

**Exploring the Role of Parkinson's-Linked Leucine-Rich Repeat
Kinase-2 in the Immune System**

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ABSTRACT

The mechanisms by which Leucine-Rich Repeat Kinase-2 (LRRK2) mutations in humans are linked to the risk of Parkinson disease (PD), Crohn's disease and leprosy remain elusive. We hypothesized a shared role for LRRK2 in immune system functions.

I discovered robust LRRK2 expression in mammalian leukocytes, foremost within cells of the innate immune system. For example, human lymph nodes, spleen, and distal ileum exhibited abundant LRRK2-positive macrophages and granulocytes, findings that were confirmed by FACS of cells collected from the same organs of adult mice. Microscopy studies revealed robust LRRK2 reactivity in infiltrating, myeloperoxidase-positive granulocytes and CD68-positive macrophages in inflammatory conditions, such as viral encephalitis, idiopathic radiculitis, terminal ileitis and abscess formation. Analysis of midbrains from idiopathic *versus* genetic variants of Parkinson disease (PD) revealed rare, anti-LRRK2-positive reactivity that was confined to intravascular leukocytes. Neuronal LRRK2 signals were seen in forebrain regions, consistent with reports in the literature. To explore an immunological role for *Lrrk2*, we first examined bone marrow-derived macrophages from PD-linked R1441C knock-in mice and wild-type (WT) animals. Following stimulation with bacterial and viral pathogens, *Lrrk2* expression was increased in cells from both genotypes.

In subsequent *in vivo* experiments using an established, nasal inoculation model of newborn mice with a virulent microbe, we detected a role for WT *Lrrk2* in modifying disease outcomes, such as after reovirus (type-3 Dearing) infection. There, *Lrrk2* deficiency conferred increased vulnerability to elevated viral protein levels in the brain and greater mortality rates from encephalitis in mice. In contrast, we observed an initially protective role for the PD-linked G2019S mutant in the same reovirus inoculation model; there, lower viral titers were

recorded in the lungs and brain of acutely infected, *Lrrk2* knock-in mice at days 3 and 11 post-inoculation (dpi), respectively. Paradoxically, in related survival studies, we observed a significantly higher (rather than the expected reduced) mortality rate during the ensuing weeks in female mice that carried the G2019S *Lrrk2* mutation. Related screening for cytokine dysregulation in infected tissues of mutant mice revealed significant changes in select signaling molecules, e.g., MIG and IP10.

These collective results suggest a role for mammalian LRRK2 in the innate immune system following the encounter of a virulent pathogen, which is associated with a female sex bias. An early mechanistic clue points at changes in cytokine production by infected tissue, a second at the degradation efficiency of viral proteins. We speculate that *LRRK2* alleles function in the regulation of the host's innate response to invading pathogens, which may help explain its association with three human disorders, each of which is pathogenetically associated with one or more environmental trigger. Future studies will test LRRK2's function in other infection paradigms, expand on mechanisms underlying genotype-dependent differences in inflammation, and determine the effects of LRRK2's kinase inhibition in in vivo models.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BMDM	Bone-marrow-derived macrophages
bp	Base pair
BSA	Bovine serum albumin
CD	Crohn's disease
CBX	Cerebellar cortex
CHO	Chinese hamster ovary (cells)
CN1	Cranial nerve 1
CN5	Cranial nerve 5
CNS	Central nervous system
CREB	The cAMP responsive element binding protein
CSF	Cerebrospinal fluid
CSF3R	Colony Stimulating Factor 3 Receptor
CTX	Cerebral cortex
DA	Dopamine
ddH ₂ O	Double-distilled water
dLRRK	Drosophila LRRK2 homolog
dpi	Days post inoculation
EM	Electron microscopy
ERK	Extracellular Signal-regulated Kinase

ES cells	Embryonic stem cells
ELISA	Enzyme-linked immunosorbent assay
4E-BP	Eukaryotic initiation factor 4E (eIFE)- binding protein (4E-BP)
FACS	Fluorescence-activated cell sorting
GDP	Guanosine 5'-diphosphate
GFP	Green fluorescent protein
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine-5'-triphosphate
HAND	HIV-1- associated neurocognitive disorders
HEK cells	Human embryonic kidney
HET	Heterozygous
HIV-1	Human immunodeficiency virus 1
HK	Heat-killed (as in HK listeria or shigella)
HMW	High molecular weight
hPBMC	Human peripheral blood mononuclear cell
HT	Hypothalamus
Iba1	Ionized calcium binding adaptor molecule 1
IC ₅₀	Concentration of an inhibitor to reduce the response by half
IFN-gamma	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL	Interleukin

IL-6ST	Interleukin 6 signal transducer
iNOS	Nitric oxide synthase
IP-10	Interferon gamma-induced protein 10
JAK	Janus kinase
JAM-A	Junctional adhesion molecule A
KC	Keratinocyte chemokine
KI	Knock-in
KO	Knock-out
LB	Lamellar bodies
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LRRK2	Leucine-rich repeat kinase 2
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MB	Midbrain
M-CSF	Macrophage colony-stimulating factor
MDP	Muramyl dipeptide
MEF	Mouse embryonic fibroblast
MEK	MAPK/ERK kinase
mEPSCs	Glutamatergic miniature excitatory postsynaptic currents
MIG	Monokine Induced by Interferon
Min	Minutes
MIP	Macrophage inflammatory protein

MJFF	The Michael J. Fox Foundation
MPO	Myeloperoxidase
MyD88	Adaptor protein Myeloid Differentiation primary response gene-88
NF-kappaB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T-cells
NgR	Nogo receptor
NP	Nucleoprotein
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
OB	Olfactory bulb
OE	Olfactory epithelium
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Parkinson disease
Pfu	Plaque forming unit
PGK-neo	Phosphoglycerate kinase I promoter-neomycin resistance gene
Pi	Post inoculation
PKA	Protein kinase A
PMA	Phorbol 12-myristate 13- acetate
P/M	Pons/medulla
P-s15	Phospho (ribosomal protein) s15
STAT1	Signal transducer and activator of transcription 1
SNpc	Substantia nigra pars compacta

RBC	Red blood cell
RNAi screen	RNA-mediated interference screen
ROS	Reactive oxygen species
S	Seconds
SDS	Sodium dodecyl sulfate
TAK1	Transforming Growth Factor β -activated kinase
Tat protein	Trans-activating protein
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TH	Tyrosine hydroxylase (in IHC staining)
TH	Thalamus (in brain anatomy)
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-alpha	Tumor necrosis factor-alpha
TRAF6	TNF receptor-associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon- β
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPS	Ubiquitin-proteasome system
WBC	White blood cell
WT	Wild type

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CHAPTER I. INTRODUCTION

1.1 Parkinson Disease - The Role Of Environment And Genetics

Parkinson disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer's, affecting 1-2% of the population older than 65 years (Xiromerisiou et al. 2010; Healy et al. 2008). PD is characterized as a movement disorder with motor signs, i.e., tremor, rigidity, bradykinesia, as well as postural instability (i.e., impaired gait control and stance) and responsiveness to dopaminergic therapy (Kalia and Lang, 2015) (Cilia et al. 2014). PD is also accompanied by non-motor symptoms such as loss of olfaction, constipation, genitourinary complications (e.g., erectile dysfunction), sleep disorders (including rapid-eye movement sleep disorder), fatigue, apathy, depression and anxiety, and less frequently, pain, paresthesia (Chaudhuri et al. 2006; Shulman et al. 2001).

Progressive loss of dopaminergic neurons in the *Substantia nigra* and fibrillar, intracellular aggregates of proteins such as α -synuclein in Lewy bodies and Lewy neurites are pathological hallmarks of PD (Healy et al. 2008). The first decade of PD involves up to 60% loss of pigmented neurons in the *S. nigra* of affected patients while healthy individuals of comparable age show only <5% neuronal loss in the same brain region as a result of aging (Fearnley & Lees 1991).

It is estimated that up to 5-10% of clinically diagnosed PD are associated with mutations in known genes leading to monogenic forms of the disease (Xiromerisiou et al. 2010). Recessive forms of PD are caused by mutations in select genes, e.g., *PARKIN*, *DJ-1*, and *PINK1* (reviewed in Marras et al., 2016). Autosomal-dominant forms of parkinsonism can be caused by mutations in the genes encoding leucine-rich repeat kinase-2 (*LRRK2*) and *SNCA* (α -synuclein) (Zimprich et al. 2004). There, α -synuclein- and LRRK2-linked PD cases

commonly show Lewy bodies as part of their neuropathology (Zimprich et al. 2004; Kalia & Lang 2016). PD cases due to recessive deficiency of the Parkin protein generally do not show Lewy body pathology (Pramstaller et al. 2005).

Mutations in *LRRK2*, first discovered in 2004, are the commonest cause of both inherited and sporadic PD (Mata et al. 2006; Greggio & Cookson 2009; Klein & Schlossmacher 2007; Marín 2006; Taylor et al. 2006). Mutations in *LRRK2* mostly lead to typical, late-onset parkinsonism. Intriguingly, a mutation in the kinase domain of *LRRK2*, G2019S, is found to occur in 40% of PD patients of Arab descent and 20% of Ashkenazi Jewish ethnicity (Klein & Schlossmacher 2007). Unlike other autosomal dominant PD genes, penetrance rates for *LRRK2* mutations are relatively low, namely at a mean of 22-32%, based on studies in multiple ethnicities (Marder et al. 2015); these findings suggest the possibility that *LRRK2* may act as susceptibility rather than causative allele (Goldwurm et al. 2007), and that disease modifiers (genetic or environmental in nature) are essential, contributing factors in the development of *LRRK2*-linked PD (Goldwurm et al. 2007; Kitada et al. 2012).

1.2 LRRK2 in Parkinson Disease, Structural, and Functional Insights

1.2.1 LRRK2 Structural Domains

In situ hybridization experiments revealed high expression of *LRRK2* mRNA in the striatum and parts of the neocortex in rodents but showed a slightly lower level of expression of the transcript in the caudate and putamen in humans. Unexpectedly, the dopamine neurons of the *S. nigra* in both rodents and primates did not show any detectable signal for *LRRK2* mRNA (Zimprich et al. 2004; Galter et al. 2006). *LRRK2* transcript levels in

striatal tissue were not variable between PD patients and chosen control subjects (Galter et al. 2006).

LRRK2 gene is classified as a member of the Roco protein-encoding gene family characterized by Roc GTPase and COR domains but remains of unknown function (Marín 2006; Maekawa et al. 2010). The gene locus for *LRRK2* is PARK8 on human chromosome 12 (and mouse chromosome 15) (Tong et al. 2009; Tong & Shen 2009). This gene has 51 exons encoding a 286 kDa protein (Klein & Schlossmacher 2007) (Zimprich et al. 2004). Human *LRRK2* protein is composed of leucine-rich repeat domain (LRR), Roc GTPase domain, COR domain, a kinase domain (MAPKKK) of the tyrosine kinase-like (TKL) superfamily, and finally, a C-terminal WD40 domain (Mata et al. 2006; Greggio & Cookson 2009; Klein & Schlossmacher 2007).

LRR and WD40 domains have a high net positive charge of +20 and +21, respectively, at physiological pH level. This suggests that these domains are possibly involved in interactions with negatively charged proteins, phospholipids or nucleic acids. The mitogen-activated protein kinase (MAPK) domain of *LRRK2* is involved in three-tiered cascades of activation-loop phosphorylation and hence the name MAPKKK is referred to these three kinase cascade events. The physiological substrates of this kinase are not yet known (Mata et al. 2006; Greggio & Cookson 2009; Kubo et al. 2010; Gardet et al. 2010). However, a recent report from Martin and colleagues indicated that ribosomal s15 is an authentic, physiological substrate of *LRRK2* kinase activity (Martin et al. 2014). Phosphorylation of T136 site on s15 appeared to be imperative for G2019S *LRRK2* driven neurotoxicity. The authors suggested that G2019S *LRRK2*-mediated neurotoxicity is mediated through ribosomal s15 by augmenting translation of both CAP-dependent and independent mRNAs.

Roc domain is a GTPase domain, similar to small GTPase of the Ras superfamily (Marín 2006). The Roc domain possesses catalytic activity that hydrolyzes GTP to GDP. GTPase activity is thought to govern the autophosphorylation of Lrrk2 and hence regulating its MAPK domain-like activity (Mata et al. 2006; Greggio & Cookson 2009). The LRRK2 monomer has a low GTPase activity compared to its dimer. As such, LRRK2 is proposed to function via dimerization through the COR domain (Berger et al. 2010). Another proposed mechanism for GTPase regulation is through GTPase-activating proteins (GAP), such as ArfGAP1, and guanine nucleotide exchange factor (GEF), such as ARHGEF1 (Haebig et al. 2010).

In human brain and cultured human cells, LRRK2 is found in cytoplasmic compartments, autophagic vacuoles and multivesicular bodies (Alegre-Abarrategui et al. 2009; Melrose 2008). In human genomic reporter cell models, LRRK2 is shown to locate to membrane microdomains such as the neck caveolae, microvilli/filopodia and intraluminal vesicles of multivesicular bodies (Alegre-Abarrategui et al. 2009; Melrose 2008).

1.2.2 LRR Motif And Relevance To Potentially Functional Insights Into LRRK2 Protein

An LRR motif can be found in many immune receptors of both animals and plants. This characteristic motif is composed of hydrophobic leucine residues, which also serve as a large binding surface (Padmanabhan et al. 2009). The leucine-rich repeats range from 2 to 45 repeats in humans (Ng & Xavier 2011; Ng et al. 2011). The LRR domain of the human LRRK2 protein contains thirteen repeats (Li et al. 2014). It is thought that these repeat molecules confer unique binding specificities (Padmanabhan et al. 2009).

In plants, LRRs are found both intracellularly and extracellularly; in the extracellular space, they are integrated into trans-membrane receptors. Plant-associated pattern-recognition receptors (PRR) identify the microbe-associated molecular pattern (MAMP), which regulate the first line of defense in plants' innate immune system (Padmanabhan et al. 2009). If a pathogen escapes the general detection by PRR, intracellular LRR receptors, such as NB (nucleotide-binding)-LRR receptors, recognize specific pathogen components and as such resemble the action of mammalian immune molecules (Meyers et al. 2003). NB-LRR receptors then generate a downstream signal leading to cell death, thereby controlling the threat of systemic disease by effectively isolating the infected cells (Padmanabhan et al. 2009).

Intramolecular changes occurring within plant-derived NB-LRR proteins represent a means of self-regulation and activation of the host's immune response. The NB domain has catalytic activity and generates ADP from ATP following the formation of an NB-ATP complex. Consequently, NB acts as a 'molecular switch' in activation of the host's defense signaling. LRRs also tend to bind other LRR proteins and form complexes (Padmanabhan et al. 2009). In the case of LRRK2, dimerization is suggested to be the active form of the protein exhibiting increased kinase activity (Berger et al. 2010). Accordingly, it is suggested that dimerization may be a regulating mechanism in controlling LRRK2's kinase activity as well.

A canonical LRR domain can recognize the pathogen directly or indirectly. Direct recognition can resemble the function of mammalian TLRs in detecting specific molecular microbe-determinants. Indirect recognition can be accomplished by other domain(s) of the

LRR-containing protein, or other host factors, which may form a complex with an LRR receptor. Efficient signaling of some mammalian nucleotide and oligomerization domain (“NOD”) containing-like receptors require chaperones such as HSP90 and SGT1; these interact with NOD1, NOD2, NALP3 and NALP12 proteins (Ye & Ting 2008). Some chaperones, such as SGT1, interact with multiple regions of the LRR-containing protein. The assembly of LRR-proteins with chaperones may assist to couple immune signaling with MAMP recognition (Padmanabhan et al. 2009).

The LRR domain of toll-like receptors (TLRs) and NOD-like-receptors (NLRs) in humans recognize specific structures present in microbes, such as bacteria, viruses, fungi and parasites. With exception of NLRs and TLRs, the function of most of the 375 human LRR proteins is not clear (Ng et al. 2011; Ng & Xavier 2011). Ng and colleagues classified 230 LRR genes for expression in 79 specialized tissue types using human microarrays (Ng et al. 2011; Ng & Xavier 2011). Interestingly, their findings unveiled high expression levels of select LRR-carrying proteins among neuronal tissues (39 genes, ~17%) in addition to immune organs (93 genes, ~ 40%). Furthermore, they experimentally tested the expression level of a subset of LRR-genes with a suspected role in immunity, which had not been previously characterized with an immune function. The authors infected human primary macrophages with clinically relevant bacteria, such as *Mycobacterium tuberculosis*, *Salmonella enteric serovar typhi* (*S. typhi*), *Listeria monocytogenes* and enterohemorrhagic *Escherichia coli* (EHEC) for different duration. Their experiments revealed elevated mRNA expression for a subset of those genes, which included *LRK2*. Moreover, other cellular processes, to which LRR-containing proteins contribute, include autophagy, ubiquitin-associated processes,

neuronal development, nuclear mRNA shuttling and cell death. (Ng et al. 2011; Ng & Xavier 2011)

1.2.3 LRRK2 Mutations

There are more than 50 known variants of *LRRK2*; however, not all confer a pathogenic phenotype. Among these, 16 distinct mutations have been identified to be associated with PD, and they mostly cluster in the C-terminus of the protein (Klein & Schlossmacher 2007). A schematic diagram of LRRK2 protein structure and select, *bona fide* pathogenic mutations are shown in Figure 1.2.1 (Lewis & Manzoni 2012).

Mutations in the GTPase domain of LRRK2, such as R1441C, were previously reported to result in the accumulation of multivesicular bodies, large autophagic vacuoles encompassing incompletely digested materials, and increased levels of p62. As well, skein-like cellular lesions were observed in cells expressing these mutants (Alegre-Abarregui et al. 2009; Alegre-Abarregui & Wade-Martins 2009). On the contrary, *LRRK2* knockdown via siRNA showed an increase in autophagy-linked LC3 turnover marking enhanced autophagic activity (Alegre-Abarregui et al. 2009; Alegre-Abarregui & Wade-Martins 2009). GTPase mutations of R1441C/G may lower the enzymatic activity of the domain, while R1441H may elevate it (Liao et al. 2014).

The G2019S mutation of the LRRK2 kinase domain is the most frequent and best-studied variant of *LRRK2* causing PD (Klein & Schlossmacher 2007). The missense mutation G2019S in its MAPKKK domain confers enhanced kinase activity onto LRRK2, suggesting a gain of function event, which may be implicated in neurotoxicity (Plowey et al. 2008). However, depending on the population studied, the frequency of mutations is variable. *LRRK2* screening in a Canadian PD cohort conducted by Grimes et al. did not detect any of

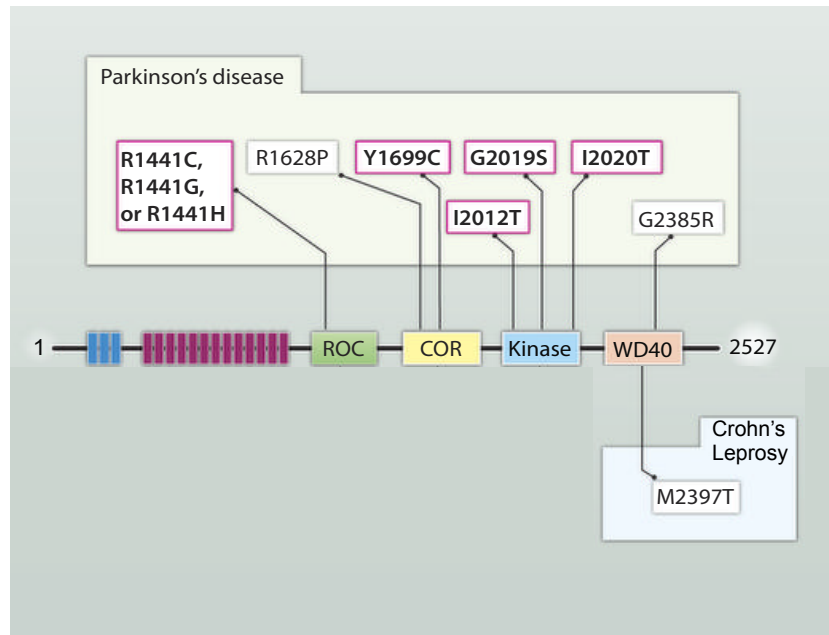


Figure 1.2.1. Schematic diagram of LRRK2 protein structure. Some common pathogenic mutations of LRRK2, as implicated in PD, are shown in red boxes. Diagram was slightly modified from (Lewis & Manzoni 2012).

the known mutations in seven *LRRK2* exons or any novel mutations in the gene (Grimes et al. 2007).

When differentiated SH-SY5Y cells were transfected with *LRRK2* cDNA bearing the mutant G2019S, significant neurite retraction was observed. The control samples containing wild-type *LRRK2* or the kinase-dead K1906M transfected cells did not show this effect (Plowey et al. 2008). The *LRRK2*-dependent neuritic changes happen during development and presence of growth substrate (Sepulveda et al. 2013). The level of LRRK2 increases as synaptic structures and functional maturation takes place (Sepulveda et al. 2013). The kinase function of LRRK2 is linked to synaptic endocytosis, possibly through endophilin A phosphorylation cycle (Matta et al. 2012), to cytoskeleton remodeling through phosphorylating ezrin/radixin/moesin, and to the translational regulation of select mRNAs through phosphorylating eukaryotic initiation factor 4E-BP (Martin et al. 2014).

Researchers have identified select phosphorylation sites on LRRK2 that are subject to auto-phosphorylation and targeting by other kinases (West et al. 2007; Doggett et al. 2012; Dzamko et al. 2010). The GTPase and kinase domains of LRRK2 possess a series of autophosphorylation sites that form clusters within these catalytic domains (West et al. 2007; Webber et al. 2011; Greggio et al. 2009; Gloeckner et al. 2010). Most of LRRK2 phosphorylation sites were found using *in vitro* settings; Sheng Z et al. have recently identified S1292 as an *in vivo* autophosphorylation site for LRRK2 (Sheng et al. 2012). Sheng and colleagues also postulated phosphorylation of S1292 residue offered a measure of LRRK2 kinase function; phosphorylation of S1292 is also modulated by various PD linked mutations by which it may attribute to PD pathogenesis.

The same team transfected HEK293 cell line with constructs expressing FLAG-tagged WT *LRRK2*, G2019S or kinase dead mutant LRRK2 (D1994A). Following the extraction of the proteins from the cells and gel electrophoresis, the LRRK2 gel bands were digested into peptides and then processed by mass spectrometer along with the standard peptides for analysis. LC-MS results revealed S1292 residue as an autophosphorylation site on LRRK2. The S1292 residue, in LRRK2 protein, is located between the LRR and GTPase domains. Subsequent Western blotting of cell lysates with an antibody directed against S1292 LRRK2 yielded a signal corresponding to transfection of cells with WT and G2019S *LRRK2* but not for the kinase-dead mutant D1994A or the S1292A mutant (i.e., control mutants). Related experiments carried out in HEK293 cells further showed augmented phosphorylation of S1292 residue for G2019S, R1441C/G and N1437H versus WT *LRRK2* expression. Animal brain lysates also demonstrated a >10-fold increase in phosphorylation of S1292 within G2019S *versus* WT *LRRK2* BAC transgenic mice.

Transfection of rat primary hippocampal neurons with double mutant G2019S/R1441G constructs produced retracted neurites compared to transfection with WT or single mutant G2019S or R1441G constructs. A kinase-dead mutant, D1994A as well as a S1292A mutant construct effectively rescued the neurite shortening, thereby denoting S1292 phosphorylation was indispensable for modulation of neurite morphology (Sheng et al. 2012).

Sheng and colleagues generated a new inhibitor for LRRK2, G1023, that abolished pS1292 levels in brains and spleens of BAC-transgenic mice carrying the G2019S mutation. This new inhibitor was also effective in eliminating phosphorylation of another LRRK2 phosphorylation site, S935, which had not been previously reported as a residue for

autophosphorylation (Dzamko et al. 2010). The G2019S LRRK2 overexpressing mice exhibited shortened neurite length, which was ameliorated by treatment with G1023 (Sheng et al. 2012).

1.2.4 Protein Interactors and Related Functional Activity

The molecular mechanisms by which PD genes are thought to cause neurodegeneration include protein aggregation (α -synuclein), impaired ubiquitin–proteasome-mediated protein degradation, lysosomal dysfunction, excess oxidative stress and mitochondrial abnormalities (Zimprich et al. 2004). The essential, cellular function(s) of *LRRK2* is(are) largely unknown (Marín 2006). Cell culture assays have shown *LRRK2* to interact with Parkin, an E3 ubiquitin ligase. Given the fact that protein aggregates and Lewy bodies are present in some *LRRK2* mutation cases, this may be indicative of a potential role for LRRK2 in ubiquitin-mediated metabolism (Zimprich et al. 2004; Marín 2006). Venderova et al. reported, using a *Drosophila melanogaster* model of *LRRK2*-PD, genetic interactions between *PINK1*, *DJ-1*, or *Parkin* and *LRRK2* (Venderova et al. 2009).

Furthermore, *in vitro* assays have shown α -synuclein and Alzheimer's-linked tau to reciprocally induce fibril formation. A-synuclein is the primary component of Lewy bodies, and tau can be found co-localized with a subset of Lewy bodies in idiopathic PD (Zimprich et al. 2004). In theory, the kinase activity of LRRK2 may be responsible for phosphorylation of these two proteins that consequently lead to their misprocessing and accumulation (Zimprich et al. 2004; Taymans & Cookson 2010). A recent study suggested that *SNCA* gene expression occurs downstream of *Lrrk2* kinase activity (Carballo-Carbajal et al. 2010). Other

interactors of *LRRK2* found *in vitro* include moesin, Rab5, α and β -tubulin and heat shock protein 90 (Hsp90) (Melrose 2008).

1.3 LRRK2-PD Pathogenesis and Diverse Phenotypes

Lewy bodies are commonly found in *LRRK2*-linked PD brains. However, patients with *LRRK2* mutations of R1441C, Y1699C and I2020T do not exhibit the presence of Lewy bodies, and instead show other abnormalities such as neurofibrillary tau tangles (NFTs) (Kett & Dauer 2012). This observation suggests that different mutations may employ distinct pathological mechanisms, which ultimately lead to neurodegeneration.

Zimprich et al. studied 46 families with various mutations in the *LRRK2* gene and reported strikingly diverse pathologies. The *post mortem* analysis of affected carriers showed brainstem dopaminergic degeneration that accompanied abnormalities consistent with Lewy body-positive PD of the brainstem, diffuse Lewy body disease (with central nervous system-wide pathology), nigral degeneration without distinctive histopathology, and progressive supranuclear palsy-like pathology (Zimprich et al. 2004; Kalia & Lang 2016). Hence, these findings place *LRRK2* as a central player in elucidating the etiology of several parkinsonism-associated neurodegenerative diseases (Zimprich et al. 2004; Marín 2006).

1.4 Leprosy And Crohn's Disease And Their Association With *LRRK2*

1.4.1 *LRRK2* As A Risk Factor For Crohn's Disease

Inflammatory bowel disease (IBD) is an immune-related disease that mainly affects small intestine and colon. IBD is classified into two main types of Crohn's disease (CD) and ulcerative colitis.

The clinical symptoms of Crohn's disease include weight loss, vomiting, abdominal pain, diarrhea, and gastrointestinal bleeding (Duerr et al. 2006). CD is an autoimmune disease where the gastrointestinal tract is attacked by the host's immune system as a result of microbial colonization and/or invasion leading to inflammatory conditions.

In a study conducted by Barrett et al., the *LRRK2* locus was identified as a novel susceptibility gene for CD, but less likely, for ulcerative colitis. By genome-wide association, they identified more than thirty distinct susceptibility loci for CD (Barrett et al. 2008). Genome-wide studies are a useful tool offering a systematic assessment of the contribution of common variants to create the general genetic architecture of a multifactorial disease. Being a complex disease, a large number of single nucleotide polymorphisms (SNPs) were generated as risk factors of CD. One of such SNPs was rs11175593 (odds ratio of 1.54 and p -value of 3.08×10^{-10}), occurring in non-coding region of chromosome 12, which is upstream of *LRRK2* (Barrett et al. 2008)

Polymorphisms associated with autophagy pathway genes *ATG16L1* and *IRGM1* also confer susceptibility to development of CD (Cadwell et al. 2009; Hampe et al. 2007). Also, mutations in the 'nucleotide-binding, oligomerization domain containing-2' protein (encoded by *NOD2/CARD15*) are also associated with susceptibility to CD (Stockton et al. 2004; Ogura et al. 2001; Maeda et al. 2005). Interestingly, the structure of *NOD2* contains six leucine-rich repeats (LRRs), and the protein is predominantly found in peripheral blood leukocytes. *NOD2* encodes a cytosolic bacterial sensor involved in immune responses and activation of nuclear factor-kB (NF-kB) signaling pathways when encountering bacterial ligands.

LRRK2 and NOD2 are also found to form a complex with small GTPase Rab2a in intestinal Paneth cells wherein this complex function in directing of lysozyme-containing cargo to lysozyme-containing dense core vesicles (DCVs) (Zhang et al. 2015; Rocha et al. 2015). Lysozymes are secreted from Paneth cells into the extracellular space to serve as an anti-bacterial barrier in the gut mucosa. Lysozymes are glycoside hydrolases that break bacterial cell wall, and Paneth cells are a type of epithelial cells found in the small intestine with roles in immunity and stem cell maintenance. Disruption of the lysozyme cargo complex with LRRK2, NOD2, and Rab2a (due to mutations in any of these three proteins) are followed by lysosomal degradation of the lysozymes. It may be plausible to think that LRRK2 functions similar to an NLR protein at least in the context of Paneth cells. Reduced secretion of lysozymes by Paneth cells is linked to reduced immunity and susceptibility to Crohn's disease (Zhang et al. 2015; Rocha et al. 2015).

The presence of LRRK2 was previously detected in the *lamina propria* of human intestinal tissue (Gardet et al. 2010). Gardet et al. reported increased expression of LRRK2 in inflamed intestines of Crohn's disease patients. They showed expression of LRRK2 in macrophages, B-cells, and CD103-positive dendritic cells. Collectively, their work suggested that LRRK2 was involved in immune responses via the interferon (IFN)- γ pathway. Inflamed intestines of CD patients showed increased levels of pro-inflammatory cytokines, such as TNF- α and IFN- γ . Data suggested that IFN- γ stimulation of THP-1 cells (a human macrophage-derived cell line) as well as human peripheral blood mononuclear cells (PBMC) increased *LRRK2* mRNA and protein expression as detected by quantitative PCR and flow cytometry, respectively (Gardet et al. 2010).

1.4.2 LRRK2 Gene Is Identified As A Risk Factor For Leprosy

In a genome-wide association study conducted by Zhang et. al, a single-nucleotide polymorphism (SNP) in the locus for *LRRK2* gene was also shown to confer susceptibility to the chronic infectious disease of leprosy (Zhang et al. 2009). Leprosy is caused by *Mycobacterium leprae*; the microorganism primarily attacks macrophages and Schwann cells. The disease manifests itself with damage to skin, peripheral nerves, resulting in characteristic deformities and disabilities that contribute to social stigmatization for patients (Schurr et al. 2006; Alcaïs et al. 2005). Multibacillary and paucibacillary variants are the two clinical subtypes of leprosy (Zhang et al. 2009; Schurr & Gros 2009). Zhang et al. proposed, based on their data, a gene-interaction network involving five genes, including *LRRK2*, which conferred susceptibility to leprosy. Implicated gene variants were generally associated with both multibacillary and paucibacillary forms; however, *LRRK2* showed a trend toward association with multibacillary subtype only. Previous studies reported variants of *PARK2* gene encoding Parkin, and its co-regulated gene, *PACRG*, as a risk factor for infection with *M. leprae*; it is also thought that the two proteins both take part in regulation of innate immunity (Zhang et al. 2009; Schurr et al. 2006; Alcaïs et al. 2005).

Further studies revealed that *LRRK2* association with leprosy involved a specific subtype of leprosy, one with “type-1 reactions” (T1R), which are characterized by excessive inflammatory reactions (Fava et al. 2016). Variations in some of the genes conferring susceptibility to leprosy were also linked to inflammatory bowel disease (Schurr & Gros 2009). Interestingly, the reported *LRRK2* SNP linked with T1R-specific leprosy was M2397T, which was the same mutation linked to Crohn’s disease (Fava et al. 2016). This indicated that both T1R-specific leprosy and Crohn’s potentially shared the same pathogenic pathway

downstream of this *LRRK2* mutation. The new linkage of these two infectious disorders with the PD gene *LRRK2* has important implications for the strategy of genetic analysis of complex infectious diseases (Buschman & Skamene 2004).

Understanding the genetic predisposition to infectious diseases can point to specific alleles and pathways that can potentially be targeted for pharmacological treatments (Schurr & Gros 2009; Alter et al. 2008; Alcaïs et al. 2009; Burgner et al. 2006). Recent *in vivo* studies and *post-mortem* analysis in PD provided evidence of neuroinflammatory processes in patients (Hirsch & Hunot 2009; Simón-Sánchez et al. 2009; Reale et al. 2009). 90% of PD cases are idiopathic PD, and environmental factors are thought to contribute to these cases. Braak and his colleagues suggested a dual-hit hypothesis proposing that PD is triggered by environmental pathogens that enter a susceptible host via the nasal and/or gastrointestinal routes. Impairment of olfaction, vagal dysfunction, and REM sleep behavioral disorder occur long before impaired motor symptoms in PD (Hawkes et al. 2009; Hawkes et al. 2007). According to Braak et al., after the initial entry of a pathogen or toxin into the host via these two routes (olfactory and GI tract), a PD-linked pathogen can travel to the temporal lobe or to the parasympathetic motor neurons of the vagus nerve in the medulla, respectively, thus subsequently reaching the pons and midbrain (*S. nigra*) to cause neuronal degeneration (Hawkes et al. 2009; Hawkes et al. 2007). As a result, the focus of my research was –in part– to delineate the role of *LRRK2* in leukocytes to shed light on its contribution to inflammation, which may benefit our understanding of PD, leprosy as well as CD pathogenesis.

1.5 Lrrk2 And Immune Regulation

1.5.1 A Developmental Role For LRRK2 In Immune Cells

Lrrk2 expression, outside the CNS, was first reported in mouse kidneys (Tong et al. 2010) and spleens (Maekawa et al. 2010) during aging. *Lrrk2* mRNA and protein underwent a constant reduction throughout aging (Kubo et al. 2010; Maekawa et al. 2010). The Lrrk2 expression in the spleen was attributed to CD19⁺ B-cells. Among B-cell subsets, LRRK2 expression was exclusive to B-2, but not B-1, type cells. B-1 cells are most abundant during early development while conventional B-2 cells are present after birth. Interestingly, stimulation of B-2 cells markedly down-regulated Lrrk2 expression. (Kubo et al. 2010)

Analysis of transcriptomes has indicated the presence of *LRRK2* mRNA in a variety of organs including select immune cells (Su et al. 2004). Following our finding (Hakimi et al. 2011) of LRRK2 expression in human and mouse leukocyte subtypes, Thevenet et al. looked further into the LRRK2 expression of monocyte subtypes (Thévenet et al. 2011). Thevenet et al. characterized *LRRK2* mRNA and protein expression in human peripheral blood mononuclear cells (PBMC) subpopulations. They found expression of LRRK2 in CD19⁺ B cells and CD14⁺CD16⁺ monocytes but not in T-cells (CD3⁺/CD4⁺/CD8⁺) nor natural killer cells (CD3⁻CD14⁻CD16⁺) (Thévenet et al. 2011).

Among monocytes subpopulation, the CD14⁺ monocytes, which contained the LPS co-receptor exhibited robust levels of the *LRRK2* transcript (Thévenet et al. 2011). Among CD14⁺ monocytes, both CD14⁺CD16⁻ and CD14⁺CD16⁺ monocytes had comparable levels of *LRRK2* mRNA. However, the protein levels of LRRK2 in CD14⁺CD16⁺ monocytes were dramatically increased by 14-16 folds compared to CD14⁺CD16⁻ monocytes. (Thévenet et al.

2011) Of note, regulation of LRRK2 levels in macrophages through maturation elicits the opposite effect to that in B-cell through aging.

Monocytes with CD14⁺CD16⁺ expression represent non-classical mature monocytes (as opposed to classical CD14⁺CD16⁻ monocytes) and constitute <10% of all monocytes subpopulations under normal conditions (Thévenet et al. 2011). Elevated numbers of CD14⁺CD16⁺ cells are linked with inflammatory conditions such as rheumatoid arthritis, Atherosclerosis, HIV and severe bacterial sepsis (Ziegler-Heitbrock 2007).

IFN- γ activation of monocytes is known to change the ratio of CD14⁺CD16⁻/CD14⁺CD16⁺ in monocytes and render them more adhesiveness (Thévenet et al. 2011). IFN- γ -induced upregulation of LRRK2 was concomitant with the maturation of monocytes to CD14⁺CD16⁺. Further results indicated that maturation of monocytes to CD14⁺CD16⁺ was dependent on the kinase activity of LRRK2. (Thévenet et al. 2011)

Collectively, these results indicated a developmental role for LRRK2 in immune cell differentiation (in macrophages) and during aging (in B-cells). Both Gardet et al. and Thévenet et al. found several consensus IFN-stimulated response elements and - γ activated sequences in the *LRRK2* gene (Gardet et al. 2010; Thévenet et al. 2011). IFN- γ plays a crucial role in innate immunity and maturation of monocytes (expressing I-CAM, CD14, CD16), which is increased in inflammation and infectious diseases.

1.5.2 Involvement Of LRRK2 In Immune Signaling

As mentioned previously, recruitment and activation of microglia have been documented in *postmortem* PD brains (Sanchez-Guajardo et al. 2013). There are several reports now that have investigated microglia to find an immunologic role for LRRK2 in the

CNS (Puccini et al. 2015; Moehle et al. 2012; Daher et al. 2014). The major findings from these reports are discussed in Chapter IV: Discussion. Briefly, their primary results indicated that LRRK2 has a pro-inflammatory role in microglia since genetic deletion or inhibition of LRRK2 elicited protective effects and reduced inflammatory responses in particular after LPS treatment. In support of this novel (non-neuronal) concept, Schapansky et al. since demonstrated that immune activation of monocyte-type cell lines led to increased Lrrk2 phosphorylation and dimerization concomitant with its membrane recruitment, where autophagosome membrane association was most abundant (Schapansky et al. 2014).

Their initial experiments revealed that activation of immune cells with LPS resulted in increased Lrrk2 expression and membrane localization in murine macrophage RAW264.7 and microglial BV2 cell lines. Schapansky et al. had indicated in their previous report (Berger et al. 2010) that LRRK2 is predominantly found in a monomeric and soluble form in the cytoplasm – under inactivated conditions. LRRK2 dimers, although constituting only a small portion of the total LRRK2 pool, are functionally more relevant regarding their activity (Berger et al. 2010).

Treatment of RAW264.7 macrophages with LPS led to the up-regulation of S935 Lrrk2 phosphorylation, which was followed by its dimerization (Schapansky et al. 2014). Both cytosolic and membrane fractions showed enhanced Lrrk2 dimerization; though the fold increase in Lrrk2 dimer content was higher in membrane fractions. Given the importance of phagocytosis in immune response, Schapansky et al. examined the effect of Lrrk2 inhibition on this cellular process. Their results collectively did not indicate a phagocytic role for Lrrk2 (Schapansky et al. 2014).

One of the very first reports that indicated an immune role for LRRK2 was from Podolsky's team who showed IFN- γ inducing LRRK2 expression and leading to activation of NF- κ B pathway (Gardet et al. 2010). Following this finding, Kuss and colleagues reported ERK5 to positively regulate LRRK2 in this signaling pathway (Kuss et al. 2014). ERK5 was speculated to act downstream of IFN- γ and upstream of LRRK2. LRRK2 negatively regulated PKA, which was responsible for phosphorylation and activation of NF- κ B (Russo et al. 2015). PKA also has a role in cytoskeletal dynamics, neuronal development and synaptogenesis, which are all related to PD (Parisiadou et al. 2014).

LRRK is modulated by I κ B kinases, which phosphorylate LRRK2 at S935 and S910 following MyD88-dependent Toll-like-receptor (TLR) stimulation (Dzamko et al. 2012). TLRs are embedded in the cell membrane and involved in pathogen recognition and innate immunity. In TLR signaling mediated through MyD88 adaptor protein, TAK1 phosphorylates κ B kinases IKK α and IKK β , which collectively yield modulation of LRRK2 phosphorylation.

Based on an RNAi screen of *D. melanogaster*, Liu et al. found "Nuclear factor of activated T-cells" (NFAT) as an interactor of LRRK2. NFAT is a group of transcription factors with important roles in immune signaling. NFAT1 controls the expression of IL-2, IL-6, and TNF- α and has a pivotal role in T-cell function and cytokine production. Also, NFAT1 modulates immunity mediated by dendritic cells, macrophages, and neutrophils (Liu et al. 2011; Liu & Lenardo 2012; Hogan et al. 2003).

Under normal conditions, NFAT is found in the cytoplasm and is highly phosphorylated. During pathogen encounter, antigen recognition causes an influx of calcium in the cells, which promotes dephosphorylation by calcineurin. NFAT is then transferred from cytoplasm to nucleus to induce transcription of target genes that activate the defensive signaling

cascade (Liu & Lenardo 2012). The function of NFAT is regulated by its phosphorylation state, nuclear translocation and repressor complexes (Liu et al. 2011). LRRK2 negatively regulates transcription factor NFAT localization and activity (Liu et al. 2011). It is also important to note NFAT is involved in regulation of both inflammatory and non-inflammatory cytokines. Hence, the type of pathogenic stimulus may be a determinant of NFAT's activity in the cells as well (Liu & Lenardo 2012).

1.6 Lrrk2-PD Mouse Models – Focus On Experimental Animals

Human LRRK2 protein has 86.7% homology with murine Lrrk2 (Herzig et al. 2011). In delineating the physiological role of LRRK2 in the immune system, we have utilized *Lrrk2* mutant PD mouse models in the majority of our experiments for both *in vivo* and *in-vitro* studies. Because of their relevance to studies carried out during my research project, descriptions of these mouse models are summarized below and in Table 1.6.1. Known mutation outcomes and possible pathophysiological mechanisms relating to each of the chosen *Lrrk2* mutations (*Lrrk2* KO, R1441C, and G2019S) are also covered below.

1.6.1 Lrrk2 Knock-Out Mouse Model

The *Lrrk2* deficient mouse model that I used in my Research was generated and characterized in: (Tong et al. 2010). These mice appeared normal and lacked any neuronal loss and abnormality in the brain. Tong's report as well as those by others, who generated similar mouse lines, indicated that *Lrrk2* deficiency caused abnormality in murine kidneys (Herzig et al. 2011; Tong et al. 2010). Kidneys of *Lrrk2* KO mice developed lipofuscin deposits and increased LAMP1, LAMP2 and ubiquitin immunostaining, which collectively indicated

Table 1.6.1. Description of *Lrrk2*-PD Mouse Models - experimental animals. Results summarized from (Tong et al. 2010; Tong et al. 2012; Tong et al. 2009; Herzig et al. 2011; Beccano-Kelly et al. 2014). These mouse models were used for our animal studies throughout.

<i>Lrrk2</i> -PD mouse model	Outcomes/findings	Conclusion/clinical relevance	Reference
<i>Lrrk2</i> knock-out	- Normal locomotor activity - Absence of neuronal loss and abnormality - Abnormality in kidneys of the mice: lipofuscin deposits and increased LAMP1, LAMP2 and ubiquitin immunostaining, synucleinopathy, apoptotic cell loss and inflammation - Biphasic changes in autophagic activity in kidneys: increased autophagic influx in younger mice and decreased autophagic influx in older mice - Increased Kim-1 levels by 10-folds in kidneys - Diastolic hypertension - Enlarged lamellar bodies in alveolar type II epithelial cells of lungs	- Impairment in autophagy-lysosomal pathway in kidneys - Chronic renal injury - Alteration in morphology (and release) of lamellar bodies of lung epithelial cells with possible implications on mucosal immune defense	(Tong et al. 2010; Herzig et al. 2011; Tong et al. 2012)
R1441C <i>Lrrk2</i>	- Absence of neuronal loss and abnormality - Normal locomotor activity in absence of drug treatment - Impaired locomotor activity when stimulated with DRD2 ligands AMPH and catecholamine	- Impairment (attenuation) of dopamine transmission through dopamine D2 receptor	(Tong et al. 2009)
G2019S <i>Lrrk2</i>	- Normal locomotor activity - Absence of neuronal loss and abnormality - Diastolic hypertension - Increased glutamatergic release and decreased synapsin-1 phosphorylation in the cortical neurons	- Aberration in synaptic function and glutamate release in cortical neurons	(Herzig et al. 2011; Beccano-Kelly et al. 2014)

impairment in the autophagy-lysosomal pathway (Herzig et al. 2011). A type of 'synucleinopathy,' apoptotic cell loss and inflammation were also noted in the renal tissues of these mice. (Tong et al. 2010)

Biphasic changes in autophagic activity in *Lrrk2* null mice indicated an increased autophagic influx in younger mice, which was decreased in older mice (Tong et al. 2012). The initial enhancement of autophagy in the absence of *Lrrk2* implies that *Lrrk2* is a suppressor of autophagy. The subsequent attenuation of autophagy in aged mice means possible recruitment of compensatory mechanisms to overcome adverse consequences of *Lrrk2* deficiency (Cookson 2015). Related to this observation, brains of Alzheimer's and PD patients have revealed the accumulation of autophagosomes and autolysosomes in autopsy (Nixon et al. 2005; Nixon 2006). Cathepsin D and lipofuscin are also elevated in these diseased brains (Nixon et al. 2005).

Besides, Herzig and colleagues reported increased diastolic hypertension in *Lrrk2* KO mice compared to WT control (Herzig et al. 2011). Furthermore, kidney injury molecule-1 (Kim-1) levels were significant and 10-fold higher in *Lrrk2* KO kidneys compared to WT animals at 7-months of age indicating chronic injury as a consequence of *Lrrk2* absence. Kim-1 is a sensitive and specific marker used to determine renal injury (Ichimura et al. 2004).

1.6.2 R1441C *Lrrk2* Mouse Model

The R141C *Lrrk2* mutant mouse model that I used in my research was generated and characterized in: (Tong et al. 2009). A common amino acid substitution occurs at the R1441 residue of the LRRK2 GTPase domain. In PD cases with R1441C amino acid substitution,

variable neuropathology is documented in patients. To delineate the related pathophysiological mechanisms of Dardarin (an alternate name for LRRK2), Tong et al. generated R1441C *Lrrk2* KI mouse model and characterized it in their 2009 report (Tong et al. 2009).

Tong's characterization of R1441C KI mice indicated impairment (namely attenuation) of dopamine transmission through dopamine D2 receptor (Tong et al. 2009). R1441C KI mice appeared normal and aged without any locomotor deficit; however, the drug-induced locomotor activity of these mice was impaired when DRD2 was stimulated using AMPH and catecholamine (Tong et al. 2009).

Further analysis of the brains by immunohistochemistry and immunoblots showed the R1441C KI mice were devoid of aberrant protein accumulation such as Lewy bodies, ubiquitin-positive inclusions, and neurofibrillary tangles, all of which are found in the disease cases (Zimprich et al. 2004). Pathogenic phosphorylation of α -synuclein, ubiquitin and tau were also absent in the mutant mice (Tong et al. 2009). No evidence of gliosis was marked in this mouse line as analyzed using GFAP markers. These data point to the impaired function of D2 the receptor in mice carrying this GTPase genetic alteration. A pathway where the functions of D2 the receptor, AMPH and catecholamine exocytosis converge in dysfunction of *Lrrk2* is intriguing but not known yet. (Tong et al. 2009)

1.6.3 G2019S *Lrrk2* Mouse Model

The G2019S *Lrrk2* mutant mouse model used for my Research Project was generated by Novartis Pharma and characterized in: (Herzig et al. 2011). Similar to the previous mouse models, characterization of PD-linked G2019S *Lrrk2* mouse revealed the absence of brain

neuropathy and any unusual locomotor activity in younger animals; however, they observed an abnormal (unexpectedly, hyperkinetic) locomotor responses in older mice when compared to WT mice. Herzig and colleagues also reported diastolic hypertension in G2019S *Lrrk2* mice compared to WT control. This phenotype was also present in the *Lrrk2* KO mice but not in their KD mice. The authors mention that the increased blood pressure is unlikely to be a result of kinase-dependency since it was present in both *Lrrk2* deficient mice and G2019S *Lrrk2* mice (Herzig et al. 2011).

Further research on G2019S *Lrrk2* mouse model indicated aberrations in glutamate transmission as reported by (Beccano-Kelly et al. 2014). These authors chose cortical neurons for their cell culture model because their input in the striatum is regulated by dopamine from the nigrostriatal pathway; the critical pathway affected in PD. Interestingly, the investigators found significant glutamatergic release, ~ 37%, in the neurons of G2019S *Lrrk2* mice. Relative to non-transgenic control, the G2019S mutation did not affect synaptic density thereby suggesting an increase in release probability. It is noteworthy that one copy of the G2019S mutant allele generated a more potent effect (i.e. more severe dysregulation) than overexpressing transgenic LRRK2 by three folds.

In addition, synapsin-1 phosphorylation was significantly decreased in G2019S neurons compared to control neurons as measured for the two phosphorylation sites of S9 and S603 (Beccano-Kelly et al. 2014). Synapsins are common pre-synaptic vesicle proteins that are substrates for a number of kinases and regulate vesicle exocytosis (Jovanovic et al. 2001). It is also noteworthy that S603 on synapsin-1 is a phosphorylation site-specific to CaMKII. A link between CaMKII and LRRK2 pathogenesis may be an interesting investigation to

mechanistically explore the role of synapsin-1 phosphorylation in altered excitatory transmission. (Beccano-Kelly et al. 2014)

1.7 Neuroinflammation And Relevance To PD Experimental Designs

1.7.1 Influenza Virus Infection And Parkinson-Like Symptoms

Jang and colleagues postulated that protein misfolding, and aggression of neurological diseases can originate from the insult of neurotropic viruses in general (Jang et al. 2009). In their 2009 report, they described infection of the central nervous system by H5N1 influenza A virus using a C57BL/6J mouse as a host model organism. They showed the virus to travel from the peripheral, enteric nervous system to the central nervous system, and the infected mouse developed Parkinson-like symptoms.

The main infection site of influenza type A virus is the lungs. The famous 1918 influenza pandemic was reported to have additionally caused neuroinflammation and other CNS abnormalities (Ravenholt & Foege 1982). The 2008 report from The World Health Organization indicated the lethality of H5N1 avian influenza to have reached 61% in the 433 human cases identified at that time (Jang et al. 2009).

Jang and colleagues intranasally inoculated adult mice, 6-8 weeks of age, with LD₅₀ of H5N1. The disease manifested itself in mice with progressive weight loss, motor dysfunction, neurologic symptoms and death (in half of the mice). Infection progression in mice was studied using an antibody against H1N5 nucleoprotein (NP) in IHC experiments; EM was used for validation studies later.

The virus was detectable in the nervous system structures as early as 3 dpi initially in the enteric (mesenteric and myenteric plexi) and peripheral (DRG neurons) nervous systems and slightly later in the central nervous system. In the brainstem, the solitary nucleus at 3 dpi, and vagal and hypoglossal nuclei at 7 dpi showed signs of infection. Pons and midbrain (LC and SNpc at 7 dpi), olfactory bulb (periglomerular and mitral cells) and spinal cord also showed reactivity for the viral protein. The influenza protein was found both in neuronal and microglial nuclei and cytoplasm. Infection was resolved in the surviving animals by day 21.

The authors posited that the mode of CNS virus entry included cranial nerves and a hematogenous spread. Interestingly, areas of infection correlated with augmented phosphorylation of α -synuclein as determined by the anti-phospho-Ser129 antibody. Hyperphosphorylation of α -synuclein was observed in the olfactory bulb, cortex, hippocampus, midbrain, brainstem of the infected animals; this was not present in uninfected animals. Serine 129 is a key residue on α -synuclein that is hyperphosphorylated in many synucleinopathies (Anderson et al. 2006). Hyper-phosphorylation promotes the pathological aggregation of α -synuclein (Anderson et al. 2006). Infected animals, as well exhibited elevated levels of active caspase-3 indicating apoptotic cell death signaling. Two months later, 17% dopamine cell loss in the SNpc and activated microglia were observed in surviving animals.

Chronic neuroinflammation is documented in patients with parkinsonism who previously suffered from encephalitis; however, evidence of viral components is not always found in these brains. Persistent inflammation, after the pathogen is cleared, supports a "hit-and-run" mechanism. Consistent with this notion, non-neurotropic viruses can still

stimulate cytokine secretion in the brain as experimented by Kobasa D and colleagues who used an H1N1 variant of influenza virus on macaques (Kobasa et al. 2007).

1.7.2 Reo Virus Pathogenesis, Mechanisms, And Receptors

1.7.2.1 Background And Rationale For Reovirus Infection Model And Mechanisms

Summary of Rationale: Aspects of reovirus biology intersect with immune functions in mammals, which have potential relevance to pathways that LRRK2 has recently been shown to be involved in, such as induction of NFκB signaling in infected tissues and cellular apoptosis. Therefore, given the neurotropism of some reovirus strains (and the relevance of influenza-type infections in the development of Parkinsonism), we decided to include reovirus models as an important platform to further test an immune function for endogenous Lrrk2 in our experimental animals.

Reovirus has been isolated from many mammalian species including humans and mice (Danthi et al. 2010). The word “reo” stands for respiratory-enteric-orphan virus and is named so as it is not associated with disease in humans (Gauvin et al. 2013). Infection with reovirus elicits mild flu-like symptoms in some, but not the majority of, individuals (Norman & Lee 2000). Reovirus is ubiquitous in the environment, and 50% of people are positive for the virus antibody by the age of 20-30 years; the presence of the virus antibody indicates previous reovirus infections (Norman & Lee 2000). Some reports indicate 70-100% of people to contract reovirus infection throughout their lifetime (Norman & Lee 2000; Minuk et al. 1987; Minuk et al. 1985).

Reoviruses are non-enveloped viruses whose genome consists of 10 double-stranded RNA segments surrounded by protein shells constituting the virus outer capsid and core (Antar et al. 2009). Oral administration of the reovirus Serotype 3 Dearing (T3D) into newly born mice leads to encephalitis, as the virus travels from GI to the CNS (Antar et al. 2009). The primary site of infection of mammalian reovirus is the lungs; however, the virus is found in other organs such as heart, spleen, gastrointestinal tract and brain as the pathogen causes systemic infection (Morrison et al. 1991). ReoT3D virus infection model is a well-established infection model used by researchers. The reoT3D virus is a neurotropic virus and is categorized as Biosafety Level 2. Furthermore, as opposed to pathogen mimics, which investigators have recently used in determining *Lrrk2* function, reoT3D virus is physiologically relevant to humans, as mentioned earlier. Accordingly, we utilized reoT3D virus for *in vivo* (as well as some *in vitro*) studies of our *Lrrk2* mouse models to explore the immunologic role of *Lrrk2*.

T3D reovirus causes cell death by apoptosis, necrosis, and autophagy (Gong & Mita 2014). Reovirus utilizes mitochondrial and death receptor (such as TNF superfamily of death receptors) mediated pathways to activate apoptotic cell death in the host (Clarke et al. 2005)(Tyler et al. 2001).

Apoptosis is an important mechanism of T3D reovirus-mediated infection of the host (Clarke et al. 2005). Reovirus-induced apoptosis is associated with the viral $\mu 1$ protein (Danthi et al. 2008). Literature also indicates a role for viral $\sigma 1$ s in the induction of apoptosis and cell cycle arrest at the G2/M boundary (Boehme et al. 2013). Following attachment with host cell membrane, the virus is thought to internalize by clathrin-mediated endocytosis (Danthi et al. 2010). The viral $\sigma 3$ protein is then cleaved from the virus outer capsid by acid-

sensitive cysteine proteases present within the endocytotic vesicles. After this initial disassembly, the virus membrane-penetration protein, called $\mu 1C$, punctures the endocytotic membrane facilitating the extrusion of the viral core into the cytosol. In this process, viral $\mu 1$ is further cleaved releasing the ϕ fragment to the cytosol as well. The viral particles then activate NF- κ B signaling to induce apoptosis (Danthi et al. 2010). In agreement with this, inhibition of endocytosis pathway or NF- κ B signaling by experimental means is shown to hinder virus-induced apoptosis (Danthi et al. 2010). Furthermore, phagocytosis of T3D reovirus is observed in L cells, and viral particles are found in lysosomal structures being downstream of the phagocytic pathway (Silverstein & Dales 1968). Related to LRRK2, recent reports denote lysozyme sorting, pathogen phagocytosis, NF κ B signaling and apoptosis to be all associated with LRRK2 function as well (Rocha et al. 2015; Gardet et al. 2010).

Reovirus exploits Ras signaling through Ras/p38, in a strain-dependent manner, and inhibition of PKR activity (Clarke et al. 2005). PKR is activated in response to viral RNA, and Ras signaling is implicated in the antiviral activity of PKR. Phosphorylation of PKR is associated with its active form that in turn mediates eIF-2 α phosphorylation and activation (Norman & Lee 2000). Active eIF-2 α prevents translation of viral transcript to abrogate infection. Reovirus-induced activation of Ras signaling hinders phosphorylation of PKR thereby facilitating the synthesis of viral proteins (Norman & Lee 2000). In contrast, other published data indicates that PKR can enhance reovirus infection in cultured cells (Smith et al. 2006). Thus, there may be positive and negative aspects to PKR effects on reovirus replication. Related to LRRK2, regulation and function of this protein involve kinases such as PKA and κ B kinases IKK α and IKK β with roles in neuronal development and immunity

respectively (Russo et al. 2015; Dzamko et al. 2012). In addition, ribosomal s15 is reported as a physiologic substrate of LRRK2 kinase function (Martin et al. 2014). Increased protein synthesis mediated by s15 is thought to lead to G2019S LRRK2 neurotoxicity.

Similar to TLRs, the host cytoplasmic RIG-I, MDA5 and IPS-1 are involved in sensing reoviral RNA and subsequent phosphorylation of IRF3 and its nuclear translocation to induce transcription of IFN-related genes (Sherry 2009). MDA5 is thought to activate NF κ B pathway in the process as well. Depending on the virus strain, reoviruses use distinct mechanisms to subvert the host's Type I interferon response. Binding of reovirus σ 3 protein to its double-stranded RNA hinders the activation of host's PKR which is involved in the antiviral response. The virus then uses the protein synthesis machinery to translate its mRNA and propagate in the host (Sherry 2009).

IFN- α and β stimulate the PKR activity (Replication & Chiocca 2002). Activation of Ras signaling is associated with suppression of IFN- β response in transformed cells (Shmulevitz et al. 2010). Reovirus triggers distinct signaling through MAPK such as SAPK/JNK and downstream c-Jun transcriptional activity (Clarke et al. 2005). Increased phosphorylation and activity of c-Jun is paralleled with increased apoptotic signaling in virus infection (Clarke et al. 2001). In addition, ERK, but not p38, activity is reported in T3D reovirus infection of HEK293 cells (Clarke et al. 2005). Ras signaling inhibits RIG-I function through MEK/ERK pathway, which ultimately results in a deficient IFN- β response by the cells (Shmulevitz et al. 2010). Related to LRRK2, ERK5 positively regulates LRRK2 in IFN- γ induction of LRRK2 expression (Kuss et al. 2014).

In agreement with the utilization of apoptotic pathways by reovirus, anti-apoptotic measures, such as genetic deletion of caspase 3 in mice, attenuate reovirus infection and damage in the host. Furthermore, T3D reovirus capacity to induce apoptosis has also been studied as a therapeutic tool, oncolysis, for cancer cell growth and tumors in humans; this research has now extended to at least 32 clinical trials (Clarke et al. 2005; Gong & Mita 2014). Interestingly, T3D reovirus has a distinct preference for growth and replication in transformed cells as opposed to non-transformed normal cells (Gong & Mita 2014). This property further makes T3D reovirus an attractive therapeutic agent that preferentially target cancerous cells.

Furthermore, functional microtubules in the host cells are involved in normal replication of the virus (Mainou et al. 2013) but not required. There is no effect on virus yield by colchicine or other drug treatments that totally blocks microtubule formation – inclusion bodies are not formed, but the yield is unaffected (Dales 1963; Spendlove et al. 1963). Reovirus is found in the proximity of microtubule structures and microtubule motor dynein 1. Related to LRRK2, multiple reports indicate ROC GTPase domain of LRRK2 to interact with tubulin molecules and to regulate actin and microtubule dynamics. In vitro studies have shown LRRK2 phosphorylation of tubulin and regulation of cell motility (Parisiadou & Cai 2010).

1.7.2.2 ReoT3D Virus Receptors

The mechanism by which T3D reovirus invades the nervous system is not yet defined. Cell surface sialic acid is reported to act as a coreceptor for reovirus facilitating its entry into the cell. Reovirus σ 1 protein binds with sialic acid and enhances cell death as a result of infection (Connolly et al. 2001). The hematogenous spread of reovirus is facilitated by

junctional adhesion molecule A (JAM-A). JAM-A belongs to immunoglobulin family of proteins and is expressed as a cell-surface receptor on blood cells. After reovirus entry into the host's cell, JAM-A β 1 integrin promotes the virus toward endocytic signaling pathway (Maginnis et al. 2006). Reovirus invasion triggers NF- κ B signaling in neurons.

Interestingly, however, incubation of T3D reovirus in JAM-A knockout mice still produces systemic infection meaning alternate mode(s) of viral entry exists in neurons, which are not known yet (Antar et al. 2009). Accordingly, Konopka-Anstadt and colleagues report NgR1 receptor as a determinant of T3D reovirus infection in CNS as found by a whole-genome siRNA screen (Konopka-Anstadt et al. 2014).

NgR1 is the second known protein receptor for reovirus (Konopka-Anstadt et al. 2014). The exact mechanism of action of NgR1 is unknown. NgR1 is comprised of 8 LRR domains and has a glycosylphosphatidylinositol (GPI)-linkage on its C-terminus anchoring it to the cell membrane. LRR proteins, such as different TLRs, exert a role in pathogen sensing. Likewise, the authors speculate that NgR1 might serve a similar role in reovirus infection. In contrast to JAM-A receptor, NgR1 receptor does not have a transmembrane integrin and thus for effective downstream signaling it depends on recruitment and function of other cellular receptors/components (Konopka-Anstadt et al. 2014). It is possible for LRRK2 to play a role at this junction since mutations in LRRK2 modify reovirus disease outcomes based on our cumulative data.

NgR1 is expressed on the axonal surface of mature neurons in the cerebral cortex, thalamus, CA3 regions of the hippocampus and cerebral Purkinje cells (Antar et al. 2009). Immunoglobulin, LRR-containing proteins and Nogo-A family of proteins are some ligands of the NgR1 receptor. Nogo (reticulon-4) protein in humans is expressed in neurons and exerts

an inhibitory function on neuritic growth. NgR1 signaling influences axonal growth during neuronal maturation (Hu & Strittmatter 2004). Sequestering NgR1 ligands or obstructing the receptor itself has been associated with neurite regrowth and extension (Fournier et al. 2002). Related to this, LRRK2 mutations are reported to induce neuritic shortening and growth abnormalities (Sepulveda et al. 2013). LRRK2 interacts with cytoskeletal proteins and is thought to modify actin dynamics (Gandhi et al. 2008).

CNS NgR1 expression is low at birth, but increases with aging; likewise, myelination and the myelin-linked ligands increase developmentally (Wang et al. 2002). Reovirus has a greater propensity for unmyelinated axons, which are more abundant in newborn mice (Wang et al. 2002). Maturation of CNS causes diversification of the cellular environment and an increase in the number and types of myelin-associated ligands, which collectively may outcompete reovirus for the NgR1 receptor. As such encephalitis does not manifest in adult mice while it does in newborn mice; this may partly explain the age-dependent effect of reovirus infectivity (Konopka-Anstadt et al. 2014).

Overall, literature reviews suggest that NgR1 is a tissue-specific receptor in CNS whose pattern of expression in the brain strikingly matches the pattern of T3D reovirus infection as observed by histology data (Antar et al. 2009; Wang et al. 2002). Reports indicate that intracranial injection of reovirus T3D in mice leads to injury in certain regions of the brain namely hippocampus, thalamus, and cortex (Clarke et al. 2009). It is worth mentioning that there is ongoing research looking at tropism in an intracranial injection model, which infects different regions than the respiratory infection model, i.e., prominence in the hippocampus in the former but not in the latter. Factors responsible for reovirus neurotropism and distinct pattern of brain infection are not elucidated. Konopka-Anstadt et al. postulated that

NgR1 is a neural pathogen recognition receptor and thus a determinant of T3D reovirus viral pathogenesis. The results obtained by Konopka-Anstadt team shows that T3D reovirus uses distinct viral components in binding to different host cell receptors. The ability of the reovirus to exploit its various viral components in interacting with various host factors may allow its successful internalization and dissemination across multiple tissue types (Konopka-Anstadt et al. 2014).

OBJECTIVES AND HYPOTHESES

An interaction between environmental and genetic factors is implicated in PD pathogenesis reviewed in: (Schlossmacher et al. 2017). Analysis of *post mortem* PD brains have indicated an influx of leukocytes and the recruitment and activation of microglia (Sanchez-Guajardo et al. 2013). Recent GWAS studies have found LRRK2 variants as a genetic risk factor for leprosy and Crohn's disease, both of which are immune diseases. (Zhang et al. 2009; Barrett et al. 2008) Given the low expression of LRRK2 in brainstem neurons and the low penetrance of *LRRK2* mutations in developing a PD phenotype (Marder et al. 2015), we investigated a role for LRRK2 outside the central nervous system. Concurrent with our findings, recent evidence from various teams suggest a role for LRRK2 in the immune system.

For example, recent reports indicate that LRRK2 has a pro-inflammatory role in microglia because inhibition of LRRK2 led to protection via a reduced inflammatory response after LPS treatment (Puccini et al. 2015; Moehle et al. 2012; Daher et al. 2014). In support of an immunological role for LRRK2, Schapansky et al. have also demonstrated increased *Lrrk2* phosphorylation and dimerization following immune activation of monocytes (Schapansky et al. 2014). LRRK2 is a target of IFN- γ and involved in activation of NF- κ B (Gardet et al. 2010). LRRK2 negatively regulates NFAT activity, which is involved in immune signaling of dendritic cells, macrophages, and neutrophils (Liu et al. 2011; Liu & Lenardo 2012; Hogan et al. 2003). Hence, we focused on LRRK2's immune functions.

SUMMARY OF RATIONALE

We posit that environmental factors, e.g., xenobiotic pathogens, may influence the expression of a mutant *LRRK2*-linked phenotype in carriers. Following the characterization

LRRK2 expression in various human tissues and organs. Next, I focused on identifying leukocyte subtypes of peripheral human blood and mouse immune organs that express *Lrrk2* under physiological conditions. Furthermore, we investigated *Lrrk2* expression in various inflammatory paradigms in mouse monocytes / macrophages. The second objective of the thesis was to determine whether *Lrrk2* deficiency alters the innate immune response in mice following a systemic reovirus infection. Lastly, the third objective of the thesis was to determine whether the Parkinson's-linked G2019S mutation in *Lrrk2* affects the host's immune response(s) following invasion by a neurotropic virus.

RESEARCH HYPOTHESES

We hypothesize that LRRK2 is expressed in leukocytes and upregulated by microbial stimulation (relating to the first objective). LRRK2 has a pathogen-sensing role akin to NLR-type proteins that aids in the clearance of virulent xenobiotics, and its absence will make *Lrrk2*-deficient mice more susceptible to reovirus infection (relating to the second objective). The G2019S *Lrrk2* knock-in mutation in mice enhances the immune response to a systemic reovirus infection and thus alters the disease outcome of a systemic viral infection (relating to the third objective).

CHAPTER II. MATERIALS AND METHODS

2.1 Human Specimen Collection and Ethics Approval

Phlebotomy was performed using the cubital vein of healthy adults (n=10) to collect 20 cc of peripheral blood in EDTA vacutainers (Becton Dickinson and Company, Franklin Lakes, NJ). Blood collection was conducted at The Ottawa Hospital (Ottawa, ON). Immortalized, human lymphoblasts were obtained from Epstein-Barr Virus (EBV)-transformation of specimens collected from PD and healthy control subjects. EBV lymphoblasts from PD

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Table 3.1.1. Specifications of human specimens used for histological experiments.

Case ID	Tissue/organ	Diagnosis	Age (year)	Gender	Source
CA195C	parietal lobe; brain	HIV	N/A	N/A	Wimar Dumaop; CNTN
CA195N	pons; brain	HIV	N/A	N/A	Wimar Dumaop; CNTN
CA246A	frontal lobe; brain	HIV	N/A	N/A	Wimar Dumaop; CNTN
CA246M	substantia nigra; brain	HIV	N/A	N/A	Wimar Dumaop; CNTN
CE147C	parietal lobe; brain	HIV	N/A	N/A	Wimar Dumaop; CNTN
CE147M	substantia nigra; brain	HIV	N/A	N/A	Wimar Dumaop; CNTN
CA308C	parietal lobe; brain	HIV control	N/A	N/A	Wimar Dumaop; CNTN
CA308M	substantia nigra; brain	HIV control	N/A	N/A	Wimar Dumaop; CNTN
CC144B	frontal lobe; brain	HIV control	N/A	N/A	Wimar Dumaop; CNTN
CC144M	substantia nigra; brain	HIV control	N/A	N/A	Wimar Dumaop; CNTN
CC202C	parietal lobe; brain	HIV control	N/A	N/A	Wimar Dumaop; CNTN
CC202M	substantia nigra; brain	HIV control	N/A	N/A	Wimar Dumaop; CNTN
AU09_0063_P	Spleen	N/A	73	M	Dr. J. Woulfe; OHRI
AU09_0084_I	spleen	N/A	55	M	Dr. J. Woulfe; OHRI
AU09_0089_J	spleen	N/A	38	M	Dr. J. Woulfe; OHRI
S10_13147_1A	gut	N/A	90	F	Dr. J. Woulfe; OHRI
S10_13147_2A	gut (appendix ?)	N/A	90	F	Dr. J. Woulfe; OHRI
S10-14494-1J	gut	N/A	N/A	N/A	Dr. J. Woulfe; OHRI
S10_13147_1Q	lymph nodes	N/A	90	F	Dr. J. Woulfe; OHRI
S10_13147_10	lymph nodes	N/A	90	F	Dr. J. Woulfe; OHRI
S10_13147_1P	lymph nodes	N/A	90	F	Dr. J. Woulfe; OHRI
S10_13144_1P	lymph nodes	N/A	82	F	Dr. J. Woulfe; OHRI
Au_09_0129_I	lung	N/A	85	F	Dr. J. Woulfe; OHRI
Au_09_0129_M	lung	N/A	85	F	Dr. J. Woulfe; OHRI
AU_11_000_25-1O	lung	N/A	86	M	Dr. J. Woulfe; OHRI
AU_11_000_25-1D	kidney	N/A	86	M	Dr. J. Woulfe; OHRI
Au_09_0131-E	kidney	N/A	80	F	Dr. J. Woulfe; OHRI
Au_09_0131-F	kidney (?)	N/A	80	F	Dr. J. Woulfe; OHRI

Case ID	Tissue/organ	Diagnosis	Age (year)	Gender	Source
AU-11-000043	brain	numerous cerebral metastases, hypoxic-ischemic changes, incidental Lewy body disease	72	F	Dr. J. Woulfe; OHRI
CP-12-000023	brain	infarcts, hepatic encephalopathy	75	M	Dr. J. Woulfe; OHRI
CP-12-000034	brain	multiple cerebral infarcts	64	M	Dr. J. Woulfe; OHRI
NP-11-000001	brain	Parkinson's disease/dementia with Lewy bodies	81	F	Dr. J. Woulfe; OHRI
NP-11-000067	brain	incidental Lewy body disease	90	F	Dr. J. Woulfe; OHRI
NP-12-000002	brain	infarct, left frontal lobe	64	M	Dr. J. Woulfe; OHRI
NP-12-000013	brain	metastatic melanoma	73	F	Dr. J. Woulfe; OHRI
NP-12-000014	brain	corticobasal degeneration	72	F	Dr. J. Woulfe; OHRI
NP-12-000053	brain	Parkinson's disease/dementia with Lewy bodies	88	M	Dr. J. Woulfe; OHRI
NP-12-000060	brain	dementia with Lewy bodies	86	F	Dr. J. Woulfe; OHRI
FN-12-000080, 1R-11	brain	idiopathic PD	32	M	Dr. J. Woulfe; OHRI
96.225, 32	brain	acute encephalitis - rabies infected	N/A	N/A	Dr. J. Woulfe; OHRI
X2274	brain	A53T-SNCA linked PD	N/A	N/A	Dr. J. Woulfe; OHRI
NP-11-000036, 1A-13	midbrain	progressive supranuclear palsy (PSP)	73	F	Dr. J. Woulfe; OHRI
NP-11-000036, IN-8	striatum	progressive supranuclear palsy (PSP)	73	F	Dr. J. Woulfe; OHRI
SP-12-003421, 1A-3	olfactory epithelium	healthy/control (?)	50	M	Dr. J. Woulfe; OHRI
A99 331.15	brain	N/A	N/A	N/A	Dr. M Schlossmacher
A97 320.3	enteric nervous system	PD	N/A	N/A	Dr. M Schlossmacher
A0-110-10	brain	N/A	N/A	N/A	Dr. M Schlossmacher
BN06-04, A20	brain	N/A	N/A	N/A	Dr. M Schlossmacher
A01-39, 1	brain	N/A	N/A	N/A	Dr. M Schlossmacher
SG2	enteric nervous system	N/A	N/A	N/A	Dr. M Schlossmacher

Case ID	Tissue/organ	Diagnosis	Age (year)	Gender	Source
BN343-116	midbrain	PD	N/A	N/A	Walter J. Schulz-Schaeffer
BN379-25	olfactory epithelium	PD	N/A	N/A	Walter J. Schulz-Schaeffer
BN379-61	gut	PD	N/A	N/A	Walter J. Schulz-Schaeffer
BN343-40	gut	PD	N/A	N/A	Walter J. Schulz-Schaeffer
BN343-34	pancreas	PD	N/A	N/A	Walter J. Schulz-Schaeffer
BN399-11	olfactory epithelium	PD	N/A	N/A	Walter J. Schulz-Schaeffer
NP-10-000092, 1O-34	midbrain	vasculitis	61	F	Dr. J Michaud; OHRI
NP-10-000092, 1W-3	nerve root	vasculitis	61	F	Dr. J. Michaud; OHRI
91-5160; Derm	skin	Leprosy	47	M	Dr. J Michaud; OHRI
6.3143	skin lesion	Leprosy Lepromatous (treated with BAAR auZiehl)	N/A	N/A	Dr. J. Michaud; OHRI
7.2102	skin lesion	Leprosy Borderline	N/A	N/A	Dr. J Michaud; OHRI
12.3732	skin lesion	Leprosy Tuberculoid	N/A	N/A	Dr. J. Michaud; OHRI

2.2 Animal Research Ethics

Approval for all animal experimentation was obtained from the University of Ottawa Animal Care Committee. The research was conducted in accordance with approved Protocols: NSI-74 and NSI-84.

2.3 Animal models for LRRK2 mutations

Transgenic *Drosophila melanogaster* expressing human, wild-type *LRRK2* cDNA were generated and characterized by (Venderova et al. 2009). Transgenic flies expressed wild type human LRRK2 in the retina. Male transgenic flies were used as positive controls for the presence of LRRK2 in WB experiments while their non-transgenic male littermates were used as negative controls in related experiments.

The *Lrrk2* deficient mouse model was generated and characterized by (Tong et al. 2010), and kindly provided by Dr. Jie Shen. *Lrrk2* deficient mice were used as negative control in related experiments. Briefly, Tong et al. generated the *Lrrk2* KO mice by targeting the promoter and exon 1 of *Lrrk2* in their strategy (Tong et al. 2010). Their linearized targeting vector contained PGK-neo cassette, which was then transfected into MKV6.5 ES cells by electroporation technique. The ES cells were harvested from F1 hybrid of 129 and B6 background mice. Following transfection, ES cells with desired homologous recombination were selected, and the presence of neo was confirmed in them by Southern blots. The resultant F1 and F2 generations from this genetic modification were tested for the targeted allele. The homozygous F2 mice carrying PGK-neo cassette were mated with CaMKII-cre transgenic mice to complete generation of the KO mice. In this final crossing of the mice, cre facilitated removal of the floxed PGK-neo cassette from the homozygous *Lrrk2*

KO mice. The absence of *Lrrk2* mRNA and protein, in these animals, were validated in Northern and Western blots respectively.

R1441C and G2019S *Lrrk2* genocopy the commonest disease variants of *LRRK2* in humans. We were provided with R1441C *Lrrk2* KI mouse model from our collaborator, Dr. Jie Shen, who created the animals and characterized them in (Tong et al. 2009). In generating the R1441C *Lrrk2* KI mice, Tong and colleague initially amplified a 1.7 kb genomic DNA of B6 mice which contained exon 31 (and some of intron 31) of the *Lrrk2* gene. The R1441C mutation was accomplished in the amplicon by site-directed mutagenesis. This new DNA segment was then introduced to MKV6.5 ES cells (from the B6/129 background) via electroporation. Homologous recombination led to the integration of the desired DNA fragment, which was in turn determined by the presence of the neo marker in Southern blots. The PGK-neo-cassette was subsequently removed from the cells by transfection with Cre plasmids. Injection of the resultant ES cells into mouse blastocysts led to the development of chimeric embryos. The resultant offspring were then crossed with F1 mice of the same background.

The G2019S *Lrrk2* mouse model was generated by Novartis Pharma, characterized by (Herzig et al. 2011) and provided to us via an MTA. Genomic DNA from Balb/c mouse was isolated and used to amplify the mouse *LRRK2* containing exon 41. The mutation of G2019S in *Lrrk2* was introduced in exon 41 of the mouse *Lrrk2* by site-directed mutagenesis. The modified gene was cloned to generate the final *Lrrk2* plasmid using restriction enzymes. Mouse ES cells were transfected with the *Lrrk2* plasmid using electroporation. Homologous recombination led to the integration of the modified *Lrrk2* DNA into the ES cells, which exhibited neomycin resistance. Subsequent selection of cells with neomycin resistance was

performed to excise the neomycin cassette from the cells, which integrated the modified DNA. The ES cells with G2019S *Lrrk2* expression were then injected into blastocysts from G57Bl/6J mouse background. Next, the blastocysts were implanted in B6CF1 females to develop the pregnancy and give birth to chimeric offspring. The mice with G2019S *Lrrk2* were backcrossed with G57Bl/6J mice to generate pure background.

2.4 Leukocytes Enrichment and Sorting

Human peripheral blood mononuclear cells (PBMC) were separated from venous blood collections of healthy donors using Ficoll gradient (Sigma-Aldrich, St Louis, MO). Ficoll is a hydrophilic polysaccharide that allows separation of different blood components upon centrifugation. Red blood cells and neutrophils were also removed from the PBMC layer of blood/Ficoll gradient using this method, Next; PBMCs were washed in PBS and centrifuged at 250 x g for 10 min, followed by aspiration. For ICC/IHC experiments, PBMCs were resuspended in plasma, saved from initial blood separation steps, and were smeared onto slides. For leukocyte sorting, the PBMCs were resuspended in buffer (PBS, 0.5% BSA, and 2 mM EDTA, pH=7.2) and allowed to incubate with Microbeads (Miltenyi Biotec Inc., Auburn, CA) that magnetically label various cell surface markers. Next, MACS® Cell Separator (Miltenyi Biotec Inc., Auburn, CA) was employed to separate different leukocyte subtypes based on the Microbeads labeling (CD14+ monocytes, CD19+ B-cells, and CD4+ as well as CD8+ T-cells). Following fixation and permeabilization, the isolated cells were stained with LRRK2 antibody and processed further for flow cytometry. FACS protocols were adapted from (Vranjkovic et al. 2011).

Select mouse organs were harvested from 13-week old wild-type C57BL/6 mice. Tissue homogenates were made and passed through a 70 µm nylon mesh cell strainer to produce single cell suspensions. Following plating, the cells were probed for various cell-surface leukocyte markers as well as intracellular LRRK2 protein as described in (Hakimi et al. 2011). Anti-mouse antibodies CD11b (PE) (macrophage marker), CD45R (B220) APC-eFluor® 780 (B-lymphocyte marker), TCRβ APC-eFluor® 780 (T-lymphocyte marker), Ly-6G (Gr-1) Cy5 (granulocyte marker) and CD11c PE-Cy7(dendritic cell marker) were utilized for cell type sorting. Lrrk2 was probed using anti-LRRK2 antibody (MJFF-1 clone, C5-8 antibody) which interacted with the secondary antibody anti-rabbit IgG (H+L) Alexa Fluor® 488. The samples were processed by flow cytometry at the University of Toronto (Toronto, ON).

2.5 Bone Marrow-Derived Macrophage Cultures and Stimulation

Procedures for harvesting BMDM were described in Hakimi M et al., 2011. Briefly, bone marrow cells were flushed from femur and tibia bones of mice from different genotypes. The cells were plated in complete growth medium (RPMI 1640 medium, 10% fetal calf serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 100 U/ml penicillin and 100 µg/ml streptomycin antibiotics) and 30% L929 supernatant. The cell line L929 produces M-CSF, which is used for maturation of the bone marrow cells into macrophages. The cells were incubated at 5% CO₂ and 37°C with their medium being replenished on day 3 and 7. BMDM were stimulated on day 7 with the following agonists (Invivogen, San Diego, CA): 50 µg/ml C12-iE-DAP, 50 µg/ml L18-MDP, 10 µg/ml MDP, 10 µg/ml FK156, 50 µg/ml rapamycin, 100 ng/ml LPS, 5mM ATP, 1-15 µM nigericin, 1 µM CpG and heat-killed listeria (8×10^8 CFU/ml, MOI 25). Heat killed listeria were obtained from Laboratory of Dr. Dana

Philpott as described in (Travassos et al. 2004). BMDM stimulation continued for 16 hours, after which cells supernatants were aliquoted and stored at -20°C for next experiments. The cells supernatant were analyzed using ELISA techniques for determining their cyto- and chemokine profile.

In experiments pertaining to the measurement of *Lrrk2* mRNA in mouse BMDM, some modifications were made to the cell culture and stimulation. The BMDM cultures were established using 10n/ml GM-CSF in the medium and the stimulation continued for 5 hours on day 7. The stimulants (InvivoGen, San Diego, CA) targeted various TLR ligands and were as following: 5 µg/ml Pam3CSK4, 10 µg/ml Poly(I:C), 1 µg/ml LPS, 4 µg/ml imiquimod (R837), and 10µg/ml CpG.

2.6 Antibodies

Rabbit polyclonal antibodies to LRRK2 and LC3 were obtained from Novus Biol (Littleton, CO). The WB concentration used was 1:3000 for both antibodies. Rabbit monoclonal antibodies to *Lrrk2* (5 clones: MJFF-1 to -5) were obtained from the Michael J. Fox Foundation (New York, NY). The working concentration of these antibodies was 1:1000 for WB and 1:200 for FACS. An affinity-purified rabbit polyclonal anti-human *Lrrk2* antibody ("HL-2") was prepared at OPEN Biosystems Inc (Huntsville, AL.) against residues 2508-2527 and used at a concentration of 1:3000 for WB and 1:300-1:1000 for IHC. The antibody specificity was validated are reported in JNT, 2011. LRRK2 antigen peptide for the HL-2 antibody was obtained from the laboratory of Dr. Mathew LaVoie (Boston, MA). Mouse monoclonal antibodies anti-GBA1 (WB working concentration 1:1500) and anti-β-actin (WB working concentration 1:5000) antibodies were purchased from Abcam (Cambridge, MA).

Anti-CD4+, CD8+, CD14+ and CD19+ antibodies that were bound to microbeads were obtained from Miltenyi Biotec (Auburn, CA); they were used for cell sorting and FACS based on the company's protocols. Human anti-CD3, CD8, CD20, CD68 and MPO antibodies were obtained from Pathology Department of University of Ottawa (Ottawa, ON) Anti-Iba1 antibody was purchased from Wako Pure Chemical Industries, Ltd. (Richmond, VA) and was used at 1:250 concentration in IHC. Syn-1 antibody was used for detection of α -synuclein. Antibody against reovirus T3D was affinity-purified from polyclonal rabbit sera (Gauvin et al. 2013) and provided by Dr. Earl Brown (Ottawa, ON). Each of these antibodies was used at 1:5,000 concentration in IHC experiments. Biotinylated secondary anti-rabbit, -mouse and -goat antibodies were purchased from Vector Laboratories (Vector Laboratories Inc., Burlingame, CA) and used at 1:225 concentration for IHC protocols.

2.7 Reverse transcription and qPCR of mRNA

Human EBV-transformed lymphoblasts were used as positive control for *LRRK2* in related experiments. The presence of *LRRK2* was first verified in the lymphoblasts, from healthy donors, by isolating mRNA using Trizol Reagent (Invitrogen, Carlsbad, CA) and manufacturer's instructions. The extracted mRNA was next reverse transcribed, and PCR amplified using distinct primers for *LRRK2*. *GAPDH* and *18S* mRNAs were used as internal controls in the PCR experiment. Reverse transcription of the extracted mRNA was carried out using Superscript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). Briefly, sample RNA was added to DNase I in RNase free water and heated at 37°C for 20 min. The temperature was raised to 70°C and maintained for 10 min proceeded by addition of random hexamers. The reaction was maintained at 70°C for another 5 min following by a

quick chill on ice. Next, 5X First-Strand Buffer (from the kit) along with dNTP and SuperScript II RT were added to the reaction tube and mixed thoroughly. The reaction tube was kept at 25°C for 10 min then heated to 42°C for 50 min. Finally the reaction was inactivated by heating the samples at 95°C for 5 minutes. The products were saved for processing by PCR and gel electrophoresis as explained in the next section.

Cultures of bone-marrow derived macrophages were established from wild-type mice; their mRNA was next extracted by the RNeasy MiniKit purchased from Qiagen, MA. Mouse *Irrk2* was probed using the following primers: Forward-5'-TCTGGCTGGAACCCTGCTAT-3' and Reverse-5'-AACTGGCCATCTTCA TCTCC-3'. mRNA from experimental and unstimulated samples were quantified using the $\Delta\Delta CT$ method; 18S RNA was used as a reference.

2.8 Cytokine and Chemokine Measurement By ELISA

ELISAs were conducted to measure the levels of cyto- and chemokines released from cultured BMDM, of different genotypes and treatments, into the cells media. ELISA kits from R & D Systems (Minneapolis, MN) were utilized, and the experimental protocols followed the manufacturer's instructions. Briefly, after 16-hour treatment of BMDM with inflammatory stimuli, the conditioned media were collected in tubes. The 96-well ELISA plates were coated with the accompanying cyto-/chemokine antibodies at 4°C overnight. Serial dilutions (1:2) and replicates of each conditioned media sample and the kit standards were made using the kit diluent. All samples and standards were incubated in the coated plates for 2 hours at room temperature followed by aspiration and washes. Conjugates were added to the plate and incubated for 2 hours at room temperature followed by aspiration and washes. The Substrate Solution was dispensed into the plate and allowed to

incubate for 30 min at dark. Following that, Stop Solution was added to each well, and the results were read using spectrophotometer at 450 nm. ELISA protocols used for determination of α -synuclein were previously described in (Mollenhauer et al. 2008; Cullen et al. 2009; Cullen et al. 2011).

2.9 Cytokine and Chemokine Measurement By Multiplex

Mouse brain and lung homogenates were prepared in PBS as described in Tissue homogenization and plaque assay section. Tissue homogenates were next centrifuged at 13000 rpm for 10 min at 4°C. 75 μ l of each sample supernatant was transferred into a new tube and was shipped on dry ice to Eve Technologies (Calgary, Alberta, Canada) as per company's instruction. The multiplex assay to measure the cytokine and chemokine of various mouse tissues was performed by the company that utilizes Multiplexing LASER Bead Technology and robotic and automated systems improving the consistency of the results. Prior to the assay, samples were filtered through a Durapore 1.2 μ m filter then a Durapore 0.22 μ m filter. Each analyte is conjugated to distinct color fluorescent-bead. Bio-Plex 200 is used as the bead analyzer, which detects and measures various analyte levels using its flow cytometry and dual-laser systems. Analyte quantification is facilitated using the company standard curves.

2.10 Genotyping

2.10.1 Crude DNA Extraction

Crude DNA extraction was carried out using a small piece of mouse tail or ear digested in a buffer containing 100mM NaCl, 10mM Tris-HCl pH=8, 25mM EDTA pH=8, 0.5%SDS and 200 µg/ml proteinase K. Each animal sample was digested in 20 µl of the buffer and incubated at 55°C for 2.5 hours. The digested samples were then diluted with 180 µl distilled water, mixed by vortex and heated at 95°C for 10 minutes to inactivate proteinase K. Subsequent centrifugation of the samples, using a counter-top centrifuge at top speed, yielded crude DNA in the supernatant.

2.10.2 PCR and Gel Electrophoresis

Polymerase chain reaction was then performed using 0.5 µl of the crude genomic DNA, 0.5 µl of each required primer (i.e. forward and reverse), 5 µl of GoTaq Green Master Mix (Promega Corporation, Madison, WI) and 3.5 µl of nuclease-free water to make up a total reaction volume of 10 µl. The PCR reaction was then performed using the cycling conditions specified for the genotyping protocol explained below. The gel electrophoresis was run using 5-8 µl of each reaction product on a 1.5-2% agarose gel. The gel was prepared in advance and contained agarose in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) and 0.5 µg/ml ethidium bromide for DNA visualization. The electrophoresis was conducted at 110V for about 20 minutes or until appropriate band separations were made. The PCR products were then visualized under UV illumination, and their corresponding sizes were assessed using 100bp DNA Ladder-RTU (GENEDIREX, Las Vegas City, NV).

For genotyping the R1441C *Lrrk2* mouse colony, the following primer set was used: forward primer 5'-CTGCAGGCTACTAGATGGTCAAGGT-3' and reverse primer 5'-CTAGATAGGACCGAGTGTCTGCAGAG-3'. The PCR cycling condition was 94°C for 5 min followed by 35 cycles of 94°C for 30 s; 66°C for 30 s; 72°C for 1 min, followed by 72°C for 10 min in the thermal cycler. The expected product lengths were 386 bp for wild-type *Lrrk2* and 545 bp for R1441C *Lrrk2*. A wild-type mouse sample showed a single band at 386 bp, and a homozygous R1441C *Lrrk2* mouse sample showed a single band at 545 bp while a heterozygous R1441C *Lrrk2* mouse sample contained both 386 bp and 545 bp bands.

For genotyping the *Lrrk2* KO mouse colony, the following primer set was used: mF80 (WT forward primer): 5'-GGCTCTGAAGAAGTTGATAGTCAGGCTG-3', neoLKF1 (KO forward primer): 5'-CCCAAGCTAGCTTTATCGATAAGCTTGA-3' and mR52 (common reverse primer): 5'-CTGTACACTGGCAACTCTCATGTAGGAG-3'. The PCR cycling condition was 95°C for 5 min, followed by 40 cycles of 95°C for 40 s, 66°C for 30 s, 72°C for 1 min and 30 s and a final 10 min incubation at 72°C in the thermal cycler. Expected products were a single 375 bp band for wild type, a single 253 bp band for KO and both 375 bp and 253 bp bands for the homozygous *Lrrk2* KO mice.

For genotyping the G2019S LRRK2 mouse colony, the following primer set was used: *Lrrk2.for.2lox* (forward primer) 5'-GCTGCAGGATGGGAATAAAG-3' and *Lrrk2.lox.rev* (reverse primer) 5'-TGGGGGGAAAATCAGCTTTC-3'. The PCR cycling condition was 95°C for 5 min, followed by 35 rounds of incubation at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. A final 10 min incubation at 72°C was followed. The PCR products were 300 bp for WT, 468 bp for homozygous G2019S *Lrrk2*. The heterozygous G2019S *Lrrk2* sample showed both 300 bp and 468 bp bands.

The genotyping protocol for determination of mouse sex was adapted from (Clapcote & Roder 2005). The primers were designed using specific genes of X and Y chromosomes. Primer sequences were: forward primer 5'-CTGAAGCTTTTGGCTTTGAG-3' and reverse primer 5'-CCACTGCCAAATTCTTTGG-3'. PCR was carried out at 94°C for 5 min, 35 rounds of incubation were conducted next with the following setting: 94°C for 20 s, 54°C for 1 min, and 72°C for 40 s. A final incubation at 72°C was next carried out for 10 min. A female sample showed a 331 bp band while a male sample showed the 331 bp band as well as a 302 bp band.

RT-PCR was conducted on human EBV-lymphoblast mRNA to verify the presence of *LRRK2*. PCR was performed on the reverse transcribed products using primers designed for different regions of *LRRK2* as described in (Hakimi et al. 2011). The primer sequences were as follows: set 1: Forward-5'-AGGTGGGTTGGTCACTTCTG-3', Reverse-5'-ATGAGTGCATGGCATCAAAA-3'; set 2: Forward-5'-AGATGCCAATCAAGCAAAGG-3', Reverse-5'-AAGGACCAAGCCAAGAAGGT-3'; and set 3: Forward-5'-TTCTTCCCCTGTGATTCTCG-3', Reverse-5'-GCTGTCCAACAACAAGCTGA-3'. The PCR was performed at 45°C for 30 min proceeded by ~ 35 cycles at 94°C for 2 min, 62°C for 30s, 72°C for 1 min and a final incubation of 72°C for 10 min. The product lengths for *LRRK2* primers were 273 bp, 263 bp, 236 bp respectively for each primer set. The product lengths for *GAPDH* and *18S* primers were 176 bp and 178 bp respectively.

2.11 Immunohistochemistry Followed By Microscopy

The IHC protocols were previously described in (Schlossmacher et al. 2002; Schlossmacher & Shimura 2005; Cullen et al. 2011; Hakimi et al. 2011). Mouse skulls, brains,

and other organs were dissected and fixed in formalin for 5 days with formalin solution being changed every day for skulls. The skulls were decalcified using 12.5% formic acid treatment for 5-7 days. All tissues were paraffin embedded and serially cut using a vibratome. The brain and skull sections were cut into 40 μm thick slices and then mounted on glass slides at the University of Ottawa Laboratory for Pathology Services.

The immunohistochemistry protocol was carried out by first deparaffinization and rehydration of tissue slides using the following solutions successively: 100% histoclear, 50% histoclear:50% ethanol, 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol and ddH₂O. For each solution, the slides were dipped 20 times, left in the solutions for 1 minute and then dipped for another 20 times before proceeding to the next one. The slides were quenched in 3% H₂O₂ in 100% methanol for 10 minutes, followed by rinsing in ddH₂O for 5 min twice. An antigen retrieval step was carried out by placing the tissue slides in 10% Antigen Retrieval Citra Solution (concentrated 10X) (BioGenex, Fremont, CA) and heating in the microwave for about 10 minutes.

Immunocytochemistry of human blood specimens was performed with the following modifications from the protocol outlined above. Blood samples were prepared from healthy adult males and smeared onto microscope slides as a film. After being air-dried, the specimens were fixed in 100% methanol (not formalin) for 2 min proceeded by drying. Note, the hydration step was excluded from the protocol for blood smears and the quenching step was modified to 3% H₂O₂ in 20% methanol for 15 min. Antigen retrieval step was also modified to 1% Citra Solution kept at 37°C water for 35 min.

The slide tissues were incubated in blocking solution containing 10% goat serum in TBS (50 mM Trizma base, 140 mM NaCl, pH7.5) for 30 minutes at room temperature. Primary

antibody application was carried out using optimized concentration of the desired antibody in 1% goat serum dissolved in TBS. The primary antibody incubation took place at 4°C overnight. The slides were then rinsed by placing them in TBS solution for 5 min twice. The Primary antibodies were probed with biotinylated secondary antibodies, which were purchased from Vector Laboratories (Burlingame, CA). Appropriate secondary antibody dilution, usually 1:225, was made in TBS containing 10% goat serum. The slides were incubated with the secondary antibody for 30 min at room temperature, followed by rinsing with TBS solution as before.

A signal amplification step was performed by mixing reagents A and B of avidin-biotin-peroxidase kit, ABC kit, (Vector Laboratories Inc., Burlingame, CA) in TBS and applying them to the slides for 30 min at room temperature. The slides were then rinsed with Tris solution (50 mM Trizma base (Sigma-Aldrich, USA), pH7.5) twice. The signal was developed by placing the slides in 0.055M DAB (3,3'-diaminobenzidine substrate) dissolved in 50 ml Tris and 16.5 µl of 30% H₂O₂. Note the developing solution was filtered through no.1 Whatman paper prior to slide incubation. After developing the stains, DAB was deactivated by rinsing the slides in ddH₂O. Hematoxylin counterstain was carried out by dipping slides in the hematoxyline solution (Fisher Scientific) followed by rinsing with 3 successive baths of water. If necessary, a destaining step was carried out using acid alcohol (80% ethanol, 0.01M HCl). Finally, the tissue slides were dehydrated by similarly dipping the slides in the solutions described in rehydration step in reverse order; i.e. starting with ddH₂O and proceeding to 50% ethanol, 75% ethanol, 95% ethanol, 100% ethanol, 50% histoclear:50% ethanol and 100% histoclear. The slides were coverslipped using Permount adhesive (Fisher Scientific, USA) and results were then analyzed using microscopy.

Note Iba-1, hematoxylin and eosin as well as select anti-reovirus staining was carried out using an automated system operated by the Pathology Department of University of Ottawa (Ottawa, ON). The personnel at the Pathology Department, namely Mrs. Li Dong, Mrs. Ana Giassi, Mrs. Saeeda provided assistance with these operations and throughout with the specimens slides preparation.

2.12 Western Blotting

The WB protocols were previously described in (Schlossmacher et al. 2002; Schlossmacher & Shimura 2005; Cullen et al. 2011; Hakimi et al. 2011). Cells were rinsed with PBS and centrifuged at 14000 rpm for 10 min. After aspiration, the cells were lysed in 150 μ l buffer containing; 140 mM NaCl, 50 mM Tris-HCl, 0.5% Triton X-100, 2 mM EDTA and 1X protease inhibitor. The protein content of samples was later normalized using Pierce BCA Kit (Fisher Scientific Inc., Waltham, MA). Alternatively, 40 μ l of lysis buffer was used per 1 million cells. Each *Drosophila* sample for WB consisted of 4 fly heads, which were homogenized in 40 μ l of lysis buffer solution. The cell lysis took place on ice for 15 min and then centrifuged at 14000 rpm for 30 min. The proteins were collected from the supernatant of the cell lysate.

The protein aliquots were next mixed with Sample Loading buffer/dye, which contained sodium dodecyl sulfate (SDS) and glycerol. The samples were boiled for 5 minutes, then spun down using a countertop centrifuge and transferred to the ice to cool down. The samples were loaded at 20-30 μ g of protein per lane in a 4-12% bis-tris (SDS-PAGE) gel (Invitrogen, Carlsbad, CA). Fly samples were loaded at 10 μ g per lane and the Precision Plus

Protein all blue standards (Bio-Rad Laboratories, Hercules, CA) at 5-10 µl per lane. The samples were run under denaturing and reducing conditions using 1X Nupage MES SDS Running Buffer (Invitrogen™ Novex, Carlsbad, CA) at 125V for 1-1.5 hour. The proteins were next transferred from the gel onto a PVDF membrane (Millipore, Billerica, MA) in 1X Transfer Buffer (Invitrogen™ Novex, Carlsbad, CA) at 30V for 1 hour.

The protein membrane was blocked with 5% fat-free milk in 1X Phosphate Buffered Saline Tween (PBST) for 1 hour at room temperature. Optimized primary antibody dilution was made and applied to the membrane at 4°C overnight. Following rinses with PBST, secondary biotinylated antibody, which was conjugated to HRP, was applied to the membrane for 1 hour at room temperature. After final rinses with PBST, the membrane was subjected to Supersignal West Pico Chemiluminescent substrate (Thermo Scientific, Waltham, MA) as per manufacturer's instruction. The proteins were finally visualized by exposing the membrane to X-ray film and developing the film using the KONICA MINOLTA Processor (Wayne, NJ).

2.13 Viral Stock Preparation and L929 Cells

ReoT3D viruses (N.B. Field's and Jaclick's strains) and mouse L929 cells were gifts from Dr. Earl Brown. Jay Majithia, MSc, provided assistance with viral quantification of the samples. The L929 cell line was originally derived from connective (areolar and adipose) tissues of the mouse. The viruses were propagated in L929 cells supplemented with MEM growth medium containing 5% fetal bovine serum and penicillin-streptomycin antibiotics. The viruses were released from cultured L929 cells by freeze-thaw cycles, as a cell lysis method, and collected in the supernatant as described in (Gauvin et al. 2013).

2.14 Viral Inoculation of Newborn Mice and Survival Study

Viral dilutions were made to deliver 1.7×10^5 pfu reoT3D in PBS to 1-day old suckling mice from the *Lrrk2* KO and G2019S LRRK2 colonies versus their corresponding WT mice. 1.7×10^5 pfu reoT3D corresponded to the lethal dose (LD_{50}) as was previously established in (Gauvin et al. 2013). In intranasal inoculation of mice with the virus, the animals were first anesthetized with isoflurane in 5% oxygen and then the virus was delivered by pipetting 10 μ l of the viral aliquot on their nose pad. Mock infection contained the same volume of cells media diluted in PBS and was similarly introduced to 1-day old suckling mice that served as control groups. The animals were sacrificed at specific time points post-infection, and various organs were harvested for analysis by IHC and plaque assays.

The survival study was conducted using suckling mice from the homozygous and heterozygous *Lrrk2* KO and homozygous G2019S *Lrrk2* colonies versus their corresponding WT mice. Two different strains of reoT3D virus, the N.B. Field's and Jaclick's strains, were used in the *Lrrk2* KO colony and each of which contained 1.7×10^5 pfu as was predetermined by plaque assays. The animal survival was monitored for at least 25 days post infection.

2.15 Tissue Homogenization and Plaque Assay

PBS was added to mouse organs in 4:1 ratio (ml PBS/g tissue) prior to homogenization with metal beads in a ROCHE Cell MagNA Lyser (Roche, Indianapolis, IN) at 7,000 rpm for 10 sec. Subsequent centrifugation of the homogenate samples collected the viral content in the supernatant which was used for viral titer determination next. For the plaque assay, serial dilutions of each organ homogenate were made, and each different titer was placed

on L929 cells in duplicates. The cells were supplemented with growth medium in 2% agar for the next 8 days. A final overlay with Neutral Red (Thermo Fisher Scientific, Fair Lawn, NJ) was used to visualize the plaques resulted from the reovirus. The viral titers were determined as per gram of tissue.

2.16 Quantification of the Viral Staining in Murine Tissues Using Aperio Imagescope Software

The IHC slides of mouse brains were scanned using Aperio ScanScope Console (Leica Biosystems, Buffalo Grove, IL). The slides were viewed and analyzed using Aperio ImageScope software. Positive Pixel Count v9.1 algorithm was used to quantify neurons that were positive for reoT3D virus infection. Parameters were set to distinguish positivity from weak to moderate to strong using different color representation. Similarly, the parameter for negativity (lack of infection) was marked by a yet different color. The quantification of the mouse brain IHC signal was performed by Ms. Irene Harmsen.

2.17 Statistical Analyses

Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA). Multiple t-tests were performed for analysis of data, and statistical significance was determined using the Sidak-Bonferroni method unless otherwise stated for individual results (as noted in legends of the corresponding figures). A p -value of ≤ 0.05 was considered significant. Mouse survival data were additionally analyzed by statisticians at the Center for Practice Changing Research (OHRI, Ottawa, ON). The Log Rank Test was performed to assess the differences statistically. Multivariable Cox Regression modeling was used to calculate

and p-values for genotype while controlling for dose. Multivariable Cox Regression modeling with a robust sandwich covariance matrix estimate to account for the correlation within the family (random effect) was used to calculate p-values as the litter sizes varied in the experiments. A p -value of ≤ 0.05 was considered significant as aforementioned.

2.18 Source of Funding

This research project was supported by the Michael J. Fox Foundation for Parkinson's Research (Consortium Grant on Lrrk2 Biology to M. Schlossmacher, J. Shen and D. Park, 2009-2011; Consortium Grant on LRRK2 Function in Immunity to M. Schlossmacher, J. Tomlinson, D. Philpott, 2013-2015), Canadian Institute of Health Research (Team Grant on LRRK2 in Inflammation to D. Park, M. Schlossmacher, D. Gibbings, S. Hayley, D. Philpott, E. Schurr and J.D. Rioux, 2014-2018), and the Parkinson Research Consortium (Larry Haffner Fellowship and Crabtree Family Fellowship to M. Hakimi).

CHAPTER III. RESULTS

3.1 Examining LRRK2 Expression In Various Human Tissues And Conditions

3.1.1 LRRK2 Expression Is Detected In Mammalian Leukocytes

Our initial investigation for LRRK2 expression took place in human peripheral blood obtained from healthy donors. Blood smears were prepared on slides and processed by immunocytochemistry for detection of proteins of interest. Staining with anti-LRRK2 antibody, HL-2, revealed LRRK2 reactivity in cytoplasmic regions of some white blood cells (WBC). White blood cells are less abundant but larger in size than red blood cells, which are disc-shaped and typically measure 6-8 μm in diameter. Morphological analysis of LRRK2-positive WBCs suggested LRRK2 expression mainly in granulocytes and monocytes and sometimes lymphocytes. Granulocytes have a 13-16 μm diameter with multi-lobed/fragmented nucleus; monocytes have a 14-16 μm diameter with kidney-shaped nucleus while lymphocytes are typically 8 μm in diameter and have a round nucleus with little cytoplasm (Figure 3.1.1. A-B). Erythrocytes and platelets, which are typically positive for α -synuclein staining (Scherzer et al. 2008) were devoid of any LRRK2 staining under these conditions.

Examining *post-mortem* sections of human midbrain and ileum of the gastrointestinal tract from neurologically healthy donors revealed LRRK2 reactivity in leukocytes within capillaries (Figure 3.1.1C-D). Notably, LRRK2 reactivity was not detected in enteric neurons of the submucosal and myenteric plexi or any neuromelanin-carrying cells of the *S. nigra*. These ganglionic and neuronal cells were stained positive for α -synuclein and tyrosine hydroxylase on parallel sections (data not shown).

FIGURES AND TABLES

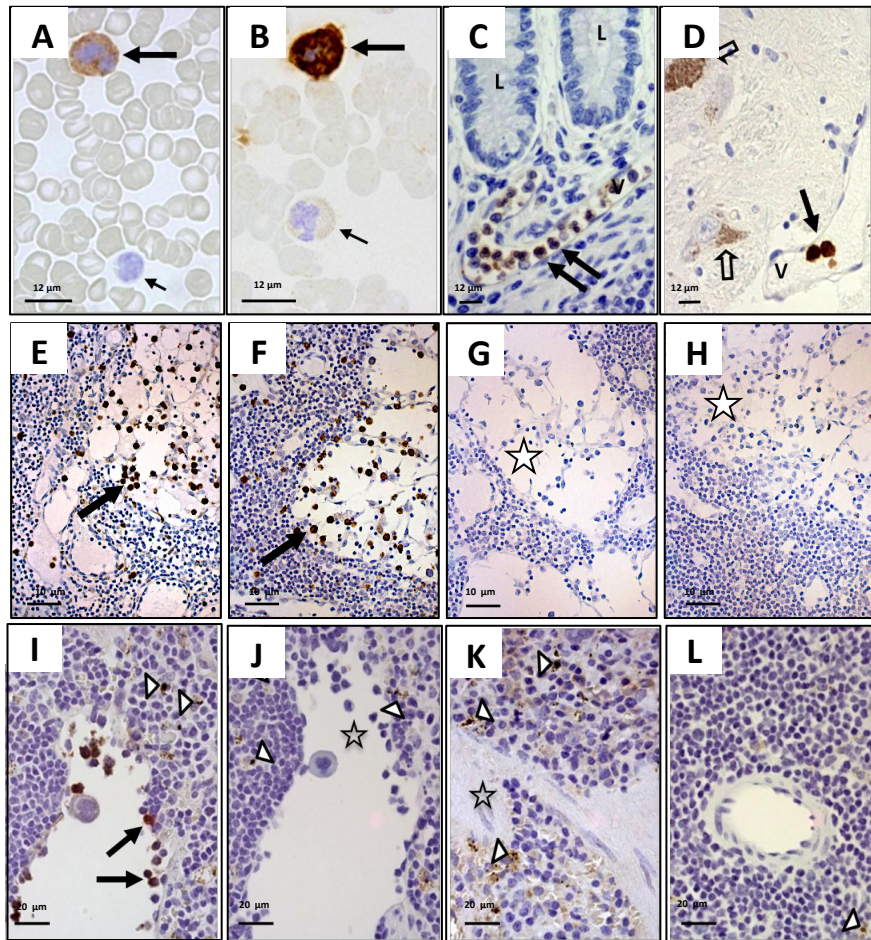


Figure 3.1.1. Detection of LRRK2 reactivity in mammalian tissues. LRRK2 staining was observed in certain leukocytes from human blood smears (A-B, n=2) and gastrointestinal tract (C, n=3) of healthy donors and midbrain of PD patient (D, n=3). The LRRK2 antibody (HL-2) specificity was validated using lymph node (n=2) staining with HL-2 (E), HL-2 + BSA (F) and HL-2 + HL-2 antigen peptide (G-H). As expected LRRK2 staining, which was seen with the antibody alone or antibody and control BSA staining was diminished by >90% in the preabsorption experiment (G-H). In addition, the specificity of LRRK2 antibody was validated in splenic sections of WT (I-J, n=4) and LRRK2 null (K-L, n=4) mice. Application of anti-LRRK2 antibody (I and K) yielded signal in only WT. Negative controls (J and L) for each genotype of WT and KO were processed using identical staining technique without the primary antibody. As expected negative controls were devoid of LRRK2 signal. N ≥3 for each sample.

Symbols and abbreviations: large $\longrightarrow$: LRRK2 reactivity, small $\longrightarrow$: poor/no LRRK2 reactivity, $\Rightarrow$: autofluorescent neuromelanin in DA cells, $\triangle$: lipofuscin granules, $\star$: unstained leukocytes, V: vasculature, L: lumen. Images were obtained using original magnifications of 63x (A-D), 40x (I-J) and 20x (E-H). Some of these data were published in (Hakimi et al. 2011).

The specificity of our affinity-purified polyclonal anti-LRRK2 antibody, HL-2, was validated in both human and mouse tissues using the same IHC protocol (Figure 3.1.1E-L). The HL-2 antibody was raised against amino acid 2508-2527 of the human wild-type LRRK2 (Berger et al. 2010). IHC staining of human lymph node sections revealed a robust signal for HL-2 antibody in cells of the nodes' sinusoidal system (Figure 3.1.1E). Application of BSA (a control peptide) did not affect HL-2 reactivity (Figure 3.1.1F), as expected. Application of the HL-2 antigen peptide diminished the antibody reactivity by >90%; indicating successful reabsorption and specificity of the antibody (Figure 3.1.1G-H).

Next, we conducted IHC on sections of spleens from *Lrrk2* KO (n=4) and WT (n=4) mice (Figure 3.1.1I-L). In contrast to *Lrrk2* KO spleen staining, HL-2 showed reactivity in select splenic leukocytes of WT mice. *Lrrk2* deficient spleens only exhibited autofluorescent lipofuscin granules, as expected; presence of lipofuscin was previously described in this mouse model (Tong et al. 2012). Furthermore, omitting the primary antibody generated no signal in sections of either genotype, as anticipated. These results indicate validation of our LRRK2 antibody specificity and identify LRRK2 reactivity in certain pools of leukocytes present in the CNS vasculature, ileal capillaries as well as sinusoidal system of lymph nodes.

3.1.2 LRRK2 Is Expressed in Monocytes, B-Lymphocytes, and T-Lymphocytes

To further explore LRRK2 expression by leukocytes, we initially examined mouse tissues for relative *Lrrk2* levels in various subpopulations of WBCs. We harvested immune organs (ex. mesenteric lymph nodes, ileal Payer's patches and spleen) from WT mice and prepared tissue homogenates from them to be analyzed by flow cytometry. We co-stained cells with anti-LRRK2 antibody and cell surface markers of various leukocyte subtypes. FACS staining revealed detection of murine *Lrrk2* in B- and T-cells, macrophages, dendritic cells and

neutrophils to varying degrees (Figure 3.1.2). There, the expression of *Lrrk2* was ≥ 2 folds higher in neutrophils compared to other leukocyte subpopulations in both mesenteric lymph nodes and spleen.

Next, we substantiated our findings from mouse tissues in human specimens, where we similarly investigated LRRK2 expression in peripheral blood drawn from healthy adult donors. The blood was enriched for peripheral blood mononuclear cells (PBMC) using a Ficoll gradient and then processed by FACS staining with anti-LRRK2 antibodies, including with monoclonal rabbit antibodies (MJFF 1-5) that had recently been generated by the Michael J. Fox Foundation for Parkinson's Research (MJFF, New York, NY). As published in (Hakimi et al. 2011), approximately 25% of all PBMCs were strongly positive for LRRK2 staining and about 27% of all CD14⁺ monocytes showed a robust signal for LRRK2 reactivity as determined by FACS analysis.

SDS/PAGE and Western blotting techniques were used to further confirm the presence of LRRK2 in PBMC lysates using a number of different monoclonal, anti-LRRK2 antibodies (Figure 3.1.3 A-D). In addition, *LRRK2* mRNA was also detected in human EBV-transformed lymphoblasts using distinct primer pairs that each probed a different region of the *LRRK2* sequence (Figure 3.1.4).

In the Western blots, the full-length LRRK2 monomer was detected as a single band of ~260 kDa, or a series of bands >260 kDa, in cell lysates (Figure 3.1.3). Variable higher molecular weight (HMW) LRRK2 species were observed in the monoclonal MJFF antibody-stained blots. Red blood cell lysates did not show any signal for LRRK2 confirming earlier IHC observations (Figure 3.1.5A). Head lysates of transgenic flies expressing human *LRRK2* cDNA (tg) versus WT littermate flies were used as positive and negative controls, respectively, for

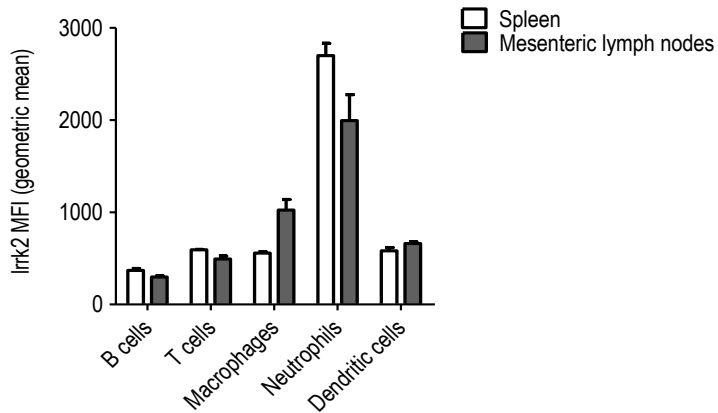


Figure 3.1.2. Lrrk2 expression in various leukocytes of mouse. Spleen and mesenteric lymph nodes were harvested from WT mice (n=3) followed by homogenization to make single cell suspension for each immune organ. The tissues were then processed by FACS using Lrrk2 antibody MJFF-4 and markers of leukocyte subtypes for B220+ B-cells, TCR β + T-cells, CD11b+/CD11c+ macrophages, Gr-1+ neutrophils, and CD11c+ dendritic cells. Lrrk2 expression was expressed as mean % in various leukocytes as shown above. Published in (Hakimi et al. 2011).

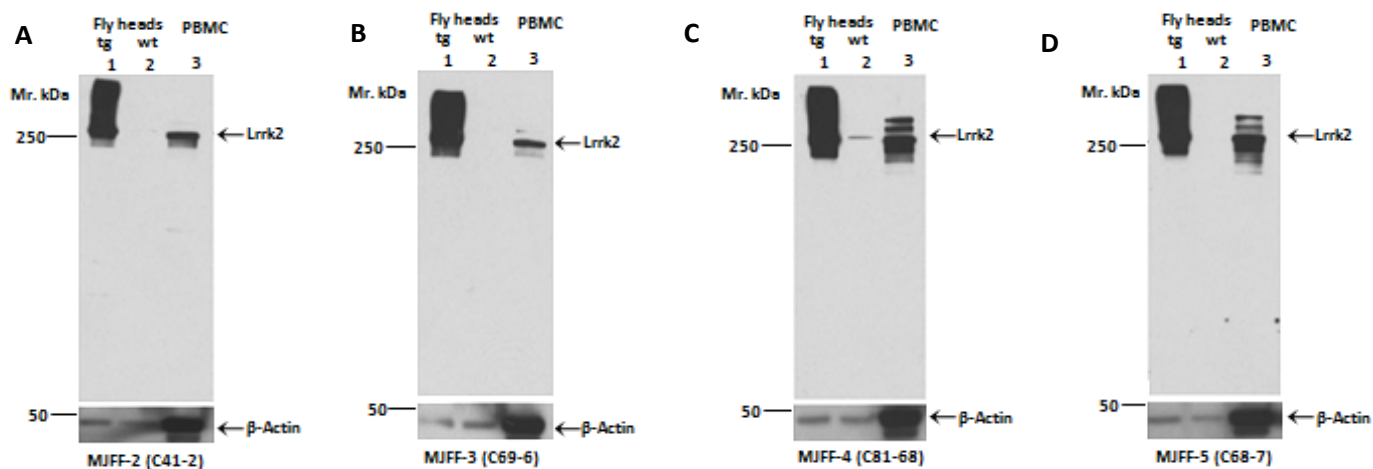


Figure 3.1.3. Expression of LRRK2 in white blood cells. LRRK2 protein was detected in immunoblots of peripheral blood mononuclear cell lysates that were enriched and collected from healthy donors (A-D, $n > 5$ donors). LRRK2 protein (260 kDa) in PBMCs and human LRRK2 transgenic flies (positive control) was detected using four different monoclonal rabbit anti-LRRK2 antibodies (MJFF, New York, NY). Higher molecular weight LRRK2 species was also detected in both PBMC and transgenic flies lysates. Wild type flies were used as negative control. Published in (Hakimi et al. 2011).

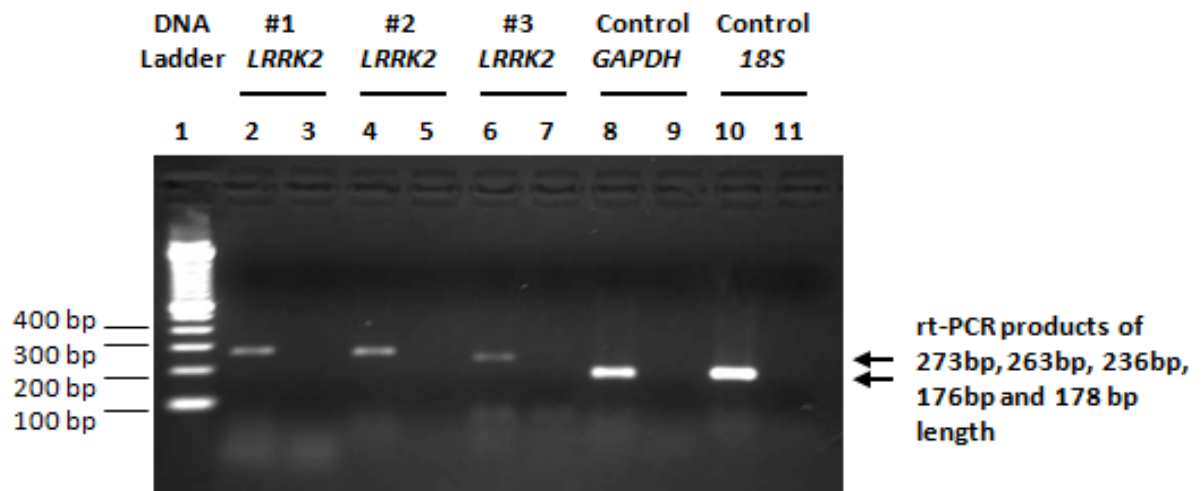


Figure 3.1.4. Detection of *LRRK2* mRNA transcript in EBV-transformed lymphoblasts. Three different *LRRK2* primer sets were to detect *LRRK2* mRNA in EBV-transformed lymphoblasts that were generated from a healthy donor (E). *GAPDH* and *18S* mRNA were used as control there. This blot is representative of at least 3 separate experiments. Published in (Hakimi et al. 2011).

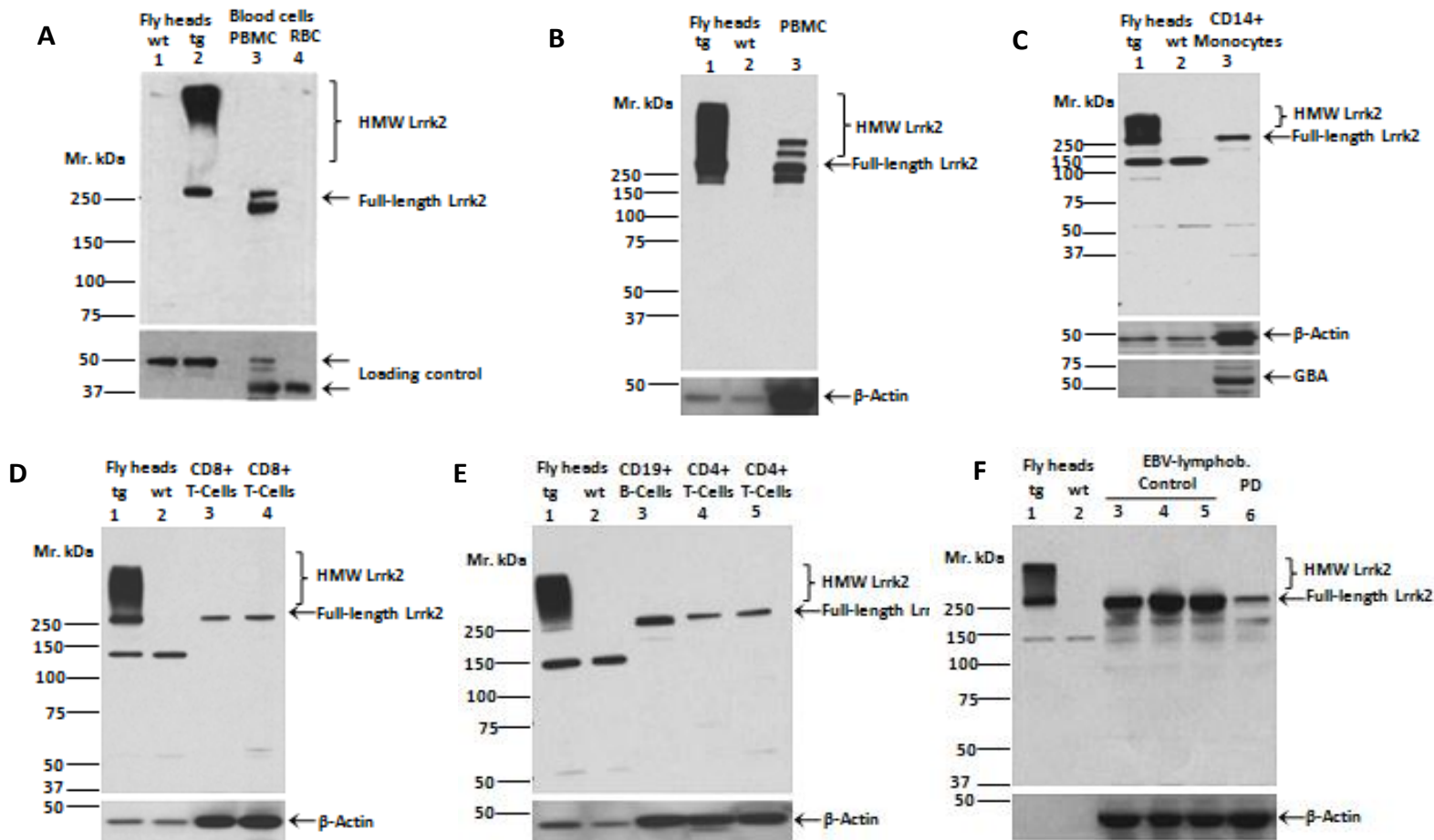


Figure 3.1.5. Expression of LRRK2 in CD14+ monocytes, CD4+ and CD8+ T-lymphocytes and CD19+ B-lymphocytes. Subpopulation of leukocytes were isolated from PBMCs (n=5 donors) using cell sorting and cell surface markers for each cell type as explained in Methods. The cells were then lysed and processed by reducing SDS/PAGE. The subsequent immunoblots showed robust signal for LRRK2 protein in PBMC (but not red blood cells (RBC), A-B), CD in CD14+ monocytes, CD4+ and CD8+ T-lymphocytes and CD19+ B-lymphocytes (C-E). EBV-transformed lymphoblast derived from B-lymphocytes also showed strong signal for LRRK2 (F). Anti-acid β-glucosidase (GBA) antibody was used as an additional marker for monocyte identity (C). LRRK2 protein was probed using antibodies HL-2 (A), MJFF-1 (B) and NB300-268 (C-F). β-actin marker was used as a loading control in blotting membranes. Published in (Hakimi et al. 2011).

LRRK2 expression on immunoblots (Figure 3.1.3 and 3.1.5).

Monocytes isolated from PBMC using CD14+ marker also yielded a robust signal for LRRK2 in their lysates (Figure 3.1.5C). These were also positive for the presence of acid- β -glucosidase protein (~62 kDa), as expected for monocytes and macrophages (Cullen et al. 2011). Similar cell sorting with other leukocyte markers and Western blotting of cell lysates detected LRRK2 in CD19+ B-cells and CD8+ and CD4+ T-cells (Figure 3.1.5D-E). Interestingly, EBV-transformed lymphoblasts obtained from B-cells of human control subjects showed relatively higher LRRK2 expression when compared with that of PD patients (Figure 3.1.5F). We concluded that endogenous LRRK2 represents a common constituent of WBCs, in particular in circulating monocytes and neutrophils.

3.1.3 Robust LRRK2 Expression Is Found In Sections of Inflamed Human Brain and Organs of the Immune System

Using specific antibodies for immunohistochemistry and indirect immunofluorescence (IIF) microscopy, we also found that LRRK2 is a relatively common constituent of myeloperoxidase (MPO)-positive granulocytes and CD68-positive macrophages in various human organs (Figure 3.1.6). Parallel sections of human lymph nodes stained with different leukocyte markers generated LRRK2 reactivity most prominently in macrophage- and granulocyte-type cells (Figure 3.1.6). Similarly, sections of human spleen, lung, kidney, inflamed ileum and *Mycobacterium leprae*-infected skin (Figure 3.1.7) all showed expression of LRRK2 in human cells with a granulocyte- and macrophage-type appearance. The latter was confirmed by IIF experiments in sections of ileum and lymph nodes (not shown).

In addition, we found that LRRK2 is highly expressed in invading leukocytes in sections of neural tissue affected by a range of infectious/inflammatory conditions (See Table 3.1.1). For example, various regions of HIV-infected brains showed robust signal for LRRK2 (Figure 3.1.8A); such strong LRRK2 reactivity was not seen in healthy control or PD brain sections (Figure 3.1.8E-F). In addition, robust LRRK2 signal was noted in sections of midbrain and a cervical nerve root of a single patient with systemic vasculitis (Figure 3.1.8B-C). Furthermore, strong LRRK2 reactivity was also seen in rabies-infected human brain areas (Figure 3.1.8D).

3.2 Examining Lrrk2 Induction In Response To Immune Stimulation In Vitro

3.2.1 Lrrk2 Induction Is Paralleled by Activation and Recruitment of Microglia and Macrophages in the Central Nervous System

LRRK2-associated PD is classically thought to manifest itself in the central nervous system, where marked loss of dopaminergic neurons occurs in the midbrain, which promotes parkinsonism (Zimprich et al. 2004). However, both the protein and mRNA of *LRRK2* are expressed at low levels as observed by the investigators (Galter et al. 2006). In sections of chronic neurodegenerative diseases, e.g., PD and atypical parkinsonism, we found few LRRK2-positive, CD68- and MPO-positive leukocytes outside the cerebral vasculature. To confirm the expression of endogenous Lrrk2 in invading and resident immune cells of the brain, we analyzed mice that were injected into their midbrain with saline, LPS or LPS together with interferon- γ (IFN- γ) (Figure 3.2.1). Five days after injection, we recorded significantly higher signals for endogenous Lrrk2 after LPS (versus saline) administration in both invading macrophages and CD11-positive microglia, an effect that

was markedly enhanced by co-injection of LPS and IFN- γ , two agents known to increase *Lrrk2* transcription (Figure 3.2.1). This is consistent with the data published by A. West's team (Moehle et al. 2012).

3.2.2 *Lrrk2* Is Upregulated Following Microbial Infections

Given the importance of monocytes and tissue macrophages (derived from circulating monocytes) in Crohn's disease (CD) and the high expression of LRRK2 in human monocytes (above), we next investigated *Lrrk2* expression and behavior in mouse macrophages. We harvested BMDM from femurs and tibias of WT and R1441C *Lrrk2* mouse littermates (see Method section). Next, we treated BMDM cultures with inflammatory stimuli for 5 hours to examine *Lrrk2* behavior by isolating and quantifying mRNA using RT-PCR. Murine *Lrrk2* mRNA was significantly upregulated in macrophages treated with TLR4 ligand (LPS; >4-fold), TLR7 ligand (R837; >2-fold), and TLR9 ligand (CpG; >3-fold) as shown in Figure 3.2.2A. In contrast, *Lrrk2* mRNA level was significantly decreased in treatment with TLR-2 ligand (Pam3CSK4; <0.5-fold) compared to unstimulated control treatment.

Next, we examined the effect of viral transduction on *Lrrk2* protein expression in BMDM using eGFP cDNA-carrying lentiviral preparations. Transduced BMDM cultures were treated with various immune stimulants for 16 hours, followed by protein extraction and Western blotting. Viral induction led to increased *Lrrk2* levels (normalized by β -actin signal) in all transduced cells compared to non-transduced cells; this occurred regardless of the type of stimulant used (Figure 3.2.2B). Both homozygous and heterozygous R1441C *Lrrk2* genotypes and WT BMDM generated consistently comparable results. These results highlighted the induction of murine *Lrrk2* at both mRNA and protein levels in response to

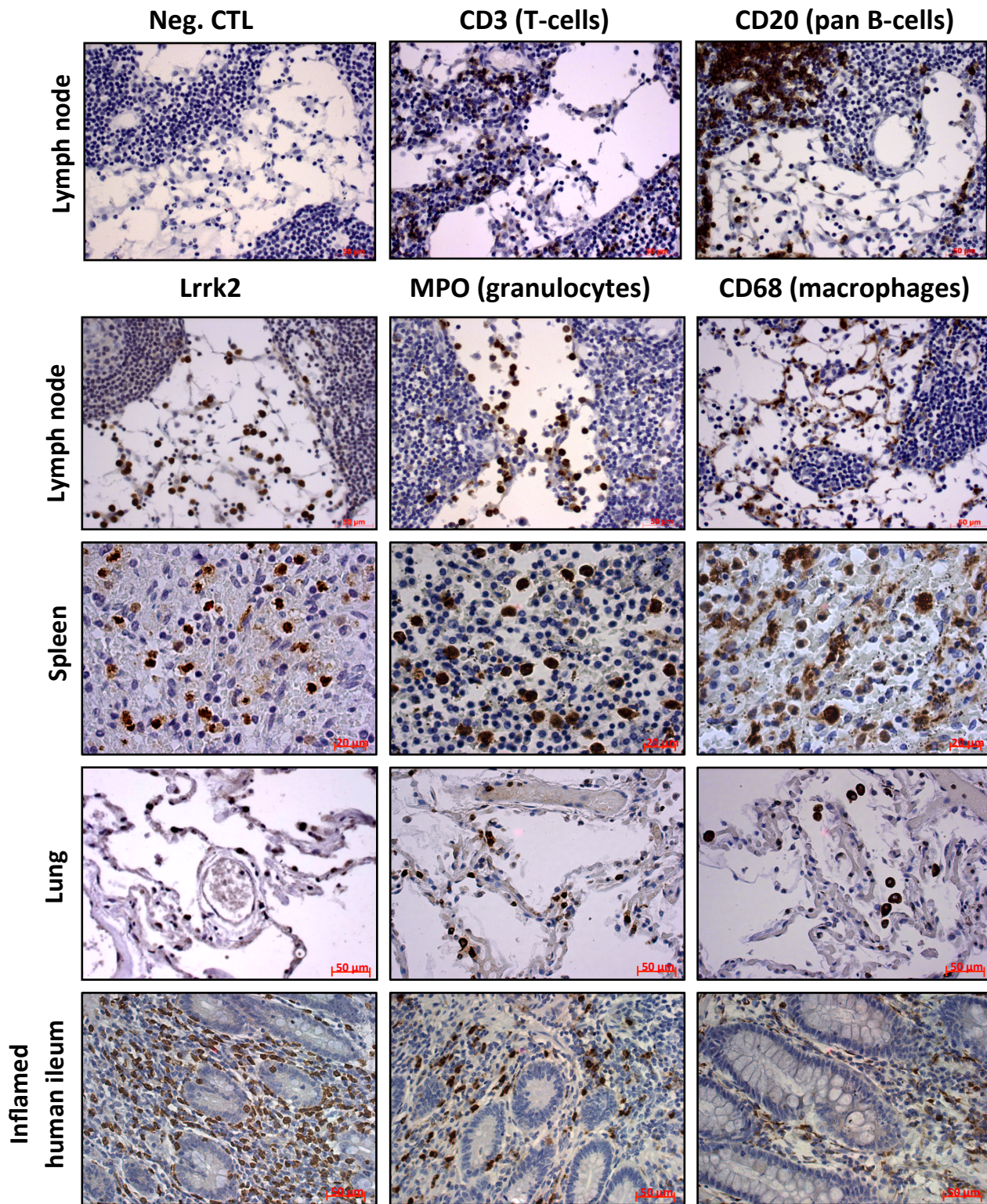


Figure 3.1.6. Characterization of select human organs with leukocyte markers. Tissues were fixed in 10% formalin and staining was carried out by routine IHC. Serial sections were stained with anti-LRRK2, anti-MPO (myeloperoxidase, a marker of granulocytes) and anti-CD68 (macrophages). Lymph nodes were additionally stained with anti- CD3 (T-cells) and CD20 (B-cells) antibodies. Neg. CTL refers to negative control where the use of primary antibody was omitted. Parallel sections stained with commercial anti-LRRK2 Ab (NB300-268) generated similar images. Note, anti-LRRK2 reactivity (brown, DAB) is closest to that of granulocytes and macrophages, which we confirmed by double staining using indirect immunofluorescence microscopy (not shown). N for each organ/tissue were as following: lymph node (n=2), spleen (n=3), lung (n=2), ileum (n=2). Images were obtained using original magnification of 40x for spleen and 20x for all other organs.

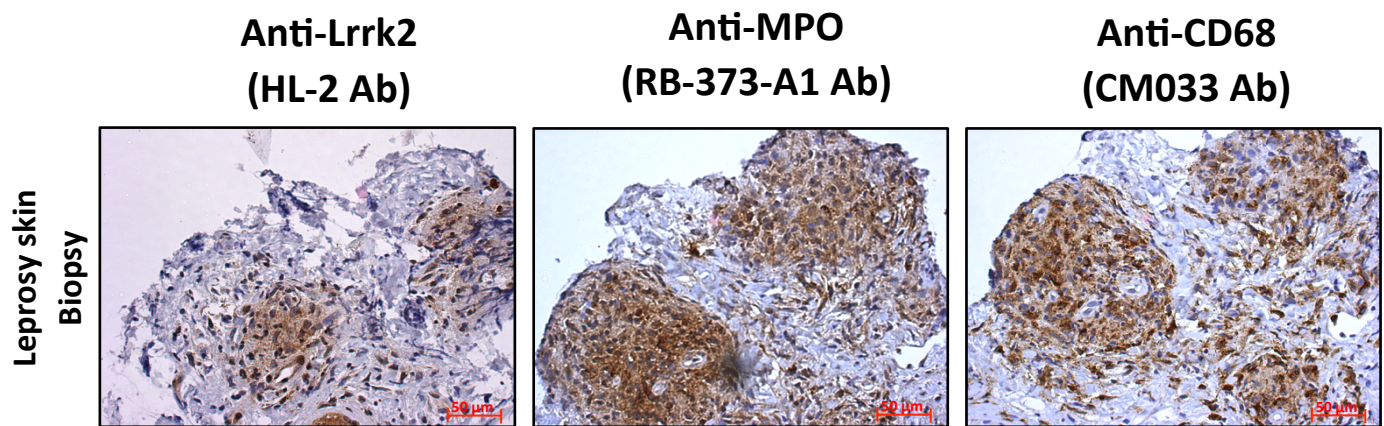


Figure 3.1.7. Characterization of *M. leprae*-infected skin with leukocyte markers. Human tissues (n=4 donors) were fixed in 10% formalin and staining was carried out by routine IHC. Serial sections were stained with anti-Lrrk2, anti-MPO (myeloperoxidase, a marker of granulocytes) and anti-CD68 (macrophages). Parallel sections stained with commercial anti-Lrrk2 Ab (NB300-268) generated similar images. Note, anti-Lrrk2 reactivity (brown, DAB) is closest to that of granulocytes and macrophages, which we confirmed by double staining using indirect immunofluorescence microscopy (not shown). Images were obtained using original magnification of 20x. Images were generated by Ms. M. Hakimi, Drs. J. Michaud and J. Woulfe (Division of Neuropathology).

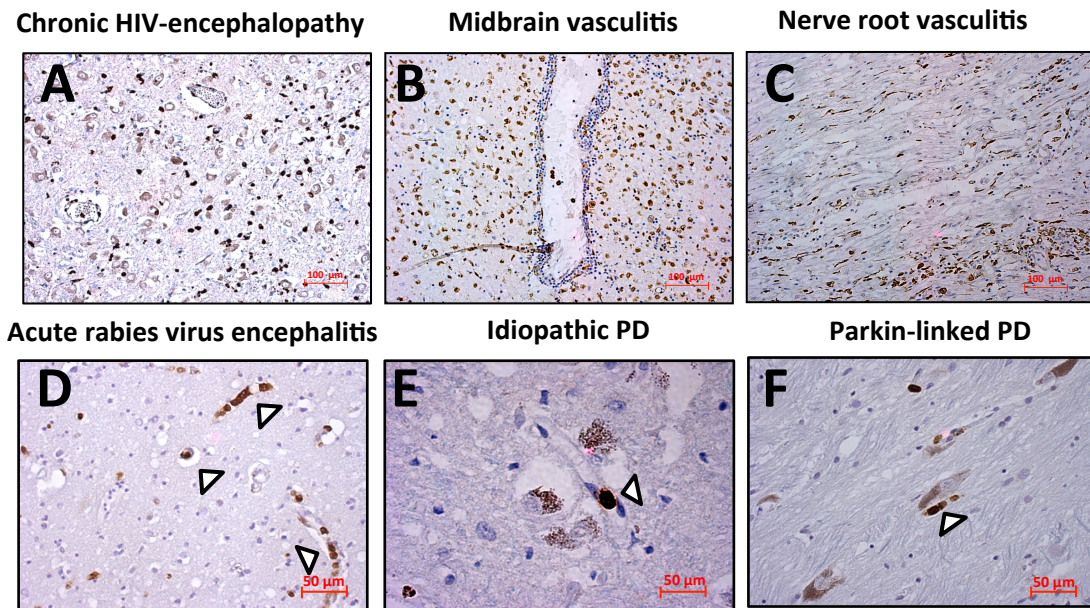


Figure 3.1.8. Characterization of *post mortem* human brain (and nerve root) with anti-LRRK2 antibody. Lrrk2 expression was prominently detected in recruited leukocytes (white arrowheads show representative antibody reactivity), as seen in chronic HIV encephalopathy (A, n=3), vasculitis (B-C, n=1 each) and acute rabies encephalitis (D, n=1). Note also the presence of intravascular LRRK2-positive leukocytes in midbrain structures of sporadic (n>3) and inherited PD (n=2). Images were generated by M. Hakimi, Dr. M Schlossmacher and Dr. J. Woulfe. Rabies-infected sections are a gift by Dr. M. Frosch (Mass. Gen. Hosp.), Parkin-deficient brain was provided by Dr. JP vonSattel (Columbia Univ.), and HIV-infected brain was from Dr. E. Masliah of UCSD. Images were obtained using original magnification of 40x (E) and 20x (D, F) and 10x (A-C).

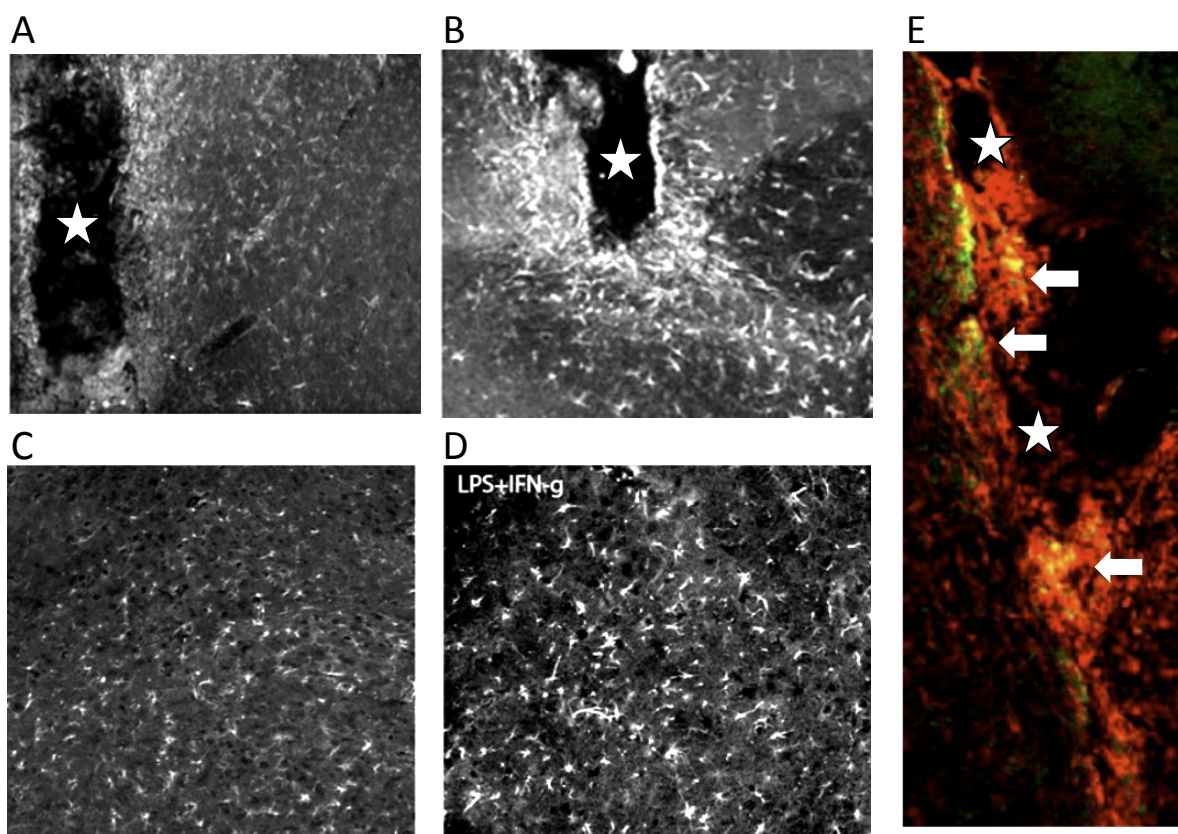


Figure 3.2.1. Endogenous *Lrrk2* reactivity in glia including CD11b⁺-cells in the *S. nigra* of wild-type mice (A-E). Animals were stereotactically injected with saline (A, E, $n \geq 3$) or lipopolysaccharide (LPS; B, C, $n \geq 3$), or with LPS plus interferon-g (D, $n \geq 3$). (LPS and IFN- γ are known upregulators of *Lrrk2* transcription in isolated macrophages). Asterisks in A, B and E denote needle tracks. Representative images are shown from four pilot experiments. Note, that LRRK2 is detectable in the vicinity of all needle tracks and markedly enhanced by LPS (0.5 mg/ml; compare B vs. A). *Lrrk2* expression is also seen away from the needle track (>500 μ m) and further augmented by IFN- γ (D vs. C). A pool of LRRK2-positive (green) cells is stained by anti CD11b (red; arrows in E). Experiments were performed by Dr. S. Hayley team (Carleton University, ON) . The initial preliminary work was conducted by M. Hakimi.

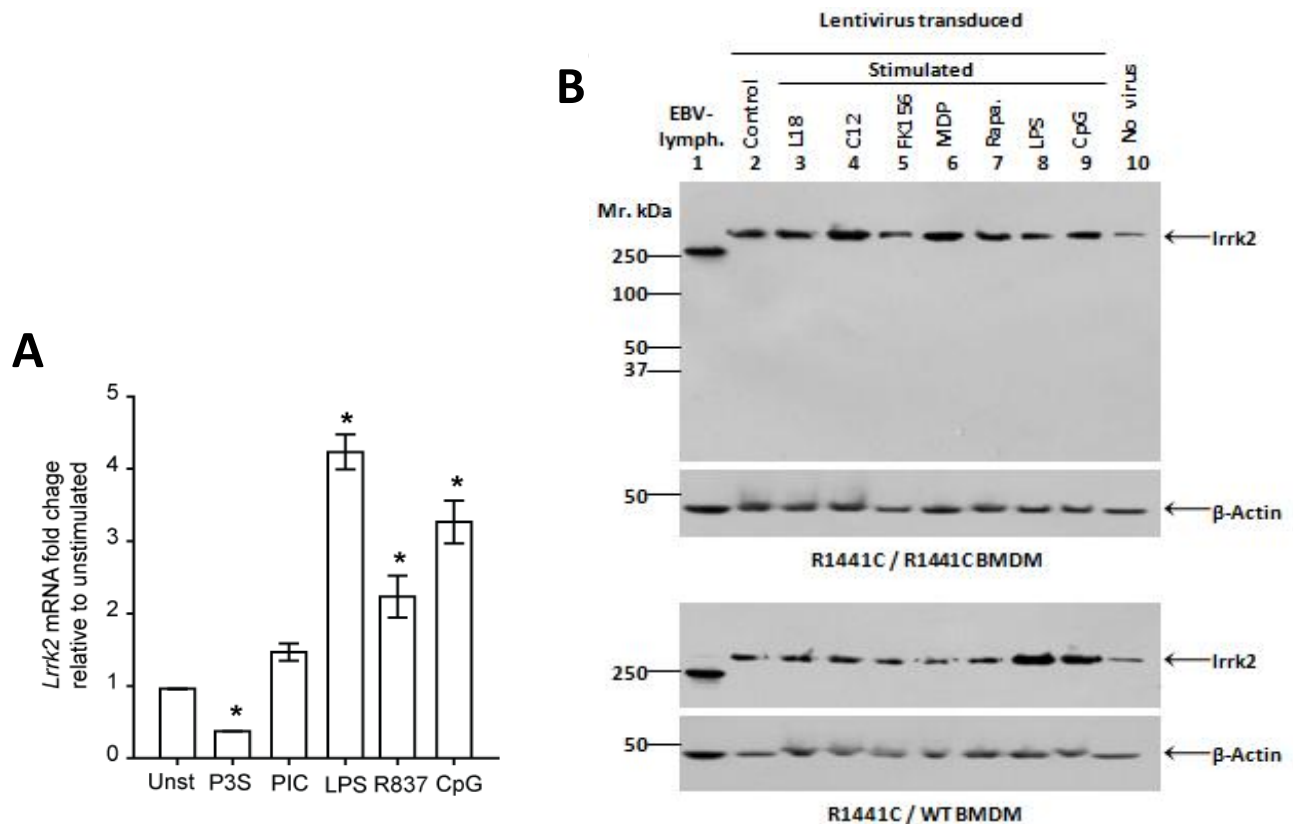


Figure 3.2.2. Induction of *Lrrk2* following pathogenic stimuli. Activation of TLR2, 3, 4, 7 and 9 were conducted using Pam3CSK4 (P3S), Poly I:C (PIC), LPS, R837 and CpG respectively in WT BMDM (n=3 animals) for 5 hours (A). The macrophages were then lysed and processed for quantification of *Lrrk2* transcript by $\Delta\Delta$ CT method and comparison with 18S in all samples. LPS, R837 and CpG led to significant induction of *Lrrk2* mRNA while P3S led to significant attenuation of *Lrrk2* mRNA compared to unstimulated macrophages as determined by ANOVA and Tukey test; $P < 0.05$. All pathogenic treatments elevated $\text{TNF}\alpha > 30$ folds indicating successful stimulation of BMDM (data not shown). Experiment was conducted by Dr. Denis Gris (University of Sherbrooke, QC). Activation of BMDM and induction of *Lrrk2* protein were also studied in cultures derived from R1441C mice (B, n=4). BMDM were transduced with a lentivirus for 48 hours followed by treatment with various stimuli for 16 hours as depicted in (B). Rapa stands for rapamycin. Lentivirus transduction of BMDM led to strong *Lrrk2* protein signal relative to cells with no virus treatment. Anti- β -actin antibody was used as loading control. Published in (Hakimi et al. 2011).

inflammatory modulators.

3.2.3 *Lrrk2* Deficiency and R1441C *Lrrk2* Mutation: Screening For Macrophage Regulation of Cytokines Following Immune Stimulation

Regulation of cytokines is important in the immunologic response by macrophages and their communication to other cells. Following the finding of human and murine macrophages as a leukocyte population with fairly robust LRRK2 levels, bone marrow cells were isolated from the femurs and tibias of *Lrrk2* KO and R1441C *Lrrk2* KI mice and their corresponding control wild-type littermates. Isolated bone marrow cells were cultured and differentiated into macrophages using L929 supernatant, as described above. Next, BMDM were subjected to inflammatory stimulants such as live viruses (ex. T3D reovirus) or ligands that mimicked bacterial or viral pathogens. The stimulation treatment had continued for 16 hours before cells supernatants were collected for cytokine analyses. The cells supernatants were used for individual ELISA or multiplex assays that detected different cyto- / chemokine levels using corresponding sets of standards.

In examining the supernatants of *Lrrk2* KO and WT BMDM, significant elevation of IL-6 and KC was noted following treatments with heat-killed listeria, LPS, and LPS combined with nigericin (Figure 3.2.3 A-B). Such IL-6 and KC up-regulation (in treatments mentioned earlier) was previously reported by other researchers (Saïd-Sadier et al. 2010). However, these experiments also showed that endogenous *Lrrk2* did not affect IL-6 and KC regulation under these conditions.

In further examining the supernatants of R1441C *Lrrk2* and WT BMDM cultures, other cytokine levels were upregulated following treatment with heat-killed bacteria, LPS and nigericin, and reovirus (Figure 3.2.3 C-F). However, in comparison to WT, we found that

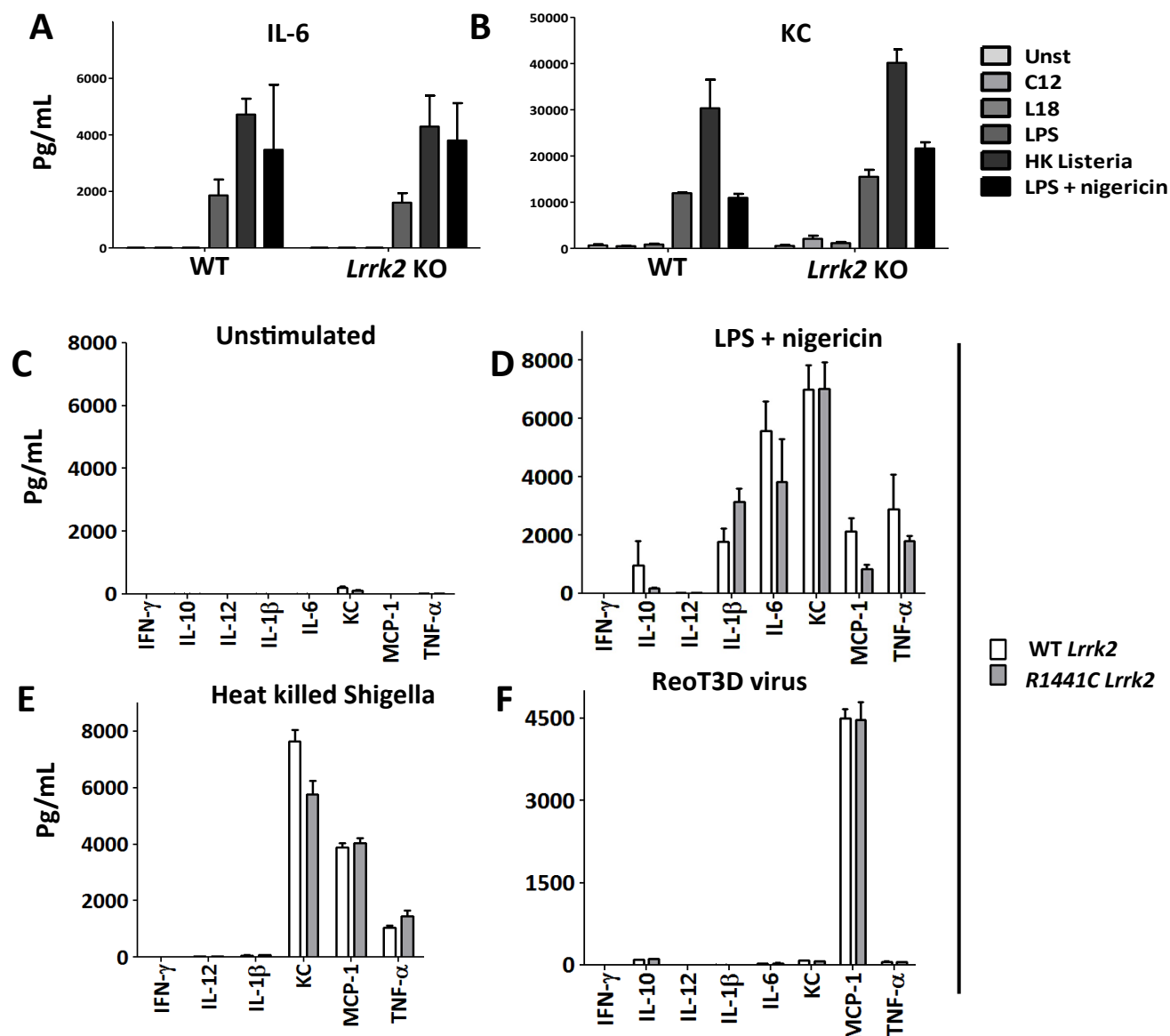


Figure 3.2.3. Screening the effects of *Lrrk2* deletion and R1441C *Lrrk2* mutation on cytokine response during immune stimulation. BMDMs from *Lrrk2* KO mice (n= 2, A-B), R1441C-*Lrrk2* mutant mice (n=4, C-F) and their corresponding age- and sex-matched WT mice (n=2 and 4 respectively) were stimulated for 16 hours with various microbial antigens in quadruplicate wells. Cytokine release was measured in the cells supernatants by individual ELISAs and cytokine multiplex for *Lrrk2* KO and R1441C *Lrrk2* samples respectively. Unstimulated cells showed absence of cytokine release constituting a control/baseline for microbial stimulations. In *Lrrk2* KO vs. WT comparison, the levels of IL-6 (A) and KC (B) released by macrophages indicate lack of genotype-specific response for LRRK2 - published in (Hakimi et al. 2011). In R1441C *Lrrk2* vs. WT, LPS plus nigericin initiated IL-6, IL-1 β and KC secretion (D). Shigella caused the release of KC, MCP-1 and TNF- α (E) and reovirus exposure led to secretion of MCP-1, as expected (F). Note that *Lrrk2* GTPase mutation (R1441C) in MDM cells had no detectable effect on cytokine production and release in this and related experiments.

R1441C *Lrrk2* mutation did not lead to statistically significant changes in the release of detectable IL-1 β , TNF- α , IL-6, MCP-1 (and of other cytokines; not shown) measured.

3.3 Characterization Of *Lrrk2* Deficient Mouse Model In An In Vivo Reovirus Infection

Because our collective results showed strong expression of mammalian *Lrrk2* in mature monocytes and granulocytes, these data suggested to us that *Lrrk2* may play a role in the host's innate (rather than adaptive) immune response. In collaboration with Dr. Earl Brown (a virologist from BMI, University of Ottawa), I next tested whether *Lrrk2* affects the response to systemic infection by the neurotropic virus, respiratory orphan (reo) Serotype 3 Dearing (T3D) virus, in mice carrying different *Lrrk2* genotypes. Note, the employment of reoT3D virus was designed to test a function for *Lrrk2* in innate immune defenses against microbes rather than to test whether a reovirus causes PD.

Loss of olfaction and constipation are reported in PD patients, in fact, the neuronal structures of these organs are affected by the disease before the neurodegeneration of the brain (and the related motor-symptoms) are often diagnosed (Chaudhuri et al. 2006; Shulman et al. 2001; Scheperjans et al. 2015). Changes in gut microbiota of PD patients are also reported (Scheperjans et al. 2015). As proposed by the dual-hit hypothesis of Braak and colleagues, nasal route and gut both provide entry for a pathogen/toxin into susceptible individual, which leads to migration of these agents into the midbrain over time and finally the development of PD as the final outcome (Hawkes et al. 2007; Hawkes et al. 2009). Hence, the intranasal inoculation of reoT3D virus in *Lrrk2* mouse models is relevant to understanding the etiology of *LRRK2*-PD. Further reasons for suitability of this infection model for our study were previously covered in the Introduction section.

Previous experiments with reoT3D virus, which was carried out in WT mice (of the Balb-c strain), established the LD₅₀ of the virus to be in the 10⁵ pfu range (Gauvin et al. 2013). Infiltration of leukocytes was recorded in the lung and liver of virally infected pups (Figure 3.3.1A). Penetrance by and expression of the virus in enterocytes of the ileum of the young pups at days 3-7 were also observed as shown in Figure 3.3.1B. Immunostaining of sections of the CA3 area of the hippocampus and cerebellum from reoT3D-infected mice revealed expression of viral proteins in neurons of 13 day-old pups, as detected by a specific anti-reoT3D antibody (Figure 3.3.1B). These results identified the model as exceptionally suitable to test a function for endogenous *Lrrk2* in innate immune defenses.

For the objective of this research, we compared WT to *Lrrk2* knockout mouse pups initially. In these experiments, *Lrrk2* deficient mouse pups, as well as control wild-type pups (WT), were intranasally inoculated with a reoT3D virus dose (1.7 x 10⁵ pfu), which was previously determined to efficiently cause encephalitis after dpi >8 (Gauvin et al. 2013). For the first cohort/pilot study in homozygous and wild-type mice (in 2013), mouse litters were treated with various doses of the virus just to confirm the mortality seen in Balb-c strain of mice (Gauvin et al. 2013) was valid in our C57/B6 strain of mice as well (Figure 3.3.10). A more thorough and rigorous study of viral inoculation using a fixed dose of virus was then conducted in the second study cohort, which was generated by the crossing of heterozygous pairs (in 2014), for which the data were analyzed in more detail including the effect of sex.

Two key parameters used to evaluate the degree of infection were 1) survival; and 2) viral load. The disease manifestation and survival rate were evaluated by changes in weight, postural instability, lack of gait control (i.e., increased or decreased movement), other changes (such as tremulousness), and death due to encephalitis. Different mouse tissues

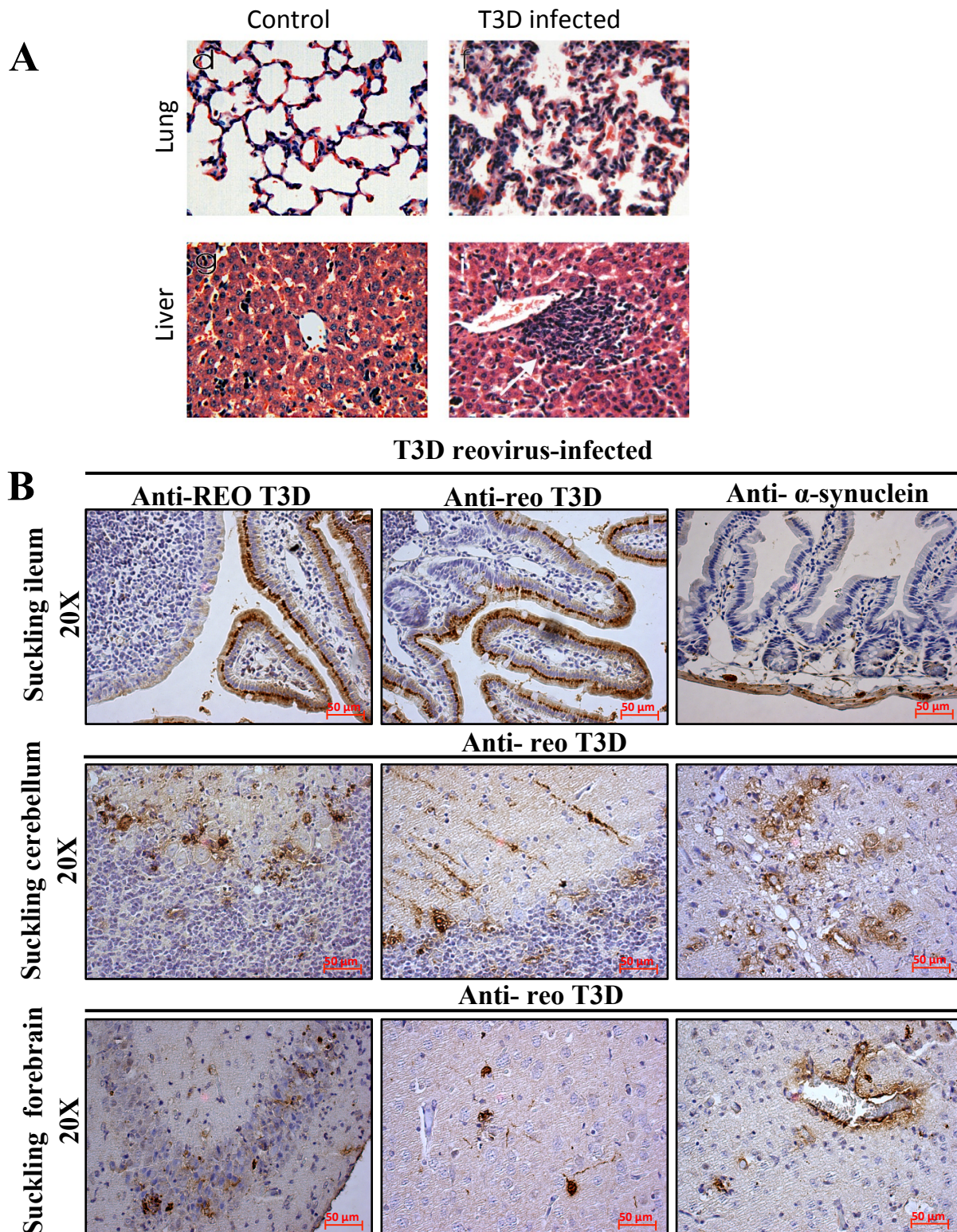


Figure 3.3.1. Pathology of T3D reovirus infected lung, liver, gut and brain in WT mice. Two-day old suckling mice ($n \geq 2$) were infected by nasal inoculation with T3D reovirus and processed for routine immunohistochemistry using anti-reo T3D antibody 13 dpi (A-B). Areas of leukocytic infiltration of infected lung and liver are shown by white arrows, A (Gauvin et al. 2013). Note, immunoreactive changes (brown, DAB) are seen in infected epithelial cells of ileum, in Purkinje cells and neurites of the cerebellum (areas of neuronal death), in CA3 neurons of the hippocampus, as well as in neurons and arterioles of the cortex (B) (images were generated by M. Hakimi and Dr. E. Brown).

(e.g., whole skull, lung, gut, liver, spleen, kidney) were harvested at different time points; 0, 3, 5, 7, 8, 11 days post inoculation (dpi) for each genotype. A control group composed of mock treated litters was generated for each genotype and tissues were similarly collected at various time points. Harvested organs were processed for histological studies and determination of viral titers both with an appropriate number of animals. All experimental techniques for the viral infection model were developed by Dr. Brown's team, as described in (Gauvin et al. 2013).

3.3.1 *Lrrk2* Deficiency Confers Minor Susceptibility To Higher Viral Load In Mouse Lungs at the end of pneumonitis

Viral growth in organs of *Lrrk2* KO versus WT mice was assessed by plaque assays following intranasal inoculation of the mice with 1.7×10^5 pfu of the reoT3D virus. In establishing the reovirus infection model in *Lrrk2* KO mouse colony, select organs were initially harvested from animals that were infected and later collected for different time points. Plaque assays conducted in those organs determined viral titers, as shown in Figures 3.3.2. As expected, the highest load of virus was found in lungs as it was the primary organ site of infection (Figure 3.3.2). Furthermore, the results show that the virus was able to reach other organs, such as liver, kidney, gut and spleen, at the early time point of 3 days post infection (dpi). Virus accumulation in various mouse organs, such as liver, kidney, and brain continued through day 5 pi (Figure 3.3.2 D). As expected from our previous studies (Gauvin et al. 2013), most organs began to effectively clear the virus in the course of infection. This was seen for example in the viral titers of the liver of KO mice, which significantly decreased on day 7 when compared to day 3 pi (P-value <0.03), Figure 3.3.2 B.

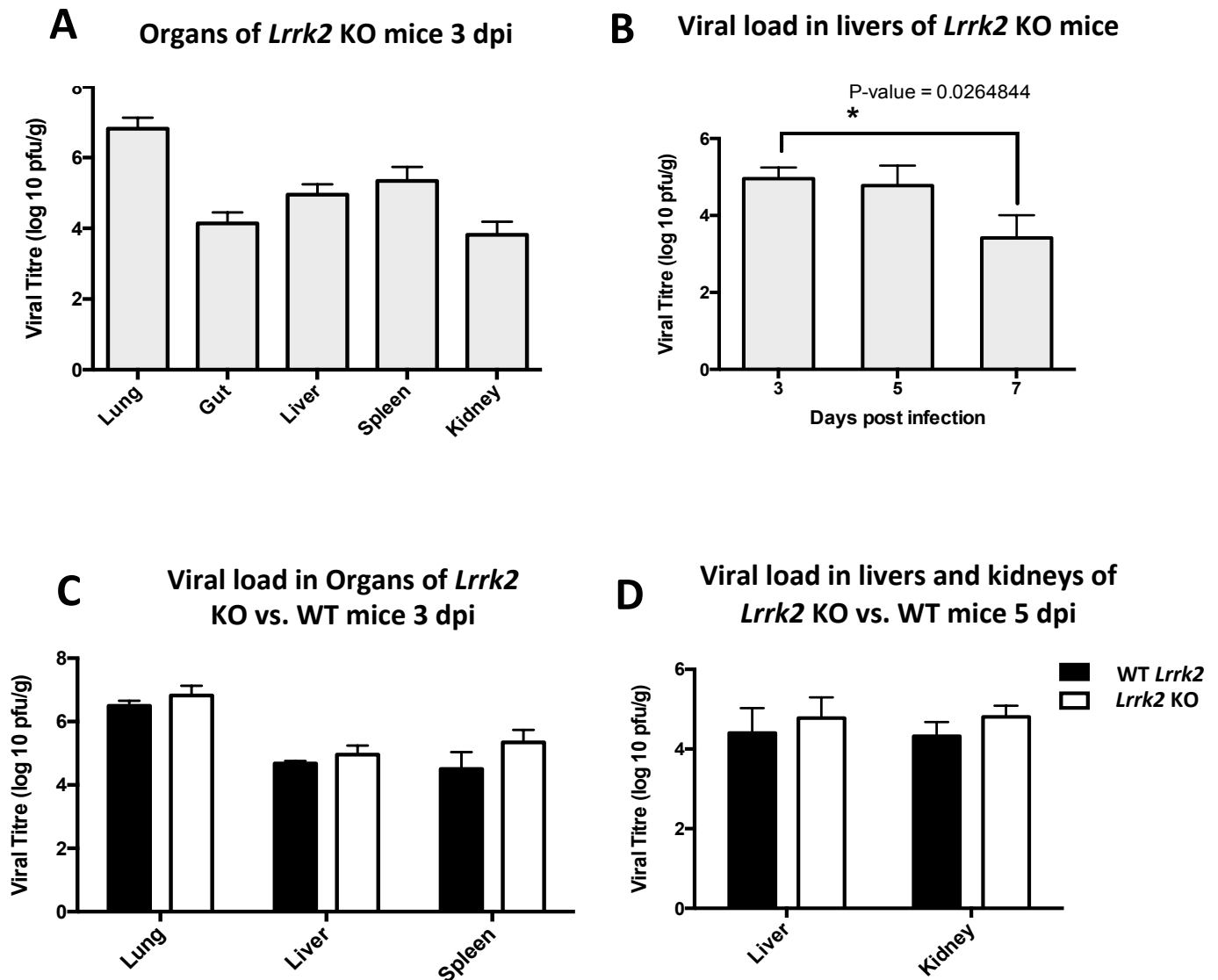


Figure 3.3.2. T3D reovirus titers in organs of *Lrrk2* KO and WT mice. Suckling pups from both mouse genotypes were inoculated with 1.7×10^5 pfu of the virus. Various organs were harvested and homogenized for different time points post infection (n=3 for most organs). Note significant reduction of virus titer in the livers of the *Lrrk2* KO mice from day 3 today 7 post treatment (p-value = 0.0264844). Absence of *Lrrk2* did not produce statistically significant differences in viral titers of the organs analyzed for D3 and D5 compared to WT (C-D).

The focus of the next experiments was to compare virus burden between the genotypes in homogenates of lungs (the primary organ of infection) and brains (encephalitis) of animals from a separate cohort of inoculated mice at the early time points, 3 dpi, 8 dpi, and the late time point, 11 dpi (Figure 3.3.3). On day 3, viral titer was the highest in the lungs during the course of infection, and both genotypes had similar virus load. On days 8 and 11, both genotypes had decreased T3D reovirus in their lungs; however, the *Lrrk2* null lungs had significantly more virus than the WT lungs on day 11 (P-value: 0.0156391). The viral load in the brains of both murine genotypes increased over time (3-11 dpi), but it did not significantly differ between *Lrrk2* KO and WT tissues.

3.3.2 ReoT3D Virus Pathology Is Observed In *Lrrk2*-Deficient and WT Mice

IHC results of *Lrrk2* KO and WT mice revealed reovirus reactivity in lungs and olfactory epithelium (OE) of mice 3 dpi (Figure 3.3.4). Analysis of the mouse skulls on dpi 1-7 showed viral reactivity predominantly in OE, and to a lesser extent in the associated lamina propria as well as in extra- (and in rare cases, intra-) cranial segments of nerve 1 (CN1), which were positive for the presence of reoviral protein. Gradual movement during 2-4 dpi of the virus from the epithelium toward the olfactory bulb was observed in skull sections. Microglial recruitment/activation was detected at this time point in the lamina propria and adjacent structures, as determined by a microglial (and macrophage) marker, Iba1. By dpi 5, some destruction of OE (cell loss) and reduced viral signal in this region were noted, and repair of the OE usually detected on dpi 7. All viral staining was confirmed by appropriate controls (e.g., untreated groups and mock treated groups).

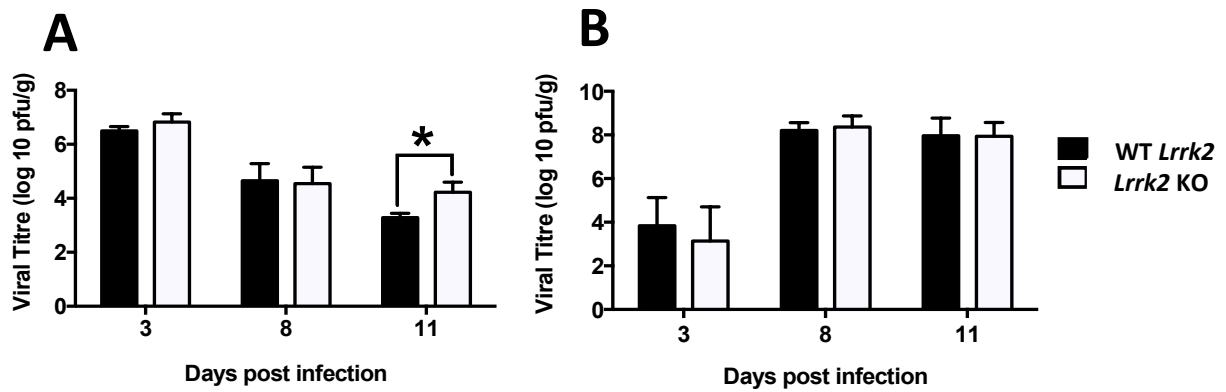


Figure 3.3.3. Viral load in lungs (A) and brains (B) of *Lrrk2* KO and WT mice. The mouse organs were harvested on days 3, 8 and 11 post treatment with 1.7×10^5 pfu T3D reovirus. n=3 to 5 per genotype per treatment (dpi) for the lungs and n=3 to 7 per genotype per treatment (dpi) for the brains. Note significant increase of the virus titer in D11 lungs of the KO animals, P-value = 0.0156391.

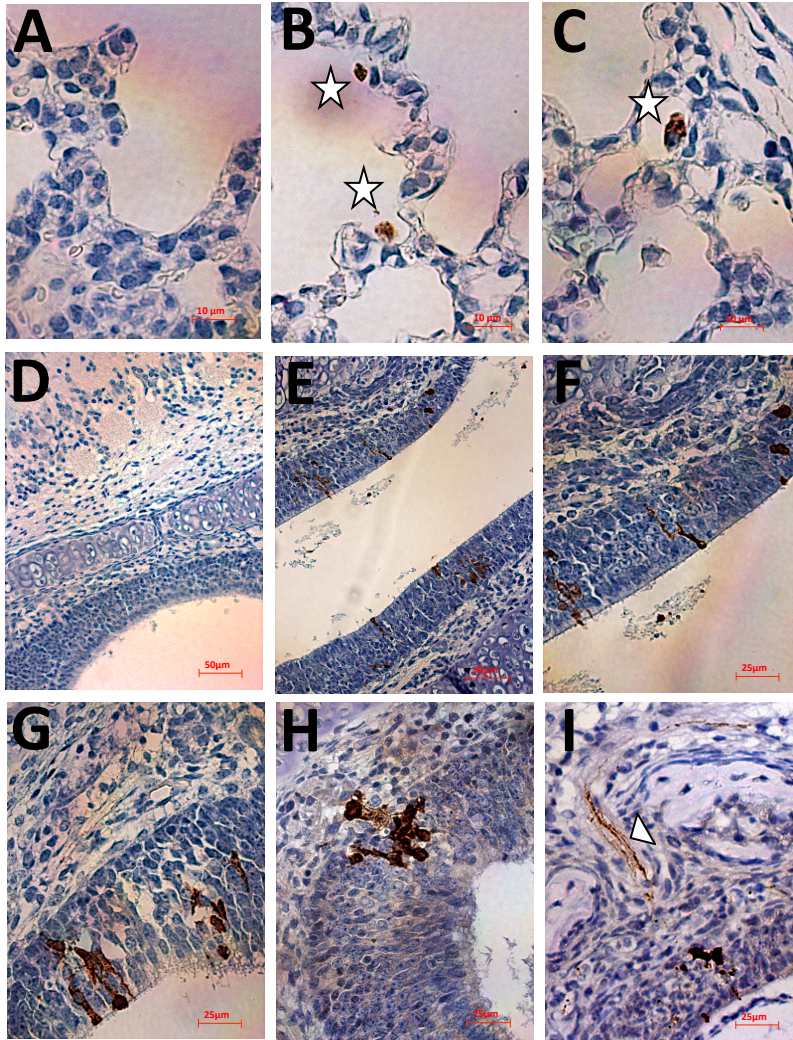


Figure 3.3.4. Reo virus staining of *Lrrk2* KO mouse lungs and olfactory structures 3 dpi. One day old mouse pups were inoculated with 1.5×10^5 pfu T3D reovirus and sacrificed 3 dpi for assessing virus infection. Virus reactivity is seen in lungs of infected pups (B-C, n=2), representative positive cells are shown by the star. Mock infected lungs (A, n=2) did not shown any reo antibody reactivity. Prominent virus staining is shown in olfactory epithelium of infected pups (E-I, n=2) compared to mock treatment (D, n=2). Uptake of the virus by the olfactory receptor cells (ORC) is depicted in all images of infection (E-I). Marked staining of CN1 with reovirus is shown by arrow heads (I). Images were obtained using original magnifications of 63x (A-C), 40x (F-I) and 20x (D and E).

Note, images are shown from *Lrrk2*-deficient animals. Corresponding images obtained from WT mice showed the same results.

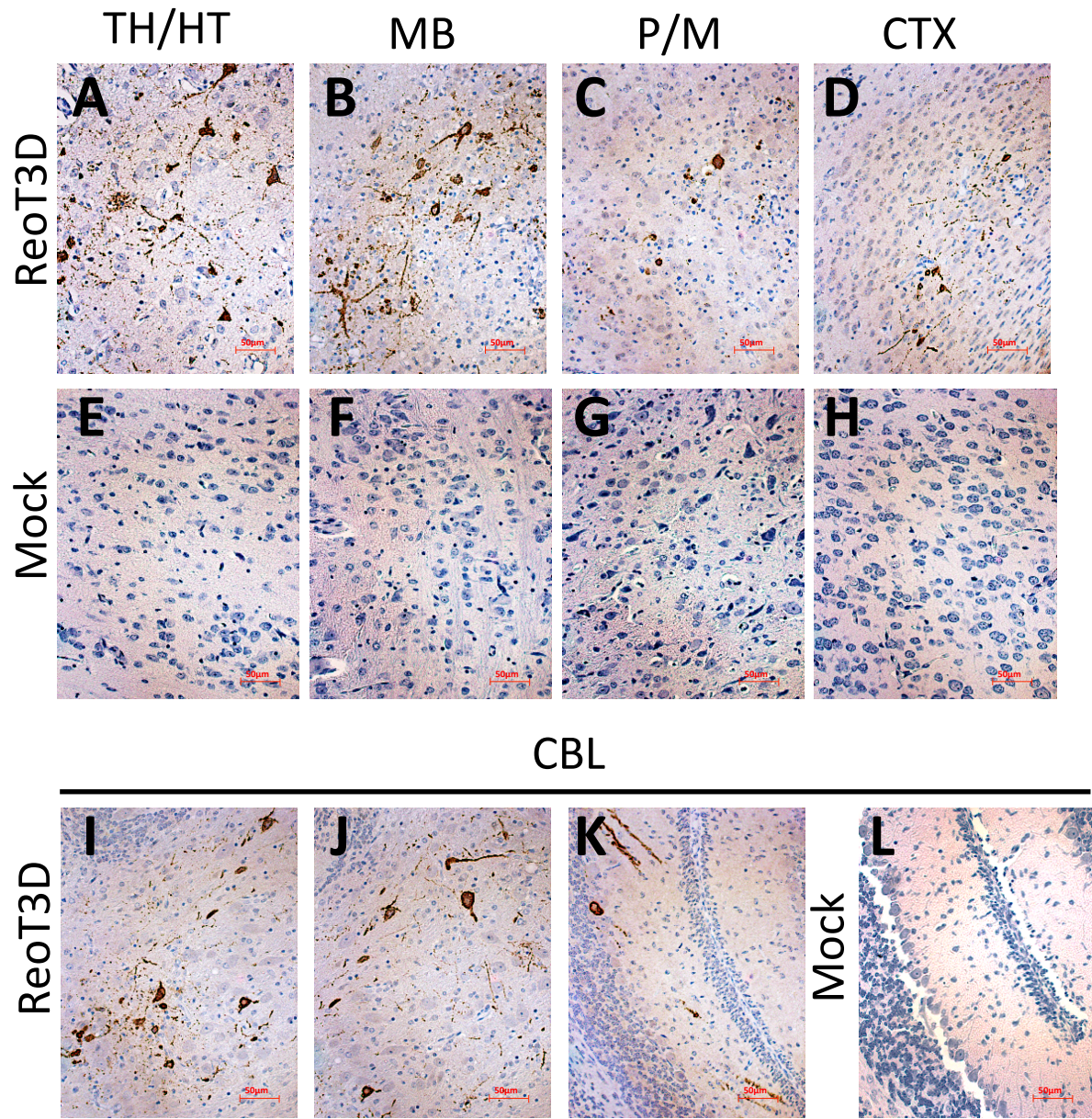


Figure 3.3.5. Reovirus staining of *Lrrk2* KO mouse brain 11 dpi. Virus reactivity was seen in brain stem pons and medulla (P/M), cerebral cortex (CTX), cerebellum (CBL) and most abundantly in thalamus/hypothalamus (TH/HT) and midbrain (MB) of mice ($n>5$) 11 dpi. Reo stain was seen in neuronal and microglial structures and leukocytes. Mock-treated animals ($n>5$) were used as control for infection and were devoid of any reo staining as shown. Images were obtained using original magnification of 20x.

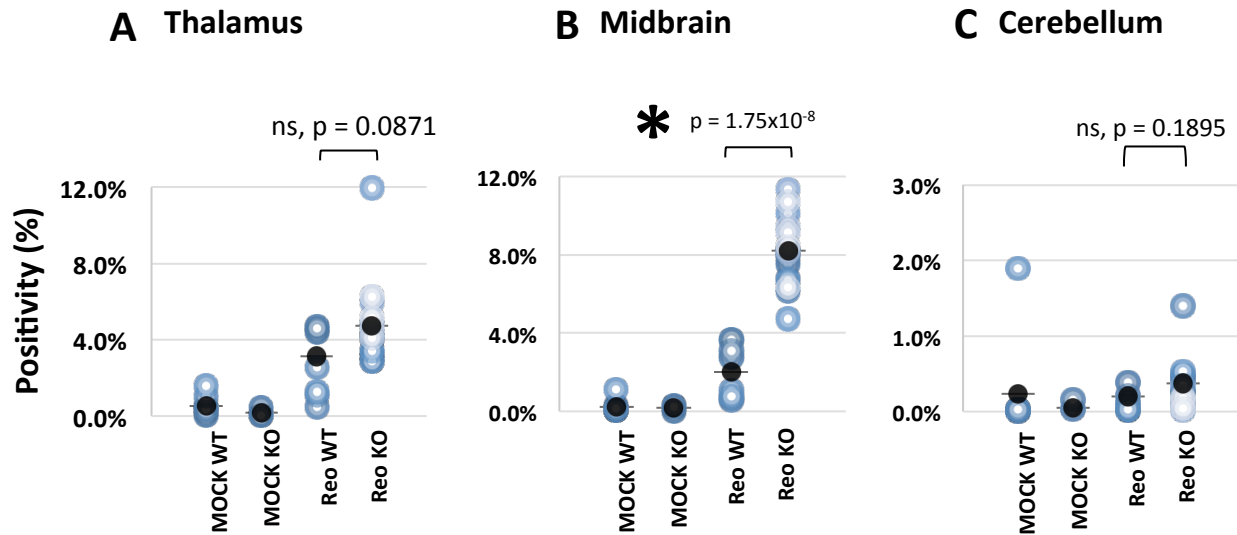


Figure 3.3.6. Quantification of the IHC data from the brains of *Lrrk2* KO vs. WT mice 11 dpi.

(A) Percent infection in thalamus. Positivity (%) vs Treatment-Genotype categories; comparing MOCK WT, MOCK KO, Reo WT, Reo KO. No significance between Reo WT and Reo KO; $p = 0.087115$ using Student's t-Test with two-tailed distribution, two-sample unequal variance. Category average denoted with black markers. Separate samples denoted with blue markers: MOCK WT std dev = 0.005157, $n = 8$ (representative of 2 animals); MOCK KO std dev = 0.001594, $n = 4$ (representative of 2 animals); Reo WT std dev = 0.018902, $n = 8$ (representative of 2 animals); Reo KO std dev = 0.020400, $n = 18$ (representative of 2 animals).

(B) Percent infection in midbrain. Positivity (%) vs Treatment-Genotype categories; comparing MOCK WT, MOCK KO, Reo WT, Reo KO. Significance between Reo WT and Reo KO; $p = 1.75 \times 10^{-8}$ using Student's t-Test with two-tailed distribution, two-sample unequal variance. Category average denoted with black markers. Separate samples denoted with blue markers: MOCK WT std dev = 0.003568, $n = 8$ (representative of 2 animals); MOCK KO std dev = 0.001009, $n = 4$ (representative of 2 animals); Reo WT std dev = 0.012622, $n = 8$ (representative of 2 animals); Reo KO std dev = 0.016523, $n = 18$ (representative of 2 animals).

(C) Percent infection in cerebellum. Positivity (%) vs Treatment-Genotype categories; comparing MOCK WT, MOCK KO, Reo WT, Reo KO. No significance between Reo WT and Reo KO; $p = 0.189594$ using Student's t-Test with two-tailed distribution, two-sample unequal variance. Category average denoted with black markers. Separate samples denoted with blue markers: MOCK WT $n = 8$ (representative of 2 animals), MOCK KO $n = 4$ (representative of 2 animals), Reo WT $n = 8$ (representative of 2 animals), Reo KO $n = 18$ (representative of 2 animals). Quantification of IHC data and statistical analysis were performed by I. Harmsen. Animals treatments and tissue processing were performed by M. Hakimi.

Table 3.3.1. Forest plot of odds ratios for T3D reovirus protein positivity in organs of *Lrrk2*-null vs. WT mice. CC: cerebral cortex, TH: hypothalamus, MB: midbrain, BT: brainstem other than midbrain.

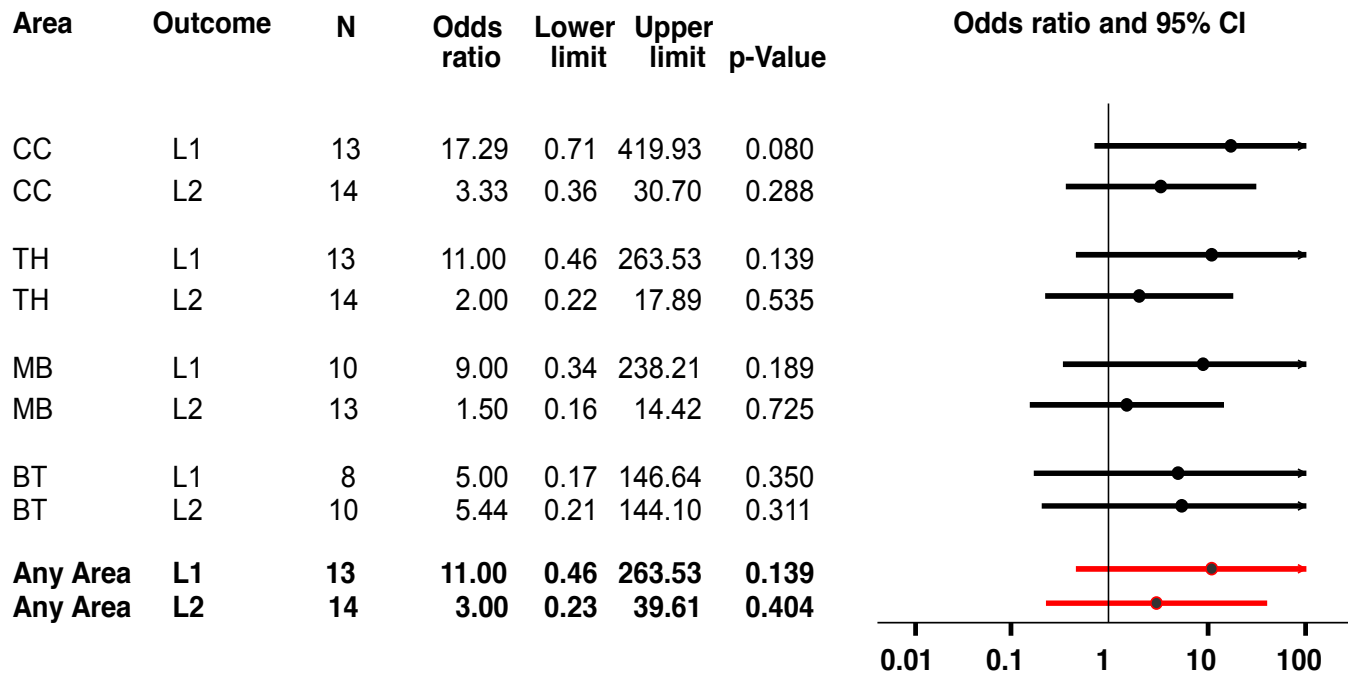


Table 3.3.2. Number of experimental samples used for calculation of odds ratio for T3D reovirus protein positivity in organs of *Lrrk2*-null vs. WT mice. These data pertain to the results presented in Table 3.3.1. CC: cerebral cortex, TH: hypothalamus, MB: midbrain, BT: brainstem other than midbrain. Statistical analysis for this data Table was conducted by Centre for Practice-Changing Research (OHRI, ON). Quantification of data was performed by I. Harmsen. Animal inoculation and tissue preparation were conducted by M. Hakimi.

Brain Area Level 1	KO total (n)	KO reo (n)	WT total (n)	WT reo (n)
CC (L1)	5	5	8	3
CC (L2)	6	4	8	3
TH (L1)	5	5	8	4
TH (L2)	6	4	8	4
MB (L1)	4	4	6	3
MB (L2)	5	3	8	4
BT (L1)	3	3	5	3
BT (L2)	3	3	7	4
All brain areas taken together (L1)	5	5	8	4
All brain areas taken together (L2)	6	5	8	5

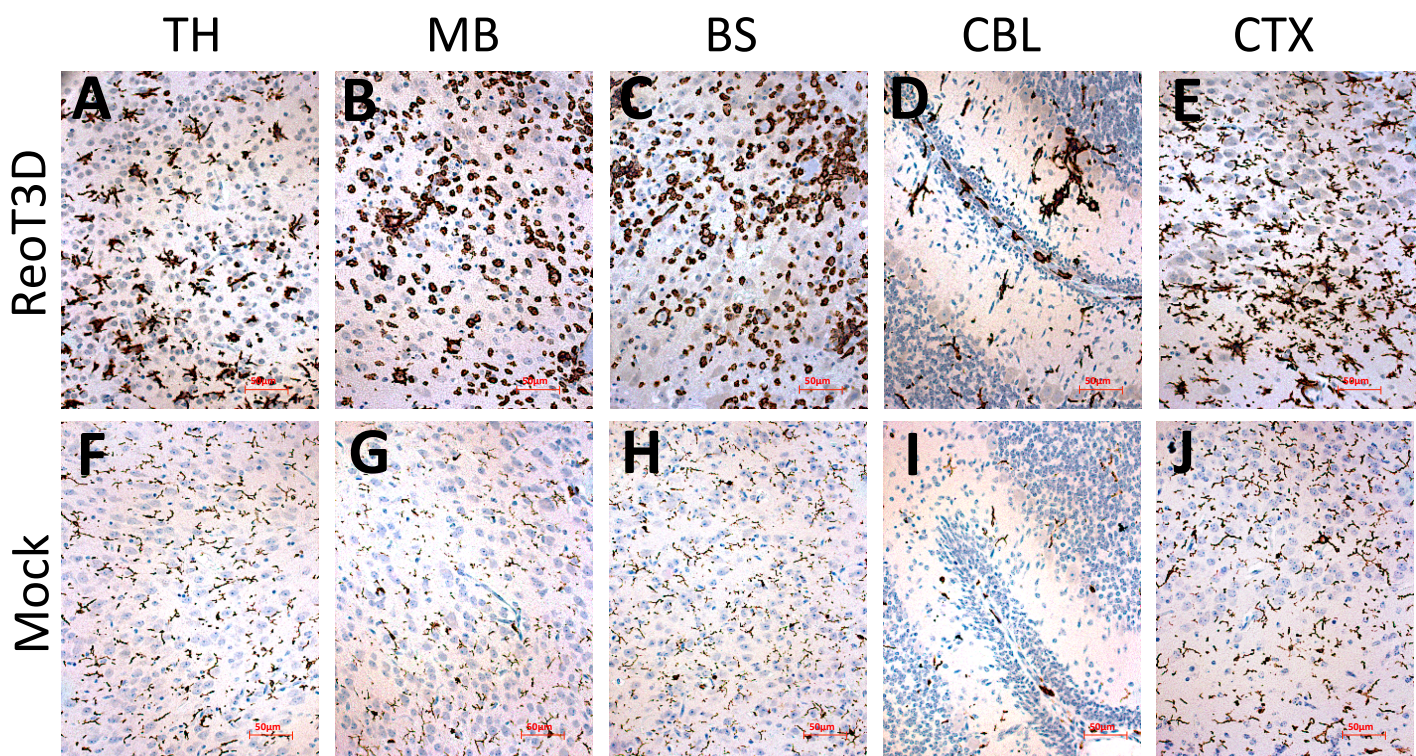


Figure 3.3.7. Upregulation of microglial response in reo infection of *Lrrk2* KO mice 11 dpi. Profound signal for microglial marker Iba1 was seen in reoT3D infected animals (n=2) which is indicative of microglial recruitment and activation in all brain regions examined as shown. Mock treated animals (n=2) did not show such intense Iba-1 signal (F-J). Images were obtained using original magnification of 20x.

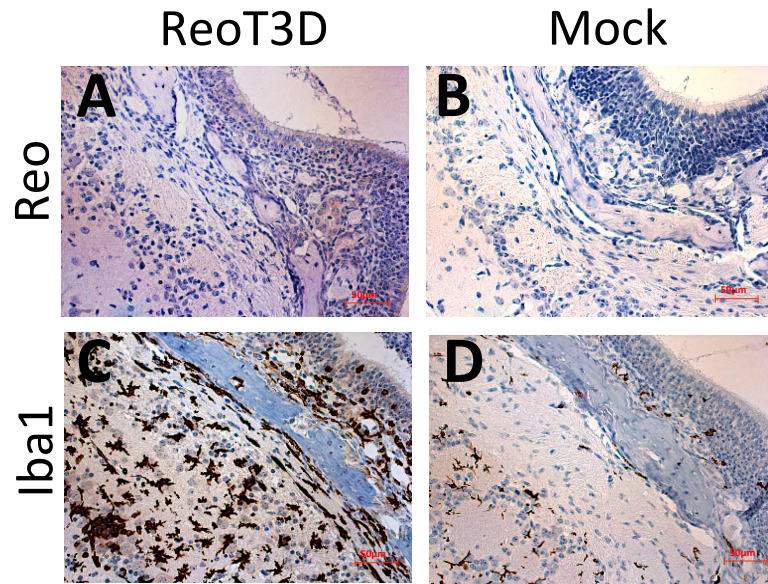


Figure 3.3.8. Sustained induction of microglial signal in olfactory epithelium (OE) and bulb (OB), despite clearance of viral signal, in reo infected *Lrrk2* KO mice 11 dpi. Reo staining of infected animals (n=2) show recovery of the nasal epithelium and clearance of viral signal in both OE and OB (A) compared to mock-infection (n=2). In contrast, profound Iba1 microglial signal persists in these regions of earlier virus infection (C). Images were obtained using original magnification of 20x.

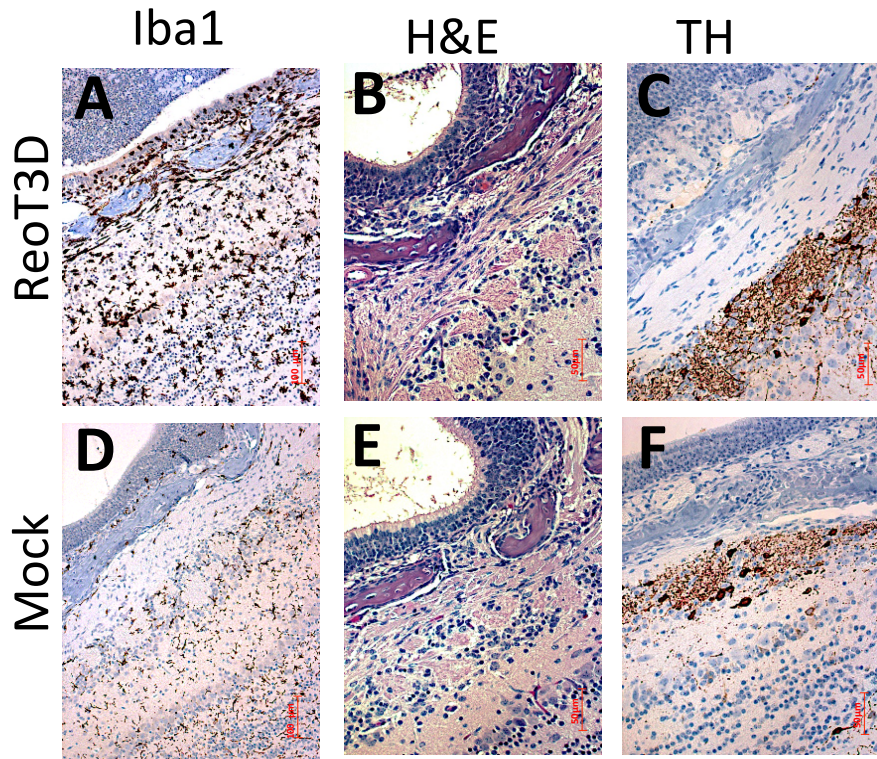


Figure 3.3.9. Histological examination olfactory epithelium and bulb in *Lrrk2* KO mice 11 dpi. Robust microglial Iba1 signal persisted in the regions of earlier infection. H&E and dopamine (using TH antibody as a marker) staining showed by and large the olfactory epithelium and bulb to be rather normal in reo infection (n=2) compared to mock treatment (n=2). Images were obtained using original magnification of 10x (A, D) and 20x (B-C, E-F).

By day 11 pi, the virus –using hematogenous spread- reached many brain regions and caused extensive infection in areas of the cortex, thalamus, superior midbrain, CA1, CA3 (of the hippocampus), and cerebellum; representative images are shown in Figure 3.3.5. Note, that a similar distribution of virus staining and spread were seen for WT animals as well.

Select regions of *Lrrk2* WT and KO brains revealed reproducible, high infection rates: i.e., thalamus/hypothalamus, midbrain (in particular superior and inferior colliculi), pons, and to a lesser extent, the cerebellum. To better assess the data from the immunohistochemical experiments, the signal from related experiments were quantified using Aperio ImageScope software (Positive Pixel Count v9.1' algorithm; see Figure 3.3.6. The rate of neuronal infection at the time of euthanasia (11 dpi) was higher in KO mice for three regions examined. Mean percentage counts for anti-reo antibody-positivity in KO (versus WT) mice of thalamus, midbrain, and cerebellum measured 4.72(3.12), 8.20(2.00) and 0.37(0.20), respectively; corresponding *P*-values for the differences (0.17-6.20%) were calculated at 0.0871, 1.75×10^{-8} and 0.1895, respectively, in these three regions. Of the three brain regions analyzed, the midbrain generated significantly higher viral staining for the *Lrrk2* deficient animals. Of note, these findings were confirmed in a subsequent, separate cohort of inoculated animals from a HET X HET crossing experiment (conducted by I. Harmsen, E. Brown and M. Schlossmacher), and all data were included in the analysis for odds ratios (Table 3.3.1).

In concert with previous findings, infiltration of leukocytes was evident in regions of the brain that coincided with virus infectivity as indicated by H&E staining. Staining with a microglial marker (Iba1) showed strong upregulation/activation of microglia in various regions of the infected brains as expected (Figure 3.3.7). Interestingly, while day 11 pi

showed recovery of OE and lack of viral staining there, microglial activation remained at a heightened state in this region (Figure 3.3.8 and Figure 3.3.9).

Collectively, these collective results suggested to us a possible hematogenous route for T3D reovirus spread from the lung and GI tract into the brain, and to a lesser extent, a route via CN 1 from the OE into the brain, as previously proposed by (Gauvin et al. 2013).

3.3.3 *Lrrk2* Null Mice Show Reduced Survival Following Viral Inoculation

Nasal administration of T3D reovirus evokes a set of behavioral changes in infected mouse pups, such as weight loss, impaired mobility, ataxia, etc. Disease staging for infected mice for both genotypes is summarized in Table 3.3.3. Note, that video recordings were also made for reoT3D-infected *Lrrk2* KO versus WT mice (in addition to mock treated groups for each genotype) to capture and describe changes in the behavioral phenotype of mice and to potentially use them for scoring purposes in the future.

Next, the survival of *Lrrk2* deficient animals was compared with control WT mice as another outcome of the reoT3D virus infection model. As mentioned previously, the first cohort of survival study (2013) was conducted using various doses of the virus in *Lrrk2* KO vs. WT mice (Figure 3.3.10), which was to validate the mice mortality reported by (Gauvin et al. 2013). For the second cohort of the study (2014), a large number of mouse pups (n=85) were generated from crossing HET x HET parents, which led to all three desired genotypes (WT, KO, and HET) for the study (Figure 3.3.11). These animals were inoculated with a fixed dose of virus as opposed to the first cohort. The effects of heterozygosity for the *Lrrk2* gene were also assessed in this study. One-day-old pups were inoculated with 1.7×10^5 pfu virus as before, and their subsequent survival was monitored after that. Deletion of the *Lrrk2*

Table 3.3.3. Disease staging of T3D reovirus in *Lrrk2* KO and WT suckling pups.

0- 5 dpi	Normal, healthy looking, well-groomed, huddling (no obvious rhonchi or sneezes noted).
6-8 dpi	Hyperactive, agitation with spinning.
9-14 dpi	Shivering, lethargic, inaction, rest tremor, broad based stance, very ataxic gait with poor righting, death around day 12 - neurological signs of an encephalitis.

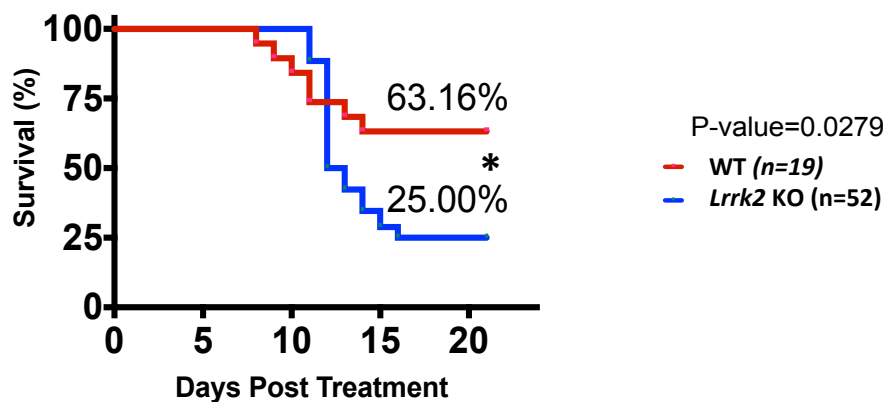
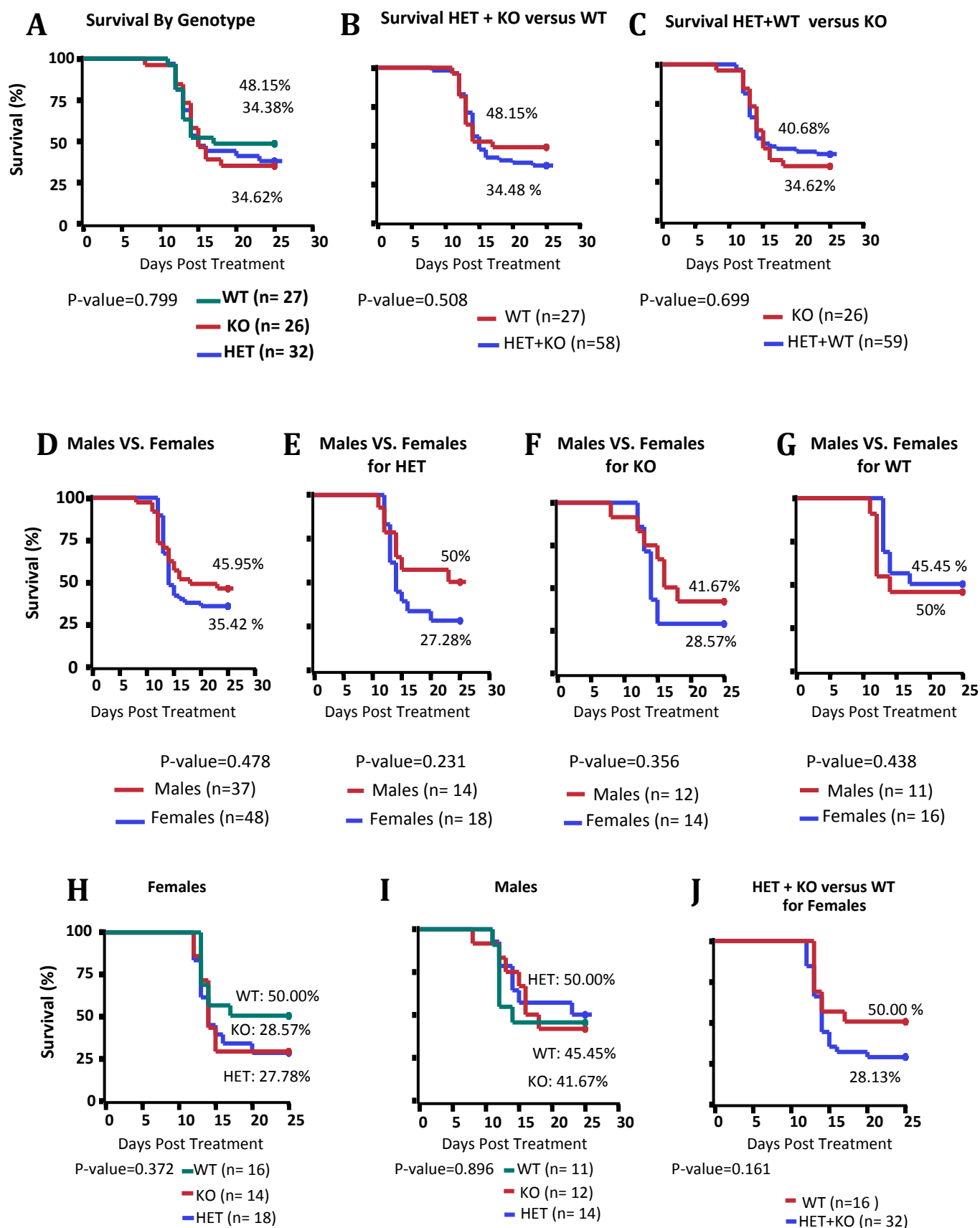


Figure 3.3.10. Survival study of *Lrrk2* KO versus WT mice in reo virus infection model (pilot study: 2013 Cohort). Mice were inoculated various doses of T3D reovirus and their survival was monitored for the effects of *Lrrk2* mutation. The cumulative survival for each *Lrrk2* genotype is shown here. A concentration-specific virus inoculation study was conducted in the 2014 Cohort. The results are shown in Kaplan-Meier plots and Log Rank test was used throughout for statistical analysis.



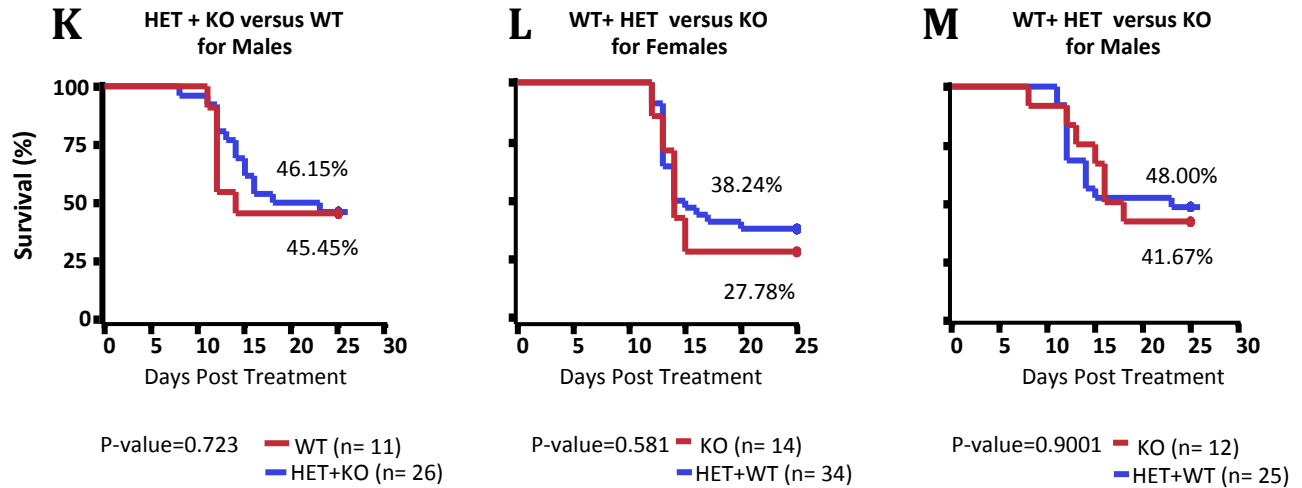


Figure 3.3.11. Survival study of *Lrrk2* KO versus WT mice in reo virus infection model. Mice were inoculated with 1.7×10^5 pfu of T3D reovirus and their survival was monitored for the effects of *Lrrk2* genotypes: homozygous *Lrrk2* KO vs. heterozygous *Lrrk2* KO (HET) vs. WT *Lrrk2*. The contribution of sex was also assessed in this study. The results are shown in Kaplan-Meier plots and Log Rank test was used throughout for statistical analysis; P-values are indicated below each plot. Collectively the survival difference of mice with variable LRRK2 genotypes did not produce statistical significance. Likewise, the genotypic variations did not significantly affect survival within each sex (male vs. female) analyzed.

gene in pups demonstrated decreased survival (34.62%) when compared to the WT control pups (48.15%) (Figure 3.3.11A). However, the genotype-dependent decrease in survival did not reach statistical significance (P-value= 0.799).

As seen in Figure 3.3.11A, animals with one copy of *Lrrk2* generated a response that appeared in between that recorded for KO and WT animals in their survival rate until day 23. However, after day 23 the heterozygous animals' mortality shifted toward that of the KO animals. When heterozygous animals were grouped with the KO animals, they generated a smaller P-value (0.508) than when being grouped with the WT (P-value= 0.699). This may indicate that the heterozygous animals behave more like the KO animals in this infection setting.

The effect of sex on animals' mortality was also examined in this cohort. Overall, females showed a trend for greater mortality (35.42%) compared to males (45.95%) of all genotypes, Figure 3.3.11D. Within the HET and KO genotypes, females once again showed the same reduced survival trend Figure 3.3.11E-F. In contrast, WT mice showed rather comparable death rates for both sexes generating P-value=0.438 (Figure 3.3.11G).

Within each sex (either males or females), the survival rates were similarly analyzed for *Lrrk2* genotype variations (Figure 3.3.11H-I). The KO animals showed greater mortality overall than the WT within each sex but more so for females.

In addition, the KO and WT mice exhibited greater differences in females as opposed to males (Fig. 10A-B). In other words, heterozygosity seems to not alter the survival of males as opposed to (possibly) in females by almost two folds, corresponding to the survival of 50.00% versus 27.78% respectively (Figure 3.3.11E). Nevertheless, statistical significance was not recorded for this difference.

The survival of HET pups appeared to segregate best with the KO pups and females as opposed to males (Figure 3.3.11H-I). Note that HET mice grouped with the KO mice and compared to WT group generated smaller P-values than when they were grouped with the WT and compared with the KO group. The difference between the HET+KO versus WT mice was greater in females than males, P-values of 0.161 versus 0.723 respectively, but the study was underpowered to demonstrate any statistical significance.

3.3.4 Risk Assessment For Viral Infection Outcomes in *Lrrk2*-Deficiency Indicates Potential Vulnerability For Viral Protein Burden, Neuronal Loss, And Mortality

The odds ratio (OR) estimates the association between exposure to an event and the outcome in helping to determine the causal effect between the two (Szumilas 2010). OR values can be used to statistically identify the extent to which a certain exposure or risk marker (ex., carrying a specific LRRK2 mutation) can be a risk factor for a certain outcome (ex., death from an infection) (Szumilas 2010). By that token, different risk factors can be quantitatively compared and ranked for the outcome of interest. In the calculation of odd ratios, an OR value of 1 means that there is no association between the exposure and outcome. An OR value of greater than 1 means that there is a higher probability of the outcome, and similarly, an OR value of less than 1 means that there is a lower probability of the outcome (Cummings 2009). To better substantiate our understanding of the outcomes of *Lrrk2* deficiency in T3D reovirus infection experiments, we measured the odds ratios for the results pertaining to viral protein positivity (Table 3.3.1 and Table 3.3.2), viral titers (Table 3.3.4), mortality (Table 3.3.5), and loss of neuronal expression (Table 3.3.6).

Table 3.3.4. Forest plot of odds ratio for T3D reovirus titer in organs of *Lrrk2*-null vs. WT mice.

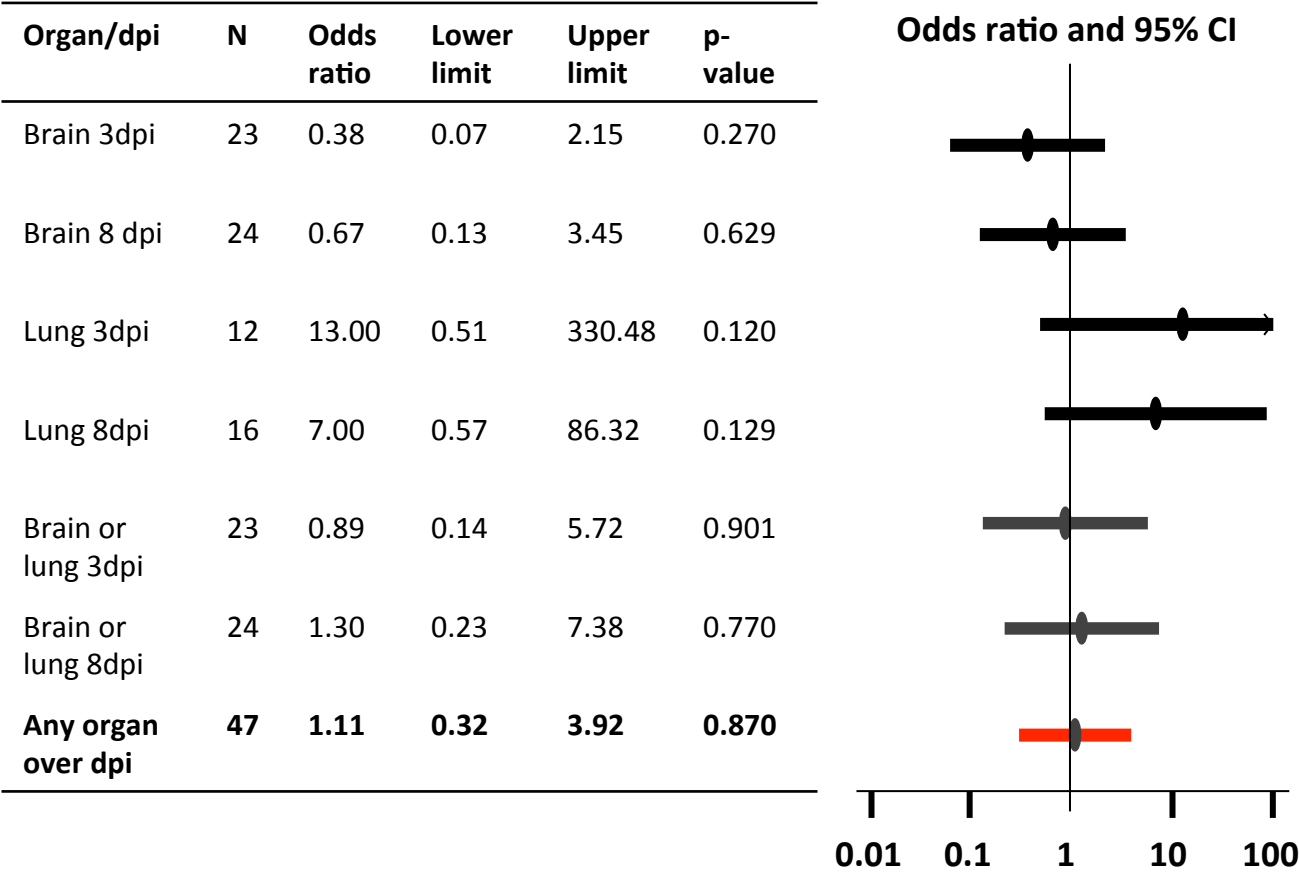
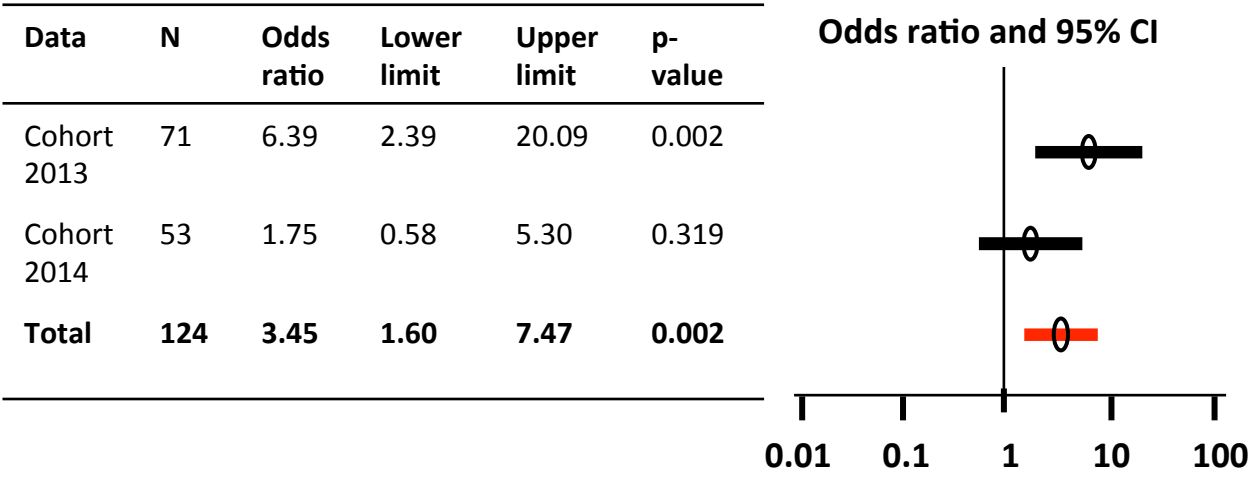
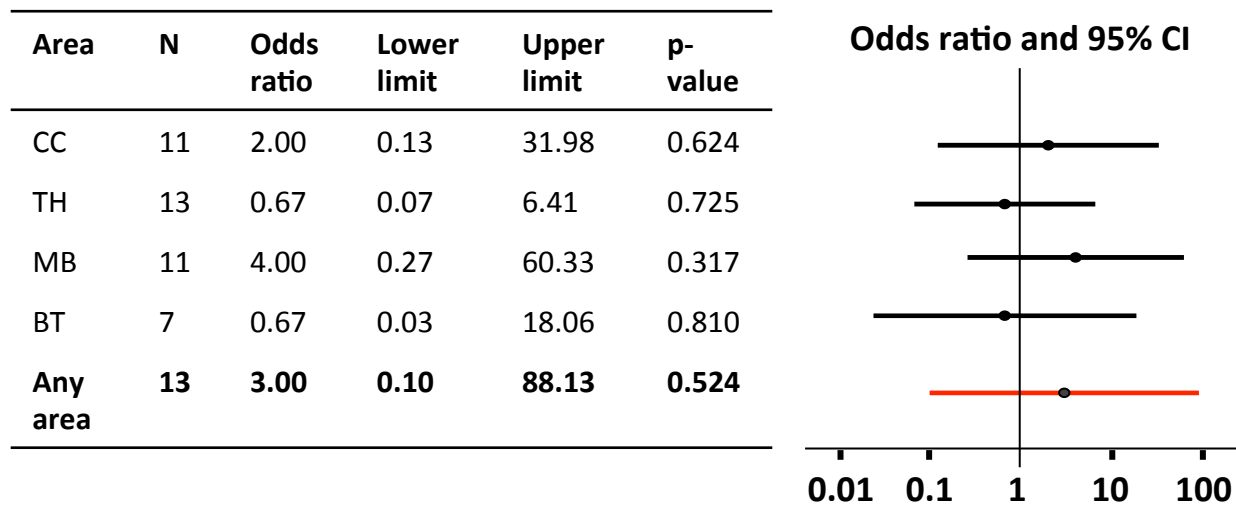


Table 3.3.5. Forest plot of odds ratio for mortality in *Lrrk2*-null vs. WT mice during T3D reovirus infection.



Statistical analysis for the results presented in Tables 3.3.1-3 was conducted by the Centre for Practice-Changing Research (OHRI, ON).

Table 3.3.6. Forest plot of odds ratio for loss of neuronal expression of A60 in *Lrrk2*-null vs. WT mice 11 dpi with T3D reovirus. CC: cerebral cortex, TH: hypothalamus, MB: midbrain, BT: brainstem other than midbrain. Statistical analysis for this data Table was conducted by Centre for Practice-Changing Research (OHRI, ON). Quantification of data was performed by I. Harmsen. Animal inoculation and tissue preparation were conducted by M. Hakimi.



Brain Area Level 2	KO total (n)	KO reo (n)	WT total (n)	WT reo (n)
CC	5	4	6	4
TH	6	2	7	3
MB	5	4	6	3
BT	2	1	5	3
Any Area	6	6	7	6

In assessing viral protein positivity in brains of reovirus challenged mice (11 dpi), anti-reo T3D antibody was in IHC experiments. Each brain was analyzed for two different levels, which corresponded to sagittal sections with the various distances from the brain midline. As explained previously, the histology slides were processed for quantification of the IHC staining using Aperio ImageScope software (conducted by Ms. Irene Harmsen). Next, the quantified IHC staining results were used to calculate OR for various regions of mouse brains (Table 3.3.1 and Table 3.3.2). All four brain regions, i.e., cerebral cortex, thalamus, midbrain as well as pons and medulla, generated an OR value greater than 1, meaning positive association between viral protein reactivity and *LRRK2* genotypes, as shown in Table 3.3.1. These OR results were based on increased viral staining observed in the knock-out brains shown in Figure 3.3.6.

Similarly, neuronal loss in reovirus-challenged mice was assessed by A60 antibody, which was used as a marker of neuronal expression in IHC experiments (Table 3.3.6). Quantification of neuronal staining was performed using Aperio ImageScope software. Thalamus and brainstem (pons and medulla regions) generated an OR close to 1.00 (0.67) indicating a lack of relative association between neuronal loss and *Lrrk2* genotype. In contrast, midbrain and cerebral cortex sections generated an OR greater than 1 (4.00 and 2.00, respectively), thereby indicating a positive association between neuronal loss and *Lrrk2* deficiency. Among the four brain regions analyzed, midbrain had the highest OR of neuronal loss, which is in agreement with previous results from virus staining in the brain sections analyzed (Figure 3.3.6). Midbrain, but not thalamus or cerebellum, had generated significant reovirus protein reactivity in *Lrrk2* KO mice 11 dpi compared to WT.

In assessing ORs for overall virus burden in the lungs and brains of all genotyped mice analyzed (Table 3.3.4), only the lungs generated an OR greater than 1 (13.00 and 7.00), but this was not significant (refer to Table 3.3.4) Thus a positive association for *Lrrk2* deletion and increased virus burden was only present in the lungs at the later time point, and not the brains. This was due to our plaque assay results (Figure 3.3.3), where a significant virus titer difference was recorded for 11 dpi *Lrrk2* KO lung tissue only compared to WT.

The virus-induced mortality recorded in the two survival cohorts, which I had performed, generated an OR of 3.45 for *Lrrk2* deficiency; note, its corresponding OR limit range (1.60-7.47) did reach significance ($P=0.002$) (Table 3.3.5). This finding was indicative of a positive association between *Lrrk2* deletion and mortality; thus *Lrrk2* deficiency is predicted to be disadvantageous for the animal in making it more prone to virus-induced death compared to WT.

Collectively, the assessment of ORs in *Lrrk2* deficiency vis a vis reovirus-associated disease outcomes suggested that the gene deletion represents a risk factor for the animals' worse outcome(s). The absence of *Lrrk2* negatively affected virus burden, neuronal loss and mortality in mice.

3.3.5 *Lrrk2* Deficiency Is Linked to Cytokine Alterations In Lungs And Brain Of Virus-Infected Mice

To revisit the issue of inflammatory signaling responses between the genotypes, we next compared organ lysates for their cytokine levels. The lungs (3 dpi) and brains (11 dpi) of *Lrrk2* deficient animals versus wild-type animals were studied for cytokine modulation

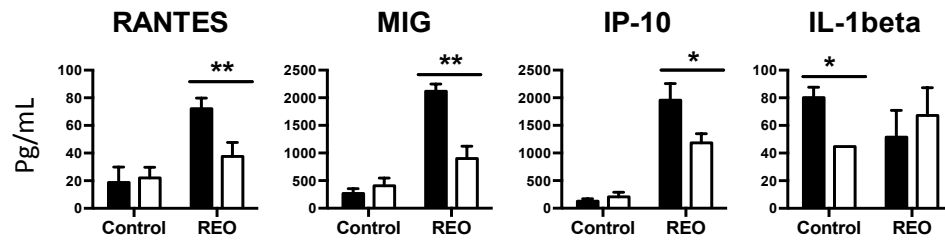
post viral inoculation, as described earlier. The reason for selection of these time points was that the viral titers were the highest in these organs during these particular times. The tissue homogenates from these organs were analyzed for 32 cyto-/chemokine levels processed by Eve Technologies. However, not all 32 cytokines were within the detectable range of the experimental procedure, thus only successfully detected cytokines are reported and analyzed here.

Figure 3.3.12-14 show the results of detected cytokines in tissue homogenates. The lungs of *Lrrk2* deficient animals showed statistically significant decreases in levels of RANTES (p-value = 0.009689), MIG (p-value = 0.001279) and IP-10 (p-value = 0.01696) after infection compared to those of WT mice. Also, the basal level of IL-1 β was significantly lower in the gene knock-out animals without infection; however, the viral inoculation model did not differentiate the genotypes for this cytokine as its level increased similarly in both groups. In contrast to the lungs, the brains showed significant increases in IP-10 (p-value = 0.01730) and IL-12(p-70) (p-value = 0.01859) as well as IL-4 (p-value = 0.01644) levels in *Lrrk2* KO animals treated with reoT3D virus. The results from other cytokines did not show genotype-dependent differences (Figure 3.3.13-14).

3.3.6 A-Synuclein Processing Of *Lrrk2* KO Mice Is Not Different From The WT Mice During Reovirus Infection

As a side experiment, the brains of *Lrrk2* KO versus WT mice were analyzed for their levels of total α -synuclein, which is a major component of Lewy bodies, a hallmark of PD (aggregated in Lewy bodies). Changes identified as “synucleinopathy” are found in many (but not all) cases *LRRK2*-PD cases (Kalia et al., 2016) and thus represent an area of interest

A Lungs lysate



B Brain lysate

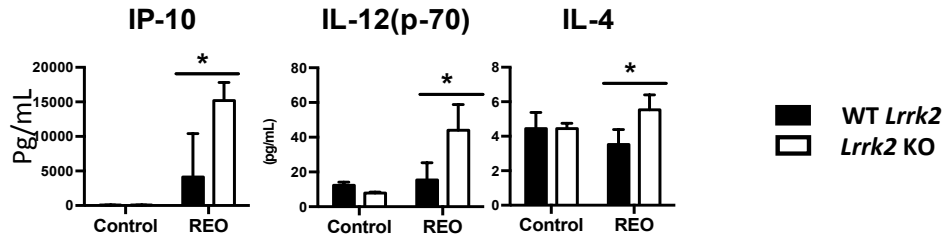


Figure 3.3.12. Results from pilot studies showing cytokine regulation in the lungs and brains of *Lrrk2* KO vs. WT mice during reovirus infection. The lungs (3dpi) and brains (11 dpi) were harvested from animals inoculated with 1.7×10^5 pfu of the T3D reovirus (n=3 per genotype). Cytokine levels were measured in tissue homogenates using multiplex assay. KO Lungs showed significantly decreased RANTES, MIG and IP-10 in infected animals and IL-1beta in mock treatments (i.e. basal condition). KO brains showed significantly increased IP-10, IL-12(p-70) and IL-4. Statistical significance is annotated by asterisks and their corresponding P-values are as following: RANTES: 0.009689, MIG: 0.001279, IP-10: 0.01696, IL-1beta: 0.02184, IP-10: 0.01730, IL-12(p-70): 0.01859, IL-4: 0.01644.

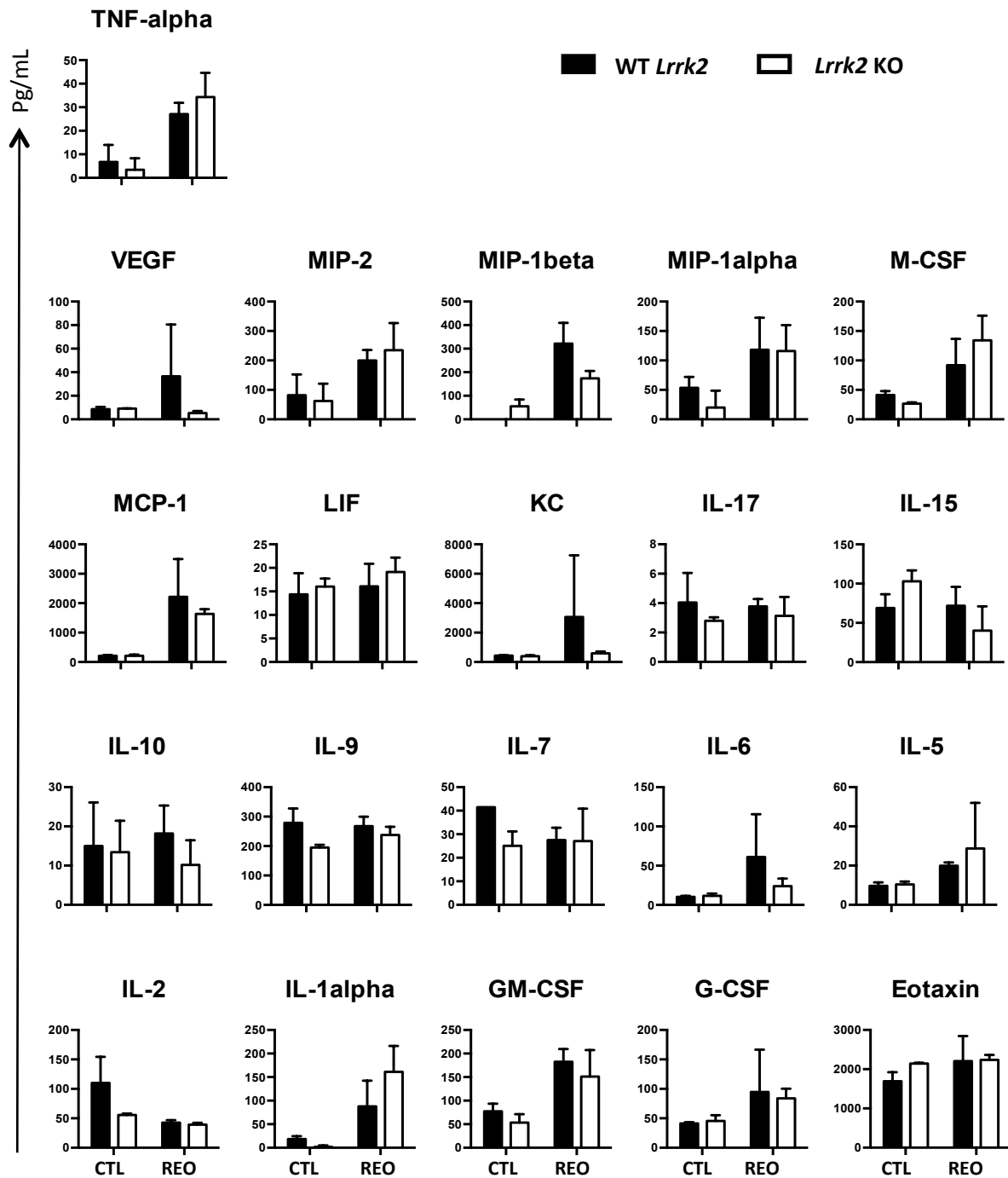


Figure 3.3.13. Results from pilot studies showing cytokine regulation in the lungs of *Lrrk2* KO vs. WT mice 3 dpi. The lungs were harvested from animals inoculated with 1.7×10^5 pfu of the T3D reovirus (n=3 per genotype). Cytokine levels were measured in tissue homogenates using multiplex assay.

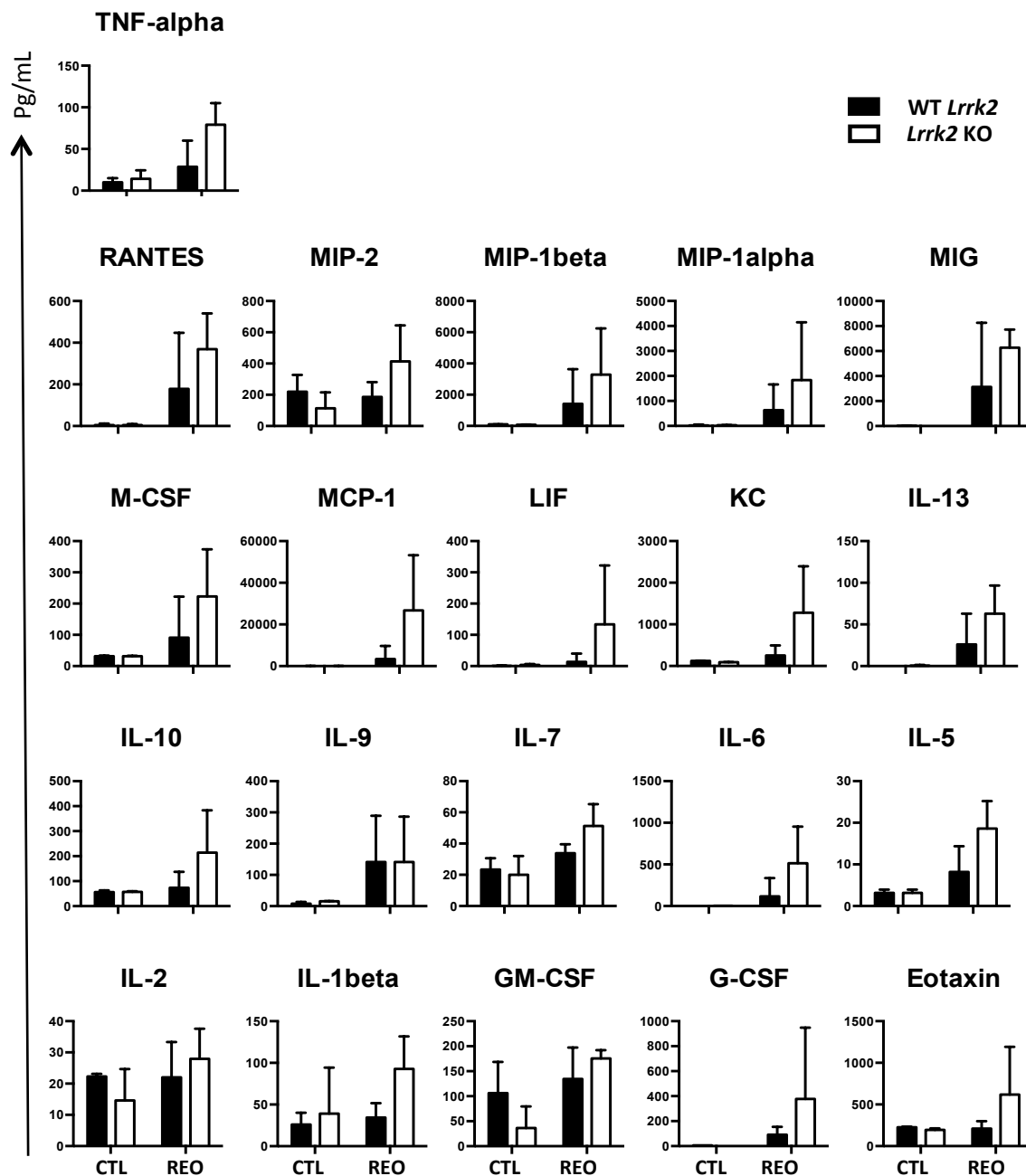


Figure 3.3.14. Results from pilot studies showing cytokine regulation in the lungs of *Lrrk2* KO vs. WT mice 11 dpi. The brains were harvested from animals inoculated with 1.7×10^5 pfu of the T3D reovirus (n=3 per genotype). Cytokine levels were measured in tissue homogenates using multiplex assay.

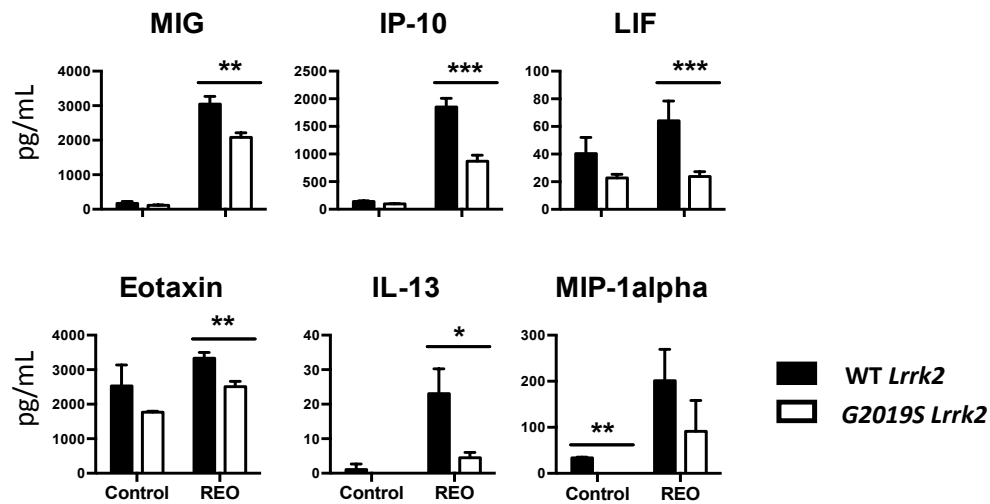


Figure 3.3.15. Preliminary data showing cytokine regulation in the lungs of G2019S *Lrrk2* vs. WT mice during reovirus infection. The lungs (3dpi) were harvested from animals inoculated with 1.7×10^5 pfu of the T3D reovirus (n=4 per genotype). Cytokine levels were measured in tissue homogenates using multiplex assay. G2019S *Lrrk2* Lungs showed significantly decreased MIG, IP-10, LIF, Eotaxin and IL-13 in infected animals and MIP-1alpha in mock treatments (i.e. basal condition). Statistical significance is annotated by asterisks and their corresponding P-values are as following: MIG: 0.002905, IP-10: 0.0008524, LIF: 0.009306, Eotaxin: 0.003365, IL-13: 0.01190, MIP-1alpha: 0.001278.

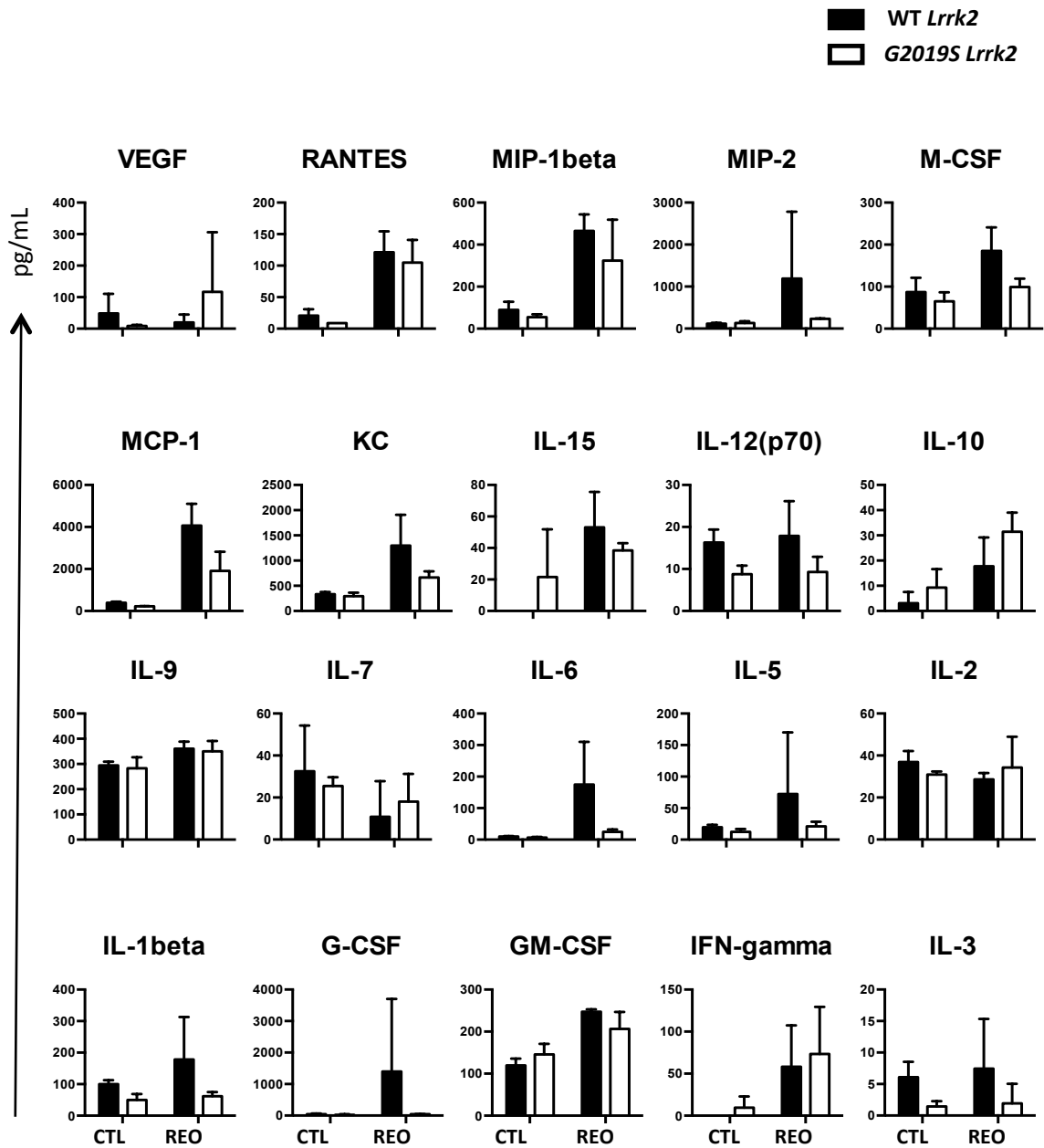


Figure 3.3.16. Preliminary data showing cytokine regulation in the lungs of *G2019S Lrrk2* vs. WT mice 3 dpi. The lungs were harvested from animals inoculated with 1.7×10^5 pfu of the T3D reovirus (n=4 per genotype). Cytokine levels were measured in tissue homogenates using multiplex assay.

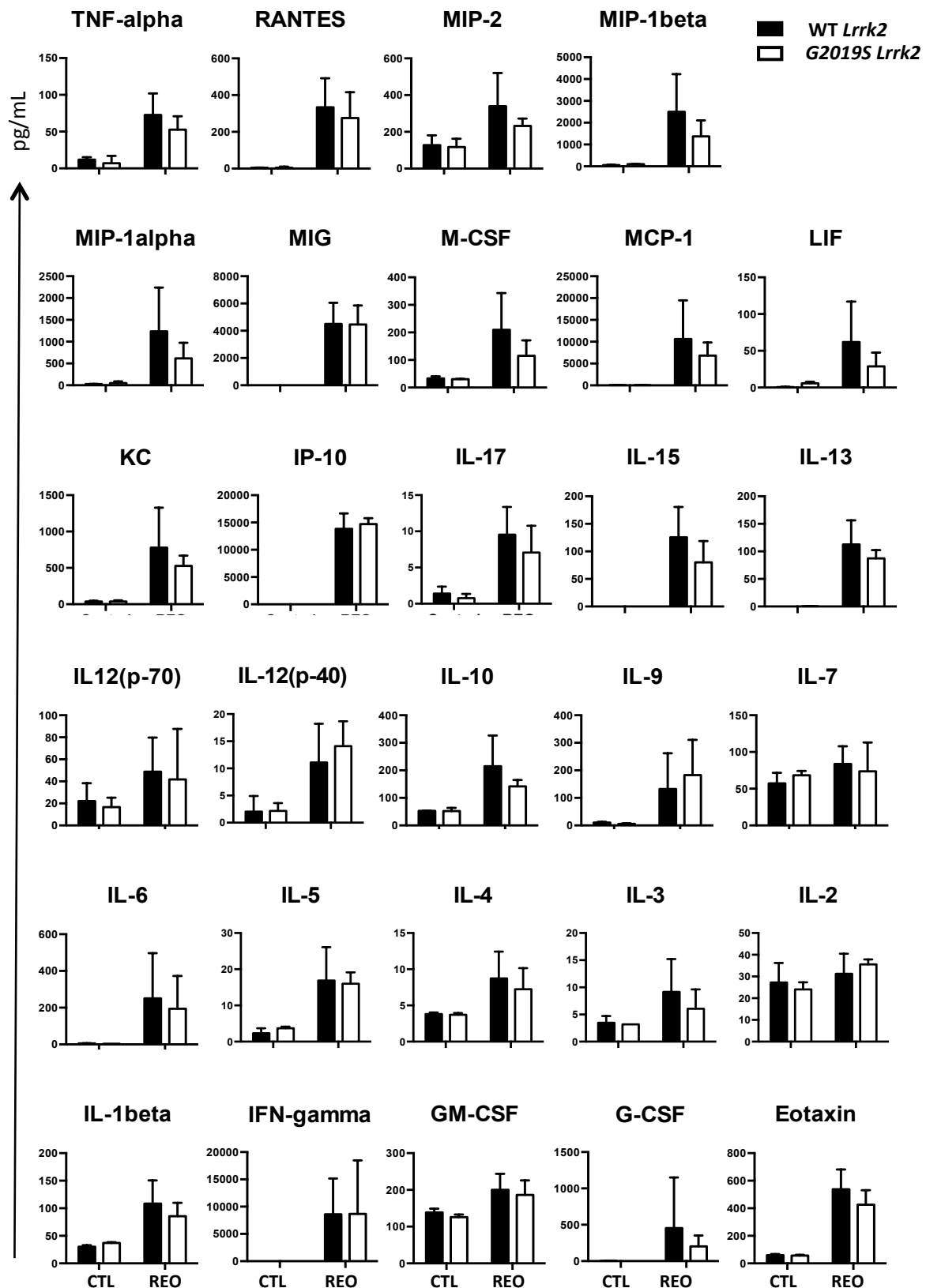


Figure 3.3.17. Preliminary data showing cytokine regulation in the brains of G2019S *Lrrk2* vs. WT mice 11 dpi. The brains were harvested from animals inoculated with 1.7×10^5 pfu of the T3D reovirus (n=4 per genotype). Cytokine levels were measured in tissue homogenates using multiplex assay.

in the pathological consequences of altered function downstream of mutant LRRK2. Accordingly, the measurement of total α -synuclein concentration, as performed by ELISA, was intended to link the findings from our encephalitis in *Lrrk2* model to the topic of synucleinopathy in PD. The results are shown in Figure 3.3.18. A significant increase in total α -synuclein levels was detected on 11 dpi compared to 8 dpi in both mouse genotypes Figure 3.3.18A. The viral infection by itself did not affect the levels of α -synuclein compared to mock treatment in either genotype during the time frame observed (and for the number of samples analyzed) Figure 3.3.18B. Furthermore, no genotype-dependent response was detected for total α -synuclein levels at either time points, as shown in Figure 3.3.18A-B.

3.4 Characterization Of G2019s *Lrrk2* Mouse Model Using In Vivo Reovirus Infection

3.4.1 G2019S *Lrrk2* Mutation Is Associated With Lower Viral Burden In The Lungs and Brain of Mice

Plaque assays from the lungs and brains of infected animals yielded an unexpected, but significant decrease in viral load in mutants' lungs at the early time point (3 dpi) and mutants' brains at the late time point (11 dpi) during the course of reoT3D virus infection – all compared to control WT mice (Figure 3.4.1). Note that the significant decrease is consistent in the two organs that were of primary interest to us (one, the site of initial lower airway infection; two, the organ responsible for mortality from encephalitis after the systemic infection had been cleared). Thus, the focus for viral titers determination had been kept on the brains and lungs of our mice.

From this observation and previous titration of viral load in mutant animals, we speculated that WT murine *Lrrk2* functions in an intermediate (i.e., in 'a neutral

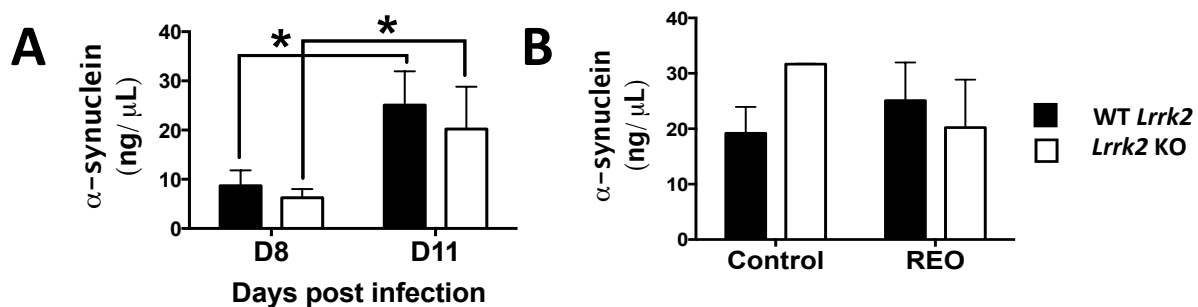


Figure 3.3.18. Alpha-synuclein changes in *Lrrk2* KO vs. WT mouse brains during reovirus infection. Mice were inoculated with 1.7×10^5 pfu of the T3D reovirus (REO) or media (control) and their brains were next collected, homogenized and analyzed for alpha-synuclein levels using ELISA. $n=2$ per genotype for control and $n=4$ per genotype for reo treated brain homogenates. Alpha-synuclein from brain homogenates of both virus infected WT and *Lrrk2* KO was significantly increased 11 dpi compared to 8 dpi; P-values were 0.0129836 and 0.0193842 for WT and *Lrrk2* KO animals respectively (A). Virus infection did not differentially affect alpha-synuclein levels 11 dpi when compared to control mock-treatment for the same time-point. ELSIA protocols were previously described in (Mollenhauer et al. 2008; Cullen et al. 2009; Cullen et al. 2011). ELISAs were performed by C. Jagow. Animal inoculations, tissue preparations and statistical analysis were conducted by M. Hakimi.

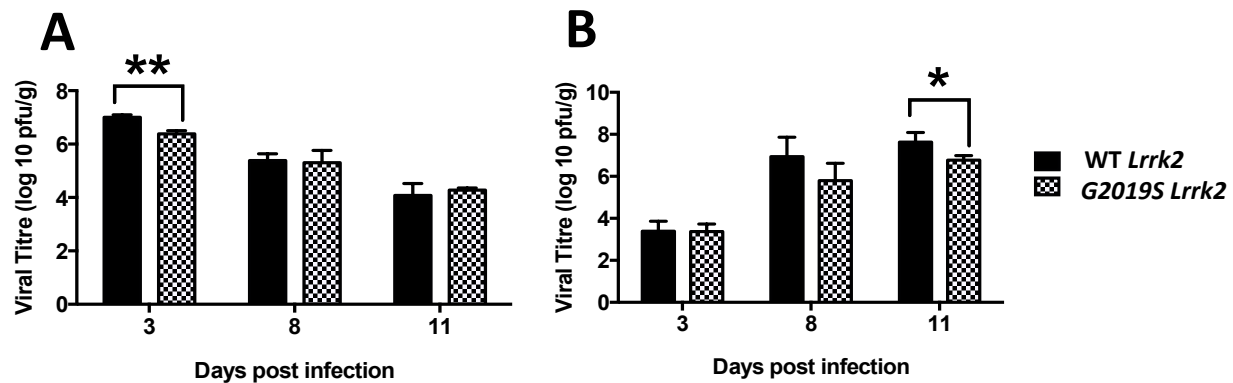


Figure 3.4.1. Viral load in lungs (A) and brains (B) of G2019S *Lrrk2* and WT mice. The mouse organs were harvested on days 3 , 8 and 11 post treatment with 1.7×10^5 pfu T3D reovirus. n=3 to 6 per genotype per treatment (dpi) for the lungs and the brains. Note significant decrease of the virus in D3 lungs (P-value: 0.00211764) and D11 brains (P-value: 0.0199893) of the mutant animals.

homeostasis zone') between G2019S and KO mutants in controlling inflammation. We, therefore, examined the effects of the G2019S mutant in another cohort of animals regarding their survival from encephalitis.

3.4.2 ReoT3D Virus Pathology Is Observed In G2019S *Lrrk2* Mutant and WT Mice

Based on our results of *Lrrk2* effects in the comparison of KO *versus* WT mice provided us with a plausible platform against which the behavior of PD-relevant pathogenic forms of *Lrrk2* can be studied. G2019S *LRRK2* mutation is the commonest variant associated with human PD, accounting for as many as 30-40% of all cases in certain populations, and ~1-2% of cases in North America. Accordingly, the same T3D reovirus infection model (1.7×10^5 pfu) was utilized to study immune function and infection outcomes of G2019S *Lrrk2* versus WT mice.

Similar to pathology data obtained in the *Lrrk2* KO colony, the G2019S mouse colony revealed infection of OE with T3D reovirus as of 3 dpi. Viral reactivity was observed in olfactory receptor cells, olfactory knobs, and axons of CN1 (Figure 3.4.2). A marked increase in microglial recruitment/activation was noted in OE of G2019S *Lrrk2* animals compared to mock-treatment (Figure 3.4.2).

Analysis at 11 dpi of mouse skull sections revealed robust virus reactivity in the thalamus, midbrain, pons and cerebellum (Figure 3.4.3 and Figure 3.4.4-5). Viral reactivity was seen in neurons, degenerating axons and dendrites, select glia and cellular debris including in the cortex, Purkinje cells of the cerebellum, microglia as well as in neuronal inclusions.

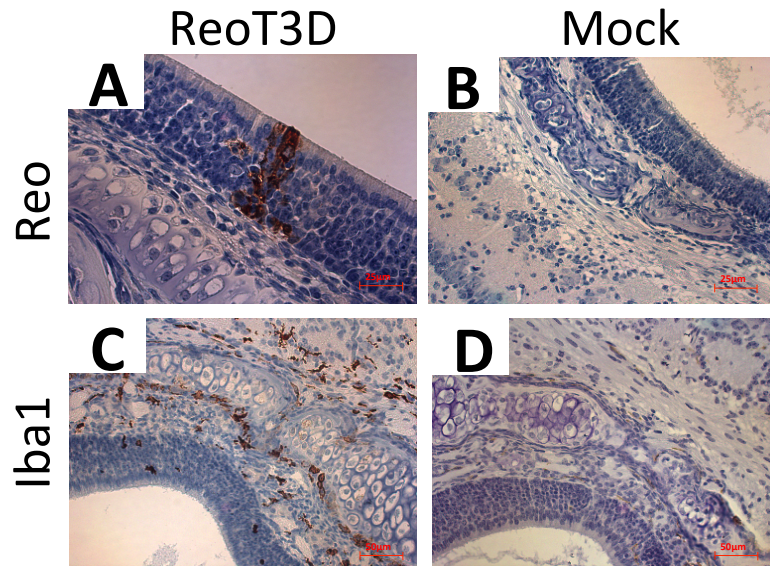


Figure 3.4.2. Reo virus staining of G2019S *Lrrk2* mouse olfactory epithelium and bulb 3 dpi. One day old mouse pups were inoculated with 1.5×10^5 pfu T3D reovirus and sacrificed 3 dpi for assessing virus infection. Prominent virus staining (Reo) is seen in olfactory epithelium of infected pups (A, n=2) but not in mock treatment (B, n=2). Uptake of the virus by the olfactory receptor cells (ORC) is depicted in A. Robust Iba-1 microglial signal was observed in OE and vicinity of OB of infected mice (C) but not in mock-treatments (D). Images represent G2019S *Lrrk2* mutant tissue. Images were obtained using original magnifications of 40x (A) and 20x (B-D).

Note, all IHC images represent G2019S *Lrrk2* mice tissue. IHC staining of WT mice tissue generated similar results to mutant mice, thus for consistency and conciseness of the thesis report images of WT IHC results are omitted here and onward.

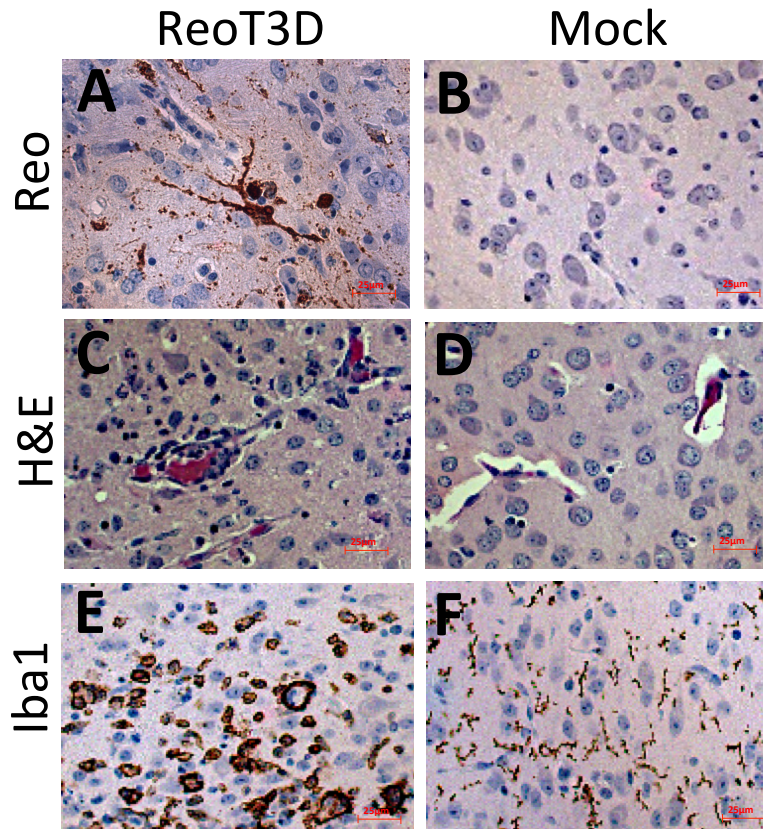


Figure 3.4.3. Histological characterization of midbrain (tectum) in reo virus infected G2019S *Lrrk2* mice 11 dpi. Virus reactivity, leukocyte infiltration and microglial reactivity were observed in virus infected mice (n=2) using anti-Reo antibody, H&E stain and anti-Iba1 antibody respectively. Mock-treatments of mice (n=2) were used as control for infection and were devoid of any reo staining and inflammatory markers. Images were obtained using original magnification of 40x.

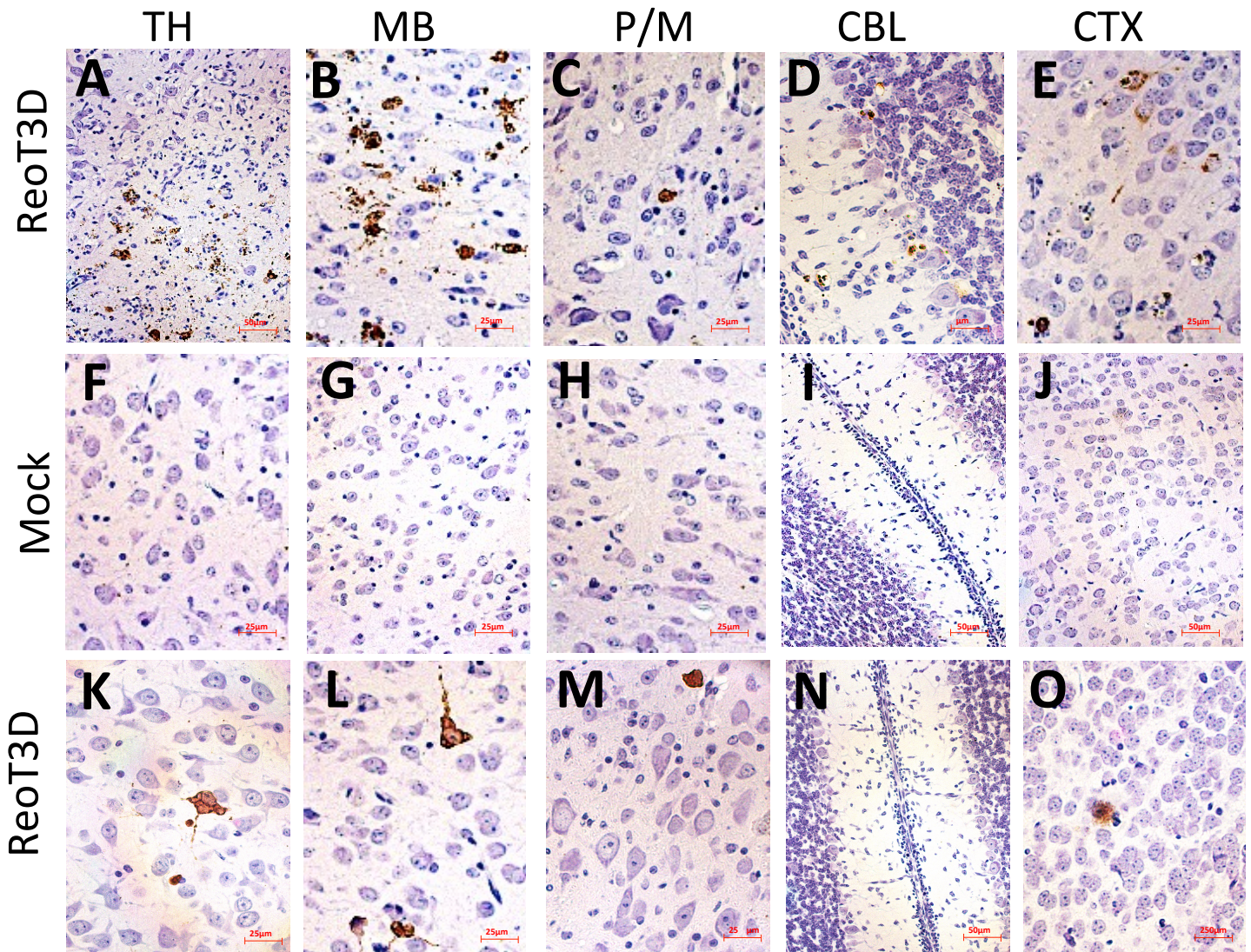


Figure 3.4.4. Reovirus staining of G2019S *Lrrk2* vs. WT mouse brains 11 dpi. Virus reactivity was seen in different regions of mouse brains of both G2019S *Lrrk2* (n=2) and WT (n=2) genotypes as shown. Both genotypes generated similar virus staining (A-D: G2019S *Lrrk2*, K-O: WT). Mock-treated animals (n=2) were used as control for infection and were devoid of any reovirus staining (F-J). Images were obtained using original magnifications of 20x (A, I-J, N) and 40x (B-H, K-M, O).

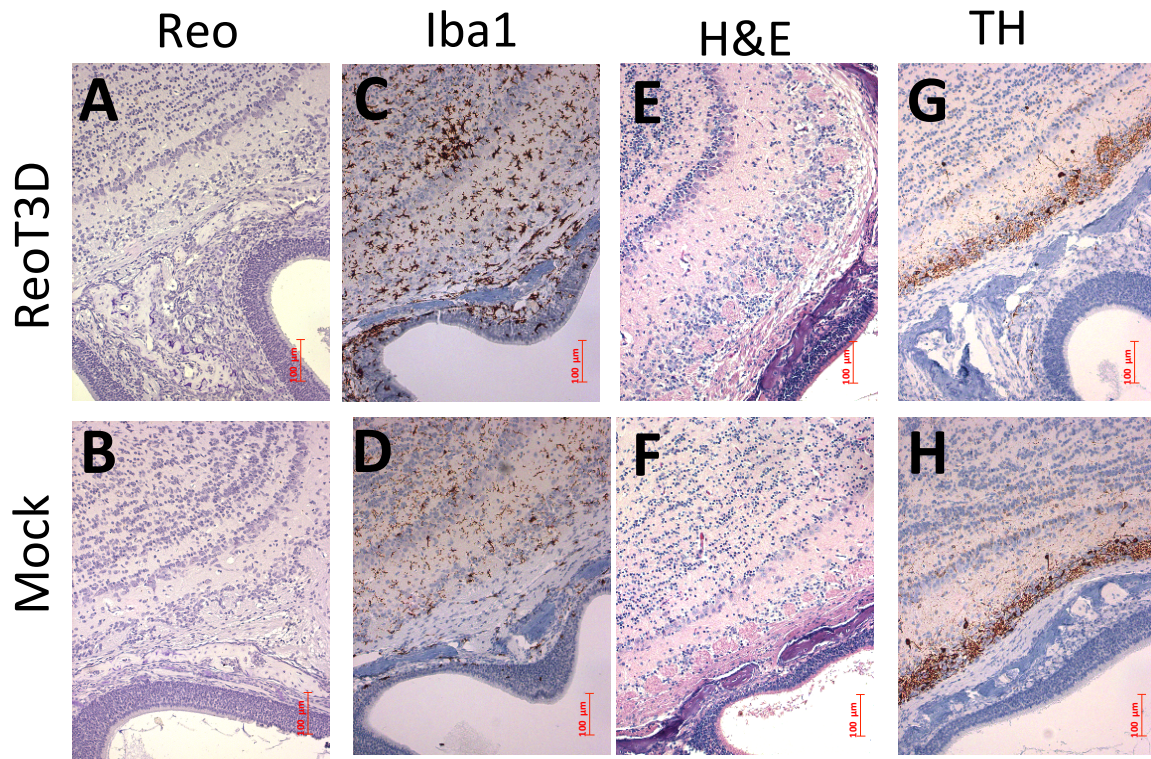


Figure 3.4.5. Histological examination olfactory epithelium and bulb in G2019S *Lrrk2* mice 11 dpi. Reo staining of infected animals (n=2) show recovery of the nasal epithelium and clearance of viral signal in both OE and OB (A). In contrast, profound Iba1 microglial signal persists in these regions of earlier infection (C). H&E and dopamine (using TH antibody as a marker) staining showed the olfactory epithelium and bulb to be normal in reo infection (n=2) compared to mock treatment (n=2) by and large. Images were obtained using original magnification of 10x (A-H) and 20x (I-J).

All G2019S *Lrrk2* mouse pathology slides and their staining results were comparable/similar to those of WT, thus for consistency and conciseness, all IHC data shown represent those from G2019S *Lrrk2* tissue slides (reovirus *versus* mock infection). We did not see any detectable differences regarding the viral protein load in G2019S *Lrrk2* animals compared to WT.

As expected, marked leukocyte infiltration was also observed in the brains of G2019S *Lrrk2* animals treated with the virus but not in mock-treated animals; this leukocyte infiltration was seen in the regions of the brain that corresponded with presence of detectable virus expression (Figure 3.4.3)

In addition and as expected, enhanced microglial recruitment/activation was observed in various regions of the brain in the infected animals (Figure 3.4.3); Iba1-positive cells were scattered throughout the brain and included areas in the vicinity of vasculatures. Furthermore, total α -synuclein staining of virus-infected brain regions was similar to that of mock-infected brains for all *Lrrk2* genotypes studied (not shown).

Interestingly, brain analyses of mice that survived reoT3D infection beyond 21 dpi, of which several were examined by us (n=4 skulls), exhibited minute amounts of viral reactivity (sacrificed on 30 dpi), which indicated a rather small spread of virus to the brain to begin with and/or a very efficient virus clearance by the host. Iba1 staining, likewise, revealed a select few regions that were positive for continuously activated microglia.

3.4.3 G2019S *Lrrk2* Mutation Decreases Survival Of Females But Not Males In T3D Reoviral Infection Model

Our above data suggested a possible protective role for the PD-linked G2019S *Lrrk2* by rendering the mice to have better control over virion replication in both their lungs and brains, as mentioned. That said, the next goal of the project was to assess the possible contribution of this observation to the overall survival of the mutants *versus* WT animals in our discovery cohort. Counterintuitively, the survival study (n=150 animals enrolled) revealed decreased percentages for the mutant animals' survival, 41.67% *versus* 51.69% (Figure 3.4.6A). The latter had the expected rate from our chosen LD₅₀. However, this difference of ~10 per cent in the survival of inoculated suckling mice was not statistically significant.

Investigating survival rates of all females *versus* all males, regardless of genotypes, revealed the two sexes survived at similar rates in our chosen infection paradigm (Figure 3.4.6B). Within the WT colony, all females taken together had a slightly better survival (57.14%) compared to males (45.83%) Figure 3.4.6C. In contrast, within the G2019S *Lrrk2* mutant colony, males had a better survival (48.48%) compared to females (33.33%) Figure 3.4.6D. Accordingly, the males from either genotype survived very similarly in the infection model as shown in Figure 3.4.6E. Intriguingly, the G2019S *Lrrk2* mutation generated a significantly decreased survival for mutant (33.33 %) females *versus* WT (57.14 %) females (Figure 3.4.6.F). The G2019S *Lrrk2* mutation produced similar survival rates for mutant males (48.48 %) *versus* WT (45.83%) males (Figure 3.4.6E). In brief, the G2019S *Lrrk2* mutation appears to act in a gender-specific manner worsening female mice's survival in the reovirus infection paradigm Figure 3.4.6F. Intriguingly, a female gender bias has recently

been published in three cohorts of LRRK2 G2019S carriers that developed PD, as reviewed in: (Schlossmacher et al. 2017).

3.4.4 A-Synuclein Processing Of G2019S *Lrrk2* Mice Is Not Different From WT Mice During T3D Reovirus Encephalitis

Brain lysates of mice from 11 dpi were processed for determination of total α -synuclein by ELISA (Figure 3.4.7). The reoT3D infection did not affect the levels of α -synuclein concentration compared to mock treatment in either genotype. Furthermore, the G2019S mutation of *Lrrk2* did not elicit any changes in α -synuclein levels compared to WT mice under these experimental conditions.

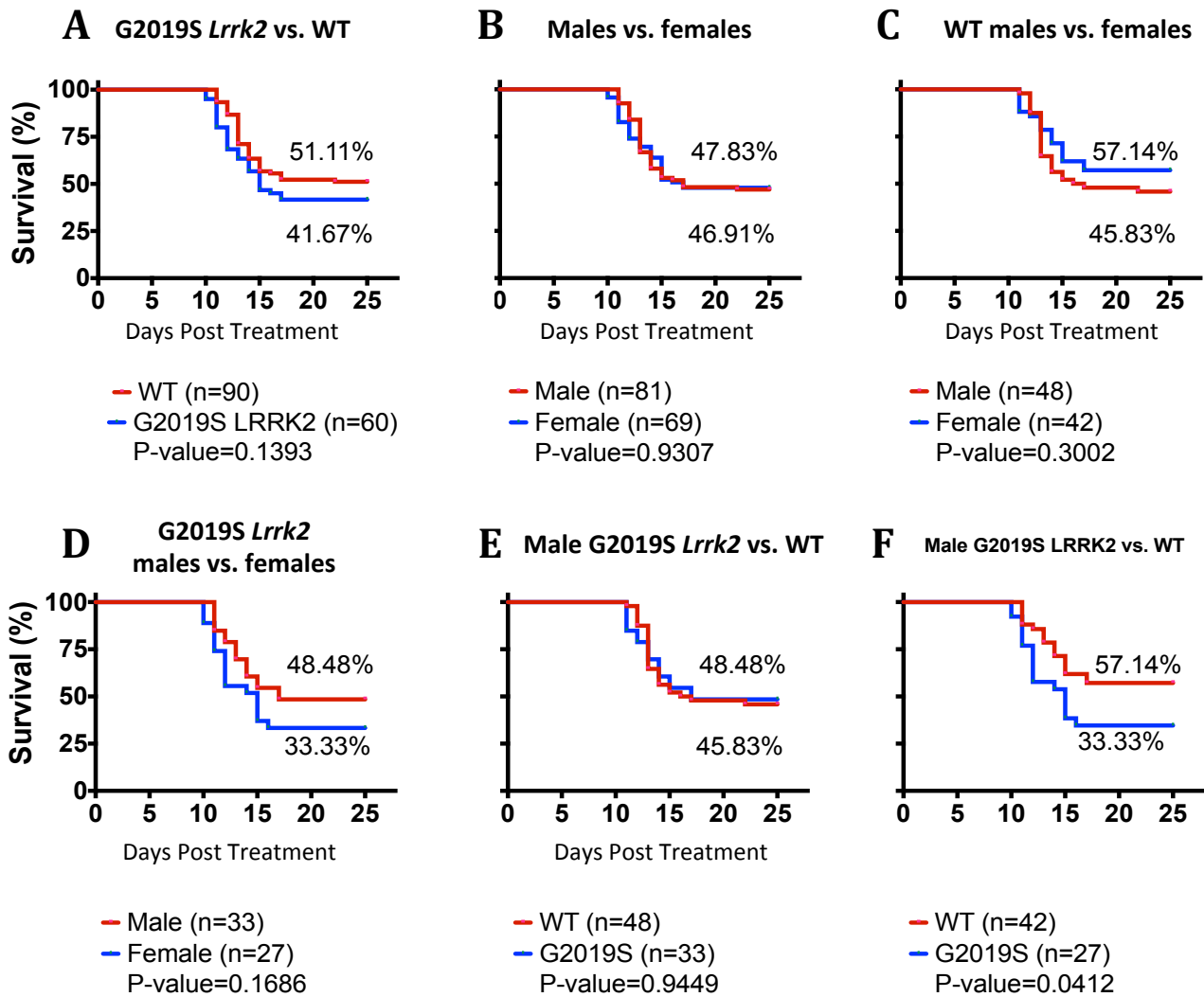


Figure 3.4.6. Survival study of G2019S *Lrrk2* versus WT mice in reo virus infection model. Mice were inoculated with 1.7×10^5 pfu of T3D reovirus and their survival was monitored for the effects of *Lrrk2* mutation. The contribution of sex (male vs. female) was also assessed in this study. The results are shown in Kaplan-Meier plots and Log Rank test was used throughout for statistical analysis; P-values are indicated below each plot. In comparison to WT, G2019S *Lrrk2* mutation was shown to significantly reduce the survival of females but not males (C, p-value = 0.0412).

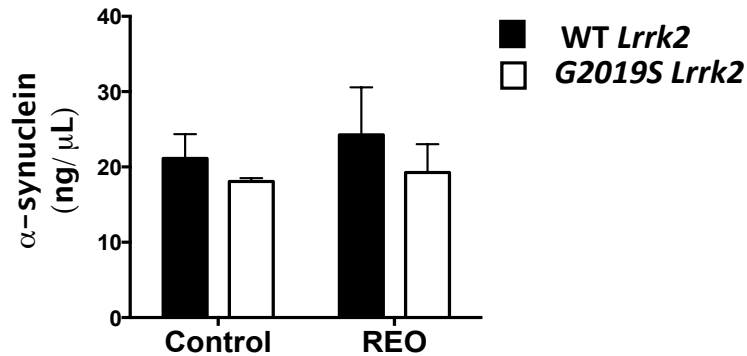


Figure 3.4.7. Absence of alpha-synuclein changes in G2019S *Lrrk2* vs. WT mouse brains during reovirus infection. Mice were inoculated with 1.7×10^5 pfu of the T3D reovirus (REO) or media (control) and their brains were next collected, homogenized and analyzed for alpha-synuclein levels using ELISA. $n=2$ per genotype for control and $n=4$ per genotype for reo treated brain homogenates. Virus infection did not differentially affect alpha-synuclein levels 11 dpi when compared to control mock-treatment for the same time-point. ELISA protocols were previously described in (Mollenhauer et al. 2008; Cullen et al. 2009; Cullen et al. 2011). ELISAs were performed by C. Jagow. Animal inoculations, tissue preparations and statistical analysis were conducted by M. Hakimi.

CHAPTER IV. DISCUSSION

4.1 Lrrk2 Expression Levels in Various Mammalian Tissues

Difficulty in the reliable detection of LRRK2 expression in the nervous system has been reported by many researchers in the field. The consensus among researchers suggests that LRRK2 has low expression in the CNS, in particular under physiological condition, and shows exceptionally low levels in the brainstem, which is the major site of pathology in PD subjects. Our data have also confirmed limited expression levels of LRRK2 in the neurons of both central and peripheral nervous systems. Low brain expression of LRRK2/Dardarin in addition to its yet unknown physiological function further compounds studying this protein in the central nervous system and PD etiology. These facts, in addition to increasing evidence of inflammation in PD, initially led us to explore this protein's function in the periphery instead.

Experiments with human blood specimens led us to the detection of robust LRRK2 expression in leukocytes that are part of the innate immune system, such as monocytes and neutrophils, and to a lesser extent, of adaptive immunity, such as B- and T-cells. Our IHC, FACS and WB results from human and murine studies indicated that Lrrk2 reactivity is mostly found in macrophages and granulocyte cells (Figure 3.1.6); this was confirmed by analyses of various mammalian organs, i.e., lymph nodes, spleen, lung, kidney, gut, and skin.

Interestingly, our histology experiments on inflammatory human conditions suggested marked upregulation of LRRK2 in recruited WBCs within inflamed ileum, *Mycobacterium leprae*-infected skin, vasculitis-affected nerve roots and corresponding inflamed midbrain, rabies-infected brain, and HIV-infected brains. Our results thus suggested to us that human LRRK2 expression is strongly linked to virulent infections. That provided, it is plausible to

speculate that LRRK2 mutants alter the protein's role in the process of inflammation in a bi-directional manner (Figure 3.4.8), i.e., where its absence leads to a reduced host response, and the G2019S mutant augments LRRK2 function.

It is noteworthy to mention here that we did not detect any altered LRRK2 reactivity in immune cells of PD brains, which were comparable to control brains regarding their IHC signal for LRRK2. This may be because the inflammation in PD is not as pronounced as it is in HIV encephalopathy, rabies virus infection and during acute vasculitis of the human brain, or that IHC is not sensitive enough to discern a robust difference.

Nevertheless, a commonality between these inflammatory brain conditions and PD is the presence of activated microglia (Hirsch & Hunot 2009; Simón-Sánchez et al. 2009; Reale et al. 2009). Given recent evidence pointing to an immune role of *Lrrk2* in microglia, Puccini et al., investigated contribution of *Lrrk2* inhibition to Tat-induced neuroinflammation and neurotoxicity model of HIV-1-related neurodegeneration (Puccini et al. 2015). LRRK2 was reported to interact with 14-3-3, which is elevated in HIV-1-associated neurocognitive disorders (HAND) and was also identified as a biomarker for HAND diagnosis (Morales et al. 2012).

Aging in human immunodeficiency virus 1 (HIV-1)-infected patients is a risk factor for developing HIV-1-associated neurocognitive disorders (HAND) (Heaton et al. 2010). Immune cells of CNS in HAND patients are found to be positive for the presence of HIV-1 trans-activating (Tat) protein (Lu et al. 2011). HIV-1 Tat is one of the early RNA binding proteins that regulates transcription of the viral genome (Kure et al. 1990). Immune activation, microgliosis, cytokine and chemokine perturbations, immune cell infiltration of CNS and neuronal death are some of the pathological aspects of HAND (Puccini et al. 2015).

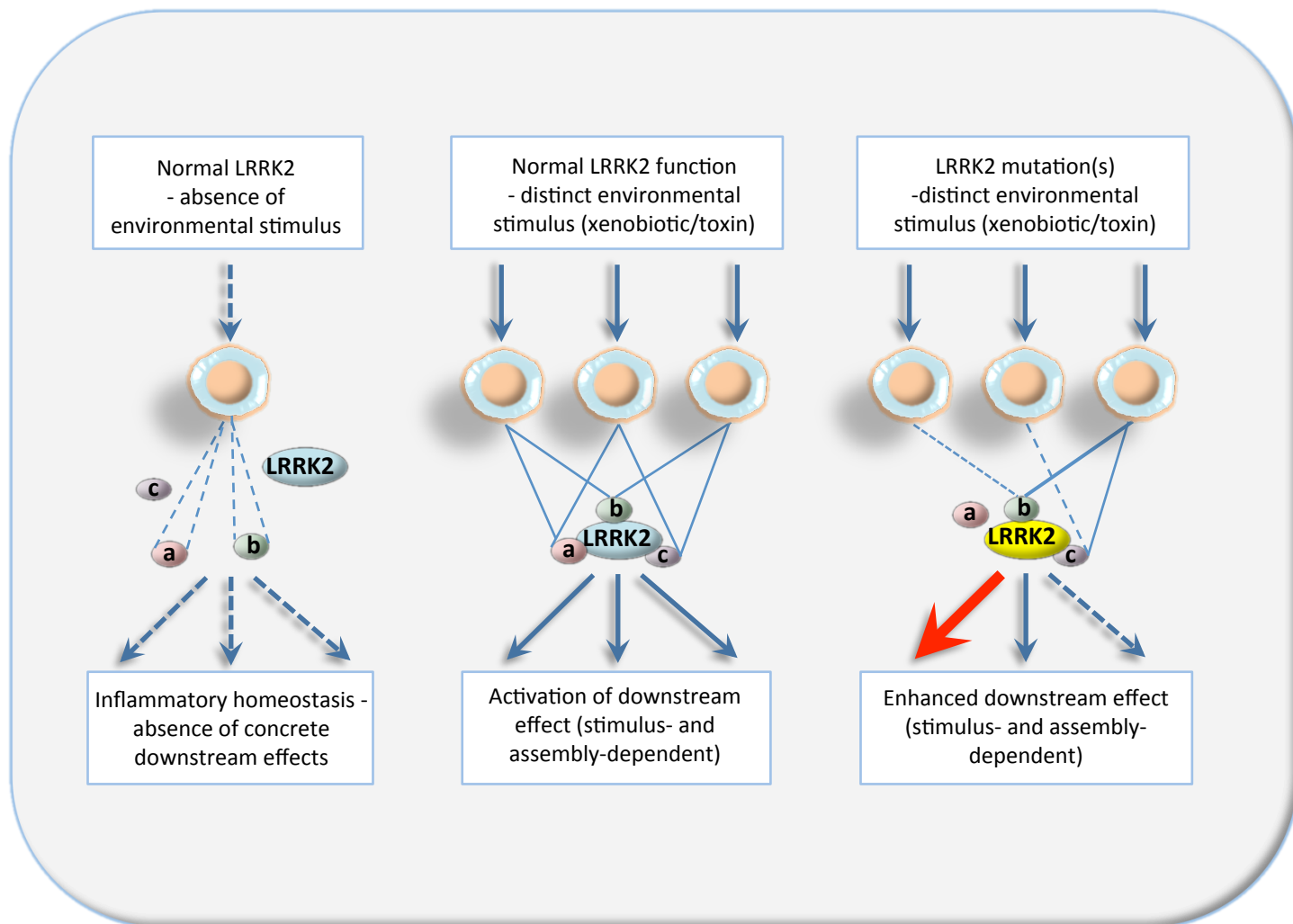


Figure 3.4.8. Model for LRRK2 functions downstream of environmental stimuli. Schematic drawing of Lrrk2 assembly in response to select environmental stimuli, which may help explain its function in inflammatory pathways. A-C represent different interactors of LRRK2 that depend upon specific environmental triggers to associate with LRRK2. Drawing is modified from (Lewis & Manzoni 2012).

Stereotactic cortical injection of 3 μ L of Tat-induced neuronal toxicity and apoptosis in WT mice. The KO mice exhibited lowered levels of Iba-1 microglia, CD11 peripheral leukocytes, and infiltration of neutrophils and monocytes all compared to WT. Tat treatment of mice showed synaptic integrity was preserved in LRRK2 deficient but not in WT mice as it was seen by the loss of synaptic markers (Puccini et al. 2015) These experiments described a protective role for *Lrrk2* deficiency under these conditions.

Collectively, it appears therefore that *Lrrk2* inhibition is protective when the environmental stimulus does not represent a virulent microbe (but rather a mimic), and in contrast, that *Lrrk2* absence is detrimental when the challenge to the host is a real xenobiotic, as shown above (Figure 3.4.8). Furthermore, our results revealed a gain-of-function effect for the p.G2019S mutant in LRRK2 in the context of infection of peripheral organs. However, this 'protective effect' was offset by a negative outcome of disease in female G2019S mutant carriers when the infection reached the nervous system (Figure 3.4.6). A major shortcoming of this study is the lack of a well-defined mechanism(s) for endogenous LRRK2's function in innate immunity within our chosen disease model; its delineation was beyond the scope of my Research Project and the time limit of my study plan. As such, possible mechanisms to underlie the function for LRRK2 were researched in the literature and presented here throughout the Discussion, as they may be informative / explanatory in the context of my findings.

4.2 Induction of *Lrrk2* Expression by Immune Stimulation

Our preliminary results from LPS injection into midbrains of WT mice showed robust signals for endogenous *Lrrk2* in both invading macrophages and CD11-positive microglia

compared to control saline injections. We found this phenotype to be more pronounced by co-injection of LPS and IFN- γ , two agents known to increase *Lrrk2* transcription (Figure 3.2.2). This work is further pursued now by the Dr. Shawn Hayley group at Carleton University to test immune responses involving *Lrrk2*.

Our experiments in mouse BMDM showed induction of *Lrrk2* at both mRNA and protein levels in response to inflammatory modulators. There, murine *Lrrk2* mRNA was significantly upregulated in macrophages treated with ligands for TLR4, TLR7, and TLR9 (Figure 3.2.2A). In contrast, *Lrrk2* mRNA level was significantly decreased in treatment with a TLR2 ligand compared to unstimulated treatment. Viral induction of BMDM led to robustly increased levels of *Lrrk2* protein (Figure 3.2.2B).

The mechanism(s) by which LRRK2 may respond to TLR signaling triggered by bacterial *versus* viral ligands may markedly vary. In TLR signaling of macrophages, $\text{I}\kappa\text{B}$ kinase family regulate LRRK2 in a MyD88-dependent way, as reported by Dzamko and colleagues (Dzamko et al. 2012). They showed that $\text{I}\kappa\text{B}$ kinase regulated LRRK2 phosphorylation at S935 and S910 in BMDM and murine macrophage cell line RAW264.7 Toll-like receptors (TLRs) are cell membrane bound receptors, which are activated by pathogen sensing. TLRs form an essential arm of innate immunity. TLRs signaling through MyD88 adaptor protein leads to activation of TAK1, a kinase upstream of canonical ($\text{IKK}\alpha$ and $\text{IKK}\beta$) κB kinases activation, which results in TAK1 phosphorylation of $\text{IKK}\alpha$ and $\text{IKK}\beta$ and perpetuation of the TLR signaling (Hacker & Karin 2006). Canonical $\text{I}\kappa\text{B}$ kinases govern NF κB -dependent gene transcription while non-canonical $\text{I}\kappa\text{B}$ kinases negatively regulate activation of canonical $\text{I}\kappa\text{B}$ kinases. Members of the $\text{I}\kappa\text{B}$ kinase family phosphorylated LRRK2 at LRRK2 S935 and S910

independent of LRRK2 kinase activity and interaction with 14-3-3 protein (Dzamko et al. 2012; Nichols et al. 2010).

In stimulation of mouse BMDM by bacterial lipopeptide Pam3CSK4 (an agonist of TLR1/2 complex) and LPS (agonist of TLR4), a transient phosphorylation of p105 (mediated by IKK α and IKK β activity), JNK and p38 MAP kinases was seen, as expected (Dzamko et al. 2012). Note, non-TLR agonists TNF- α , NOD1, and NOD2 did not affect the phosphorylation state of Lrrk2 in BMDM cultures (Dzamko et al. 2012).

The concrete nature of an external stimulus and the type of LRRK2 mutation are potentially two key determinants regarding the specific pathologies that could be expected in PD, CD, and leprosy. Different mutations in *LRRK2* may differentially affect this protein's stability and abundance, its function and signaling pathways involved (Lewis & Manzoni 2012). Distinct mutations can cause alterations in localization and in LRRK2 binding to its partners/substrates, and may also change its encoded enzymatic activity(ies) in a cell/tissue-specific manner (Lewis & Manzoni 2012). A schematic model of how diversely LRRK2 may function, in this regard, is shown in Figure 3.4.8.

Recent evidence (Martin et al. 2014) indicated that Lrrk2 was involved in regulation of protein synthesis by ribosomes, through which it may auto-regulate its own protein expression when triggered by immune stimulants. Martin and colleagues found that ribosomal subunit protein s15 is an authentic physiological phosphosubstrate of Lrrk2 (Martin et al. 2014). Phosphorylation of T136 residue on s15 protein appeared to be imperative for G2019S *Lrrk2* driven neurotoxicity. G2019S *Lrrk2* neurotoxicity was mediated through ribosomal protein s15 by augmenting translation of both CAP-dependent and independent mRNAs. We have not yet examined s15 metabolism in our studies.

Certain LRRK2 mutations can affect its protein abundance and stability, which may have important implication for LRRK2-dependent immune response (Liu et al. 2011). Most of the *LRRK2* SNPs found as risk alleles for IBD occur in the non-coding regions of the gene. However, one coding variant was located in an exonic region, rs376186, and generated a protein with lower stability. The authors speculated that carriers of this SNP might have reduced levels of LRRK2. The point mutation resulting from the SNP rs376186 caused substitution of methionine for a threonine, generating the high-risk allele M2397. Compared to the low-risk allele p.T2397, patients with a homozygous mutation of M2397 had significantly less LRRK2 protein levels. The authors measured the abundance of LRRK2 in B lymphocytes isolated from the peripheral blood of carriers that were patients. Liu and colleagues found LRRK2 to negatively NFAT localization and activity. They also found M2397 LRRK2 exhibited slightly lower inhibitory effect on NFAT's activity as determined by the preliminary experiments of Liu and colleagues (Liu et al. 2011), which could possibly be explained by its faster turnover in cells.

Intriguingly, the LRRK2 mutant M2397T was also recently linked to T1R-specific leprosy which is characterized by excessive inflammatory responses even after antibiotic treatment (Fava et al. 2016). This indicated that both T1R-associated leprosy and Crohn's potentially share the same pathogenic pathway involving this LRRK2 variant. The novel linkage of these two infectious disorders with the PD gene *LRRK2* has important implications for the strategy of further genetic analyses of complex infectious diseases (Buschman & Skamene 2004).

4.3 Characterization of *Lrrk2* Mutants Regarding Cellular and Tissue Cytokine Regulation

Both peripheral inflammation and central nervous system inflammation are implicated in parkinsonism (Garcia-Esparcia et al. 2014). Interestingly, PD patients with sleep disturbances, depressive and cognitive disorders are found to contain high levels of TNF- α in their peripheral blood (Menza et al. 2010). Polymorphism in IL-1 β , IL-6 and TNF- α are linked with susceptibility to PD (Håkansson et al. 2005; Chen et al. 2008).

The fact that inflammatory markers and immune mediators are found both in the PD central nervous system and peripheral blood mononuclear cells, a notion of systemic inflammation is inferred in parkinsonism (Garcia-Esparcia et al. 2014). The driving force for chronic inflammation in PD remains unknown. It is important to note that the chronic activation of the immune system in PD is not comparable to acute administration of MPTP in mice, which are used as PD models by some investigators (Garcia-Esparcia et al. 2014).

In examining the BMDM supernatants from *Lrrk2* KO versus R1441C *Lrrk2* versus WT mice, we did not detect any significant genotype-dependent response for cytokines levels produced from stimulations with heat-killed bacteria, reovirus, LPS, and nigericin. Other investigators have shown discordant cytokine response in treatments of BMDM from various *Lrrk2* genotypes. Some, such as (Puccini et al. 2015) showed *Lrrk2* dependent cytokine modulation while others showed absence of any genotype-dependent cytokine signaling. The differences in cytokine results among different research teams could possibly be attributed to various factors, such as by differences in animal model background/strain, sex of animals, type, and dosage of specific immune stimulus, methodological approaches, sensitivity of cytokines detection kit/method, etc. Nevertheless, it might be important to

consider the possibility that cytokine regulation may not be a direct target of *Lrrk2* function, at least under the experimental conditions we used *ex vivo*.

While the G2019S *Lrrk2* mutation in my pilot studies did not detectably affect brain cytokine levels, the *Lrrk2* KO mutation significantly increased brain IP-10, IL-12(p-70) and IL-4 levels in the reovirus infection paradigm. In contrast to brain, the *Lrrk2* KO mutation significantly decreased lung levels of RANTES, MIG and IP-10 in reovirus infection and IL-1 β under basal/unstimulated conditions. Similarly, G2019S *Lrrk2* mutation significantly reduced lung levels of MIG and IP-10 but also LIF, eotaxin and IL-13 in reovirus infected mice and of MIP-1 α under basal/unstimulated conditions. This might imply that distinct triggers may elicit discordant outcomes in an organ-specific manner, for example, changes in IP-10 levels were found in both brains and lungs of *Lrrk2* KO mice as well as lungs of G2019S *Lrrk2* mice although the direction of changes for IP-10 was different for brain versus lung tissues. IP-10 is interferon γ -induced protein 10 kDa and MIG is a monokine induced by IFN- γ (Farber 1997), as is the case for *Lrrk2* as well. The levels of IFN- γ itself were either below detection level or did not generate significant genotype-dependent difference in our multiplex ELISAs. Both MIG and IP-10 are natural ligands of CXCR3 receptor, and their immune role is mostly specific to active T-lymphocytes (Farber 1997).

Thus, our multiplex-based cytokine analyses of brains and lungs indicated some significant genotype-dependent changes in select cytokines from reovirus infection of *Lrrk2* KO *versus* WT *versus* G2019S mice (Figures 3.3.12 and Figure 3.3.15). Note, that these results were preliminary and most cytokines that we measured did not elicit such genotype-dependent changes in either organ. It is also noteworthy that when these cytokine responses were grouped according to their viral titers in an additional cohort of animals

during subsequent studies (i.e., normalized by their viral titers), the statistical significance was not present anymore (Shutinoski B and Schlossmacher MG; unpublished data). Nevertheless, we recognize the possibility that transient cytokine changes, regardless of the contributing organ source, could have differed in infected host tissues from *Lrrk2* mutant mice, but evaded detection, but which nevertheless could have potentially influenced pathogenic mechanisms.

Recent findings suggested that the cytokine expression in PD is region dependent in brain (Garcia-Esparcia et al. 2014). Garcia-Esparcia and colleagues conducted a comprehensive study of expression profile of cytokines and immune mediators of 56 PD versus 43 control subjects. They investigated variation of immune response in four distinct brain regions: SNpc, putamen, frontal cortex area 8 and angular gyrus area 39. The authors measured expression of 25 mRNAs of various immune mediators including pro- and anti-inflammatory cytokines, Toll family, and cathepsins. Garcia-Esparcia's findings highlighted that PD encompasses different immune responses in various brain regions all occurring at the same time in the same person (Garcia-Esparcia et al. 2014).

4.4 Characterization of T3D Reovirus Pathology In *Lrrk2* Mouse Models Including Viral Protein Levels

Serotype-specific infection and disease outcomes were reported for various types of reovirus (Konopka-Anstadt et al. 2014). The presence of various cellular environments, tissue types, and distinct receptor types requires neurotropic viruses such as reovirus to develop complex mechanisms to overcome these host barriers to successfully disseminate their virions throughout the host's body (Konopka-Anstadt et al. 2014).

Analysis of IHC data obtained from *Lrrk2* KO and G2019S *Lrrk2* mice revealed reovirus reactivity in lungs and olfactory epithelium of mice at 3 dpi (Figure 3.3.4). While virus started to be progressively cleared in the lungs after day 3, the virus kept rising in the brain, as published by Gauvin et al. Viral reactivity was observed in olfactory receptor cells, olfactory knobs and axons as the virus marched toward olfactory bulb, only to be efficiently engaged by the host's immune system in the lamina propria, within and outside CN1, and near the cribriform plate.

Olfactory binding proteins are located in olfactory mucosa and normally transport odorant molecules, which come in contact with the mucous surface, to olfactory receptor cells (ORC) (Gire et al. 2013). ORCs are bipolar first order neurons, which are embedded in olfactory epithelium. The dendrites of ORCs form expanded structures known as olfactory knobs, which then come into contact with olfactory cilia in the OE's mucosa. Formation of odorant and olfactory receptor complex causes activation of the ORC and its associated G-protein complex followed by activation of second messenger systems such as cAMP. The activity of Adenylyl cyclase converts ATP to cAMP by cleavage of pyrophosphate bond. The subsequent intracellular influx of Ca^{2+} and Na^{+} ions and exit of Cl^{-} ions generate membrane depolarization, which is transmitted along the axons to relay neuronal signal further. Axons of the ORCs penetrate the cribriform plate and synapse with second order neurons, i.e., mitral and tufted cells located within the glomeruli of olfactory bulb. (Gire et al. 2013)

As opposed to other cranial nerves, where bundles of neuron axons give rise to a single cranial nerve, ORC axons give rise to clusters of smaller nerves known as cranial nerve 1. Mitral and tufted cells axons form nerve bundle that extends along the olfactory tract *en*

route to relay sensory information to forebrain structures. In our experiments, we have not seen any convincing spread of virus into the brain via CN1.

Related to the organization of olfactory system, our IHC results clearly showed virus reactivity in cranial nerve 1 as early as dpi 3, but effectively disappeared by dpi 7. By 11 dpi, virus reactivity had reached hippocampal regions CA1 and CA3, cerebellum, cerebral cortex, thalamus, midbrain and pons (Figures 3.3.4, 3.4.3). Although some of these forebrain regions, such as hippocampus and thalamus and cerebral cortex, which the virus infects, are also regions targeted by the olfactory signal transduction system, the majority of the seeding of the brain (beyond very rare detection in the olfactory bulb) in the chosen reovirus model occurred via the blood stream (Gauvin et al. 2013). In our histology experiments, H&E stains (and cresyl violet staining; not shown) served as a surrogate marker of inflammation in virus infected mouse specimens. In concert with previous finding, infiltration of leukocytes was evident in regions of the brain that coincided with virus infectivity as indicated by H&E staining (Gauvin et al. 2013; Antar et al. 2009).

The ability of reovirus to exploit its different viral components in interacting with various host factors may allow its successful internalization and dissemination across multiple tissue types. Detailed information on known reovirus mechanisms in mediating infection was covered Introduction section. The hematogenous spread of reovirus is facilitated by junctional adhesion molecule A (JAM-A). The mechanism by which T3D reovirus invades the CNS when mediated via a neural route is however not understood. Konopka-Anstadt et al. postulated that NgR1 was a neural pathogen-recognition receptor and thus a determinant of T3 reoviral pathogenesis. NgR1 is a tissue-specific receptor in CNS whose pattern of expression in the brain strikingly matches the pattern of T3 reovirus

infection as observed by histology data (Antar et al. 2009; Wang et al. 2002). Factors responsible for reovirus neurotropism and the distinct pattern of brain infection were not elucidated in my studies.

Binding of reovirus $\sigma 3$ protein to its double-stranded RNA hinders the activation of host's PKR which is involved in the antiviral response. The virus then uses the protein synthesis machinery to translate its mRNA and propagate within the host (Sherry 2009). Reovirus invasion triggers NF- κ B signaling in neurons. Host RIG-I, MDA5, and IPS-1 are involved in sensing reoviral RNA and subsequent phosphorylation of IRF3 and its nuclear translocation to induce transcription of IFN-related genes (Sherry 2009). Depending on the virus strain, reovirus serotypes use distinct mechanisms to subvert the host's Type I interferon response.

In addition, both Gardet A et al. (Gardet et al. 2010) and Thevenet J et al. (Thévenet et al. 2011) found several consensus interferon-stimulated response elements and γ activated sequences in the *LRRK2* gene. As mentioned previously, *Lrrk2* induction was triggered by IFN- γ (but not IFN- α or IFN- β) pathway (Gardet et al. 2010). Kuss and colleagues found that ERK5 controls *Lrrk2* in IFN- γ pathway (Kuss et al. 2014). ERK5 has regulatory effects on monocyte enhancer factor 2, which is linked to PD as well (Yin et al. 2012). Formation of IFN- γ homodimers leads to recruitment of JAK, Janus tyrosine kinase, which phosphorylates STAT1 homodimer (De Weerd & Nguyen 2012). STAT1, signal transducer, and activator of transcription 1 is a transcription factor whose activation leads to expression of certain genes provoked by IFN- γ signaling pathway (De Weerd & Nguyen 2012). IFN- γ induces expression of ERK5 downstream of JAK/STAT1; ERK5 then upregulates expression of *Lrrk2* (Kuss et al. 2014). In related work in the lab, my colleagues have recently identified that endogenous

Lrrk2 constitutively regulates the phosphorylation of STAT-1 in BMDMs and brain lysates and that the ratio of p-STAT1/total STAT protein is enhanced by the G2019S mutant, but reduced in cells from *Lrrk2* KO animals (not shown; Shutinoski B et al.; manuscript in preparation).

Related to Lrrk2's mechanisms of action, treatment with *Salmonella typhimurium* bacteria in mouse macrophage cell line RAW 264.7 showed localization of Lrrk2 with the engulfed bacteria in the cytosol (Gardet et al. 2010). This was indicative of a possible role for LRRK2 in pathogen phagocytosis. Knock-down of Lrrk2, via siRNA, led to reduced ROS production, a key anti-bacterial response of host cells, in the macrophages (Gardet et al. 2010).

4.5 Contribution of Microglia to Reovirus Pathogenicity and Relevance to LRRK2-Linked PD

Chronic inflammation attributes to PD etiology (Russo et al. 2014). Infiltration of macrophages and activation of microglia at the site of DA neurodegeneration has been documented in PD cases. Prolonged immune signaling of microglia contributes to tissue injury and loss of DA neurons in PD (Hirsch & Hunot 2009). Activated microglia encompasses their secretion of inflammatory mediators such as cytokines, ROS and NO. Kim et al. postulate that microglial neurotoxicity profoundly contributes to progression of neurodegenerative diseases (Kim et al. 2000). Expression level and activation state of microglia may be crucial determinants of microglia-mediated neurodegeneration (Kim et al. 2000).

Microglial recruitment/activation was investigated in reovirus infection of *Lrrk2* KO and G2019S *Lrrk2* mice using microglial marker Iba1 in IHC experiments. On day 3, virus-treated animals did not show changes in Iba1 staining in the brain overall compared to mock treatment as expected since virus infection had not yet been manifested in the brain. Virus reactivity was restricted to OE and CN1 at this time point. This observation was partly consistent with findings of Andrew West's team who reported attenuation of microglial activity to result from *Lrrk2* inhibition (Moehle et al. 2012). By implication, A. West's team predicted G2019S mutation of *Lrrk2* to augment microglial response; in that manner, our data from OE of G2019S *Lrrk2* can provide an in vivo evidence for their prediction.

West and colleagues showed inhibition of *Lrrk2* to lead to significant reduction of TNF- α mRNA and protein which they used as markers of microglial inflammatory response (Moehle et al. 2012). They conducted these experiments in wild-type mice that were stereotactically injected with LPS in their SNpc. They performed inhibition of *Lrrk2* kinase by either *Lrrk2* inhibitors L2in1 or Sunitinib. Note, both *Lrrk2* inhibitors are the most specific inhibitory molecules yet described for this protein; however, both are also reported to act on other kinases. In short, their findings highlighted the requirement for kinase activity in *Lrrk2* to induce an inflammatory response in microglia.

Our IHC results from virus-treated animals 11 dpi showed strong upregulation/activation of microglia in various regions of the infected brains from all *Lrrk2* genotypes (Figures 3.3.6 and 3.4.5), but we have not yet carried out detailed, time-dependent studies employing multiple markers of microglial activation states among the different genotypes. Interestingly, while OE was healed and cleared of virus as early as dpi 7,

and certainly by day11 pi, heightened microglial response was sustained at this region (Figure 3.3.8 and Figure 3.4.5).

Quantification of virus staining in *Lrrk2* KO vs. WT brains 11 dpi showed the midbrain to contain significantly higher virus signal than thalamus/hypothalamus and other regions in *Lrrk2* deficient animals (Figure 3.3.6.). This meant that absence of *Lrrk2* was contributing to midbrain pathology, the site of PD-linked cell loss (where little detectable LRRK2 protein is found in neurons from both primates and non-primate mammals (Galter et al. 2006)). By extension, the G2019S mutation, a potentially hyperactive model of LRRK2, lowered viral replication in the brain. In fact, our plaque assay data showed significant reduction in viral load of G2019S *Lrrk2* brains compared to WT at 11 dpi. This apparent discrepancy could possibly be explained if the G2019S mutant promotes an enhanced host response by invading WBCs and overactivation of resident microglia.

Reovirus reactivity in the brain was detected in different cell types including foremost neurons, leukocytes, and rarely, microglia. Each one of these cell types may have had its own distinct contribution to reovirus spread and pathogenicity. The *Lrrk2* expression is three to four folds higher in cultured microglia than isolated neurons (Schapansky et al. 2014). That provided, it is also plausible to speculate that LRRK2 mediates immunoregulatory function through microglia and contribute to inflammation-driven neurodegeneration in PD. Furthermore, heterogeneous distribution of neurons or microglia that express *Lrrk2* in mouse brain could underlie altered, regional susceptibility to inflammatory response.

Findings from Kim et al. showed that midbrain has the most abundance of microglia compared to other brain regions (Kim et al. 2000). This distinct abundance of microglia in midbrain accounted for LPS-induced dopaminergic neurodegeneration found in their study

with rats. Staining with OX-42, a marker of microglia uncovered a greater number of activated microglia in midbrain, which was 4 fold higher than hippocampus and eight folds greater than cortex. It is important to iterate that LPS is a TLR-4 ligand because neurons do not have TLR-4 receptors while microglia do (Lehnardt et al. 2003). Therefore, LPS-induced cytotoxicity in the brain is likely driven by microglia but not neurons (He et al. 2013).

In corollary with findings from A West and colleagues, I Russo et al. demonstrated attenuation of LPS-induced IL-1 β and COX-2 mRNAs and proteins in *Lrrk2* deficient microglial cultures of primary (Russo et al. 2015). IL-1 β and COX-2 induction was used as indicators of NF- κ B activation. Russo et al. denoted that LRRK2 has inhibitory effect on PKA through which it modulated NF- κ B signaling. NF- κ B signaling controls inflammation culminated in induction of IL-1 β , COX-2 and other inflammatory factors (Russo et al. 2015). NF- κ B is commonly present as a p65:p50 heterodimer (Napetschnig & Wu 2013). Under unstimulated conditions, PKA phosphorylates NF- κ B p50 to activate binding of the p50:p50 homodimer to DNA thereby repressing gene expression. During this time, the cytosolic NF- κ B p65:p50 heterodimer is bound to I κ Bs inhibitory proteins via the p65. Upon stimulation, IKK complex phosphorylates p65, which leads to degradation of the inhibitory protein complex and translocation of the p65:p50 dimer to the nucleus. Next, p65:p50 dimer replaces the p50:p50 homodimer and triggers NF- κ B-dependent gene expression. (Napetschnig & Wu 2013)

PKA was also reported to affect formation of synapses, which has implication for *LRRK2*-PD (Parisiadou et al. 2014). A recent report indicated that *Lrrk2* regulates intracellular distribution of PKA subunit PKARII β and localizes it to spinal shaft where PKA activity

resulted in abrogated actin polymerization (Parisiadou et al. 2014). *Lrrk2* GTPase domain directly interacted with PKA subunit PKARII β (Parisiadou et al. 2014).

In brief, a literature review on characteristics of *Lrrk2* deficiency in mice or cell culture indicated a possible pro-inflammatory role for endogenous *Lrrk2* in the context of microglial function. Collectively, it was suggested that *Lrrk2* KO animals exhibited dampening of inflammation, as imposed by a microbial mimic. However, the outcome of a virulent reovirus infection is worse in the absence of *Lrrk2*.

4.6 Absence of Aberrant α -Synuclein Expression in Reovirus Infection of *Lrrk2* Mutant Mice

Our results from brain α -synuclein ELISAs showed a significant increase in α -synuclein levels at 11 dpi compared to 8 dpi in mice. Nevertheless, neither *Lrrk2* genotypes (*Lrrk2* KO vs. G2019S vs. WT) nor virus infection had any effects on α -synuclein processing as measured 11 dpi. Furthermore, our IHC experiments showed α -synuclein staining of virus-infected brains were similar to that of mock-infected brains for all *Lrrk2* genotypes studied. This was expected because α -synuclein is a long-lived protein, and thus, accumulation and detection of any aberration in α -synuclein would require a longer time frame (Li et al. 2004). Tong and colleagues who generated and characterized our *Lrrk2* KO mice, also suggested that *Lrrk2*-pathological events in the kidneys (such as of a potential synucleinopathy driven by *Lrrk2* mutation) were age-dependent and may lead to neurodegeneration through impairment of the protein degradation machinery (Tong et al. 2012; Tong et al. 2010)

Genetic deletion of *Lrrk2* in mouse model caused accumulation of α -synuclein and apoptotic cell death in aged mice (Tong et al. 2010). Perhaps early, as opposed to late, α -synuclein aggregates have a protective and similar role to amyloid β fibrils in Alzheimer's disease, wherein the fibrils create a physical mesh-like barrier against pathogens thereby sequestering them from damaging viable neurons (Kumar et al. 2016). In addition, amyloid β was previously shown to have antimicrobial properties (Kumar et al. 2016). Perhaps as PD progresses in the brain, dying neurons may release α -synuclein aggregates that can activate neighboring microglia. The activated microglia, in turn, can induce inflammation in a *Lrrk2*-dependent pathway. It is critical to note the strong possibility that LRRK2 does not function in isolation from other PD-linked genes. As such, contribution of α -synuclein to immune responses that are modulated by LRRK2 cannot be neglected in understanding the exact pathogenesis.

Reports indicated that treatment with α -synuclein aggregates led to microglial induction of IL-1 β and NF- κ B activation as well as loss of TH-positive cells in WT, but not *Lrrk2* KO cells and animals (Russo et al. 2015; Daher et al. 2014). These findings suggested that α -synuclein aggregates were toxic and activated *Lrrk2*-mediated inflammatory response involving NF- κ B (PKA, IL-1 β), which may be a *Lrrk2*-pathomechanism in PD.

4.7 LRRK2 Co-Regulation of Reovirus Growth in Lungs of *Lrrk2* Mutant Mice

Our results from plaque assays indicated that, during the course of infection, T3D reovirus titers reached the highest replication phase in the lungs at 3 dpi; thereafter, a constant decrease of viral titer was seen on days 8 and 11. In comparison to the lung viral titers of WT mice, *Lrrk2* KO mutation induced a significant increase of virions at 11 dpi while

G2019S *Lrrk2* mutation induced a significant decrease of virions at 3 dpi. G2019S *Lrrk2* mutation also induced a significant reduction of virions, similar to the lungs, in the brains at 11 dpi.

Ablation of *Lrrk2* gene in mice was previously reported to cause enlarged secondary lysosomes of kidney and lamellar bodies by >50% (LB) in alveolar type II (ATII) cells of the lungs (Herzig et al. 2011). Lung LBs are vesicular organelles containing phospholipid surfactants, which are released to alveolar surface for lowering the surface tension (Dietl & Haller 2005). Pulmonary surfactant is composed of hydrophobic phospholipids (90%) and proteins (10%): SP-A, B, C and D (Chroneos et al. 2010).

Miklavc's team demonstrated enhanced fusion of LB with cell membrane in *Lrrk2* KO rats when stimulated with ATP compared to WT. In contrast to this observation, surfactant secretion assay revealed significantly lower exocytosed phospholipids from the KO ATII cells. Miklavc and colleagues emphasized a role for *Lrrk2* in modulating Ca^{2+} homeostasis, which was required for LB exocytosis from ATII cells.

Surfactant proteins SP-A and SP-D are hydrophilic proteins with a role in innate immune system (Han & Mallampalli 2015). They bind to a wide variety of pathogens including yeast, fungi, bacteria and viruses and take part in their phagocytosis in a Ca^{2+} - dependent manner and coordination with other receptors such as TLR. By implication, reduction of surfactants or other dysregulation of surfactants, as were reported in *Lrrk2* KO rats, inevitably dampen line of defense in alveoli. The link between immunologic functions of LRRK2 and surfactant proteins SP-A and SP-D is unknown but is certainly tantalizing in understanding this lung phenotype of *Lrrk2* mutant mice. SP-A and SP-D interact with a variety of viruses, including influenza A and herpes simplex virus type 1, and hinder their entry by binding to them and

physically sequestering them. Of note, reduced binding of SP-A and D with hemagglutinins of influenza A are thought to be a causal factor in past influenza epidemics (Glasser & Mallampalli 2012). SP-A or SP-D knock-out mice are reported to exhibit susceptibility to infection and increased inflammatory cytokine production (Glasser & Mallampalli 2012).

By extension, our results indicate that it is possible for *Lrrk2* to interact synergistically with immune functions of SP-A and/or SP-B when T3D reovirus is exposed to pulmonary surfactant. Despite persistent presence of both surfactant proteins, reo virions significantly accumulated in the lungs of *Lrrk2* KO mice. Interestingly, G2019S mutation of *Lrrk2* may enhance the synergistic function with SP-A and SP-B as they significantly reduce reovirus growth in the lungs of G2019S *Lrrk2* mice.

4.8 Reo Virus Pathogenesis in Other Organs of *Lrrk2* Mutant Mice

Nasal inoculation of T3D reovirus caused systemic infection in young pups; as a result, the virus was found in all tissues of the affected host. For this research project, we focused our efforts on the brains and lungs for aforementioned reasons. However, it is possible that the virus pathogenesis exhibits *Lrrk2*-dependent phenotypes in other organs as well, especially since we have already shown robust LRRK2 expression in these organs. Our preliminary work showed a trend for parallel viral replication in various organs of mice including spleen, liver, kidney and gut. Similar to the lungs results, kidney, spleen, liver showed a trend for mildly augmented, viral growth in *Lrrk2* KO mice compared to WT at different time points. Continuation of viral titer determination in these organs and comprehensive analysis of virus pathogenesis in these tissues will offer valuable insights and complement our current knowledge about *Lrrk2*'s contribution to reovirus-induced disease in our models. This investigation will be part of future studies.

The gut luminal surface presents an interface between host and environment, which may contain pathogenic agents. In context of CD, Gardet et al. showed LRRK2 to be a pathogen-responsive protein, which increased its expression level and affected ROS production in the disease state (Gardet et al. 2010). In experimental colitis model (induced by 3% dextran sodium sulfate (DSS)), *Lrrk2* KO mice showed exacerbated disease symptoms and NFAT1 translocation to the nuclear region compared to WT mice (Liu et al. 2011). NFAT signaling is involved in immune responses evoked by various stimulants including fungal zymosan, TLR agonists, and viruses such as HIV-1 and Kaposi's sarcoma-associated herpesvirus (Farrow et al. 2011; Minematsu et al. 2011; J. Zhang et al. 2015; Liu et al. 2011). That provided, involvement of NFAT signaling can be investigated in the context of reovirus infection model of *Lrrk2* mutant mice. Induction of TAK1 upregulates NFAT activity and NFκB pathway (Li et al. 2015). As mentioned previously LRRK2 negatively regulated PKA, which is involved NFκB pathway as well.

Liu et al. proposed that *Lrrk2* interacted and functioned synergistically with the repressor complex NRON to mediate an inhibitory effect on NFAT's function. Under normal conditions, NFAT is found bound to NRON-LRRK2 in cytoplasm and is highly phosphorylated (Liu & Lenardo 2012). During pathogen encounter, antigen recognition causes an influx of calcium in the cells, which promotes NFAT dephosphorylation by calcineurin. NFAT is then transferred from cytoplasm to nucleus to induce transcription of target genes that activate the defensive signaling cascade. (Liu & Lenardo 2012)

Investigators who generated and characterized our *Lrrk2* KO mouse model found kidney pathologies in these mice which included cell death, accumulation, and aggregation of

synuclein and impairment of UPS and lysosomal-autophagy pathways in kidneys of these mice (Tong et al. 2010). Kidney injury molecule-1 (Kim-1) levels were also significantly higher in *Lrrk2* KO kidneys compared to WT indicating chronic renal injury (Tong et al. 2010). In addition, renal biomarker lipocalin-2 levels were significantly decreased in urine and serum of *Lrrk2* KO rats (Ness et al. 2013). Lipocalin 2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is another marker of renal injury and is expressed in myeloid and epithelial cells. (Ness et al. 2013)

Splenocytes of KO rats displayed significant alterations in white blood cells ratios as analyzed by flow cytometry (Ness et al. 2013). The *Lrrk2* KO rats showed significant increases in CD4+ and CD3+ T-cells and CD11b cells while a significant decrease in B-cells. In infection with rat-adapted influenza virus (RAIV) and *Streptococcus pneumonia* Type 14 bacteria, the KO animals generated significantly higher percentages of CD4+, CD8+, and CD3+ T-lymphocytes but significantly lower percentage of CD11b compared to uninfected rats. (Ness et al. 2013)

Intriguingly, though, Ness and colleagues also found *Lrrk2* KO lungs to contain significantly more bacterial colonies compared to WT. It is noteworthy that absence of *Lrrk2* does not always confer protection as proposed by a number of different teams such as A West, E Greggio and HA Gelbard (Moehle et al. 2012; Russo et al. 2015; Daher et al. 2014; Puccini et al. 2015). In that manner, Ness's report is in agreement with our observation regarding increased propensity of *Lrrk2* KO tissue for viral growth. In our virulent infection paradigm, the deletion of *Lrrk2* conferred enhanced vulnerability, as opposed to protection, which was observed when non-virulent, pathogen-associated stimuli were used.

4.9 Considerations Regarding Reovirus versus LPS Infection in *Lrrk2* Mouse Models

Some important distinctions need to be addressed when comparing our reovirus infection with LPS infection, which other investigators have primarily used to show an immunologic role for LRRK2.

First, LPS is a mimic of bacterial pathogen while reovirus is a whole live pathogen that is commonly encountered by humans; in that manner, the virus is physiologically more relevant to humans. Second, it is very likely that LPS activates different signaling pathways than the virus does in the host. Reports from mouse intranasal LPS (a bacterial mimic) versus poly(I:C) (a viral mimic) inoculation indicated that different pathways were involved for each stimulus leading to inflammation and airway hyperresponsiveness (Starkhammar et al. 2012). LPS acts through MyD88-dependent TLR-4 receptor signalling while poly(I:C) acts through TLR-3 signalling. LPS enhances recruitment of neutrophils while poly(I:C) enhances recruitment of lymphocytes in lung tissues (Starkhammar et al. 2012).

Third, the host factors used by the pathogens are different for LPS versus reovirus. LPS TLR-4 receptor is mainly expressed on microglia; its expression is very limited in neurons. Thus, LPS-induced neurodegeneration in the brain is in fact driven by microglial toxicity. The receptors for reovirus are found in neurons such as NgR1 as well as in leukocytes such as JAM-A receptor. Thus, reovirus-induced toxicity can attribute to both neuronal and leukocytic/immune pathways.

Fourth, the mode of pathogen delivery to animals can affect the pathology development in the host. Brain LPS administration has been mainly conducted by stereotactic surgery in pre-determined areas of the brain by the LRRK2 researchers. Such introduction of LPS in animals is further compounded with inevitable outcomes of

mechanical intracranial injury, which accompanies some inflammatory response on its own. In contrast, our reovirus was administered by intranasal inoculation, which is a natural route of infection.

Fifth, *Lrrk2* animal models used by researchers can further cause variability or complicate disease outcomes measured in LPS versus reovirus infections. The age, sex, background/strain and the type of *Lrrk2* genetic manipulations of animal models are other factors that generate variability in experimental results among these investigators. For example, we used young mouse suckling for our reovirus infection model while most of LPS infections are carried out in adult *Lrrk2* mouse models.

By inference, assigning a protective *versus* pathogenic role for LRRK2 is context-dependent. Comprehensive analysis of various disease outcomes (ex. neuronal loss, inflammation, survival) measured for multiple pathogens will give us a better idea of the overall pathogenicity of a specific LRRK2 mutation. In short, the behavior of LRRK2 and its mutations appears to be influenced by the nature of pathogen and by other host factors (Figure 3.4.8).

4.10 Understanding The Relation Between *Lrrk2* Mutations And Survival In Infected Mice

In assessing ORs for viral protein reactivity, viral burden, neuronal loss, and mortality, a positive association was seen for these disease outcomes and *Lrrk2* deficiency. Plaque assay results showed the lungs and brain of *Lrrk2* KO animals to contain increased T3D reoviral titers compared to WT. *Lrrk2* deficient animals, in particular females, also showed a trend toward reduced survival. In contrast, the lungs and brain of G2019S *Lrrk2* mice contained

reduced T3D reoviral titers compared to WT. Though, surprisingly, G2019S *Lrrk2* females showed significantly reduced survival. A critical consideration should be made about *Lrrk2* mutations and virus-induced mortality. Death is a complex disease outcome, which can arise from a combination of different factors. The exact relation between reovirus load and survival is not clear and in fact, it was beyond the scope of our research project. While it is plausible to link the increased viral load to reduced survival in *Lrrk2* KO animals, this notion is not plausible and does not afford an explanation for the case of G2019S *Lrrk2* animals. The viral load was negatively correlated with the survival of female G2019S *Lrrk2* mice. It will be important to determine whether other PD-linked LRRK2 mutants (as restaged in mice) will generate the same outcome.

Reovirus infection combined with pathological functions caused by a mouse genetic mutation may lead to activation of several different pathways in the course of disease development. Some pathological events/signaling pathways could happen in parallel as opposed to one leading in another. It is also crucial to make the distinction between direct, signal-induced changes *versus* trigger-independent, constitutive changes in LRRK2 signaling that could underlie the range of pathological mechanisms seen in distinct disease outcomes.

Although viral load is a strong prognostic factor in many diseases, there are cases where alterations in viral load are independent of disease progression. For example, low human papillomavirus (HPV) viral load is associated increased disease progression in early uterine cervical cancer (Kim et al. 2009). Concordantly, higher viral load of HPV-16 positively correlates with increased survival of patients diagnosed with tonsil cancer (Cohen et al. 2008). Another example is the case of severe acute respiratory syndrome (SARS) coronavirus infection, where high initial viral load of SARS virus is independent of survival

outcomes based on preliminary data published by (Chu et al. 2004). As well, a decline in CD4+ T-cell counts in HIV-1 AIDS occurs independently from HIV-1 viral titers in the early virus infection (Deeks et al. 2004). Moreover, analysis of asymptomatic period of HIV-1 AIDS shows that HIV-1 infectiousness is associated with variable viral titer within and between patients (Fraser et al. 2007).

Taken together, reovirus load (in the brain) does not appear to be a prognostic factor for survival from a neurotropic infection in the G2019S *Lrrk2* mice. Note that we do not yet know the mechanistic functions of *Lrrk2* in the context of reovirus infection to explain the changes in disease outcome. For that reason, I have interpreted our findings throughout the text using the current literature in the field. Nevertheless, our results showed novel aspects of *Lrrk2* function that are highly relevant to LRRK2 pathologies in humans. In doing so, we have succeeded –for the first time- to generate distinct differences in disease outcomes in vivo that discern loss- and gain-of-function effects for two *Lrrk2* mutations versus the WT genotype (i.e., KO and p.G2019S), which may be a crucial stepping-stone for future mechanistic studies. Distinct LRRK2 mutants, for example, the p.G2019S, may interfere with various stages of virus life cycle. Blocking or delaying certain virus life cycle stages such as its dis/assembly or genome replication can lead to lower viral titers. Future mechanistic studies will determine how LRRK2 is involved in regulating viral load.

We could speculate that a reduced viral titer is independent of survival outcome only when it affects the brain (but not other organs). An interesting gain-of-function effect of the p.G2019S mutant emerges from our observations, which could help explain why it may be so frequent in nomads of Northern Africa – pointing at possible links between geography, environment, and genotype. Future investigations will further analyze as to how lower titers

in the p.G2019S-expressing mice are linked to altered immune signaling, glial and leukocyte activity in infected areas, to reduced neuronal survival in female animals, and thus, to significantly altered host survival with a sex bias.

4.11 Sex Disparity in Survival of *Lrrk2* Mutant Mice Following Reovirus Inoculation

The survival study although did not indicate statistically significant differences for *Lrrk2* KO versus WT survival, overall it did show a clear and reproducible trend toward reduced survival for the KO mutants (34.62% versus 48.15% WT) as a result of virus infection. This was more pronounced for females (28.57%) as opposed to males (41.67%), Figure 3.3.11F. Intriguingly, G2019S *Lrrk2* mutation generated similar survival rates for male mutants (48.48 %) versus WT (45.83%). More importantly, G2019S *Lrrk2* mutation produced significantly decreased survival for female mutants (33.33 %) versus WT (57.14 %) Figure 3.4.6F. In brief, G2019S *Lrrk2* mutation appeared to act in a sex-specific manner exacerbating females' survival in virus infection paradigm.

Some disease aspects of PD appear to be different for men versus women (Saunders-Pullman et al. 2011). Idiopathic PD manifests more frequently as a severe disease phenotype in men while it is associated with more therapeutic complication in females (Saunders-Pullman et al. 2011). While the occurrence of idiopathic PD is more frequent in males (male: female ratio of 3:2) (Cilia et al. 2014), certain genetic PD subtypes seem to be less so. With respect to *LRRK2*-PD, females have a higher disease incidence (male:female ratio of up to 1:2) than idiopathic PD and an earlier age of onset (Saunders-Pullman et al. 2011). In certain populations such as Italians and Ashkenazi Jews, *LRRK2*-PD is even more prevalent in females than males (Cilia et al. 2014; Saunders-Pullman et al. 2011).

Sex-specific outcomes of LRRK2 function can be due to a number of different gender-specific factors in the CNS and immune system of humans. The cumulative effect of these factors may culminate in females' susceptibility to LRRK2-related PD. For instance, dopamine receptors D2 and D1 exhibited sex-differentiated functions in mediating reward-enhancing effects of bupropion, but not nicotine, with females possessing enhanced vulnerability (Barrett et al. 2016). Deficiency in dopamine receptor D3 induced significant hypoalgesia in females compared to males when stimulated with acute thermal pain (Liu et al. 2016).

Interestingly, castration of young male mice produced PD-like phenotype as marked by augmented levels of iNOS, CD11b microglia, α -synuclein and DA neurodegeneration (Khasnavis et al. 2013). Despite increased CD11b microglial levels, glial activation was attenuated. In agreement with the role of sex hormones in the brain, administration of 5 α -dihydrotestosterone pellets in castrated young mice exhibited a rescue effect and ameliorated the PD-like pathology in mice. These investigators proposed castrated male mice as a new animal model for PD research (Khasnavis et al. 2013).

In addition, analysis of *Lrrk2* KO rats revealed significant reduction of prolactin (involved in lactation in females) and elevation of testosterone (Ness et al. 2013). By inference, it is plausible to suggest that testosterone is negatively regulated by *Lrrk2* and may be protective against neurodegeneration.

Related to immunologic role of LRRK2 in Crohn's and leprosy, autoimmune diseases are more prevalent in women while infections are more common in men (Ngo et al. 2014). Sex disparity in immune diseases may pertain to sex-specific difference in immune response, reproductive system, sex steroid hormones and epigenetics (Ngo et al. 2014). Different sex

hormones have variable effects on innate and adaptive immunity (Giefing-Kroll et al. 2015). For example, testosterone has an immunosuppressive effect while estradiol has a stimulatory (enhancing) effect on immune cells (Giefing-Kroll et al. 2015). Of note, no sex bias has been published in *LRRK2*-associated leprosy or CD.

Moreover, aging and epigenetics further affect sex hormones influence on immune response. Menopause is accompanied by reduction of estradiol, which may make postmenopausal women vulnerable to some immune conditions (Giefing-Kroll et al. 2015). In general, the basal immune states of females seem to be more elevated/sensitive, which may offer an evolutionary advantage to females, so they can mediate faster clearance of viruses during demanding stages of their life-cycle such as pregnancy, which is critical to survival of the offspring and species. The same elevated immune state may, on the other hand, sometimes lead to a disadvantage for women in contracting autoimmune diseases more frequently than men. This emphasizes the importance of understanding of how environmental pathogens affects the homeostatic functions of immune system in developing diseases.

4.12 Conclusion

Since its discovery in 2004, mainstream research into *LRRK2* function has focused on the brain. Animal models utilized for studying the role of *LRRK2* have therefore been designed in this context (i.e., a primary neuronal function). However, results in this line of investigation have not been able to explain the role of mutant *LRRK2* in dopamine neurodegeneration (Greggio et al. 2012). Findings from multiple recent reports, together with our results suggest: that *LRRK2* plays a key role in the innate immune system of

mammals (Ness et al. 2013; Fava et al. 2016; Dzamko & Halliday 2012); that for *Lrrk2* proteins to show a robust effect in mice, an environmental trigger is essential (Herzig et al. 2011); and that genotypic variants may alter the course of infections in more than one organ system, such as the distal ileum (CD), peripheral nerves (leprosy) and the brain (parkinsonism) (Hakimi et al. 2011).

Taken together, our results showed robust expression of mammalian LRRK2 in both circulating and tissue-resident leukocytes of various organs including the CNS in humans. Inflammation caused by viruses, bacteria and microbial structures (or their mimics) was observed to induce LRRK2 expression in both human and mouse tissues including the CNS. Various mechanisms were discussed in detail for how LRRK2 could modify inflammatory responses based on recent literature reviews and our preliminary cytokine results.

The collective *in vivo* experiments with mutant *Lrrk2* mouse models indicated a prominent role for *Lrrk2* in modifying disease outcomes in systemic reovirus infections. To our knowledge, this is the first time a live virus is reported to worsen multiple disease outcomes in a *Lrrk2* genotype-dependent manner *in vivo* in mice. We found *Lrrk2* deficiency conferred vulnerability to increased virus burden and mortality in mice. Our results also suggested a partially protective, systemic role for the PD-linked G2019S mutation of *Lrrk2* by rendering the mice to have a higher resistance to infectious virion replication in both their lungs and brains, as mentioned. Counterintuitively, the survival study revealed higher mortality rates for females -but not male animals- that bear the G2019S *Lrrk2* mutation. This indicated to us that this *Lrrk2* (G2019S) mutation behaved in a sex-specific manner, and, excitingly, is reminiscent of the emerging data for a sex bias in humans, i.e., regarding the age of onset for PD (that is lower) and penetrance rate (that is higher) in female G2019S

carriers. In comparison to WT, the point mutation G2019S of *Lrrk2* generated a more pronounced effect / phenotype in our murine encephalitis model than did the deletion mutant of *Lrrk2*. In any case, the point mutation is physiologically more relevant in humans as it genocopies the common PD-linked mutation of *LRRK2*. Future studies should test other mutants (e.g., R1441C and a kinase-dead mutant). Similar experiments to our reovirus infection model are currently being pursued in the lab using other xenobiotic platforms, such as with *Salmonella* and *Listeria* bacterial strains.

An immediate implication of our findings is emphasizing safety concerns regarding development of LRRK2 kinase inhibitors as a therapeutic intervention for PD, and possibly, for other LRRK2-related diseases. The literature suggests that G2019S mutation of LRRK2 confers 2-3 fold increase in kinase function when compared to the wild-type protein (West et al. 2005; Greggio et al. 2006). As well, the kinase function of LRRK2 mediates toxicity as found in cell culture and mouse models of G2019S *Lrrk2* (Greggio et al. 2006). Furthermore, mutations in other region of LRRK2, such as in the GTPase domain, seem to additively enhance kinase activity of this protein (West et al. 2007). If LRRK2 function were to be reduced to below WT levels, increased vulnerability to infection may be a severe side effect of drugs that target its kinase activity in human subjects, which could compromise their overall health and treatment effects. Our model allows the testing of selective inhibitors of its kinase activity to test such scenarios in preclinical animal studies. Therefore, due to possible adverse effects of LRRK2 inhibition in the lung even without infection (Fuji et al. 2015) and following microbial exposure (our results), the overall safety of inhibitors in chronic treatment paradigms remains questionable.

A common shortcoming in LRRK2 research is lack of reliable anti-LRRK2 antibody, especially one that is useful in mice. An observation that others and we have made is that most anti-LRRK2 antibodies do not work well in mice. In the experiments with viral infection of mice, we did not routinely measure *Lrrk2* mRNA or protein in these samples, which could also be a factor in the outcome of all readouts. Thus, this was a caveat that similar future work should focus to rectify. Also, generation of reliable antibodies to murine *Lrrk2* is of great importance to the pre-clinical research field. Furthermore, due to limitations of my research time and animal facility space, in our *in vivo* experiments with reovirus, we did not get the chance to investigate the age-specific effects of the virus in escapees and its link to neurodegeneration later in life. Aging is an essential requirement for Parkinson disease susceptibility.

Environmental factors, such as pathogens, may well influence the penetrance of LRRK2 mutation in carriers. To first explore proof-of-principle within the innate immune system, we focused our study on an acute model of infection. In contrast, PD is a chronic condition and thus investigating mouse models in chronic infection paradigms is warranted for future studies.

Since LRRK2 mutations give rise to PD with diverse phenotypes, it is possible that various mutations of this gene utilize different pathogenic mechanisms. Accordingly, our results showed LRRK2 deletion and G2019S *Lrrk2* mutation have opposing effects on tissue virus burden in the mice. For that reason, studying animal models with other common LRRK2 mutations such as R1441C LRRK2 is helpful in understanding the pathophysiological mechanisms of LRRK2 to determine whether the same outcome differences occur as in the G2019S animals. As explained previously, disease determinants (such as for reovirus and

LRRK2 pathogenicity) comprise of both host and viral factors, which collectively give rise to a cascade of tissue changes. The molecular and cell-specific signaling pathways that become activated upon virus invasion when *Lrrk2* is mutated are of great interest and importance in further delineating the functions of WT and mutant *Lrrk2* proteins here. Due to time restrictions, I did not get a chance to explore these signaling pathways, but they should be followed up in future studies. For example, such signaling pathways could include immune/disease modulators that are recently reported to involve LRRK2 function and/or reovirus pathogenicity, such as signaling pathways in MyD88-dependent-TLR, TAK1, PKA, NF- κ B, IFN- γ , NFAT, Nlrp3 inflammasomes, P38 MAPK, ERK5, Ras signaling, PKR, virus receptors such as NgR1 and JAM-A, virus phagocytosis and exocytosis, as highlighted above.

Note, the contribution of sex to *Lrrk2*- and reovirus-related pathogenesis was only considered toward the end of my Research Project; it is likely that other immune investigations will corroborate a sex-specific behavior for LRRK2. In that manner, any future endeavors on LRRK2 research should certainly take sex into account when designing and analyzing experiments, both *ex vivo* and *in vivo*.

Furthermore, our results could have been more comprehensive if we measured actual murine *Lrrk2* kinase and/or GTPase activities in our experimental paradigms. The lack of knowledge on widely agreed upon, authentic substrates of LRRK2 kinase and GTPase functions in mammalian organs has further complicated the field of LRRK2 research.

Building on my cumulative findings of LRRK2 expression in distinct leukocyte subtypes, its cellular induction following inflammatory stimuli, and *Lrrk2*'s involvement as a modulator of reovirus encephalitis in mice, future investigations will explore two objectives: one, its potential interactors; and two, the downstream signaling events of endogenous *Lrrk2*.

Building on the concept of complex disease etiologies and therefore, further incorporating gene-environment interactions (Schlossmacher et al. 2017) in future PD research activities, scientists may finally elucidate the factors that influence the penetrance of *bona fide* LRRK2 mutations and the mechanisms that underlie the development of PD.

SUMMARY

Taken together, LRRK2's function proves to be multimodal, and possibly, cell-/tissue-specific, but most likely, stimulus-dependent as well. We hypothesized a role for LRRK2 in immune system functions, which most likely is of relevance to the pathogenesises of PD, Crohn's disease and leprosy.

I discovered robust LRRK2 expression in mammalian leukocytes, foremost within cells of the innate immune system. LRRK2 expression was further shown to be inflammation-driven in both human and mouse tissues including the CNS. The *in vivo* experiments with mutant *Lrrk2* mouse models showed a prominent role for *Lrrk2* in modifying disease outcomes in systemic reovirus infections. To our knowledge, this is the first time a live virus is reported to worsen multiple disease outcomes in a *Lrrk2* genotype-dependent manner *in vivo*.

Absence of *Lrrk2* led to increased vulnerability to elevated viral protein levels in the brain and increased death due to encephalitis in mice. In contrast, the PD-linked p.G2019S mutant was shown to be initially protective as lower viral titers were recorded in the lungs and brain of infected mutant mice. Paradoxically, a significantly higher (rather than the expected reduced) mortality rate was also observed in G2019S *Lrrk2* female mice. This observed sex-specific outcome in G2019S *Lrrk2* mice is a novel finding which is also reminiscent of the sex bias reported in humans carrying G2019S LRRK2 mutation.

Further characterizing LRRK2's behavior, in an immune system context, will likely offer more valuable knowledge in delineating the pathogenesis of PD as well as of CD and leprosy. Such newly gained insights could help better direct therapeutic efforts that are urgently needed for more effective treatments of these devastating human diseases and their complications.

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PUBLICATION RECORD AND AWARDS BY CANDIDATE

PUBLICATIONS

- **Hakimi M**, Selvanantham T, Swinton E, Padmore RF, Tong Y, Kabbach G, Venderova K, Girardin SE, Bulman DE, Scherzer CR, LaVoi MJ, Gris D, Park DS, Angel JB, Shen J, Philpott DJ, Schlossmacher MG. (2011) **Parkinson's disease-linked LRRK2 is expressed in circulating and tissue immune cells and upregulated following recognition of microbial structures.** J Neural Transm. 118:795-808.
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- Mollenhauer B, Trautmann E, Otte B, Ng J, Spreer A, Lange P, Sixel-Döring F, **Hakimi M**, Vonsattel JP, Nussbaum R, Trenkwalder C, Schlossmacher MG. (2012) **α -Synuclein in human cerebrospinal fluid is principally derived from neurons of the central nervous system.** J Neural Transm. 119:739-46.

AWARDS

- The Parkinson Research Consortium Fellowship (the Crabtree Family Fellowship) 2011
- The Parkinson Research Consortium Fellowship (the Larry Haffner Parkinson Research Fellowship) 2012-2013

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Parkinson's disease-linked *LRRK2* is expressed in circulating and tissue immune cells and upregulated following recognition of microbial structures

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Abstract Sequence variants at or near the leucine-rich repeat kinase 2 (*LRRK2*) locus have been associated with susceptibility to three human conditions: Parkinson's disease (PD), Crohn's disease and leprosy. As all three disorders represent complex diseases with evidence of inflammation, we hypothesized a role for *LRRK2* in immune cell functions. Here, we report that full-length Lrrk2 is a relatively common constituent of human peripheral blood mononuclear cells (PBMC) including affinity isolated, CD14⁺ monocytes, CD19⁺ B cells, and CD4⁺ as well as CD8⁺ T cells. Up to 26% of PBMC from healthy donors and up to 43% of CD14⁺ monocytes were stained by anti-Lrrk2 antibodies using cell sorting. PBMC lysates contained full-length (>260 kDa) and higher molecular weight Lrrk2 species. The expression of *LRRK2* in circulating leukocytes was confirmed by microscopy of human blood smears and in sections from normal

midbrain and distal ileum. Lrrk2 reactivity was also detected in mesenteric lymph nodes and spleen (including in dendritic cells), but was absent in splenic mononuclear cells from *lrrk2*-null mice, as expected. In cultured bone marrow-derived macrophages from mice we made three observations: (i) a predominance of higher molecular weight Lrrk2; (ii) the reduction of autophagy marker LC3-II in ^{R1441C}Lrrk2-mutant cells (<31%); and (iii) a significant up-regulation of *lrrk2* mRNA (>fourfold) and protein after exposure to several microbial structures including bacterial lipopolysaccharide and lentiviral particles. We conclude that Lrrk2 is a constituent of many cell types in the immune system. Following the recognition of microbial structures, stimulated macrophages respond with altered *lrrk2* gene expression. In the same cells, *lrrk2* appears to co-regulate autophagy. A pattern recognition receptor-type function for *LRRK2* could explain its locus' association with Crohn's disease and leprosy risk. We speculate that the role of Lrrk2 in immune cells may also be relevant to the susceptibility of developing PD or its progression.

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Introduction

Parkinson's disease (PD) is a progressive, late-onset disorder of the nervous system of unclear pathogenesis. Several loci, multiple genotypes and selected environmental triggers have been identified in the aetiology of this complex disorder (Klein and Schlossmacher 2007). Previously published autopsy studies of brain from PD patients and rodent models have provided evidence for neuroinflammation (Hirsch and Hunot 2009; Reale et al. 2009), including activation of microglia and infiltration of the midbrain by CD4⁺ and CD8⁺ lymphocytes (Brochard et al. 2009).

Heterozygous missense mutations of the *LRRK2* gene represent a common cause of sporadic and heritable PD (Mata et al. 2006; Greggio and Cookson 2009; Simón-Sánchez et al. 2009; Marín 2006; Taylor et al. 2006). Intriguingly, the G2019S variant of Lrrk2 (the protein is also referred to as Dardarin) can be found in ~40% of all PD patients of Arab background and in ~20% of PD subjects with Ashkenazi Jewish heritage (Ozelius et al. 2006). Depending on ethnicity and geography, the frequency of bonafide mutations in the *LRRK2* gene can vary considerably (Grimes et al. 2007). In contrast to other genes linked to late-onset neurological disorders, the mean penetrance rate for certain pathogenic *LRRK2* mutations (e.g., G2019S) can be relatively low (~30%) (Goldwurm et al. 2007). When considered together, these results suggested to us that *LRRK2* may act chiefly as a susceptibility gene, and that a “second hit” is required to express the PD phenotype in an autosomal dominant-type manner (Klein and Schlossmacher 2007).

In situ hybridization and Northern blotting results revealed surprisingly low signals for *LRRK2* mRNA in mammalian brain with modest amounts found in the putamen and caudate, and very low levels recorded in critical PD sites including the Substantia nigra of human midbrain (Galter et al. 2006; Zimprich et al. 2004). In contrast, *LRRK2* transcripts are more abundant in kidney, lung, spleen and testis of mammals (e.g. Maekawa et al. 2010).

In their first report on the discovery of *LRRK2*, Zimprich et al. identified an unexpected heterogeneity of neuropathological findings in several mutation-carrying patients. The postmortem analysis of their initial cases and of subsequently examined material revealed three distinct

phenotypes: (i) brainstem synucleinopathy (= typical PD); (ii) diffuse Lewy body pathology (= definite dementia with Lewy bodies); and (iii) nigral degeneration without a distinctive histopathology. Rarely, a tangle bearing, progressive supranuclear palsy-type pathology was diagnosed (Zimprich et al. 2004; Wider et al. 2010). This range of expressivity in neurodegeneration thus positioned *LRRK2* mutations farther away from the dysregulation of α -synuclein (*SNCA*) metabolism than mutational events affecting the *SNCA* gene and the *GBA1* gene (e.g., Neumann et al. 2009; Cullen et al. 2011).

The *LRRK2* gene contains 51 exons that encode a 2,527 amino acid-long, >260 kDa large protein (Marín 2006). Full-length (FL) Lrrk2 contains a leucine-rich repeat (LRR) domain, a Roc GTPase domain, a COR domain, a mitogen-activated protein kinase (MAPK) domain, and a C-terminal WD40 domain (Mata et al. 2006; Greggio and Cookson 2009; Cookson 2010). Its LRR and WD40 elements are thought to be involved in interactions with negatively charged proteins, phospholipids or nucleic acids. Lrrk2 protein is associated with the cytoplasm, the plasma membrane, autophagic vacuoles and mitochondria (West et al. 2005; Alegre-Abarrategui et al. 2009; Melrose 2008). The physiological and pathological substrates of Lrrk2's MAPK-type activity are under intense investigation (Mata et al. 2006; Greggio and Cookson 2009). Its GTPase function is thought to control MAPK activity including auto-phosphorylation (Mata et al. 2006; Greggio and Cookson 2009). Berger et al. (2010) recently demonstrated that Lrrk2 multimers (e.g. dimer) are more likely to be associated with membrane structures, to show more efficient binding of GTP, and to confer greater kinase activity.

Mutations in the GTPase domain (e.g. R1441C) cause accumulation of autophagic vacuoles with increased levels of p62 (Alegre-Abarrategui et al. 2009). Intriguingly, Tong et al. (2010) recently created knock-out mouse models of *lrrk2*, which—unexpectedly—revealed no neurological or neurochemical defects, but showed impaired protein degradation via the autophagy-lysosome pathway, in particular in the kidneys. Accumulation of lipofuscin, a rise in ubiquitinated proteins, dysregulation of LC3-II and p62, and up-regulation of pro-inflammatory as well as pro-apoptotic proteins were recorded in 20 month old *lrrk2*^{-/-} mice. These findings identified an essential role for *lrrk2* in lysosomal autophagy outside the nervous system, and—if confirmed by other *lrrk2*-null models—raise further questions as to the precise mechanism(s) by which *LRRK2* mutations confer neurological disease in humans.

In a recently published genome-wide association study, single nucleotide polymorphisms at the *LRRK2/MUC19* locus were identified as modulators of Crohn's disease (CD) susceptibility (Barrett et al. 2008). CD is classified as

an autoimmune disease where the terminal ileum comes under attack by the host's immune system in response to microbial colonization and/or invasion, thereby resulting in chronic inflammation. To date, 30 distinct loci have been involved in CD susceptibility including genes encoding innate pattern recognition molecules, lymphocyte differentiation factors, autophagy components, and regulators of secondary immune responses (van Limbergen et al. 2009). Among the group of 'pattern recognition receptors', mutations in the gene encoding nucleotide-binding domain-contacting-2 (Nod2) are strongly associated with CD risk (Stockton et al. 2004; Ogura et al. 2001; Maeda et al. 2005). The structure of Nod2 contains six LRRs. Nod2 is predominantly expressed in peripheral leukocytes and is involved in the activation of nuclear factor- κ B (NF- κ B) signaling following the recognition of bacterial lipopolysaccharides (LPS).

In an unrelated genome-wide association study of leprosy susceptibility, Zhang et al. (2009) identified single-nucleotide polymorphisms at the *LRRK2* locus as risk modifiers. Leprosy (aka Hansen's disease) is caused by infection with *Mycobacterium leprae*, which shows tropism for macrophages and Schwann cells (Schurr et al. 2006; Alcaïs et al. 2005). Zhang et al. proposed a gene-interaction network involving multiple loci including *LRRK2* that when altered confers susceptibility to leprosy. Importantly, variations in several of the genes associated with leprosy risk were previously linked to inflammatory bowel disease, including CD (Schurr and Gros 2009; Buschman and Skamene 2004).

Given the evidence reviewed above, we speculated that the function of endogenous *Lrrk2* may: (i) be similar to a LRR domain-carrying member of the pattern recognition receptor family (e.g. Nod2); and (ii) play an important role in autophagy by cells of the innate or adaptive immune system, or both. To date, three studies have provided evidence for a role of *Lrrk2* in immune system function. Maekawa et al. (2010) found high *Lrrk2* expression in one specific subtype of murine B lymphocytes (i.e. B1 cells). The same group reported that *Lrrk2* mRNA and protein levels progressively decrease in mouse spleen during aging. This decline was not observed in kidneys, lungs or brain (Maekawa et al. 2010; Kubo et al. 2010). More recently, Gardet et al. (2010) identified several other leukocyte populations with relevance to CD pathogenesis that expressed *Lrrk2* and provided evidence that the gene's transcription is up-regulated through an interferon- γ (IFN- γ)-mediated signaling pathway.

In this study, we extend these findings and show that FL *Lrrk2* is a constituent of human peripheral blood mononuclear cells (PBMC) including monocytes, T cells and B cells. We confirm the expression of *LRRK2* in circulating leukocytes by microscopy of peripheral blood smears, in

sections from normal midbrain and distal ileum, and by isolating cells from organ homogenates of mesenteric lymph nodes and spleen. We also find that cultured bone marrow-derived macrophages (BMDM) from mice show robust basal levels of higher molecular weight (HMW) *Lrrk2*; there, we also provide evidence for autophagy dysregulation in ^{R1441C}*Lrrk2* mutant knock-in mice, and demonstrate the up-regulation of *Lrrk2* mRNA and protein in BMDM after exposure to microbe-associated pathogens.

Materials and methods

Human blood and tissue collection

Venous blood was collected by routine phlebotomy from healthy adult donors ($n = 10$) at The Ottawa Hospital using EDTA as an anticoagulant. Blood collection vacutainers were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Epstein-Barr Virus (EBV)-transformed lymphoblasts generated from healthy control donors ($n = 4$) and PD subjects ($n = 2$) were obtained from the Harvard NeuroDiscovery Center and Dr. I. Irrcher, respectively (Aleyasin et al. 2010). Sections of formalin-fixed, paraffin-embedded human tissues (encompassing midbrain, distal ileum, spleen, mesenteric lymph nodes; $n = 2$ each) were collected during routine autopsies from neurologically healthy subjects at The Ottawa Hospital and kindly provided by Dr. J. Woulfe. Human specimens were collected according to ethics board-approved institutional guidelines.

Animal models expressing mutant *Lrrk2* genotypes

The generation of *Lrrk2* knock-in mice carrying the ^{R1441C}*Lrrk2* variant and of germ line deleted *Lrrk2*^{-/-} mice (targeting the promoter and exon 1) were previously reported (Tong et al. 2009, 2010; Tong and Shen 2009). These mice as well as human *LRRK2* cDNA-transgenic flies and their littermates (carrying the same regulatory domains but without *LRRK2* cDNA in their transgene construct) were used as positive and negative controls in microscopic and biochemical experiments. The generation and characterization of these flies were previously reported (Venderova et al. 2009).

Sorting of leukocytes

Collection of PBMC from human donors (20–60 ml), their separation from red blood cells and granulocytes using Ficoll gradients, and affinity-isolation of CD14⁺ monocytes, CD19⁺ B cells, and CD4⁺ as well as CD8⁺ T cells were carried out, as described. Leukocyte subtypes were

magnetically labeled using MicroBeads directed against cell surface markers and processed by MACS® Separator. Selected cells were eluted, fixed, permeabilized, stained with primary and secondary antibodies, and gated by FACS, as reported (Vranjkovic et al. 2011). In related experiments, homogenates of select wild-type (WT) mouse organs (age 13 weeks; C57BL/6 strain) were dissected; single cell suspensions were prepared by mechanically passing tissue lysates through a sterile cell strainer (70 µm nylon mesh) using a pestle. Cells were then seeded in 96-well plates, and stained with anti-Irrk2 antibody MJFF-1 (clone, C5–8) along with antibodies directed against various sub-populations of leukocytes in each organ. Anti-mouse antibodies CD11b (PE) (a marker of macrophages), CD45R (B220) APC-eFluor® 780 (a marker of B cells), TCRβ APC-eFluor® 780 (a marker of T cells), Ly-6G (Gr-1) Cy5 (a marker of granulocytes) and CD11c PE-Cy7 (a marker of dendritic cells) were used to sort different cell types. Detection of Irrk2 intracellular staining was carried out by applying secondary antibody (anti-rabbit IgG (H + L) Alexa Fluor® 488) at optimized concentrations. Subsequent flow cytometry analysis determined the relative expression levels of proteins of interest (Le Bourhis et al. 2009).

Bone marrow-derived macrophage cultures

Protocols for obtaining BMDM from mice were adapted from those by Werts et al. (2007). Briefly, BMDM were cultured in RPMI 1640 medium, fetal calf serum (10% v/v), penicillin (100 U/ml), streptomycin (100 µg/ml), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), and supplemented with conditioned media of a macrophage colony-stimulating factor (M-CSF)-expressing cell line (L929, 30% v/v). BMDM were plated and maintained at 5% CO₂ at 37°C, and stimulated on day 7. A separate set of macrophages were transduced with lentivirus. Recombinant viral particles were produced by co-transfection of HEK293T cells with lentiviral vector (pHR-SIN-CSGWΔNotI-GFP-LC3, from C. Münz) and the helper plasmids pCMVΔR8.91 and pMDG. The supernatants of HEK293T cells that contained lentiviral particles were collected and added to BMDM. The transduction medium contained polybrene (8 mg/ml). Macrophages were centrifuged at 2,000g for 90 min. After over-night incubation at 37°C in 5% CO₂, fresh medium was added to the cells and transduction was repeated after 24 h, as described by Travassos et al. (2010).

Stimulants used to activate BMDM included C12-iE-DAP, L18-MDP (Nod1 and Nod2 ligands, respectively; 50 µg/ml each), MDP (muramyl dipeptide; 10 µg/ml), FK156 [D-lactyl-L-alanyl-D-glutamyl-(L)-mesodiamino-

pimelyl-(L)-glycine; 10 µg/ml], rapamycin (50 µg/ml), LPS (lipopolysaccharide; 100 ng/ml), ATP (adenosine triphosphate; 5 mM), or nigericin (1–15 µM), heat-killed (HK) listeria (8 × 10⁸ CFU/ml, MOI 25), CpG (synthetic oligonucleotides with unmethylated CpG dinucleotides serving as TLR9 ligand; 1 µM). All stimulants were purchased from Invivogen (San Diego, CA, USA) except HK bacterial lysates, which were prepared, as described (Travassos et al. 2004). Cells supernatants were collected 16 h after stimulation and analyzed using ELISA kits from R&D (Minneapolis, MN, USA).

For quantification of mouse *Irrk2* mRNA, BMDM were harvested from femur of WT mice and differentiated in medium containing 10 n/ml GM-CSF for 7 days. BMDM were stimulated for 5 h with different Toll-like receptors (TLR) agonists: Pam3CSK4 (P3S; 5 µg/ml) targeting TLR2; Poly (I:C; PIC; 10 µg/ml) for TLR3; LPS (1 µg/ml) for TLR4; R848 (4 µg/ml) for TLR7; and CpG (10 µg/ml) for TLR9. Pam3Cys, poly(I:C), ultrapure LPS, imiquimod (R837), and mouse CpG-B (ODN 1826) were purchased from InvivoGen (San Diego, CA, USA).

Antibodies, immunoblotting and microscopy

Rabbit polyclonal antibodies to Lrrk2 (#NB300-268) and LC3 (#NB100-2220) were from Novus Biol (Littleton, CO). Each of these two antibodies were used at a dilution of 1:3,000 for Western blotting (WB). Five rabbit monoclonal antibodies to Lrrk2 (MJFF-1 to -5) were provided by the Michael J. Fox Foundation (New York, NY, USA). For WB, these antibodies were used at a dilution of 1:1,000; for FACS at 1:200. An affinity-purified rabbit polyclonal anti-human Lrrk2 antibody (“HL-2”) was prepared at OPEN Biosystems Inc (Huntsville, AL, USA) against residues 2,508–2,527 and used at a dilution of 1:3,000 for WB and 1:300–1:1,000 in immunohistochemistry (IHC). SDS/PAGE, WB and IHC protocols were previously described (Schlossmacher et al. 2002; Schlossmacher and Shimura 2005; Cullen et al. 2009). Staining of peripheral blood smears was carried out as per our IHC protocol using the avidin–biotin–peroxidase (ABC) method and 3,3′-diaminobenzidine substrate for color (brown) visualization of antibody reactivity. The ABC kit was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). During fixation of smears, methanol was substituted for formalin.

RT- and qPCR to quantify mRNA

Human *LRRK2* mRNA was isolated from preparations of white blood cells and analyzed by RT-PCR using three distinct pairs of primers: #1: AGGTGGGTTGGTCAC

TTCTG (forw.), ATGAGTGCATGGCATCAAAA (rev.); #2: AGATGCCAATCAAGCAAAGG (forw.), AAGGACCAAGCCAAGAAGGT (rev.); #3: TTCTTCCCCTGTGATTCTCG (forw.), GCTGTCCAACAACAAGCTGA (rev.). Mouse mRNA was purified from BMDM using RNeasy MiniKitCat# 74104 (Qiagen MA). Murine *lrrk2* primers were: TCTGGCTGGAACCCTGCTAT (forw.); and AACTGGCCATCTTCA TCTCC (rev.). Quantification of mRNA was performed using the $\Delta\Delta$ CT method (relative to 18S RNA) and compared to unstimulated controls.

Cytokine and chemokine quantification by ELISA

Cyto-/chemokine antibody kits were purchased from R&D Systems (Minneapolis, MN, USA). ELISAs were used to measure the levels released into the conditioned media of BMDM from genotyped mice. BMDM were stimulated with inflammatory reagents for 16 h prior to collection of their supernatants. 96-well plates were coated with cyto-/chemokine antibodies including keratinocyte-derived chemokine (KC) and IL-6.

Results

Detection of Lrrk2 reactivity in circulating leukocytes

To screen for evidence of *LRRK2* expression in cells of the immune system, we first examined peripheral blood smears from healthy adult controls by immunocytochemistry using a panel of anti-Lrrk2 antibodies (Fig. 1). There, affinity-purified polyclonal HL-2 reacted with cytoplasmic and membrane-associated structures in a subset of leukocytes that were 10–12 μ m in size and showed morphological characteristics of monocytes (Fig. 1a). Smaller cells that appeared lymphocytic in nature with a larger nucleus-to-cytoplasm ratio stained infrequently (see also below). Longer development of sister slides generated an occasional but faint HL-2 immunoreactivity in typical granulocytes, which we identified by counterstaining with hematoxylin (Fig. 1b); in contrast, erythrocytes and platelets, which were readily detectable by anti-SNCA antibodies (Scherzer et al. 2008), were never stained by anti-Lrrk2.

We next examined neural and gastrointestinal tissue sections for Lrrk2 reactivity by routine IHC with anti-Lrrk2 (HL-2). Examining sections of distal ileum (Fig. 1c), ascending colon and human midbrain (Fig. 1d) from neurologically healthy donors, we detected occasional intravascular leukocytes that stained for Lrrk2. Under these conditions, no HL-2 reactivity was seen in enteric neurons of the submucosal and myenteric plexi or in any neuromelanin-carrying cells of the S. nigra (Fig. 1c, d). However, in parallel sections these ganglion cells and neurons

were labelled by anti- α -synuclein and anti-tyrosine hydroxylase antibodies, respectively (not shown).

Given the controversy in the PD field regarding the specificity of many anti-Lrrk2 antibodies (Biskup et al. 2007), we validated the staining obtained by anti-Lrrk2 HL-2 using sections of genotyped mouse spleens, which were processed like human tissue (above) and examined using the same IHC protocol. There, HL-2 identified a subset of mononuclear splenic leukocytes in WT mice ($n = 4$; Fig. 1e, f), but not in *lrrk2*-null animals ($n = 4$; Fig. 1g, h). In sections from *lrrk2*-deficient mice, only autofluorescent intracellular granules (representing lipofuscin) were visible. Signals were also absent when the primary antibody was omitted, as expected (Fig. 1f, h). We concluded from these results that antibody HL-2 reactivity was specific and that Lrrk2 is present in select populations of circulating leukocytes from human blood, including in capillaries of tissues that are affected by the pathogenesis of CD and PD.

Quantification of Lrrk2-expressing mononuclear cells from human blood

To better characterize the subpopulations of leukocytes that express Lrrk2 protein, we first enriched PBMC by routine Ficoll preparation, and then carried out fluorescence-based automated cell sorting (FACS; see “Materials and methods”) with anti-Lrrk2 antibodies (Fig. 2). For the purpose of optimizing conditions for anti-Lrrk2 to bind and capture permeabilized cells, we also explored the use of several monoclonal rabbit antibodies, which recently became available (MJFF 1-5; see below). Panels in Fig. 2a1-2 show absence of Lrrk2 signal in PBMC when no anti-Lrrk2 antibody was present (0.58%), as expected; in contrast, 25.71% of PBMC were stained by anti-Lrrk2 MJFF-1. Similarly, panels in Fig. 2b1-2 show percentage of Lrrk2-positive cells in CD14+ monocytes at 0.81% when no anti-Lrrk2 antibody was present. In contrast, 43.17% were stained when anti-Lrrk2 MJFF-1 was employed. From these results we concluded that $\leq 26\%$ of all PBMC were Lrrk2-reactive, and that within the pool of CD14+ monocytes $\sim 43\%$ were Lrrk2-positive.

Detection of full-length Lrrk2 in monocytes, B cells and T cells

Detection of Lrrk2 in PBMC was further substantiated by SDS/PAGE and immunoblotting when we probed lysates with a panel of antibodies (Fig. 3; Suppl. Figure 1A–D). There, we specifically detected the presence of the FL-Lrrk2 monomer (>260 kDa) and of a 245–250 kDa variant in PBMC lysates, as detected by anti-Lrrk2, HL-2. No Lrrk2 signal was seen in red blood cell (and granulocyte) lysates (Fig. 3a). Monoclonal MJFF-1 confirmed these

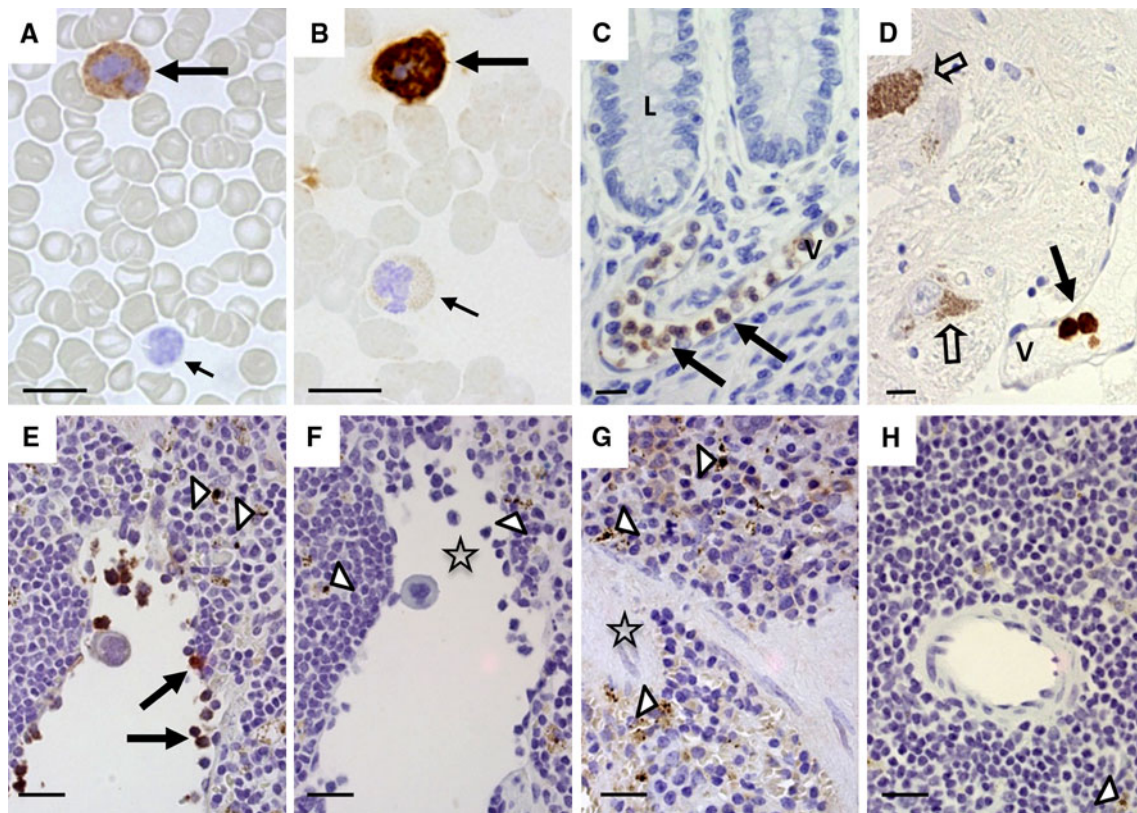


Fig. 1 Lrrk2 reactivity in mammalian leukocytes using immunocytochemistry and immunohistochemistry. **a–d** Lrrk2 reactivity was detected in select leukocytes of peripheral blood smears (**a, b** healthy controls; $n = 5$), and in intravascular leukocytes of the submucosa in the distal ileum (**c**; neurological controls; $n = 2$) and human midbrain (**d**) as denoted by *large arrows*. *Small arrows* point to unstained and poorly stained leukocytes; *open arrows* identify autofluorescent neuromelanin in dopaminergic cells of the midbrain. *V* indicates vessel; *L* lumen of the gut. Bar length equals 12 μm ; original magnification, 60 $\times$. Lrrk2 signal was generated by affinity-purified, polyclonal anti-Lrrk2 antibody [“HL-2” raised against a synthetic

peptide comprising residues 2508–2527 of human, wild-type Lrrk2 (Berger et al. 2010)]. **e–h** The specificity of the Lrrk2 antibody used in **a–d** was confirmed in spleen sections from age-matched, wild-type (**e, f**) and *lrrk2* knock-out (**g, h**) mice ($n = 4$ each). Parallel sections were developed with anti-Lrrk2 HL-2 as primary antibody (**e, g**) or without any primary antibody present (**f, h**). *Arrows* depict lrrk2-immunoreactive leukocytes in the spleen; *open arrow heads* show autofluorescent cytoplasmic granules seen irrespective of antibody use; *open asterisks* denote groups of unstained leukocytes (**e–h**). Bar length equals 20 μm ; original magnification, 40 $\times$

findings (Fig. 3b), and also clearly detected HMW Lrrk2 species. The specificity of our antibodies was confirmed in all WB experiments using lysates of fly heads that were either transgenic for *LRRK2* cDNA (tg) or from control (WT) littermates (Fig. 3a–f). Four additional monoclonal rabbit antibodies to Lrrk2 produced identical results to those shown in Fig. 3a, b although with variable staining of HMW Lrrk2 (Suppl. Figure 1A–D).

Next, we confirmed the presence of endogenous Lrrk2 protein in circulating monocytes, which was suggested by our analysis of blood smears and FACS data (above). As shown in Fig. 3c, CD14⁺ monocytes isolated by magnetic beads contained FL-Lrrk2. The identity of these sorted cells was further confirmed by the presence of the ~62 kDa acid- β -glucosidase protein, a well established constituent of monocytes (lowest panel in Fig. 3c) and tissue macrophages.

Following affinity enrichment by magnetic beads, we also detected FL-Lrrk2 in CD8⁺ and CD4⁺ T cells as well as in CD19⁺ B cells (a pan-B cell marker; Fig. 3d, e). In comparing their adjusted signals, we found that members of the B cell family consistently showed higher Lrrk2-to- β -actin ratios than CD4⁺ or CD8⁺ T cells in peripheral blood specimens from healthy adults (examined under basal rates). The highest relative expression of endogenous *LRRK2* in the myeloid lineage was recorded in EBV-transformed lymphoblasts (Fig. 3f; Suppl. Figure 1A). The expression of murine *lrrk2* in these different subpopulations of white blood cells (monocytes; B cells; T cells) was confirmed in leukocytes isolated from tissue homogenates of WT mice, e.g. mesenteric lymph nodes, spleen (Suppl. Figure 2A) and Peyer’s patches (not shown). Lrrk2 signals in granulocytes varied greatly in our experiments (see below).

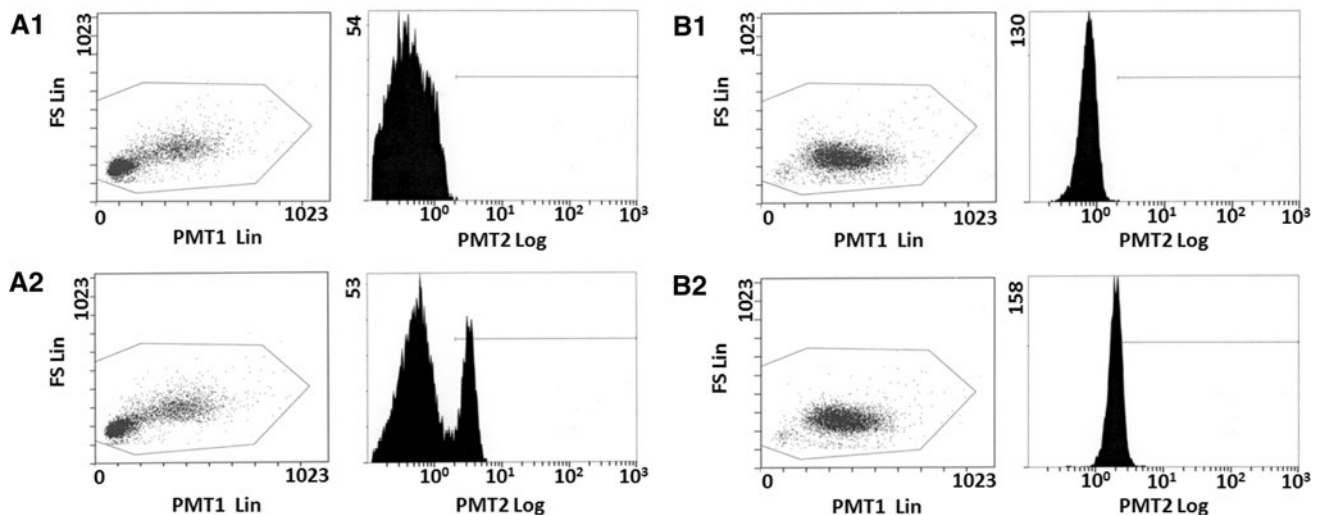


Fig. 2 *Lrrk2* reactivity in human peripheral blood mononuclear cells and isolated monocytes. *Lrrk2* reactivity was detected in peripheral blood mononuclear cells (PBMC) (**a1**, **a2**) and CD14+ monocytes (**b1**, **b2**) that were isolated by magnetic bead separation from healthy adult controls ($n = 3$). FACS analysis of fixed and permeabilized cells was carried out using monoclonal anti-*Lrrk2* antibody MJFF-1(C5-8). The fluorophore used was Alexa Fluor® 488. For each pair of the diagrams, the left panel shows forward and side scatter of the ungated

cell population, and the right panel is a flow histogram obtained for the intracellular staining of *Lrrk2*. PBMC and monocytes were collected from two different individuals. Note that the background signal was 0.58% in PBMC (**a1**) and 0.81% in monocytes (**b1**) in unstained cells. However, following the optimization of primary and secondary antibody concentrations the percentage of *Lrrk2*-positive cells was determined to be 25.71% for the pool of PBMC (**a2**) and 43.17% for CD14+ monocytes (**b2**)

Lrrk2 is expressed in tissue macrophages, induced by pathogens and involved in autophagy regulation

Given the robust expression of the *LRRK2* gene in human monocytes, we next explored its presence in tissue macrophages (Fig. 4); the latter are derived from circulating monocytes and play a key role in the pathogenesis of CD and leprosy. To this end, we isolated and cultured primary BMDM from femurs and tibias of both WT mice and R^{1441C} *lrrk2*-mutant animals (heterozygous and homozygous) using a published ‘macrophage colony stimulating factor’-based protocol (see “Materials and methods”). When probing BMDM cultures, which were prepared in parallel from age-matched mice of three different genotypes, using SDS/PAGE/WB, we found comparable and robust amounts of HMW *lrrk2* in all lysates examined (Fig. 4a); when probing BMDM lysates from *lrrk2*-deficient mice we observed no HMW *lrrk2* signal, as expected (not shown). These results suggested two things: similar expression and stability rates for the endogenous R^{1441C} *lrrk2* mutant protein when compared with WT *lrrk2*; and a predominance of HMW *lrrk2* (>290 kDa) in cultured murine macrophages versus the FL *Lrrk2* species seen in circulating human monocytes (>260 kDa).

When we probed BMDM with autophagy markers, we observed variable results for LC3-I and p62 proteins in their lysates, but found a consistent reduction in LC3-II levels in all R^{1441C} *lrrk2*-expressing macrophages (Fig. 4b). Quantifying the ratio of LC3-II-to- β -actin signals, we

recorded a $\leq 31\%$ reduction in R^{1441C} *lrrk2* mutant versus WT cells. The expression of endogenous *lrrk2* in tissue macrophages was confirmed independently in cells isolated from homogenates of spleen, lymph nodes (Suppl. Figure 2A) and Peyer’s patches (not shown).

Given the robust expression of *lrrk2* protein in isolated macrophages from multiple murine tissues, we next explored the possible induction of *lrrk2* transcription following the exposure of BMDM to established inflammatory stimuli through ‘pattern recognition receptors’ (Fig. 4c). Using rt-qPCR, we recorded specific down- (rather than up)-regulation of *lrrk2* mRNA following the activation of Toll-like receptor (TLR)-2 by Pam3CSK4. However, in parallel we recorded the specific and significant up-regulation of *lrrk2* mRNA in BMDM that were stimulated with ligands for TLR4 [(i.e., LPS); >4-fold], for TLR7 (with R837; >2-fold), and for TLR9 [with CpG molecules; >3-fold] (Fig. 4c). These transcriptional changes were observed as early as 5 h after the addition of stimulants signalling microbial invasion.

To determine whether viral transduction of BMDM also induced the transcription of *lrrk2* and—if so—whether increased mRNA signals also translated into increased protein levels, we next exposed cultured macrophages to eGFP-LC3 cDNA-carrying lentiviral preparations. When examining transduced BMDM versus virus-free sister cultures 16 h later by SDS/PAGE/WB with anti-*Lrrk2* (and anti- β -actin), we consistently saw a rise in *lrrk2* following viral transduction (Fig. 4d; compare lanes 2 and 10). The

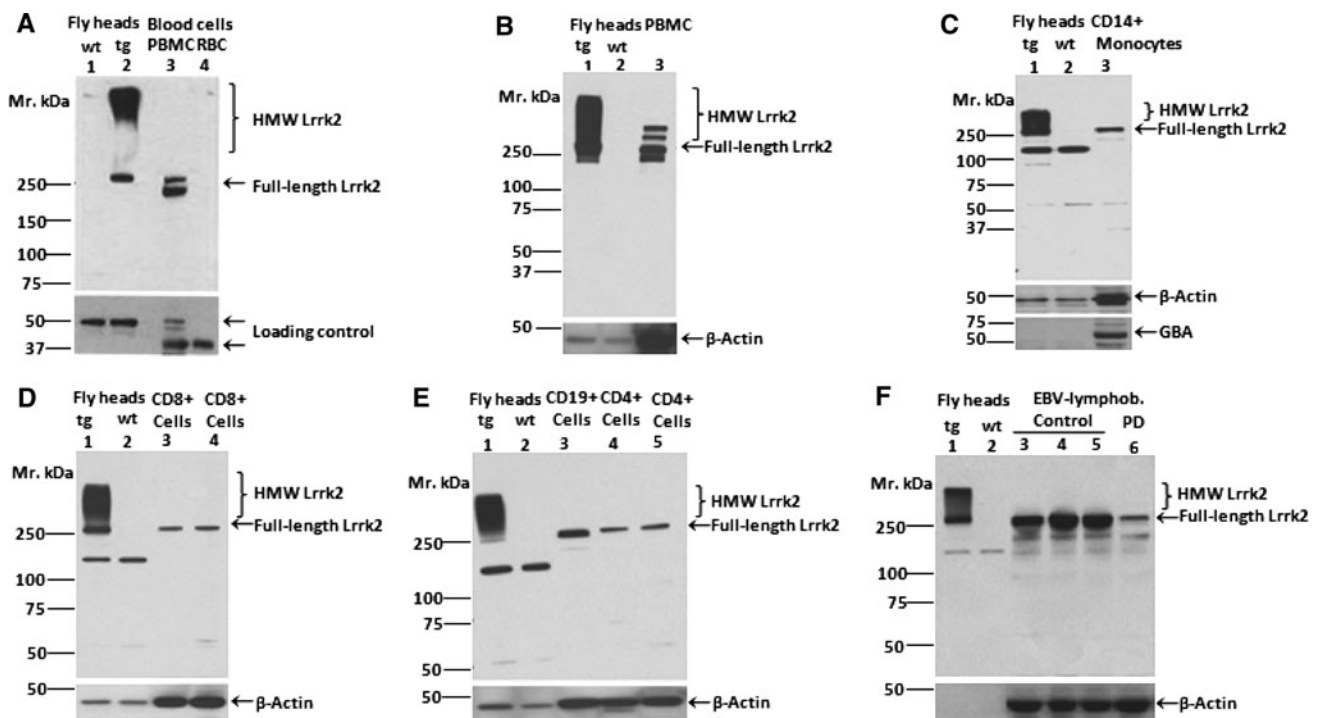


Fig. 3 Detection of full-length Lrrk2 protein in isolated monocytes, B cells and T cells. **a–f** Peripheral blood mononuclear cells (PBMC; **a**, **b**) were obtained by Ficoll separation from healthy adult controls ($n = 5$) and separated from erythrocytes (RBC) and granulocytes. PBMC were further processed using antibodies to CD14 (monocyte marker; in **c**), CD4 and CD8 (both T cell markers; in panels **d**, **e**), and to CD19 (B cell marker; **e**), which were conjugated to and immobilized by magnetic beads (see “Materials and methods”) (Vranjkovic et al. 2011). Isolated subpopulations of leukocytes were lysed and then subjected to denaturing SDS/PAGE under reducing conditions, followed by Western blotting. Transgenic flies expressing full-length *hLRRK2* cDNA (*tg*) and their non-*hLRRK2* littermates (*wt*) were used as positive and negative controls, respectively. EBV-transformed, B cell derived lymphoblasts from healthy control and Parkinson’s

disease donors ($n = 5$) were run in parallel as additional controls (panel **f**). Immunoblot in **A** was probed with affinity-purified, polyclonal anti-human Lrrk2 antibody (HL-2) raised against residues 2508–2527; probing with a monoclonal rabbit anti-Lrrk2 antibody [MJFF-1(C5-8)] is shown in **b**. Polyclonal anti-Lrrk2 antibody NB300-268 was used for membranes **c–f**. Anti-acid β -glucosidase was used as an independent marker of peripheral monocytes (see bottom panel in **c**). All membranes were stripped and reprobed with anti- β -actin antibody as loading control. Note, the robust and specific detection of endogenous, full-length Lrrk2 proteins (245–260 kDa) and higher molecular weight (HMW) species thereof in human PBMC, monocytes, B cells, T cells and EBV lymphoblasts. There, CD19⁺ B cells showed a slightly stronger signal for Lrrk2 when compared with isolated CD4⁺ T cells enriched from the same donor (**e**)

effect was the same for ^{R1441C} mutant and WT*lrrk2*-expressing alleles; furthermore, the *lrrk2*-to- β -actin ratio remained elevated in all virally transduced BMDM irrespective of the nature of additional stimuli (Fig. 4d; compare signals in lanes 2–9 vs. lane 10). We concluded from these results that our mRNA and protein data in BMDM cultures were consistent; they revealed a modest but significant up-regulation of *lrrk2* following the recognition of most microbial structure-associated stimuli tested.

Lrrk2 expression does not control IL-6 and KC cytokine release by stimulated macrophages

To investigate the involvement of Lrrk2 in cytokine production and/or release rates, we also screened conditioned media of BMDM cultures following the addition of C12 (a Nod1 ligand), L18 (Nod2 ligand), LPS, and heat-killed (HK) listeria. There, we focused on interleukin-6 (IL-6)

and keratinocyte chemokine (KC), two cytokines that have been previously linked to macrophage function (Fig. 5; Suppl. Figure 2B, C). When we first compared the effect of ^{R1441C}Lrrk2 mutant versus WT*lrrk2*-expressing cells, we saw some variability but no consistent changes in IL-6 and KC concentrations in BMDM supernatants (Suppl. Figure 2B, C). We next explored the effect of presence versus deficiency in Lrrk2 (Fig. 5). There, we recorded the significant and multi-fold rise in IL-6 and KC concentrations in the supernatants of stimulated BMDM 16 h after exposure to LPS, HK listeria and LPS plus nigericin (an NLRP3 inflammasome stimulator), as expected (Saïd-Sadier et al. 2010). However, in parallel we did not detect a *lrrk2* genotype-dependent difference in IL-6 and KC levels (e.g. compare bars for WT versus KO mice in Fig. 5a, b). We concluded from these cytokine screening experiments that the expression of WT (or mutant) *lrrk2* did not measurably alter the release of IL-6 and KC under these conditions.

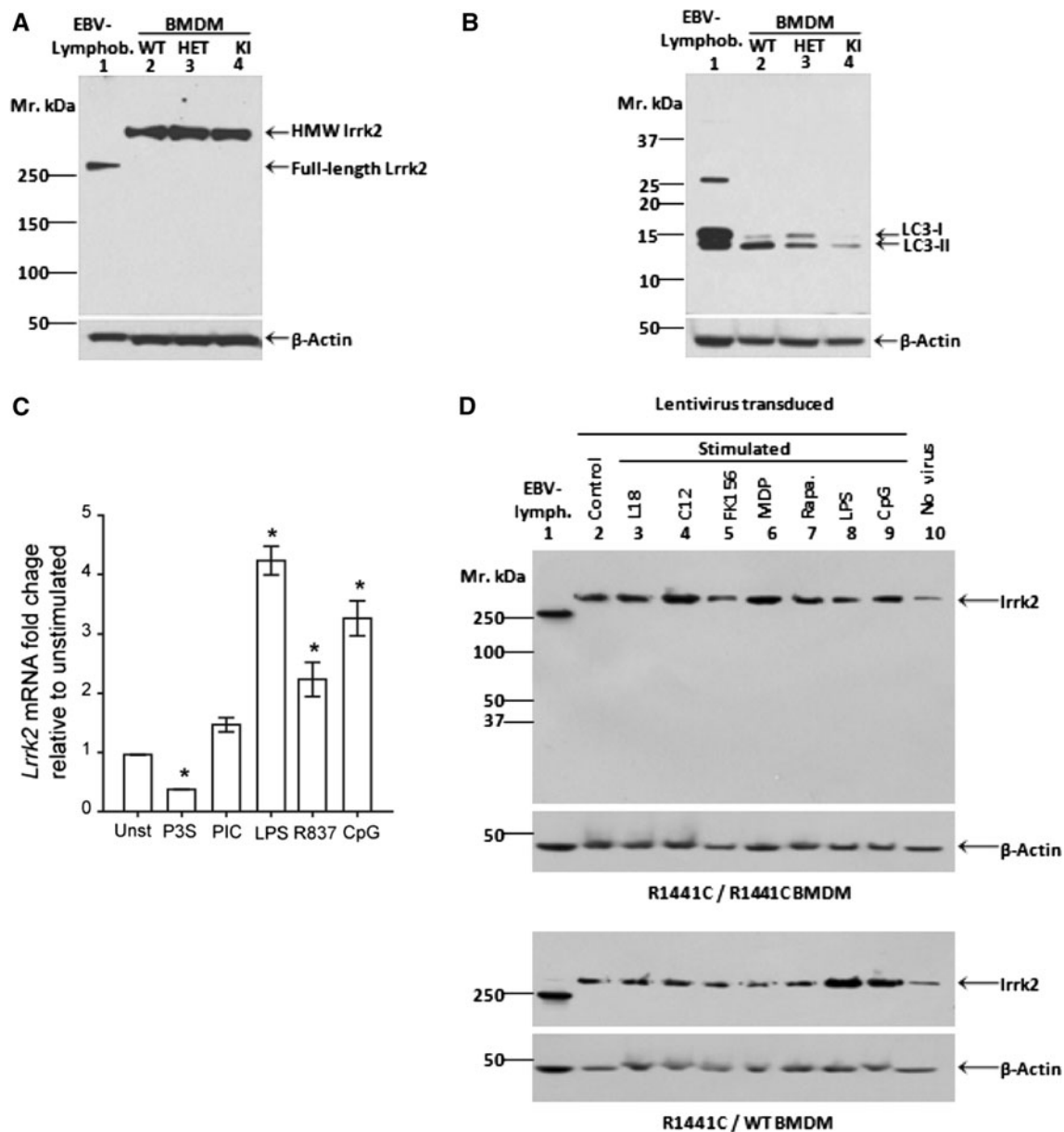


Fig. 4 A likely role for *lrrk2* during autophagy and pathogen response in macrophages. **a**, **b** SDS/PAGE under denaturing, reducing conditions followed by immunoblotting of murine bone marrow-derived macrophages (BMDM) that were cultured for 48 h without stimulation. Lysate of human EBV-transformed lymphoblasts from a control donor was loaded as control. Lanes 2–4 show lysates of BMDM prepared in parallel from age-matched mice of three distinct *lrrk2* genotypes: wild type (WT); heterozygous (HET); and homozygous knock-in (KI) mice expressing mutant ^{R1441C}*lrrk2*. Note, the robust expression of HMW *lrrk2* in murine BMDM, as detected by monoclonal anti-*Lrrk2* antibody [MJFF-4] in **a**. Sister aliquots of the same lysates were probed with an antibody to autophagy markers LC3-I and LC3-II (**b**). All membranes were stripped and reprobed with anti-β-actin antibody as loading control. The reduction of LC3-II levels in mutant ^{R1441C}*lrrk2*-expressing BMDM compared to WT cells measured ≤31% (see text for details). **c** BMDM from WT mice were stimulated for 5 h with different Toll-like receptor (TLR)

agonists: Pam3CSK4 (P3S) for TLR2; Poly I:C (PIC) for TLR3; LPS for TLR4; R837 for TLR7; and CpG for TLR9. Quantification of *lrrk2* mRNA was performed using the ΔΔCT method (relative to 18S) and compared to levels in unstimulated BMDM cells. Asterisks denotes significant *lrrk2* gene expression change ($p < 0.05$ using ANOVA followed by Tukey test, $n = 3$). For comparison, *TNFα* was upregulated in all samples >30-fold (not shown). **d** BMDM cultures from homozygous (R1441C/R1441C) and heterozygous (R1441C/WT) mice were stimulated with different inflammatory agonists. Macrophages (2 million plated per well, 24 h before transduction) were transduced with a lentivirus expressing eGFP-LC3 48 h prior to stimulation. Transduction by virus caused an upregulation in *lrrk2* protein levels when compared with untransduced BMDM (compare lanes 10 with lanes 2). EBV-lymph and Rapa refer to EBV-transformed lymphoblast and treatment with rapamycin, respectively. See “Materials and methods” for further details

Discussion

The pathogenetic mechanisms leading to typical PD remain unknown. Prevailing concepts have favored either an environmental factor or genetic contributors as the primary event in its aetiology, but few models have explored the impact of both in *in vivo* models (Aleyasin et al. 2010). The discovery that late-onset PD could be caused by the inheritance of a mutation in the *LRRK2* gene leading to familial as well as sporadic forms has provided us with an opportunity to explore the pathophysiological events underlying this complex disease. However, as summarized in the introduction, more questions have arisen than been answered surrounding the function(s) of *LRRK2* (reviewed in: Cookson 2010); most poignantly, to date no model has adequately explained how a gene, which is expressed at exceedingly low levels in brain nuclei that are severely affected by the process that leads to PD (e.g. *S. nigra*), can promote a neurological disorder when mutated.

Here, we provided experimental evidence to support the following two conclusions: one, *Lrrk2* is a relatively abundant protein in mammalian leukocytes of circulating blood and organs of the immune system; and two, *lrrk2* expression is altered in cultured macrophages following their recognition of distinct microbe-associated structures. We arrived at these two conclusions based on several, complementary approaches. Our findings are in agreement with the recently published, elegant study by Gardet et al. These authors demonstrated the involvement of *LRRK2* in the IFN- γ response after leukocyte invasion and pointed out how it possibly relates to CD pathogenesis (Gardet et al. 2010). In their study, *LRRK2* was up-regulated in intestinal tissues of CD patients with expression detected in macrophages, B cells and CD103⁺ dendritic cells of inflamed intestines. Gardet et al. (2010) further showed that exogenous expression of *Lrrk2* promoted transcriptional activation of the NF- κ B pathway, and that its down-regulation facilitated enhanced bacterial survival in a *S. typhimurium* cell infection assay. However, we believe our data further expand on these findings by adding three additional observations regarding *Lrrk2* biology: one, we demonstrated—to our knowledge for the first time—that the expression of ^{R1441C} mutant *Lrrk2* in a primary, non-neural cell model (i.e. BMDM) reveals an autophagy defect; two, we showed that the up-regulation of *lrrk2* also occurs in response to viral transduction of a host cell by lentiviral particles (and likely, in B-lymphocytes transformed by EBV); and three, when screening for a *lrrk2* genotype-dependent effect on IL-6 and KC cytokine release, we found that these two signaling molecules (and their pathways) are not regulated by leukocytic *lrrk2*.

Below, we mention five specific limitations of this study followed by a discussion of five reasons why we consider

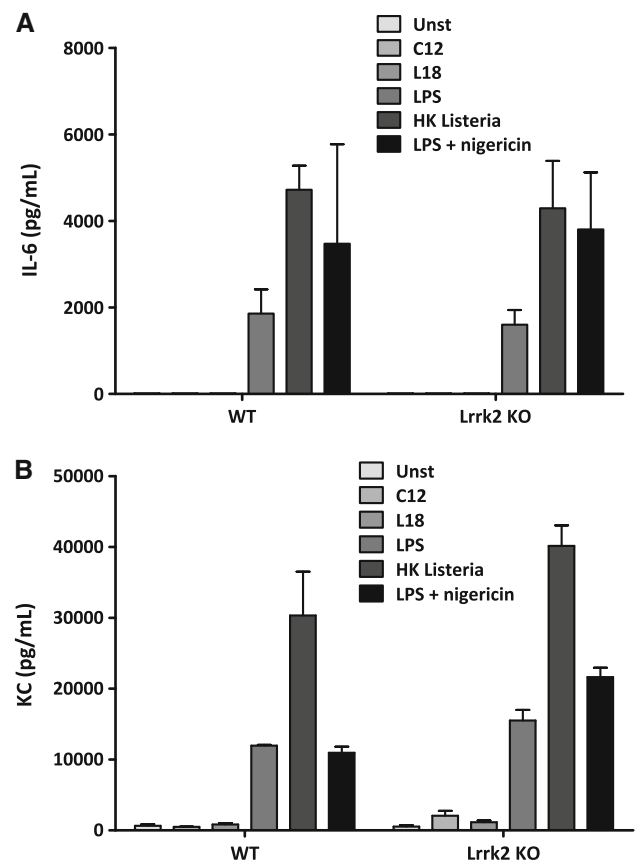


Fig. 5 *Lrrk2* protein expression does not control IL-6 and KC cytokine signaling. **a, b** Screening of *lrrk2* genotype-dependent cytokine signaling by cultured BMDM following exposure to different stimulants was explored by probing the release of interleukin-6 (IL-6; pg/ml in **a**) and keratinocyte-derived chemokine (KC; pg/ml in **b**) by ELISA. BMDM were isolated from *lrrk2* knock-out (KO), and from age-matched, wild-type (WT) littermates (12 weeks old; $n = 2$ for each genotype) and cultured for 16 h (each supernatant was analyzed in quadruplicates). Similarly, stimulants were added 24 h after plating. No specific *lrrk2* expression-associated differences were detected in the conditioned media for the production and release rate of KC and IL-6 (for additional genotypes, see also Suppl. Figure 2)

our *Lrrk2* findings relevant to the pathogenesis of PD. One, although we consistently demonstrated the expression of *LRRK2* in CD19⁺ B cells, CD4⁺ T cells (in contrast to Gardet et al. 2010; Maekawa et al. 2010), CD8⁺ T cells, CD14⁺ monocytes, macrophages and dendritic cells, we obtained highly variable—and thus, inconclusive—results regarding granulocytes, which were: mostly negative in staining of human blood smears; positive in cellular isolates from murine spleen and mesenteric lymph nodes analyzed by FACS; and negative in genotyped spleen sections examined by IHC. These differences may be due to low *LRRK2* expression rates in healthy donors, species differences in accessible epitopes of granulocytic *Lrrk2*, or subtle changes in the binding strength of the antibodies used in our different protocols. In future work, we will

revisit the degree of *LRRK2* expression in granulocytes focusing on mRNA-based techniques (e.g. rt-qPCR).

Two, in our IHC work of brain tissue, where we used a protocol that includes formalin-fixed, paraffin-embedded material and two antigen retrieval steps, we have not yet identified staining of microglia (or of any other glia) with our panel of anti-Lrrk2 antibodies, in contrast to a report by Miklossy et al. (2006). As before, differences in antibody specificity and antigen accessibility could account for this mismatch in results. To convincingly answer the question of expression of *lrrk2* in resting or activated microglia, which are derived from the monocytic lineage, and to exclude any false positive signals due to antibody cross-reactivity, future studies will employ the induction of microglial activation in WT *lrrk2* versus *lrrk2*-deficient mice using established inflammatory paradigms (Mount et al. 2007).

Three, although we recorded a mean, $\leq 31\%$ reduction in basal LC3-II (but not LC3-I) levels in BMDM from ^{R1441C}*lrrk2*-expressing mice when compared with age-matched WT littermates processed at the same time, more autophagy markers need to be examined in primary cells (and tissue) using a larger series of animals and comparing unstimulated versus stimulated conditions than we did here (mice, $n = 6$). If our data were to be confirmed, i.e. reduced autophagy rates can be seen in ^{R1441C}*lrrk2*-expressing BMDM and tissue homogenates as concluded from our LC3-II results, then the effect of this point mutation could be similar to the recently observed dysregulation of autophagy described by Tong et al. (2010) in *lrrk2*-deficient mice. Such a reduction in autophagic efficiency due to the loss of dimeric WT *lrrk2* activity is principally consistent with the results of Gardet et al. (2010) who recorded increased bacterial survival rates in macrophages after *lrrk2* knock-down. Effective phagocytosis following pathogen opsonization requires intact autophagy. In parallel, future studies need to systematically screen additional cytokines (and signaling pathways) beyond those carried out so far to better define the full complement of cellular changes downstream of *lrrk2* gene expression in activated immune cells.

Four, in our work we have not yet defined the post-translational nature of the HMW *lrrk2* band(s) seen with multiple specific antibodies in PBMC and, even more strikingly, in BMDM. We performed our SDS/PAGE experiments under reducing and denaturing conditions, thereby probably but not definitively precluding dimer (or higher order multimer) stability. We predict that the relative abundance of *Lrrk2* protein in the monocyte/macrophage lineage will significantly aid its biochemical exploration in the future, including the delineation of post-translational modifications, of its still elusive binding partners, and of authentic substrates for its kinase activity (Cookson 2010).

Fifth, one could argue that the precise single nucleotide polymorphisms at or near the *LRRK2* locus, which were associated with CD and leprosy risk in genome-wide association studies, have not yet been independently validated, thereby theoretically minimizing the general applicability of our cellular studies. Intriguingly, most recently *LRRK2/MUC19* was identified as one of seven CD loci (out of 30 examined) that was positively associated with risk modulation for yet another human condition, i.e. ankylosing spondylitis, which often occurs together with CD (Danoy et al. 2010). Moreover, dysregulation of monocytes, macrophages, B cells and T cells plays a pivotal role in the pathogenesis of CD, ankylosing spondylitis and leprosy (Schurr and Gros 2009).

Importantly, five arguments can be made in favor of studying *LRRK2* biology in leukocytes as a suitable cell model for PD pathogenesis and to functionally compare its role with other CD- and leprosy-associated genes in suitable paradigms. One, in vivo studies and post-mortem analyses of PD subjects have generated robust evidence from multiple centers in favor of ongoing inflammation (Hirsch and Hunot 2009; Mutez et al. 2010). Furthermore, the infiltration by CD4⁺ and CD8⁺ lymphocytes and the activation of microglia in PD brain and related models have been well documented (Brochard et al. 2009). In addition, the analysis of peripheral cytokine concentrations in PD brain and related models have revealed abnormal levels for MCP-1 (monocyte chemoattractant protein-1), MIP-1 (macrophage inflammatory protein-1), IFN- γ , IL-8, IL-1 β , IL-6, and TNF- α (Reale et al. 2009; Chen et al. 2008) among others, thereby justifying the systematic exploration as to the possible reason(s) for their dysregulation by immune cells (Mutez et al. 2010).

Two, the mammalian immune system undergoes significant changes during the process of aging including for example in the rate of *lrrk2* expression (Maekawa et al. 2010) and by inference, in WT *lrrk2* activity, which could further influence the age-of-onset, the expressivity of the PD phenotype, or its progression rate.

Three, the central themes in the aetiology of typical, late-onset PD, which Jellinger (2011) recently referred to as a “progressive multisystem (multiorgan) disorder”, have not yet been successfully integrated. However, its environmental and genetic contributors are not mutually exclusive, but rather represent the foundation of many human disorders (Klein and Schlossmacher 2007; Klein et al. 2011). The immune system governs the host's susceptibility to pathogen invasion (e.g. viruses, bacteria, fungi etc.), controls its tolerance of possible colonization, and determines the response mounted by the host to eliminate any threat of invasion. Nucleotide exchange domain- and LRR domain-carrying proteins (“NLR”) encoded by the genome play an evolutionarily conserved

role in immune surveillance from plants to mammals (reviewed in: Takken and Tameling 2009; Taxman et al. 2010). Together with TLR proteins, NLR molecules are functionally understood as pattern recognition receptors. NLR proteins oversee inflammasome assembly and control the pathogen-induced regulation of cytokine release (Lamkanfi and Dixit 2010). Based on the cellular expression pattern and functional domain similarities between Nod2 and Lrrk2, we seek to further test our hypothesis of a NLR-type function for Lrrk2.

Fourth, if established successfully, such a *LRRK2*-dependent regulation of immune cell function (Mutez et al. 2010; Gardet et al. 2010) during the interaction between host and environment would be expected to significantly vary based on geography. This scenario—together with other factors—could help explain several known, previously difficult to explain aspects of *LRRK2*-linked PD, e.g. variable rates of penetrance in carriers of the same mutation in different regions of the world, differences in the phenotypic conversion rate among carrier members of the same family, variable rates of disease progression, and possibly, even the pleomorphic pathology (Zimprich et al. 2004) that could have been triggered by distinct agents in the environment.

Lastly, and most strikingly, a key role for Lrrk2 function at the interface between host and environment, which we propose here, could provide a new platform to revisit Braak's hypothesis as it relates to the pathogenesis of typical PD. Braak et al. (2003) postulated that an elusive environmental factor (microbial or otherwise) is essential for pathology to ensue. At stage 0, these authors described the earliest signs of synucleinopathy in the submucosal plexus of the gut and in the olfactory bulb, two structures in close proximity to the environment. The convergence of several independent pieces of evidence from the fields of CD pathogenesis, leprosy genetics and *LRRK2* research related to leukocyte function promises to bring together five previously irreconcilable elements in the aetiology of PD: one, the importance of the environment; two, the evolution of its neuropathology; three, the significance of genetic susceptibility; four, the role of the immune system; and five, the process of aging in a long lived host.

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