Interferon gamma induces leucine-rich repeat kinase LRRK2 via extracellular signal-regulated kinase ERK5

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Für Susanne

„Lerne alt zu werden mit einem jungen Herzen“ (JWG)

I Abstract

The gene encoding leucine-rich repeat kinase 2 (*LRRK2*) is associated with familial and sporadic Parkinson's disease (PD), the second most common neurodegenerative disease. However, the role of LRRK2 in PD pathophysiology remains elusive. Recently, it has emerged that LRRK2 is highly expressed in the immune system including monocytes and macrophages and plays important roles in the immune system. LRRK2 has been reported to be induced by interferon-γ (IFN-γ) in macrophages, but the signaling pathway is not known.

In this work, it was shown that LRRK2 is indeed induced upon IFN-γ stimulation. Importantly, IFN-γ-mediated induction of LRRK2 was suppressed by both pharmacological inhibition and RNA interference of the extracellular signal-regulated kinase 5 (ERK5). This was confirmed by LRRK2 immunocytochemistry, which also revealed that the morphological responses to IFN-γ were suppressed by ERK5 inhibitor treatment. Both human acute monocytic leukemia THP-1 cells and primary human peripheral blood monocytes stimulated the ERK5-LRRK2 pathway after differentiation into macrophages. Moreover, a transcriptome analysis of IFN-γ stimulated THP-1 cells co-treated with ERK5 inhibitors identified several targets putative downstream targets of LRRK2, which have to be further verified.

In conclusion, this works establishes that LRRK2 is induced via a novel, ERK5-dependent IFN-γ signal transduction pathway. Moreover, it adds evidence that LRRK2 is involved in morphological alterations upon macrophage activation. The trancriptome analysis provides a list of candidate genes that may provide leads for future investigations to unravel LRRK2 immune cell function. In total, this is pointing to new functions of ERK5 and LRRK2 in human macrophages.

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V List of Abbreviations

A	Alanine
aa	Amino acid
AKAP	A kinase anchoring protein
ANK	Ankyrin
APC	Antigen presenting cell
APS	Ammonium persulfate
ARM	Armadillo
α-Syn	α-synuclein
AD	Alzheimer's disease
BCA	Bicinchoninic acid
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
C	Cysteine
CBD	Corticobasal degeneration
CD	Crohn's disease
CD	Cluster of differentiation
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computer tomography
D	Aspartic acid
DA	Dopaminergic neurons
DC	Dendritic cell
DJ-1	Daisuke-Junko-1
DAPK1	Death associated protein kinase 1
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
DTT	1,4-Dithiothreitol
E	Glutamic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EGF	Epidermal growth factor
EndoA	Endophilin A
ERK	Extracellular-signal-regulated kinase
ER	Endoplasmatic reticulum
FCS	Fetal calf serum
FACS	Fluorescence-activated cell sorting
G	Glycine
g	Gravitation constant
GAK	cyclin G-associated kinase
GAP	GTPase-activating proteins
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Gamma-interferon activation site
GBA	Glucocerebrosidase
β-GCase	β-glucocerebrosidase

GD	Gaucher's disease
GEF	Guanine nucleotide exchange factors
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPe	Globus pallidus externus
GPi	Globus pallidus internus
GTP	Guanosine-5-triphosphate
GWAS	Genome wide association study
H	Histidine
H_2O_2	Hydrogen peroxide
HLA	Human leucocyte antigen
HRP	Horseradish peroxidase
h	Hour
I	Isoleucine
IN-1	LRRK2-Inhibitor-1
IL	Interleukin
IBD	Inflammatory bowels disease
IFN	Interferon
IFNGR	Interferon-gamma receptor
IRF	IFN regulator factor
ISRE	IFN-stimulated response element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K	Lysine
Kb	Kilo basepairs
kDa	Kilodalton
KO	Knock-out
l	Liter
LB	Lewy Body
LC3	Microtubule-associated protein 1A/1B-light chain 3
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LRRK1/2	Leucine rich repeat kinase 1/2
m	Milli
M	Molar
MAPK	Mitogen activated protein kinase
MAPT	Microtubule-associated protein tau
MAO	Monoamine oxidase
MASL	Malignant fibrous histiocytoma amplified sequences with leucine-rich tandem repeats
MCP	Monocyte chemotactic protein
MDM	Monocytes-derived macrophage
MHC	Major histocompatibility complex
MIP	Macrophage Inflammatory Protein
MLK	Mixed lineage kinase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple system atrophy
min	Minute
N	Asparagine
n	Nano
NFAT	Nuclear factor of activated T-cell
NKT	Natural killer T
NP-40	Nonident P-40

NRON	ncRNA repressor of the nuclear factor of activated T cells
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PET	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
PINK	PTEN-induced putative kinase 1
PKA	Protein kinase A
PMA	Phorbol-13-myristate-12-acetate
PVDF	Polyvinylidene difluoride
PSP	Progressive supranuclear palsy
PTP	Protein tyrosine phosphatase
PUT	Putamen
Q	Glutamine
Rab	Ras-related in brain
RANTES	Regulated on activation, normal T cell expressed and secreted
RIPA	Radioimmunoprecipitation assay
RIPK	Receptor-interacting protein kinase
ROC	Ras of complex
ROCO	ROC-COR
ROS	Reactive oxygen species
RT	Room temperature
RT	Reverse trancriptase
SDS	Sodium dodecyl sulfate
S	Serine
SNps	Substantia nigra pars compacta
STAT	Signal transducer and activator of transcription
STN	Nucleus subthalamicus
T	Threonine
TAE	Tris-acetate-EDTA
Tat	HIV-1 transactivator of transcription
TBST	Tris buffered saline with tween
TEMED	N,N,N',N'-Tetramethylethyldiamine
TH	Tyrosine-hydroxylase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR-1	Tumor necrosis factor receptor
U	Units
UPS	Ubiquitin-proteasome-system
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
Y	Tyrosine

1 Introduction

1.1 Parkinson's disease

1.1.1 Parkinson's disease – History and relevance

The increase in life expectancy, despite all obvious benefits, will cause public health-care systems worldwide to face dramatic problems, especially with the simultaneously present demographic change. Age-related diseases are going to affect a much greater proportion of our societies than they already do nowadays and will further aggravate these demographic problems. Along with the bundle of economic, social and political problems comes the tremendous scientific challenge and necessity to approach age-related neurodegenerative diseases such as Alzheimer's (AD) or Parkinson's disease (PD) in the hope of ultimately deciphering their mechanisms and finding a way for cure.

Since the famous work "An assay on the Shaking Palsy" in 1817 by J. Parkinson, from whom the eponym PD originates, a lot of research has been conducted to advance the understanding of PD. One hallmark discovery was in 1912, when Friedrich Lewy discovered the presence of so called "Lewy bodies" (LBs). Not much later, in 1921, Konstantin Tretiakoff identified the substantia nigra as the predominantly affected brain area. Another important finding, especially in regard to medication, was the work of Arvid Carlsson, who majorly contributed to the understanding of the role of dopamine in the diseased PD brain (Fahn, 2008; Goetz, 2011).

In 1997, α-synuclein (α-Syn) was discovered to be the major component of LBs and, moreover, α-Syn was shown to harbor PD causing mutations (Polymeropoulos et al., 1997; Spillantini et al., 1997). Soon, more PD causing genes were found and subsequent studies on their functions further improved our understanding[1]. Still, a causative cure does not exist and the more we dissect the disease, the more we begin to realize its complexity and the difficulties there are still to face in the next years to come.

[1] (see chapter 1.1.5)

1.1.2 Clinical features of Parkinson's disease

PD is the second most common progressive, neurodegenerative disease just after AD. Roughly 1% of the population above the age of 60 is affected. The prevalence of the disease rises to ca. 4-5 % at the age of 85 clearly indicating that PD is an age-related disease. In fact, aging is still considered as the main risk factor to develop PD (de Lau and Breteler, 2006). The mean age of onset is 70 years, but about 4% of PD patients are affected by early-onset PD before reaching the age of 50. Interestingly, epidemiological studies found that both incidence and prevalence are roughly 2 times higher in men than in women (Haaxma et al., 2007). PD is the most common form of Parkinsonism, which includes other disease types that display similar clinical features: Progressive supranuclear palsy (PSP), multiple system atrophy (MSA) and corticobasal degeneration (CBD) are collectively categorized as atypical Parkinsonism. Another group of Parkinsonism is drug-induced Parkinsonism (Jankovic, 2008).

Although more sophisticated methods such as positron emission tomography (PET) scans and single photon emission computer tomography (CT) are emerging, the general clinical diagnosis of PD still relies on visible clinical symptoms (Jankovic, 2008; Seppi and Poewe, 2010). The four cardinal symptoms of PD are: Tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability (Fig. 1.1).

Fig. 1.1: Illustration of Parkinson's disease

The four cardinal symptoms of PD are tremor at rest, rigidity, bradykinesia and postural instability. They were already well described by physicians in the 19th century as depicted by this drawing from a case from "A Manual of Disease of the Nervous System (1886) by W.R. Gowers.

Moreover, freezing (motor blocks) and a flexed posture belong to the classic features of PD. Bradykinesia refers to slowness of movements and is very characteristic for PD. In general, bradykinesia is typical for basal ganglia disorders and includes the impairment of planning, initiating and executing movements as well as coordinating simultaneous or sequential tasks. Early manifestations are often slow movements in daily life. Other prominent manifestations of bradykinesia are monotonic and hypophonic dysarthria (impairment in the motoric execution of speech), hypomimia (loss of facial movements/expression) and general loss of spontaneous movements and gestures. Emotional triggering of patients can overcome the slowness in bradykinesia, indicating that the impairment is not purely caused by a loss of neuronal motor programs but rather the inability to initiate them. Rest tremor, which affects most of PD patients, is usually a repetition of unilateral, involuntary movements most prominent in the distal parts of an extremity but can also involve hands, legs, lips, jaw and chin. However, tremor disappears during movement or sleep. Rigidity, which is defined as an increased resistance during passive movements, can affect all distal and proximal parts of both legs and arms. Especially in the shoulder, it is one of the most frequent initial manifestations of PD. Postural instability is caused by a loss of postural reflexes and therefore is prominent at later stages of disease. Other typical secondary motor symptoms include dysphagia (difficulties in swallowing), sialorrhoea (increased saliva production) and shuffling gait. Common non-motor symptoms of PD include cognitive decline, depression, autonomic dysfunctions, sleep disorders and sensory abnormalities. Dementia is reported in about 30% of cases at later stages (Jankovic, 2008).

The classic treatment for PD is levodopa, which is a dopamine precursor capable to cross the blood-brain barrier and generally leads to an improvement in motor symptoms. However, it does not slow down disease progression and is also known to cause side-effects such as motor fluctuations and dyskinesias. Other therapeutic strategies include dopamine agonists, in order to directly stimulate postsynaptic dopamine receptors, Catechol O-Metyltransferase (COMT) inhibitors, which lead to a reduced breakdown of dopamine and levodopa in the liver, and monoamine oxidase (MAO) inhibitors, which do the latter within the terminals of dopaminergic neurons. In many cases, surgical lesions of specific regions or deep-brain electrical stimulation can further improve motor symptoms (Bowes, 2013).

1.1.3 Neuropathology of Parkinson's disease

The neuropathology of PD is characterized by two hallmarks: First, the selective death of nigrostriatal dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), and second, the presence of LBs in surviving cells. LBs are intraneuronal proteinacious cytoplasmic inclusions (Fig. 1.2). The archetypal LB is a round or spherical eosinophilic inclusion, with α-Syn as main component. LBs are present in different brain areas, including the brainstem but also cortical brain regions, mainly depending on the degree of disease. Because LBs were observed in pre-clinical brains in non-dopaminergic structures, brainstem synucleinopathy was suspected to progress rostrally to the SN at later disease stages. Therefore, it was attempted to classify LB pathology in a process of six stages, in which the lower brainstem is affected first, followed by the basal ganglia up to cerebral cortex (Braak et al., 2002). However, other studies question the so-called "Braak-staging", as it is often not very accurate since LB pathology is generally very heterogenic (Jellinger, 2012). In a unified 4-stage system, pathology starts in the olfactory bulb, followed by predominantly affected brainstem *or* limbic system, followed by a stage where *both* of the latter are affected and finally when also neocortical regions display LB pathology (Beach et al., 2009). Yet, the functional role of LBs in PD pathogenesis remains elusive as it is still unclear if LB resemble cause or correlation (Shults, 2006).

The second hallmark of PD pathology is the decay of DA in the SNpc. Because the DA neurons are pigmented with neuromelanin the characteristic gross pathology of PD is the depigmentation of the SNpc (Marsden, 1983). However, there is no general loss of neurons in PD as compared to other neurodegenerative diseases such as AD. More importantly, loss of DA neurons results in striatal dopamine deficiency. Typically, at disease onset, already 80% of putamenal dopamine is depleted, and roughly 60% of SNpc neurons have perished. Adjacent areas from which neurons project to the caudate are much less affected (Uhl et al., 1985). Interestingly, the decay of neurons in PD seems to be a "dying back" process, meaning that the DA nerve terminals are primarily or earlier affected than the cell body itself (Bernheimer et al., 1973; Braak et al., 2004; Morfini et al., 2007). The presence of LBs and neuron loss in SNpc is required for a definite diagnosis. Yet, this analysis has to be carried out post-mortem.

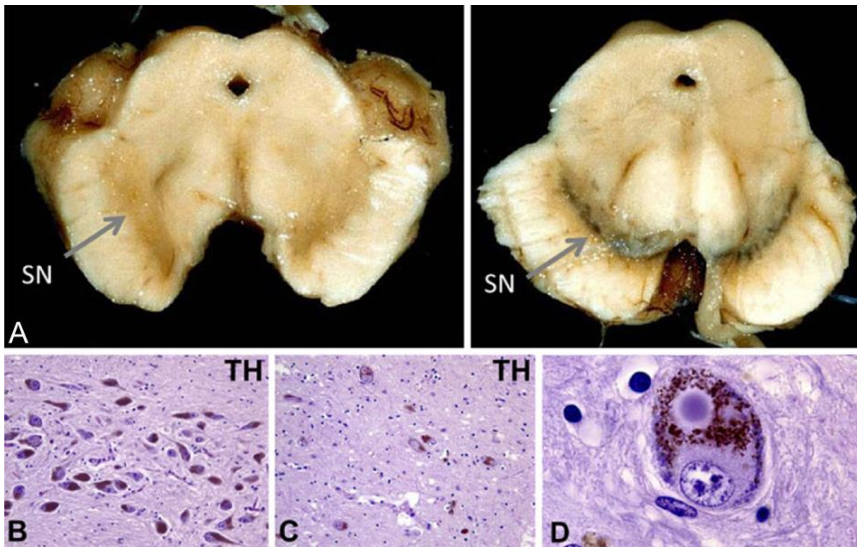

Fig. 1.2: Hallmarks of PD pathology

In advanced PD (left), loss of dopaminergic neurons in the SN is visible by depigmentation in midbrain sections, whereas such loss is absent in healthy (right) individuals (A). The substantia nigra (SN) is characteristically populated by tyrosine-hydroxylase (TH) positive neurons (B). In PD, the number of TH positive neurons is greatly diminished (C). Surviving neurons display so called "Lewy bodies" (LBs), which are intraneuronal proteinacious cytoplasmic inclusions (D) (Illustration from Mandel et al., 2010).

Death of dopaminergic neurons in the SNpc is the pathological hallmark of PD, but other areas of the brain are also affected by neurodegeneration and LB formation. This includes the noradrenergic (locus coerulus), cholinergic (nucleus basalis) and serotonergic (raphe) systems, the olfactory bulb, the cerebral cortex and the autonomic nervous system (Hornykiewicz and Kish, 1987). Although aging, the biggest PD risk factor, is clearly involved in PD, the pattern of neuronal loss is clearly distinct from healthy elderly brains (Fearnley and Lees, 1991). The decay of DA neurons in the SNpc entails the motoric dysfunctions that are central in PD. Because the DA neurons mainly project to the putamen, their death causes a decrease of dopamine-release in the putamen, which in turn reduces DA input on downstream centers of motor control in thalamic or cortical regions of the brain (Fig. 1.3) (Dauer and Przedborski, 2003).

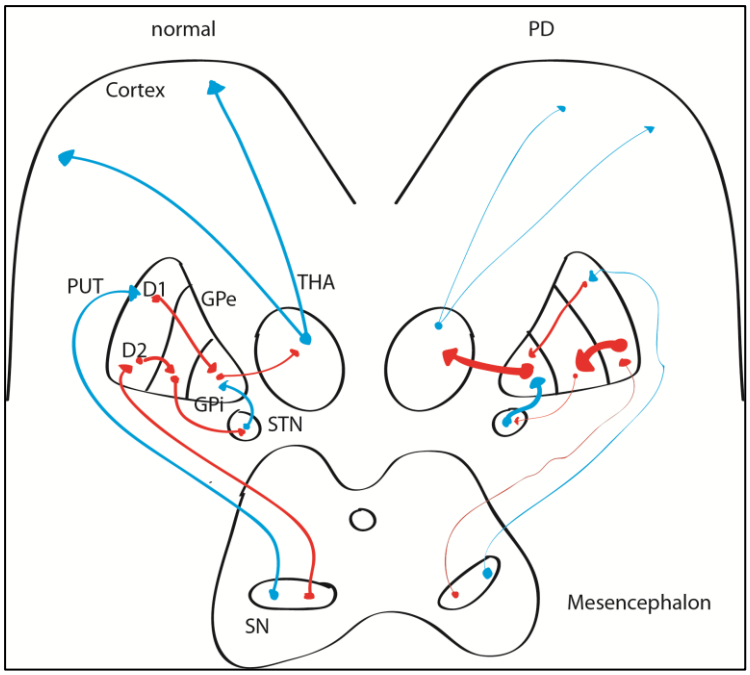

Fig. 1.3: Neuronal pathways affected in PD pathophysiology

Dopaminergic (DA) neurons from the substatia nigra (SN) project to parts of the basal ganglia, including the putamen (PUT), which in turn projects to downstream brain areas that are important in motor control, including the nucleus subthalamicus (STN), the thalamus (THA), the globus palidus (GP, GPe globus pallidus externus; GPi globus pallidus internus) and the cerebral cortex. The normal situation is depicted on the left-hand side, while the right-hand side represents the pathophysiological alterations in the PD brain. Here, the selective loss of DA neurons in the SN in the midbrain and the decay of the nigrostratial pathway are causing a decrease in dopamine-release in the putamen. Hence, this loss of DA input results in decreased activation of downstream centers of motor control in the thalamus and cortex.

1.1.4 Etiology of Parkinson's disease: Environmental contributions

PD is commonly regarded as a multifactorial disease, both genetic and environmental factors impact on the risk of developing PD. As an environmental factor, rural residency was identified to increase the risk of developing PD, possibly due to the contact with herbicides, fungicides and pesticides that are used in agricultural industry (Barbeau et al., 1987; Hancock et al., 2008). The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is probably the most famous example of chemically induced parkinsonism. MPTP is highly lipophilic and can therefore easily cross the blood brain barrier (BBB). Within the brain, it is metabolized by glial cells. The oxidized product MPP+ is selectively taken up via the dopamine transporter (DAT) by DA neurons, thus specifically resulting in the death of this population of neurons. Mechanistically, MPP+ is believed to cause impairment of mitochondrial function by inhibition of the complex I of the electron transport chain, which

in turn causes increase in free radicals and cell death. (Langston et al., 1984; Porras et al., 2012).

Interestingly, also the commonly used pesticide and insectizide rotenone induces Parkinsonism (Cannon et al., 2009). Rotenone is also a complex I inhibitor, but in contrast to MPTP or 6-OH dopamine, it does not selectively accumulate in DA neurons. Yet, the rotenone model does not ideally reproduce PD pathophysiology, due to major limitations in terms of high variability in both percentage of animals that develop lesions but also variability in the degree of severity of these lesions (Hirsch et al., 2003; Hoglinger et al., 2006; Schmidt and Alam, 2006). Moreover, manganese, a constituent of several pesticides and herbicides, is another example of a neurotoxin causing Parkinsonism by damaging basal ganglia (Lucchini et al., 2009). Head injuries with amnesia or loss of consciousness are associated with an increased risk (Goldman et al., 2006). On the other hand, coffee drinking and cigarette smoking are believed to lower the risk of PD by an unknown mechanism (Checkoway et al., 2002; Prediger, 2010). Physical activity is also believed to reduce the risk of developing PD (Chen et al., 2005).

1.1.5 Etiology of Parkinson's disease: Overview of genetic contributions

Although an increased prevalence of PD among relatives of patients was already observed 100 years ago (Gowers 1888), clear evidence linking genetics to PD was long missing and PD had been considered a "non-genetic" disorder (Farrer, 2006). This perspective changed dramatically with the discovery of familial forms of PD. Familial PD is caused by certain mutations in specific genes. Within the last 15 years, a lot of genes have been postulated to be connected with PD using different methods (linkage analysis, genome sequencing and genetic association). At that time, loci associated with PD were systematically classified as PARK genes (PARK 1-x). However, not all the discovered loci could be confirmed. Nowadays, only a few genes are generally regarded to be undoubtedly monogenetic causes of PD (Tab. 1.1). These include two dominantly inherited forms of PD, namely PARK1/4 (encodes α-Syn) and PARK8 (encodes Leucine-rich repeat kinase 2, LRRK2). PARK2 (encodes Parkin), PARK6 (PTEN-induced putative kinase 1, PINK1), PARK7 (Daisuke-Junko-1, DJ-1) and PARK9 (ATPase 13A2, ATP13A2) are recessively inherited.

The respective genes of the PARK loci PARK9, PARK14 and PARK15 are ATP13A2, PLA2G6 and FBOX7. In comparison to the aforementioned genes, these genes display autosomal-

recessive parkinsonian syndromes with more complex phenotypes and widely unknown pathogenesis.

For the remaining PARKs, the status is not so distinct. Some PARK loci such as PARK5 (UCHL1), PARK13 (HTRA2) and PARK18 (EIF4G1) could not be confirmed, raising doubts about their implications in PD. Other loci (e.g. PARK3, PARK10 and PARK12) remain quite mysterious in regard of the attributed gene, clinical phenotype and neuropathology.

Tab. 1.1: Confirmed monogenic forms of PD

(Table adapted from Gasser et al., 2011; Klein and Westenberger, 2012; Martin et al., 2011)

Locus	Gene	Clinical appearance	Inheritance	Mutations/comments	References
PARK1/4	*SNCA*	Early onset PD	Autosomal dominant	SNCA gene duplication or triplication, rare point mutations: A53T, A30P, E54K	(Polymeropoulos et al., 1997; Singleton et al., 2003)
PARK2	*Parkin*	Early onset PD	Autosomal recessive	Over 100 mutations, point mutations, indels and exon rearrangements	(Kitada et al., 1998)
PARK6	*PINK1*	Early onset PD	Autosomal recessive	Over 20 mutations, point mutations, indels and exon rearrangements	(Valente et al., 2004)
PARK7	*DJ-1*	Early onset PD	Autosomal recessive	Rare, 3 point mutations and genomic deletions	(Bonifati et al., 2003)
PARK8	*LRRK2*	Classical PD	Autosomal dominant	Confirmed point mutations include G2019S, I2020T, Y1699C, N1437H, R1441C, R1441G	(Paisan-Ruiz et al., 2004; Zimprich et al., 2004)
PARK9	*ATP13A2*	Atypical PD	Autosomal recessive	Complex phenotype with parkinsonism, spasticity and dementia	(Ramirez et al., 2006)
PARK15	*FBX07*	Atypical PD	Autosomal recessive	Rare, complex phenotype	(Di Fonzo et al., 2009)
Gaucher's locus	*GBA*	Gaucher's disease or PD	Risk factor (5 fold)	Multiple rare variants with medium effect strength	(Sidransky et al., 2009)

1.1.5.1 α-Synuclein

PARK1/4 encodes α-Syn, which is central in PD pathology. It is the key component of LBs, the hallmark of PD. Further, it was demonstrated to be involved in dominantly inherited familial forms of PD (Polymeropoulos et al., 1997). Classic linkage analysis lead to the discovery of three specific point mutations, namely A30P, A53T and E46K and more recently discovered mutations H50Q and G51D (Appel-Cresswell et al., 2013; Kruger et al., 1998; Polymeropoulos et al., 1997; Proukakis et al., 2013; Zarranz et al., 2004). Moreover, genomic

multiplications (duplications and triplications) of α-Syn are known to cause PD (Singleton et al., 2003).

α-Syn is expressed throughout the whole vertebrate brain and localized to neuronal synaptic terminals, where it is associated with vesicles and the plasma membrane (Irizarry et al., 1998). The physiological role of α-Syn remains unclear, yet increasing evidence suggests a role in neurotransmitter release (Nemani et al., 2010). *In vitro*, α-Syn exhibits a high tendency to aggregate and it is classically found to be aggregated in LBs. Moreover, most mutations of α-synuclein or its or multiplications seem to increase this tendency to polymerize into oligomers and fibrillar aggregates (Conway et al., 1998; Conway et al., 2000). The inclusions are thought to be generated by an aggregation cascade, starting with α-Syn monomers, which assemble to form oligomers followed by aggregation to mature fibrils which are ultimately are incorporated into LBs. Interestingly, α-Syn as extracellular oligomer or aggregate, also seems to be involved in the initiation or aggravation of neuroinflammatory processes caused by activation of microglia within the SNpc (Austin et al., 2011; Kim et al., 2013; Lee et al., 2010b; Zhang et al., 2005b) However, whether neuroinflammation is indeed caused by exceeding extracellular α-Syn species or a secondary response to neuronal death is still under debate.

1.1.5.2 Parkin/PINK1

PARK2/*Parkin* encodes a 465 amino acid (aa) cytosolic protein, which acts as an E3 ligase. E3 ubiquitin ligases are enzymes mediating the final step in protein ubiquitination. Parkin is also found to be associated with cellular membranes including mitochondria (Shimura et al., 2000). PD inheritance follows an autosomal recessive pattern. PD pathology appears usually early (at 17-24 years), and in most cases without LB formation (Farrer et al., 2001). Up to date, more than 100 putative pathogenic mutations have been discovered, thus making Parkin-linked PD the most common autosomal-recessive form of PD. Parkin takes part in a patho-mechanism where neuronal death is thought to be caused by impaired mitophagy, in which interestingly also a second disease-causing PARK gene, PINK1, is involved. In this process, PINK1 is stabilized by mitochondrial depolarization, recruits Parkin to damaged mitochondria, which in turn then initiates mitophagic clearance (Matsuda and Tanaka, 2010; Narendra et al., 2008; Narendra et al., 2010). PD-associated Parkin mutations were shown to derogate this process (Geisler et al., 2010). Interestingly, mutations in the PARK2 locus were

found to be associated with increased susceptibility to leprosy (Mira et al., 2004). Accordingly, more recently Parkin was found to play a role in ubiquitin-mediated autophagy of *M. tuberculosis* (Manzanillo et al., 2013). PINK1-associated PD is the second most common autosomal-recessive early onset (30-50 years) form of PD, and its overall clinical picture is similar to PD caused by Parkin mutations. PINK1 is a 581 aa mitochondrial protein, for which many missense and nonsense mutations have been reported (Hatano et al., 2004; Valente et al., 2004).

1.1.5.3 DJ-1

Mutations in DJ-1 cause autosomal-recessive early onset (20-40 years) PD. DJ-1 is an 189 aa cytoplasmic protein, but it has also been found to be associated with mitochondria and the nucleus (Junn et al., 2009; Zhang et al., 2005a). Although the precise mechanisms of DJ-1 biology remain unclear, proposed functions include a role in regulating antioxidative signaling and neuroprotective effects by dampening mitochondrial oxidative stress (Canet-Aviles et al., 2004; Kahle et al., 2009; Taira et al., 2004; Takahashi-Niki et al., 2004) Interestingly, DJ-1 was reported to be a regulator of pro-inflammatory responses, as astrocytes from DJ-1 KO mice show increased nitric oxide (NO) production upon TLR4 stimulation (Waak et al., 2009).

1.1.5.4 GBA

The glucocerebrosidase (GBA) gene, coding for β-glucocerebrosidase (β-GCase) is known to be involved in Gaucher's disease (GD), a lysosomal storage disease. It recently has been discovered to also play a role in PD (Aharon-Peretz et al., 2004; Hruska et al., 2008). Although mutations in GBA do not give rise to Mendelian PD like the genes mentioned above, they increase the chances to develop PD about 5 fold (Sidransky et al., 2009). Thus, mutations in the GBA gene are considered to be a big risk factor. As lipid accumulation is the pathological hallmark of GD, GBA is thought to be involved in lysosomal degradation. Interestingly, GD was initially believed to be a purely macrophage-specific sphingolipidosis and patients display a predisposition to infections caused by an impaired microbicidal capacity (Marodi et al., 1995). A more recent study reports a diverse dysregulation of the immune system, including increased numbers of CD4+ cells, a reduction in CD4+ CD8+ precursors and an increased antigen presentation (Liu et al., 2012).

1.1.5.5 PD associated risk factors

Most monogenetic forms of disease were discovered early by classic approaches, but more recently, genome wide association studies (GWAS), which generally investigate the involvement of common variants in disease by comparing the frequencies of given allels between patients and healthy controls, further added genes to the list of PD associated genes (Tab. 1.2). Additional to the two loci containing SCNA and LRRK2, which involvement in familial PD was already known, GWAS studies revealed involvement of genes including microtubule-associated protein tau (MAPT) and human leucocyte antigen (HLA-DR) (Hamza et al., 2010; Simon-Sanchez et al., 2009): MAPT is a known key-player in other neurodegenerative diseases especially AD. HLA-DR, interestingly, serves as a long missed genetic link between PD, neuroinflammation and innate immunity. HLA-DR encodes for the major histocompatibility complex class II (MHCII) DR, which is a cell surface receptor that interacts with T-cell receptors and has been implicated in autoimmune diseases. However, this connection remains to be further investigated.

Tab. 1.2: Genetic risk factors associated with PD identified by GWAS
(Adapted from Gasser et al., 2011; Trinh and Farrer, 2013)

Gene	References
SNCA	(Satake et al., 2009)
LRRK2	(Satake et al., 2009)
MAPT	(Simon-Sanchez et al., 2009)
PARK16	(Satake et al., 2009)
HLA-DRA	(Hamza et al., 2010)
GAK	(Pankratz et al., 2009)

1.2 Leucine rich repeat kinase 2 (LRRK2)

1.2.1 Clinical implications of LRRK2

Mutations in *LRRK2* are associated with both familial and sporadic forms of PD. It is generally believed that 5% - 15% of all familial and 1-5% of all sporadic cases of PD are caused by *LRRK2* (Di Fonzo et al., 2005; Kumari and Tan, 2009; Nichols et al., 2005). As a dominant form of PD, the PARK8 locus was first mapped in a large Japanese family by a classic linkage analysis (Funayama et al., 2002). Soon after the disease causing mutation was

found within the gene encoding *LRRK2* (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). At that point, the authors named the protein dadarin, which derives from the Basque word for tremor. The penetrance of *LRRK2* mutations generally increases with age; however this can be different among individuals (Healy et al., 2008; Kay et al., 2005). Nowadays, at least six disease missense mutations have been identified that clearly segregate with disease: G2019S, I2020T, R1441C/G/H and Y1699 (Tab. 1.3). Other mutations such as G2385R and N1437H are likely candidates, but as for those and others, the final proof of pathogenicity is difficult (Klein and Westenberger, 2012).

The most common *LRRK2* mutation, G2019S, is associated with both familial and sporadic forms of PD. This mutation is responsible for up to 7% of familial cases and 1% - 2% of sporadic forms. However, prevalence differs among populations, e.g. in more isolated population such as the Ashkenazi Jewish and North African Arab populations, the prevalence is 40% (Ozelius et al., 2006). Although the penetrance of G2019S is not very high (roughly between 35% and 70%), it is thought to be a true disease causing gene because it is found very rarely in control groups. Whereas G2019S mutation carriers typically show LB pathology, this is not the case in R1441C, Y1699C and I2020T mutation carriers (Poulopoulos et al., 2012a; Poulopoulos et al., 2012b). Interestingly, pathogenic mutations seem to cluster in the LRRK2 kinase domain (G2019S, I2020T) and the ROC/GTPase domain (R1441C/G/H) or in the COR domain (Y1699C), which is connecting these two catalytic domains.

In general, clinical signs and symptoms of LRRK2-related PD as well as age of onset closely resemble the picture from typical sporadic late-onset PD, which includes brainstem LB pathology (Ross et al., 2006). However, reports from some familial mutations have revealed a more pleomorphic pathology even within families with the exact same mutation. Although some reports claim that LRRK2 is present in LB or Lewy neurites (Perry et al., 2008; Zhu et al., 2006), this issue is still under debate (Giasson et al., 2006; Melrose et al., 2007). Interestingly, LRRK2 has also been implicated in the pathogenesis of other diseases including leprosy (Zhang et al., 2009) , Crohn’s disease / inflammatory bowels disease (IBD) (Franke et al., 2010; Liu and Lenardo, 2012) and cancer (Saunders-Pullman et al., 2010). This indicates that these diseases might share pathways involving LRRK2 function.

Tab. 1.3: LRRK2 mutations with their respective impact on enzymatic activity

Table adapted from (Esteves et al., 2014; Paisan-Ruiz et al., 2013; Rudenko et al., 2012b)

LRRK2 mutation	Affected domain	Enzymatic impact
N1437H	ROC	unknown
R1441G	ROC	deceased GTPase activity
R1441C	ROC	deceased GTPase activity
R1441H	ROC	deceased GTPase activity
Y1699C	COR	deceased GTPase activity
G2019S	kinase	increased kinase activity
I2020T	kinase	None
I2012T	kinase	None
G2385R	WD40	decreased kinase activity

1.2.2 LRRK2 structure

The *LRRK2* gene is located on chromosome 12 and spans a genomic region of 144 kb with 51 exons, encoding a transcript that is translated to a 2527 aa protein. LRRK2 is a cytosolic protein with a molecular weight of approximately 286 kDa. It has multiple functional domains (Fig.1. 4): A serine/threonine protein kinase domain, a GTPase domain called ras-of-complex (ROC) domain and a C-terminal-of-ROC (COR) domain, which is the linker region between kinase and GTPase domain (Bosgraaf and Van Haastert, 2003; Gilsbach and Kortholt, 2014). This structural feature of a ROC-COR domain together with a kinase domain classifies LRRK2 as a so called “ROCO kinase”. Other classifications group LRRK2 to the mitogen-activated protein kinase kinase kinase (MAPKKK) family, to the mixed lineage kinases (MLK) or to the receptor-interacting protein (RIP) kinases (Marin, 2006). Other ROCO family members include LRRK1, the one mammalian homologue of LRRK2, the death associated protein kinase 1 (DAPK1) and the malignant fibrous histiocytoma amplified sequences with leucine-rich tandem repeats (MASL1) that lacks a kinase domain (Civiero et al., 2014; Marin et al., 2008). Despite 70% homology in the ROC, COR and kinase domain, no PD-causing mutations have been found in LRRK1 (Taylor et al., 2007). This might indicate that LRRK1, despite structural similarities, has different functional roles (Civiero and Bubacco, 2012; Reyniers et al., 2014). LRRK2 also harbors several putative protein-protein interaction domains: N-terminal armadillo and ankyrin repeats, an N-terminal region

containing a leucine-rich repeat (LRR) motif and WD40 repeats at the C-terminus, which are suggested to form a beta-propeller structure (Deng et al., 2008; Gilsbach and Kortholt, 2014). LRRK2 can form homo- and heterodimeric complexes (Dachsel et al., 2010; Klein et al., 2009; Sen et al., 2009), but also the opposite has been observed (Ito and Iwatsubo, 2012).

Recently, a study suggested that LRRK2 is predominantly monomeric in the cytoplasm, but forms complexes when associated with membranes (James et al., 2012). Taken together, these combined structural features suggests that LRRK2 might have the ability to function as a scaffold for other signaling proteins, which themselves regulate LRRK2 or receive LRRK2 specific input, thus LRRK2 has the ability to integrate and modify multiple signaling pathways (Esteves et al., 2014).

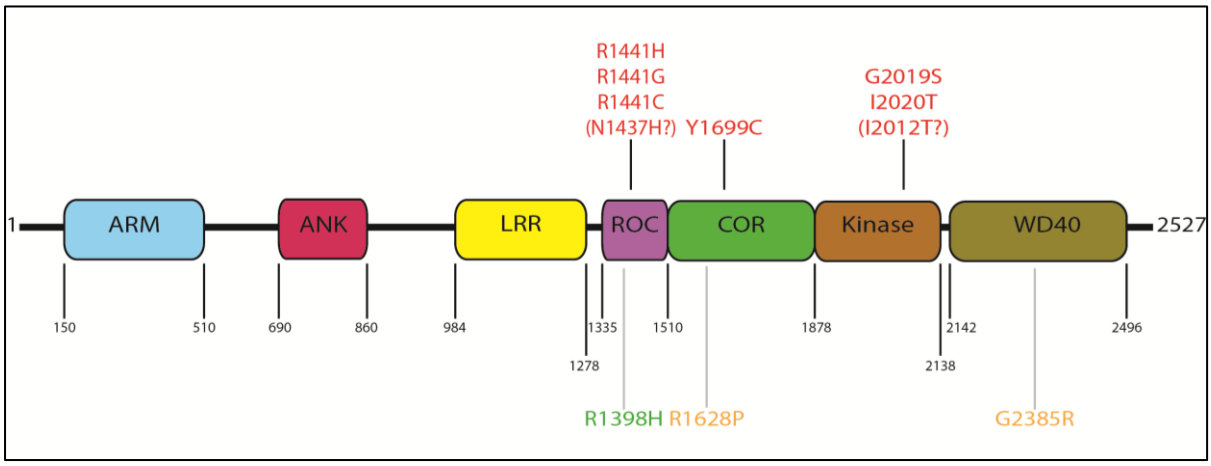

Fig. 1.4: Schematic representation of LRRK2

LRRK2 is a multi-enyzyme protein with several different domains. The central region contains a GTPase domain called ROC (Ras of complex) and a kinase domain, which are connected by the COR (c-terminal of ROC) domain. Moreover, LRRK2 is composed of multiple protein-protein interaction domains (from N-terminal to C-terminal): ARM (armadillo), ANK (ankyrin), LRR (leucine rich repeat) and WD40 domain. Pathogenic PD mutations are depicted in red; in brackets are those which need further confirmation. Variants that increase the lifetime PD risk are marked orange; a variant that is reported to play a protective role is colored in green.

1.2.3 Kinase function of LRRK2

Protein kinases catalyze the transfer of a phosphate group from ATP to the hydroxyl group of a peptide substrate at a serine/threonine/tyrosine site. Protein phosphorylation is an important post-translational protein modification, especially in signal transduction. It allows a specific and tight control over diverse biological functions by modulating the activity

of enzymes and transcription factors and can impact on cellular localization of target proteins. The kinase domain of LRRK2 shares homology with MLKs (West et al., 2005), which are part of the MAPK family. MAPKKKs are known to initiate and transduce a broad variety of cellular responses to various stimuli such as mitogens, growth factors, stress and cytokines (Gallo and Johnson, 2002). However, if LRRK2 truly is a MAPKKK is under debate, because the precise mechanisms of activation and downstream targets are not well understood (Biskup and West, 2009).

Among kinases, dimerization is a common feature. Dimerization is thought to facilitate auto-regulation and may modulate downstream signaling molecules. For LRRK2, several *in vitro* studies provide evidence for its ability to form a homodimer (Civiero et al., 2012), but also the formation of heterodimers with other ROCO kinases has been observed (Klein et al., 2009). In both cases, the dimerization occurs in the ROC-COR region, however, also the WD40 domain is crucial for dimerization (Jorgensen et al., 2009). Interestingly, dimerization has been reported to be important for kinase activity (Greggio et al., 2008; Sen et al., 2009).

Since two mutations, including the most common variant G2019S, are located in the kinase domain, the idea that altered kinase activity causes pathogenicity is a prominent target of investigation. In fact, G2019S substitutes a serine for a highly conserved glycine within the active site, which has repeatedly been reported to cause a significant albeit only moderate increase in kinase activity. The other mutation within the kinase domain, I2020T, but also R1441C/G/H, which are located in the GTPase domain, have all been reported to impact on kinase activity, yet these studies are not coherent (Rudenko and Cookson, 2014; West et al., 2005).

Tight regulation of kinases activity is critical due to deleterious effects caused by aberrant kinase signaling. And indeed, abnormal phosphorylation is a common feature in neurodegenerative diseases, including PD (Tenreiro et al., 2014). Regulation can be accomplished by activator or inhibitor proteins as well as small molecules or phosphorylations. LRRK2 indeed has been found to be a highly phosphorylated protein (Kamikawaji et al., 2009). Because LRRK2 harbors a kinase domain itself, it might be involved in its own regulation. Many autophosphorylation sites, that are believed to play a role in kinase regulation, have been identified under *in vitro* settings mainly within the ROC GTPase domain (Webber et al., 2011). Also a mutual interaction between kinase domain and GTPase domain was investigated. Similar to the control of other small GTPases, it was proposed that

GTPase activity was regulating the kinase output (Ito et al., 2007; Taymans et al., 2011). Moreover, it has been shown that the kinase domain can phosphorylate various autophosphorylation sites within the ROC domain (Greggio et al., 2007) and even impact on GTPase binding activities (Webber et al., 2011). Further complexity of this matter was added by two observations that also the WD40 domain impacts on kinase function: First, loss of WD40 domain ablates the kinase function, and second, the G2385R polymorphism, located within the WD40 domain, leads to a decrease in kinase activity (Jaleel et al., 2007; Rudenko et al., 2012b).

1.2.4 GTPase function of LRRK2

In addition to its kinase domain, LRRK2 possess a GTPase domain. LRRK2 shares sequence homology with the Ras-related small GTPase family (Bosgraaf and Van Haastert, 2003). Specifically, the enzymatic activity seems to be mediated by the ROC domain, which binds and hydrolyses GTP in a fashion just like the Ras-related small GTPase Rac1 (Guo et al., 2007). Ras-GTPases are molecular switches that regulate many cellular functions by changing between a GTP-bound active and a GDP-bound inactive conformation. This regulation is further controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). While numerous reports show that LRRK2 can bind GTP, GTPase activity has been more difficult to detect (Ito et al., 2007; West et al., 2007), which might be due to the absence of certain GEF or GAPs under *in vitro* conditions. Some candidate GEFs and GAPs for LRRK2 have already been suggested. ARHGEF7 interacts with LRRK2 and was proposed to acts as GEF for LRRK2 (Haebig et al., 2010). Moreover, ArfGAP1 and LRRK2 reciprocally regulate their activity (Xiong et al., 2012). This finding is extended by another study showing that this regulation is mediating neuronal toxicity (Stafa et al., 2012). Pathogenic mutations within the ROC domain R1441C/G/H have been shown to impair GTPase domain without affecting kinase domain (Lewis et al., 2007; Li et al., 2007). However, the overall data suggests, that LRRK2 GTPase activity is in general rather weak and the effects of the respective mutations are inconsistent (Anand and Braithwaite, 2009).

1.2.5 Anatomical and cellular localization of LRRK2

In order to elucidate the physiological and pathogenic function of a protein, the expression pattern can give important clues. LRRK2 is expressed in most organs, including

the central nervous system (CNS), but the highest expression levels can be detected in kidney, lungs, spleen and the immune system (Biskup et al., 2007; Hakimi et al., 2011; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). Within the brain LRRK2 is predominantly expressed in cerebellum, brainstem, midbrain, hippocampus, cortex, striatum and olfactory bulb. Interestingly, the levels of LRRK2 are comparatively low in the SN whereas in dopamine-receptive areas LRRK2 levels are much higher (Biskup et al., 2006; Han et al., 2008; Higashi et al., 2007). LRRK2 expression is thought to be primarily neuronal rather than glial, although systematical studies investigating the expression pattern of LRRK2 hardly focus on glia cells. This view was at least partially challenged by the discovery that LRRK2 can be induced in microglial cells upon LPS stimulation (Moehle et al., 2012). Studies from rodents suggest that LRRK2 is not essentially involved during embryonic brain development. Being not detectable before birth, LRRK2 expression then peaks at 9 weeks of age and remains constant during aging (Biskup et al., 2007; Giesert et al., 2013; Westerlund et al., 2008). Because LRRK2 and LRRK1 expression patterns do not overlap, a redundant function in the pathogenesis of PD seems unlikely, which is in line with the absence of disease causing mutation within LRRK1 and the distinct cellular functions of LRRK2 and LRRK1 (Giesert et al., 2013)

LRRK2 is mainly located in the cytoplasm. It has been shown to co-localize with various intracellular membranes and vesicular structures. These include endosomes, lysosomes, multivesicular bodies, the mitochondrial outer membrane, lipid rafts, microtubule-associated vesicles, the Golgi apparatus and the endoplasmatic reticulum (ER) (Esteves et al., 2014; Hatano et al., 2007). LRRK2 possess no hydrophobic transmembrane domain or any obvious organelle-specific targeting sequences, therefore it is believed that LRRK2 is rather membrane associated than membrane integrated. This association to a broad variety of membrane structures suggests a functional role in multiple pathways including the regulation of autophagy, microtubule dynamics and mitochondrial function.

1.2.6 LRRK2 – In search for physiological roles

As mentioned above, LRRK2 is expressed throughout the body and, more importantly, has been reported to be associated with various different cellular compartments. This might be simply due to difficulties to define the “true” localization pattern of LRRK2, but could also just reflect the involvement of LRRK2 in multiple pathways.

LRRK2 knock-out (KO) mice breed and live normally, which indicates the LRRK2 might not be critically involved in neuronal survival or its function can be compensated in vivo (Yue, 2009). LRRK2 KO mice have an intact dopaminergic system and are not hypersensitive to treatment with MPTP (Andres-Mateos et al., 2009; Hinkle et al., 2012).

As for the generated transgenic overexpression LRRK2 rodent models, the current data is controversial. Although the majority of models have failed so far to robustly mimic a PD phenotype and at most only partially recapitulate PD pathology, some studies report DA neuron death (Chen et al., 2012) even including the motor behavioral and histopathological features of disease (Li et al., 2009). This discrepancy between studies might be due to different expression strength because of the usage of different promotors and also inconsistent use of murine and human genes (Sloan et al., 2012; Xu et al., 2012). Yet, because there is no evidence for LB pathology in any transgenic LRRK2 mouse model, these models cannot be regarded as serving as a true recapitulate of a PD phenotype.

Within the past few years, a myriad of functions has been attributed to LRRK2; complicating the efforts to unravel LRRK2 pathobiology in PD. LRRK2 seems to be involved in many different biological roles, which are at least partially interconnected, increasing the challenge of focusing on the most physiological relevant ones (Fig. 1.5). In the following subchapters, some the main emerging roles of LRRK2 biology shall be introduced.

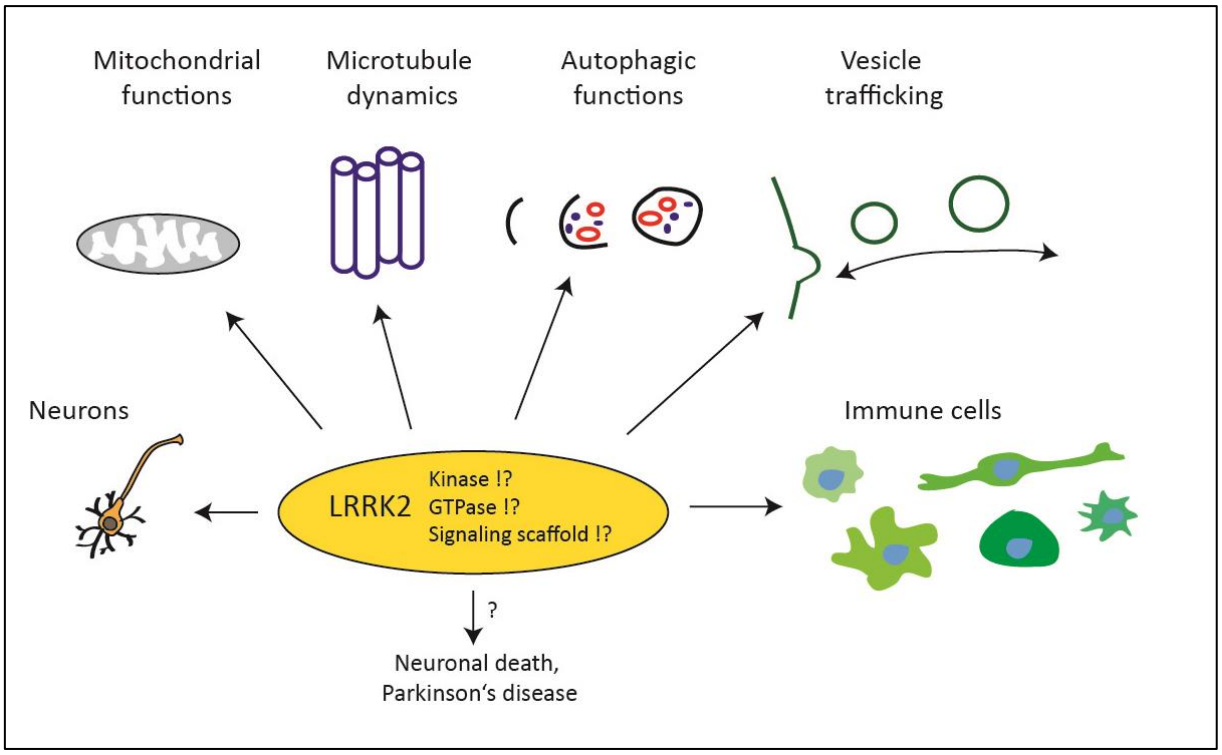

Fig. 1.5: Overview of physiological roles of LRRK2

For LRRK2, various physiological roles have been suggested. Emerging themes for LRRK2 include mitochondrial functions, microtubule dynamics, autophagic functions and vesicle trafficking. These themes have at least partial mutual influence and they are all believed to be involved in neuronal decay underlying PD. LRRK2 might influence these functions by either kinase or GTPase function or as a scaffold for signaling complexes. Analysis of LRRK2 function has previously focused on neuronal function, but more recently roles for LRRK2 in immune cells are emerging, including immune cell specific functions.

1.2.6.1 LRRK2 in vesicle trafficking and dopamine homeostasis

Since the decay of DA is central in PD pathology, a connection between LRRK2 and dopamine homeostasis and vesicle trafficking seems plausible. And indeed, already early studies found LRRK2 localized to vesicles (Biskup et al., 2006). Moreover, knockdown and overexpression of LRRK2 has been shown to modulate synaptic vesicle endocytosis (Heo et al., 2010a; Heo et al., 2010b; Shin et al., 2008) and LRRK2 silencing has been reported to induce an alteration in vesicle distribution and recycling dynamics (Piccoli et al., 2011). This regulation might be both dependent on kinase activity (Cirnaru et al., 2014) and protein interactions at its WD40 domain (Piccoli et al., 2014). Another piece of evidence that LRRK2 is involved in synaptic vesicle endocytosis was presented by demonstrating that LRRK2 can phosphorylate EndophilinA (EndoA) (Matta et al., 2012).

Although loss of DA neurons was not observed in most transgenic mouse models, these studies do provide evidence for disruption in DA neurotransmission. One indication for this came from the observation that different LRRK2 transgenic mice show L-dopa-sensitive motor deficits. Moreover, a reduction in both extracellular striatal dopamine levels as well as a decrease in dopamine metabolites was found (Chen et al., 2012; Li et al., 2009). This might be caused by altered dopamine release and re-uptake. In fact, LRRK2 R1441C transgenic mice have both impaired dopamine and catecholamine release (Tong et al., 2009). LRRK2 WT, on the other hand, has been reported to cause an enhanced dopamine release (Li et al., 2010). Re-uptake of DA seems also to be affected, as reduced DAT (dopamine active transporter) levels are present in the G2019S LRRK2 transgenic mice (Chen et al., 2012), and differences in extracellular dopamine levels were nullified by DAT inhibition (Zhou et al., 2011). More recently, LRRK2 was reported to regulate synaptogenesis during development and dopamine receptor activation by anchoring PKA subunits PKACα and PKARIIβ to the dendritic shaft thus controlling PKACα activity during synaptic transmission. Noteworthy, the R1441G mutant impaired this interaction. Thus, LRRK2 was postulated to function as a neuronal a kinase anchoring protein (AKAP), fine tuning dendritic spine formation during development and synaptic transmission (Parisiadou et al., 2014).

Although it seems very plausible to search for a role of LRRK2 in the “pathogenic vicinity” of PD, much more research is required to approve the above mentioned findings. For example, a dysregulation in DA neurotransmission could very well also be not specifically and directly influenced by LRRK2 but rather via indirect effects of altered endosomal trafficking or impaired cytoskeletal dynamics.

1.2.6.2 Functional interaction between LRRK2 and α-Syn

Forms of direct interaction between α-Syn and LRRK2 have long been postulated and might seem likely for the following reasons: First, at least partially, they seem to have a functional overlap since they both are reported to play a role in microtubule dynamics and assembly (Caesar et al., 2013; Parisiadou and Cai, 2010). Secondly, approximately 60% of all SNCA-positive LBs also contain LRRK2 (Perry et al., 2008) or are rimmed by it (Vitte et al., 2010). Third, LRRK2 co-immunoprecipitates with α-Syn from post-mortem brains with LB pathology. Besides these pieces of evidence for spatial interactions, some studies contribute data for functional interactions: Overexpression of LRRK2 has been reported to induce α-Syn

expression via an extracellular signal-regulated kinase (ERK) meditated pathway (Carballo-Carbajal et al., 2010), and also facilitates α-Syn release and its subsequent uptake by neighboring cells (Kondo et al., 2011). The idea that LRRK2 phosphorylates α-Syn at S129, which is believed to promote the formation of filaments and oligomers (Qing et al., 2009) has so far not been confirmed. More puzzling, in human carriers of LRRK2 G2019S α-Syn aggregation was lower when compared with idiopathic PD patients (Mamais et al., 2013). In total, the sum of studies indicates a possible interaction of LRRK2 with α-Syn, however the real nature of a putative *in vivo* interaction is an open question and under debate. For example, early studies reported that LRRK2 regulates the progression of neuropathology of α-Syn transgene mice (Lin et al., 2009), while other similar studies could not find such interactions (Daher et al., 2012).

1.2.6.3 LRRK2 and mitochondria

Mitochondrial dysfunction is generally believed to be a trigger for PD. Accordingly, PINK1 and Parkin have been demonstrated to mediate autophagy of impaired mitochondria (Narendra et al., 2008; Narendra et al., 2010). Although LRRK2 is not involved in this process, it has been found in fractions of the outer membranes of mitochondria (Biskup et al., 2006). Some studies report perturbed mitochondrial function and morphology in skin biopsies of G2019S carriers (Mortiboys et al., 2010), however in mouse neurons structural deficits of mitochondria seem to be absent despite overexpression of mutant or WT LRRK2 (Lin et al., 2009). Other studies investigated mitochondrial responses to oxidative stress. Overexpression of either mutant or WT LRRK2 increases the deleterious effects of H_2O_2 in a dopaminergic cell model (Heo et al., 2010b) and LRRK2 overexpression alone was shown to increase ROS levels (Niu et al., 2012). This might be caused by a loss of protective antioxidant function, as LRRK2 mutations have been shown to reduce activity of peroxiredoxin 3, an antioxidant enzyme involved in controlling levels of hydrogen peroxide (Angeles et al., 2011). Although a general protective role for LRRK2 to attenuate oxidative stress has long been proposed (Liou et al., 2008), much further research is needed to clearly decipher the functional connection of LRRK2 in this context.

Mitochondria are very dynamic cell organelles. While fusion and fission of mitochondria is important to maintain a healthy and homogenous population, disturbed mitochondrial dynamics can have deleterious effects for the cell (Chan, 2012). Several studies suggest that

LRRK2 is regulating mitochondrial dynamics. For example, LRRK2 has been reported to directly regulate dynamin-like protein (DLP-1), which is a fission protein and partially co-localizes with LRRK2. LRRK2 overexpression causes mitochondrial fragmentation, which is increased by expression of the G2019S or R1441C LRRK2 mutant (Niu et al., 2012; Wang et al., 2012). Moreover, LRRK2 has been reported to interact with dynamin-related GTPases that regulate mitochondrial membrane fission (Drp1) and fusion (mitofusins and optic atrophy 1 (OPA1)) (Stafa et al., 2014). In conclusion, LRRK2 interactions with mitochondrial dynamics might contribute to PD pathology.

1.2.6.4 LRRK2 cytoskeletal organization

Another major emerging theme for LRRK2 is involvement in cytoskeletal dynamics. More specifically, LRRK2 was shown to co-immunoprecipitate with α/β-tubulin heterodimers and was found to co-localize with α/β-tubulin in primary neurons as well (Gandhi et al., 2008). This interaction seems not to be limited to neurons (Caesar et al., 2013). The nature of interaction is still not fully understood, but one study suggests that LRRK2 is phosphorylating β-tubulin, with the pathogenic mutation G2019S ultimately leading to a microtubule overstabilization, which in turn affects cell function and viability (Gillardon, 2009). Accordingly, the opposite phenotype, reduced tubulin polymerization, has been observed in LRRK2 KO mice (Lin et al., 2009; Maekawa et al., 2012). Additional to its interaction with tubulins, LRRK2 also has been shown to interact with actin. Specifically, mutant LRRK2 induces accumulation of polymerized actin as well as phosphorylated ERM (ezrin, radixin, moesin), which are actin cross-linking proteins. Upon LRRK2 KO, this effect is reversed (Parisiadou et al., 2009). Tau, which in general contributes to microtubule stability and is known for its involvement in AD, has also been suspected to interact with LRRK2. Specifically, LRRK2 has been shown to phosphorylate tubulin associated tau (Kawakami et al., 2012), but not the single molecule, and both G2019S and I2020T mutations increase tau-phosphorylation. Moreover, LRRK2 G2019S mice display increased levels of phosphorylated tau when compared to WT animals, whereas LRRK2 null mice exhibit less phosphorylated tau (Parisiadou et al., 2009). Concluding from these studies that connect LRRK2 with structural and regulatory components of the actin cytoskeleton and the microtubule network, it might seem plausible that LRRK2 also impacts on neurite morphology, where reorganization or establishment of cytoskeletal structures is crucial. In fact, both LRRK2 overexpression and

deficiency has been observed to alter neurite branching as well as neurite length (MacLeod et al., 2006; Plowey et al., 2008).

1.2.6.5 LRRK2 in autophagy

Protein aggregates and proteinacious inclusion bodies are general hallmarks in neurodegenerative diseases such as AD and PD. Impairment of the two major cellular protein degradation pathways, the ubiquitin proteasome system (UPS) and lysosomal protein degradation or autophagy, is widely established to be associated with neurodegeneration (Dennissen et al., 2012; Hara et al., 2006; Nixon, 2013). Therefore, it is plausible that PD associated genes might play a role in either the UPS or autophagy. And indeed, LRRK2 has been observed in cellular organelles involved in autophagy. This includes co-localization of LRRK2 with multivesicular bodies (MVB) and autophagic vacuoles (Alegre-Abarrategui et al., 2009), but also endosomal vesicles (Biskup et al., 2006), which are fed into autophagic degradation. In that sense, dysfunction in the endocytic pathway could contribute to an autophagic phenotype. This in fact has been proposed in a study where LRRK2 overexpression led to a slowdown of endocytosis in a mutant and kinase independent fashion (Shin et al., 2008). LRRK2 has also been demonstrated to interact with Rab proteins, small GTP binding proteins. Rab7 is known to be involved in the fusion of endosomes and autophagosomes with lysosomes. LRRK2 is localized to the endosomal-lysosomal compartment in tissues with LB pathology where it is co-localized with Rab7 (Higashi et al., 2009). A similar interaction between LRRK2 and Rab7 has been observed in a fly model (Dodson et al., 2012). Interestingly, RAB7L1, a related Rab protein that lies in the PARK16 locus, has been associated to interact with LRRK2 causing a protein sorting defects (MacLeod et al., 2013). In LRRK2 null mice, elevated levels of LC3-II, an autophagy marker reflecting either an increase in autophagic induction or a reduction in clearance of autolysosomes, have been found in the kidney. However, the mice brain was not affected by this. This might indicate that brain-specific mechanisms can compensate for LRRK2 function, or that loss of LRRK2 or its mutated forms have only subtle pathogenic effects, which may take long ageing in order to impact on neuronal viability. Upon pharmacologically inhibition of LRRK2, autophagy is stimulated as indicated by an increase in LC3-II levels (Manzoni et al., 2013). Moreover, overexpression of G2019S LRRK2 increases the number of autophagic vacuoles in neurons, possible thereby also affecting neurite length (MacLeod et al., 2006; Plowey et al.,

2008). If this is reflecting a cytoskeletal defect, dysregulated autophagy or even an upstream cytotoxic effect remains to be elucidated. Lastly, while LRRK2 has been reported to be removed from the cytosol by chaperone mediated autophagy (CMA), the degradation of the G2019S variant was significantly less efficient (Orenstein et al., 2013). As a secondary effect, jamming the CMA with mutated LRRK2 could compromise neuronal removal of α-Syn as well.In conclusion, LRRK2 seems to be involved in autophagic processes, however if these effects are of a very direct or rather indirect nature is not yet fully understood.

In regard to the complex structure of LRRK2, the myriad of functions might after all not surprise. In search of the LRRK2 functions, LRRK2 might truly offer a great variety of roles, surely also depending on tissue, cellular localization, the cellular state etc. However, the above mentioned themes for LRRK2 could also indirectly converge on the same pathway.

1.2.7 LRRK2 in the immune system

Classically, LRRK2 function has been studied mostly in neuronal systems or at least with the aim to understand neuronal LRRK2 function. More recently, an emerging body of evidence is drawing growing attention towards the role of LRRK2 in immune cells and its association with the immune system. More clinical evidence for this role came with the discovery that LRRK2 is not only associated with PD, but also with leprosy (Zhang et al., 2009), a chronic infectious disease caused by *Mycobacterium leprae*, and with CD (Barrett et al., 2008), a subtype of inflammatory bowel disease, that affects the gastrointestinal tract. Interestingly, also Parkin was found to be a risk factor for leprosy (Mira et al., 2004). From a structural point of view, LRRK2 belongs to the receptor-interacting protein kinase (RIPK) family, which are known to play roles in immune signaling pathways (Ofengeim and Yuan, 2013; Zhang et al., 2010).

Probably the simplest albeit important observation is that LRRK2 expression level in immune cells actually surpass that in neurons, as shown by analysis of mRNA levels in different tissues (Miklossy et al., 2006). Indeed, expression is high in spleen tissue, more specifically in CD19+ B-lymphocytes, but much lower in CD3+ T-lymphocytes (Maekawa et al., 2010). Analysis of primary human peripheral blood mononuclear cells (PBMCs) revealed high LRRK2 expression in CD19+ B-lymphocytes, CD14+ monocytes and dendritic cells (Gardet et al., 2010; Hakimi et al., 2011; Thevenet et al., 2011). Considering monocyte subpopulations, the highest LRRK2 levels were observed in CD14+/CD16+ monocytes, which

are a more mature or activated monocyte population (Hakimi et al., 2011). Tissue macrophages, which differentiate from circulating monocytes before being recruited to their site of action, also express profound levels of LRRK2, as evident in the immortalized RAW264.7 macrophage cell line, in phorbol-12-myristate-13-acetate (PMA) differentiated THP-1 macrophages as well as in primary mouse bone marrow-derived macrophages (BMDMs) (Dzamko et al., 2012; Liu et al., 2011). Moreover, LRRK2 is also present in microglia, the resident brain macrophages, as it has been assessed in humans, mice and primary mouse cultures (Gillardon et al., 2012; Miklossy et al., 2006; Moehle et al., 2012).

Interestingly, these high LRRK2 levels are even further increased upon stimulation of these cells. Upon treatment with Interferon-γ (IFN-γ), both mRNA and protein levels are robustly increased in CD19+ B-lymphocytes, CD11b+ monocytes and CD3+ T-lymphocytes (Gardet et al., 2010).

IFN-γ is a classic macrophage activating factor, primarily produced by natural killer (NK) cells and T-lymphocytes. Its main role is to modulate both innate and adaptive immune responses by causing the induction of various target genes (Schroder et al., 2004). Next to IFN-γ, stimulation of the toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) was also reported to modulate LRRK2 levels. However, despite numerous reports investigating the LPS mediated effects, the results seem inconclusive: LPS has been demonstrated to increase LRRK2 levels (Hakimi et al., 2011), to induce the down-regulation of LRRK2 within 1 h (Liu et al., 2011) or not to affect LRRK2 levels at all (Dzamko et al., 2012). Next to these inconsistent findings LRRK2 has also reportedly shown to be phosphorylated at S910 and S935 upon TLR signaling, supposedly through activity of the inhibitor of NF-κB kinase (IKK) (Dzamko et al., 2012). Although the physiological role of this phosphorylation remains elusive, LRRK2 overexpression studies propose that alterations in S910 and S935 can modulate subcellular localization (Deng et al., 2011a; Nichols et al., 2010). Localization patterns of LRRK2 might be critically for functional aspects as it translocates to bacterial membranes during infections (Gardet et al., 2010). Moreover, LRRK2 has been reported to modulate expression of pro-inflammatory cytokines including IL-6, IL-12 and TNFα through activation of NF-κB. Specifically, murine microglia overexpressing the LRRK2 mutant R1441G display an increase in TNFα production in comparison to WT cells (Gillardon et al., 2012), whereas LRRK2 knockdown or kinase inhibition reduced the microglial TNFα production after LPS stimulation (Moehle et al., 2012). However, similar experiments using murine BMDMs found no

differences in regard to cytokine secretion (Dzamko et al., 2012; Hakimi et al., 2011). If this reflects a possible functional difference of LRRK2 in microglia and macrophages is an open question. Interestingly, LRRK2 has been demonstrated to be a negative regulator of nuclear factor of activated T-cell 1 (NFAT1), which is known to orchestrate the expression of various cytokines including TNFα and IFN-γ. Mechanistically, LRRK2 decreases the transcriptional activity of NFAT1 by interfering with its nuclear translocation. This is accomplished via association with the ncRNA repressor of the nuclear factor of activated T cells (NRON) complex, which is directly interacting with NFAT1, inhibiting its nuclear translocation.

In conclusion, LRRK2 is highly expressed in different immune cells and may function as a transcriptional regulator by modulating immune functions of transcription factors such as NFAT1 and NF-κB. Other studies have proposed a role in phagocytosis. Upon pharmacological kinase inhibition, microglial phagocytosis was reported to be inhibited (Marker et al., 2012). Accordingly, LRRK2 deficiency impairs the production of reactive oxygen species (ROS) during phagocytosis (Gardet et al., 2010). However, more detailed studies are required to rule out the precise nature of these interactions.

1.2.8 Neuroinflammation and neuronal death in PD: LRRK2 intersections

Neuroinflammatory events have long been observed in PD (Banati et al., 1998; Imamura et al., 2003; McGeer et al., 1988). While it is obvious that LRRK2 plays an important role in PD, together with the now emerging role in the immune system, it is worthwhile to acknowledge the involvement of inflammation in PD, especially since the familial form of PD caused by LRRK2 very closely resembles PD pathology in sporadic cases.

As long as it remains unclear if neuroinflammation is preceding neuronal death or instead is secondary to neurodegeneration, both possibilities should be considered. Yet, maybe the simplest idea is that LRRK2 directly causes neuronal death which subsequently initiates a secondary inflammatory cascade. Indeed, several studies have found LRRK2 to exert toxic effects on primary neurons (Greggio et al., 2006; Lee et al., 2010a; Ramsden et al., 2011; Smith et al., 2006). A common theme in these studies using overexpression models of LRRK2 mutants was that LRRK2 toxicity was mediated by its kinase activity. Hence, toxicity was reduced when kinase inhibitors were applied. Yet, as appealing this straightforward mechanism might seem, there are some controversies as well. Toxicity was found to be mediated not only by kinase activity but also by GTPase activity (Stafa et al., 2012; Xiong et

al., 2010), adding complexity to the issue in regard of their mutual regulation. More confusingly, it was recently reported that not kinase activity but rather protein levels are crucial for toxicity in neurons (Skibinski et al., 2014). In conclusion, up to date precise mechanism of how LRRK2 could contribute to neuronal toxicity are missing, and importantly, most evidence is based on overexpression experiments demonstrating toxicity.

Another possibility of how LRRK2 could contribute to neurodegeneration is by cell non-autonomous mechanisms of neighboring non-neuronal cells. This brings microglia into play, which are actively screening the brain for pathogens and cell debris. The presence of activated microglia, the activated state of the resident macrophages of the brain, within the SNpc of PD patients was repeatedly observed (Banati et al., 1998; Imamura et al., 2003; McGeer et al., 1988). Moreover, a great number of clinical and animal studies found an increase in various inflammatory mediators including cytokines and chemokines involved in PD pathology (Tab. 1.4). Activated microglia can mediate neurotoxicity by production of cytokines, chemokines, complement proteins and nitric oxide (Cunningham, 2013; Hanisch and Kettenmann, 2007).

Interestingly, microglia are present in particular high densities in the SNpc (Kim et al., 2000) and can be activated by toxins, pathogens or endogenous proteins. Whereas temporal activation is apparently beneficial, chronically reactive microglia have detrimental effects causing neuronal decay and ultimately initiating a vicious cycle: Cell debris from dying neurons but also α-Syn species, neuromelanin, ATP and matrix metalloproteinase-3 (MMP-3) further increases microglial activation (Davalos et al., 2005; Kim et al., 2005; Wilms et al., 2003; Zhang et al., 2005b), which then leads to aggravated neuroinflammation. Beside the numerous triggers for microglia activation, there are also inhibitory neuronal-glia interactions. The CD200-CD200R interaction has been shown to be essential for maintaining an unperturbed CNS environment, whereas its disruption leads to microglial activation and ultimately to neurodegeneration (Lyons et al., 2007; Wang et al., 2011). Interestingly, LRRK2 is highly expressed in primary microglia and more importantly, has been also reported to be involved in inflammatory responses. Specifically, in murine LRRK2 KO microglia, inflammatory responses were attenuated when provoked by insults with TLR4 agonist such as LPS (Kim et al., 2012; Moehle et al., 2012). In addition, LRRK2 kinase inhibition has been reported to protect primary axons from microglia phagocytosis after treatment with HIV-1 transactivator of transcription (Tat) protein, which is known to induce neurotoxicity through

monocyte activation (Marker et al., 2012; Sui et al., 2006). However, despite numerous indications for the importance of LRRK2 function in relation to PD, detailed studies dissecting the mechanisms and roles of LRRK2 in microglia in a pathogenic setting are still missing.

Apart from neural causes for neurodegenerative diseases in the neuronal vicinity, systemic body infections and inflammation can exacerbate symptoms (Cunningham, 2013). The primarily underlying process of systemically induced neurotoxicity is the transformation of “primed” microglia by systemic triggers into “activated” microglia, which in turn increase neurodegenerative effects. The BBB regulates the passage of substances from the blood to the brain. Yet, there are several routes bypassing the BBB termed humoral mechanisms (Fig. 1.6). First, the circumventricular organs (CVO) are not surrounded by the BBB, thus provide an entry site for inflammatory substances (Schulz and Engelhardt, 2005). Second, endothelial cells, which are the main component of the BBB, can be stimulated by peripheral stimuli subsequently leading to a synthesis of molecules within the CNS (Dantzer and Kelley, 2007; McCusker and Kelley, 2013). Third, the choroidplexus, which also serves as a part of the BBB, has been reported to transiently alter its transcriptome profile upon peripheral LPS stimulation (Marques et al., 2009). This might then in turn disturb brain homeostasis. Lastly, some cytokines and amines are suggested to cross the BBB simply by specific transporters (Banks, 2005).

Moreover, a neural pathway can mediate inflammatory signals from the periphery to the brain. This pathway is mainly represented by the vagus nerve, which is part of the parasympathetic nervous system. Peripheral stimulation of the vagus nerve can result in a rapid increase of CNS cytokines (Cunningham et al., 2009). Accordingly, IL-1β receptors are expressed on vagal ganglia in vicinity to lympathic nodes (Maier et al., 1998).

Additional to the humeral and neural pathways, immune cells may directly enter the brain. In general, the BBB controls immune cell entry into the CNS (Engelhardt, 2008). In regard to PD, CD4+/CD8+ T-cells infiltration into the SN was observed in both post-mortem PD brains and MPTP mouse models (Brochard et al., 2009). Moreover, monocytes can be recruited to the brain by secretion of monocyte chemotactive protein (MCP-1) by activated microglia during peripheral inflammation (D'Mello et al., 2009; Kerfoot et al., 2006). Logically, also the disruption of the BBB as a result of chronic inflammation, presents a possibility of how systematic inflammation can translate into the CNS. Yet, if disruption of

the BBB is generally part of PD pathology is under dispute (Bartels et al., 2008; Chung et al., 2010; Kortekaas et al., 2005).

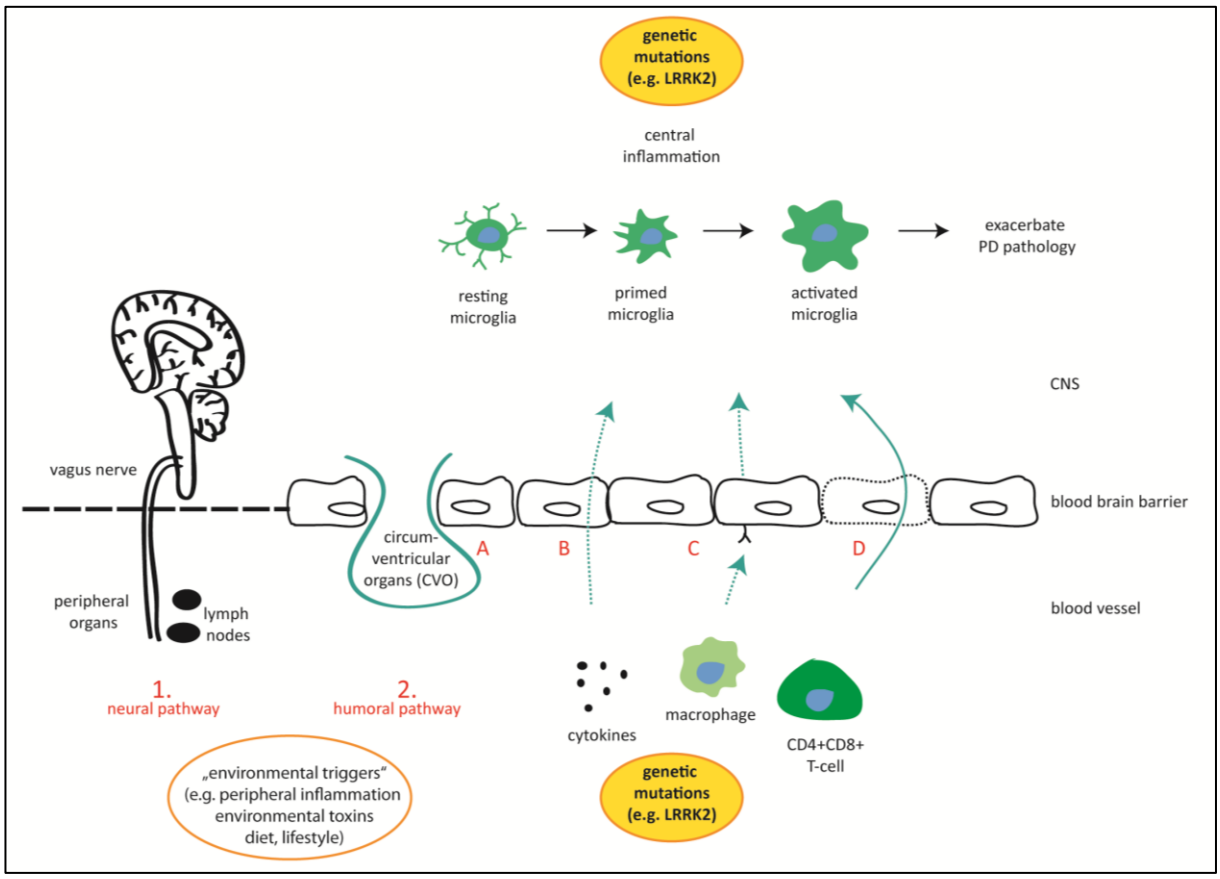

Fig. 1.6: Peripheral influence on CNS inflammatory events in PD

Neuroinflammatory events in PD are mainly represented by microglia causing exacerbation of PD pathology. While intrinsic factors are believed to be involved in priming of microglia in central inflammation, accumulating evidence suggests that environmental triggers (e.g. peripheral inflammation) or genetic predispositions contribute to activate primed microglia. These triggers have different routes to impact on CNS inflammatory events bypassing the blood-brain barrier (BBB). The neural pathway (1) is mainly represented by the vagus nerve. It serves as an afferent for neuronal transmission of peripheral inflammatory signals from lymph nodes or peripheral organs. The humoral pathways are mechanism for peripheral stimuli to bypass the BBB to enter the CNS. Some areas of the CNS like the circumventricular organ lack BBB thus providing an entry site (A). Some molecules may cross the BBB by specific transporters (B). Moreover, endothelial cells of the BBB may be involved in signal transduction of peripheral stimuli (C). BBB breakdown can cause cytokine and immune cell extravasation into the CNS. As LRRK2 is involved in both central and peripheral immune system, potential implications in PD pathology are numerous.

Conclusively, the crosstalk between periphery and the CNS is far less restricted than traditionally assumed, yet the mechanisms in PD are not very well understood. While it is

commonly questioned that systemic inflammation alone can give rise to PD, a "multiple-hits hypothesis" is emerging: Systemic insults prime the system making it more sensitive and vulnerable to other disease-causing factors, which for themselves would not have been pathogenic. Set aside systemic alterations of the immune system in elderly people, chances to receive such a "hit" would logically increase with time and indeed aging, the main risk factor for PD, has been associated with a chronic state of neuroinflammation (Frank et al., 2006; Perry et al., 1993). Moreover, studies show that a systemic inflammation causes exaggerated brain inflammation and accelerated pathology in animal models of chronic neurodegeneration (Cunningham et al., 2009; Perry et al., 2007). As for PD specifically, studies using 6-OHDA-treated animals report an exacerbation in neurodegeneration (Pott Godoy et al., 2008). Moreover, systemic LPS administration induces neuroinflammation in mice but does not result in decay of DA neurons in the SNpc (Jeong et al., 2010).

Tab. 1.4: Evidence for inflammatory processes in PD pathology

Process	Location	Reference
Activated microglia	Brain	(Banati et al., 1998; Imamura et al., 2003; McGeer et al., 1988)
COX-2	Brain	(Knott et al., 2000)
IFN-γ	Brain	(Barcia et al., 2011; Hunot et al., 1999; Mogi et al., 2007; Reale et al., 2009)
IL-1β	Brain/CSF	(Hunot et al., 1996; Hunot et al., 1999; Mogi et al., 1996)
IL-2	Brain/Serum	(Mogi et al., 1996; Stypula et al., 1996)
IL-6	Brain/Serum/CSF	(Blum-Degen et al., 1995; Dobbs et al., 1999; Mogi et al., 1996; Muller et al., 1998; Reale et al., 2009)
IL-8	Serum	(Reale et al., 2009)
iNOS	Brain	(Hunot et al., 1996; Knott et al., 2000)
MCP-1/CCL2	Serum	(Reale et al., 2009)
MIP1-α	Serum	(Reale et al., 2009)
RANTES	Serum	(Reale et al., 2009; Rentzos et al., 2007)
TNF- α	Brain/Serum/CSF	(Boka et al., 1994; Dobbs et al., 1999; Hunot et al., 1996; Mogi et al., 1994)
TNFR-1	Brain	(Boka et al., 1994; Mogi et al., 2000)

In conclusion, neuroinflammation is a central aspect in PD. However, if inflammation precedes and initiates neurodegeneration or is a secondary event that is exacerbating the progression of cell death remains an important but open question that deserves more detailed research. Moreover, systemic inflammation may not cause PD but seems to

increase the likelihood of developing PD by shifting a predisposed CNS in a more unfavorable state. Yet, the role of LRRK2 in this context remains to be further investigated.

1.2.9 Interferon-γ: Signaling and biological implications of a LRRK2 inducer

The already high expression levels of LRRK2 in immune cells such as macrophages and B-cells can be further increased by stimulation with IFN-γ (Gardet et al., 2010). This indicates that LRRK2 plays an important role in these cells in general and in IFN-γ response specifically. Noteworthy, IFN-γ has also been found to be elevated in the PD brain (Hunot et al., 1999; Mogi et al., 2007).

Interferons were originally discovered as agents that interfere with viral replication (Isaacs and Lindenmann, 1957). Later, they were classified according to their sequence homology and receptor specificity. IFN-γ is the only type II interferon and structurally unrelated to other interferons. The active cytokine is a soluble dimer that binds to its own receptor and is encoded on an individual chromosomal locus. IFN-γ is predominantly produced in CD4+ Th1 lymphocytes, CD8+ cytotoxic T cells, NK cells, but also in natural killer T (NKT) cells, B cells and antigen presenting cells (APCs), which includes monocyte/macrophages as well as dendritic cells (DCs) (Bach et al., 1997; Frucht et al., 2001; Schroder et al., 2004). The production and secretion of IFN-γ is regulated by APCs through secretion of IL-12 and IL-18, classically as recognition of a pathogen to initiate an immune response (Schindler et al., 2001). Negative regulators of IFN-γ on the other hand include IL-4, IL-10 and glucocorticoids (Schroder et al., 2004).

To initiate IFN-γ signaling, IFN-γ must bind to the IFN-γ receptor (IFNGR), which consists of two ligand binding IFNGR1 chains in association with two IFNGR2 chains that are in turn associated with the downstream signaling machinery (Fig. 1.7). Because both IFNGR subunits are lacking intrinsic kinase activity, signal transduction relies on the association of janus tyrosine kinase (JAK) 1 and JAK2 and their respective cytosolic downstream target signal transducer and activator of transcription 1 (STAT1). As many other cytokines, growth factors and hormones, IFN-γ predominantly signals through this so called JAK/STAT pathway (Subramaniam et al., 2001) (Schroder et al., 2004). Upon ligand binding, a conformational change induces JAK2 autophosphorylation and activation, which causes JAK1 transactivation. This in turn leads to the phosphorylation of the IFNGR1, enabling binding of STAT1. Hence, the receptor recruited STAT1 homodimer is phosphorylated causing its dissociation from the

receptor. Then, the released homodimer is translocated to the nucleus and binds to gamma-interferon activation site (GAS) to induce or reduce the expression of IFN-γ-regulated genes (Ramana et al., 2000).

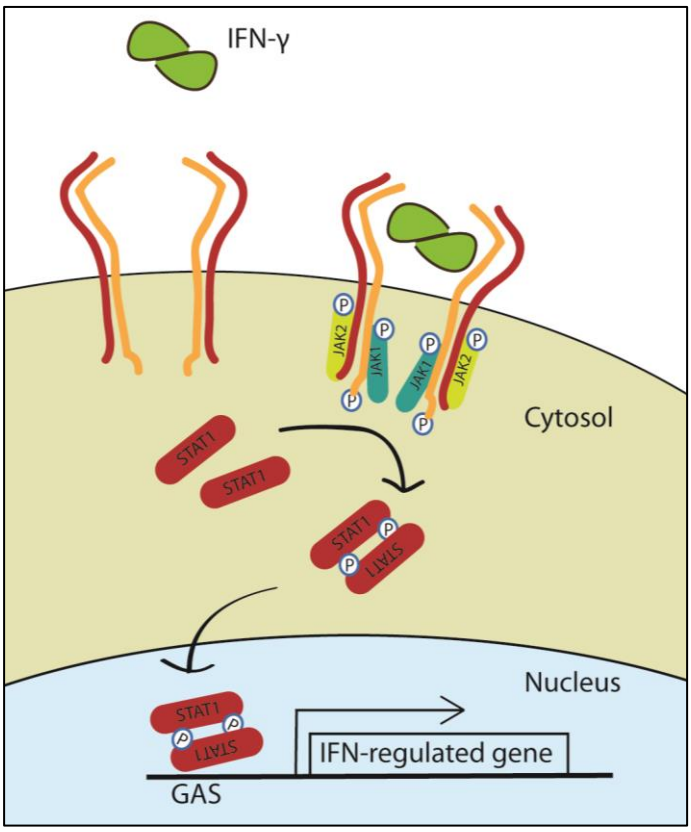

Fig. 1.7: Classical IFN-γ signaling

IFN-γ classically signals through the JAK/STAT pathway. Upon ligand binding to the IFN-γ receptor (IFNGR), which is composed of two subunits of both IFNGR1 (orange) and IFNGR2 (red), a conformational change occurs that leads to the autophosphorylation of JAK2. JAK2 in turn phosphorylates JAK1, which again phosphorylates the IFNGR1 cytosolic docking sites for STAT1. This receptor recruited STAT1 homodimer is phosphorylated and translocated to the nucleus. There, it is binding to IFN-γ activation sites (GAS) to activate transcription of IFN-γ effector genes.

IFN-γ primarily signals through STAT1, which is indicated by the fact the IFNGR1 KO mice phenocopy STAT1 KO mice to a high degree (Meraz et al., 1996). A smaller proportion of STAT1 homodimers are associated with IFN regulator factor (IRF)-9, which induces expression of genes at IFN-stimulated response element (ISRE) promotor regions. The first wave of IFN-γ induces transcription factors, like IRF-1, that also bind to ISRE. Termination of signaling occurs at different stages: IFN-γ-IFNGR1 complex internalization and degradation, inducible feedback inhibitors of the Jak1/2 kinases (e.g. SOCS1), Jak dephosphorylation by protein tyrosine phosphatases (PTPs) and analogous STAT1 dephosphorlyation by nuclear

PTPs (Endo et al., 1997; Farrar and Schreiber, 1993; Haque et al., 1997; McBride et al., 2000). Involved in both innate and adaptive immunity, IFN-γ plays an important role during viral, bacterial and protozoan infections. IFN-γ exerts its diverse functions through transcriptional regulation of various cellular processes which include pathogen recognition, antigen processing and presentation, antiviral responses, anti-proliferative effects, apoptotic effects, antimicrobial mechanisms and immunomodulation (Tab. 1.5).

The role of LRRK2 in the IFN-γ response is not yet understood. However, some studies provide insights for potential functional interactions. For example, the production of ROS species, which is a general IFN-γ response, is reduced upon silencing of LRRK2 (Gardet et al., 2010). The nuclear factor κB (NF-κB), an important transcription factor for immune responses, is activated upon IFN-γ stimulation (Pfeffer, 2011). Overexpression of LRRK2 in in HEK293T cells suggest that this might depend on LRRK2 (Gardet et al., 2010). In microglia, pharmacological inhibition of LRRK2 kinase using the IN-1 inhibitor lead to a reduction of inflammatory cytokine expression, including IL-6 and MCP1, which are part of the IFN-γ response (Biondillo et al., 1994; Marker et al., 2012; Moehle et al., 2012). The reduced cytokine expression was accompanied by impaired microglial phagocytosis, a process which is also modulated by IFN-γ in microglia (Chan et al., 2001). Moreover, in monocytes, kinase inhibition of LRRK2 was reported to cause reduced up-regulation of CD14, CD16 and MCH-II upon IFN-γ treatments suggesting a functional role of LRRK2 in monocyte maturation (Thevenet et al., 2011). The inducible nitric oxide synthase (iNOS) is induced during IFN-γ and LPS responses to kill pathogenic bacteria (Lowenstein et al., 1993). Upon LRRK2 kinase inhibition, NO production is attenuated.

In conclusion, this demonstrates that LRRK2 is tightly connected to IFN-γ induced responses in immune cells like macrophages or microglia. However, more research is required in order to gain a better understanding of the exact mechanisms of LRRK2 immune biology.

Tab. 1.5: INF-γ regulated genes and their roles in IFN-γ responses

(Table adapted from Schroder et al., 2004)

IFN-γ effects	Regulated genes	Functions
Antigen processing and presentation: Class I	LMP-2/-7 PA28 TAP1/2 MHC I heavy chain β_2.microglobulin Tapasin	Enzymatic proteasome subunits Nonenzymatic proteasome activator Transmembrane pump for peptide transport Structural component of class I MHC Structural component of class I MHC Chaparone involved in peptide loading
Antigen processing and presentation: Class II	MHC II α/β chains Ii chain DMA/DMB ves B/H/L CIITA	Structural component of class II MHC Transmembrane chaperone, induces CLIP generation CLIP removal to enable peptide loading Lysosomal proteases transactivator, induces transcription of class II MHC
Antiviral	PKR ADAR GBP1/2	Inhibits viral protein synthesis by eIF-2 phosphoryl. Deaminase, editing of viral mRNA, inhibit replication Unknown antiviral effects
Antiproliferate	PKR p21, p27 p202 Mad1 c-myc	Inhibits cellular proliferation by eIF-2 phosphoryl. CDK inhibitors, causing cell cycle arrest Strong cell cylcle repressor, binds E2F4 Antagonizes c-myc function, inhibits proliferation Down-regulated by IFN-γ
Pro-Apoptotic	IRF-1 Caspase 1 PKR DAPs Cathepsin D Fas/Fas ligand TNFα receptor	Tumor suppressor, induces apoptosis Protease, generation of active IL-1β and IL-18 Mediates TNF-α apoptosis by unkown mechanism Mediates apoptosis, mechanism elusive Mediates apoptosis, mechanism elusive Increase sensitivity to apoptosis Increase sensitivity to apoptosis
NO intermediates	iNOS/NOS2 Argininsuccinate synthetase GTP-cyclohydroxylase I	Inducible producer of nitric oxide (NO) Involved in NO production Involved in NO production
ROS species	gp91 / gp67	Subunits of NADPH oxidase, role in ROS production
Antimicrobial	NRAMP1 FcRγI C2,C4 CR3	Confers resistance to intracellular pathogens Binds extracellular pathogens in adaptive immunity Part of the complement system Part of the complement system
Immuomodulation; Th development; Leukocyte trafficking	IL-12 CXCL9,10 CCL2,3,4,5/MCP1 ICAM-1 VCAM-1 B7.2	NK cell activator and role in Th1 phenotype Chemoattractants (T-cells and monocytes) Chemoattractants (T-cells and monocytes) Adhesion molecule Adhesion molecule APC surface molecule involved in T cell activation

1.3 Objectives

Aiming to understand the role of LRRK2 in PD pathogenesis, early research has been focused on neuronal cell autonomous mechanisms with unfortunately little success. More recently, accumulating evidence suggests that LRRK2 plays an important role in the immune system, which might contribute to the cause of PD. One important finding was that LRRK2 expression, already relatively high in immune cells such as monocytes and macrophages, can be further increased upon stimulation with IFN-γ (Gardet et al., 2010). In this work, initial experiments were performed in order to

1. **Verify if LRRK2 is induced by IFN-γ in macrophages.**

Co-treatments with the LRRK2 kinase inhibitor IN-1 aimed to

2. **Identify LRRK2 kinase functions in macrophages.**

Yet, they led to the surprisingly abolishment of this induction. While the hypothesis of an auto-regulation of LRRK2 was early disproved, subsequent investigations were carried out in order to

3. **Investigate the mediation of IFN-γ signaling in LRRK2 up-regulation in macrophages.**

Finally, in order to gain more insides in the role of LRRK2 biology, a transcriptome analysis of macrophages treated with IFN-γ with and without pharmacological inhibitors was carried out aiming to

4. **Identify genes that might play a role in LRRK2 induction and/or macrophage biology.**

2 Material and Methods

2.1 Materials

2.1.1 Chemicals

1,4-Dithiothreitol (DTT)	Carl Roth GmbH
2-propanol	Merck KGaA
40% Acrylamide/Bis soulution 19:1	Bio-Rad laboratories GmbH
Agarose	Biozym Scientific
Ammonium persulfate (APS)	Sigma Aldrich Chemie GmbH
Accutase	Invitrogen GmbH
β-Mercaptoethanol	Carl Roth GmbH
Bicinchoninic acid (BCA) Protein Assay Kit	Pierce Protein
BIX02189	Selleckchem
Bovine Serum Albumin (BSA)	Carl Roth GmbH
Bradford Protein Assay Kit	Pierce Protein
Bromphenol blue (sodium salt)	Merck KGaA
Calcium chloride	Merck KGaA
CD14 microbeads	Miltenyi Biotec
Cyclosporin A (CsA)	Sigma Aldrich GmbH
CZC-25146	Calbiochem
Complete protease inhibitor	Roche Applied Science
Dimetyl sulfoxide (DMSO)	Sigma Aldrich Chemie GmbH
Dulbecco's minimal essential medium (DMEM)	Biochrom AG
D-MEM/F-12 medium	Thermo Fisher Scientific
DNA 1kb ladder	Fermentas GmbH
DNA loading buffer (6x)	Fermentas GmbH
dNTPs	Fermentas GmbH
EGF (human)	Peprotech
Ethanol	Merck KGaA
Ethidium bromide (1% in water)	Merck KGaA
Ethylene glycol tetraacetic acid (EGTA)	AppliChem GmbH
Ethylenediaminetetraacetic acid	Merck KGaA
Fetal bovine serum (FCS)	PAA Laboratories GmbH
Ficoll	Biochrom AG
FuGENE 6	Roche Applied Science
Gammunex (10%)	Grifols
Granulocyte macrophage colony-stimulating factor (GM-CSF)	ImmunoTools
Glucose	Carl Roth GmbH
Glycerol	AppliChem GmbH
Glycine	Carl Roth GmbH
GoTaq Polymerase Kit	Promega
H-1152	Selleckchem

Hepes	Sigma Aldrich Chemie GmbH
High Fidelity cDNA Synthesis kit	Roche Applied Science
Hoechst 33342	Invitrogen GmbH
Holo-transferrin (human)	Sigma Aldrich Chemie GmbH
Hydrochloric acid (HCl)	Merck KGaA
Hydrogen peroxide (30%)	Carl Roth GmbH
Interferon alpha (IFN-α)	PBL Interferon Source
Interferon beta (IFN-β)	Peprotech
Interferon gamma (IFN-γ)	Peprotech
Interleukin 4 (IL-4)	ImmunoTools
Insulin (human)	Sigma Aldrich
Immobilon Western HRP Substrate	Milipore GmbH
Iscove's Modified Dulbecco's Medium (IMDM)	Thermo Fisher Scientific
JAK IN-1	Calbiochem
K-252a	Calbiochem
Lipopolysaccharide (LPS) from Escherichia coli 055:B5	Sigma Adrich Chemie GmbH
LRRK2 IN-1	Tocris/R&D Systems
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH
Methanol	Merck KGaA
N,N,N',N'-Tetramethylethyldiamine (TEMED)	Merck KGaA
Non-fat milk powder	Edeka
Nonident P-40 (NP-40)	United States Biological
PeniceillinG/Streptomycin sulfate 100x	Biochrom AG
Phorbol-13-myristate-12-acetate (PMA)	Tocris/R&D Systems
Poly-D-lysine	Sigma Aldrich
Potassium Chloride (KCl)	Merck KGaA
Potassium hydroxide (KOH)	Carl Roth GmbH
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth GmbH
Protein A-agarose	Millipore GmbH
Protein G-agarose	Millipore GmbH
QIAprep RNA kit	Qiagen GmbH
OptiMEM	Invitrogen GmbH
RPMI	ATTC
Sodium azide (NaN_3)	Sigma Aldrich Chemie GmbH
Sodium chloride (NaCl)	Merck KGaA
Sodium dodecyl sulfate (SDS)	Sigma Aldrich
Sodium hydroxide (NaOH)	Merck KGaA
di-sodium hydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH
Sodium nitrite ($NaNO_2$)	Merck KGaA
Sodium sulfate (Na_2SO_4)	Carl Roth GmbH
Sodium thiosulfate ($Na_2S_2O_3$)	Carl Roth GmbH
Sorbitol	Carl Roth GmbH
Sucrose	Carl Roth GmbH
Sunitinib	Sigma-Aldrich
TAE-684	Selleckchem
Tumor necrosis factor alpha (TNFα)	ImmunoTools

Transcriptor High Fidelity cDNA Synthesis kit	Roche Applied Science
Tris base	Carl Roth GmbH
Trypsin-EDTA (10x)	Invitrogen GmbH
Tween 20	Merck KGaA
Western Blocking Reagent Solution	Roche Applied Science
XMD 8-92	Tocris/R&D Systems

2.1.2 Consumables

4-20% gradient SDS-PAGE gels	Expedeon Ltd
Cell culture consumables	BD Biosciences, Corning and Greiner Bio-One
Coverslips	Carl Roth GmbH
Disposable pipettes	Corning
Glass slides	Langenbrinck
Microtiter plates	Greiner Bio-One GmbH
Object glass	Langenbrinck
PCR reaction tubes	PeqLab
Pipette tips	Sarstett; Biozym Scientific
Polypropylene tubes	Greiner Bio-One GmbH
Polyvinylidene diflouride (PVDF) membrane	Millipore GmbH
Reaction tubes	Greiner Bio-One GmbH
Scalpels	Braun
Syringe filters (0.45 µm)	Thermo Fisher Scientific
Whatman paper	Schleicher und Schuell

2.1.3 Buffer recipes

APS:	10% (w/v) ammonium persulfate in ddH_2O
Antibody solution:	5% Roche-Block solution, 0.02% sodium azide in TBST
Blocking solution:	5% (w/v) skimmed milk powder in TBST or as antibody solution
BSA solution:	5% (w/v) bovine serum in TBST
FACS buffer:	1x PBS, 2% FCS, 5mM EDTA
Laemmli buffer (6x):	12% SDS, 60% Glycerol, 375mM Tris, 12% β-mercaptoethanol, 0.01% bromphenol blue, pH 6.8
PBS:	150mM NaCl, 2.2mM KH_2PO_4, 7.8mM Na_2HPO_4, pH 7.4
Resolving gel buffer:	6-15% (w/v) acrylamide 19:1, 0.375 M Tris-HCl, 0.1% (w/v) SDS, 0.01 (w/v) APS, 0.001% (v/v) TEMED, pH 8.8
RIPA buffer:	150mM NaCl, 50mM Tris, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, pH=8
Running buffer:	25mM Tris, 192mM glycine, 3.5mM SDS, pH 8.3
Stacking gel buffer:	4.6% (w/v) acrylamide 19:1, 0.13M Tris-HCl, 0.1% (w/v) SDS, 0.01 (w/v) APS, 0.001% (v/v) TEMED, pH 6.8
Stripping buffer	2% (w/v) SDS, 100mM β-mercaptoethanol, 62.5mM Tris-HCl (pH 7.6) in ddH_2O
TAE buffer	40mM Tris-HCl, 20mM acetic acid, and 1mM EDTA

TBS buffer:	50mM Tris-HCl, 150mM NaCl
TBST:	50mM Tris-HCl, 150mM NaCl, 0,1% (v/v) Tween-20
Transfer buffer:	25mM Tris, 192mM glycine, 20% (v/v) methanol

2.1.4 Devices

Agarose gel chamber	Peqlab
Axiolmager microscope with ApoTome Imaging system	Carl Zeiss Microimaging GmbH
Autoclave (VX-150)	Systec
Blotting Chamber	Bio-Rad laboratories
Cell incubator (Heracell 240)	Thermo Fisher Hereaus
Centrifuges:	
(Biofuge pico and fresco)	Thermo Fisher Hereaus
(Centrifuge 5810R)	Eppendorf
(Evolution Rc)	Thermo Fisher Sorvall
(Micro 22R)	Hettich
(Multifuge3 S-R)	Thermo Fisher Hereaus
Developer	Fujifilm
Film cassettes	Dr Groos-Suprema
Flow Cytometer CyAn ADP	Beckman Coulter
96well plate reader	Bio-Rad laboratories GmbH
Milli-Q Synthesis	Millipore Corporation
Nanodrop (ND1000)	Peqlab
Pipetboy	Integra Bioscience
Power supply (EV231)	Consort
Scanner (Epson Perfection V700 Photo)	Epson Corporation
SDS-PAGE gel chamber (Perfect Blue Twin S)	Peqlab
Stereo microscope MZ7	Leica
Sterile bench (Herasafe)	Thermo Fisher Heraeus
Stirer (RH basic)	IKA
Thermocycler (2720)	Applied Biosystems
Thermomixer (Comfort)	Eppendorf
UV-table	Vilber Lourmant

2.1.5 Antibodies

Primary antibodies are listed in Tab. 2.1. Secondary peroxidase conjugated antibodies for immunoblotting were purchased from Jackson ImmunoResearch Laboratories, secondary Alexa-Flour conjugated antibodies for immunocytochemistry were bought from Invitrogen GmbH.

Tab. 2.1: Antibody list

Antibody	Dilution	Producer	Application
CD11b (PB), clone ICRF44	1:40	Biolegend	FACS
CD11c (APC), clone MJ4-27G12.4.6	1:10	Milteny	FACS
CD14 (FITC), clone M5E2	1:20	BD Pharmingen	FACS
HLA-A,B,C (PE), clone W6/32	1:30	Biolegend	FACS
HLA-DR (PB) , clone L243	1:60	Biolegend,	FACS
ERK5	1:2000	Abcam (Ab 40809)	Immunoblot
LRRK2	1:5000	Abcam (Ab133518) UDD3	Immunoblot
LRRK2 pS910	1:2000	Epitomics; # 5098-1 UDD1	Immunoblot
LRRK2 pS935	1:2000	Epitomics; # 5099-1 UDD2	Immunoblot
STAT1	1:10.000	Cell Signaling; #9172	Immunoblot
STAT1 pS701	1:10.000	Cell Signaling; #9167	Immunoblot
STAT1 pS727	1:10.000	Cell Signaling; #9177	Immunoblot
Vinculin	1:500.000	Sigma-Aldrich; # V 9131	Immunoblot

2.1.6 Primers

Primers (Tab. 2.2) were purchased from Sigma.

Tab. 2.2: Primer list

Primer	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
β-actin	AAGATCAAGATCATTGCTCCTCC	GTCATAGTCCGCCTAGAAGCA
C1QA	ATATCGCTGGCCTCTATGGT	CCCTTGGTGCCTTTAATTCCC
C1QB	GGCCTAATCGATATCTCCCA	TTGTAGTCTCCCGATTCACC
C1QC	CTGAGGACATCTCTGTGCCA	GTTCTCCCTTCTGCCCTTTG
HLA-DR	TTATTCTTGTCTGTTCTGCCTC	CATGTTCTTCTTTGATAGCCCA
LRRK2	AAACTCTGTGGACTAATAGACTGCGT	TTTAAGGCTTCCTAGCTGTGCTGTC
MARCH1	CTGTCACATTCCACGTAATCGCG	ACCTCCTGTGAAGCCAATGGCT

2.2 Cell culture

2.2.1 General cell culture techniques

Cell line stocks were kept in gas-phase liquid nitrogen. In order to freeze cells, cell pellets were resuspended in freezing medium (50% RPMI, 40% FCS, 10% DMSO) and directly placed in the -80 freezer in a "Mr. Frosty" (Thermo Scientific) freezing container to allow optimal cooling rates. The next day, cells were put in gas-phase liquid nitrogen for long time storage. For THP-1 cells, pellets of 1-5 10^6 cells per vial were used in order to allow quick recovery of cells after re-thawing. For thawing, vials were resuspended in 10 ml of pre-warmed (37°C) medium, followed by a centrifugation step in order to reduce DMSO contamination, as it has been described to allow differentiation of THP-1 cells. Cell viability was checked regularly before splitting. Early passage cells were proliferating in spherical clusters, which was less prominent with extended passage number.

2.2.1 THP-1

The human leukemia cell line THP-1 was maintained roughly at 5x10^5 cells/ml in Roswell Park Memorial Institute (RPMI, ATCC) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. Cells were split twice a week. To induce differentiation into a more macrophage-like state, cells were seeded in six well plates (1.5x10^5 cells/well) and treated with 10-100 ng/ml phorbol-12-myristate-13-acetate (PMA, Tocris) for 24-48 hours. FCS was heat inactivated for 30 minutes at 56°C.

2.2.2 C13-NJ

The microglia cell line C13-NJ was maintained in Dulbecco's Modified Eagle Medium (DMEM; ATCC) supplemented with 10% fetal calf serum FCS at 37°C and 5% CO_2. Cells were split twice a week using trypsin (5 minutes at 37°C) to avoid over-confluent growing.

2.2.3 HeLa

The breast cancer cell line HeLa was maintained in DMEM (ATCC) supplemented with 10% fetal calf serum FCS at 37°C and 5% CO_2. Cells were split twice a week using trypsin for 5 minutes at 37°C to avoid over-confluent growing.

2.2.4 NIH3T3-tCD40L

The mouse NIH3T3 fibroblast cell line stably transfected with CD40L (t-CD40L) was kept in D-MEM/F-12 medium supplemented with 10% FCS, 200µM L-glutamine and 100 U/ml Penicillin/Streptomycin.

2.2.5 Monocyte-derived macrophages (MDMs)

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque density centrifugation from either venus blood or buffy-coat products donated from healthy individuals. Blood donations were obtained from German Red Cross, Tübingen. Collection and subsequent processing of the blood products was approved by the ethics commission of the medical faculty of the University of Tübingen. In brief, blood was diluted 1:1 with PBS and 35 ml of the diluted cell suspension was layered on 15 ml of Ficoll-Paque in a conical 50 ml Falcon tube. After 30 minutes centrifugation at 400 g at 20°C in a swinging-bucket rotor (no brake!) the interphase containing the mononuclear cell layer (lymphocytes, monocytes and thrombocytes) was carefully collected. The cells were washed with PBS/EDTA following centrifugation at 300 g for 10 minutes at 20°C. Optionally, cells were additionally washed with PBS following centrifugation at 200 g for 10 minutes to reduce platelet contamination. Erythrocyte contamination was removed by RBC lysis. The cells were then washed in PBS and resuspended in RPMI (ATCC) containing 10% pooled heat inactivated normal human serum and 1% Penicillin/Streptomycin (100 I.U./ml) and seeded into six well plates at the concentration of 10-15x10^6 cells/well. Non-adherent cells were removed by gentle but intensive washing with PBS after 2 hours of incubation at 37°C. Adherent cells were allowed to further differentiate for 7 days into MDMs in the presence of 100 U/ml GM-CSF, which was added at day 1, 3 and 5 of differentiation.

Cyro-preserved PBMCs from PD patients as well as age matched controls were obtained from the Neuro-Biobank Tübingen. After thawing, cells were counted and cell viability was determined using the trypan blue exclusion method. When needed, cells were further washed to reduce platelet contamination, and then cells were processed as described above.

2.2.6 Generation of CD40L-activated B cells (CD40B cells)

CD40B cells were generated as previously described (Adamopoulou et al., 2007). In brief, for generating the CD40B cells, fresh PBMCs (8x10^6 cells/well) were cultured on γ-irradiated

(96 Gy) CD40L-transfected NIH3T3 cells in the presence of IL-4 (4 ng/ml) and Cyclosporin A (CsA; 0.7 µg/ml) in 4 ml of B cell medium consisting of IMDM supplemented with 10% pooled human serum, 50 µg/ml himan holo transferrin, 5 µg/ml human insulin and 100 U/ml Penicillin/Streptomycin. The expanding cells were transferred onto freshly prepared NIH3T3t-CD40L cells and fed with cytokine-replenished medium every 3-4 days. CD40B cells were expanded in the culture conditions described above for 14 days after initial activation and cryo-preserved until used.

2.2.7 Generation of monocyte-derived dendritic cells (DCs)

Monocytes were isolated from fresh PBMCs using human CD14 microbeads. CD14+ monocytes were cultured in PRMI 1640 containing 10% FCS supplemented with GM-CSF (100 ng/ml) and rhIL-4 (40 ng/ml) for 5 days. DC maturation was induced by the addition of 5 µg/ml LPS for 24 hours. Cells were harvested and either used directly or cryo-preserved until used.

2.2.8 Isolation of T cells

Human CD3+ and CD4+ T cells were isolated from fresh whole PBMCs by magnetic cell separation using antibody-conjugated microbeads. CD3+ and CD4+ T cells were negatively enriched using the Pan T cell isolation kit and the CD4+ T cell isolation kit II, respectively according to the manufacturer's instructions (both MACS cell sorting systems, Miltenyi Biotec).

2.3 Methods

2.3.1 RNA isolation

RNA was isolated using the RNeasy Mini kit according to manufacturer's instructions (Qiagen). Briefly, cells were washed with PBS and then lysed with RLT (+β-ME) buffer (350 µl/well) directly in the 6 well plate on ice. For complete cell lysis, cells were deep frozen (-80°C) for at least 1 hour. Lysate was then thawed on ice and 1 volume of 70% Ethanol was added. Next, the ethanol-RLT mixture was loaded on QIAgen spin columns and total RNA was bound to the silica membrane. RNA was purified by subsequently washes with RW1 and RPE. Finally, RNA was eluted with 30-50 µl of RNAase free water. RNA quality and yield was checked by NanoDrop analysis.

2.3.2 cDNA synthesis

For cDNA synthesis, total RNA (300 – 1000 ng per reaction) was reverse transcribed with anchored oligo-dT primers using the Transcriptor High Fidelity cDNA Synthesis kit (Roche):

Component	Volume [µl]	Final concentration
Total RNA	Variable	300-1000 ng
Anchored-oligo(dT) Primer	1	2.5 µM
Water	Add to 11.4	

Template-primer mixture was allowed to denature at 65°C for 10 minutes and transferred to ice immediately. Next, the rest of the components of the reverse transcription (RT) mix were added (listed below).

Component	Volume [µl]	Final concentration
Reaction buffer (5x)	4	1x (8 mM MgCl2)
RNase Inhibitor	0.5	20 U
Deoxynucleotide Mix	2	1 mM each
Reverse Transcriptase	1.1	10 U
Final volume	20	

Next, the RT mix was allowed to incubate at 55°C for 30 minutes for reverse transcription. In order to terminate the reaction, reverse transcriptase was inactivated by heating to 85°C for 5 minutes. The cDNA was then diluted by adding 200 µl RNase free water and subjected for PCR.

2.3.3 RT-PCR

cDNA was used as template for transcript amplification in a standard PCR reaction, according to manufacturer's instructions (Promega):

1 5µl	cDNA template
5 µl	5xGoTaq buffer
0.125 µl	GoTaq polymerase
1 µM	primers (forward and reverse)
0.2 mM	dNTPs

After RT-PCR, amplified DNA fragments were separated in 1.5% (w/v) agarose gels containing ethidium bromide. DNA bands were detected with a Vilber Lourmat.

2.3.4 SDS-PAGE

Naïve suspension THP-1 cells were collected in reaction tubes, centrifuged (300 g for 5 minutes at 4°C) and washed with cold PBS before subjected to cell lysis. Adherent cells were briefly washed with PBS and then directly lysed in the six well plate on ice using 100 µl/well RIPA lysis buffer containing 1x complete protease inhibitor (Roche) and optionally complete phosphatase inhibitor (Roche) when lysates were used for the detection of protein phosphorylations. After 15 minutes of lysis on ice and occasional shaking, cell debris was removed by centrifugation (15 minutes at 14,000 g and 4°C). Protein concentration of lysates was determined using the BCA protein assay kit (Thermo Scientific). Protein samples were then boiled in Laemmli buffer at 95°C for 5 minutes and either stored at -20°C or directly subjected to SDS-PAGE. For SDS-PAGE, generally 15 µg of denatured proteins were loaded and separated on self-made gels consisting of 6-15% polyacrylamide. Gels were run with initial 30 minutes at 110 V followed by 140 V until the loading dye front has run out of the gel.

2.3.5 Immunoblotting

Next, size separated proteins were transferred onto methanol activated polyvinylidene difluoride (PVDF) membranes using 100 V constant for 2 hours or at 20 V overnight. Optionally, successful transfer of proteins was checked using reversible Ponceau S staining. PVDF membranes were pre-incubated 2-16 hours at 4°C in 5% skim-milk/TBST, 5%BSA/TBST or 5% Western Blocking Reagent/TBST and then incubated with primary antibody in Western Blocking Reagent at 4°C overnight. After intensive washing (at least 3 times 10 minutes), incubation with secondary HRP-conjugated antibodies was performed for 2 hours at room temperature followed by a second round of intensive washing. All incubation and washing steps were carried out on a rocking table. Detection of proteins was conducted with the Immobilon Western chemiluminescent HRP substrate on Amersham Hyperfilm ECL. The films were scanned, optionally quantified by densitometry using ImageJ software. For reprobing, membranes were incubated for 30 minutes at 56°C in stripping buffer and washed in TBST for at least 3 times 10 minutes. After this membranes were ready for blocking and subsequent incubation with a primary antibody.

2.3.6 Immunocytochemistry

THP-1 cells were seeded on poly-D-lysine (Sigma) coated coverslips and treated with PMA for 48 hours to allow cell differentiation and then treated as indicated. Then, cells were fixed with ice-cold Methanol for 10 minutes at -20°C, and blocked 2 hours with 10% normal goat serum (NGS). Primary antibody incubation was performed in 1% BSA in PBS overnight at 4°C. After washing, cells were then incubated with secondary Alexa-Flour conjugated antibodies in the dark for 1 hour at room temperature. Nuclei counterstaining was applied using Hoechst 33342 (2 µg / ml in PBS) for 10 minutes at room temperature. Cover slips were mounted in fluorescent mounting medium (Dako) onto microscope slides. Cells were analyzed with ApoTome Imaging system and processed with AxioVision software (Zeiss).

2.3.7 FACS analysis

Phenotypic characterization of naïve (untreated) or PMA-differentiated THP-1 cells was determined by flow cytometry. Naïve THP-1 cells in suspension were harvested from culture flasks and washed with FACS buffer. PMA-differentiated adherent THP-1 cells were detached from culture flasks using treatment with Accutase for 5 minutes at 37°C and gentle rocking.

Cells were harvested and washed in FACS buffer. FcR blocking step was performed using human pooled IgG (10% Gamunex) for 15 minutes at RT. After washing with FACS buffer, cells ($1x10^6$) were stained with the following mouse anti-human fluorochrome conjugated monoclonal antibodies: APC-CD11c (Miltenyi Biotec), FITC-CD14 (Becton-Dickinson), PB-CD11b, PE-HLA-A,B,C and PB-HLA-DR (all from Biolegend) for 25 minutes on ice. Cells were washed twice with FACS buffer to remove unbound antibody and then analyzed on a Cyan ADP flow cytometer. For each sample, 50 000 events were collected and analyzed using Summit 4.3 software (Beckman Coulter).

2.3.8 Lenitviral mediated gene knockdown

For RNAi gene knockdown experiments, MISSION shRNA Lentiviral Transduction Particles (Sigma-Aldrich) were purchased and used according to manufacturer's instructions. A set of 4 MISSION shRNA Lentiviral Transduction Particles with different sequences targeting ERK5 were added to the cells in two different multiplicity of infection (MOI) ratios: 0.5 and 1.5. To enhance transfection efficiency spin infection (45 minutes at 500 g at 35°C) in the presence of 8 µg/ml polybrene was applied. Cells were then seeded in 6 well plates to recover. 48 hours after transduction, 1 µg/ml Puromycin was added and stable transduced cells were allowed to proliferate to generate pooled stable knockdowns. Knockdown efficiency was then assessed by Immunoblot analysis of early passage pooled knockdowns.

2.3.9 Trancriptome analysis

For transcriptome analysis, THP-1 cells were seeded in quintuples in six well plates and treated as indicated. One well from the same plate was lysed and protein lysates subjected for Immunoblotting (as described above) control in order to confirm the inhibitor effects on IFN-γ treatments. The remaining quadruples of each condition were used to isolate RNA (as described above) and quality was checked. From that point, quadruples of samples were handed over to the IZKF Microarray Facility Tübingen in order to conduct an Affymetrix Whole Genome mRNA Expression Profiling. Prior to microarray analysis, quality of samples was double checked using the Nanodrop ND-1000 and Agilent Bioanalyzer 2100. Raw data in form of Affymetrix CEL files and tables of normalized and summarized signal intensities as well as analyzed data was then provided by the Microarray Facility (http://www.microarray-facility.com).

3 Results

3.1 LRRK2 is expressed in the immune system

In order to understand the biology of a protein of interest and ultimately its role in disease, it is crucial to investigate its expression pattern. LRRK2 is expressed throughout the body including the in PD pathology affected brain areas. Interestingly, much higher concentrations of LRRK2 have been found within the immune system, including dendritic cells, monocytes, macrophages, microglia and B-cells (Gardet et al., 2010; Hakimi et al., 2011; Maekawa et al., 2010; Miklossy et al., 2006; Thevenet et al., 2011). Therefore, a closer investigation of LRRK2 expression in immune cell biology can yield valuable insights into PD pathogenesis.

Here, LRRK2 expression levels were analyzed in a panel of different primary cells and cell lines. Immunoblot analysis from different primary human cells confirmed high expression levels of LRRK2 in B-cells and dendritic cells and to a much lesser content also in CD3+ cells. Interestingly, CD4+ T-cells were almost devoid of LRRK2 expression by comparison to B-cells and dendritic cells (Fig. 3.1 A). Previous studies suggested that in microglia, monocytes and macrophages induction of LRRK2 expression is induced by pro-inflammatory stimuli, such as IFN-γ and LPS (Thevenet et al., 2011). Indeed, stimulation with IFN-γ for 24 hours led to an increase in LRRK2 protein levels from basal levels when primary monocytes were stimulated immediately after isolation (Fig. 3.1 B). Monocyte-derived macrophages (MDMs) differentiated by treatment with granulocyte macrophage colony-stimulating factor (GM-CSF) already express moderate LRRK2 levels in the untreated condition, while IFN-γ stimulation strongly induces LRRK2 levels (Fig. 3.1 B). This might indicate that the differentiation state of the cells is influencing expression strength of LRRK2 upon IFN-γ stimulation. Next, the microglia cell line C13-NJ was analyzed for LRRK2 expression. Surprisingly, neither in the untreated condition nor when being stimulated with LPS or IFN-γ LRRK2 expression was detected (Fig. 3.1 C). Expression of LRRK2 in the widely used cervical cancer line HeLa was also not detectable (Fig. 3.1 D), despite the application of various treatments.

Taken together, this data strengthens the reports of LRRK2 being highly expressed within the immune cells (with the exception of the C13-NJ microglia cell line) and robustly inducible upon IFN-γ treatment.

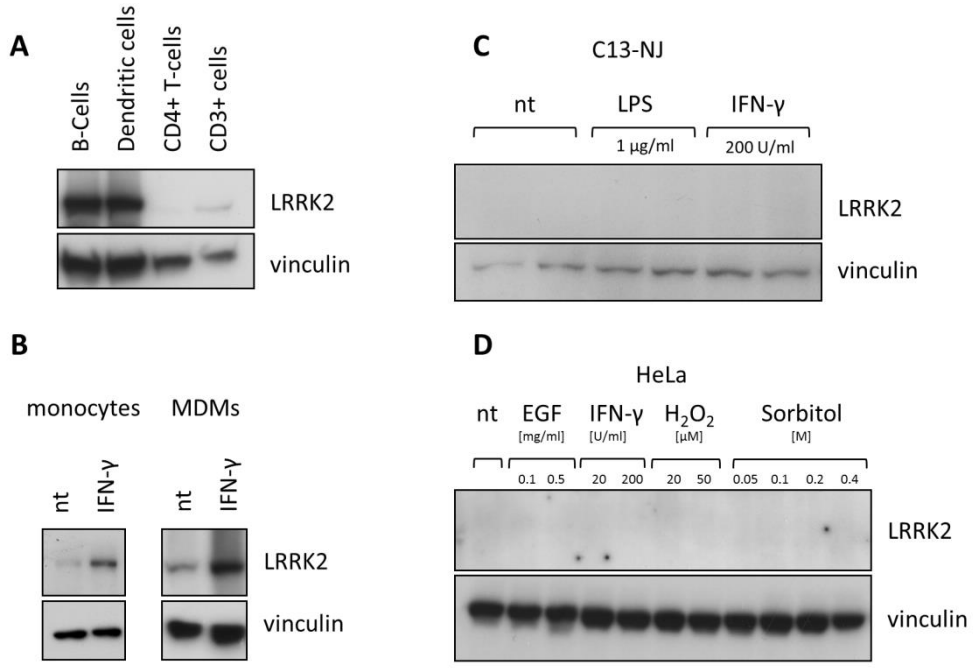

Fig. 3.1: LRRK2 is expressed in the immune system

A: CD3+, CD4+ T cells and dendritic cells (DCs) were isolated from PBMCs by MACS cell separation and DCs were further differentiated *in vitro*. CD40L-activated B cells were generated by co-culture with irradiated CD40L transfected NIH3T3 cells. Cells were lysed and proteins were subjected to immunoblot analysis of LRRK2 levels. While B-cells and dendritic cells display high levels of LRRK2, CD3+ cells and CD4+ T-cells are almost devoid of LRRK2 **B**: Human primary monocytes were isolated from PBMCs by adherence followed by a direct stimulation with IFN-γ (200 U/ml for 24 hours) or differentiated into monocyte-derived macrophages (MDMs) for 7 days in complete medium containing human serum and GM-CSF (100 U/ml) and then treated with IFN-γ (200 U/ml for 24 hours). Untreated controls were included in both cases. Cell lysates were subjected to immunoblot for LRRK2. Both undifferentiated monocytes as well as MDMs displayed increased LRRK2 expression upon IFN-γ stimulation. **C**: Microglia C13-NJ cells were treated with LPS, IFN-γ as indicated for 24 hours or left untreated, cell lysates probed for LRRK2 by immunoblot. C13-NJ did not express detectable levels of LRRK2 **D**: HeLa cells were treated 24 hours as indicated with different concentrations of EGF, IFN-γ, H_2O_2 or Sorbitol and cell lysates were subjected for immunoblot. Yet, LRRK2 could not be detected.

3.2 IFN-γ stimulation of macrophage-like cells induces LRRK2

The monocytic cell line THP-1 is a widely used model to study monocyte and macrophage biology (Daigneault et al., 2010; Tsuchiya et al., 1980). PMA treatment is the most common among a panel of other treatments that can be utilized to promote monocyte differentiation towards a more macrophage-like cell status.

Upon PMA (10 ng/ml for 48 hours) stimulation, the formerly naïve suspension cells became adherent, stopped dividing and were metabolizing slower (Fig. 3.2 A I+II). Subsequent stimulation with 200 U/ml IFN-γ led to a morphological change, the roundish macrophages acquired an elongated “spindle-like” appearance (Fig. 3.2 A III). Next, FACS analysis of CD14, CD11b, CD11c and MHC1 surface markers was carried out in order to confirm that the observed morphological changes were indeed accompanied by a differentiation from naïve monocyte to macrophage characteristics (Fig. 3.2 B). As expected, the morphological changes were associated with up-regulation of surface expression of the aforementioned markers for monocyte to macrophage differentiation. Moreover, RT-PCR and immunoblot analysis revealed, that exposure to IFN-γ strongly induced LRRK2 mRNA (Fig. 3.2 C) and protein (Fig. 3.2 D) expression, as expected, when compared to the much lower basal levels of LRRK2 mRNA and protein, as it has also previously shown (Thevenet et al., 2011).

In conclusion, PMA differentiated THP-1 cells undergo morphological changes, up-regulate specific macrophage markers and induce LRRK2 expression upon IFN-γ stimulation. LRRK2 expression levels in naïve and differentiated THP-1 cells as well as their inducibility by IFN-γ resembles the situation in MDMs, thus making them a well suited model to study LRRK2 biology in macrophages.

3.3 LRRK2 kinase inhibitor IN1 blocks induction of LRRK2

As described above LRRK2 has been shown to play a role in monocyte/macrophage biology and is robustly induced by IFN-γ (Fig. 3.2 C and D). However, still very little is known about LRRK2 kinase function in this context. In order to study the effects of LRRK2 in macrophages, the initial aim was to pharmacologically block LRRK2 kinase function in IFN-γ treated THP-1 cells by administration of the inhibitor LRRK2-IN-1 (Deng et al., 2011a). Strikingly, when IN-1 and IFN-γ were co-administered, not only LRRK2 kinase function was inhibited as assed with immunoblot probing for LRRK2 auto-phosphorylation at S935. More importantly, total LRRK2 protein induction was reduced on both mRNA and protein level (Fig. 3.2 C and D). Yet, the inhibitory effect on LRRK2 protein induction appeared to be stronger than on the mRNA level. Thus, LRRK2 expression might be both regulated at the transcriptional and post-transcriptional level.

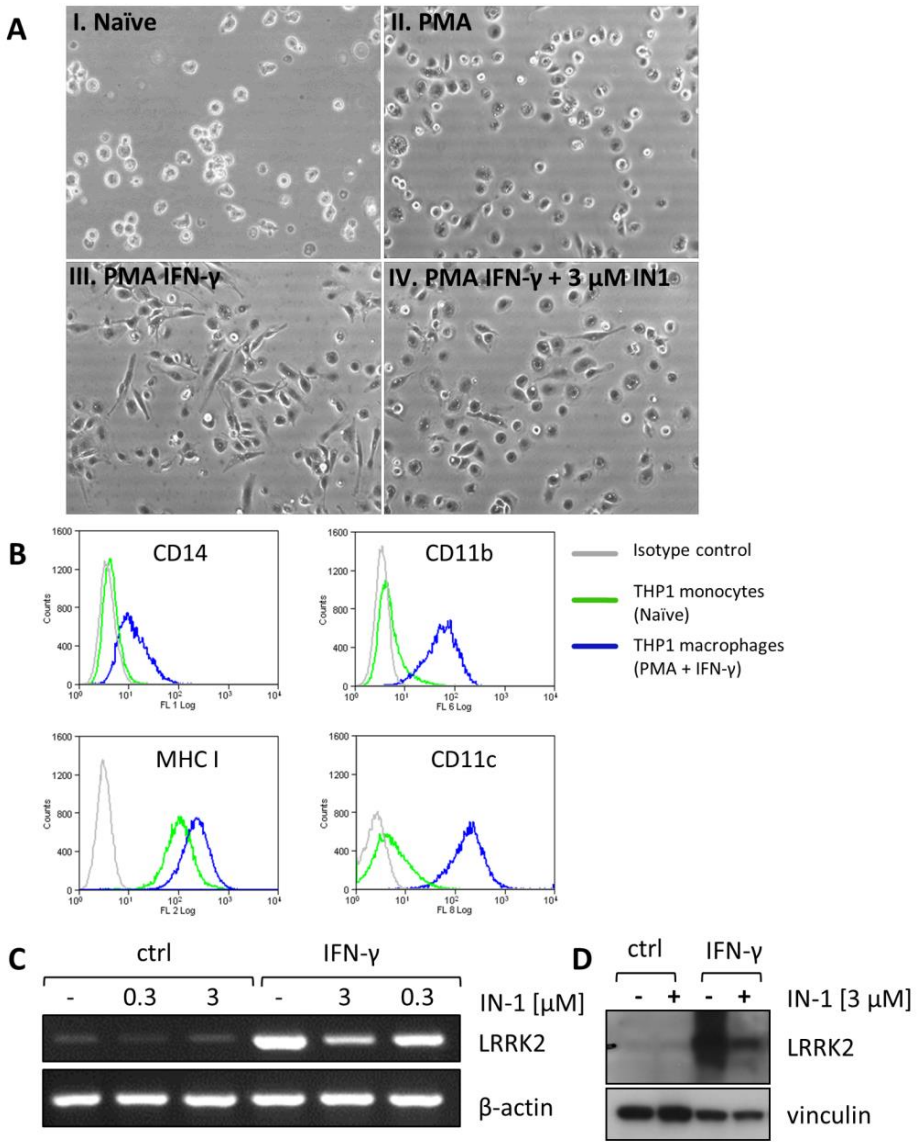

Fig. 3.2: IFN-γ stimulation of PMA-treated THP-1 cells induces LRRK2

A: Naïve THP-1 (I) cells were differentiated with 10 ng/ml PMA for 48 hours and subsequently (II) treated with 200 U/ml IFN-γ for 24 hours (III) in combination with concurrent treatment of 3 µM LRRK2 kinase inhibitor IN-1 (IV). Living cells are depicted as bright field 10x micrographs. Upon PMA differentiation suspension cells became adherent. Subsequent IFN-γ treatments induced an elongated morphology, which was partly repressed by IN-1 treatment **B**: Naïve THP-1 monocytes and THP-1 macrophages (PMA differentiation with 10 ng/ml for 48 hours followed by stimulation with 200 U/ml IFN-γ for 24 hours) were subjected to FACS analysis of CD14, CD11b, CD11c and MHC1 surface expression. PMA + IFN-γ treated THP-1 cells displayed an up-regulation of surface macrophage differentiation markers when compared to naïve THP-1 cells. **C, D**: THP-1 cells were differentiated with 10 ng/ml PMA for 48 hours and then treated with 200 U/ml IFN-γ or not (-) for 24 hours in combination with indicated concentrations of LRRK2 kinase inhibitor IN-1. RNA was extracted and subjected to RT-PCR to investigate LRRK2 mRNA levels (**C**), β-actin served as internal control. Additionally, THP-1 cells were subjected to Western blot analysis (**D**) of LRRK2 levels. IFN-γ stimulation led to an increase in LRRK2 levels, which was inhibited by co-treatments with the LRRK2 kinase inhibitor IN-1 on both mRNA and protein level.

As the initial goal was to study LRRK2 kinase function in macrophages, at that point, a mechanism was hypothesized in which kinase function is involved in its own transcriptional regulation. Therefore, experiments were performed to investigate the inhibitory effect of IN-1 on LRRK2 induction upon IFN-γ stimulation in more detail.

LRRK2 induction was found to be inhibited in a dose-dependent manner: 0.3µM IN-1 had almost no effect after 24 hours, but a visible reduction of LRRK2 up-regulation was detected after 48 hours. At 3µM, IN-1 had clear inhibitory effects on LRRK2 induction already after 24 hours (Fig 3.3 A). In fact, at the high dose of 3µM IN-1, reduction of IFN-γ stimulated LRRK2 protein was already emerging (Fig 3.3 A and B). Although IN-1 was considered as a selective LRRK2 inhibitor, all pharmacological approaches suffer from cross-reactivity resulting in unspecific off target effects. For example, a general shut-down of the JAK-STAT1 pathway, which is the classical signal cascade in IFN-γ signaling, would cause inhibition of all classically IFN-γ induced genes. In order to address this issue, proximal IFN-γ signaling events were checked by measuring the phosphorylation state of STAT1 at Y701. As expected, IFN-γ stimulation lead to a strong phosphorylation at STAT1 Y701, however this was not affected by IN-1 treatment (Fig. 3.3 C). Thus, IN-1 did not interfere with proximal IFN-γ signaling itself. This suggests that rather a downstream step in the signal transduction pathway is affected by IN-1. According to this rationale, application of the selective JAK inhibitor JAK IN-1, which is blocking the immediate effector of IFN-γ signaling, blocked STAT1 Y701 phosphorylation and also LRRK2 up-regulation in a dose-dependent manner (Fig. 3.3 C). Combining LRRK2 IN-1 and JAK IN-1 practically abolished LRRK2 induction. IFN-β, which signals through a different receptor as IFN-γ but shares the STAT1 module, did not consistently up-regulate LRRK2 under these conditions and only caused mildly elevated STAT1 Y701 phosphorylation (Fig. 3.3 C).

In conclusion, LRRK2 expression seems to be under control of a specific IFN-γ signal transduction pathway in macrophage-like cells, which can be suppressed by IN-1.

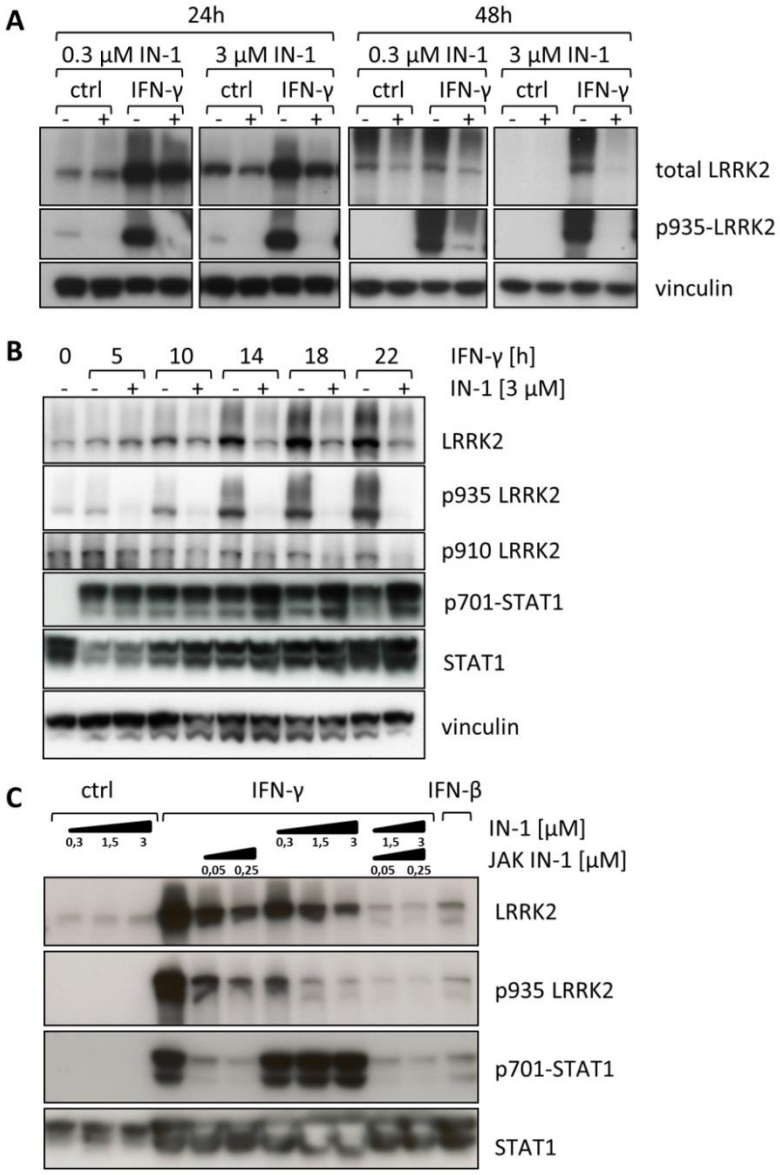

Fig. 3.3: LRRK2 kinase inhibitor IN-1 blocks induction of LRRK2 without interfering with proximal IFN-γ signaling

THP-1 cells were differentiated into macrophages with 10 ng/ml PMA for 48 hours and then treated with 200 U/ml IFN-γ or not (ctrl) in the presence of the indicated concentrations of IN-1. **A, B**: After the indicated time points, cells were lysed and proteins subjected to immunoblot analysis of (phospho-) LRRK2 and (phospho-) STAT1 as well as vinculin for loading control. LRRK2 levels were increased upon IFN-γ stimulation, which was reduced by IN-1 co-treatment. Also, autophosphorylation of LRRK2 was inhibited upon IN-1 treatments without affecting proximal IFN-γ signaling as observed by phospho STAT1. **C**: Differentiated THP-1 cells were stimulated or not (ctrl) with IFN-γ or IFN-β, together with increasing concentrations of LRRK2 IN-1 or JAK IN-1, as indicated, and cell lysates subjected to Western blot analysis as above. LRRK2 levels were increased upon IFN-γ stimulation, which was reduced by IN-1 co-treatment. Also, autophosphorylation of LRRK2 was inhibited upon IN-1 treatments without affecting proximal IFN-γ signaling as observed by phospho STAT1. As expected, JAK IN-1 inhibited LRRK2 up-regulation by interfering with proximal IFN-γ signaling as assessed by phosphor STAT1. Combined treatments of JAK IN-1 and IN-1 had a strong inhibitory effect.

3.4 Other LRRK2 inhibitors fail to suppress IFN-γ mediated induction of LRRK2

As expounded above, IN-1 inhibits IFN-γ mediated induction of LRRK2. This suggests that LRRK2 kinase function is regulating LRRK2 expression by an unknown mechanism. In order to verify this hypothesis, a panel of additional LRRK2 inhibitors was tested (Tab 3.1), including CZC-25146 (Ramsden et al., 2011), TAE684 (Zhang et al., 2012), H-1152 and Sunitinib (Nichols et al., 2009) as well as the staurosporine analog K-252A (Kase et al., 1987). THP-1 cells were differentiated with PMA (10 ng/ml for 48 hours) to macrophages and stimulated with 200 U/ml IFN-γ in combination with the indicated increasing concentration of the aforementioned LRRK2 inhibitors. Cells were harvested and subjected to immunoblot analysis for LRRK2 expression. Surprisingly, all of these inhibitors failed to show the same LRRK2 suppressive effect as IN-1, although LRRK2 kinase activity inhibition was effective for all inhibitors used as detected with immunoblot probing for LRRK2 auto-phosphorylation at S935 (Fig. 3.4). Notably, similar to IN-1, practically none of the additionally administered inhibitors interfered with the initial and proximal IFN-γ signaling, as confirmed by STAT1 Y701 phosphorylation. As shown above (Fig. 3.3 C), blocking of the proximal IFN-γ signaling is inhibiting LRRK2 induction as well. The only exception to this was a high dose treatment (1µM) of K-252a (Covy and Giasson, 2009), which resulted in a strong decrease in STAT1 Y701 phosphorylation. Therefore, the reduction of LRRK2 up-regulation by K-252a is likely caused by inhibition of proximal IFN-γ signaling.

In conclusion, this clearly demonstrates that LRRK2 kinase function and respectively its inhibition is not causatively connected to the observed inhibited up-regulation of LRRK2. Therefore, IN-1 must impact differently on IFN-γ mediated LRRK2 induction.

Tab. 3.1: Various LRRK2 kinase inhibitors

Several inhibitors are listed with their respective half maximal inhibitory concentration (IC_{50}) targeting LRRK2 kinase activity (Covy and Giasson, 2009; Zhang et al., 2012).

Inhibitor	**K-252A**	**Sunitinib**	**H-1152**	**TAE648**	**CZC-25146**	**LRRK2 IN-1**
IC_{50}: LRRK2	25nM	79nM	244nM	7.8nM	4.76nM	13nM
Derivate	Staurosporine	Indolinon	Quinoline	Pyrimidine	Pyrimidine	Pyrimidine

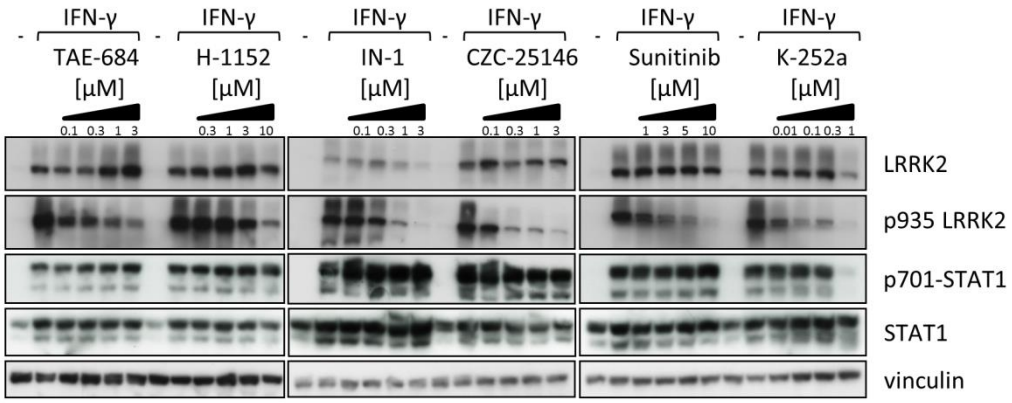

Fig. 3.4: Among a panel of LRRK2 inhibitors, only IN1 blocks LRRK2 induction by IFN-γ

THP-1 cells were differentiated with 10 ng/ml PMA for 48 hours and then treated with 200 U/ml IFN-γ or not (-) in the presence of increasing concentrations of inhibitors, as indicated. After 24h, cells were harvested and lysates were subjected to immunoblot analysis of (phospho-) LRRK2 and (phospho-) STAT1. Vinculin probing confirmed equal protein loading and general cell viability. Although all inhibitors at appropriate concentrations effectively inhibit LRRK2 kinase activity as confirmed by LRRK2 autophosphorylation at p935, only IN-1 treatment blocks total LRRK2 levels as well. Only K-252a blocks LRRK2 induction at the highest, probably by interfering with proximal IFN-γ signaling as indicated by reduced phosho STAT1.

3.5 LRRK2 up-regulation is reduced by pharmacological inhibition of ERK5

Because LRRK2 kinase inhibition could not be causative for the IN-1 inhibitory effects on LRRK2 up-regulation upon IFN-γ stimulation, another pathway had to be affected by IN-1 treatments. IN-1 is considered to be a highly specific LRRK2 kinase inhibitor in general. However, IN-1 has been reported to impact on at least 14 other kinases (Tab. 3.2) that in principle could all be involved in inhibiting the up-regulation of LRRK2 induction. Some targets could be directly excluded because of overlapping inhibitory profiles with other LRRK2 kinase inhibitors that did not reduced LRRK2 up-regulation upon IFN-γ stimulation. For example, polo-like kinase 4 (PLK4) and tyrosine kinase, non receptor, 1 (TNK1) are both inhibited by IN-1 and CZC-25146 and therefore could be excluded as a candidate mediator of LRRK2 induction. Other candidates could be omitted because their respective IC_{50} was relatively high and IN-1 effects had already been observed at relative low concentrations. This left just few targets with increased probability of being involved in interference of LRRK2 up-regulation, including both doublecortin-like kinase 1 and 2 (DCLK1 and DCLK2), ribosomal protein S6 kinase, 90kDa, polypeptide 6 (RPS6KA6) and extracellular-signal-

regulated kinase 5 (ERK5). The latter of these shortlisted candidates was chosen to be investigated first because of two reasons: First, IN-1 cross reactivity was best described for ERK5 (Deng et al., 2011a) and second, several ERK5 inhibitors were commercially available (Deng et al., 2011b; Tatake et al., 2008). Moreover, MAPK signaling has been reported to play a role in macrophage IFN-γ responses (Matsuzawa et al., 2012; Valledor et al., 2008). Therefore, we tested a selective ERK5 inhibitor, namely, XMD8-92 (hereafter XMD) for the possibility that ERK5 kinase activity is involved in the IFN-γ-mediated up-regulation of LRRK2 (Yang and Lee, 2011). THP-1 cells were differentiated with PMA (10 ng/ml; 48 hours) and stimulated with IFN-γ (200 U/ml) in combination with IN-1 or XMD over a 1-day time course. Cell lysates were then subjected to immunoblot analysis for LRRK2 (Fig. 3.5 A). Strikingly, XMD inhibited the IFN-γ mediated induction of LRRK2 very similar to IN-1. Moreover, just as IN-1, XMD did not interfere with proximal IFN-γ signaling, as confirmed by unaltered STAT1 phosphorylation at Y701 and S727 throughout the time course (Fig. 3.5 A).

Tab. 3.2: Cross-reactivity profiles of kinase inhibitors: IN-1, XMD, CZC-25146 and BIX 02189

Data has been taken from indicated publication to display the respective inhibitory capacities of the aforementioned kinase inhibitors. Inhibitory efficiencies are listed as half maximal inhibitory concentration (IC_{50}), as dissociation constant (K_d) or as percentage of inhibition at the concentration of 3 or 10 µM, respectively. Targets have been selected from the IN-1 screen (Deng et al., 2011a), the corresponding values for CZC-25146, XMD and BIX02189 have been integrated (Deng et al., 2011b; Ramsden et al., 2011; Tatake et al., 2008). [ND = not detected, NA = not addressed]

Inhibitors	**IN-1**		**CZC-25146**	**XMD**	**BIX02189**	
Targets	K_d [µM]	IC_{50} [µM]	IC_{50} [µM]	K_d [µM]	[% activity] at 3µM	[% activity] at 10µM
AURKA	1.60	0.87	not affected	NA	NA	NA
AURKB	ND	5.92	not affected	NA		57
CHEK2	ND	6.51	NA	NA	91	
DCLK1	0.005	NA	NA	0.001	NA	NA
DCLK2	0.016	0.045	NA	0.010	NA	NA
LRRK2	0.020	0.003	0.005	NA	NA	NA
MAPK7/ERK5	0.028	NA	NA	0.019	IC 50	59nM
MKNK2	0.038	4.47	NA	Cross reactive	91	
MYLK	ND	8.2	NA	NA	NA	NA
NUAK1	ND	3.67	NA	NA	NA	NA
PLK1	0.075	9.83	NA	NA		95
PLK4	0.016	NA	Cross reactive	NA	NA	NA
RPS6KA2	1.00	NA	Not affected	NA	NA	NA
RPS6KA6	0.62	NA	NA	Cross reactive		6
TNK1	0.023	NA	Cross reactive	0.029	NA	NA

To further validate the effect of ERK5 kinase inhibition in IFN-γ mediated LRRK2 up-regulation, we used another, unrelated inhibitor of the ERK5 module, BIX02189, (Tatake et al., 2008) on IFN-γ stimulated macrophage-like cells. Immunoblot analysis revealed that also BIX02189 suppressed the IFN-γ mediated induction of LRRK2 protein in a concentration-dependent manner (Fig 3.5 B). Next, the suppressive effects on LRRK2 up-regulation were aimed to be further analyzed by LRRK2 immunocytochemistry (Fig 3.5 C). Cytoplasmic, diffuse LRRK2 staining was already visible in untreated cells, but signal intensities were greatly elevated in THP-1 cells stimulated with IFN-γ. As expected, LRRK2 was up-regulation was inhibited by both IN-1 and XMD. In addition to cytoplasmic LRRK2, small punctate structures positive for LRRK2 in perinuclear areas were observed, however to an equal extent in all different treatment conditions, thus were not further investigated. IFN-γ treatment caused morphological alterations in the macrophage-differentiated THP-1 cells. Whereas unstimulated cells had an overall roundish appearance, the cells acquired an elongated, spindle-like morphology and were highly immunoreactive for LRRK2. Interestingly, these morphological changes were suppressed along with the return to basal LRRK2 levels in the presence of both IN-1 and XMD (Fig 3.5 C). However, treatments with the LRRK2 kinase inhibitor CZC-25146, which is not cross-reactive for ERK5 and therefore did not suppress LRRK2 induction, did also not suppress these morphological changes (not shown).

In conclusion, the pharmacological data strongly suggests that inhibition of ERK5 is leading to the inhibitory effect on LRRK2 induction upon IFN-γ stimulation.

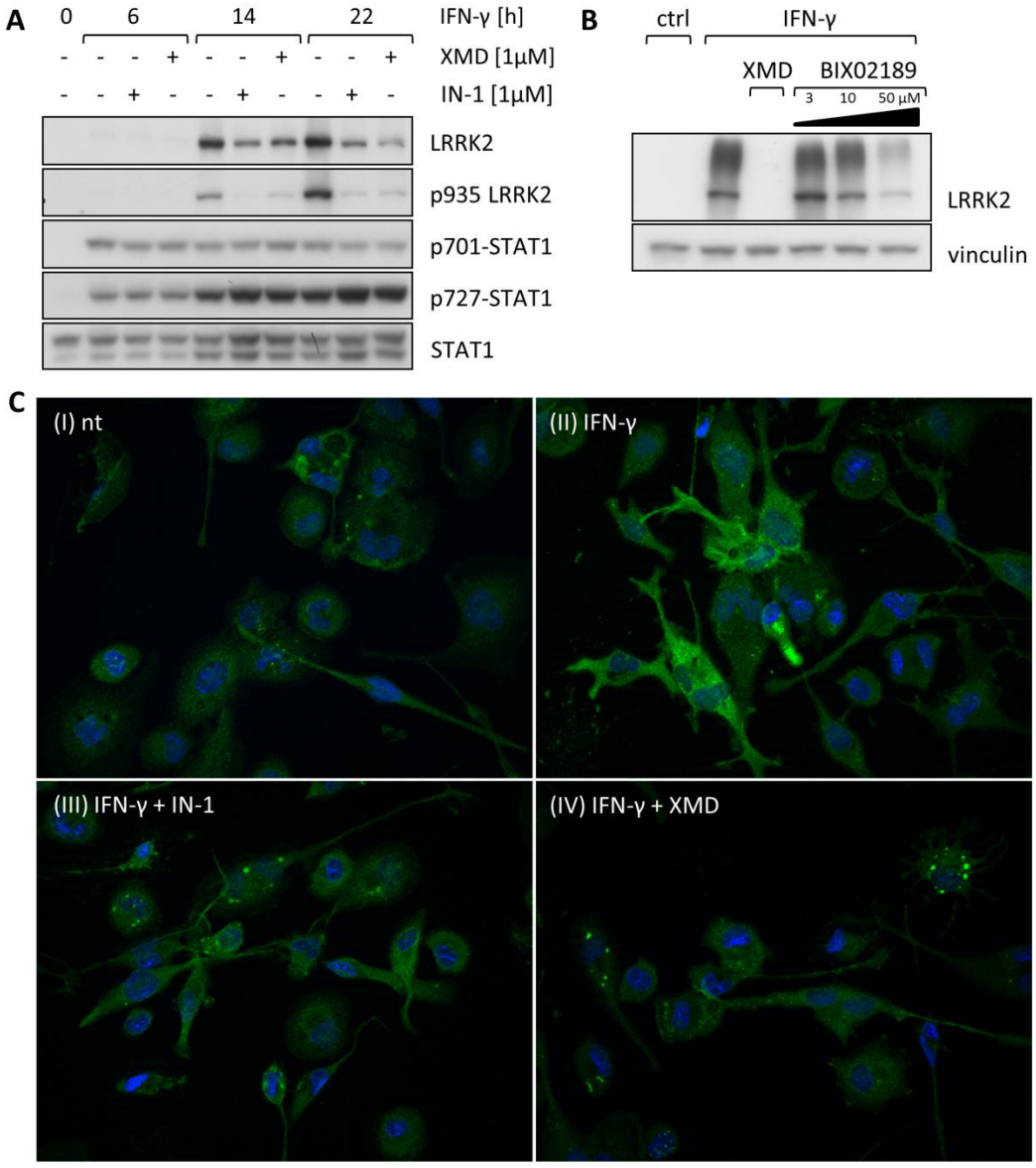

Fig. 3.5: LRRK2 up-regulation and morphological changes are reduced by pharmacological inhibition of ERK5

THP-1 cells were differentiated with 10 ng/ml PMA for 48 hours and then treated with IFN-γ. **A**: Cells were treated with 1 µM IN-1 and 1 µM XMD in the presence of 200 U/ml IFN-γ for the indicated time points and then subjected to immunoblot for analysis of (phospho-) LRRK2 and (phospho-) STAT1. IN-1 and XMD have very similar inhibitory effects on IFN-γ mediated LRRK2 induction without affecting proximal IFN-γ signaling **B**: Cells were treated for 24 hours with IFN-γ together with 1 µM XMD and the indicated concentrations of BIX02189. Next, cells were lysed and proteins subjected to immunoblot analysis for LRRK2 and vinculin as a loading control. BIX02189 is inhibiting LRRK2 up-regulation at higher concentrations. **C**: Differentiated THP-1 cells were left untreated (I) or treated for 24 hours with 200 U/ml IFN-γ (II) or in the presence of 1.5 µM either IN-1 (III) or XMD (IV). Then cells were fixed and immunostained with anti-LRRK2 (green), with DAPI (blue) as nuclear counterstain. IN-1 and XMD have very similar inhibitory effects on IFN-γ mediated LRRK2 induction; both as well reduce the number of spindle-like, activated macrophages.

3.6 LRRK2 up-regulation is reduced by knockdown of ERK5 and correlates with ERK5 expression levels

To validate the pharmacological evidence for ERK5 mediated LRRK2 up-regulation upon IFN-γ stimulation RNAi knock down experiments were conducted. However, acute transient transfections (lipofection, nucleofection) with several small interfering RNAs against ERK5 were difficult to obtain and yielded inconclusive results. Therefore, lentiviral vectors harboring ERK5-directed shRNA were purchased and used to transduce naïve THP-1 cells to generate stable knockdown (KD) clone pools. These cells were then differentiated with PMA for 48 hours and then stimulated with IFN-γ for to analyze if a reduction of ERK5 levels impacts on LRRK2 induction. Indeed, control cells displayed the IFN-γ concentration-dependent augmentation of LRRK2 protein, whereas in ERK5 KD cells LRRK2 induction was attenuated (Fig. 3.6 A). Two different ERK5 KD clones were analyzed, namely clone #10 with a moderate knockdown efficiency and clone #5 with a stronger degree of ERK5 knockdown. Interestingly, the ERK5 knockdown clone #5 showed a greater reduction of IFN-γ induced LRRK2 protein than clone #10, which only mildly affected LRRK2 induction. Thus, it seems as if residual quantities of ERK5 are capable of mediating the effects on LRRK2 induction.

More experimental indication that LRRK2 induction by IFN-γ is mediated by ERK5 is provided by comparing inducibility of LRRK2 between naïve and differentiated cells (Fig. 3.6 B). Here, we found that the PMA pre-treatment in fact induced ERK5 expression, which was a pre-requisite for subsequent IFN-γ-mediated LRRK2 induction. Accordingly, naïve THP-1 cells did not up-regulate LRRK2 upon IFN-γ stimulation. Moreover, PMA differentiation alone led to a modest elevation of LRRK2 protein levels, which was boosted further by IFN-γ. Interestingly, the type I interferons IFN-α and IFN-β did not lead to induction of LRRK2 at the used concentrations. This indicates that LRRK2 up-regulation is specific for the sole member of type II interferon, IFN-γ.

Taken together, these data provides additional evidence that ERK5 is mediating LRRK2 protein induction by IFN-γ.

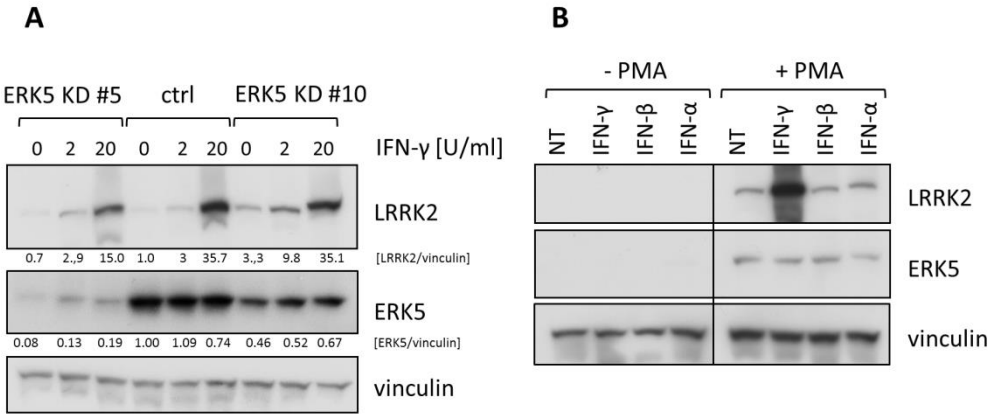

Fig. 3.6: LRRK2 up-regulation is reduced by RNAi knockdown of ERK5 and correlates with ERK5 expression levels.

A: THP-1 cells were differentiated with 10 ng/ml PMA for 48 h. ERK5 knockdown clones and control cells clones were stimulated with indicated doses of IFN-γ for 24 hours. Next, lysates of harvested cells were subjected to immunoblot probing of LRRK2 and ERK5 as well as the loading control vinculin. Densitometric analysis of band strength was carried out using ImageJ. LRRK2 up-regulation is reduced by ERK5 knockdown (KD), correlating with KD efficiency. **B**: THP-1 cells were differentiated with 10 ng/ml PMA for 48 hours or left untreated (naïve) followed by the stimulation of the respective IFNs (IFN-γ, IFN-β and IFN-α each 200U/ml). Next, cells were subjected to immunoblot as described above. Whereas naïve THP-1 cells did not display visible LRRK2 or ERK5 levels, moderate levels were detected upon PMA differentiation. LRRK2 levels were further increase upon IFN-γ treatment, but not upon stimulation with IFN-γ or IFN-α.

3.7 LRRK2 is induced by IFN-γ via ERK5 in primary human macrophages

THP-1 cells are a very useful model to study human macrophage biology because of their advantages in availability and homogeneity. A disadvantage is their nature as cancer cells. Therefore MDMs were used to check if ERK5 mediated induction of LRRK2 protein levels by IFN-γ stimulation can also be observed in primary cells. Human monocytes were isolated from PBMCs by adherence and allowed to differentiate to macrophages *in vitro* with GM-CSF treatment for 7 days and subsequently stimulated with IFN-γ. In accordance to the experiments using THP-1 cells, immunoblot analysis revealed that IFN-γ indeed leads to an increase in LRRK2 expression in MDMs as well (Fig. 3.7: A). More importantly, the ERK5 inhibitor XMD as well as IN-1 suppressed the IFN-γ mediated induction of LRRK2, just like in THP-1 cells. Thus, this is providing evidence that ERK5 is mediating LRRK2 induction also in primary human macrophages.

Recently, the transcriptional profile of PBMCs form PD patients with LRRK2 mutations has been reported to display dysregulated IL and TGF-β signaling (Monahan et al., 2008). Hence,

a dysregulation in macrophage IFN-γ mediated LRRK2 induction was hypothesized. In order to gain more insight in the pathogenic role of LRRK2, we wanted to evaluate if MDMs differentiated from PBMCs from PD patients displayed an alteration in IFN-γ mediated LRRK2 inducibility. Therefore, as an initial set of experiments from a small number of PD patient samples, PBMCs were differentiated into MDMs and subsequently treated with IFN-γ (Fig. 3.7: B) However, no differences were observed in regard to inducibility of LRRK2 by IFN-γ. Yet, due to the small number (n=2), these preliminary results still lack statistical power for a final statement.

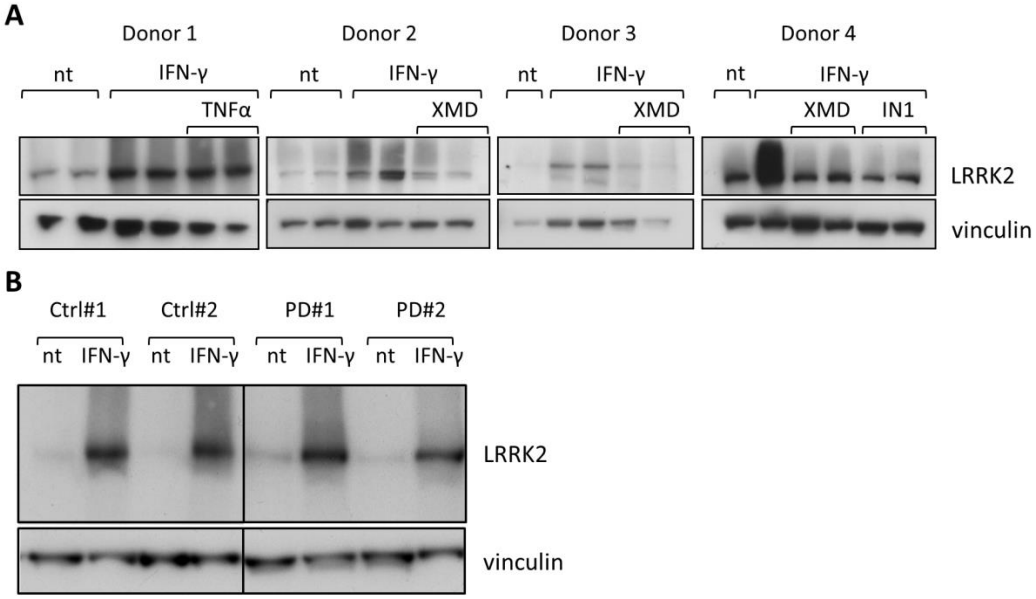

Fig. 3.7: LRRK2 is induced by IFN-γ via ERK5 in primary human monocyte-derived macrophages (MDMs)

A: Human primary monocytes were isolated from PBMCs from four individual donors by adherence and differentiated into MDMs for 7 days in complete medium containing human serum and GM-CSF (100 U/ml).Then, cells were treated with IFN-γ (200 U/ml for 18 hours) in the presence or absence of inhibitors, as indicated. Untreated controls were included in both cases. Cell lysates were subjected to immunoblot for LRRK2 and the loading control vinculin. LRRK2 was up-regulated upon IFN-γ stimulation, co-treatments with IN-1 or XMD had an inhibitory effect on LRRK2 induction **B**: For patient material and respective controls, experiments were conducted as described above. In this set of initial experiments, no differences in induction of LRRK2 levels were observed when comparing MDMs from PD patient and healthy controls, respectively.

3.8 Transcriptome analysis and verification of LRRK2 co-regulated genes in THP-1 and primary human monocyte-derived macrophages

Despite increasing efforts, the role of LRRK2 in the immune system and more specifically in macrophages remains poorly understood. In order to gain new insights in the role of LRRK2 in macrophage biology, we performed a transcriptome analysis of PMA-differentiated THP-1 cells treated with IFN-γ in combination with IN-1, XMD or DMSO as control, respectively. RNA was isolated from the cells and triplicates of RNA were subjected for Microarray analysis was carried out by the microarray facility, Tübingen. In order to control the inhibitory effects of IN-1 and XMD, lysates from the same experiment were subjected to Western blot analysis and probed for LRRK2, confirming the reduced up-regulation of LRRK2 upon IFN-γ stimulation (Fig. 3.8 A). Raw data from micro array experiments was analyzed by the microarray facility. A large number of hits were identified that were affected by the treatments. They were subdivided in different categories, namely genes that were a) up-regulated by IFN-γ and reduced upon inhibitor co-treatment, b) not affected by IFN-γ treatment but affected by inhibitor treatment or c) down-regulated by IFN-γ but not further affected by either of the inhibitors. In this work, only hits from category a) were further investigated. Top fold changes of these genes are summarized below (Tab. 3.3).

Confusingly, LRRK2 itself was not regulated as expected on mRNA level by inhibitor co-treatments. Although there was a robust induction in LRRK2 mRNA, as expected, no inhibitory effects could by observed upon co-treatments with IN-1 or XMD in any of the triplicates subjected to transcriptome analysis (data not shown). This might indicate that different mechanisms are mediating the inhibitor effects, possibly affecting LRRK2 protein levels and mRNA levels independently. However, because at least LRRK2 protein levels were clearly reduced in IN-1 and XMD treated cells (Fig. 3.8 A), the obtained hits were further investigate in order to reveal a possible relation to LRRK2 biology.

C1QA, C1QB and C1QC are part of the complement system and were among the genes that were identified in the transcriptome analysis (Tab. 3.3). The complement system plays an important role in both innate defense and adaptive immune response. This is achieved by efficient proteolytic cascades ultimately terminating in opsonization and destruction of pathogens as well as the generation of an inflammatory response by producing pro-inflammatory mediators (Dunkelberger and Song, 2010). Verification of microarray targets was carried out by RT-PCR analysis. And as expected, THP-1 cells treated with IFN-γ in

combination with IN-1 and XMD respectively displayed the same expression pattern as already observed in the transcriptome analysis: C1QA, C1QB and C1QC mRNA levels were induced when treated with IFN-γ and this induction was reduced by IN-1 or XMD co-treatments as assessed by RT-PCR (Fig. 3.8 B). Moreover, membrane-associated ring finger (C3HC4) 1 (MARCH1), a membrane-bound E3 ubiquitin ligase, was verified to be regulated in the same fashion as predicted from the transcriptome analysis (Fig. 3.8 B). MARCH1 down-regulates the surface expression of MHC class II molecules by directing them to the late endosomal/lysosomal compartment (De Gassart et al., 2008; Thibodeau et al., 2008), thus directly impacting on antigen presentation. Therefore, the potential influence on human leukocyte antigen (HLA) expression by MARCH1 was investigated (Fig. 3.8 C + D). As expected, HLA was strongly induced by IFN-γ, but not affected by inhibitor treatments on mRNA levels. Moreover, also surface expression was found to be unaltered as assessed by FACS analysis. Conclusively, MARCH1 did not impact on HLA surface expression.

Tab. 3.3: Selected microarray hits of IFN-γ induced genes inhibited by XMD and IN-1

Listed are genes from the transcriptome analysis that were just up-regulated by IFN-γ and inhibited by both IN-1 and XMD, just like LRRK2.

Gene Symbol	Gene Title	RefSeq Transcript ID
MTHFD2L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	NM_001144978
MARCH1	membrane-associated ring finger (C3HC4) 1	NM_001166373
EOMES	eomesodermin homolog (Xenopus laevis)	NM_005442
IL1R2	interleukin 1 receptor, type II	NM_004633
C1QC	complement component 1, q subcomponent, C chain	NM_001114101
C1QA	complement component 1, q subcomponent, A chain	NM_015991
C1QB	complement component 1, q subcomponent, B chain	NM_000491
S100B	S100 calcium binding protein B	NM_006272
CD226	CD226 molecule	NM_006566
CDK5RAP2	CDK5 regulatory subunit associated protein 2	NM_001011649
CCL1	chemokine (C-C motif) ligand 1	NM_002981
CLEC10A	C-type lectin domain family 10, member A	NM_006344
CLEC5A	C-type lectin domain family 5, member A	NM_013252
FFAR2	free fatty acid receptor 2	NM_005306
APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	NM_145699

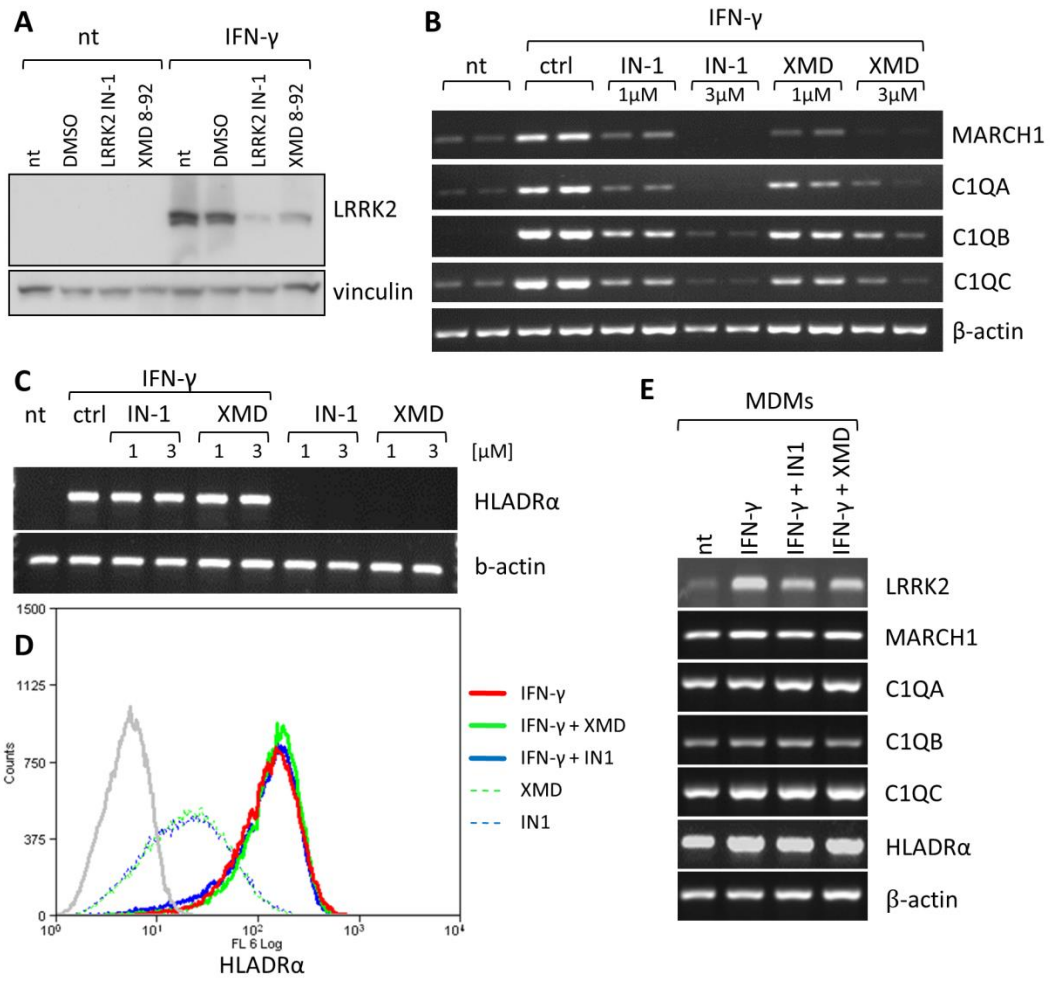

Fig. 3.8: Transcriptome analysis and verification of LRRK2 co-regulated genes in THP-1 and primary human monocyte-derived macrophages (MDMs)

THP-1 cells were differentiated with 10 ng/ml PMA for 48 h and then treated with IFN-γ or not (nt) in combination with 1 µM of XMD, IN-1 or the equivalent DMSO amount as solvent control. RNA in triplicates from each condition was sent for RNA transcriptome analysis. **A:** As a control, lysates from cells treated as described above were subjected to immunoblot analysis and probed for LRRK2 and vinculin as loading control. As expected, LRRK2 protein induction by IFN-γ was inhibited by both IN-1 and XMD. **B, C**: THP-1 cells were treated as above with the indicated inhibitor concentrations and RNA was isolated for RT-PCR for verification of selected micro array hits, namely MARCH1, C1QA, C1QB and C1QC. While the latter 4 follow the same pattern as LRRK2, HLADRα did not. β-actin served as loading control. **D**: THP-1 cells were treated as above and stained for HLADRα surface expression and subjected to FACS analysis. HLADRα expression was up-regulated by IFN-γ but unaffected by IN-1 or XMD. **E**: Human primary monocytes were isolated from PBMCs by adherence followed by a differentiation into MDMs for 7 days in complete medium containing human serum and GM-CSF (100 U/ml) and then treated with IFN-γ (200 U/ml for 24 h) in presence of the indicated concentrations of inhibitors. RNA was isolated for RT-PCR to evaluate mRNA levels of the listed micro array hits. While LRRK2 was up-regulated by IFN-γ and inhibited by XMD and IN-1 treatments, MARCH1, C1QA, C1QB, C1QC and HLADRα did not display differences upon inhibitor treatments.

Finally, targets from the THP-1 transcriptome analysis were aimed to be verified in primary human MDMs. However, although LRRK2 was induced upon IFN-γ stimulation and less so when co-treated with XMD or IN-1, an inhibitor effect on other targets could not be observed in primary MDMs (Fig. 3.8 E). Next to LRRK2, only moderate up-regulation of HLADrα and C1QC was observed in these cells. Other targets (MTHFD2L, EOMES, IL1R2 and CD226) could not be confirmed to be regulated as predicted from the transcriptome analysis, however, it is possible that this might be due to technical obstacles in RT-PCR analysis (genomic amplification, primer design etc.), therefore should not completely omitted from this point on. CDK5RAP2, CCL1, CLEC10A, CLEC5A, FFAR2 and APOBEC3A as well as not listed hits have not been further investigated.

In conclusion, some targets of the transcriptome analysis could be confirmed by RT-PCR analysis in THP-1 cells, however, not in primary MDMs. Confusingly, LRRK2 mRNA was not affected by inhibitor treatments although protein levels were shown to be reduced, as expected. MARCH1 was not found to be functionally active in terms of down-regulation of HLA surface expression and the role of C1QA, C1QB and C1QC in THP-1 cells remains to be investigated, however, in regard to the results from the primary MDMs, functional studies might be more relevant when directly investigated in a primary cell system.

4 Discussion

4.1 LRRK2 is expressed in the immune system

Since the discovery that mutations in LRRK2 can cause PD, the majority of research has focused on elucidating cell autonomous mechanisms to explain the neuronal death in the SNpc. Yet, the role of LRRK2 in immune cells and the respective potential in PD pathology has long been overlooked. While LRRK2 expression is detectable in neurons, the expression of LRRK2 is much higher within the immune system, including dendritic cells, monocytes, macrophages, microglia and B-cells (Gardet et al., 2010; Hakimi et al., 2011; Maekawa et al., 2010; Miklossy et al., 2006; Thevenet et al., 2011). Therefore, LRRK2 seems to play an important role in immune cell biology. Yet, it is arguable that LRRK2 might have different roles depending on the cell type and therefore LRRK2 in the immune system is not necessarily related to brain PD pathology. Alternatively, LRRK2 could influence PD pathogenesis via the immune system. This view is supported by the general involvement of inflammatory processes in PD pathology on the one hand and the role of other PD risk genes in immune cell biology on the other. For example, activated microglia are repeatedly observed within the SNpc of PD patients (Banati et al., 1998; Imamura et al., 2003; McGeer et al., 1988) and beside LRRK2 also GBA, DJ-1, Parkin and α-Syn are reported to play a role in immune cell biology (Dzamko and Halliday, 2012; Liu et al., 2012; Manzanillo et al., 2013; Marodi et al., 1995; Mira et al., 2004; Pandey and Grabowski, 2013; Roodveldt et al., 2008; Russo et al., 2014; Trudler et al., 2014; Waak et al., 2009).

While the CNS has long been regarded as an immune privileged organ, it is now established that several humoral and neural routes are providing ways of interaction and therefore possibilities of how systemic inflammation can contribute to PD pathology (Su and Federoff, 2014). Moreover, an increase in permeability of the BBB caused by persistent systemic inflammation is believed to raise the flux of inflammatory factors and immune cells from the periphery into the CNS, including T cell infiltration (Kannarkat et al., 2013). But even under homeostatic conditions, the brain hosts several myeloid cell types at specific locations, executing homeostatic and surveillance tasks. This includes microglia in the brain parenchyma, and macrophages at the choroid plexus, the meninges and in the perivascular spaces (Prinz and Priller, 2014). Therefore, non-cell autonomous mechanisms like systemic inflammation might cause or at least contribute to the decay of dopaminergic neurons and

PD pathology, e.g. by transforming primed microglia into an activated state. Thus, increasing efforts are made to investigate the role of LRRK2 within the (peripheral) immune system in order to elucidate possible links to PD pathogenesis.

In this work, the presence of LRRK2 in primary monocytes, macrophages, B cells and dendritic cells was detected by immunoblot analysis, which is in line with previous findings. LRRK2 expression was found to be much lower in CD3+ and CD4+ T cells. This is in line with a previous study reporting a moderate but lower expression level of LRRK2 in T cells in comparison to B cells (Hakimi et al., 2011). Surprisingly, the microglia cell line C13-NJ was devoid of LRRK2, although other reports have found LRRK2 to be expressed in primary microglia (Marker et al., 2012; Miklossy et al., 2006; Moehle et al., 2012). This difference might reflect that C13-NJ cells do not fully resemble primary microglia. Accordingly, LRRK2 expression in C13-NJ has not yet been reported elsewhere. Moreover, LRRK2 expression could not be detected in the cervical HeLa cell line. Various stimuli, which were triggering growth- (EGF) immune- (IFN-γ) or oxidative stress- (H_2O_2) or osmotic stress- (Sorbitol) responses, were also ineffective in inducing LRRK2 expression. This was unexpected as endogenous expression of LRRK2 in HeLa cells is generally considered to be low, yet detectable (Hatano et al., 2007). An explanation for this discrepancy could be that the experimental conditions for LRRK2 detection in this work were optimized for the detection of the high levels of LRRK2 in macrophages, thus indicating that LRRK2 levels in HeLa cells are much lower than in immune cells. As IFN-γ signaling is predominantly restricted to immune cells, this could also suggest that regulation of LRRK2 expression may vary between different cell types. Whether this also results in different functions of LRRK2 in immune or e.g. neuronal cells remains an open question.

In conclusion, this work confirms the notion that LRRK2 is highly expressed in the immune system. In macrophages, it is even further inducible by external stimulation with IFN-γ. Noteworthy, LRRK2 is found in a variety of different immune cells, yet not in all. For example, the absence of LRRK2 expression in T-cells might provide clues to its role in immune cell biology when compared to cells that display high levels of LRRK2, like dendritic cells, macrophages and monocytes. While the latter three have long been considered to be professional antigen presenting cells (APCs), increasing evidence suggests that also B-cells are APCs (Chen and Jensen, 2008; Hume, 2008; Randolph et al., 2008; Unanue, 1984; Yuseff et al., 2013). This might indicate a role for LRRK2 in antigen presentation, which is a

particularly an interesting discovery because common variations in the HLA regions have been associated with sporadic PD (Ahmed et al., 2012; Hamza et al., 2010).

4.2 IFN-γ stimulation of macrophage-like cells induces LRRK2

IFN-γ is involved in multiple biological processes and is the classical macrophage activating factor. It induces a robust pro-inflammatory profile including the production of IL-1β, IL-12, TNF-α and iNOS (Lowenstein et al., 1993; Mills, 2012). In this work, IFN-γ was found to induce LRRK2 in PMA differentiated THP-1 macrophages, in accordance to previous findings (Gardet et al., 2010; Thevenet et al., 2011). LRRK2 induction coincides with the up-regulation of other markers for differentiation from monocytes to macrophages including CD14, CD11b, CD11c and MHCI as well as with respective morphological changes. Thus, LRRK2 might play a role either in differentiation or maturation of monocytes to macrophages, as suggested in preceding studies (Thevenet et al., 2011). As an increase of LRRK2 is present on both mRNA and protein level, a transcriptional up-regulation is likely. Yet, additional posttranscriptional mechanisms or reduced protein turnover might also contribute to the observed LRRK2 induction.

In conclusion, the IFN-γ induced up-regulation of LRRK2 on mRNA and protein levels, the induction of macrophage markers as well as the morphological changes are all in line with previous findings. Moreover, THP-1 cells seem to present a very suitable model to study LRRK2 biology in macrophages. They resemble key features of macrophages and express high endogenous LRRK2 levels, which is a great advantage to many other systems that use artificial overexpression of LRRK2.

4.3 LRRK2 kinase inhibitor IN-1 blocks induction of LRRK2

Kinase inhibitors present an elegant and efficient method to study kinase activities in various biological systems and offer options to interfere with cellular functions in order to cure or mitigate pathological conditions. This kind of approach is believed to entail clinical potentials because G2019S, the most common LRRK2 mutation, has been shown to have a moderately increased kinase activity that is associated with its pathogenicity (Bonifati, 2006; Lee et al., 2012; Rudenko et al., 2012a; Smith et al., 2006; West et al., 2005). Recently, the development and characterization of the LRRK2 inhibitor IN-1 has opened new possibilities for functional studies of LRRK2 kinase (Deng et al., 2011a). Here, co-treatment with IN-1 lead

to a reduction in LRRK2 up-regulation upon IFN-γ stimulation in macrophages. This surprising piece of evidence led to the preliminary hypothesis that LRRK2 kinase function might be involved in regulating its own expression. IN-1 treatment did impact on mRNA and protein levels indicating that it primarily impairs the transcriptional up-regulation of LRRK2.

Moreover, IN-1 also suppressed the capacity of PMA differentiated THP-1 cells to undergo morphological changes upon IFN-γ stimulation into spindle-like, activated macrophages. This could be caused by either suppression of LRRK2 kinase activity, total LRRK2 protein levels or possibly also off-target effects of IN-1. Interestingly, LRRK2 has been reported to be involved in cytoskeleton and actin dynamics in general, which play a central role in cellular morphological changes. Specifically in regard to immune cells, LRRK2 has been shown to be involved in microglial morphological responses upon LPS stimulations (Marker et al., 2012; Moehle et al., 2012; Parisiadou and Cai, 2010). Because proximal IFN-γ signaling was not affected by IN-1 treatments as checked by phosphorylation status of STAT1, this data suggested that a downstream step in the signal transduction pathway had to be affected. Effective inhibition of IFN-γ signaling using JAK IN-1 added further proof that LRRK2 is indeed induced via this pathway. Yet, the inhibitory effect of (LRRK2) IN-1 had to take place more downstream.

In conclusion, from the sole observation that IN-1 blocks LRRK2 induction, it was suspected that LRRK2 kinase activity is involved in regulating its own expression levels in macrophage upon IFN-γ stimulation. Interestingly, a similar role was already reported to occur in the murine kidney where both pharmacological kinase inhibition of LRRK2 as well as the expression of a kinase dead LRRK2 mutant down-regulated its steady state levels, with lesser effect in lung and brain (Herzig et al., 2011).

4.4 Other LRRK2 inhibitors fail to suppress IFN-γ mediated induction of LRRK2

All pharmacological approaches suffer from potential cross-reactivities. Therefore, they must be accompanied with additional experimental verification such as knockdown or knockout of the gene of interest (GOI) or usage of additional inhibitors that have non-overlapping off-target profiles. In this work, surprisingly, the application of a panel of different LRRK2 kinase inhibitors did not reproduce the IN-1 mediated reduction of LRRK2 levels. Even a high degree of kinase inhibition (e.g. high concentrations of CZC-25146 or Sunitinib), as checked by p935 LRRK2 autophosphorylation, did not alter LRRK2 induction.

Therefore, the initial hypothesis that LRRK2 kinase activity is involved in regulating its own expression levels was falsified. As an exception, an inhibitory effect on IFN-γ-stimulated LRRK induction was observed at high concentrations of K252a, which is a staurosporine analog and a potent but unspecific inhibitor of LRRK2 (Ruegg and Burgess, 1989). Thus, the K252a effect is most likely to unspecific inhibition of proximal IFN-γ signaling, as p701-STAT1 levels were dramatically diminished. In conclusion, the robust inhibitory effect of IN-1 on LRRK2 induction during IFN-γ signaling had to be caused by an inhibitory cross-reaction of IN-1.

4.5 LRRK2 induction is reduced by knockdown and pharmacological inhibition of ERK5

In search of alternative explanations for the inhibitory effects of IN-1 on LRRK2 induction upon IFN-γ stimulation, the total inhibitory profile of IN-1 was analyzed. Comparison with inhibitory profiles of IN-1 and CZC-25146 as well as consideration of respective inhibitor potencies lead to a shortlisted number of candidates including ERK5. Because the ERK5 inhibitor XMD had very similar effects as IN-1, it was hypothesized that ERK5 cross-reactivity of IN-1 is responsible for the inhibitory effects of IN-1 on LRRK2 induction upon IFN-γ stimulation. Indeed, IN-1 was previously reported to inhibit ERK5 (Deng et al., 2011a). Because IN-1 and XMD share structural similarities, a third ERK5 inhibitor, BIX02189, was used. BIX02189 had the same effect as IN-1 or XMD, thus strengthening the hypothesis that this pathway is indeed ERK5 mediated. Moreover, lentiviral knockdown experiments added additional proof by showing that a reduction in expression levels of ERK5 causes a decreased induction of LRRK2 upon IFN-γ stimulation. Another piece of evidence came from differentiation of monocytes to macrophages using PMA. While PMA treatment was observed to be accompanied by up-regulation of ERK5, untreated THP-1 cells did not display detectable ERK5 levels. Thus, LRRK2 levels were correlating with ERK5 levels, as LRRK2 up-regulation upon IFN-γ stimulation was only present in PMA pre-treated macrophages but not in untreated monocytes. In conclusion, this strongly suggests that ERK5 is mediating LRRK2 up-regulation upon IFN-γ stimulation, as demonstrated by both genetic as well as different pharmacological approaches.

Induction of LRRK2 levels upon IFN-γ treatments was confirmed by immunocytochemical analysis. Moreover, also the IN-1 and XMD inhibitory effects on IFN-γ mediated up-regulation of LRRK2 were robustly confirmed. Interestingly, the acquired morphological

changes to a more elongate, spindle-like “activated” macrophage were suppressed as well. This observation is in line with a recent study suggesting that LRRK2 is playing a role in monocyte maturation and respective morphological changes (Thevenet et al., 2011). Moreover, LRRK2 has been shown to be a negative regulator of NFAT, which are Ca^{2+}-regulated transcription factors involved in regulation of immune responses in many different cell types, including APCs (Liu et al., 2011; Muller and Rao, 2010). Therefore, the morphological alterations in macrophages might be caused by a NFAT dependent mechanism.

However, the assumption that LRRK2 kinase is involved in the regulation of these changes was rejected because treatment with a different LRRK2 inhibitor, CZC-25146, did not suppress either LRRK2 induction or morphological changes. This might be caused by several reasons: First, other genes that are co-regulated with LRRK2 and which expression or kinase activity was also inhibited upon inhibitor treatments could theoretically be responsible for the morphological differences. Therefore, kinases that were inhibited in activity or reduced in expression by both XMD and IN-1 but not upon CZC-25146 treatment might be involved. In fact, the transcriptome analysis from this work yielded many co-regulated genes that might be responsible. Alignment of hits from a microarray analysis with co-treatments of IFN-γ and CZC-25146 would theoretically narrow down this candidate list. Yet, the simplest explanation might be that LRRK2 kinase independent functions are involved. Interestingly, data that showed specific dependence on kinase function was not presented in the study that first linked LRRK2 to NFAT immune regulation (Liu et al., 2011). Although kinase function is the primary focus of LRRK2 research, it might not be the true pathogenic output after all. LRRK2 kinase independent output could be the GTPase function of LRRK2 or its proposed role as a scaffold for signaling complexes. For example, a novel study suggests that diffuse LRRK2 levels positively correlate with earlier cell death independently of kinase activity (Skibinski et al., 2014). Another report presents a model in which LRRK2 bridges membrane and cytosolic components of Wnt signaling, serving as a signaling scaffold (Berwick and Harvey, 2012). In conclusion, the observed morphological changes in this work may possibly be caused by kinase independent functions of LRRK2 by a yet unknown mechanism. Aside from these so far speculative hypotheses, experiments using stable LRRK2 KO cells would clearly advance our understanding in regard to LRRK2 specificity of the aforementioned morphological changes.

ERK5 belongs to the MAPK family. It differs from the other MAPKs because of its structural features including an extended C-terminal tail with a transcriptional activation domain (Fig. 4.1). Upstream activation of ERK5 has been shown by a great variety of stimuli including oxidative stress, osmotic stress, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF-2) and platelet-derived growth factor (PDGF), brain-derived neurotropic factor (BDNF) nerve growth factor (NGF) and IL-6 (Abe et al., 1996; Carvajal-Vergara et al., 2005; Nithianandarajah-Jones et al., 2012). All these extracellular stimuli cause downstream activation of the MAPK cascade. Classically, this occurs via the apical MAPKK, which in the case of ERK5 are Mek kinase 2 (MEKK2) or MEKK3. This leads to the subsequent phosphorylation of MAPK/ERK kinase 5 (MEK5), which in turn activates ERK5 (Drew et al., 2012). Just like other MAPKs, ERK5 regulates a number of downstream transcription factors including the myocyte enhancer factor (MEF) family, specifically MEF2A, MEF2C and MEF2D, but also Sap1, c-FOS and c-Myc (Drew et al., 2012; Nithianandarajah-Jones et al., 2012).

MEF2 is a critical branchpoint that integrates cellular signaling and can promote both cellular survival and death. Dysregulation of MEF2 function/signaling has been proposed to underlie PD pathogenesis (Yin et al., 2012). Specifically, stress induced S-nitrosylation of MEF2C was contributing to mitochondrial dysfunction and apoptotic cell death in a PD stem cell model (Ryan et al., 2013). Therefore, it seems possible that ERK5 mediated up-regulation of LRRK2 is associated with MEF2 activity. Yet, an interaction between ERK5 and LRRK2 in neurons has not been reported and accordingly, it remains elusive how macrophage MEF2 signaling might influence PD pathogenesis.

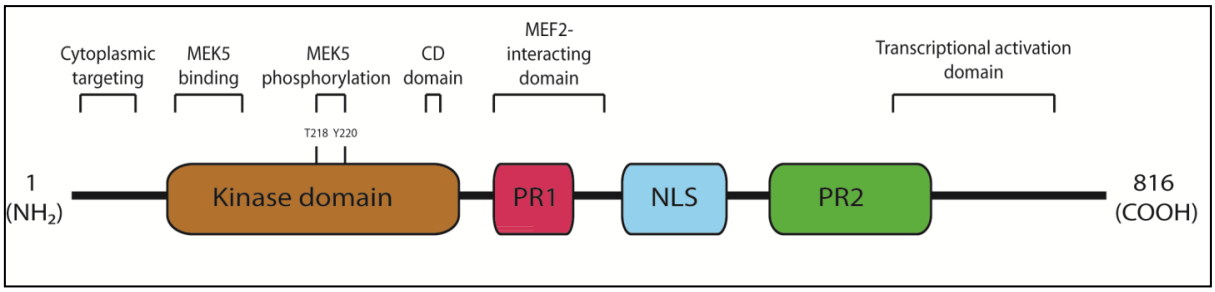

Fig. 4.1: Schematic representation of ERK5

ERK5 consists of 816 amino acids. The N-terminal contains a region for cytoplasmic targeting and a kinase domain while the extended C-terminal tail is composed of two proline rich (PR) domains including a MEF2-interacting region, a nuclear localization signal (NLS) domain and a transcriptional activation domain. A MEK5 binding domain, the respective phosphorylation sites as well as a common docking (CD) domain are located within the N-terminal kinase domain.

But how is ERK5 signaling or activity involved in IFN-γ induced up-regulation of LRRK2? Theoretically, at least three options are possible (Fig. 4.2): First, ERK5 could be directly activated during IFN-γ signaling. While canonical, STAT1-dependent IFN-γ signaling seems not likely to be affected by ERK5, putative interactions are imaginable when considering STAT1-independent IFN-γ signaling (Gil et al., 2001; Ramana et al., 2002). Several other proteins have been shown to be activated upon IFN-γ stimulation including protein-tyrosine kinase 2-beta (PTK2B), ERK1/2, the protein-tyrosine kinase FYN (Takaoka et al., 1999; Uddin et al., 1997). Thus, it would be possible that ERK5 plays a similar, yet unidentified role in STAT1-independent signaling. Interestingly, STAT3 has also been implicated as an upstream activator of MEK5 (Song et al., 2004). This might provide a link as IFN-γ has been demonstrated to activate STAT3, though with less potency when compared to STAT1 (Qing and Stark, 2004).

Second, ERK5 might be involved indirectly in IFN-γ signaling. This could be implemented by involvement in a wave of secondary IFN-γ induced genes or activation of ERK5 in a feedback loop. Because of the fact that IFN-γ is inducing at least 100 genes, the identification of the actual signaling cascade might be challenging (Waddell et al., 2010).

The two aforementioned options assume that ERK5 is activated directly or indirectly by IFN-γ signaling to mediate LRRK2 up-regulation. A third option is that ERK5 activity might modulate canonical IFN-γ mediated gene induction. For example, a modulatory effect has already been observed in regard to STAT1 phosphorylation at S727 by PI3K (Nguyen et al., 2001). Interestingly, p38, a MAPK and ERK5 family member, has also been observed to

phosphorylate STAT1 at S727 under stress conditions (Kovarik et al., 1999). Although S727 phosphorylation of STAT1 was unaffected in this work by pharmacological inhibition of ERK5, this portrays an example of how MAPKs impact on IFN-γ signaling. Moreover, other proteins like the mini chromosome maintenance protein 5 (MCM5), CREB-binding protein (CBP) and breast cancer susceptibility gene 1 (BRAC1) have also been shown to associate with STAT1 and enhance its transactivation (Ouchi et al., 2000; Zhang et al., 1996; Zhang et al., 1998). Thus, ERK5 might have a similar role.

Similar to other MAPKs, activation of ERK5 is achieved by dual phosphorylation of the T218/Y220 residues within the activation loop by MAPKK, MEK5. According to the current understanding this leads to the unfolding of ERK5 and the subsequent autophosphorylation of its C-terminus. Phoshorylated ERK5 then shuttles from the cytoplasm to the nucleus to induce transcription. In this work however, the detection of phosphorylated ERK5 from IFN-γ stimulated THP-1 cells was problematic. Assuming that ERK5 is regulating or impacting on LRRK2 transcription via its kinase function, IFN-γ stimulated cells should display an increased amount of phosphorylated ERK5, which was not the case. A possible explanation for this could be a generally insufficient quality of the used antibodies as they also failed to detect ERK5 autophosphorylation after treatment with EGF, a classic ERK inducer. A second explanation could be very transient kinetics of ERK5 activity. Higher temporal resolution might yield a better picture of ERK5 autophosphorylation. Lastly, it is also possible that ERK5 kinase activity is not at all required for transcriptional activity. Just recently it was reported that ERK5 transcriptional activity does not require kinase activity (Erazo et al., 2013).

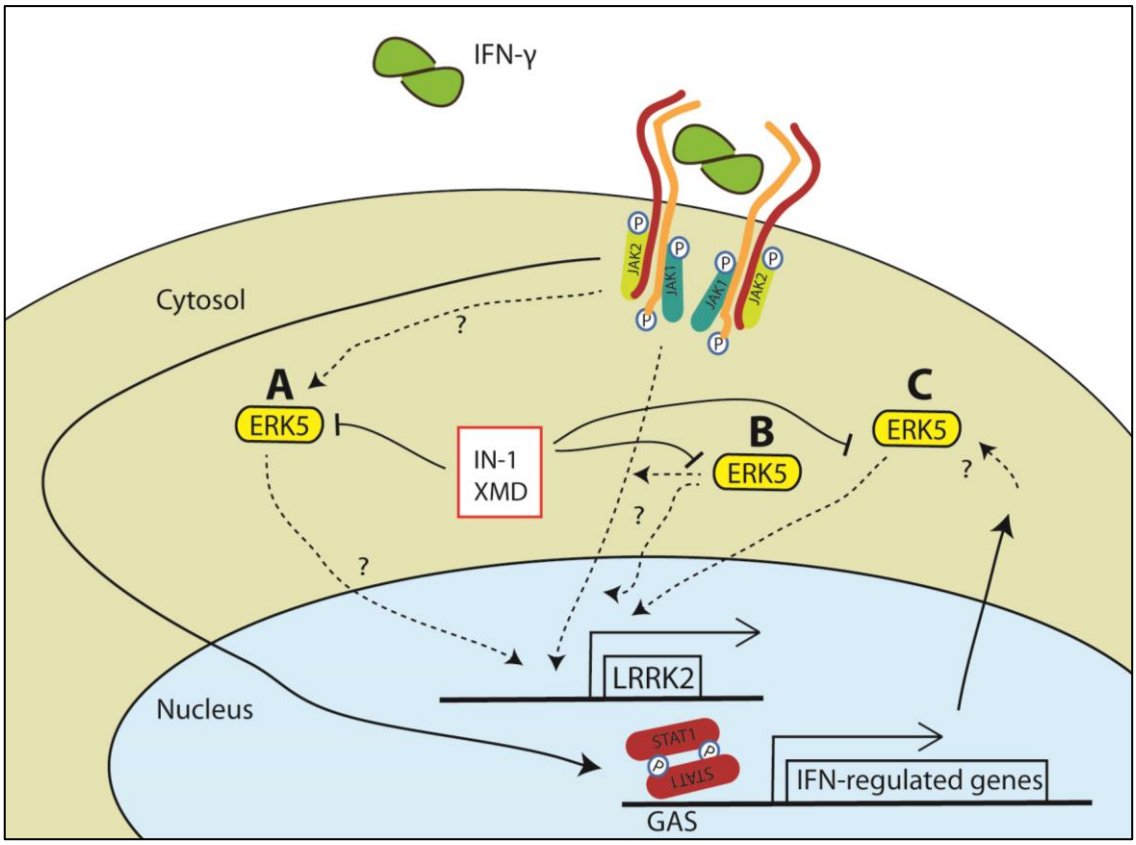

Fig. 4.2: Potential roles of ERK5 mediating LRRK2 up-regulation upon IFN-γ signaling

ERK5 inhibition or knockdown leads to a reduced up-regulation of LRRK2 upon IFN-γ signaling. LRRK2 up-regulation might be regulated by either of these hypothetical pathways. **A**: ERK5 is activated directly upon IFN-γ signaling through an unknown mechanism. Subsequently, ERK5 induces LRRK2 transcription. **B:** ERK5 is regulating LRRK2 induction by either modulating cytosolic or nuclear signaling events of STAT1 independent IFN-γ signaling. **C:** ERK5 is indirectly activated upon IFN-γ signaling, e.g. by an IFN-γ induced gene and subsequently induces LRRK2 expression.

More insights into potential mechanisms of how ERK5 might be involved in LRRK2 up-regulation could be gained by consideration of transcription factor (TF) binding sites within the LRRK2 promotor region (Fig. 4.3). The 800 bps of the upstream promotor region comprise several putative TFs binding sites, including the activator protein 1 (AP-1), IFN regulatory factor (IRF) and myocyte enhancer factor 2 (MEF2). Moreover, five IRF recognition sequence (IRS) were identified in the upstream 2000 bps of the LRRK2 promotor region (Thevenet et al., 2011). Therefore, it is possible that LRRK2 up-regulation is directly regulated by IFN-γ signaling which might be regulated by ERK5 activity at some stage. Alternatively, ERK5 itself might directly or indirectly mediate IFN-γ induced LRRK2 up-regulation. ERK5 has been suggested to interact with various TFs including MEF2, c-Myc, Sap1a, c-Fos and Fra-1 (English et al., 1998; Kamakura et al., 1999; Yang et al., 1998).

Moreover, ERK5 has also been reported to enhance AP-1 transactivation activity (Morimoto et al., 2007). Therefore, ERK5 could in principle also induce LRRK2 up-regulation via activity of MEF2 or an AP-1.

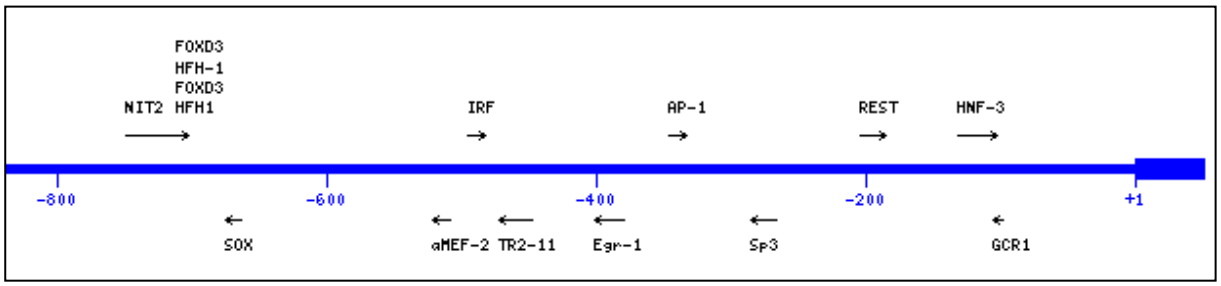

Fig. 4.3: Predicted binding sites of LRRK2 promotor region

Depicted is the 800 bp upstream promotor region of LRRK2. Several putative transcription factor (TF) binding sites are located within this region, including an AP-1, IRF an MEF-2. Promotor sequence was analyzed using the MAPPER2 (http://genome.ufl.edu/mapper) Software (filters: E value < 10; Scores > 90th percentile).

ERK5 is known to be involved in regulation of cell proliferation and differentiation in response to growth factors or cellular stress. Moreover, it has been reported to play an important role in cardiovascular development and vascular integrity as well as in cancer and tumor angiogenesis. ERK5 function in immune cells such as monocytes or macrophages has long been overlooked. Just recently, ERK5 was shown to regulate transcription factors that are crucial for monocytic differentiation (Wang et al., 2014). Further, another report describes the role of ERK5 as a regulator of monocytic TLR2 signaling. Upon inhibition of ERK5, expression of TLR2 induced by agonists of TLR1/2 and other cytokines is blocked in monocytes (Wilhelmsen et al., 2012). In macrophages, ERK5 has been shown to stimulate efferocytosis (Heo et al., 2014). Moreover, ERK5 was reported to be involved in colony-stimulating factor 1 (CSF-1) induced proliferation in a Scr-dependent fashion (Rovida et al., 2008). In conclusion, this highlights the versatile role of ERK5 in immune cell biology. In this light, transcriptional regulation of LRRK2 by ERK5 upon IFN-γ stimulation appears to be another way to orchestrate monocyte differentiation and function. While the role of ERK5 in this context is far from being understood, it opens exciting new avenues for future investigations.

4.6 LRRK2 is induced by IFN-γ via ERK5 in primary human macrophages

In order to draw relevant physiological conclusions from a biological process that has been intensively studied *in vitro* in a cell line, it is of fundamental importance to translate observations to primary human cells. Here, this was achieved by switching from a system that uses monocytic THP-1 cells differentiated into macrophages by PMA to primary human macrophages differentiated with human GM-CSF. Up-regulation of LRRK2 by IFN-γ stimulation in primary macrophages has previously been described (Thevenet et al., 2011). This was robustly confirmed by this work. The key finding of this work, ERK5 mediated LRRK2 up-regulation upon IFN-γ stimulation, was also successfully verified in the primary cells. This highlights that the results from the THP-1 cell model probably closely resemble the situation in primary MDMs.

In order to gain first insights into the clinical situation, primary macrophages from PD patients were also analyzed in this work. Although a lot of samples have been processed, only macrophages from 2 patients have been analyzed in this work. This was primarily due to technical problems with these cells. Patient cells were generally very low in number and very heterogeneous in viability and ability to differentiate into MDMs. Protein content from the used samples varied hugely, aggravating these problems. Moreover, platelet contamination was often observed and influenced culture quality significantly. Ultimately, this led to the exclusion of most of the processed samples, thus making it impossible to draw significant conclusions from this point.

4.7 Transcriptome analysis and verification of LRRK2 co-regulated genes in THP-1 and primary human monocyte-derived macrophages

An increasing amount of evidence is suggesting that LRRK2 plays an important role in immune cell responses and differentiation. In this work, a transcriptome analysis was carried out in order to gain new insights into LRRK2 regulated immune cell biology. Therefore, PMA-differentiated THP-1 macrophages were stimulated with IFN-γ in order to induce LRRK2 expression. IFN-γ treatments were also carried out in combination with the ERK5 inhibitor XMD and IN-1 in order to suppress LRRK2 up-regulation to identify novel, LRRK2 dependent immune cell functions. Because a great number of genes was found to be differentially regulated, only those were further analyzed that were found to be co-regulated with LRRK2, meaning up-regulated by IFN-γ and reduced upon inhibitor treatment. Yet, it is also

important to consider genes that are negatively regulated by LRRK2, as it has already been reported for NFAT (Liu et al., 2011).

However, the main obstacle in interpreting and validating the data from the transcriptome analysis was a problem with the internal control. Although LRRK2 protein levels were induced upon IFN-γ stimulation and this again was inhibited upon IN-1 or XMD treatment, as expected, this was not the case for mRNA levels. On mRNA level, only an increase upon IFN-γ stimulation was observed, but no inhibitory effect upon ERK5 inhibition. Because these results were again confirmed by RT-PCR, a technical error of the microarray chip could be excluded. This result is surprising because mRNA regulation of LRRK2 was repeatedly observed in earlier experiments. Thus, this could reflect that additionally to transcriptional control, translational and/or post-translational control of LRRK2 protein levels is involved. Indeed, for LRRK2, several pathways of post-translational degradation have been proposed. For example, Carboxyl terminus of HSP70-interacting protein (CHIP) was reported to bind and ubiquitinate LRRK2, thus mediating its degradation (Ko et al., 2009). Moreover, LRRK2 has also been suggested to be degraded via chaperone-mediated autophagy (CMA), a specific form of autophagy restricted to cytosolic proteins that contain an equivalent pentapeptide motif (Orenstein et al., 2013).

In conclusion, this indicates that LRRK2 regulation might be very complex and involves different cues for its turnover, which are not yet completely understood. This should be taken into consideration for the interpretation of the hits of the trancriptome analysis.

Among the candidate gene list, several genes appeared to be very interesting regarding potential roles of how LRRK2 could impact on immune cell function. In this work EOMES, IL1R2 and CD226 regulation were not confirmed by RT-PCR analysis. Yet, they might resemble interesting clues of LRRK2 implications in immune cell regulation. For example, EOMES has been reported to control maturation in both NK cells and T-cells and ablation of EOMES leads to microcephaly (Baala et al., 2007; Gordon et al., 2012; McLane et al., 2013). Yet, a regulation in monocytes/macrophages upon IFN-γ has not yet been described. IL1R2 acts as a decoy receptor for IL-1 and has been associated with many diseases including arthritis, AD and ulcerative colitis, an IBD subtype (Peters et al., 2013). Its expression has been reported in different immune cells, including monocytes and macrophages (Colotta et al., 1996). Because of its antagonizing function on IL-1 signaling, IL1R2 regulation by a LRRK2 dependent mechanism could provide another interesting site of immune cell modulation

(Weber et al., 2010). CD226 plays a role in lymphocyte adhesion and is expressed by monocytes, NK cells and T-cells. Moreover, it was suggested that CD226 activates natural killer (NK) cell-mediated cytotoxicity (Chan et al., 2014). Interestingly, it also has been proposed to regulate monocyte migration (Reymond et al., 2004). This could provide a possible link of how peripheral LRRK2 function could eventually impact on PD pathology. However, as mentioned, these hits have not yet been verified and therefore need further confirmation.

The ubiquitin E3 ligase MARCH1 and the complement components C1QA, C1QB and C1QC were candidate genes verified by RT-PCR analysis. If this relates to protein levels remains to be elucidated. MARCH1 down-regulates the surface expression of MHC class II molecules by directing them to the late endosomal/lysosomal compartment (De Gassart et al., 2008; Thibodeau et al., 2008), thus directly impacting on antigen presentation. This could present a potential new link how LRRK2 could contribute to PD pathology because HLA has recently been identified to be associated with PD (Wissemann et al., 2013). However, in this work, no influence on HLADRα by ERK5/LRRK2 inhibitor treatments was observed by either FACS or RT-PCR analysis.

C1QA, C1QB and C1QC are part of the complement system, which plays an important role in both innate and adaptive immune responses. Specifically, the C1Q proteins are structural elements that are involved in the initiation of the “classical pathway” by forming the antibody immune complex (Dunkelberger and Song, 2010). C1QA, C1QB and C1QC were successfully confirmed to be induced by IFN-γ stimulation and again reduced in their up-regulation upon co-treatment with IN-1 and XMD. However, in primary MDMs only weak induction in case of C1QA and C1QC was observed and inhibitor effects were absent. Thus, the physiological relevance of these complements was not confirmed in this work. Interestingly, in kidneys from LRRK2 KO mice, elevated levels of C1Qs were found (Tong et al., 2010). Although this represents an opposite effect of what has been observed in THP-1 cells in this work, this might indicate that LRRK2 is indeed connected with C1Q regulation and this might differ depending on the cell type. Interestingly, in other neurodegenerative diseases such as AD, the complement system seems to play an important role in disease pathogenesis (Shen et al., 2013). While a connection between PD and complement proteins as part of LBs was already reported 20 years ago, it remains unclear how LRRK2 might

regulate components of the complement system (Yamada et al., 1992). Yet, this avenue might provide important clues for the understanding of neuroinflammatory events in PD.

4.8 Discussion summary

The key finding of this work is the identification of ERK5 as a mediator of LRRK2 up-regulation upon IFN-γ signaling in macrophages (Fig. 4.4). Here, first evidence of a functional link between the MAPK ERK5 and PD risk gene LRRK2 are portrayed. Specifically, this work provides insight of ERK5 as an upstream mediator of LRRK2 induction in IFN-γ signaling. Although the precise integration of ERK5 in the IFN-γ signaling pathway is unknown, this discovery could contribute to the development of treatments for PD. A better understanding of the LRRK2 induction pathway might be of great help in regard of the current discovery that rather total LRRK2 level but not kinase activity is mediating toxicity (Skibinski et al., 2014). Thus, pharmacological modulation of LRRK2 total levels might provide a new approach in order to tackle LRRK2 pathogenicity. However, it remains to be elucidated if this pathway is also conserved in neurons or microglia, which reside in the PD affected areas, as the systematic contributions to disease by macrophages are not yet well understood.

Moreover, pharmacological inhibition of ERK5 revealed that reduced up-regulation of LRRK2 in THP-1 cells was accompanied by morphological alterations as well as dysregulation of the complement proteins C1QA, B and C and the ubiquitin ligase MARCH1 on mRNA level. Further investigations on their respective functional relevance could provide interesting leads on the role of LRRK2 in macrophage biology. While the ERK5-LRRK2 pathway has been verified using a third inhibitor and lentiviral knockdown experiments, genes that were identified from the microarray so far fully rely on a pharmacological approach. Therefore, as the LRRK2 kinase IN-1 inhibitor was shown to have side effects that also affect LPS responses, this data awaits further confirmation and cannot be directly attributed to LRRK2 function (Luerman et al., 2014).

In conclusion, in the light of the emerging role of LRRK2 in immune cell biology, this work provides novel insights into the signaling pathway that causes LRRK2 up-regulation. It is yet to be investigated if ERK5 inhibition in order to control LRRK2 levels and thereby toxicity could be a fruitful approach.

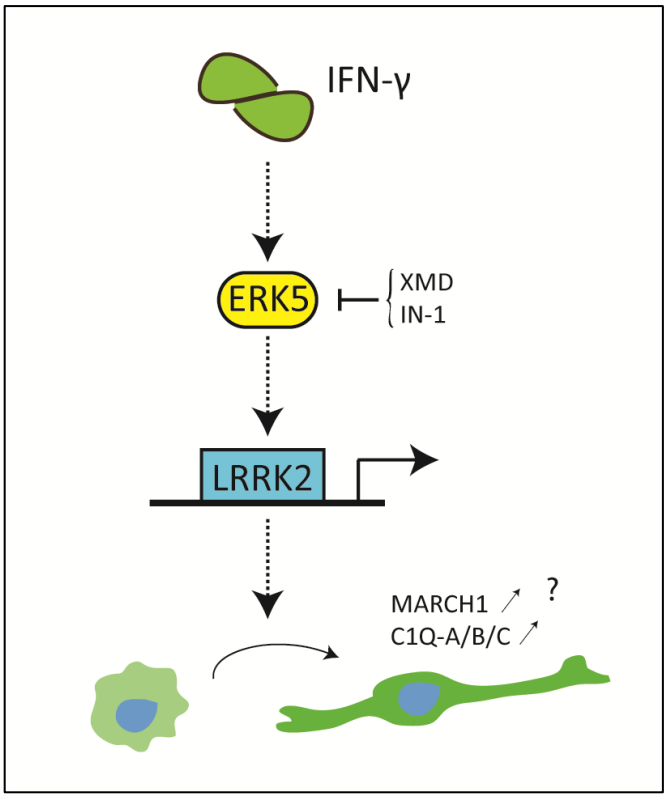

Fig. 4.4: **IFN-γ induces LRRK2 via ERK5 in macrophages**

Depicted is a simplified schematic summary of the results of this work. IFN-γ stimulation of macrophages leads to LRRK2 induction via ERK5, which is accompanied by morphological changes. Moreover, MARCH1 and C1Q-A/B/C complement proteins are transcriptionally up-regulated in THP-1 cells. Pharmacological inhibition of ERK5 by IN-1 or XMD co-treatments reduces these effects.

5 Perspectives

The discovery that ERK5 is a mediator of LRRK2 up-regulation upon IFN-γ signaling in macrophages might open a future avenue to approach LRRK2 pathogenicity. Currently, most attempts to pharmacologically modulate the course of PD aim at inhibiting the LRRK2 kinase activity. This is based on the observation that kinase-dead versions of mutant LRRK2 are less toxic compared to kinase (over-)active forms and that this could at least partially be mimicked by kinase inhibition (Lee et al., 2010a; Yao et al., 2013). Subsequently, this approach lead to the development of a wide range of different LRRK2 kinase inhibitors in the last few years (Deng et al., 2011a; Estrada et al., 2014; Estrada et al., 2012; Kavanagh et al., 2013; Kramer et al., 2012).

However, LRRK2 kinase inhibition might not be a good target after all: First, as also demonstrated in this work with IN-1, great care has to be taken in regard to off-target effects. This is a general problem because practically no inhibitor exists that is specific to only one kinase (Davis et al., 2011). Second, a recent study provides evidence that total levels are more crucial for toxicity than kinase activity (Skibinski et al., 2014). Although this exciting perspective requires more experimental confirmation, it would explain how among six disease-causing mutations only G2019S has increased kinase activity (and even this effect is comparatively weak). In line with this concept, mutations in LRRK2 in either kinase or GTPase domain could impact on LRRK2 stability and therefore on total protein levels, as previously suggested (Herzig et al., 2011). In the light of these developments, modulation of total LRRK2 levels might therefore be a future strategy to tackle the pathogenicity of LRRK2 mutations.

In order to increase chances that modulation or down-regulation of LRRK2 levels is a fruitful endeavor, certain question addressing LRRK2 expression and regulation need to be ruled out first: First of all, it is important to investigate how LRRK2 is regulated in other cell types, especially in neurons and also microglia. This question is accompanied by the general question if PD is a purely “local” pathology or alternatively, how much systemic inflammatory contributions exist. Initial experiments addressing this question would be the combination of genetic mouse models (e.g. LRRK2 overexpression or KO lines) that receive systemically inflammatory insults. In line with the “multiple hit” theory this might provide a model that reveals more about potential pathogenic mechanisms of LRRK2 in PD and neuro-

inflammatory events, while both genetic mouse models of LRRK2 and systemic inflammation so far lack appearance of clear PD pathology (Jeong et al., 2010; Sloan et al., 2012).

Another interesting question is if LRRK2 induction upon IFN-γ is also ERK5 dependent in cells other than macrophages. While the signaling pathways of LRRK2 induction are widely unknown in other cell systems, a better understanding might eventually also contribute clinical relevance. Assuming for example that LRRK2 is indeed regulated by different signaling pathways in different cell types, this might open the possibility to interfere in LRRK2 regulation in a cell-specific manner. In this context, it might also be interesting to rule out contributions of LPS to LRRK2 regulation. Currently, the physiological relevance of LPS in LRRK2 regulation might seem devious because different effects have been observed (Liu et al., 2011; Moehle et al., 2012). While Moehle et al. observed induction of LRRK2 upon LPS stimulation in primary murine microglia, the Liu et al. observed a down-regulation of LRRK2 upon LPS administration in macrophages derived from mice bone marrow (BMDM). This might reflect opposing effects on LRRK2 regulation by the same stimulant on closely related cells that mainly differ only in the localization. BMDM are peripheral cells while microglia cells are brain residents.

To further rule out that the pathway discovered in this work might provide a clinical relevant possibility to modulate LRRK2 levels, it is important to exclude negative side effects. Ideally, modulation of LRRK2 up-regulation has no side effects. ERK5 is a potential target in the treatment of some types of cancer and some studies attribute high specificity and good animal tolerance to XMD (Yang and Lee, 2011). Alternatively, miRNAs targeting ERK5 have been intensively studied and might serve as an option for pharmacological approaches (Noguchi et al., 2011). On the downside, even with a perfect pharmacological approach there might be genes that are co-regulated with LRRK2 upon IFN-γ treatment as suggested by the transcriptome analysis. In conclusion, a better understanding of pharmacological side-effects and co-regulated genes is required in order to therapeutically modulate total LRRK2 levels via a pharmacological approach.

The idea that total levels of LRRK2 are critical in PD pathology as opposed to its kinase function is in principle referring to the possibility of LRRK2 to act as a scaffold, which serves as a platform for the assembly of different proteins to perform certain functions. This ability is based on the different protein-protein interaction domains of LRRK2. Interference with this protein-protein interaction platform in order to block binding of proteins that are

mediating cytotoxicity might be a fruitful future strategy (Rudenko et al., 2012a). Therefore, this represents an interesting alternative to modulate LRRK2 function that is independent of problems related to side effects of pharmacological approaches or crucial co-regulated genes.

A major disadvantage of the transcriptome analysis performed in this study was the lack of comparison to a genetic model, especially LRRK2 knockout cells. Because of the chosen pharmacological approach it was not possible to distinguish between LRRK2 specific and LRRK2 un-specific effects. Accordingly, all hits from the transcriptome analysis could be mediated by inhibition of ERK5, independent of LRRK2. Thus, a LRRK2 knockout model, either mouse primary cells or a KO cell line, in combination with inhibitor treatments would advance this aspect.

LRRK2 has repeatedly been associated to be involved in cytoskeletal dynamics and actin interactions (Habig et al., 2013; Meixner et al., 2011; Parisiadou et al., 2009). Interestingly, LRRK2 kinase activity is also required for microglial morphological remodeling during activation upon LPS stimulation (Moehle et al., 2012). Both LPS and IFN-γ stimulations are regarded to be classical macrophage activating factors (Classen et al., 2009). In the light of the observed morphological alterations in this work, this suggests that LRRK2 might have an important role in regulating morphological alterations during classical macrophage activation. Future investigations to target the underlying mechanism would be interesting, because this function of LRRK2 seems to be conserved among both macrophages and microglia. In conclusion, this might indicate that a common function of LRRK2 in both peripheral and CNS local function may exist.

This work also provides interesting new insights into ERK5 biology in macrophages during IFN-γ signaling. Up to date, ERK5 is not directly connected to PD pathology. Therefore, it would be interesting to study *in vitro* or *in vivo* PD models for a potential modulation of ERK5 by application of ERK5 knockout or overexpression. Mechanistically, it would be interesting to investigate the upstream events that ultimately lead to downstream activity of the MEK5/ERK5 pathway. Similar, analysis of the downstream transcription factor would further complete the picture of how ERK5 is inducing LRRK2 expression. Although seemingly rather unrelated to PD, ERK5 has already been shown to be activated by oxidative stress and playing a neuroprotective role in dopaminergic cells (Cavanaugh et al., 2006). Assuming that ERK5 mediated induction of LRRK2 is a conserved mechanism also in neuronal cells this

might represent a way of how oxidative stress in the aged/disease brain could impact on ERK5 signaling and ultimately result in dysregulation of LRRK2.

Another interesting avenue is to further investigate a potential role of LRRK2 in macrophage autophagy. Because stimulation of macrophages with IFN-γ induces many different cellular responses, it is always speculative to draw causative connections from what might be just correlations. First, LRRK2 already has been connected to play a role in autophagy in different studies/models (Alegre-Abarrategui et al., 2009; Manzoni et al., 2013; Orenstein et al., 2013; Plowey et al., 2008; Saha et al., 2014). Second, macrophages display a high magnitude of LRRK2 up-regulation upon treatment with the potent autophagy inducer IFN-γ (Matsuzawa et al., 2012). Third, the highest levels of LRRK2 are expressed in immune cells, where autophagic processes play important roles such as elimination of pathogens, control of pro-inflammatory signaling, adaptive immunity and secretion of immune mediators (Deretic et al., 2013). Fourth, LRRK2 is genetically associated with CD, which pathogenesis is in turn strongly connected to impaired autophagy (Hoefkens et al., 2013; Liu and Lenardo, 2012; Nguyen et al., 2013). Taken together, this provides ground for the hypothesis that LRRK2 is playing an important yet unknown role specifically in macrophage/microglial autophagy. It would be very interesting to elucidate either peripheral or local mechanisms of the respective immune cells and their potential impact on PD pathology.

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7 Appendix

7.1 Publications and contributions

The results of this dissertation were partially published in:

Kuss, M., Adamopoulou, E., and Kahle, P.J. (2014). Interferon-gamma induces leucine-rich repeat kinase LRRK2 via extracellular signal-regulated kinase ERK5 in macrophages. J Neurochem 129, 980-987.

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