Understanding the role of the Parkinson's associated kinase LRRK2 in inflammation and infection

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To my family

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ABSTRACT

The innate immune system, which mediates pathogen clearance and tissue healing, requires tight regulation to avert self-destruction. The endo-lysosomal system is best known as a network of vesicles that targets particles for degradation and recycling. However, accumulating evidence demonstrates that the endo-lysosomal system is also a hub for innate immune signaling regulation. Defects in this system lie at the core of many pathologies with an inflammatory component including age-associated diseases such as the neurodegenerative disorder Parkinson's disease (PD). Several genetic risk factors for PD have been linked to endo-lysosomal function, one of them being mutations in the kinase Leucine Rich Repeat Kinase 2 (LRRK2). However, the roles of LRRK2 in PD development had not been completely clear.

In my thesis, I demonstrated that the Parkinson´s associated kinase LRRK2 is a negative regulator of the endo/auto-phagolysosome system, and that in humans and mice, gain-of-function mutations in this kinase results in a spontaneous antiviral innate immune response, increased release of inflammatory exosomes, accelerated inflamm-aging via cGAS-STING-IFN signaling axis and cognitive decline.

In summary, this thesis covers both fundamental and translational research aspects centered around the new discovery of LRRK2 as a key regulatory molecule linking the endo/auto-phagolysosome and innate immune system. Furthermore, the results of this thesis contributes to better understanding of the molecular aspect of inflammaging and neurodegeneration which offers a new possibility for treatment strategies.

Keywords: Innate immunity, Endo-lysosomal system, Inflammaging, Neurodegeneration, Parkinson's disease, LRRK2, Extracellular vesicles, STING-IFN axis.

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SAMMANFATTNING PÅ SVENSKA

Det medfödda immunsystemet, som förmedlar eliminering av patogener och vävnadsläkning, kräver noggrann reglering för att förhindra självdestruktion.

Det endo-lysosomala systemet är mest känt som ett nätverk av vesiklar som riktar sig mot partiklar för nedbrytning och återvinning. Men ackumulerande forskning visar att det endo-lysosomala systemet också är ett nav för reglering av medfödd immunsignalering. Defekter i detta system är kärnan i många patologier med en inflammatorisk komponent inklusive åldersrelaterade sjukdomar som den neurodegenerativa sjukdomen Parkinsons sjukdom (PD). Flera genetiska riskfaktorer för PD har kopplats till endo-lysosomal funktion, en av dem är mutationer i kinaset Leucine Rich Repeat Kinase 2 (LRRK2). Men rollen LRRK2 har i PD-utveckling har ännu inte varit helt förstådd.

I min avhandling visade jag att det Parkinsons associerade kinaset LRRK2 är en negativ regulator av endo/auto-fagolysosomsystemet, och att mutationer i detta kinas hos människor och möss resulterar i ett spontant antiviralt immunsvar, ökad frisättning av inflammatoriska exosomer, accelererat inflammationsåldrande via cGAS-STING-IFN-signalaxeln och i kognitiv försämring.

Sammanfattningsvis täcker denna avhandling både fundamentala och translationella forskningsaspekter centrerade kring den nya upptäckten av LRRK2 som en viktig regulatorisk molekyl som kopplar samman endo/auto-fagolysomet och det medfödda immunsystemet. Dessutom bidrar resultaten av denna avhandling till bättre förståelse av den molekylära aspekten av inflammation och neurodegeneration, vilket ger nya möjligheter för behandlingsstrategier.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Öberg M**, Maric I, Strömberg A, Myers C, Saffarzadeh N, Fabrikova D, Fabrik I, Rivas-Galvez L, Skibicka K, Akanbi-Kurzawa M, Paul G, Gekara NO, Härtlova A. *Parkinson's disease kinase LRRK2 accelerates aging, associated inflammation, and neurodegeneration through the STING-IFN-I axis*

Submitted Manuscript

II. Fabrik I, Bilkei-Gorzo O, **Öberg M**, Fabrikova D, Fuchs J, Sihlbom C, Göransson M, Härtlova A. *Lung macrophages utilize unique cathepsin K-dependent phagosomal machinery to degrade intracellular collagen.*

Life Sci Alliance. 2023 Jan 25;6(4):e202201535.

III. Kurzawa-Akanbi M, Whitfield P, Burté F, Bertelli PM, Pathak V, Doherty M, Hilgen B, Gliaudelytė L, Platt M, Queen R, Coxhead J, Porter A, **Öberg M**, Fabrikova D, Davey T, Beh CS, Georgiou M, Collin J, Boczonadi V, Härtlova A, Taggart M, Al-Aama J, Korolchuk VI, Morris CM, Guduric-Fuchs J, Steel DH, Medina RJ, Armstrong L, Lako M. *Retinal pigment epithelium extracellular vesicles are potent inducers of age-related macular degeneration disease phenotype in the outer retina.*

J Extracell Vesicles. 2022 Dec;11(12):e12295.

CONTENTS

ABBREVIATIONS

AGS	Aicardi–Goutières syndrome
ALRs	AIM2-like receptors
ATP	Adenosine triphosphate
BMDMs	Bone marrow derived macrophages
cGAS	Cyclic GMP-AMP Synthase
CLRs	C-type lectin receptors
COPA	Coatomer protein subunit-α
COPD	Chronic obstructive pulmonary disease
CSF	Cerebrospinal fluid
CTD	C-terminal repeat domain
DAMPs	Damage Associated Molecular Patterns
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ssDNA	Single stranded DNA
ELISA	Enzyme-linked immunosorbent assay
EVs	Extracellular vesicles
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
IFN	Interferon
IFN-Is	Type I interferons
IRF3	Interferon regulatory factor 3
ISGs	Interferon stimulated genes
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
LRRK2	Leucine Rich Repeat Kinase 2
MAMPs	Microbe Associated Molecular Patterns
M-CSF	Macrophage colony stimulating factor
MEFs	Mouse embryonic fibroblasts
mtDNA	Mitochondrial DNA
NAFLD	Non-alcoholic fatty liver disease
NFκβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	NOD-like receptors
NLRP3	NOD-like receptor family pyrin domain-containing protein 3

NTA	Nanoparticle Tracking analysis
PD	Parkinson´s disease
PI3K	Class III phosphoinositide-3 kinase
PRR	Pattern Recognition Receptors
qRT-PCR	Quantitative reverse transcript polymerase chain reaction
RA	Rheumatoid arthritis
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I like receptors
RNA	Ribonucleic acid
SAVI	STING-associated vasculopathy with onset in infancy
SDS	Sodium Dodecyl Sulfate
SLE	Systemic lupus erythematosus
STING	Stimulator of Interferon Genes
TBK1	TANK-binding kinase 1
Tbp	TATA-binding protein
TIR	Toll-IL-1 receptor
TLR	Toll Like Receptors
2.3cGAMP	2'3'-cyclic GMP-AMP

1 THE INNATE IMMUNE SYSTEM

1.1 INNATE IMMUNITY AND INFLAMMAGING

The increase of human life span is one of the major achievements of our century. However, this longevity has also brought new challenges to maintain the quality of life in the elderly[1]. Aging is accompanied by a gradual loss of physiological homeostasis, from the cellular to organismal level, and is a major risk factor for developing many chronic pathologies including age-related diseases such as cancer, metabolic disease, and neurodegeneration[2]. Therefore, there is an urgent need for a more systemic understanding of age-associated dysfunctions and for the development of therapeutic strategies to combat the effects of aging and thus enhance healthy life span and quality of life[1].

A decline of immune functions is associated with the aging process. The progressive loss of tissue homeostasis and the accumulation of damaged cells are directly associated with a chronic state of background inflammation also known as low grade chronic inflammation or inflammaging. In the majority of age-associated diseases, patients exhibit an underlying chronic inflammatory state, characterized by a local infiltration of inflammatory cells such as macrophages, and elevated levels of pro-inflammatory circulatory cytokines[3]. However, the underlying mechanisms in the transition of an initially protective to a chronic, harmful inflammatory response are unknown.

Through evolution, the maintenance of homeostasis is a key function of the innate immune system. This is achieved by sensing and response to markers of infection or endogenous stress by germ-line encoded pattern recognition receptors (PRRs) localized on the cell

surface or intracellular compartments. PRRs recognize conserved microbe-associated molecular patterns (MAMPs). In addition, PRRs can also detect endogenous danger molecules (DAMPs) generated by the host either as biproducts of metabolic pathways, or as a result of tissue damage. By detecting these outside or inside signals, the triggering of PRRs receptors leads to the transcriptional induction of pro-inflammatory cytokines, type I interferons (IFN-Is) and activation of inflammasomes, which act to eliminate danger, promote healing, and activate adaptive immunity[4, 5].

As we age, an accumulation or inadequate elimination of altered self-molecules, leads to aberrant activation of distinct innate immune pathways and development of low-grade chronic inflammation. These altered self-molecules can be derived from cell debris or metabolic biproducts or appear under pathologic conditions such as cancer and neurodegeneration.

However, the triggers and the mechanisms of chronic inflammation are incompletely understood. For example, it was previously shown that DNA damage or inefficient lysosomal degradation of misplaced DNA in the cytosol promotes the activation of cytoplasmic DNA sensors[6]. This activation leads to the production of type I IFN, which drives cellular senescence in the context of aging. Importantly, many neurodegenerative diseases are associated with mutations in proteins important for lysosomal delivery[7]. During my PhD, I have identified that one of the regulators associated with the endo/auto/phago-lysosomal compartment, the Parkinson's associated kinase Leucine Rich Repeat Kinase 2 (LRRK2), regulates peripheral systemic inflammation before the occurrence of signs of neurodegeneration. This finding offers a new possibility on how to manipulate the endo-lysosomal pathways in immune cells in the periphery to prevent neurological decline as we age **(Manuscript I)**.

1.2 THE STORY BEHIND THE PATHOGEN RECOGNITION RECETORS CONCEPT

The principles of innate immune signaling and the idea of PRRs were mounted by Charles Janeway in 1989 when he asked the simple question: "*How are T and B cells first alerted when an intruder has invaded the body?"* He pointed out that it has to be a biochemical process during an initial inflammatory response, a function of the innate immunity, which has been considered as highly unspecific and was largely ignored at that time[8, 9]. Janeway suggested an evolutionary conserved system, based on germline-encoded receptors. Proposing that those receptors recognize molecular patterns on pathogens but not on metazoan host cells and that they control the activation of the adaptive immune system lymphocytes[10].

Janeway's ideas were at first based entirely on hypothetical thoughts. These ideas were then extensively studied in his lab by his post-doc at the time, Ruslan Medzhitov; a student from Moscow who was fascinated by Janeway´s hypothesis presented in the Cold Spring Harbor Symposium: *"Approaching the Asymptote? Revolution and Evolution in Immunology"*[10]. Medzhitov used biochemistry approaches and cloned a human homolog of toll, a protein known to be involved in dorsoventral pattering in fruit flies[11, 12] . He also showed that by transfecting a constitutively active mutant version of the protein into a human cell line the NFκβ pathway (known to be involved in inflammation) was activated, and NFκβ controlled inflammatory cytokines were produced[11].

Later on, Bruno Lemaitre in Jules Hoffmans lab in Strasbourg demonstrated that the Toll receptor protein and its downstream signaling pathway are essential components of the innate immune function in fruit flies[13]. And later, Bruce A. Beutler's lab made a loss of function screen which identified Toll-like receptor 4 (TLR4) as a sensor for its ligand, bacterial lipopolysaccharide (LPS), in an experimental mouse model of septic shock[14].

The discovery of Toll-like receptors (TLRs), and their role in distinguishing common microbial molecular patterns was awarded by the Nobel prize in Physiology or Medicine in 2011. The award went to Jules Hofmann (for the discovery of Toll-innate immune function) and Bruce A. Beutler (for the discovery of TLR4, the first-known mammalian receptor protein of the innate immune system)[15]. Charles Janeway unfortunately passed away before he could get the award.

1.3 THE PATHOGEN RECOGNITION RECEPTORS (PRRS)

Since the time of Janeway, Hoffman, and Beutler, several different PRRs and their ligands have been identified. They are divided into two main groups based on their localization: the endomembrane and/or the cytosolic innate immune sensors. These include transmembrane Toll-like receptors (TLRs), C-type lectin receptors (CLRs), cytosolic Nod-like receptors (NLRs), AIM2-like receptors (ALRs), RIG-I like receptors (RLRs), and cytosolic DNA receptor (CDRs)[16]. The different pathogen recognition receptors are pictured in **figure 1**.

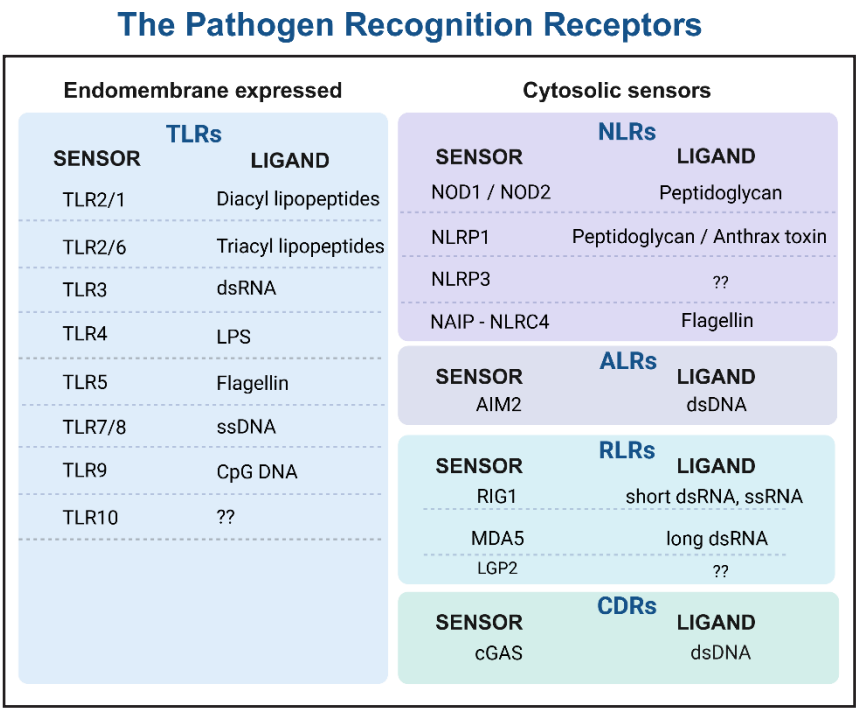

Figure 1. Overview of the Pathogen Recognition Receptors (PRRs). *The different groups of PRRs, the sensors and their ligands. Created with Biorender.com.*

1.3.1 TOLL LIKE RECEPTORS

The main group of endomembrane expressed PRRs are the TLRs. There are thirteen different TLRs (1-13) in mammals and ten of those (1-10) has been found in humans. They can be further divided into two groups depending on their localization: cell membrane and intracellular TLRs. The cell membrane TLRs include TLR1, 2, 4, 5, 6 and 10 while the intracellular TLRs include TLR3, 7, 8 and 9. Intracellular TLRs are expressed on the endoplasmic reticulum (ER), endosomes and lysosomes. The different TLRs are activated by different ligands, with LPS as an activator for TLR4 being the most well-known. Other examples include dsRNA which activates the endomembrane expressed TLR3[17]. The different TLRs and their ligands are pictured in **figure 2**.

When the signaling cascade is initiated by the binding of the ligand to the leucine rich repeat (LRR) domain on the receptors, it leads to recruitment of an adaptor protein to the C-terminal repeat domain (CTD) of the receptor. Those adaptor proteins possess a toll-IL-1 receptor (TIR) domain which transfers the signal via a series of phosphorylation events. The two main signaling pathways are then mediated via the adaptor protein Myd88 or TRIF. The Myd88 pathway is utilized by all TLRs except TLR3 and leads to the expression of pro-inflammatory genes while the TRIF pathway is utilized by TLR3 and TLR4 and leads to the expression of Type I interferons (Type I IFNs)[17]. The Myd88 and TRIF signaling pathway are pictured in **figure 2**.

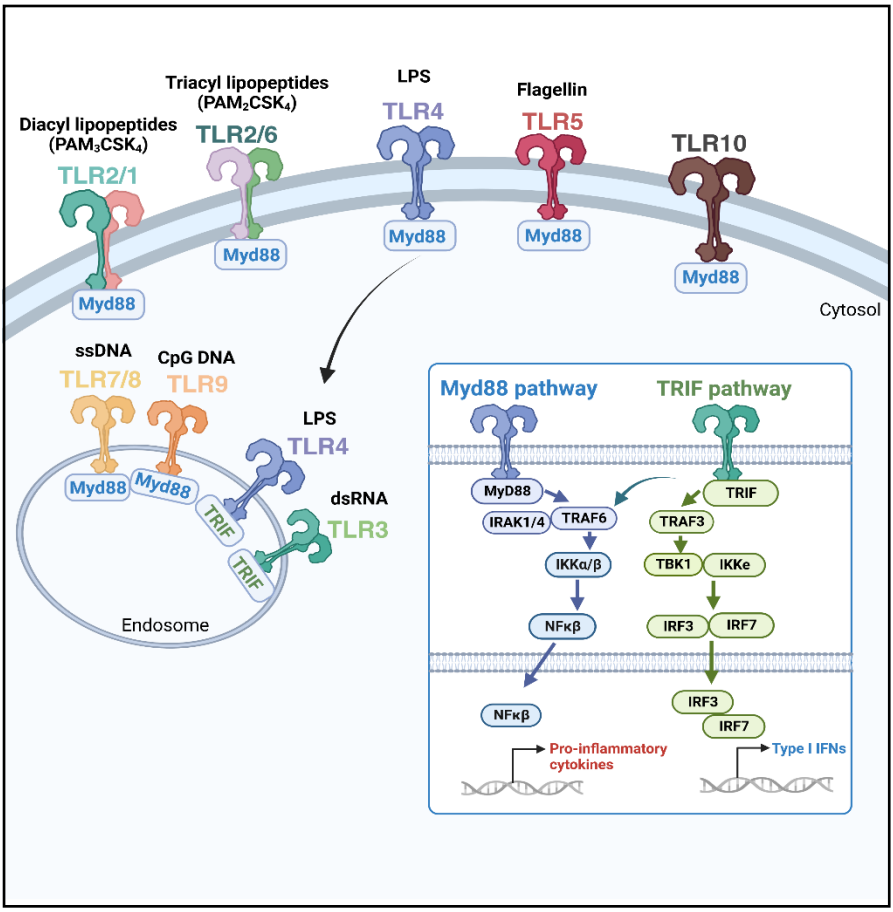

Figure 2. The Toll Like Receptors (TLRs) localization, ligands, and signaling. *The TLRs expressed on the cell surface and endomembranes with their respective ligands and signaling adaptor protein. In the box the Myd88 and TRIF pathway are pictured with arrows showing the route of phosphorylating events ending in translocation of the effector protein into the nucleus for initiation of transcription of pro-inflammatory cytokines and Type I IFNs respectively. Created with Biorender.com.*

1.3.2 NOD LIKE RECEPTORS (NLRS)

During innate immune responses to infection, cytosolic PRRs can be involved in the recognition of microbial-derived molecules. While some of the cytosolic PRRs induce transcriptional activation of cytokine expression, a distinct subset of cytosolic receptors

belonging to the Nucleotide-binding oligomerization domain-like receptors, NOD-like receptors (NLRs) regulate the secretion of certain cytokines as well as cell death. NLRs are cytosolic sensors which recognize both bacterial components as well as endogenous stress molecules such as ATP. The family includes NOD1 and NOD2 which recognize bacterial peptidoglycans and different types of inflammasomes[16]. Inflammasomes have two major functions, namely regulating the maturation and secretion of IL-1β and IL-18 family of cytokines and the initiation of a certain form of cell death, called pyroptosis. Both are mediated by the common component of inflammasomes, the protease caspase-1[18].

Of the different inflammasomes, the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) inflammasome is the most well studied. The functional regulation of an active inflammasome in a cell is a two-step process, namely priming and inflammasome assembly. The first requires a non-activating stimulus to promote the transcriptional expression of key components of the inflammasome. Once primed, NLRP3 can respond to its stimuli and assemble the NLRP3 inflammasome. These stimuli can be encountered during infection or pathologic conditions in the body that can promote the formation of an inflammasome in the absence of infection[19]. Although, the precise mechanism of the NLRP3 inflammasome activation still remains unclear, phosphorylation and actin-reorganization are crucial processes involved in the formation of NLRP3 inflammasome assembly[20, 21].

The NLRP3 inflammasome has recently gained increasing attention regarding its role in the progression of age-related diseases such as neurodegenerative and metabolic disorders[22-24]. Misfolded proteins and aberrant accumulation of certain metabolites accompanying age-related disease serve as endogenous DAMPs that have been shown to be direct activators of the NLRP3 inflammasome[25].

1.3.3 INTRACELLULAR NUCLEIC ACID SENSORS

Intracellular RNA and DNA sensors are key players of the innate immune response to viruses and other pathogens causing the secretion of type I interferons (IFN-I) as well as the expression of IFN-stimulated genes (ISG)[26]. In addition, we now know that they also recognize RNA or DNA of host origin, as a danger signal. Therefore, their activation can lead to an effective antiviral response but also to immunopathology if their functions are uncontrolled.

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are essential in sensing RNA of either viral or host origins.[27]. In this family, there are three different RNA sensors: RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). They are known to have distinct roles and substrate specificity, where RIG-I detects single-stranded RNA and short double-stranded RNA[16]. Dysregulation of RIG-I pathways may lead to severe autoimmune diseases such as Aicardi–Goutières syndrome (AGS), systemic lupus erythematosus (SLE) and other rare interferonopathies[28]. Therefore, activation of RIG-I receptors at steady state is tightly regulated by multiple mechanisms including conformal changes, posttranslational modifications, and autophagy[27].

Most mammalian cells can also detect exogeneous DNA. Bacterial, viral, or synthetic double-stranded DNA (dsDNA), and even dsDNA isolated from mammalian cells, can be recognized if it gets access to the cytosol and is over 40–50 bp in length[29]. Interestingly, over several years, a number of cytosolic DNA sensors were identified. The first one was DAI identified by Takaoka et al in 2007[30, 31], followed by AIM2 inflammasome[31-33], the DNA sensor IFI16[31, 34], the DNA sensor DDX41[31, 35] and the DNA sensor cyclic GMP-AMP Synthase (cGAS)[31, 36, 37]. However, several of these have later been questioned in their importance as DNA sensors. DAI deficient mice respond normally to plasmid-based DNA stimuli and several cells

that lack DAI show normal type I IFN induction[31, 38]. AIM-2 has been shown to not be important for the induction of type I IFNs[31, 39]. The role of DDX41 and IFI16 is so far not completely clear, but they have been suggested to work upstream of the Stimulator of Interferon Genes (STING), the initiator of Type I IFN signaling pathway[31, 40]. The essential sensor for DNA-mediated immune response, irrespective of cell type or DNA sequence, is cyclic-GMP-AMP (cGAMP) synthase (cGAS)[36, 37].

cGAS detects dsDNA present in the cytosol. When double-stranded DNA binds to the enzyme cGAS in the cytoplasm, it converts ATP and GTP into 2′3′ cyclic GMP-AMP (cGAMP) which binds and activates STING on the endosome, STING is then translocated to the Golgi leading to the phosphorylation of TANK-binding kinase 1 (TBK1) which then phosphorylates the transcription factor Interferon regulated factor 3 (IRF3). Upon phosphorylation, IRF3 translocates to the nucleus where it initiates the transcription of type I IFNs[36, 37]. The cGAS-STING signaling pathway is pictured in **figure 3**.

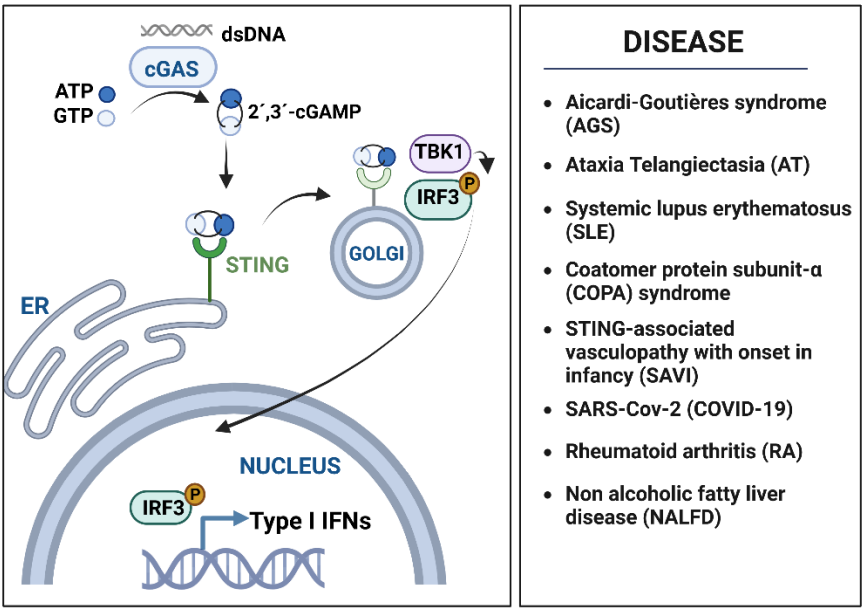

Figure 3. The cGAS-STING signaling pathway and its implication in disease. *The signaling of the cGAS-STING pathway from the binding of dsDNA to the transcription of type I IFNs and the diseases where cGAS-STING signaling has been implicated to play a role. Created with Biorender.com.*

1.4 SELF-DNA AS A DANGER SIGNAL

In the past it was believed that only foreign DNA from viruses could initiate anti-viral responses. This idea has later been challenged and it is now clear that self-DNA is also recognized.

In 2006 Kawane et al. showed that mice with induced deficiency in DNASE II develop chronic polyarthritis due to their inability to degrade mammalian DNA from apoptotic cells and erythroid precursors. This results in TNFα production which activate cells in the joint to produce inflammatory cytokines[6]. This paper demonstrated the importance of DNAses in regulating the amount of DNA present in the cytoplasm, therefore avoiding inappropriate immune activation. Next study by Yang et al. linked the DNA exonuclease; Three prime repair exonuclease 1 (*Trex1*), involved in Aicardi-Goutières syndrome, to cell division. TREX1 is the most abundant 3′–5′ DNA exonuclease in mammalian cells that specifically cleaves single-stranded DNA (ssDNA)[41]. Mutations in TREX1 DNase activity lead to the accumulation of self-DNA that cause a spectrum of type I IFN-dependent autoinflammatory and autoimmune diseases such as Aicardi-Goutières syndrome (AGS) and Systematic Lupus Erythematosus (SLE)[42]. Following reports identified that the DNA repair factors Rad51 and RPA prevents ssDNA from going to the cytosol and that TREX1 is recruited to the outer nuclear membrane to ensure immediate degradation of ssDNA[43, 44]. Further, in 2012 Ahn et al. linked STING to the inflammation driven death in DNASE II deficient mice, showing that embryonic lethality was rescued by STING deficiency[45]. These findings describe a novel mechanism that links pathways of DNA replication and repair with innate immune activation in the pathogenesis of autoimmunity.

Interestingly, in 2015 Härtlova et al. showed that lack of DNA repair or uncontrolled DNA damage can lead to the leakage of DNA into the cytoplasm where it activates the cytosolic DNA sensing STING-

mediated pathway. In other words, this showed how DNA damage alerts the innate immune system to remove damaged cells to keep tissues healthy[46]. Moreover, it shows a functional crosstalk of two ancient defense systems: the innate immunity and DNA repair. These data were further supported by detection of chromatin fragments in the cytoplasm by cGAS-STING signaling pathway[47, 48].

Notably, two other studies further identified the molecular mechanisms regulating release of nuclear DNA into the cytosol. In a mouse model of monogenic autoinflammation they showed that damaged DNA, arising from genome instability following exogenous DNA damage or spontaneously in human cancer cells, accumulates in the form of micronuclei that are released into the cytosol where they recruit cGAS and induce type I IFN[49, 50]. Additionally, it has been shown that during cellular senescence chromatin fragments can pinch of from intact nuclei and activate cytosolic cGAS-STING[51-53]. Altogether, these data provide a potential mechanistic explanation of how the immune system alerts our body via the cGAS-dependent DNA sensing pathway to detect and potentially remove a danger.

Interestingly, a recent study demonstrated how our gut microflora primes the immune system via activation of the cGAS-STING pathway for efficient anti-viral host response. Antibiotic treatment disrupts our gut microflora which leads to disruption of the cGAS-STING pathway activation and therefore increased the viral burden[54].

Originally it was anticipated that DNA sensing is excluded from the nucleus where the host cell's genomic DNA reside. In addition to nuclear DNA and DNA that has leaked into the cytosol the cGAS-STING signaling pathway can also be initiated by mitochondrial DNA (mtDNA)[55-58]. And we now also know that cGAS is not only present in the cytosol but also in the nucleus of the cell where it can sense nuclear DNA[59-61].

Together these findings show that the cGAS-STING pathway is important not only for viral infections but also for cell intrinsic

mechanism such as DNA replication, where DNA strand breaks are involved, and that dysfunctions in nucleic acid metabolism and mitochondrial integrity can activate the pathway. A summary of the papers showing a role of self DNA as danger signal is shown in **figure 4**.

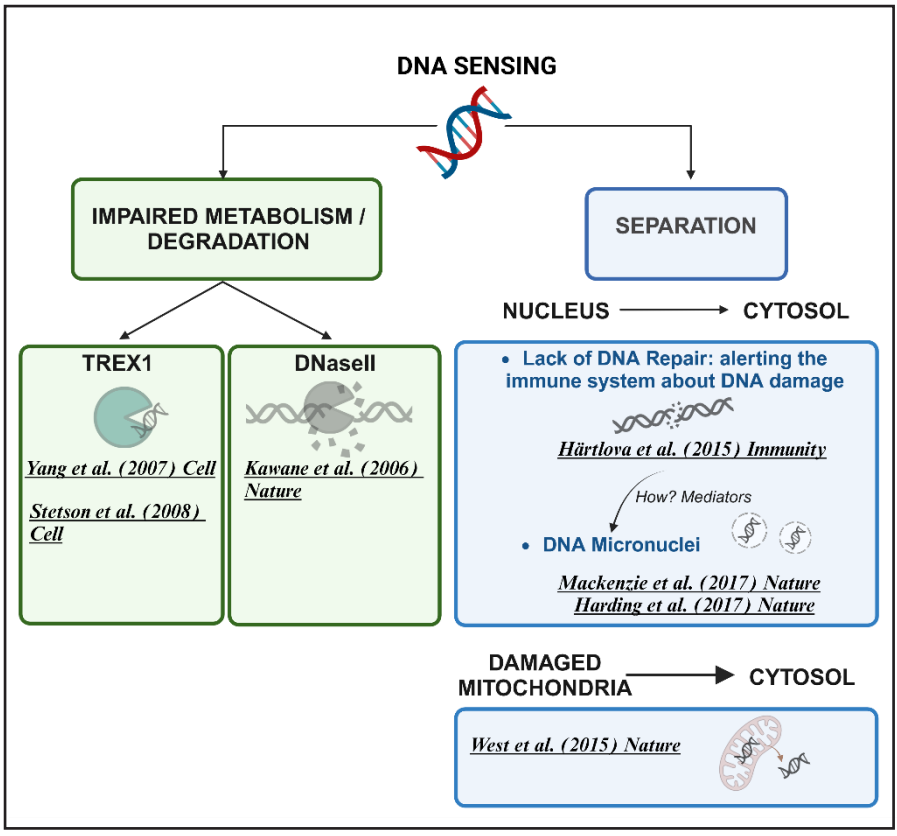

Figure 4. Self-DNA as a danger signal. *The important discoveries showing mechanisms that leads to that our own DNA is sensed and causes inflammation. Created with Biorender.com.*

1.5 CGAS-STING PATHWAY IN DISEASE

The cGAS-STING pathway have during the last decade been recognized in a multitude of autoimmune and autoinflammatory diseases including Rheumatoid arthritis (RA)[62-64], Systemic lupus erythematosus (SLE)[65-68], Aicardi–Goutières syndrome (AGS)[47, 69], and STING-associated vasculopathy with onset in infancy (SAVI)[70-72]. The pathway has also been recognized in cancers where it has both tumor-suppressive and tumor-promoting functions, in lung diseases such as Coatomer protein subunit-α (COPA) syndrome[73, 74], and Chronic obstructive pulmonary disease (COPD)[74, 75], as well as in metabolic diseases such as non-alcoholic fatty liver disease (NAFLD)[74, 76]. Mechanistically, the diseases are often attributed to increased signaling via the cGAS-STING pathway which can come both from dysfunctions in nucleic acid metabolism, mitochondrial integrity, or dysregulation of the signaling pathway itself, resulting in enhanced type I IFNs. This is the case for SLE, AGS and SAVI, which together with other genetic diseases that leads to enhanced type I IFNs have been grouped as type I interferonopathies[77].

Although we now know that the cGAS-STING pathway is also important also for sensing our own DNA and that this pathway is implicated in several diseases we still do not fully understand the role of this pathway at steady state. DNA damage and mitochondrial distress is known to increase with ageing, making the role of the cGAS-STING pathway in aging and age-related diseases an interesting aspect[78]. A recent study demonstrated the importance of cGAS-Sting pathway in age-related neuroinflammation and neurological decline. Mechanistically, they demonstrated an age dependent increase in type I IFNs in the hippocampus which was dependent on mitochondrial DNA (mtDNA)-mediated cGAS-STING activation in microglial cells[79]. In support of these data, I discovered that the STING-IFN signaling axis is important for enhanced age-related priming of our immune system and is

associated with immune cellular senescence which precedes the occurrence of neurological decline. Further, I demonstrated that the key mechanisms behind the enhanced age-related chronic inflammation (inflammaging) is an age-dependent decline in the function of the endo/exo-lysosomal system **(Manuscript I)**. Altogether, these data support an interesting hypothesis that targeting the immune system rather than directly targeting neurons is a strategy that could be employed to slow down brain-ageing and neurodegeneration.

2 ENDO/EXO-LYSOSOMAL SYSTEM

2.1 ENDO/EXO-LYSOSOMAL SYSTEM

The endo-lysosomal system is a network of interconnected vesicles by which cells engulf macromolecules and deliver them into the lysosomes for degradation and recycling[80]. The engulfment of extracellular macromolecules occurs via endocytosis or phagocytosis[81-86]. Phagocytosis is an essential process of innate immune response by which the microbe is internalized, delivered to the phagosome, killed, and digested to process antigens for presentation to T cells, to induce adaptive immunity. After internalization, the newly formed phagosome is constantly remodeled by fusion with early and late endosomes and with lysosomes, a process known as phagosome maturation. These changes ultimately deliver the engulfed pathogen into the terminal degradative compartments known as phagolysosomes[87]. Endocytosis follows similar principles, components of microbes, debris etc. are internalized, delivered to endosomes which mature and fuse with lysosomes in a process called endo-lysosomal fusion [88, 89]. On the other hand, the engulfment of unwanted macromolecules including damaged organelles inside the cell occurs via a 'self-eating' process called autophagy[90, 91]. The endo-lysosomal system performs cleaning up not only by degradation but is also closely connected and in a constant state of equilibrium with the exosome system – a secretory pathway that prevents the build-up of cellular wastes inside the cell by releasing them extracellularly via exosomes. Extracellular vesicles (EVs) are nanosized vesicles (30–100nm of diameter) derived from the endocytic pathway, and function not only as "garbage trucks" but also as "delivery trucks" enabling cells to transfer biomolecules to distal recipient cells **(Manuscript III)**[92-95] An overview of the endo-lysosomal system is shown in **figure 5**.

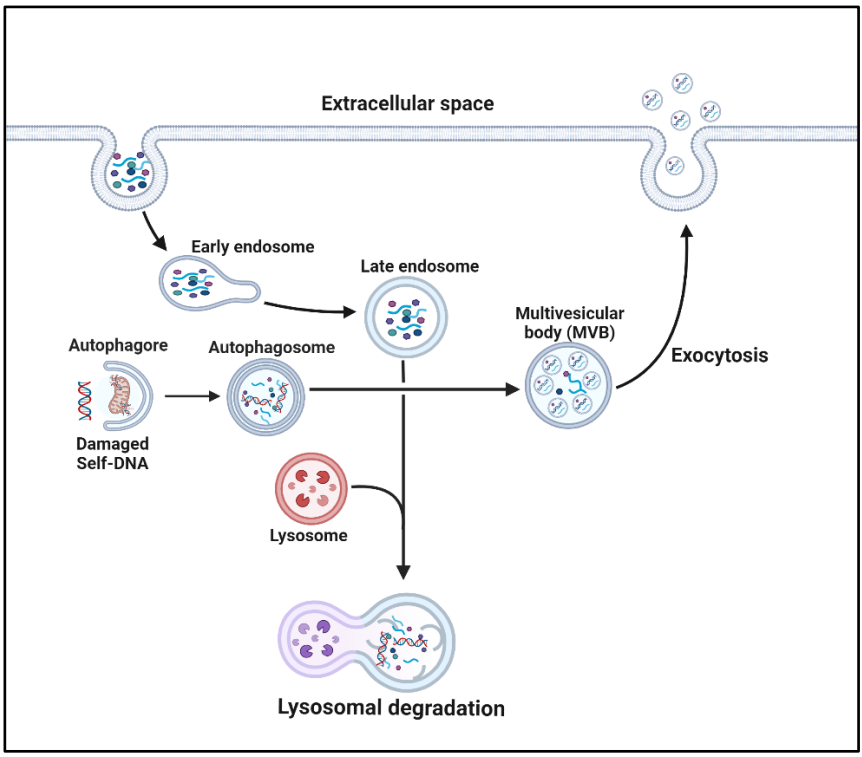

Figure 5. Endo-lysosomal system. *The process of endocytosis, phagocytosis and autophagy and the maturation culminating in fusion with the lysosome. As an alternative to fusion with lysosomes the endosomal components can be released in the form of extracellular vesicles. Created with Biorender.com.*

The different subtypes of EVs have become more defined by where they originate, for example exosomes refer to vesicles of endosomal origin while micro vesicles are vesicles that are derived from the plasma membrane. This distinction is important since it affect the composition of the EV[96]. However, the isolation techniques for different types of extracellular vesicles are so far limited as they mainly rely on differentiating different sizes of vesicles through either ultracentrifugation or size exclusion chromatography-based methods (more about this in methods section)[97]. And even though vesicle labelling techniques are being developed for differing the types of extracellular vesicles this definition can be difficult to make.

EVs have been detected in several of the bodily fluids including serum, urine, saliva, and cerebrospinal fluid (CSF)[98, 99]. Due to the possibility to detect EVs in liquid biopsies there has during the last decade been a growing interest in the role of EVs as cellular communicators and in the process of several diseases including both cancer[100-105], neurodegeneration[106] and macular degeneration **(Manuscript III)**[95].

The secretion of extracellular vesicles has been reported to increase with decreased endo-lysosomal clearance[107, 108]. Rab-GTPases have been implicated in this where Rab7 promote the fusion of late endosomes with lysosomes and thereby decrease exosome release while Rab31 has been shown to promote exosome release[92, 109, 110].

While initially thought of as a clean-up system, the endo-lysosomal system is emerging as a key hub for innate immune signaling and regulation. For instance, endo-lysosomal membranes are key signaling platforms for TLRs, especially those leading to the production of type I interferons (IFN-Is)[111]. Moreover, by targeting immune stimuli as well as innate signaling complexes for degradation, the endo-lysosomal system plays a critical role in the attenuation of many inflammatory pathways[112]. Unsurprisingly, defects in endo-lysosomal system have a causal link to many autoimmune inflammatory diseases including age-associated neurodegenerative diseases such as Parkinson´s disease (PD)[1].

Several of the genes with mutations associated with PD has been shown to regulate the endo-lysosomal system[113, 114]. GBA1 is the gene for glucocerebrosidase and is a genetic risk factor for PD. Mutations in this gene also causes Gaucher disease, a lysosomal storage disorder where deficiency in glucocerebrosidase activity leads to the accumulation of glucocerebrosidase lipids[115]. Parkin/Pink1 in important for the recruitment of autophagy components to mitochondria to target them for lysosomal degradation in a process called mitophagy[116, 117]. The most common PD-associated gene Leucine Rich Repeat Kinase 2 (LRRK2) is also emerging as a regulator of the endo-lysosomal system. Rab GTPases, in particular Rab8, and Rab10, are known physiological substrate for the protein Leucine-rich repeat serine/threonine-protein kinase 2 (LRRK2), these proteins are involved in transport of vesicles from the Golgi to the plasma membrane[118-120].

3 LRRK2: A NEW REGULATOR OF THE ENDO/EXO-LYSOSOMAL AND INNATE IMMUNE SYSTEMS

Leucine-rich repeat serine/threonine-protein kinase 2 (LRRK2) is a 285-KDa multi-domain protein with a unique multiple-domain architecture consisting of Armadillo repeats (ARM), Ankyrin repeats (ANK), leucine-rich repeats (LRR), a Ras of complex proteins (Roc) GTP-domain, a C-terminal of Roc (COR), a kinase domain and WD40 repeats[121] **(Figure 6)**. Mutations in LRRK2 are associated with the late-onset Parkinson's disease (PD), inflammatory bowel disease[122, 123], and anti-bacterial immunity[83, 124, 125]. However, the normal physiological role of mammalian LRRK2 is still not completely understood. All pathogenic PD-related mutations in this gene result in hyperactivation of the LRRK2 kinase[118, 121, 126]. The most common mutation associated with PD causes a change of a glycine to a serine in the 2019 position located in the kinase domain of the protein, this mutation is known as G2019S and gives similar symptoms as sporadic Parkinson's disease (Parkinson´s without known cause)[127, 128]. The incomplete penetrance of LRRK2 mutations – the majority of carriers may never develop PD - highlights the importance of other factors, such as lifestyle, environmental toxins, changes in the gut microbiome, and possibly infection in developing LRRK2-associated PD.

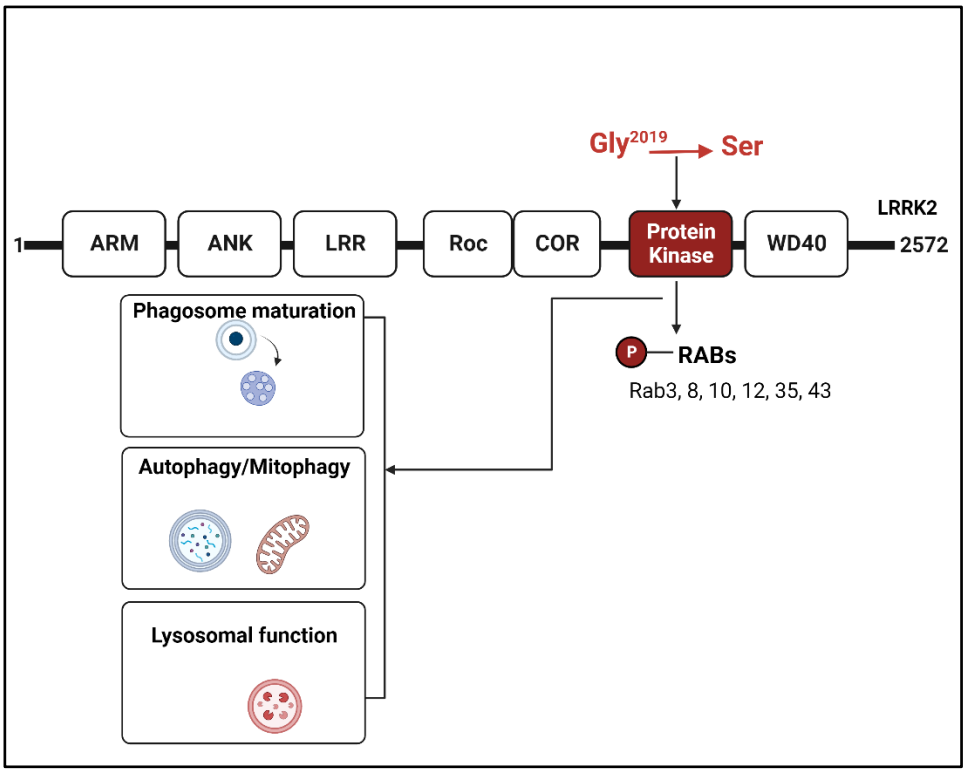

Figure 6. Structure of Leucine Rich Repeat Kinase 2 (LRRK2). *The structure of LRRK2 showing the different domains and the G2019S gain of kinase function mutation found in the kinase domain of the protein. Created with Biorender.com.*

3.1 LRRK2: LINKS TO INFECTION AND INFLAMMATION

Functionally, LRRK2 has been described in mitochondrial dynamics[129], lysosome positioning,[130-132], and autophagy[133-136], a theme similar for Parkinson's disease genes[137]. A potential role of LRRK2 in vesicle trafficking was further substantiated by the discovery that the small GTPases Rab8 and Rab10 are bona fide endogenous substrates of LRRK2[118]. These small GTPases are regulators of membrane trafficking and are also involved in cellular processes essential for immune cell activity such as phagocytosis, exocytosis, and antigen presentation[138]. This is in line with the

emerging evidence pointing to LRRK2 as a modulator of inflammation through a role in immune cells both in the central nervous system and in the periphery.

It is important to note that LRRK2 is highly expressed in peripheral organs such as the lung, spleen and kidneys, relative to the brain[139, 140]. Moreover, LRRK2 is highly expressed in different immune cells including neutrophils, monocytes, macrophages, and B cells indicating a potential role of LRRK2 in the immune system[141]. A role of LRRK2 in regulating inflammation and pathogen defense has been suggested by reports implicating LRRK2 in several bacterial infections.

Polymorphisms in the LRRK2 gene have been linked to increased susceptibility to leprosy[142]. This is supported by the observation that a loss of LRRK2 enhances *Mycobacterium tuberculosis* control in lung[83]. The study demonstrated that LRRK2 regulates phago-lysosomal fusion via recruitment Class III phosphoinositide-3 kinase (PI3K) complex and Rubicon (RUN domain protein as Beclin-1 interacting and cysteine-rich containing) to the phagosome[83]. LRRK2 has also been implicated in the control of the enteric pathogen *Salmonella typhimurium* via NLRC4 inflammasome regulation in macrophages from Lrrk2 KO mice[125]. Salmonella is known to highjack the endo-lysosomal system to replicate in the host by expressing endosomal markers on the salmonella containing vacuoles[143]. Interestingly, while loss of LRRK2 has been shown to be protective to Mycobacterium tuberculosis, it is detrimental for *Salmonella typhimurium* infection[83, 125]. Consistently, LRRK2 ablation increases the susceptibility to intestinal infection with *Listeria monocytogenes* due to a resulting lysozyme deficiency[124]. These data indicate that the outcome of infection might depend on the route of infection or that LRRK2 mediates different functions in different immune cells.

The mechanisms used by salmonella and Mycobacterium Tuberculosis to form their replicative niche seem to be partly dependent on targeting of Rab GTPases by the bacteria[143-145]. Recently endomembrane damage from membranolytic agents as well as infection with mycobacterium tuberculosis, listeria monocytogenes and candida albicans has been shown to activate LRRK2 leading to the recruitment of pRab8 and membrane repair components to damaged phagosomes[146], further highlighting a role of LRRK2 in the endo-lysosomal system.

Given the evidence discussed implicating LRRK2 in the regulation of immune responses towards pathogens. The role of LRRK2 in peripheral immune cells might contribute to the dysregulation of brain macrophage function, and thereby lead to the development of PD.

Parkinson's disease (PD) is a progressive neurodegenerative disorder mainly leading to motor symptoms due to the specific loss of dopaminergic neurons in the midbrain. Current treatments are purely symptomatic. There are no pharmacological agent available or clinical praxis that can halt or even slow the disease progress. This is largely due to the incomplete understanding of the fundamental pathogenic mechanisms. In addition to the classical motor-symptoms including resting tremor, postural instability and rigidity[147], PD patients exhibit several non-motor symptoms and comorbidities[148]. These include other neurological symptoms such as cognitive impairment and depression but also non-neurological symptoms such as GI dysfunction and constipation. Interestingly, these non-motor symptoms precede the occurrence of the motor symptoms[149-151].

One of the co-morbidities that has been linked to PD is Type II diabetes where epidemiological studies suggest that type 2 diabetes is a risk-factor for PD development[152-155]. Other co-morbidities that have been linked to PD include Anemia, Cancer, and Hypertension[155]. Although the direct link between those diseases is

not known this suggest the presence of shared pathways. More importantly it shows that in Parkinson's there are systemic changes which precede neurodegeneration, indicating that the disease may start in the periphery[155].

The idea that PD starts outside the nervous system was postulated already in 2002 by Braak et al. Due to their observation that the earliest PD lesions can be observed in the olfactory bulb and then seem to follow an structural ascending path they suggested that PD starts outside the nervous system at mucosal sites[156]. Peripheral inflammation is now known to be present in PD patients[157-160], and animal studies have shown that peripheral inflammation can contribute to the loss of dopaminergic neurons[161]. However, the mechanisms by which PD related peripheral inflammation contribute to neurodegeneration and if the peripheral inflammation is beneficial or detrimental for disease progression has previous remained largely unclear[1].

In agreement with previous findings, in my study, I showed that macrophages with the *LRRK2* gain of kinase function (*LRRK2*^{GoF}) are more protected against virus infection compared to healthy control **(Manuscript I)**[162, 163]. Our results describe that Parkinson´s monocyte derived macrophages with or without *LRRK2*^{GoF} mutation exhibit enhanced spontaneous systemic type I IFN which primes the immune cells for enhanced anti-viral response **(Manuscript I)**. We further established that this systemic activation of type I IFNs was dependent on signaling through the cGAS-STING pathway. And that the systemic response preceded the development of neuroinflammation and neurological decline **(Manuscript I)**.

Recent research indicates that signaling through cGAS-STING activates LRRK2 and that stimuli that perturbs lysosomal function converge on the same pathway to activate LRRK2[164]. I further showed that LRRK2 gain of function mutation leads to a dysfunction in endo-lysosomal fusion and results in increased number of extracellular vesicles which stimulate the cGAS-STING pathway leading to increased production of type I IFNs systemically **(Manuscript I)**. Together these findings highlight the role of LRRK2 in not only innate immune responses and inflammation but also in endo-lysosomal function, and that dysfunction of endo-lysosomal systems may promote inflammation.

3.2 LINKING THE INNATE IMMUNE SYSTEM AND ENDO-LYSOSOMAL SYSTEM TO AGING AND NEURODEGENERATIVE DISEASE

Chronic inflammation is a hallmark of neurodegenerative disease but also of aging in general[165, 166]. Aging is also the largest risk factor for developing PD. With the reduction of endo-lysosomal function during aging and its role in inflammation, this may be one of the drivers of age-related inflammation and neurodegeneration. But how the endo-lysosomal system contributes to inflammation, aging and neurodegeneration, and the molecular mechanisms involved in this are so far not completely clear.

Endo-lysosomal function has been shown to decrease with aging where autophagy and lysosomal function plays a key role. Although the role of those processes in aging and cellular senescence are still not completely understood there have been findings indicating a link between them.

Autophagy is known to decrease during aging and there have been studies showing that upregulation of autophagy genes can prolong the lifespan of mice[167, 168]. Further, knock out (KO) of certain autophagic genes such as Atg5 and Atg7 is lethal as whole-body KO and as tissue specific KO show signs of ageing in affected tissue[12, 168-170]. However, several other studies have failed to prolong lifespan with upregulation of single autophagic genes,[171] showing that ageing is more complex. Autophagy is also closely linked to lysosomal function. Lysosomal mass is increased in senescent cells, and lysosomal storage has been shown to be disrupted with ageing of yeast cells[172-174]. Further, several lysosomal genes have been shown to be downregulated with ageing[175].

In the start of writing this thesis, the paper by Ablasser group mentioned previously was published. This paper showed the importance of the cGAS-STING pathway in ageing, neuroinflammation, and neurodegeneration[79]. Our data agree with this paper showing that cGAS-STING pathway was necessary for the neurological deficits and increased type I IFNs we detected in aged mice. We further showed that the increase in type I IFNs was accelerated with the PD associated LRRK2 gain of function mutation and in PD patients **(Manuscript I)**.

Additionally, we showed that PD patients and mice with the PD-associated LRRK2 kinase gain-of-function mutation had a systemic increase of type I IFNs. Further, this systemic inflammation preceded the occurrence of brain inflammation and neurodegenerative features. Therefore, our Manuscript supports the notion that PD may start outside the brain with systemic features that later leads to changes in the CNS and to neurodegeneration.

We then coupled this age-related increase in type I IFNs to endo-lysosomal function by showing that the G2019S mutation resulted in impaired endo-lysosomal fusion. Further, aged mice with the $LRRK2^{GoF}$ mutation as well as human PD patients had increased levels of DNA containing extracellular vesicles in serum and CSF **(Manuscript I)**. Together, these findings link dysfunctions in the endo-lysosomal system to the neurodegenerative Parkinson's disease via the innate immune signaling pathway cGAS-STING.

More importantly, although accelerated in mice with the $LRRK2^{GOF}$ mutation, the decrease in endo-lysosomal fusion, the increased release of extracellular vesicles, and the associated STING-dependent increase in type I IFNs could also be detected with ageing in wild type (WT) mice. These findings indicate that these are general mechanisms associated with the ageing processes that can be accelerated by the $LRRK2^{GOF}$ mutation.

4 METHODOLOGICAL CONSIDERATIONS AND LIMITATIONS

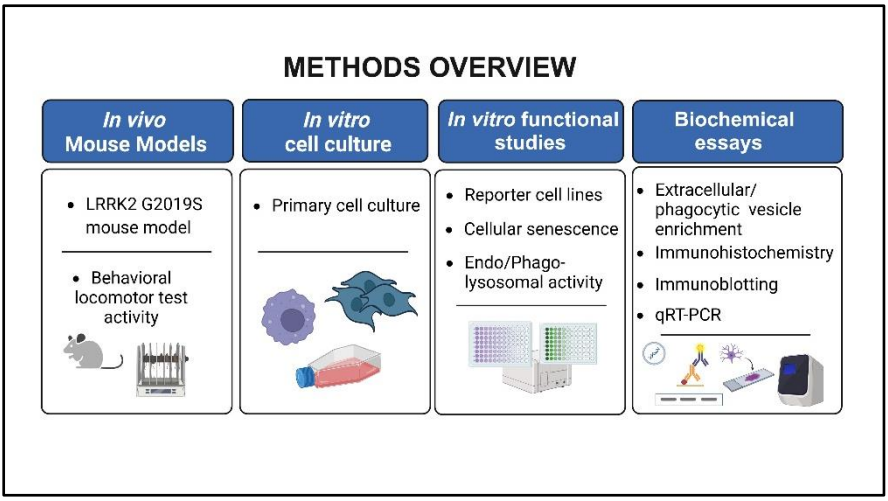

Figure 7 Methods Overview. *Summary of the methods used in this thesis. Created with Biorender.com.*

4.1 ANIMAL MODELS OF INNATE IMMUNE SIGNALLING AND LRRK2

It is important to use adequate models to study the research question that is going to be addressed. In many situations this can be done by the usage of cell-lines in in-vitro studies but on many occasions, it requires appropriate in-vivo models. In **Manuscript I** and **II** mouse models have been chosen due to the necessity of in-vivo models to study the immune-brain axis as well as tissue resident macrophages. All animal experiments have been done with regards to the 3 Rs, to Replace with cell lines, when possible, to Reduce the number of animals used and to Refine the methods used to minimize suffering for the animals.

In our study, we were interested in the role of LRRK2 in neurodegenerative disease **(Manuscript I)**. Mutations in LRRK2 that increase the kinase activity of the protein are associated with Parkinson´s disease (PD)[176]. The most common mutation is the G2019S mutation, located in the kinase domain of the protein[127, 128]. This mutation is a risk-factor for PD, but not causative. Therefore, other currently unknown factors are necessary for PD development. Mouse models for studying LRRK2 include transgenic mouse models where mutant or WT LRRK2 is overexpressed, these models have had mixed results and concerns have been raised that overexpression of LRRK2 may lead to additional changes[177]. Therefore, knock-in (KI) models with the pathogenic LRRK2 variants have been developed.

One of these KI models is the C57BL/6-Lrrk2tm4.1Arte ($LRRK2^{GOF}$) mice which have the human G2019S mutation inserted to their genome and thereby show an increased kinase activity of LRRK2. These mice serve as a good model to study how increased kinase activity in LRRK2 regulates different molecular pathways. We have seen that these mice show neurodegenerative features such as reduced motor function when aged **(Manuscript I)**. However, their symptoms do not fully manifest as the human form of Parkinson´s disease. This further highlight that other currently unknown factors contribute to disease development.

There are also knock out (KO) models of LRRK2, where the LRRK2 gene is missing. This can be useful to study the role of LRRK2 in different pathways. However, since the pathogenic mutations in LRRK2 are mainly those that increase kinase activity of the protein, and since LRRK2 has functions not related to the kinase activity, the Kinase dead models of LRRK2 may be more useful than KO models, but they are not readily available.

In **Manuscript I** and **II**, we studied innate immune signaling by utilizing different KO models where innate immune signaling

adaptor proteins are genetically deleted. In **Manuscript I**, we further crossed mice with deletions in the different immune signaling pathways with mice with the Parkinson's associated G2019S mutation to study the contribution of the immune signaling pathways to the G2019S phenotype. As mentioned in the introduction the main signaling adaptor proteins for TLRs are Myd88 and TRIF, KO-models for both of these are available. KO-models are also available for MAVS and STING, these models lack signaling through the MAVS and STING signaling pathway during sensing of RNA and DNA respectively. Together, these models allow us to pinpoint the signaling pathways involved.

4.2 BEHAVIORAL STUDIES

Behavioral studies can be used to study neurological function in mice. There are many different behaviors that can be studied, in **Manuscript I** we studied anxiety like behavior and neurological motor function. This was done in collaboration with Ivana Maric in Karolina Skibicka group. Anxiety-like behavior was studied by open-field test. Motor function was tested by open-field and Rotarod test.

4.2.1 OPEN FIELD TEST

In open field test, mice are placed in the middle of a square box with an open top. The mice are recorded for 30min and the time that the mice spend in the center and in the peripheral part of the box is measured. The distance travelled in each part of the box can also be recorded. The principle of this method is that mice are by nature curious and will explore the new environment of the box that they were placed in. When anxious, the mice will spend more time in the periphery of the box, close to the walls than in the center of the box. By measuring the time in the center and the periphery, one can get an estimate about the anxiety level of the mouse. However, since this method is based on the movement throughout the box the results can be confounded by the motor function and the energy level of the

mice, this can be somewhat corrected for by looking at the distance travelled and by adding additional behavioral studies[178]. An overview of the Open field test is shown in **figure 8**.

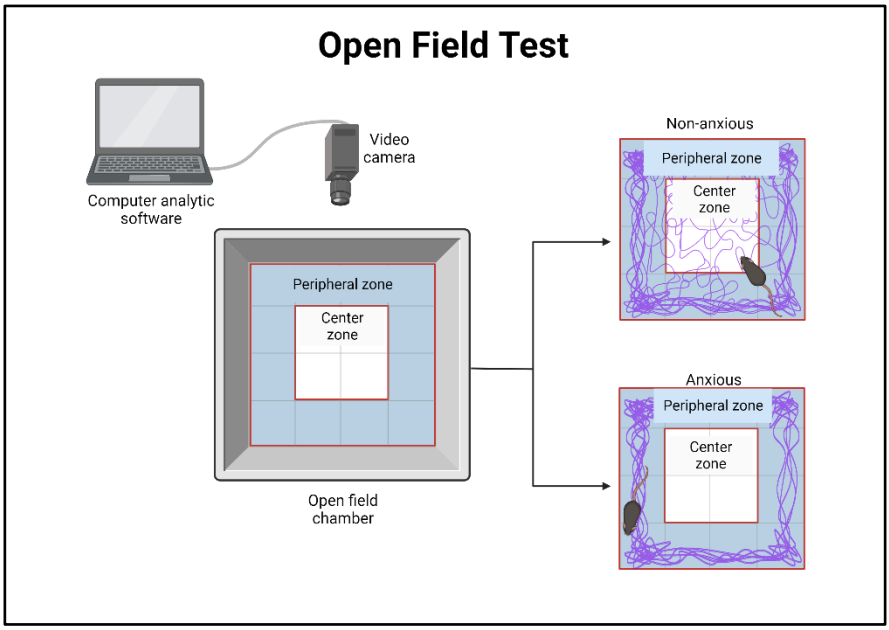

Figure 8. Open field test illustration. *Mice were placed in the center of an open field chamber and recorded while exploring the arena freely. The time and distance moved in the peripheral and center zone was measured. Created with Biorender.com.*

4.2.2 ROTAROD TEST

Rotarod test is a method to study motor function of mice. The mice are placed on a rotating rod that is rotating with increasing speed. The time and speed at which the mice fall off is recorded. For this test the mice need to be trained to use the rotating rod, it is therefore important to give the mice the same amount of training before starting the experiment. Further, the results of this test may be skewed by the size of the mice due to the design of the rotarod machine. Mice that are big may be touching the side walls of the machine and therefore be slightly restricted in their movement. This is important to keep in mind when using the rotarod test[179]. An overview of the Rotarod test is shown in **figure 9**.

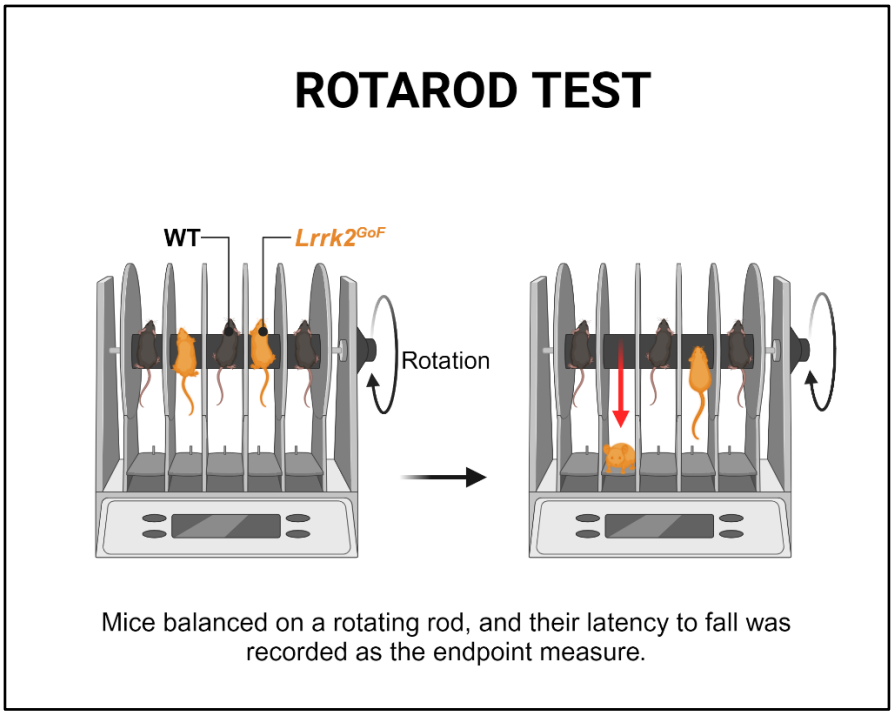

***Figure 9. Illustration of the Rotarod test**. WT (black) and LRRK2^{GOF} (orange) mice were placed on a rotating rod and the latency to fall mas measured. Adapted from Lubrich et al. 2022[180] using Biorender.com.*

4.3 CELL CULTURING AND ISOLATION OF PRIMARY CELLS FOR STUDYING BIOLOGICAL PROCESSES

Cell-lines are a good tool to study molecular mechanisms within cells, they are simple to maintain, multiply and come with less ethical considerations than animal studies. However, cell-lines are often produced from one original source and then expanded. The constant replication of the cells allows for genetic changes[181-183]. This means that a cell-line poorly reflects the cells that are found in the organism. One way to increase the resemblance to the cells in the host is to use primary cells, these are isolated from the organism directly and maintained during shorter periods. Those cells show higher resemblance with the cells in the host but do come with increased ethical aspects[183]. Primary cell lines have been used in both **Manuscript I**, **II**, and **III**.

4.3.1 BONE MARROW DERIVED MACROPHAGES

In **Manuscript I** we used bone marrow derived macrophages (BMDMs) for studying immune signaling. BMDMs are obtained by isolating the bone marrow from the femur and tibia of the hind legs of a mouse. The bone marrow myeloid progenitors are then differentiated into macrophages by addition of differentiation factors. Macrophages can be differentiated by macrophage colony stimulating factor M-CSF. Another method to differentiate the myeloid progenitors into macrophages is by using supernatants from L929 cells. L929 cells are mouse fibroblasts that produce the macrophage stimulation factor. The supernatant is generated by culturing the cells and then collecting the supernatant in batches. Studies have shown that L929 conditioned media results in macrophages that produce less pro-inflammatory cytokines upon stimulation with LPS, produce more IL-10, and have a higher metabolic activity[184]. Therefore, we in **Manuscript I**, and **II** used L929 conditioned media for macrophage differentiation. One of the

limitations with the use of L929 is that there are batch effects, different batches will have different potency. To circumvent this, we test the different batches by looking at the phenotype, inflammatory markers, and the phagocytic capacity of the cells.

4.3.2 MICROGLIA

Microglia are innate immune cells of the brain. They can be isolated by either (1) enzymatic or (2) non-enzymatic methods. Enzymatic isolation relies on the use of enzymes such as DNAse and Collagenase to dissociate the cells, this is done by incubation at 37°C. Non-enzymatic methods instead use mechanical force such as Dounce homogenizer to dissociate the cells. The non-enzymatic methods avoid the 37°C incubation which can change the activity of cells but can induce mechanical stress on cells[185, 186]. These methods can also be combined to increase cell yield. One of the main issues with these isolation methods is the activation of the microglia cells and the purity of the sample, to ensure that only microglia cells are isolated. Following the cell dissociation step myelin is removed to increase purity. This is done via centrifugation using Percoll gradients.

Today there are well established protocols and kits available to increase the purity of microglia isolation and minimize cell activation[185, 186]. In **Manuscript I** and **II**, we used well established kit (Miltenyi biotech) to isolate microglia. The purity of the sample can be further increased by the use of Cd11b positive magnetic beads. The magnetic beads are added to the cells and the mixture is then placed in a column placed on a magnet. This allows unlabeled cells to pass the column and be washed out whereas the CD11b+ cells, in this case the microglia cells, remain stuck to the column. These can then be collected. Beads were used in **Manuscript I** and **II** to increase purity of microglia.

4.3.3 SPLENOCYTES

In **Manuscript I** we used splenocytes from mice. Splenocytes are isolated from the spleen by smashing the spleen, remove the erythrocytes via lysis and culturing the remaining cells. Those cells will be a mixture of cells from the spleen, but myeloid cells can be enriched via the usage of cd11b positive magnetic beads.

4.3.4 MONOCYTE AND MONOCYTE-DERIVED MACROPHAGES

In **Manuscript I** we used monocytes and monocyte-derived macrophages. These are isolated from the blood of mice via centrifugation steps and removal of erythrocytes. Monocyte-derived macrophages are generated by culturing the monocytes in L929 conditioned medium for 5 days.

4.3.5 MOUSE EMBRYONIC FIBROBLASTS

Mouse embryonic fibroblasts (MEFs) can be proliferated for long periods and therefore used as a cell line. They are easy to maintain in culture and can be isolated from embryos from mice with different genetic mutations. Therefore, they are a good tool to study basic cellular mechanisms and how the genetic mutations affect those. Further, MEFs respond to inflammatory stimuli such as LPS and express the different PRRs. However, studies have shown that MEFs respond with different activation pattern and lower cytokine levels when stimulated with LPS compared to the response in BMDMs[187]. Therefore, studies in BMDMS are also needed when studying innate immune signaling.

4.4 REPORTER CELL LINES AS TOOL FOR STUDYING INTERFERONS

Interferons are present in very low levels in absence of infection. Therefore, they can be hard to detect by conventional method such as Enzyme-linked immunosorbent assay (ELISA). To circumvent this, we have utilized reporter cell lines from Invivogen **(Manuscript I).**

These reporter cell lines have an alkaline phosphatase coupled to the IRF-inducible promoter. Thereby presence of type I interferons or cytosolic dinucleotides will lead to the transcription of this alkaline phosphatase. The amount of the alkaline phosphatase can be measured by the addition of a colorimetric substrate called Quanti-blue which changes the color from pink to blue in proportion to the amount of alkaline phosphatase[188].

The benefit with this method is that presence of very low levels of interferons can be detected. The main drawback is that it is not a direct method, meaning that it does not measure the direct concentration of interferons. The principles of the reporter cell line used is shown in **figure 10**.

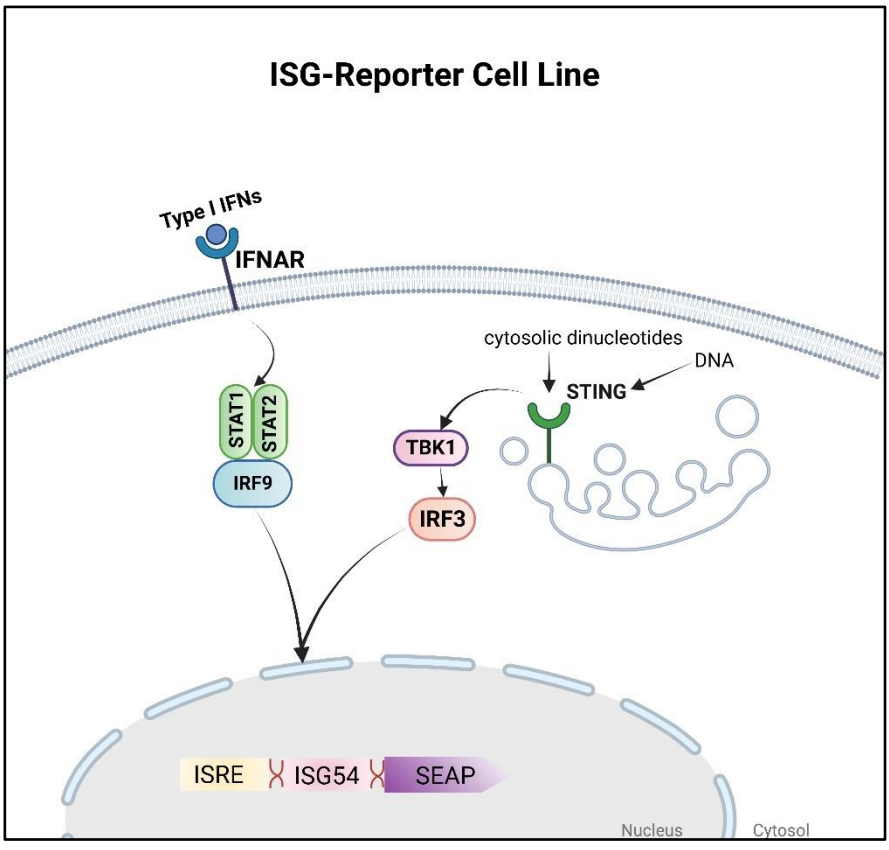

Figure 10. Principle of ISG-reporter cell line to measure type I IFN activity. *Figure showing the principle of ISG-reporter cells where type I IFNs and or cytosolic di-nucleotides can activate SEAP inserted on the ISG promoter. Created with Biorender.com.*

4.5 CELLULAR SENESCENCE

Cellular senescence can be studied by several methods. The simplest method is to look at cellular proliferation. When cells go into senescence, they stop proliferating meaning that the number of cells will increase more slowly. However, additional methods are needed to look at senescence. One is to look at β-galactosidase activity. When cells go into senescence, they increase their β-galactosidase activity. There are available kits to measure β-galactosidase in cells through colorimetric assays[189]. Further, one can look at senescence genes. Cells that are in senescence upregulate several genes including cyclin dependent kinase inhibitor 2B (CDKN2B), a cell growth regulator controlling cell cycle G1 progression[190]. Both of these methods were used in **Manuscript I** to study cellular senescence.

4.6 FUNCTIONAL ASSAYS TO STUDY PHAGOCYTOSIS AND ENDO-LYSOSOMAL FUSION

As mentioned earlier phagocytosis and endo-lysosomal fusion are necessary for the degradation of pathogens and important functions of innate immune cells. To study those events, one may use silica beads which can be coated with proteins to facilitate phagocytosis and endo-lysosomal processing and labelled with fluorescent dyes. The advantage of this is that it allows for monitoring phago/endo-lysosomal proteolysis activity, and the method is relatively simple. This method was used in **Manuscript I** and **II**.

4.7 PHAGOSOME ISOLATION

Phagosomes were isolated with the use of magnetic beads in **Manuscript II**. Briefly 1µm carboxylated magnetic beads were added to macrophages in culture. Cells were then lysed and homogenized by the help of a Dounce homogenizer. The cytosolic fraction was isolated from the homogenized cells by separating it from the nuclear fraction and cell debris via centrifugation. The cytosolic fraction containing the beads containing phagosomes were placed on a magnetic holder to isolate the phagosomes[86]. An overview of the phagosome isolation process is shown in **figure 11**.

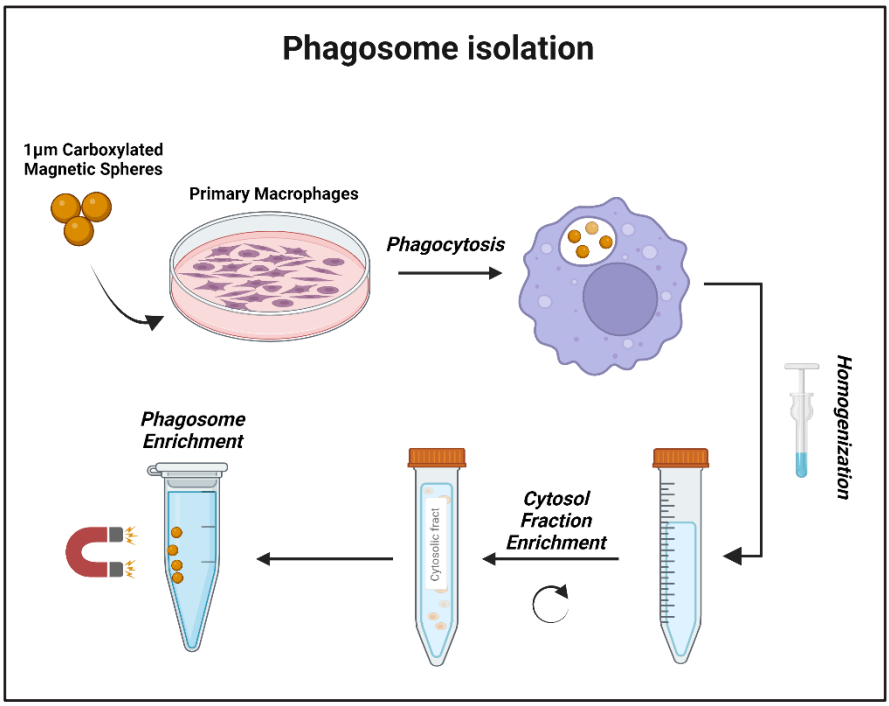

Figure 11. Phagosome isolation. *Overview over the phagosome isolation process. 1 µm carboxylated beads are added to cells in culture. The cells are lysed, homogenized, and the cytosolic fraction is then isolated via centrifugation. Phagosomes are isolated via isolation of magnetic beads. Created with Biorender.com.*

4.8 EXTRACELLULAR VESICLES, ISOLATION, AND CHARACTERIZATION

4.8.1 EXTRACELLULAR VESICLE ISOLATION METHODS

Extracellular vesicles can be isolated by several different methods. Traditionally differential ultracentrifugation has been the method of choice[97]. Here the vesicles are isolated via a series of ultracentrifugation steps for different durations. The main limitation of this method is that it requires an ultracentrifuge, is tedious, and require highly trained personnel. Another method to isolate extracellular vesicles are by the usage of density gradient centrifugation. This method also requires centrifuges capable of high speeds but less steps are needed[97].

Lately newer methods based on size exclusion chromatography have been developed. Here specialized size exclusion chromatography columns are used that separate the particles in the sample according to size. Due to the presence of a matrix, different sized particles will be eluted at different time point allowing for collection of different fractions. The extracellular vesicles will be found in some of those fractions and can be collected for downstream applications[97]. The advantage of this method is that no ultracentrifuge is needed, it is less tedious though still requiring some time, this time could however be reduced by atomization steps.

Extracellular vesicles can also be isolated via commercially available kits. Those kits are based on precipitation principles where a chemical mix is added that precipitates the vesicles which can then be pelleted at lower centrifugation speeds and then resuspended. The main advantage of this method is that it is quick and easy. The main disadvantage is that since the principle is based on precipitation, vesicles of different sizes will precipitate as well as other particles present in the sample. This will decrease the purity of the

extracellular vesicles and limit the possibility to identify different types of extracellular vesicles[97]. However, it can still give rough information about the presence of extracellular vesicles in a sample. The principles of exosome isolation are shown in **figure 12**.

Both size exclusion chromatography and commercially available isolation kits were used in **Manuscript I**. In **Manuscript III** size exclusion chromatography-based methods were used.

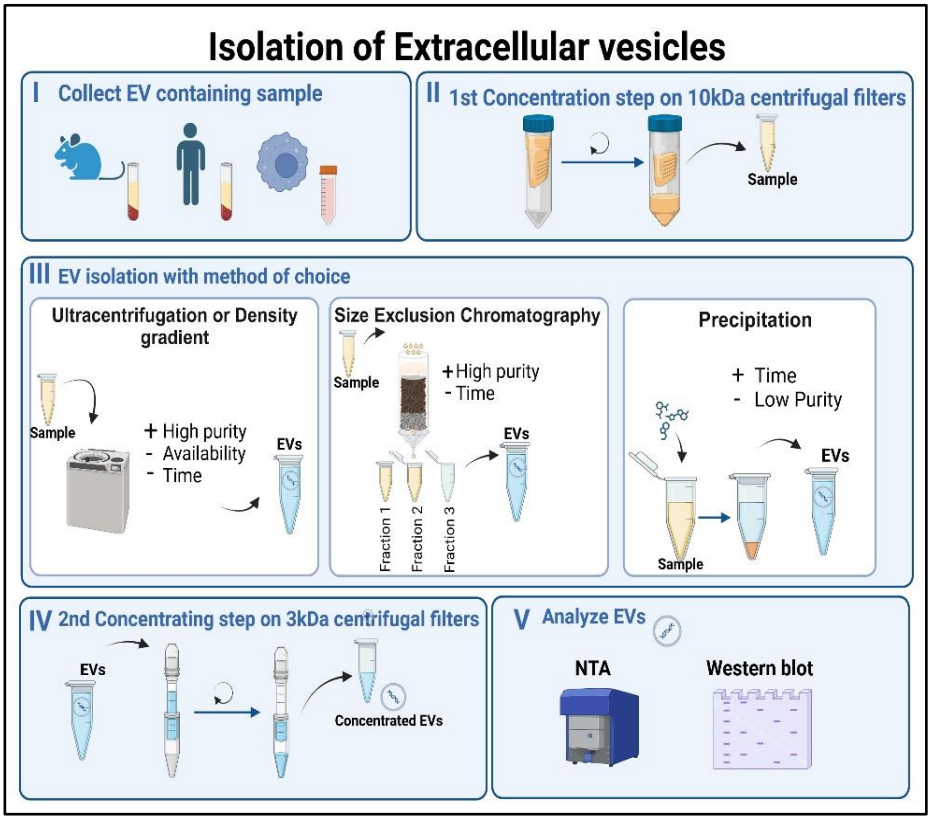

Figure 12. Principles of Extracellular Vesicle isolation. *Scheme showing the principal steps for isolation of Extracellular Vesicles (EVs). I Collection of samples containing EVs. II Concentrating sample on centrifugal units. III Isolation of EVs with isolation method of choice. IV Concentrating isolated EVs on centrifugal units. V Analyze isolated EVs and use in desired application. Created with Biorender.com.*

4.8.2 EXTRACELLULAR VESICLE CHARACTERIZATION METHODS

Extracellular vesicles can be studied and characterized by different methods to determine for example the number, size and type of extracellular vesicles.

Nanoparticle Tracking analysis (NTA) can be used to measure the number and size of the vesicles. The sample is injected into the machine where a camera takes pictures and count the number of particles and analyses their size. The size and calculations are based on reference beads that are used for normalization when starting up the machine. The benefit with this method is that it is relative quick and easy to use. The main limitation is that this machine measures all particles within the sample and is thereby unable to identify if the particles detected are extracellular vesicles or some other particles of similar size. Further, it can be difficult to obtain consistent values when measuring the same sample and between different times of measurement. Therefore, it is important to measure the samples to be compared at the same occasion and to measure each sample multiple times[191].

Western blot can be used to identify extracellular vesicles. Extracellular vesicles are enriched in the markers CD81and CD63, while they do not express nuclear markers such as Histone H3[192, 193].

Another effective method to study extracellular vesicles is via electron microscopy. However, the use of electron microscopes require highly trained personnel and electron microscopes are not readily available[193].

4.9 WESTERN BLOT AS A METHOD TO STUDY SIGNALLING PHOSPHORYLATION EVENTS

Western blot is a semi-quantitative antibody-based method to study protein amounts within cells. In principle, the samples are lysed to solubilize the proteins and treated with Sodium dodecyl sulphate (SDS) to unfold them. The proteins are then separated according to size by gel-electrophoresis. Smaller proteins will pass through the gel more easily and thereby migrate further whereas larger proteins will migrate slower through the matrix. Following the gel-electrophoresis, the proteins are transferred to a membrane with the use of electrical forces. The proteins can then be stained with specific antibodies. Usually a two-antibody system is used with a primary antibody specific for the protein of interest and a secondary antibody directed to the primary antibody. The secondary antibody is usually coupled to horse-radish peroxidase which with the help of addition of substrate allows for visualization of the signal[194]. The advantage of this two-antibody method is that it allows for amplification of the signal where multiple secondary antibodies will bind to the primary antibody.

The primary antibodies can either be directed towards an entire protein or be specific for modified versions of the protein, for example the phosphorylated protein. Using antibodies against the phosphorylated form of a protein allows for studying activation via phosphorylation events. If there is more of the phosphorylated form of a protein but the same amount of total protein between two samples this indicates that the protein in question is more activated in the sample with higher levels of phosphorylated proteins. Phosphorylation levels of proteins can be difficult to study due to that very small difference could be important. This means that the amount of total sample/protein loaded must be the same to distinguish that the difference in level is due to different amounts of phosphorylation/

specific protein and not due to different loading on the gel. This is controlled for by the usage of loading controls, a protein that will be present in the same level in all samples. Further, the exposure when developing the membrane for visualizing the proteins must be the correct one. When the exposure is too long, small differences will not be visualized due to saturation of the signal while to short exposures will lead to loss of information/no detection of the protein of interest.

One of the main drawbacks with western blot is that it is a semi-quantitative method meaning that one will not get information about the amount of protein but only about differences in amount. Further, western blot is tedious and consist of many steps. This means that at each step errors can occur resulting in that the proteins will not be detected in the end or that the result will be sub-optimal. Therefore, the method require experience and understanding of the process. For example, about which steps that are especially important, here the main one being the preparation and lysis of the samples.

4.10 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Immunohistochemistry and immunofluorescence are methods to study the expression of proteins in cells or in a tissue. They are both based on antibody labelling of the protein of interest. In principle, the cells or tissue is fixed and placed on glass slides. Unspecific binding is blocked by the usage of a blocking agent (normally serum). The primary antibody against the protein of interest is added overnight at 4°C to allow for binding to the target. The primary antibody is then washed away and a secondary antibody that binds the primary antibody is added. The fluorescent secondary antibody is then washed away, and the nucleus of the cells is stained with either DAPI or Hoechst (DNA stains). The tissues/cells can then be visualized by fluorescence microscopy. This method was used in **Manuscript I** to study brain tissue from mice as well as exosome uptake by cells.

4.11 GENE EXPRESSION ANALYSIS

Quantitative reverse transcript polymerase chain reaction (qRT-PCR) is a method used to look at the transcription of genes. The method is based on that the mRNA present in a sample is isolated and then translated into DNA using reverse transcription. Primers for a specific protein-gene of this DNA is then added allowing for transcription of that gene via the polymerase chain reaction to multiply the DNA. On the primers or in the primer mix there are labels for detection. The number of cycles needed to reach a certain level of saturation will indicate how much mRNA that was present in the sample at the beginning, with a lower CT value indicating more RNA. This is normalized to a reference gene that should be the same between the samples, for example TATA-binding protein (Tbp). By comparing two samples one can see which one that have more mRNA present, meaning more transcription of a certain gene. For q-RT-PCR to be accurate it is important that the RNA yield and purity is sufficient, and that genomic DNA is removed. The commercial kits used to isolate mRNA in **Manuscript I**, **II**, and **III** include steps to ensure removal of genomic DNA and high RNA yield and purity.

5 CONCLUSION AND FUTURE PERSPECTIVES

In summary, it is clear that age is an obvious risk factor for major neurodegenerative diseases. Yet the role of the immune system and inflammation in these diseases has not been well studied.

My work shows that genes with clear links to neurodegeneration, encode for proteins that have immune-related functions. More specifically, I show that gain of function of the Parkinson´s associated kinase LRRK2 accelerates age-related inflammation, thereby promotes neurodegeneration. Mechanistically, I further demonstrated that PD-associated inflammation is primarily due to the spontaneous activation of the cGAS-STING-IFN-I axis. I further show that this inflammation mainly starts in peripheral tissues and with age progresses to the CNS ultimately resulting in neuronal impairment. But how does inflammation from the periphery spread to the brain with age? I show that aging not only results in cell intrinsic activation of cGAS-STING-IFN-I axis within senescent cells but also in increased release of DNA containing exosomes which can horizontally transfer DNA thereby triggering the cGAS-STING-IFN-I axis in distal cells. In summary, in the **Manuscript I** we have uncovered not only how age-associated inflammation is initiated and the innate immune pathway involved but also how inflammation from the periphery may spread to the brain thereby causing neurodegeneration. A conceptual image over our findings is shown in **figure 13**.

During the coming years, I believe we will see further studies into the role of the cGAS-STING pathway in aging and neurodegeneration as well as studies on pharmacological targeting of this signaling pathway and if this can delay or halt the progression of neurodegenerative diseases. Further, I believe that we will see increased research in the field of extracellular vesicles, and their role

in signaling, and spreading of inflammation throughout the body, as well as in disease progression.

It is important to note that immunotherapies have previously been tried in neurodegenerative diseases, but they proved largely unsuccessful. With increased understanding of the molecular mechanisms behind the diseases, targeted personalized medicine can help in combating neurodegenerative disorders. However, since our study show that the inflammation seems to be driven by endo-lysosomal dysfunction and this seem to be an important driver of neurodegeneration targeting of the endo-lysosomal system may be more successful. Further research is therefore warranted in this area.

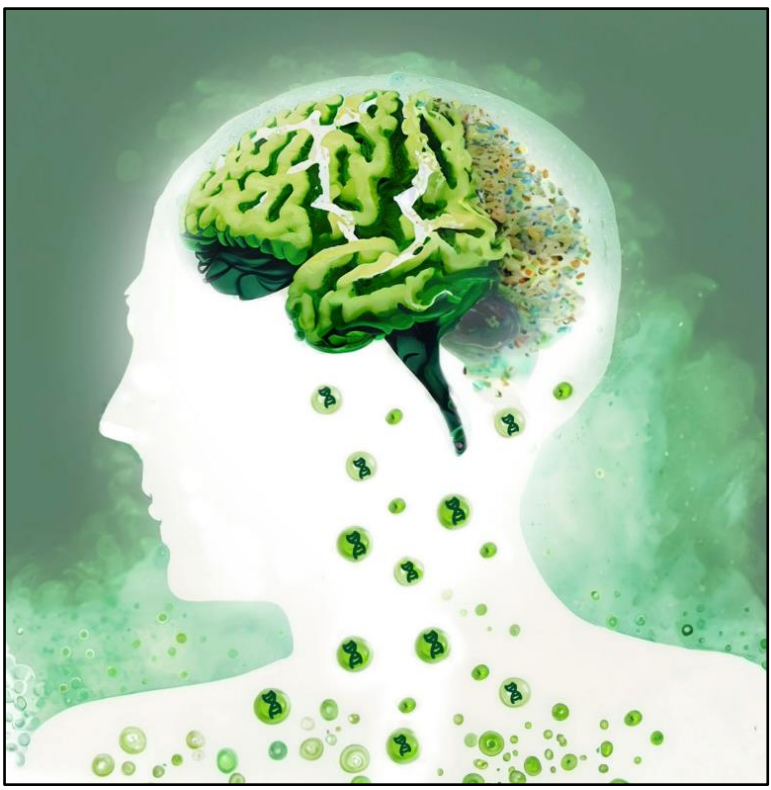

Figure 13. Conceptual image *of DNA containing vesicles spreading inflammation from the Periphery to the Brain resulting in neurodegeneration. Parts of the image was created with the assistance of AI tools.*

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