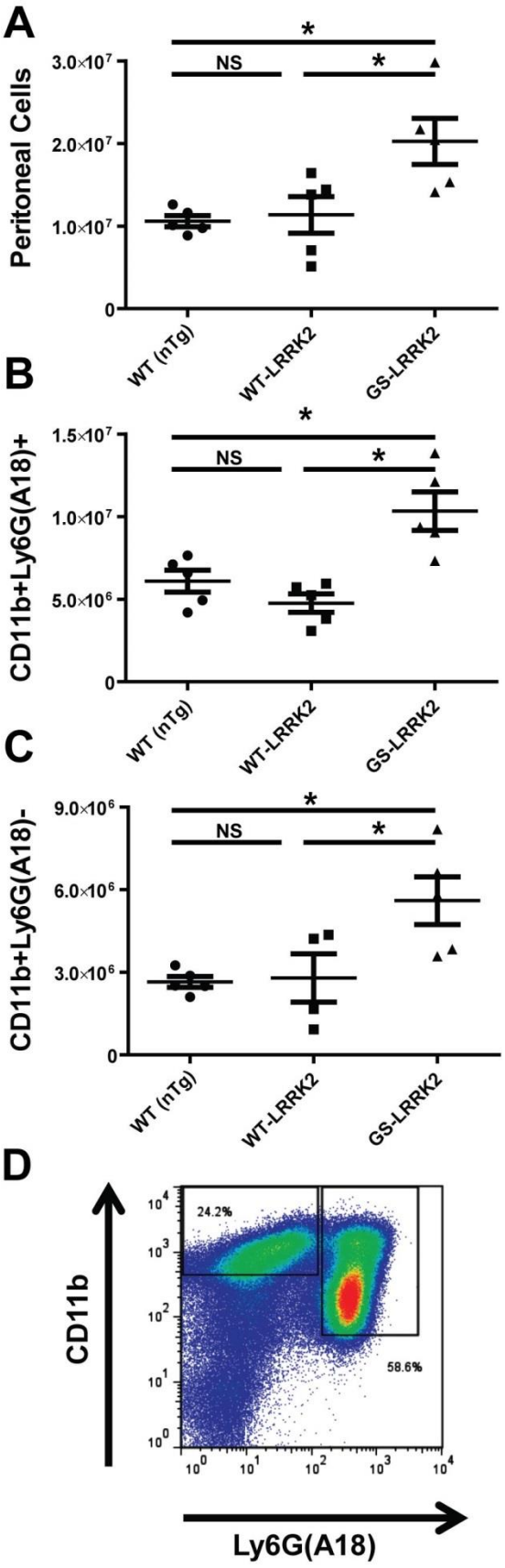

Figure 3. G2019S LRRK2 expression increases chemotaxis of innate immune cells in thioglycollate-elicited peritonitis (TIP). Total peritoneal cells from at least five male adult mice each of WT (non-transgenic, nTg), WT-LRRK2 BAC, and G2019S(GS)-LRRK2 were lavaged 16 hours after thioglycollate exposure. Red blood cells were lysed and the remaining cells analyzed as follows: **A)** Raw TIP cell counts isolated per animal, **and B, C)** sub classification of TIP cells by flow cytometry. Cell were stained for Ly6G (A18 clone) and CD11b. Quantification of CD11b+Ly6G(A18)+ cells, likely neutrophils, and quantification of CD11b+Ly6G(A18)- cells, likely monocytes/macrophages, are given. **D)** Representative flow cytometry plot used in part to calculate panels B and C. As expected, greater than 80% of TIP cells are represented by these populations (see Fig S5). *represents $p<0.05$, one-way ANOVA with Tukey's post-hoc test, and NS represents not significant ($p>0.5$). Lines are group mean and error is shown as S.E.M.

	WT	WT-LRRK2	GS-LRRK2
RBC (M/µL)	6.02 ± 1.48	6.69 ± 0.39	6.15 ± 0.70
HCT (%)	26.96 ± 8.03	30.66 ± 5.22	30.07 ± 4.49
HGB (g/dL)	9.16 ± 2.09	10.1 ± 0.66	9.45 ± 0.82
MCV (fL)	44.41 ± 4.15	45.58 ± 4.27	48.85± 5.69
MCH (pg)	15.27 ± 0.63	15.15 ± 0.93	15.35 ± 0.36
MCHC (g/dL)	34.77 ± 4.74	33.63 ± 5.44	31.75 ± 3.76
RDW (%)	30.73 ± 3.65	31.20 ± 1.60	33.45 ± 3.86
WBC (K/µL)	3.38 ± 1.95	5.28 ± 1.61	4.18 ± 1.42

TABLE 1**. Complete Blood Counts.** Adult mice (at least 5 each per genotype) from WT (non-transgenic littermates), WT-LRRK2 BAC and G2019S-LRRK2 BAC mice were analyzed for blood chemistry. RBC is red blood cells, HCT is hematocrit, HGB is hemoglobin, MCV is mean corpuscular volume, MCH is mean corpuscular hemoglobin, MCHC is mean corpuscular hemoglobin concentration, RDW is red blood cell distribution width, WBC is white blood cell count. Values reported are ± standard deviation. No significant differences ($p>0.5$, One Way ANOVA) are observed between strains for all markers.

G2019S-LRRK2-expressing myeloid cells could be different, priming them for chemotactic responses (e.g., M1 polarization). Further analysis of peritoneal white blood cells using flow cytometry demonstrated that the percentages of CD11b+ Ly6G(A18)+ or CD11b+ Ly6G(A18)- cells in white-blood cell populations are equivalent between all strains of mice (Fig. S 6). MHCII expression is often used to assess the activation state of macrophages (Kigerl et al., 2009, Mege et al., 2011, Mikita et al., 2011, Martinez and Gordon, 2014). We examined the basal activation state of the G2019S-LRRK2 CD11b+ Ly6G(A18)- cells using median-fluorescence intensity (MFI) of MHC-II reactivity (Fig S 6 D,E). No significant differences in MHC-II MFI were observed, suggesting the G2019S-LRRK2 cells are not more activated under basal conditions.

Despite the lack of activation under basal conditions, an increase in myeloid cell chemotaxis caused by G2019S-LRRK2 expression could be explained by an increased in total and/or surface expression of critical chemotaxis receptors such as CCR2. CCR2 is an essential component of myeloid cell chemotaxis in both *in vivo* LPS models and in TIP (Kuziel et al., 1997, Mack et al., 2001, Mildner et al., 2009). Using flow cytometry, the predominant populations of cells in the peritoneum, CD11b+ Ly6G(A18)- and CD11b+ Ly6G(A18)+ cells were examined for expression of CCR2 (gating strategy shown in Fig S7). According to MFI calculations from flow cytometry, the surface expression of CCR2 was comparable between all groups, despite the apparent increase in chemotaxis associated with G2019S-LRRK2 expression (Figure 4).

The G2019S-LRRK2 Mutation Enhances LRRK2 Association with Proteins Responsible for Myeloid Cell Mobility

In several cell lines, LRRK2 forms complexes with the actin cytoskeleton (Parisiadou et al., 2009, Kicka et al., 2011, Meixner et al., 2011, Caesar et al., 2015).

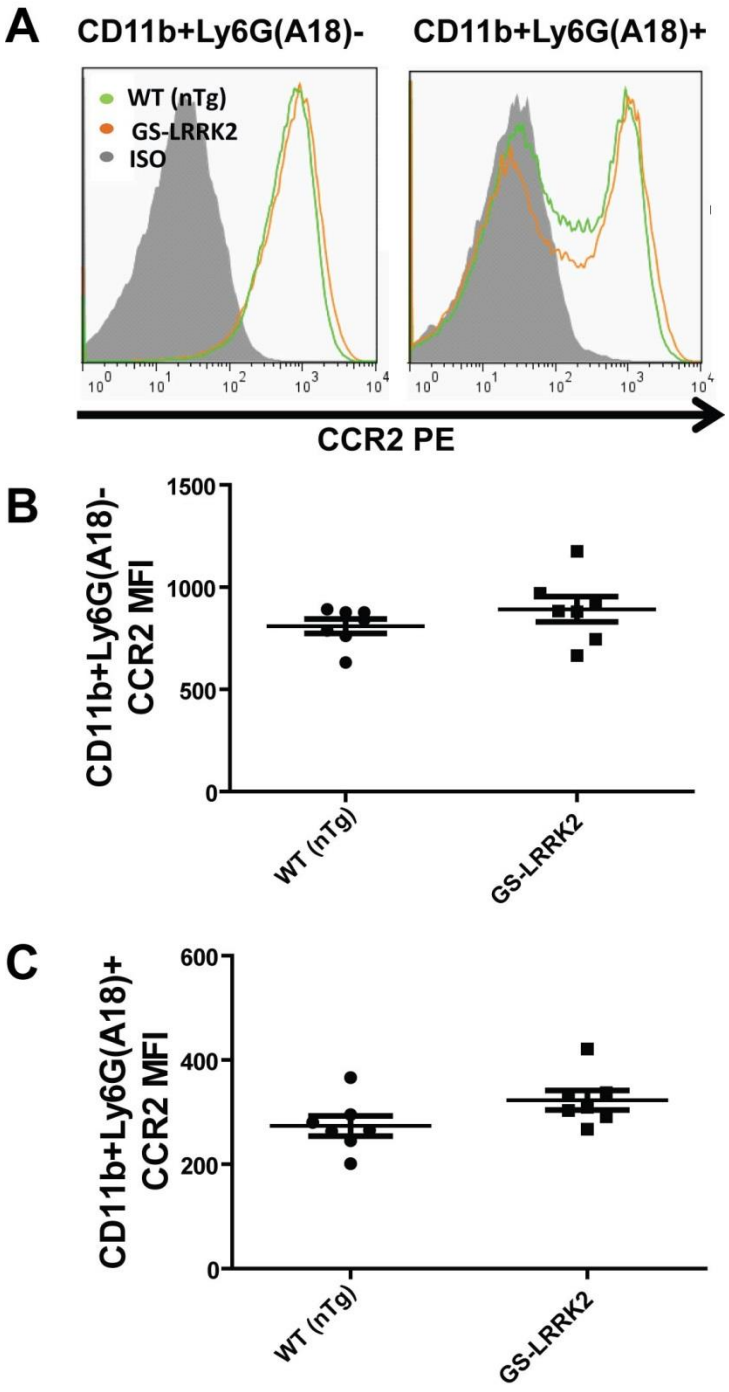

Figure 4. G2019S-LRRK2 expression does not affect CCR2 surface expression in TIP. Total peritoneal cells from at least six male adult mice from WT (nontransgenic, nTg) or G2019S (GS)-LRRK2 were lavaged 16 hours after thioglycollate exposure. Red blood cells were lysed and the remaining cells analyzed. **A)** Representative flow histograms indicating CCR2 expression in comparison to isotype (ISO) control signal, and **B)** median fluorescent intensity calculations of CCR2 from CD11b+Ly6G(A18)- and **C)** CD11b+Ly6G(A18)+ cell populations. No differences could be detected between groups, two-way Student's t test $p>0.05$, bars show group mean and error bars are S.E.M.

LRRK2 protein complexes derived from polarized myeloid cells have not been studied previously. Using M1-polarized TEPMs, we utilized the N-terminal FLAG epitope tag engineered into the BAC transgenic mice to immunoprecipitate LRRK2 protein complexes. Immunoprecipitates from non-transgenic littermate controls that do not express proteins with the FLAG epitope tag were processed in parallel to identify proteins unrelated to LRRK2-positive protein complexes. 394 proteins were detected in complex with LRRK2 through identification of high-confidence peptides via tandem mass spectrometry. However, many of these proteins were identified as possible non-specific interactions through input of the 394 proteins into a database ranking contaminants common to affinity purifications identified by mass spectrometry (www.CRAPome.org, (Mellacheruvu et al., 2013)). Proteins rarely identified in protein complexes across the 411 affinity purifications in the database (<10% chance, Fig. 5) filtered 213 proteins leaving 154 proteins identified interacting with LRRK2. 52 other proteins that include immunoglobulin from the experiment, poorly annotated proteins, and ambiguous peptides and/or database entries (listed as Gene Symbol Not Mapped) were filtered from the LRRK2-interacting set.

To confirm the mass spectrometry identifications, we repeated independent immunoprecipitation experiments from at least three TEPM cell lysates derived from at least three mice in each group. Evaluation of the LRRK2 protein complexes revealed a robust interaction between LRRK2 in complex with myosin 1f (Myo1f), myosin IIa (Myo9), and actin related complex 2 (Arp2) (Fig. 5B). In all cases G2019S-LRRK2 showed a striking enhancement of interaction with these proteins (Fig. 5B), even though both WT-LRRK2 and G2019S-LRRK2 are expressed at equivalent levels in these cells

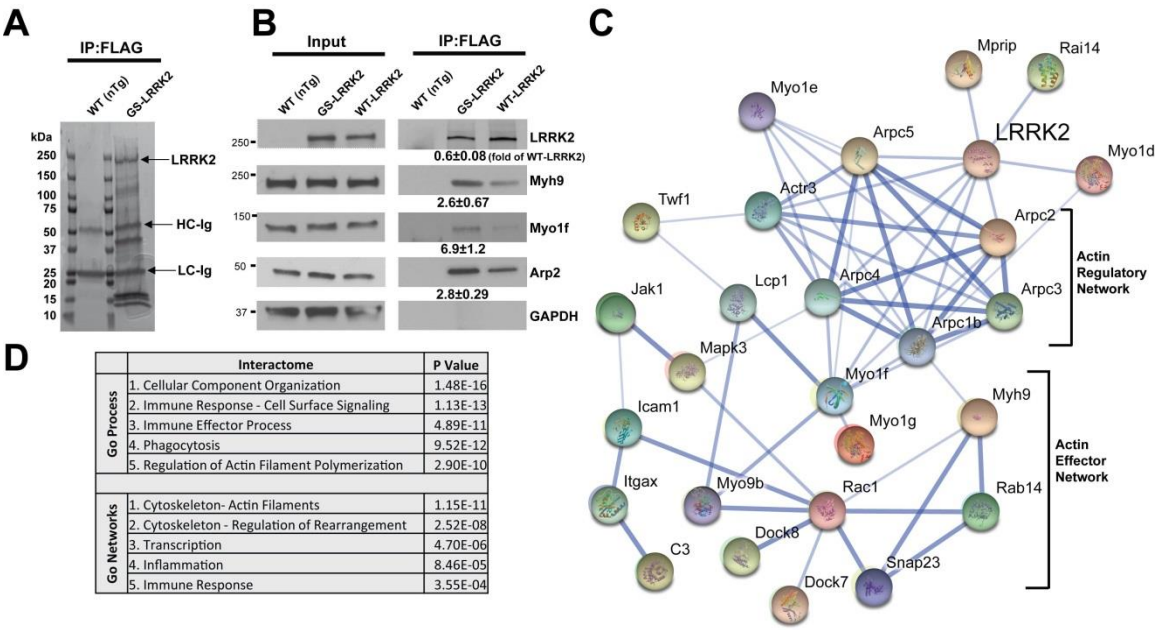

FIGURE 5. **Definition of the LRRK2:actin-regulatory complex and enhanced association caused by the G2019S LRKR2 mutation. A)** Coomassie-stained gel of immunoprecipitated proteins from M1-activated (LPS treated, 100 ng ml^{-1}) TEPM from WT (non-transgenic, nTg) and G2019S(GS)-LRRK2 mice. Immunoprecipitates on beads were washed in parallel under stringent conditions so that the only protein bands visible in the WT (nTg) lane were from FLAG antibody eluted from the resin. Labeled bands include LRRK2 protein, and the heavy-chain and light-chain (HC and LC, respectively). **B)** Representative western blots of selected components from LRRK2-positive protein complexes from three independent experiments of WT (nTg), WT-LRRK2 and GS-LRRK2 FLAG-immunoprecipitations. Immunoprecipitates from the GS-LRRK2 pull-down are compared to WT-LRRK2 pull-downs, and changes in intensity are indicated (GS-LRRK2 relative to WT-LRRK2). **C)** String-DB analysis of LRRK2 proteins in complex that segregate into the actin-regulatory network and actin-cytoskeleton effector proteins, known to control myeloid cell motility. **D)** Gene-ontology (GO) process and network analysis to detect enrichment of terms from proteins identified in LRRK2 positive protein complexes. P Values were calculated from Wilcoxon signed rank tests and corrected for multiple testing by Benjamini-Hochberg analysis.
(Fig 3 B). However, we did not identify any proteins exclusively interacting with

G2019S-LRRK2 and not WT-LRRK2, or vice-versa. Input of the list of the 154 LRRK2 interacting proteins into gene ontology and String-db analysis revealed a coordinated network of actin-regulatory proteins known to be in complex with one another. These actin-regulatory proteins comprise the actin-effector network, as indicated by graphically in Fig 5 C. Input of the 154 genes in MetaCORE GeneGO analysis corroborated a strong enrichment for the interactome related to actin-cytoskeleton control, and gene ontology networks related to the actin cytoskeleton (Figure 5 D)

DISCUSSION

In this study we have utilized several model systems and experimental approaches to help understand the effects of pathogenic G2019S-LRRK2 expression on myeloid cell function. Because studies in LRRK2 KO rodents suggest LRRK2 may affect broad changes in innate immune function, we hypothesized similar broad changes would be detectable in rodents expressing kinase-overactive G2019S-LRRK2. We discovered that most canonical myeloid cell functions were identical whether or not the cells expressed G2019S-LRRK2, and blood chemistry of G2019S-LRRK2 mice was unperturbed, in contrast to LRRK2 KO rodents. However, one aspect of myeloid cell function that is critical to inflammatory responses and may be critical to LRRK2-linked pathogenesis is enhanced chemotaxis and myeloid cell mobility. Because the action of G2019S-LRRK2 expression on chemotaxis was revealed through a diversity of chemoattractants including LPS, ADP and thioglycollate, we hypothesize that mutant LRRK2 would elicit alterations in fundamental aspects of chemotactic responses. The diversity of model systems that reveal enhanced G2019S-LRRK2 myeloid chemotactic responses suggest that the activated cells are more motile rather than enhancement of a single chemotaxis signaling pathway. Potentially related to this result, we find that in stimulated cells the G2019S-LRRK2 mutation stabilizes the association of LRRK2 with the actin-regulator network known to control the actin-cytoskeleton responsible for myeloid cell mobility (Asokan et al., Jones, 2000, Rey et al., 2002, Mukai et al., 2005, Kim et al., 2006, Sandquist et al., 2006, Dulyaninova et al., 2007, Even-Ram et al., 2007, Vicente-Manzanares et al., 2007, Sandquist and Means, 2008, Baumann, 2012, Cavnar et al., 2012, Chen et al., 2012, Choi et al., 2012, Wu et al., 2012).

In other cell systems, LRRK2 has been implicated in directing elements of the actin cytoskeleton and cell motility (Jaleel et al., 2007, Parisiadou et al., 2009, Meixner et al., 2011, Habig et al., 2013, Beilina et al., 2014, Schreij et al., 2015). Knockout of the LRRK2 homolog GbpC in *Dictyostelium* not only reduces chemotaxis but disrupts interaction and phosphorylation of Myh9 (Bosgraaf et al., 2002, Bosgraaf et al., 2005). Other proteomic screens of LRRK2 complexes using similar unbiased approaches have identified Myo1f and Arp2 as major LRRK2 interactors that we also observe in TEMP cells with mass spectrometry and western blots (Meixner et al., 2011). Arp2 expression increases the speed of cell migration or motility through increasing actin filament branching at the leading edge of the cell (Jones, 2000, Mukai et al., 2005, Baumann, 2012, Cavnar et al., 2012, Choi et al., 2012, Wu et al., 2012, Rougerie et al., 2013). Cells from Myo1f KO mice have been shown to have decreased motility (Kim et al., 2006, Chen et al., 2012), and Myh9 expression is likewise critical for cell migration and motility (Asokan et al., Sandquist et al., 2006, Dulyaninova et al., 2007, Even-Ram et al., 2007, Vicente-Manzanares et al., 2007, Sandquist and Means, 2008, Jacobelli et al., 2009, Pecci et al., 2011).

The effects of LRRK2 mutations have also been implicated in other cells systems as altering actin dynamics and cell motility. Fibroblasts derived from mice or patients with LRRK2 mutations have also indicated enhanced actin dynamics and differential LRRK2 interactions with cytoskeltal elements, as well as increased fibroblast motility (Caesar et al., 2013, Caesar et al., 2015). Additional studies are required to investigate whether G2019S-LRRK2 protein changes actin-regulatory proteins and the cytoskeleton in a manner that enhances LRRK2 association, or whether the G2019S mutation alters

intrinsic LRRK2 affinity for the proteins in the actin-regulatory network. Previously, LRRK2 kinase activity has been shown to drive toxicity and dysfunction in diverse model systems (Smith et al., 2005, West et al., 2005, MacLeod et al., 2006, Smith et al., 2006, Nichols et al., 2010, Sheng et al., 2012, Stafa et al., 2012, MacLeod et al., 2013). Future studies are also required to determine if LRRK2 may phosphorylate actin-regulatory proteins in polarized myeloid cells, and whether the effects of G2019S-LRRK2 on chemotaxis are dependent on LRRK2 kinase activity.

Myeloid cell chemotaxis is a critical aspect of inflammatory responses occurring in many disease processes. In Crohn's disease pro-inflammatory myeloid cells including macrophages can drive inflammation central to pathology underlying disease (Marks et al., 2006, Sartor, 2006, Dessein et al., 2008, Marks and Segal, 2008). In Mycobacterium *leprae* infection, macrophage recruitment to granulomas in order to contain infected cells is vital to containing infection (Turk, 1985, Adams et al., 2002, Hagge et al., 2004). In Parkinson disease (PD), neuroimaging studies reveal an abundance of activated myeloid cells throughout much of the brain (Gerhard et al., 2006), and this is confirmed in pathological studies that show an abundance of pro-inflammatory myeloid cells in affected brain regions (McGeer et al., 1988).

LRRK2 is linked to these three diseases through genome-wide association studies (GWAS), but LRRK2 is most clearly linked to PD because of familial-disease linked missense mutations (G2019S being the most common) that cause typical late-onset disease. While in a typical Western population, the prevalence of the G2019S mutation is relatively low (1-5% of PD cases), certain populations, especially Ashkenazi Jews and North African Arabs, have up to 30% of all PD cases attributable to the G2019S mutation

(Lesage et al., 2006, Ozelius et al., 2006, Healy et al., 2008, Nalls et al., 2014). These high rates of mutations in LRRK2 might be attributable to a founder effect from centuries ago (Bar-Shira et al., 2009). However, with respect to G2019S effects on myeloid cells, an increase in chemotaxis and subsequent innate immune responsiveness may be protective in some scenarios of pathogen challenge. For example, numerous studies support the conclusion that enhanced myeloid cell chemotaxis contributes to reduced mortality rates associated with bacterial sepsis (Alves-Filho et al., 2008, Paula-Neto et al., 2011, Kurihara et al., 2013, Park et al., 2013). While exaggerated chemotactic responses may offer protection from sepsis and possibly other infectious diseases, the same response may enhance neurodegeneration as observed in this study. The available tools and LRRK2 transgenic models are well suited to test this hypothesis and advance our understanding of LRRK2 function in innate immunity.

MATERIALS AND METHODS

Animals

Mouse FLAG-G2019S-LRRK2 BAC (B6.Cg-Tg(Lrrk2*G2019S)2Yue/J) and mouse FLAG-WT-LRRK2 BAC (B6.Cg-Tg(Lrrk2)6Yue/J) were developed in the laboratory of Zhenyu Yue and obtained from the Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME, USA). These mice have been previously described (Li et al., 2010, Sepulveda et al., 2013). Mice strains used in this study were maintained on the C57BL/6J strain, and upon study completion sentinels positive for G2019S-LRRK2, WT-LRRK2, or non-transgenic littermate controls were subjected to genome scanning for C57 substrain identification (Jackson Laboratories SNP panel). Mice showed >~99% identity to the 6J substrain from the analysis of ~1500 SNPs; <1% of SNPs were assigned to substrains other than 6J or 6N. Genotyping was accomplished with the forward primer GAC TAC AAA GAC GAT GAC GAC AAG and the reverse primer CTA CCA CCA CCC AGA TAA TGT C using Phusion DNA Polymerase (NEB). Since the primer pair does not distinguish the WT-LRRK2 BAC strain from the G2019S-LRRK2 BAC strain, for select breeding paris, genetic identity was confirmed by using the forward primer TAT CTC CAC TCA GCC ATG ATT ATT TAC CG and the revers primer TTG AGG GCA CTG ATG GTC CAC TG to produce a 209 bp DNA product that will be digested in half with the restriction enzyme MfeI (NEB) if the DNA is G2019S positive. Mice (and rats, described below) were maintained on an ad libitum diet with a 12-hour light and 12-hour dark cycle, and were housed according to AAALAC density guidelines.

The human BAC-G2019S-LRRK2 (NTac:SD-Tg(LRRK2*G2019S)571Cjli, Taconic Farms Sprague-Dawley outbred) were developed originally at Cornell University

by CJ Li and supported for distribution and licensing by the Michael J. Fox Foundation for Parkinson Disease research. We have previously evaluated LRRK2 expression and localization in these strains of rats (Walker et al., 2014, West et al., 2014). Genotyping of NTac:SD-Tg(LRRK2*G2019S)571Cjli was accomplished with the forward primer GAT AGG CGG CTT TCA TTT TTC C and the reverse primer ACT CAG GCC CCA AAA ACG AG using Phusion Taq DNA polymerase according to manufacturer's instructions (NEB). Rats and mice used in this study were bred and maintained at the University of Alabama at Birmingham. All protocols were approved by the University of Alabama at Birmingham Animal Care and Use Committee that is fully accredited by the AAALAC.

Animal Surgeries

Intracranial or control (saline only) injections were conducted under isoflurane anesthesia using a digital stereotaxic frame (David Kopf) with a thermal adjustable height stage (Physitemp). All rats received a single unilateral 4 µL injection over the course of 20 minutes using a 32-gauge custom needle (Hamilton) with a 110° bevel fitted to a gas-tight syringe and controlled by a Nanomite digital pump (Harvard Apparatus). 4 µL of solution containing ultrapurified LPS (20,000 Endotoxin Units, Invivogen, Santa Barbra, CA, USA) diluted into saline was injected into the right rat SNpc at empirically derived coordinates (4.65 mm anterior/posterior, 2.25 mm medial/lateral, and 7.45 mm dorsal/ventral). Scalp incisions were closed by suture, and animals were monitored for successful recovery, with food and water consumption expected in the first few hours post-surgery.

To isolate thioglycollate elicited primary macrophages (TEPMs), mice were injected in the peritoneal cavity with 1.5 mL of 4% thioglycollate broth. 72 hours later,

animals were anesthetized under isoflurane and the peritoneal cavity exposed. 10 mLs of ice cold PBS was injected into the peritoneal cavity through a 28-gauge needle. The fluid containing cells was then withdrawn through an 18-gauge needle. Cells in solution were pelleted through centrifugation at 1,000 x *g* for 10 minutes at room temperature. Red blood cells were lysed through resuspension of the cell pellet into 1 mL of red blood cell lysis buffer (Ammonium-Chloride-Potassium lysing buffer, ACK, Invitrogen) for 2 min at room temp. Cells were then centrifuged at 500 x *g* for 5 min and resuspended into 5 mLs of ice-cold phosphate buffered saline, PBS, pH 7.4. Cell number was counted using a Z2 Particle Counter (Beckman Coulter, Brea, CA, USA) set between 4 and 10 µm recording diameter. Appropriate numbers of cells were plated into 10% fetal bovine serum (FBS) in DMEM with glutamine and 100 µg/ml penicillin/streptomycin. Cells were maintained in a humidified chamber set to 5% CO_2.

Confocal and Immunohistochemistry analysis

For immunofluorescence and confocal analysis, brain sections were prepared from animals terminally anesthetized with isoflurane and transcardially perfused in 0.9% saline with 10 U/ml heparin followed by ice-cold 4% paraformaldehyde (PFA). Brains were dissected and post-fixed at 4^{o}C for 2 hours in 4% PFA, and then transferred to 30% sucrose in PBS at 4^{o}C for cryopreservation. Once saturated in sucrose, brains were flash-frozen in isopentane, cooled on dry ice to -55 ^{o} C, and stored at -80 ^{o} C until further processing. Frozen brains were embedded with tissue freezing medium on a sledge microtome chuck and sections cut to 40 µm (Leica). Sections were rinsed three times with Tris-buffered saline (TBS). All rinses and primary and secondary antibody diluents were in TBS. To allow antigen retrieval, all sections were incubated with 10 mM sodium

citrate, pH 6.0, containing 0.05% Tween-20 for 30 minutes at 37 ^{o}C with agitation. Following three consecutive five-minute washes, non-specific antigens were blocked by incubating sections for 1 hour in 5% normal serum (Equitech-Bio) from the host of the indicated secondary antibody containing 0.1% Triton X-100 at 4^{o}C with agitation. Primary antibodies were diluted in 5% normal serum and incubated on sections with agitation for 24 hours at 4 ^{o}C. Sections were washed 3 times for 5 minutes and then incubated in secondary antibody in 5% normal serum 18 hours at 4°C with agitation. The next day, sections were washed 3 times and for immunofluorescence, were mounted to SuperFrost slides (Fisher) and cover-slipped with Prolong Gold (Life Technologies). Confocal images were captured by a single observer blinded to the experimental conditions (drug treatment group and genotype status) using a Leica TCS-SP5 laser-scanning confocal microscope. The Leica LASAF software, Adobe Photoshop (contrast, brightness and color adjustments), and Adobe Illustrator were used to create figures and process images.

For DAB immunohistochemistry, sections were prepared according to the above procedure except that after antigen retrieval, sections were quenched in 0.3% hydrogen peroxide (Sigma) in methanol for 30 minutes. After final washing steps from secondary antibody incubations, the sections were incubated in Avidin-Biotin Complex (ABC) reagent (Vector Labs) for 30 minutes, washed 3 times again, then developed in ImmPACT substrate (Vector Labs, Burlingame,CA) for 2-5 minutes. The sections were placed in distilled water to terminate the DAB development reaction, rinsed 3 X in TBS, and mounted with 25% ethanol in PBS onto SuperFrost glass slides (Fisher). Following air-drying, the slides were dehydrated in ascending alcohols and three changes of xylene

and cover slipped with Permount (Fisher). Images from sections stained with DAB were captured using an Olympus BX61 microscope.

ELISA

Enzyme-linked immunoassays were accomplished by first plating 80,000 TEPM into treated wells of 24-well plates and cells maintained at 37ºC in a water jacketed incubator with 5% CO_2. Cells were allowed to rest overnight, and then washed twice with complete TPEM media to remove non-adherent cells, and allowed to rest overnight again. TEPMs were treated for 6 hours with 100 ng of LPS (500 endotoxin units) added per well. After 6 hours, the media was removed from the cells and snap frozen on dry ice. Dilutions of media to achieve signal in linear range of ELISA and multiplex ELISA kits were determined empirically prior to analysis. Ready-Set-GO anti mouse TNF ELISA (eBioscience, San Diego, CA, USA) was run according to the manufacturer's instructions. Samples were also analyzed using MILLIPLEX mouse cytokine/chemokine 32-plex magnetic bead ELISA (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

Phagocytosis Assay

250,000 TEPM cells per genotype were plated in 10-cm tissue culture-treated dishes into complete media (10% FBS in DMEM). Cells were allowed to rest overnight, and then washed twice with complete media to remove non-adherent cells. FITC-labelled Zymosan beads (Life Technologies, Carlsbad, CA, USA) were prepared by resuspending the lyophilized beads in 1 mL of sterile PBS, pH 7.4. Beads were then sonicated at 70% power 3 times for 10 seconds (Fisher Scientific 500 Sonic Dismembrator with micro sonic probe) to break up clumps of beads. Beads were applied to TEPM cells at a 10:1

bead to cell ratio in serum-free DMEM for 1 hour. Plates were then washed with 37ºC PBS 5 times, and then cells fixed with 1% PFA. FITC beads not internalized were quenched with application of Sudan Black-B reagent (Invitrogen). Nuclei were labeled with application of 10 □M Hoescht-33342 solution in PBS for 10 minutes. Combined fluorescent and phase contrast images of TEPM with beads were acquired on a Zeiss Axioobserver live cell imager (Carl Zeiss, Thornwood, NY, USA) fitted with a Colibri 2 LED cool imager and a high-speed MR2 camera (Carl Zeiss, Thornwood, NY, USA). Images were acquired using computer-assisted Mark and Find software (Axiovision v4.8) and images were analyzed by an investigator blinded to genotype and treatment condition. The number of beads internalized per TEPM was scored.

Boyden Chambers Chemotaxis assays

Chemotaxis assays were performed as previously reported (Moehle et al., 2012). 10,000 TEPM cells were plated in the upper well of an 8.0 µm pore tissue culture insert. Cells were allowed to rest for 6 hours then the insert moved into a new well of a treated 24 well plate. In the bottom of the chamber, complete TEPM media was supplemented with 100 µM ADP. TEPM cells were allowed to migrate for 36 hours during incubation at 37ºC in a humidified chamber with 5% CO_2. The number of cells migrating to the bottom well was determined by supplementing to 10 □M Hoescht-33342 and recording nuclei through automated cell counting software Mark and Find and Automatic Measurement Program, Axiovision v4.8 (Carl Zeiss, Thornwood, NY, USA).

Flow Cytometry and Cell Counts

TEPM were acquired and prepared as described above for flow cytometry experiments except the cells were collected 18 hours (instead of 72 hours) post-

thioglycollate injection. Total cell numbers were counted by hemocytometer prior to flow cytometry analysis. Cells were stained for 30 min on ice with APC labeled Ly6G (A18 clone), CD11b labeled with Alexa Fluor® 488, F4/80 antigen labeled with PE, and MHC Class II (I-A/I-E) Biotin-Streptavidin PerCP (all antibodies eBioscience, San Diego, CA, USA). For chemokine receptor experiments, cells were stained for 30 min at 37ºC with CD11b labeled with Alexa Fluor® 488, APC labeled Ly6G (A18 clone), 7-aminoactinomycin D (7-AAD) and CCR2 labeled with PE (R&D Systems, Minneapolis, MN, USA). Cells were then washed and analyzed on a FACS Caliber Flow Cytometer (BD Bioscience, San Jose, CA). Isotype-matched, fluorescently conjugated antibodies of irrelevant specificity were used as controls. Results were analyzed using FlowJo Software (Tree Star Inc, Ashland, OR).

Complete Blood Chemistry

WT, WT-LRRK2, and GS-LRRK2 mice were placed under isoflurane anesthesia. 0.5 ml of blood was drawn through intra-cardiac puncture through 1 ml syringe fitted with a 21 gauge needle. Samples were immediately placed into heparinized vaccutubes. Samples were analyzed within 24 hours on an automated hematology analyzer (Idexx Laboratories, Fremont, CA) by Animal Labs of Birmingham, LLC. Differential counts were performed manually by a licensed veterinary technician.

Immunoprecipitations

2 million TEPM cells per genotype were plated into 15 cm tissue treated culture dishes and were allowed to rest overnight then washed (as described above) to remove non-adherent cells. Cells were then treated with 100 ng / mL of LPS (500 endotoxin units per mL), or saline-control, for 6 hours. After 6 hours, cells were removed from the plate

by scraping into ice-cold PBS, pH 7.4, pelleted and then lysed into a solution of 1% Triton-x-100, Tris HCl pH 7.4, and 1x complete protease and phosphatase inhibitors (Roche, Basel, Switzerland). Cell solutions were sonicated at 10% power for ten seconds, incubated for 1 hour at 4ºC while rotating, and lysates clarified by centrifugation at 20,000 x *g* for 20 min at 4ºC. Pellets were discarded and 200 µl of M2 anti-FLAG magnetic beads (Sigma, St Louis, MO, USA) were washed 3 times in lysis buffer then added to lysates overnight on a rotating wheel. Beads were washed 2 times in lysis buffer then 2 times in lysis buffer supplemented with 350 mM NaCl. Protein complexes were eluted from the beads by adding 2x Laemmli Buffer with 5% beta-mercaptoethanol and heated to 70ºC with shaking at 1,400 rpm.

Western blots

Equal volumes of lysates per genotype condition were loaded onto and separated on 4 to 20% Tris-Glycine gradient gels (BioRad, Hercules, CA, USA). Proteins were transferred to PVDF membranes at 30 volts overnight. Membranes were blocked in 5% BSA in TBS-T then incubated in primary antibodies overnight at 4ºC. Primary antibodies were as follows: 1:2000 LRRK2 N241A/34 (Antibodies Inc, Davis, CA, USA), 1:2500 Myosin IIa (Myh9) (Cell Signaling Technology, Danvers, MA, USA), 1:5000 Arp2 (Cell Signaling Technology, Danvers, MA, USA), 1:2500 myosin 1f (Sigma, St Louis, MO, USA). Membranes were washed then incubated with appropriate secondary antibodies and imaged using Li-COR Odyssey imaging system (Li-COR Biosciences, Lincoln, NE, USA) or ECL.

Mass Spectrometry

Lysates were denatured and separated on a 7.5% Tris-glycine gel (BioRad, Hercules, CA, USA), and stained with Bio-Safe colloidal Coomassie (BioRad, Hercules, CA, USA). Bands were excised and enzymatically digested with Trypsin Gold (Promega, Madison, WI, USA) overnight according to the manufacturer's instructions, followed by acidification to pH 3-4 with 10%formic acid for analysis by liquid chromatography-mass spectrometry (LC-MS). Each peptide digest fraction was analyzed using a linear trap quadropole XL (LTQ XL) ion trap mass spectrometer equipped with a nano-electrospray source, and a Surveyor Plus binary high-pressure liquid chromatography (HPLC) pump (Thermo Scientific, Rockford, IL, USA) using a split flow configuration. Separations were carried out using a 100um x 13cm pulled tip C-18 column (Jupiter C-18 300 A, 5 µm). The HPLC was set up with two mobile phases that included solvent A (0.1% FA in ddH2O), and solvent B (0.1% FA in 85% ddH2O/15% ACN), and was programmed as follows: 15 min at 0% B (2 µL/min, load and desalt), 100 min at 0%-50% B (~0.5 nL/min, analyze), 20 min at 0% B (2 µL/min, equilibrate). During the first 15 minutes of loading and desalting, the source was set at 0.0 volts. The LTQ XL was operated in data dependent triple play mode, with a survey scan range of 300-1200 m/z, followed by an ultra-zoom scan used for charge state determination (~20k resolution @ 400 m/z) and an MS2 scan, both carried out with 2.0 Da isolation widths on the 3 top most intense ions. MS data were collected in profile mode for all scan types. Charge state screening and dynamic exclusion were enabled with a minimum signal intensity of 2000, a repeat count of 2, and exclusion duration of 90 sec for ions +/- 1.5 m/z of the parent ion. The automatic gain control settings were $3x10^4$, $5x10^3$, and $1x10^4$ ions for survey, zoom, and CID modes respectively. Scan times were set at 25, 50, and 100 ms for survey, zoom, and

collision-induced dissociation (CID) modes, respectively. For CID, the activation time, activation Q, and normalized collision energy were set at 30 ms, 0.25, and 35% respectively. The spray voltage was set at 1.9 kV following the first 15 min of loading, with a capillary temperature of 170 °C. XCalibur RAW files were centroided and converted to MzXML and the mgf files were then created using both ReAdW and MzXML2Search, respectively (http://sourceforge.net/projects/sashimi/). The data was searched using SEQUEST (v27 rev12, .dta files), set for two missed cleavages, a precursor mass window of 0.45 Da, tryptic enzyme, variable modification M at 15.9949, and static modifications C at 57.0293. Searches were performed with a mouse subset of the UniRef100 database, which included common contaminants such as digestion enzymes and human keratins. Identified peptides were filtered, grouped, and quantified using ProteoIQ v2.3.04 (Premierbiosoft, Palo Alto, CA, USA). Only peptides with charge state of $\geq$ 2+ and a minimum peptide length of 6 amino acids were accepted for analysis. ProteoIQ incorporates the two most common methods for statistical validation of large proteome datasets, false discovery rate (FDR), and protein probability (Keller et al., 2002, Nesvizhskii et al., 2003, Weatherly et al., 2005). Relative quantification was performed via spectral count, and spectral count abundances were normalized between samples (Beissbarth et al., 2004, Liu et al., 2004, Old et al., 2005). The FDR was set at <1% cut-off, with a total group probability of $\geq$ 0.7 and peptides $\geq$ 2 assigned per protein. In order to identify proteins likely to randomly or non-specifically interact with LRRK2 protein complexes, all proteins found through LC/MS/MS were filtered through the Contaminant Repository for Affinity Purification (CRAPome.org) database. Proteins that

had a >10% chance of random interaction or were not found due to improper annotation were excluded from analysis.

Statistical analysis, Unbiased Quantifications, and System Analysis- Statistical Analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc, LaJolla, CA, USA). $p<0.05$ was considered significant. For comparisons of the more than two groups, one-way ANOVA tests with Tukey's post-hoc analysis was used, and for groups showing non-normal distribution (phagocytosis assay), Kruskal-Wallis ANOVA was used with Dunn's multiple comparisons corrections. To compare two groups, a 2-way Student's T-test was used with Bonferroni corrections as appropriate. Correlations were determined by Pearson coefficients.

Stereological estimation of the total TH+ and CD68+ cells in the SNpc was performed using an optical fractionator probe (Microbrightfield, Willston, VT, USA) by an investigator blinded to experimental condition and animal genotype. Optical fractionator and dissector probes were used to count grid sizes of 50 μm^2 with density adjusted for analysis of at least 100 objects per sample.

Gene ontology analysis was performed using MetaCore GeneGO software (Thomson Reuters, New York, NY, USA).

SUPPLEMENTARY FIGURES

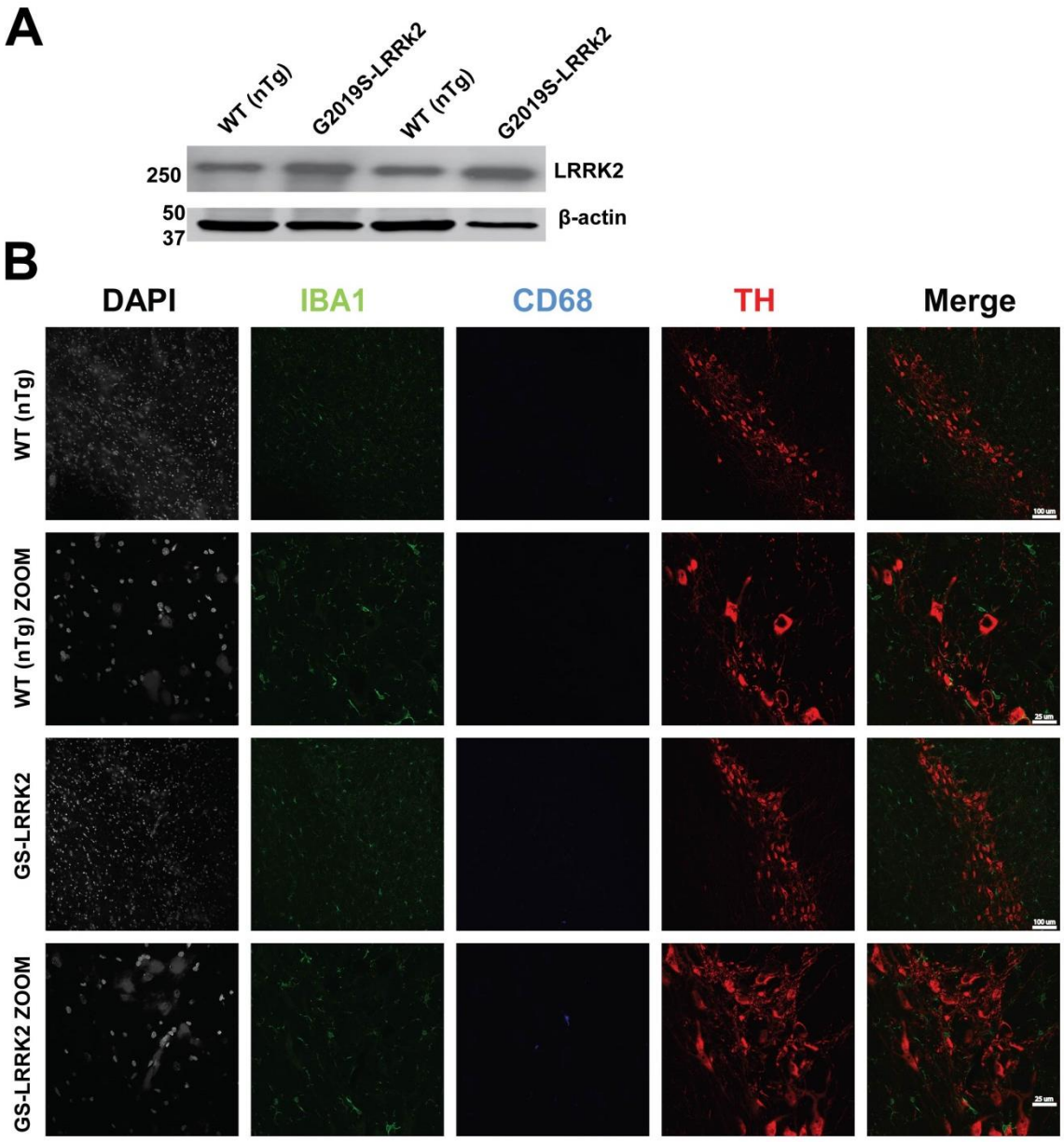

FIGURE S1**. LRRK2 expression and baseline immune markers in non-transgenic and G2019S-LRRK2 rats. A)** Representative western blots from primary macrophages derived from WT (non-transgenic, nTg) or G2019S(GS)-LRRK2 rats. Cells from GS-LRRK2 have a ~3 to ~5 fold increase in LRRK2 expression. **B)** Representative confocal images of the substantia nigra pars compacta (SNpc) of 10-12 week old WT and GS-LRRK2 rats. Ionized calcium-binding adapter molecule 1 (IBA1), cluster of differentiation protein 68 (CD68), and Tyrosine Hydroxylase (TH), show no differences between strains of animals. CD68 cells were very rarely observed through the SNpc of either WT or GS-LRRK2 rat SNpc. Scale bars for images are 100 µm and 10 µm for "Zoom" fields.

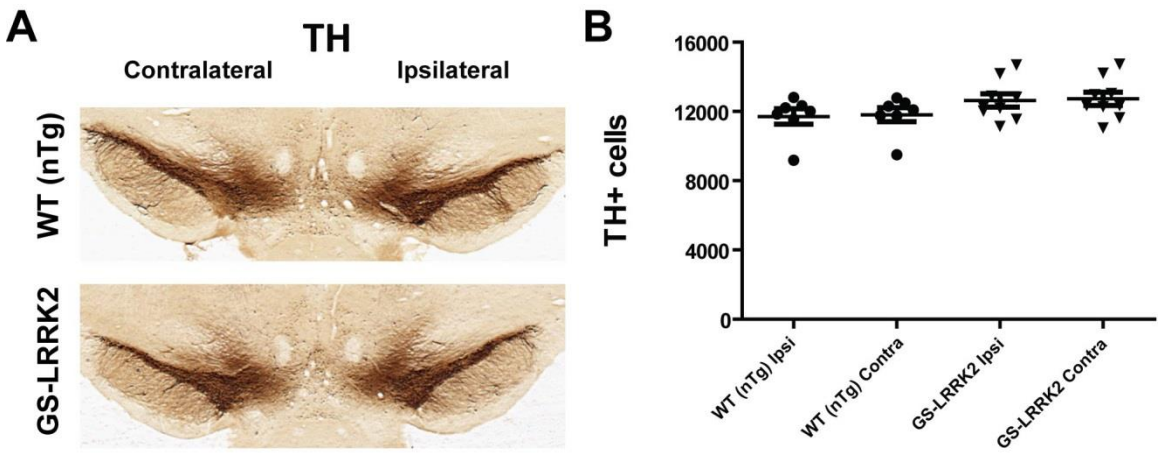

FIGURE S2**. Normal TH cell counts in adult G2019S-LRRK2 rats. A)** Representative bright-field images of the SNpc from 10-12 week old WT non-transgenic (n=8) and G2019S-LRRK2 (GS-LRRK2, n=10) rats injected with saline (ipsilateral) and sacrificed two weeks later. Sections were stained for TH. **B)** Unbiased stereological quantification of (A). No significant differences exist between the groups, and saline-injection alone did not result in cell death. $p>0.5$ calculated with one-way ANOVA, bars represent group means and error bars are S.E.M.

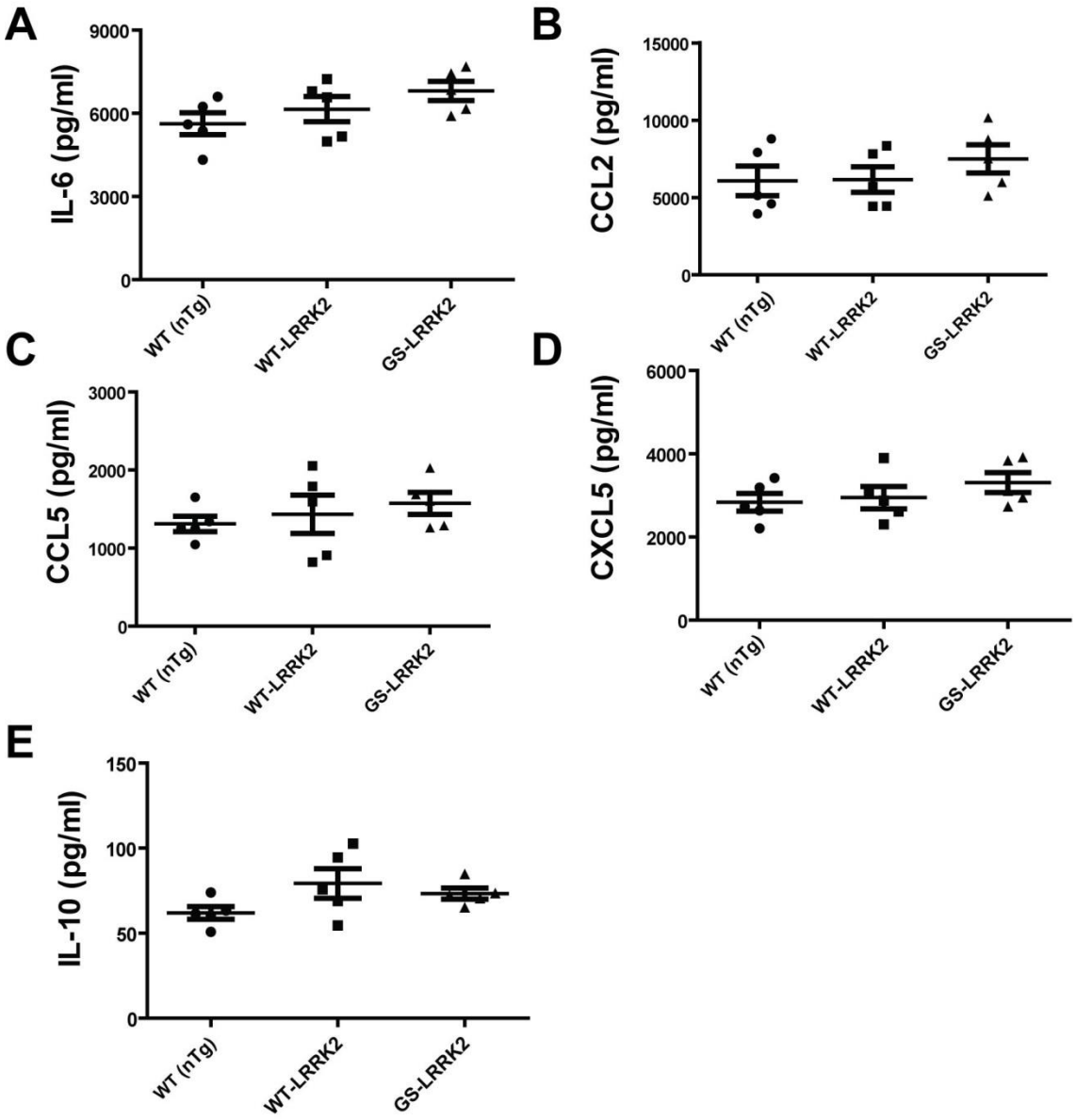

FIGURE S3**. LRRK2 does not alter cytokine or chemokine release in TEPMs.** Thioglycollate-elicited peritoneal macrophages (TEPM) cells were cultured from adult male non-transgenic littermate controls (WT, nTg), WT-LRRK2 BAC and G2019S(GS)-LRRK2 BAC mice (at least 3 animals per strain per experiment were used). TEPMs were allowed to rest overnight after plating, and then stimulated with LPS (100 ng ml^{-1}) for 6 hours. **A-E)** Quantification of secreted cytokines and chemokines as measured by a 32-plex mouse inflammation bead-array panel. Representative cytokines and chemokines are shown. There were no differences observed between groups for any detected secreted factor (one-way ANOVA, $p>0.5$). All bars represent group mean derived from at least three animals per strain, and error bars represent S.E.M.

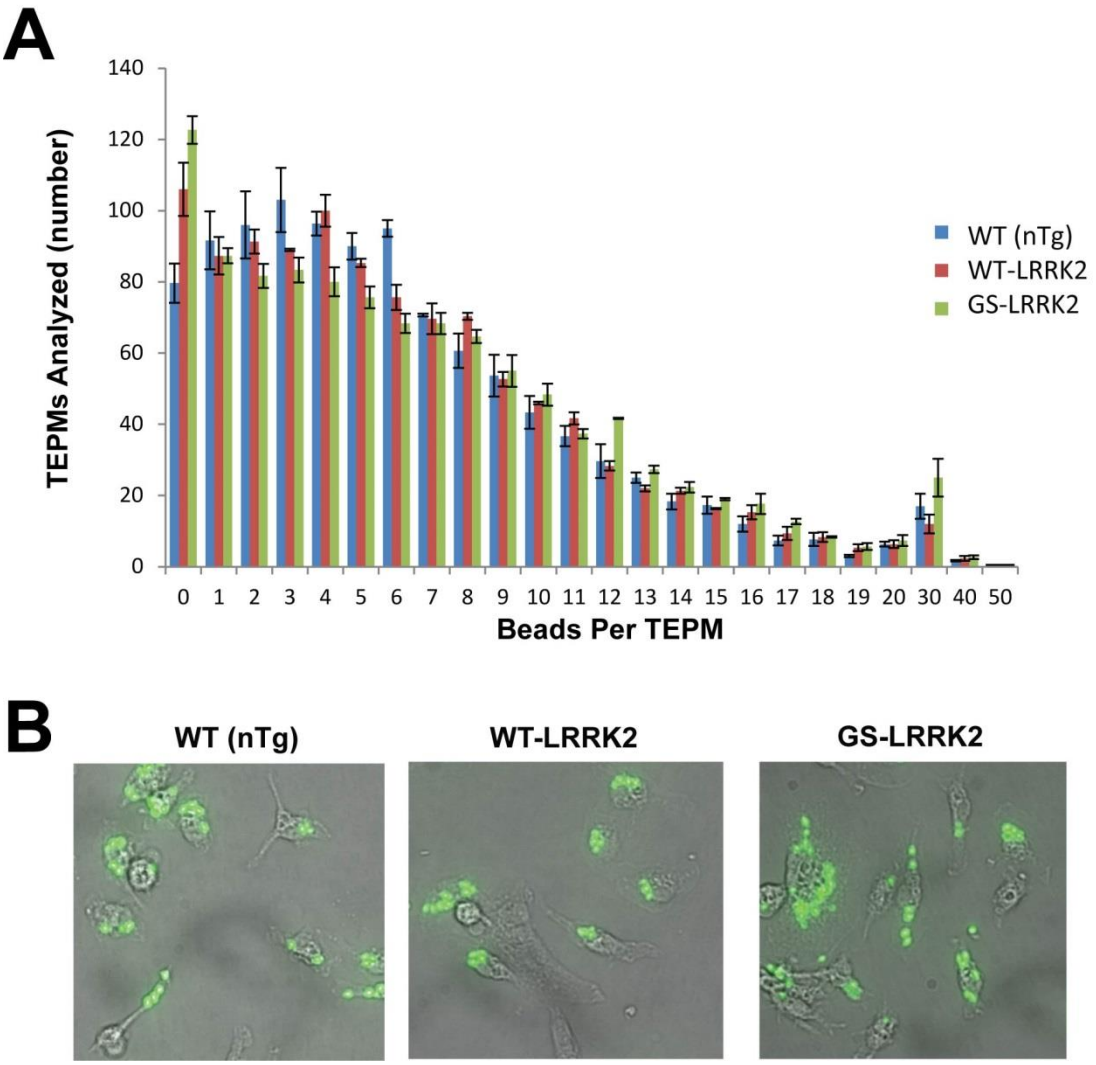

FIGURE S4. **G2019S-LRRK2 does not alter phagocytosis in TEPMs**. Thioglycollate-elicited peritoneal macrophages (TEPM) cells were cultured from adult male non-transgenic littermate controls (WT, nTg), WT-LRRK2 BAC and G2019S(GS)-LRRK2 BAC mice (at least 3 animals per strain per experiment were used). **A)** TEPMs were allowed to rest overnight after plating and Zymosan beads were added to cultures at a ratio of 10:1 (bead per cell). The numbers of beads internalized per TEPM were determined and histogram of beads phagocytized per TEPM of WT, WT-LRRK2, and GS-LRRK2 are shown. A reviewer blinded to genotype of the TEPMs counted the number of beads per cell. **B)** Representative images of TEMPs (phase-contrast) over-laid with labeled Zymosan beads (green).

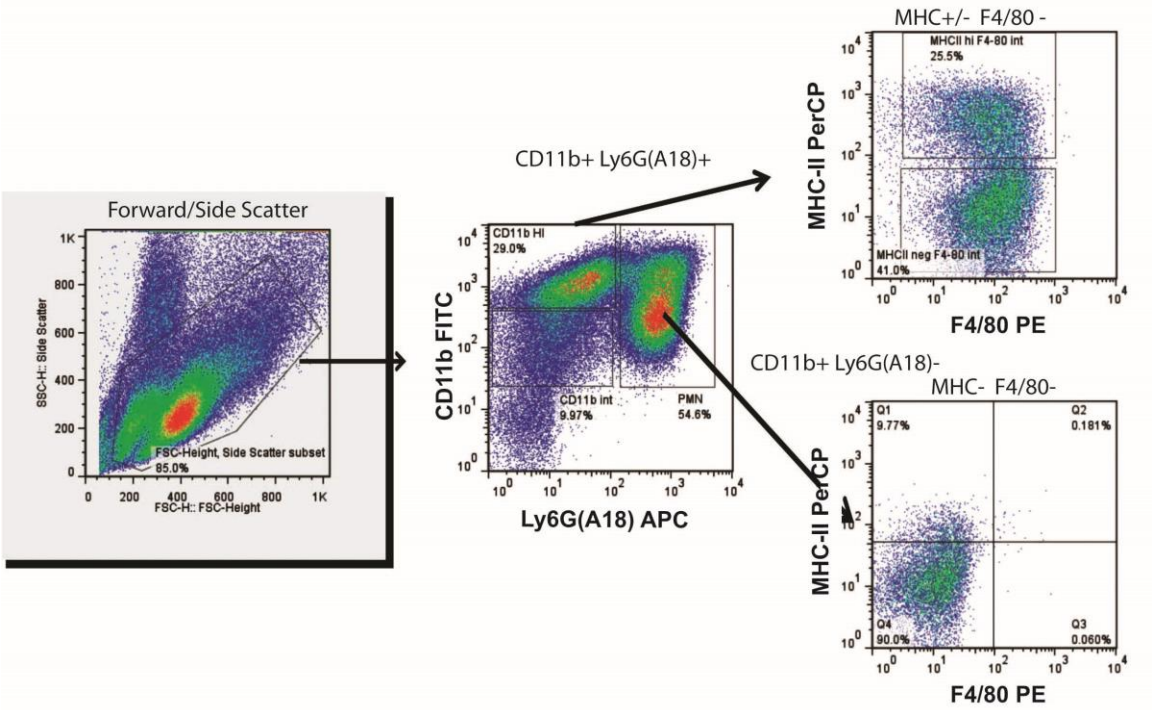

FIGURE S5**. Flow cytometry gating strategy for TIP**. Thioglycollate-induced peritoneal (TIP) cells obtained directly from lavage were first gated on forward/side scatter. Cells were then separated by CD11b and Ly6G(A18) intensities. Two populations emerged from this, CD11b+Ly6G(A18)- and CD11b+Ly6G(A18)+. The CD11b+Ly6G(A18)- was further analyzed by F4/80 and MHCII intensity. A majority (>65%) of the CD11b+Ly6G(A18)- population appeared to be weakly F4/80 positive but did not sufficiently diverge from isotype control. This is consistent with TIP cells. Only a small population (~25%) was MHCII positive, likely making this population monocytes/macrophages. The CD11b+Ly6G(A18)+ was further analyzed by F4/80 and MHCII. A majority of the CD11b+Ly6G(A18)+ (>90%) was negative for both F4/80 and MHCII, likely making these cells neutrophils.

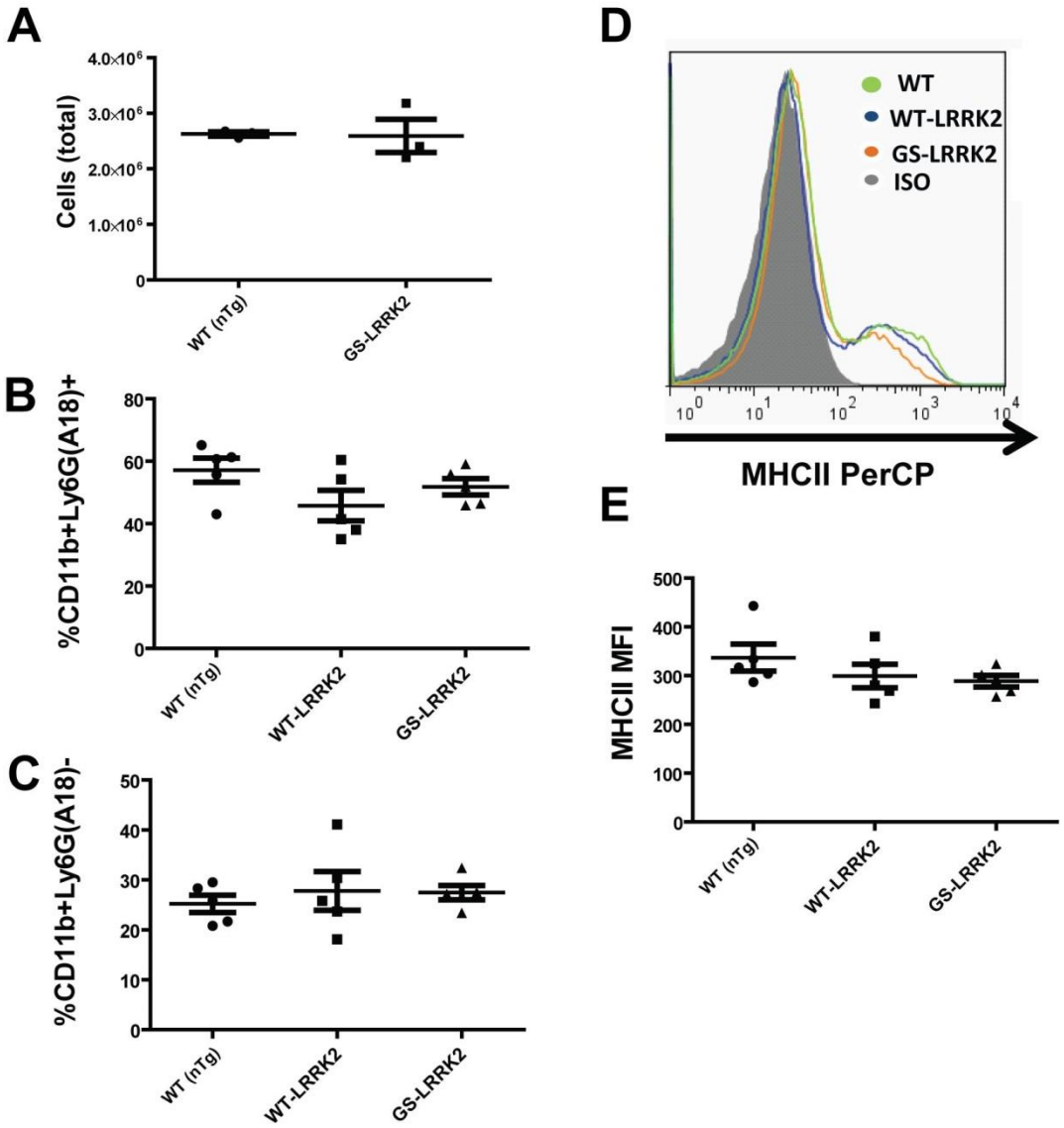

FIGURE S6**. G2019S-LRRK2 expression does not alter baseline white blood composition**. White blood cells were derived from at least three male adult mice each of WT (non-transgenic), WT-LRRK2 BAC, and G2019S(GS)-LRRK2. Red blood cells were lysed in each preparation and the remaining cells analyzed. **A)** Quantification of total white blood cells obtained by lavage of the unstimulated peritoneal cavity. **B,C)** Quantification of percentages of sub-classified white-blood cells from total cell population using flow cytometry. **D)** Representative histogram of MHCII fluorescent intensity of CD11b+Ly6G(A18)- cells from WT (nTg), WT-LRRK2, and G2019S(GS)-LRRK2 mice after TIP. **E)** Quantification of median fluorescent intensity (MFI) from panel D. No significant differences ($p>0.5$, One Way ANOVA) are observed between strains for all markers.

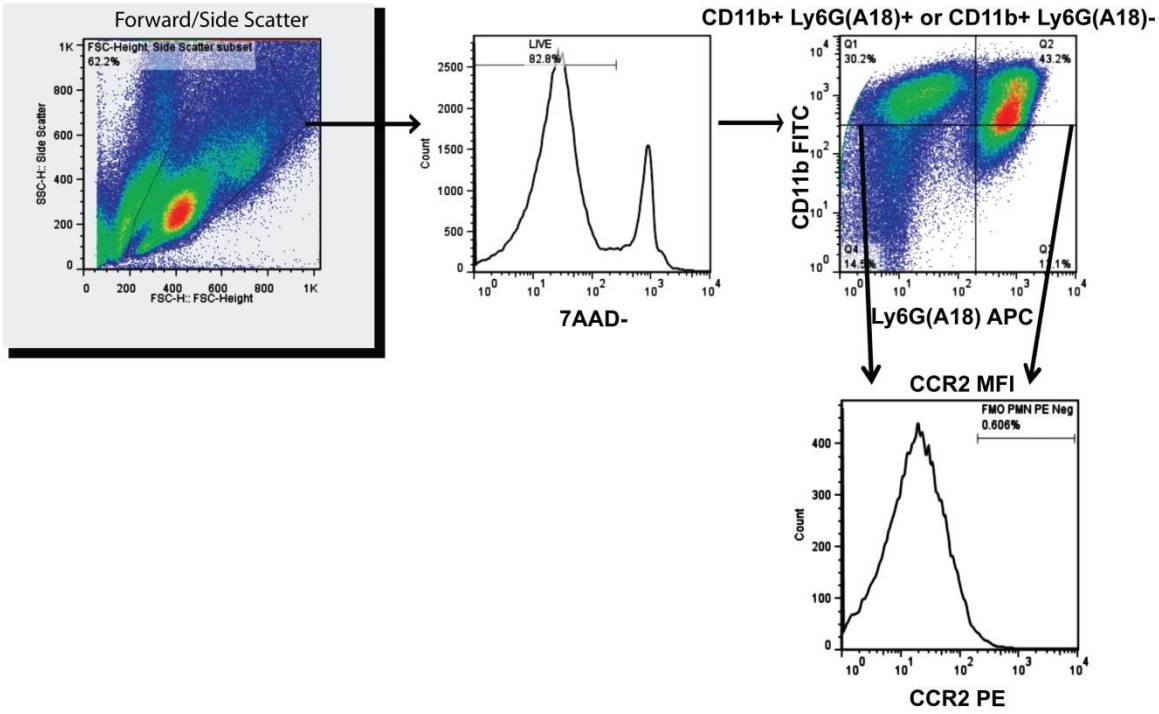

FIGURE S7**. Gating strategy for CCR2**. Thioglycollate-induced peritoneal (TIP) cells obtained directly from lavage were first gated on forward/side scatter. Cells were then gated on live cells by selecting for 7AAD- cells and separated by CD11b and Ly6G(A18) signal. The CD11b+Ly6G(A18)- and CD11b+Ly6G(A18)+ were then each analyzed by fluorescent intensity of CCR2.

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EFFECTS OF NOVEL LRRK2 KINASE INHIBITORS ON MACROPHAGE EFFECTOR FUNCTION

by

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ABSTRACT

Leucine Rich Repeat Kinase 2 (*LRRK2*) is genetically linked to Crohn's Disease and leprosy, diseases with a clear role for inflammation. Expression patterns of LRRK2 also implicate a role for LRRK2 in mature innate immune cells. Because of this, several recent reports have used pharmacological inhibitors of LRRK2 that have relatively poor selectivity or significant off target liabilities to understand what functions of innate immune cells LRRK2 kinase activity influences. These studies have implicated almost every effector function of macrophages to LRRK2 kinase activity, implicating that LRRK2 may globally effect pro-inflammatory responses. Here we utilize two novel inhibitors of LRRK2, SRI451 and Pfizer-06447475, with excellent selectivity profiles to help link LRRK2 kinase activity with functions of macrophages.

INTRODUCTION

Mutations in *leucine rich repeat kinase 2* (*LRRK2*) represent the most common known genetic form of Parkinson disease (PD) (Gilks et al., 2005, Healy et al., 2008). Subsequent genome wide association studies have linked *LRRK2* to Crohn's Disease and Leprosy, diseases that have a critical role for inflammation in the etiology of disease (Van Limbergen et al., 2009, Zhang et al., 2009). Expression patterns of LRRK2 also have implicated that LRRK2 may have a functional role in the immune system. CD14+CD16+ monocytes and macrophages of the peripheral immune system have the highest expression of LRRK2 in the immune system (Thevenet et al., 2011). LRRK2 expression and activity will increase when innate immune cells are stimulated with pro-inflammatory stimuli such as lipopolysaccharide (LPS) and interferon gamma (Gardet et al., 2010, Hakimi et al., 2011, Dzamko et al., 2012, Moehle et al., 2012, Kuss et al., 2014). These results implicate that LRRK2 expression and activity are tied to some part of the inflammatory response.

To help link LRRK2 to a particular function in the immune system, KO and pharmacological inhibitors of LRRK2, namely LRRK2-Inhibitor-1 (L2In1), have been used to dissect out what pathways are influenced by LRRK2. Recently, L2In1 was found to have significant off target effects including reducing activity of ERK5 at similar levels to LRRK2. Additionally, L2In1 treatment of LRRK2 KO microglia significantly reduces TNF release after LPS stimulation, calling into question results obtained from studies utilizing L2In1 to link LRRK2 to kinase activity to functions of immune cells (Luerman et al., 2014). KO of LRRK2 may also have potentially deleterious side effects. KO of LRRK2 alters immune homeostasis with a decrease in number of circulating white and

red blood cells, suggesting that loss of LRRK2 protein may have deleterious effects on the development of the immune system (Kim et al., 2012, Ness et al., 2013).

To help address these issues, our lab has developed a new series of inhibitors that have excellent selectivity towards LRRK2 with only 4 other kinases showing appreciable inhibition in doses >1μM (Liu et al., 2014). Additionally, Pfizer has developed a novel LRRK2 inhibitor structurally distinct from SRI451, deemed Pfizer-06447475 (Pf-06) that also has excellent selectivity for LRRK2 (Henderson et al., 2015). In this study, we utilize these two inhibitors in conjunction with primary macrophages and microglia to understand the role of LRRK2 kinase activity in innate immune cells.

RESULTS

First, we examined the ability of SRI 451 and 132 to inhibit LRRK2 in macrophages. Both drugs exhibited a dose dependent decrease in P935 signal, a pharmacodynamic phosphorylation site on LRRK2 (Fig 1A). However, concentrations of drug needed to inhibit LRRK2 was far in excess of its predicted IC_{50} based on kinase assays with recombinant protein. This may indicate that SRI451 and 132 are substrates of PGP as was predicted structurally (Liu et al., 2014). Based on the dose response curve, we treated WT and G20 macrophages with 2.5 and 5 μM SRI451, and observed a dose dependent decrease in TNF release after LPS stimulation (Fig 2B). Additionally, we observed that G2019S LRRK2 may be more sensitive to LRRK2 inhibition. To ensure that this effect was attributable to LRRK2 and not off target liabilities, we treated LRRK2 KO macrophages with SRI451 and L2In1. A decrease in TNF release was observed with L2In1 treatment as previously observed (Luerman et al., 2014). SRI451 did not have an effect within the knockout macrophages (Fig. 1C).

To reconfirm the decrease in TNF release attributable to LRRK inhibition with SRI451, we utilized a structurally distinct LRRK2 inhibitor Pf-06 (Henderson et al., 2015). Pf-06 decreases P935 by greater than 90% at both 0.5 and 1.0 μM doses (Fig 2A). We again examined the effect of LRRK2 kinase inhibition on cytokine release after LPS stimulation and did not observe a difference in TNF or IL-6 release (Fif 2D, E). We then examined other pro-inflammatory markers IBA1 and MHCII. Both of these markers increase in intensity after LPS stimulation. Pf-06 treatment did not alter the median fluorescent intensity (MFI) of these proteins after LPS stimulation (Fig 2B, C).

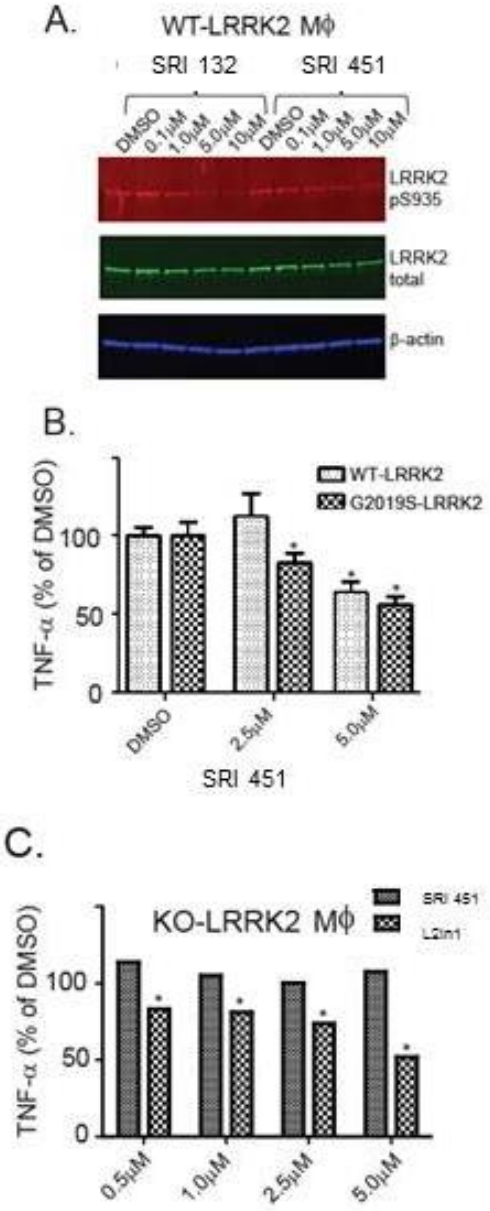

FIGURE 1. **SRI451 decreases TNF release in a dose dependent manner.** A) Wildtype macrophages treated with SRI451 and SRI132 for 72 hours then immuno-blotted for total LRRK2, P935 LRRK2, and β-actin. Compounds showed similar potency. B) Wildtype macrophages treated with SRI451 for 72 hours then stimulated with LPS. Media was analyzed via traditional ELISA. C) LRRK2 KO macrophages were treated for 72 hours with SRI 451 or L2In1 then stimulated with LPS. Media was analyzed via traditional ELISA. Error bars represent SEM. * indicates $p<0.05$

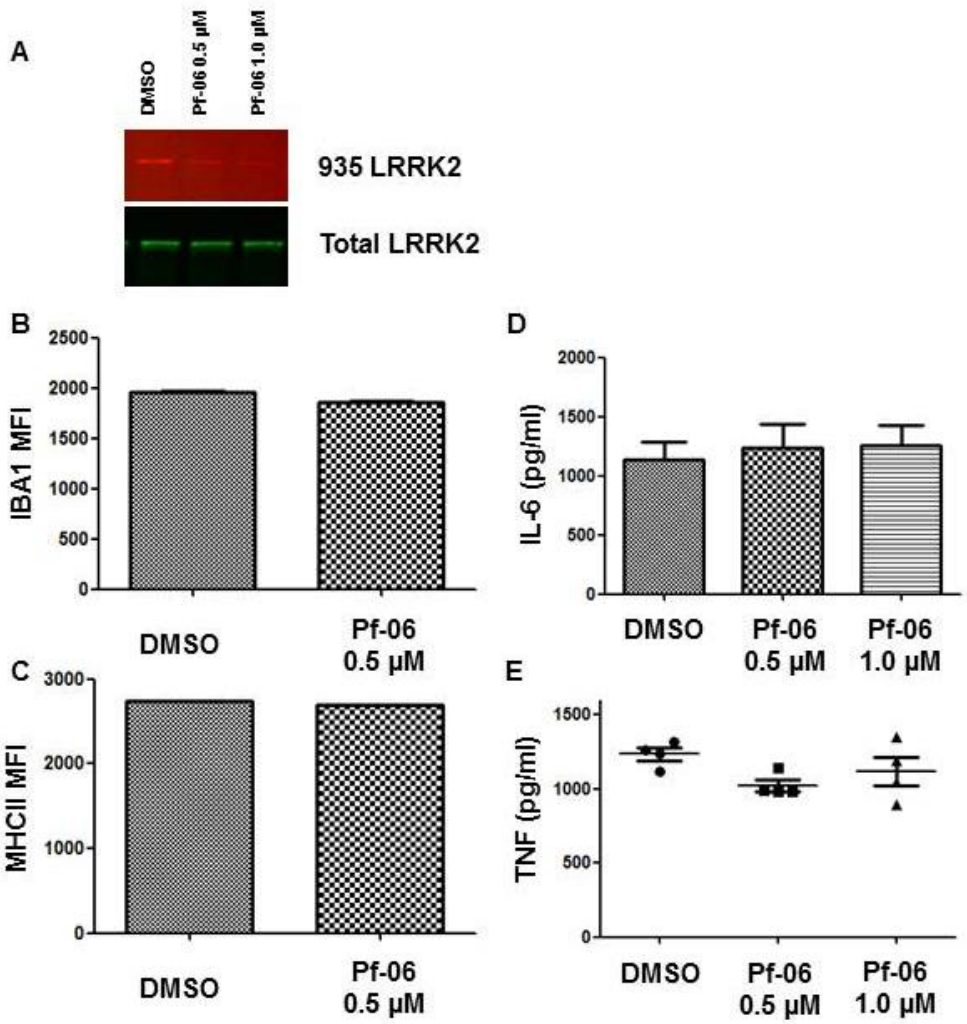

FIGURE 1. **Pf-06 Does Not Alter Expression or Release of Pro-Inflammatory Markers.** A) Wildtype macrophages treated with Pf-06 for 72 hours then immuno-blotted for total LRRK2, P935 LRRK2, and β-actin. B, C) Wildtype macrophages treated with Pf-06 or DMSO for 72 hours then stimulated with LPS for 24 hours then stained for IBA1 (B) or MHCII (C). Median fluorescent intensity is reported. D, E) Wildtype macrophages treated with Pf-06 or DMSO for 72 hours then stimulated with LPS for 6 hours. Media was analyzed for IL-6 (D) or TNF (E) by traditional ELISA. No values are significant.

DISCUSSION

Because mutations in *LRRK2* are clinically and pathologically similar to idiopathic PD, understanding physiological functions attributable to LRRK2 can potentially lead to a better understanding of pathways involved in the etiology of PD (Healy et al., 2008, West, 2014). Testing of LRRK2 inhibitors would also greatly benefit from understanding a role for LRRK2 in immune cells, as currently readouts of LRRK2 activity are tied to phosphorylation of serine residues 910 and 935 at the N terminus of LRRK2. SRI 451 seemed to implicate that LRRK2 kinase activity may influence cytokine release as previously predicted (Moehle et al., 2012). SRI451 was successful in attenuating TNF release in WT macrophages after LPS stimulation, but did not do so in LRRK2 KO implying that this decrease in TNF release was specific to LRRK2. This result appeared to replicate findings from our lab and others that LRRK2 may alter cytokine release, or possibly globally affect cell polarization to a M1 phenotype (Dzamko et al., 2012, Kim et al., 2012, Marker et al., 2012, Moehle et al., 2012, Daher et al., 2014). However, a structurally distinct LRRK2 inhibitor, Pf-06, did not replicate these findings despite achieving similar levels of LRRK2 inhibition. Neither TNF nor IL-6 release, both pro-inflammatory cytokines, were changed by LPS stimulation with pre-treatment of Pf-06. Other markers of macrophage activation, such as MHCII and IBA1 were similarly unchanged by Pf-06 treatment prior to LPS stimulation. The lack of change in either cytokine release or expression markers of activation clearly implicate that Pf-06 did not alter pro-inflammatory signaling or M1 polarization despite achieving similar LRRK2 inhibition.

At first, this seemed perplexing as SRI451's effect was attributable to LRRK2 as the drug has no effect in the knockout. A closer examination of the data reveled that SRI451 reduced LRRK2 expression by ~50% while Pf-06 had no effect on LRRK2 expression. This reduction in LRRK2 expression in SRI451 treated macrophages could have essentially made a pharmacological knockdown. Reduction of LRRK2 through knockout or knockdown has consistently decreased cytokine release and M1 polarization (Kim et al., 2012, Moehle et al., 2012, Ness et al., 2013, Daher et al., 2014). SRI451 could have produced its anti-inflammatory effects through a mechanism independent of its kinase activity through a reduction in LRRK2 expression.

Another possibility is that P935 is not an accurate correlate of LRRK2 inhibition. S935 is part of an N terminal cluster of serine residues that are constitutively phosphorylated. This cluster of sites is pharacodynamically responsive to LRRK2 inhibition, but do not track with LRRK2 kinase activity as they are unchanged by kinase activating mutations (Sen et al., 2009, Gloeckner et al., 2010, Webber et al., 2011). LRRK2 can auto-phosphorylate itself, and a number of sites have been proposed (Gloeckner et al., 2010, Webber et al., 2011, Sheng et al., 2012). Tracking LRRK2 activity through these sites may yield more reliable results. Unfortunately, antibodies have been relatively unsuccessful at picking up these auto-phosphorylation sites in tissue or cell line extracts. However, a new site, S1292, has recently been described. This site tracks with both inhibition and kinase activity, and can be detected with antibodies both in tissue and cell lines (Sheng et al., 2012). Assessing inhibition at this site may yield a better understanding of LRRK2 activity in the future.

Taken together, this data provides an important note of caution in interpreting the results of LRRK2 kinase inhibition. Inhibitors that reduce total LRRK2 may provide results that are more closely linked with loss of LRRK2 protein rather than kinase activity. Our data also reveal for a need to better understand LRRK2 kinase dependent and independent events.

MATERIALS AND METHODS

Animals

Mice were maintained on an ad libitum diet with a 12 hour light and 12 hour dark cycle, and were housed according to AAALAC density guidelines. Mouse FLAG-G2019S-LRRK2 BAC (B6.Cg-Tg(Lrrk2*G2019S)2Yue/J) and were developed in the laboratory of Zhenhue Yue and obtained from the Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME, USA). Genotyping was accomplished with the forward primer GAC TAC AAA GAC GAT GAC GAC AAG and the reverse primer CTA CCA CCA CCC AGA TAA TGT C using Phusion Dna Polymerase (NEB).

Primary Macrophage Culture

To isolate thioglycollate elicited primary macrophages (TEPMs), mice were injected in the peritoneal cavity with 1.5 mL of 4% thioglycollate broth. 72 hours later, animals were anesthetized under isoflurane and the peritoneal cavity exposed. 10 mLs of ice cold PBS was injected into the peritoneal cavity through a 28 gauge needle. The fluid containing cells was then withdrawn through an 18 gauge needle. Cells in solution were pelleted through centrifugation at 1,000 xg for 10 minutes at room temperature. Red blood cells were lysed through resuspending the cell pellet into a 1 mL of red blood cell lysis buffer (Ammonium-Chloride-Potassium lysing buffer, ACK, Invitrogen) for 2 min at room temp. Cells were then centrifuge at 500 xg for 5 min and resuspended into 5 mLs of ice-cold phosphate buffered saline, PBS, pH 7.4. Cell number was counted using a Z2 Particle Counter set between 4 and 10 μm recording diameter. Appropriate numbers of cells were plated into 10% fetal bovine serum (FBS) in DMEM with glutamine and 100

µg/ml penicillin/streptomycin. Cells were maintained in a humidified chamber set to 5% CO_2

ELISA

Enzyme-linked immunoassays were accomplished by first plating 80K TEPM into treated wells of 24-well plates and cells maintained at 37ºC in a water jacketed incubator with 5% CO_2. Cells were allowed to rest overnight, and then washed twice with complete TPEM media to remove non-adherent cells, and media changed with SRI451, SRI132 or Pf-06 added. TEPMs were grown in the presence of inhibitor for 72 hours, with media changed the day before LPS addition. TEPMs were then treated for 6 hours with 100 ng of LPS (500 endotoxin units) added per well. After 6 hours, the media was removed from the cells and snap froze on dry ice. Dilutions of media to achieve signal in linear range of ELISA and multiplex ELISA kits were determined empirically prior to analysis. Ready-Set-GO anti mouse TNF ELISA (eBioscience, San Diego, CA, USA) was run according to manufacturer's instructions.

Western Blot

Equal volumes of lysates per condition were loaded onto and separated on 7.5% Tris-Glycine gradient gels (BioRad). Proteins were transferred to PVDF membranes at 30 volts overnight. Membranes were blocked in 5% fat free milk in TBS-T then incubated in primary antibodies overnight at 4ºC. Primary antibodies were as follows: 1:2000 LRRK2 N241A/34 (Antibodies Inc), 1:1000 PS935 (Abcam), and 1:5000 β-actin (Sigma) Membranes were washed then incubated with appropriate secondary antibodies and imaged using Li-COR Odyssey imaging system (Li-COR Biosciences, Lincoln, NE, USA)

MFI Expression Analysis

250,000 TEPM cells per genotype were plated in 10-cm tissue culture-treated dishes into complete media (10% FBS in DMEM). Cells were allowed to rest overnight, and then washed twice with complete media to remove non-adherent cells. Media was then changed to contain Pf-06. TEPM were grow in the presence of inhibitor for 72 hours, then treated with 100 ng/ml LPS. After 24 hours, cells were fixed with 1% PFA for 10 min. Cells were then washed with PBS and blocked with 5% goat serum, 0.1% Triton X-100 in PBS for 1 hours. Cells were incubated with 1:1000 IBA1 (Wako) and 1:500 MHCII (eBioscience) overnight. Cells were washed then incubated with appropriate secondaries overnight and washed again. Cells were imaged on a Zeiss Axio Observer. MFI of each channel was calculated through an Axio Observer automatic measurement program.

Statistical Analysis

All statistical analysis were performed on Graph Pad Prism 5.0 software. For comparisons of the more than two groups, one-way ANOVA tests with Tukey's post-hoc analysis was used. To compare two groups, a 2-way Student's T-test was used.

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CONCLUSIONS

Both familial studies and GWAS implicate *LRRK2* in familial and sporadic PD, with mutations in *LRRK2* representing the most common genetic form of PD (Gilks et al., 2005, Nichols et al., 2005, Bras and Singleton, 2009, Nalls et al., 2014). Because of *LRRK2's* clinical and pathological overlap with idiopathic PD, *LRRK2* is also suspected of being involved in idiopathic PD as well as the clear linkages to sporadic and familial forms of PD (Healy et al., 2008). Therefore, understanding how mutations in *LRRK2* cause cellular dysfunction could provide mechanistic insight into all forms of PD.

Unfortunately, mechanisms behind neurodegeneration are poorly understood. However, mounting recent evidence suggests that inflammation may drive or exacerbate dopaminergic cell death in the SNpc (Hunot and Hirsch, 2003, Croisier et al., 2005, Qian and Flood, 2008, Hirsch et al., 2012, Deleidi and Gasser, 2013). Because *LRRK2* is associated with peripheral inflammatory disorders through GWAS, and the expression patterns of LRRK2 indicate a role for LRRK2 in the innate system, LRRK2 may prove useful as tool to understand inflammatory mechanisms in PD as well as provide mechanistic insight into how mutations in *LRRK2* cause disease.

For the first time, a specific function of immune cells has been attributed to LRRK2. The data in this thesis clearly implicates LRRK2 as a modulator of chemotaxis of innate immune cells. With diverse stimuli acting through different receptors, mutations in *LRRK2* increase the chemotactic ability of innate immune cells. The increased chemotaxis also implicates that LRRK2 may be a far downstream signaling protein, and not the MAPKKK as previously has been suggested (Bosgraaf et al., 2002, Zheng et al., 2008, Gloeckner et al., 2009). More work will be necessary to understand the exact

signaling molecules that activate LRRK2 and what LRRK2 in turn modulates through its kinase activity, GTPase activity, and protein-protein interaction domains. This chemotactic mechanism supports previous findings from Dictostylium and G2019S patient fibroblasts the implicate LRRK2 with motility, and suggests that LRRK2 may be modulating similar pathways in diverse cell types (Bosgraaf et al., 2002, Bosgraaf et al., 2005, Caesar et al., 2013).

Mutant LRRK2's increased chemotaxis implicates a possible mechanism for the inflammation noted in PD. Dopaminergic SNpc neurons appear to be particularly sensitive to pro-inflammatory mediators while other nearby dopaminergic nuclei, such as the ventral tegmental area, are often protected from pro-inflammatory mediators (Castano et al., 1998, Kim et al., 2000, Castano et al., 2002). If an insult, cell death, or dysfunction in the SNpc causes an immune response, local microglia would release chemokines to draw in additional immune cells into the area. Additional pro-inflammatory cells in the SNpc would release additional pro-inflammatory cytokines and could cause or exacerbate cell death. This recruitment of inflammatory cells to the SNpc could be the main driver of cell death in PD as a single injection of LPS into the SNpc results in the death of dopaminergic cells in the SNpc (Castano et al., 1998, 2002). However, this may be unlikely as inflammatory signaling or processes have not yet been shown to cause deposition of α-synuclein into higher order structures or Lewy Bodies.

Another mechanism for the inflammation and chemotaxis of immune cells into SNpc is through α-synuclein directly or indirectly driving these processes. Oligomers and fibrils of α-synuclein can directly drive microglial inflammation *in vitro*.(Zhang et al., 2005, Klegeris et al., 2008, Su et al., 2008, Lee et al., 2010, Beraud et al., 2011, Beraud et

al., 2013, Harms et al., 2013, Roodveldt et al., 2013) Although, it is unclear in the brain how receptors on the cell surface of microglia come in contact with α-synuclein in order to initiate pro-inflammatory signaling.

Another possibility is for α-synuclein to indirectly change neuron-microglia signaling. There are a number of ligand receptor interactions where a ligand is expressed on the cell surface of neurons and binds to a receptor on the cell surface of microglia. Often, this interaction keeps microglia quiescent. When neurons become unhealthy or stressed, they alter the expression of these neuron-microglia signaling ligands. This results in the microglia becoming more reactive to inflammatory stimuli. Of particular note, the fractalkine receptor (CXC3R1) and fractalkine ligand (CXC3L1) interaction has been shown to be protective in viral models of α-synuclein overexpression and other inflammatory models (Cardona et al., 2006, Morganti et al., 2012). In this model system, α-synuclein oligomers could decrease neuronal health, thereby activating nearby microglia and causing chemotaxis of other innate immune cells to the SNpc and leading to a pro-inflammatory response detrimental to dopaminergic neurons. Regardless of stimulus mechanism, there is some evidence in patients with PD that there is an increased chemotaxis of immune cells. In patients with idiopathic PD as well as PD patients with G2019S mutations, there was an increase in CCR2 expression and decreased number of peripheral monocytes which is consistent with increased chemotaxis of peripheral innate immune cells into a tissue (Funk et al., 2013). Our data of G2019S mutations increasing innate immune cell chemotaxis corroborates this finding in patients both with LRRK2 mutations and without.

With G2019S mutations in *LRRK2* increasing innate immune cell recruitment to the site of infection, this may implicate a mechanism for spread of the mutation, especially in some minority populations. In Ashekenazi Jews, the G2019S mutation is believed to be traced to one person between 1800 and 1500 years ago (Bar-Shira et al., 2009). This founder effect could be attributable to the original person being more likely to survive infection and be able to pass off the mutation to offspring. Increased chemotaxis of innate immune cells to the site of infection has been shown to increase survival in several models of severe infection. If the G2019S mutation confers increased chemotactic ability on innate immune cells as suggested in this thesis, this could have made the founder more able to fight off infections, survive and pass on the mutation to progeny. Replication of this founder effect proposed in Ashkenazi Jews should be repeated in other ethnic populations with high rates of LRRK2 mutations such as North African Arabs and the Basque People of Northern Spain.

Our data also implicates that loss of LRRK2 and increasing or loss of its kinase activity may have separate effects. KO of LRRK2 in mouse and rats has been associated with several potential deleterious effects including accumulation of type II pneumocytes in the lung and development of enlarged, pigmented kidneys (Herzig et al., 2011, Ness et al., 2013). In the immune system, KO of LRRK2 decreases white and red blood cell count (Ness et al., 2013). When LRRK2 KO microglia or macrophages are stimulated with inflammatory stimuli such as LPS, LRRK2 KO microglia or macrophages have an attenuated pro-inflammatory response with a decrease in chemotaxis, cytokine and chemokine release, and phagocytosis (Kim et al., 2012, Marker et al., 2012, Moehle et al., 2012, Daher et al., 2014, Schapansky et al., 2014). Whereas, overexpression of

LRRK2 or mutant LRRK2 does not recreate many of the findings of the knockout. G2019S LRRK2 does not alter immune homeostasis measured through CBC or flow cytometry. Macrophages overexpressing G2019S LRRK2 have a specific increase in chemotaxis without affecting other measures of macrophage effector functions.

Pharmacological inhibition of LRRK2 seems to support this idea. Short term pharmacological inhibition of LRRK2 does not alter cytokine release. However, when macrophages are inhibited for long periods of time, they begin to have an attenuated inflammatory response similar to that of the knockout (Liu et al., 2014). This appears to only be true of inhibitors of LRRK2 that decrease expression of the protein. For example compounds SRI-451 and SRI-132 decrease expression of LRRK2 by more than half after 72 hours, and have an attenuated pro-inflammatory response to LPS (Liu et al., 2014). Yet, Pfizer A, a compound similar to the SRI compounds in potency and specificity *in vitro*, does not lead to a decrease in LRRK2 expression and does not have an attenuated pro-inflammatory response (Henderson et al., 2015). This potential difference in loss of LRRK2 protein and loss or increase of its kinase activity needs to be further explored as it could potentially alter the interpretation of data critical to understand the function of LRRK2. One possibility to help with this problem is the generation of a kinase inactive LRRK2 mouse or rat. These LRRK2 constructs exist *in vitro* with either the D1994A or K1347A point mutations (it is important to note that these point mutations do not exist in patients and are lab derived). However, these constructs have not yet yielded a mouse that has LRRK2 expression at levels above or comparable to endogenous protein levels.

The data presented in this thesis with LRRK2 interacting with actin regulatory proteins and actin effector proteins may provide a common mechanism for LRRK2

mediated toxicity in diverse cell types. Previous unbiased mass spec studies have implicated LRRK2 interacting with elements of the actin cytoskeleton in both immortalized cell lines and neurons (Meixner et al., 2011). Additional studies in Dictostylium and patient fibroblasts have implicated LRRK2 as interacting with and altering actin dynamics in those cell types (Bosgraaf et al., 2002, Bosgraaf et al., 2005, Caesar et al., 2013, Caesar et al., 2015). Our data extends these results in primary macrophages as well as, for the first time, showing that mutations in LRRK2 increase interactions with these actin regulatory proteins and actin effector proteins, providing a possible mechanism behind LRRK2 mediated toxicity. One of the most common G2019S related phenotypes is the *in vitro* retraction of neurites in primary neurons, process known to be actin dependent (MacLeod et al., 2006, Sheng et al., 2012, Sepulveda et al., 2013). LRRK2 interacting with the same actin regulatory and effector proteins in macrophages as in neurons could result in their respective cell specific phenotypes. Changes could range from alterations to protein-protein interactions, changes in cellular localization, or phosphorylation dependent changes in activity. More work to understand how LRRK2's interaction with these actin related proteins influences these cellular phenotypes will be required.

The data provide interesting implications for the use of LRRK2 as clinical inhibitors. As already discussed, great effort may be needed to understand the loss of LRRK2 kinase activity verse loss of LRRK2 protein. Additionally, LRRK2 kinase inhibition may have therapeutic benefit outside of PD. Diseases associated with chronic, increased inflammation such as multiple sclerosis and arthritis which relies on chemotaxis of innate immune cells to sites of inflammation, as well as those

inflammatory disorders genetically associated with *LRRK2* may benefit from LRRK2 kinase inhibition. Testing of the next generation LRRK2 inhibitors may be warranted in animal models of these diseases as well as in patients, once suitable inhibitors have been developed.

In vivo changes in chemotaxis may provide an excellent opportunity for efficacy and liability testing for new LRRK2 kinase inhibitors. Currently, testing LRRK2 inhibitors in LRRK2 models of toxicity currently take over a month. To show protection from α-synuclein mediated neurotoxicity, a month of drug administration must be achieved to show LRRK2 derived protection in the model (Unpublished data, West Lab). This represents a huge cost in experimental time, throughput, and drug synthesis. Thioglycollate induced peritonitis could represent a short, 3 day or less protocol to test the efficacy of new LRRK2 inhibitors through blocking chemotaxis of innate immune cells in this model. Additionally, liability could be assessed in this model. If the novel LRRK2 inhibitors altered immune homeostasis or attenuated pro-inflammatory responses, these changes could be observed in this model as well.

Taken together, the work in this thesis supports the growing ideas of inflammatory mechanisms being involved in the etiology of PD as well as LRRK2 modulating the inflammatory process, specifically chemotaxis. This work provides a benchmark for future studies to examine the role of mutant LRRK2 in the immune system, as well as a cautionary note for the interpretation results of LRRK2 KO or pharmacological inhibition of LRRK2 in cells of the immune system. Additionally, the data implicate a common mechanism for LRRK2 in diverse cell types which represents a

novel opportunity to discover relevant substrates of LRRK2 kinase activity and possibly mechanisms behind neurodegeneration.

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APPENDIX A

REPRINT PERMISSION

Title: M1 and M2 immune activation in Parkinson's Disease: Foe and ally?
Publication: Neuroscience
Publisher: Elsevier
Date: Dec 31, 1969

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APPENDIX B

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: May 19, 2014

TO: ANDREW B WEST, Ph.D.
CIRC-552
(205) 996-7697

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Mechanisms of LRRK2 Mediated Neurotoxicity
Sponsor: NIH
Animal Project_Number: 140509088

As of May 19, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	272
Mice	B	224

Animal use must be renewed by May 8, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 140509088 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: May 19, 2014

TO: ANDREW B WEST, Ph.D.
CIRC-552
(205) 996-7697

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on May 19, 2014.

Title: Mechanisms of LRRK2 Mediated Neurotoxicity
Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC)
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