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Mitochondrial autoimmunity in Parkinson's disease

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Mitochondrial autoimmunity in Parkinson's disease

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Résumé

La maladie de Parkinson est une maladie neurodégénérative causée par la destruction des neurones dopaminergiques. La cause sous-jacente de cette perte neuronale est inconnue. La maladie de Parkinson peut se développer tôt dans la vie (forme juvénile autour de 40 ans) ou sporadiquement (après l'âge de 60 ans).PINK1 (PTEN-induced putative kinase 1) et Parkin sont des gènes liés à la forme juvénile de la maladie de Parkinson. Ils influencent la réponse inflammatoire et la dysfonction mitochondriale. PINK1 et Parkin ont été impliqués dans la régulation négative de la présentation de protéines mitochondriales (MitAP). Ces protéines répriment la présentation de protéines mitochondriales sur les molécules du complexe majeur d'histocompatibilité de classe I (CMH I) à la suite d'un stress. MitAP est dépendant de la formation de vésicules mitochondriales (MDVs). La formation des MDVs est dépendante des protéines de trafic telles que de Snx9, Rab9, et Rab7. La diminution des niveaux d'expression de ces protéines induit une inhibition de MitAP. L'instabilité mitochondriale augmente également la fuite d'ADN mitochondrial dans le cytosol (à travers un mécanisme inconnu), ce qui entraîne l'activation du capteur d'ADN cytosolique tel que cGAS / STING. L'implication de MitAP dans l'auto-immunité a été démontrée. En effet, les souris PINK1 KO infectées par une bactérie intestinale Gram négatif, développe des lymphocytes T autoréactifs spécifiques aux antigènes mitochondriaux et des symptômes de type Parkinson. Des données récentes ont par ailleurs appuyé cette hypothèse en démontrant la présence de lymphocytes T autoréactifs spécifiques de l'alpha-synucléine, une protéine dont le dysfonctionnement est fortement associé à la maladie. Ainsi, nous avons émis l'hypothèse que les patients qui souffrent de la maladie de Parkinson ont un niveau plus élevé de

MitAP. L'augmentation de MitAP, notamment au niveau des cellules présentatrices d'antigènes (CPA), entraînerait une autoréactivité mitochondriale et aussi une dérégulation inflammatoire causée par une augmentation de la production de MDVs. Cette production accrue de MDV entraînerait une augmentation des fuites d'ADN mitochondrial détecté par la voie cGAS/STING et induirait l'augmentation de la production de cytokines pro-inflammatoires impliquées dans la polarisation des lymphocytes T vers un phénotype auto-immun.

Ici, nous montrons que les patients possèdent dans le sang, des lymphocytes T autoréactifs spécifiques aux antigènes mitochondriaux et que ces cellules expriment spécifiquement l'IL-17 (un marqueur des lymphocytes Th17 et Tc17, des types de lymphocytes qui est sont fortement liés aux maladies auto-immunes). Par ailleurs, nous montrons qu'en absence de PINK1, le traitement par endotoxine bactérienne (LPS) ou par la bactérie EPEC (Enteropathogenic Escherichia coli) des CPA a comme conséquence une surproduction d'un ensemble de cytokines pro-inflammatoires. Ces cytokines (IL-6, IL-23 et IL-1β) sont impliquées dans la polarisation des cellules Th17. Nous démontrons également que les CPA PINK1 KO traitées par le LPS induisent une polarisation biaisée des lymphocytes T vers un phénotype Th17. Nous démontrons également, dans une lignée de CPA déficientes pour des gènes associés à la biogenèse des MDVs, des niveaux d'expression réduits de cytokines pro-inflammatoires après infection par l'EPEC ou traitement avec le LPS. Ces données soulignent l'importance de la voie MitAP dans la pathophysiologie de la maladie Parkinson à travers la régulation de l'auto-immunité et de l'inflammation.

Mots : MitAP, les lymphocytes T autoréactifs spécifiques aux antigènes mitochondriaux, PINK1 KO, CPAs, les lymphocytes Th17, cytokines, MDVs.

Abstract

Parkinson's disease (PD) is a neurodegenerative disease caused by the destruction of dopamine neurons, yet, the underlying cause of this neuronal loss is still unknown. Parkinson's disease can be juvenile or early onset (before age 40) and sporadically (after the age of 60). PINK1 (PTEN-induced putative kinase 1) and Parkin are genes implicated in the juvenile form of PD that have also been shown to influence inflammatory responses and mitochondrial dysfunction. PINK1 and Parkin negatively regulate mitochondrial antigen presentation (MitAP). These proteins suppress the presentation of mitochondrial proteins on Major Histocompatibility Complex (MHC) Class I following stress. MitAP is dependent on the formation of mitochondrial vesicles (MDVs). These MDVs utilize traffic proteins such as Snx9, Rab9, and Rab7. Decreased levels of expression of any of these proteins inhibit MitAP. Mitochondrial instability also increases mitochondrial DNA leakage into the cytosol (through and unknown mechanism) resulting in activation of cytosolic DNA sensors like cGAS/STING. MitAP's implication in autoimmunity has been demonstrated in PINK1 KO mice infected with gram-negative intestinal bacteria resulting in auto-reactive T-cells specific to mitochondrial antigens and Parkinson's-like symptoms. In PD patients, auto-reactive T cells specific for alpha-synuclein, a protein whose dysfunction is associated with PD, have been found, suggesting that auto-reactive T-cells are present in PD. Thus, we hypothesized that PD patients would have increased levels of MitAP in antigen presenting cells (APCs) resulting in mitochondrial self-responsiveness as well as an inflammatory deregulation. This increased production of MDVs would result in an increase mtDNA leakage and ultimately be detected by the cGAS/STING pathway inducing the production of proinflammatory cytokines. Ultimately,

these proinflammatory cytokines would induce the polarization of T-cells towards an autoimmune phenotype (Th17). Here, we show that APCs deficient in genes associated with MDVs biogenesis have reduced expression levels of pro-inflammatory cytokines after infection with Enteropathogenic Escherichia coli (EPEC) or the bacterial endotoxin LPS. We determine that in the absence of STING there is a strong inhibition of MitAP and cytokine expression in raw 264.7 macrophages after LPS treatment. Furthermore, we validate that in the absence of PINK1, LPS or EPEC treated BMDCs overproduce a set of pro-inflammatory cytokines (IL-6, IL-23 and IL-1β) that induce a biased polarization of T-cells to a Th17 phenotype. Finally, we observed mitochondrial antigen specific T-cells expressing IL-17 (a marker of Th17 and Tc17 lymphocytes, the types of lymphocytes that are strongly related to autoimmune diseases) in PD patients. This data highlights the importance of the MitAP pathway in the pathophysiology of Parkinson's disease through the regulation of autoimmunity and inflammation.

Words: MitAP, Mitochondrial-antigen autoreactive-T cell, PINK1 KO, APCs, Th17 cells, cytokines, MDVs, STING.

Table of Contents

List of Tables

List of Figures

List of acronyms and abbreviations

ACK	Ammonium-Chloride-Potassium
ADN	acide désoxyribonucléique
AECII	Type II alveolar epithelial cells
AHR	Aryl Hydrocarbon receptor
AMP	Adenosine monophosphate
APC	Antigen Presenting cells
BBB	Blood brain barrier
BMDC	Bone Marrow Derived Dendritic Cells
BSA	Bovine Serum albumin
CCCP	Carbonyl Cyanide m-Chlorophenyl hydrazone
cGAMP	Cycli GMP-AMP
cGAS	Cyclic GMP–AMP synthase
CNS	Central nervous system
CPA	Cellules présentatrice d'antigènes
CPRG	Chlorophenol red-beta-D-paglatopyranoside
CSF	Cerebrospinal Fluid
DA	Dopamine
DAMP	Damage-associated molecule pattern
DCs	Dendritic Cell
DJ-1	Protein deglycase
DN	Dopaminergic neurons
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
EPEC	Enteropathogenic Escherichia coli
ER	Endoplasmic reticulum
ETC	Electron Transport chain
FBS	Fetal bovine serum
FMO	Fluorescence minus one
gB	Glycoprotein B
GBA	Glucocerebrosidase
GI	Gastrointestinal
GM	Gut microbiome

GM-CSF	Granulocyte-monocyte colony stimulatory factor
gRNA	Guide RNA
GSH	Glutathione
GTPAse	Guanosine triphosphatase
H. Pylori	*Helicobacter pylori*
HSV1	Herpes Simplex Virus 1
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
IgMs	Immunoglobulin M
IKK	Iκb kinase
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL17	Interleukin 17
IL-17a	Interleukin 17 a
IL-17F	Interleukin 17 F
IL-18	Interleukin 18
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-23	Interleukin 23
IL-35	Interleukin 35
IL-1β	Interleukin 1 beta
IMM	Inner Mitochondrial membrane
iNOS	Inducible nitric oxide synthase
iPSC	In induced Pluripotent Stem Cell
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
IRE1	Inositol-requiring protein 1
iTReg	Inducible regulatory T-Cell
JNK	C-Jun N-terminal kinase
KD	Knock down
KO	Knock out

KI	Knock in
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	Lipopolysaccharide
LRRK2	Leucine rich repeat 2
MAPK	Mitogen-activated protein kinases
MAPKKK	Mitogen-activated protein kinase kinase kinase
MAPL	Membrane anchored protein ligase
MDV	Mitochondrial derive vesicles
MGBA	Microbiota-gut-brain-axis mgba
MHC	Major histocompatibility complex
MitAP	Mitochondria antigen presentation
MMTV	Mouse mammary tumor virus
MPP	Mitochondrial process peptides
MPT	Mitochondrial membrane permeability transition
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
MTS	Mitochondrial Targeting Sequence
MyD88	Myeloid differentiation primary response protein 88
nDNA	Nuclear DNA
NEMO	NF-kappa-B essential modulator
NFκB	Nuclear factor kappa B
NLR	NOD-like receptors
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
OGDH	Oxoglutarate dehydrogenase
OMM	Outer mitochondrial membrane
OPTN	Optineurin
PAMP	Pathogen associated molecular patterns
PARL	Presenilin associated rhomboid-like
PBC	Primary biliary cholangitis
PBMC	Peripheral blood mononuclear cell
PBS-T	Phosphate-Buffered Saline-Tween
PD	Parkinson Disease
PERK	PRKR-like endoplasmic reticulum kinase
PINK1	PTEN- induced putative kinase 1
PKB	Protein kinase b
RA	Arthritis

RIP-1	Receptor-interacting protein kinase 1
RLR	RIG-I like receptors
RNS	Reactive nitrogen species
Roc	Ras of complex
RORγT	Retinoic acid receptor-related orphan receptor-gamma-t
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
SLE	Systemic lupus erythematosus
SLP-2	Stomatin-like protein 2
SN	Substantia nigra
Snx9	Sortin nexin 9
SOD	Superoxide dismutase
STAT3	Signal transducer and activator of transcription 3
STING	Stimulator of interferon genes
TAK1	Transforming growth factor-β-activated kinase-1
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TCRαβ^{+}Tcells	T cells expressing α and β chains of the T-cell receptor
TCRγδ^{+}T cells	T-cell populations expressing γ and δ chains of the T-cell receptor
TGF-β	Transforming growth factor beta
Th1	T-helper-1
Th2	T-helper-2
Th17	T-helper-17
TIM	Translocase of the Inner Membrane
TIR	Toll-interlukin1 receptor
TIRAP	Tir domain containing adaptor protein
TLR	Toll-Like receptor
TNFR	TNF-α receptor
TNF-α	Tumor necrosis factor alpha
TOM	Translocase of the outer membrane
TRAF2	TNF-α receptor associated factor 2
TRAF6	TNF-α receptor associated factor 6
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T-cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
UPR	Unfolded protein response

α-syn	A-synuclein

Dedication

“To my great-grandparents whose patience and wisdom instilled in me all the values I need to finish this project”.

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Chapter 1 -Introduction

Inflammation has become a major signature of Parkinson's Disease, however, whether this inflammation is beneficial or deleterious reminds to be determined (1). Analysis of serum, cerebrospinal fluid (CSF), and postmodern brain samples of PD patients reveled high levels of pro-inflammatory cytokines such as TNF-α (2-4), IL-6 (5), and IL-1β (5) and peripheral immune cell infiltration (6), suggesting a major inflammatory component in the pathology of the disease. Other genetic studies demonstrated that genes coding for several cytokines and major histocompatibility complex class II (MHCII) are associated with PD (7). Innate components including cytokines and complement have been reported to be elevated in PD patients, resulting in the inflammation of the CNS, expanding from the periphery, and the enteric nervous system to the brain (6). Microglia activation along with peripheral immune activation results in a continuous inflammatory cycle active throughout the disease (7). Altogether, this data negates the theory that the brain is an immune-privileged organ and shows that the Blood-Brain Barrier (BBB) is dynamic and changes due to stress responses (8-12). Further proof of the peripheral immune system playing a role in PD includes elevation of γ/δ + T cells (a group of T-Cells that are typically $CD4^{-}CD8^{-}$ and do not express MHC receptors, which main function include immune regulation, surveillance, and homeostasis) (13) . In the peripheral blood and CSF of PD patients (14). More interestingly, PD-related genes including PTEN- induced putative kinase 1 (*PINK1*), *PARKIN*, and Leucine-rich repeat 2 (*LRRK2*) have been shown to regulate levels of cytokines and the differentiation of certain immune cells in the periphery. α-synuclein (α-syn) (another well know PD -related protein) specific T cells have been shown to be elevated in PD patients (15, 16),

supporting the potential role of autoimmunity in the disease. All this evidence suggests that inflammation plays a pivotal role in the physiopathology of PD.

1.1 Pathology of Parkinson's Disease

Parkinson's Disease is a multifactorial, progressive neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra (SN) (7). This loss of dopaminergic neurons ultimately results in a deficit of dopamine in the striatum (7). Consequences of this dopamine reduction include symptoms like bradykinesia, rigidity, and tremor (7). Non-motor symptoms include anxiety depression, sleep disturbances, constipation, and excessive salivation (7). Besides SN loss, another neuropathological characteristic of PD includes Lewy bodies, which are intraneuronal inclusion containing high levels of the alpha-synuclein (α-syn) (7).

1.2 Environmental Factors and PD.

Although there is a strong genetic basis for the disease, epidemiological studies have shown that the environment plays a crucial role in the development of the disease. Metanalysis studies have determined that rural area living, drinking well water, farming, and exposure to pesticides may be risk factors (17-21). Other environmental factors like smoking (22, 23) and mild life exercise have been shown to reduce the risk of PD (24-27). The first striking evidence of environmental factors directly resulting in PD was observed in 1982 when a group of North California substance abusers utilizing synthetic heroin contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) presented severe Parkinson-like symptoms (28, 29). Other chemicals that increase PD risk include the insecticide Rotenone (30, 31) and the herbicide Paraquat (,1´-dimethyl-4,4´-bipyridinium) (32, 33). As a result of these epidemiological studies, chemicals like MPTP and Rotenone are widely used to elicit cellular and animal models of PD. Prevalence of

infection with certain strains of bacteria, in particular, *H. Pylori* (a gram-negative bacteria) has also been correlated with PD development (34).

1.3 The mitochondria, a major player in PD

Mitochondria are cellular organelles that play an essential role in energy production and maintaining cellular homeostasis. Mitochondria are implicated in cellular signaling processes including cell survival, division, and pathogenic stress (35). Many of the neuropathological features associated with PD are rooted in the mitochondria.

1.3.1 Respiratory Chain dysfunction and PD.

Initial hints that mitochondria play a central role in the disease pathogenicity came from the discovery of MPTP. After been processed, MPTP has the ability to reduce the activity of complex I, II, and IV of the electron transport chain (ETC) in the mitochondria (36, 37). Similarly, in PD patients, a decreased in the activity of complexes I, II, and IV is observed in post mortem SN homogenates (38-40). Respiratory chain impairment results in loss of bioenergetic functions, increased oxidative stress, and impaired calcium homeostasis (36, 41). ROS generation has also tightly been linked to respiratory chain dysfunction (42). Mitochondria can catalyze nitric oxide formation, which can ultimately result in generating reactive nitrogen species (RNS). In PD patients, the equilibrium between antioxidants, (like Superoxide dismutase (SOD) and Glutathione (GSH), ROS, and RNS is disturbed (43). Interestingly, dopamine (DA) metabolism can mediate mitochondrial oxidative stress (44). DA has been shown to undergo auto-oxidation which produces active quinones (45) that interacts with ROS scavenger (46, 47), respiratory chain complexes (47), or proteins needed for mitophagy (a process by which unstable mitochondrial

are selected and targeted for lysosomal degradation via autophagy) (48, 49). PD-related genes that have been shown to affect the respiratory chain include α-syn, PINK1, Parkin, DJ-1 (50), and VPS35 (51). For instance, analysis of the 3D structure of complex I revealed abnormalities in complex I and II and depletion of complex IV in skin cells from PINK1 mutant patients (52). Parkin has been demonstrated to interact with Stomatin-like protein 2 (SLP-2), which is required for respiratory chain protein assembly (53) SLP-2 recovers complex I deficiency in IPSC-derived patient neurons with Parkin mutations (53). Abnormal complex I was also observed in DJ-1 knockout murine neurons and patient fibroblasts with VPS35 mutations (50).

1.3.2 PINK1 and Parkin: the guardians of the mitochondria

1.3.2.1 PINK1 and Parkin: structure and function

Two of the genes that have been previously shown to cause early-onset PD are *PARK2* or *Parkin* and *PARK6* or *PINK1.* PINK1 is a 581 amino acid protein kinase (54). Mutations in *PINK1* are responsible for at 1-8% of the early onset autosomal recessive PD cases (55, 56). PINK1 gene is composed of 8 exons and it encodes the only protein that contains a mitochondrial targeting sequence (MTS) at the N-terminus (57). Its kinase domain is highly conserved, and it is homologous to the serine/threonine kinases of the calcium-calmodulin family (58). PINK1 acts upstream of Parkin, where is activated by the phosphorylation of its Serine 65 (59, 60). This step is required for the activation and function of Parkin. PINK1 function in many important biochemical processes including cell respiration (61), protein folding, and degradation (62, 63). Its most important roles are in the mitochondria where it coordinates mitochondrial fission/fusion dynamics (64, 65), mitophagy (59), mitochondrial-derived vesicles formation, mitochondrial antigen presentation (MitAP), (66, 67) and calcium signaling (67).

PINK1 and Parkin have been shown to work in the same pathways using drosophila epistasis (68). Parkin is an E3 ubiquitin ligase (69), composed of 465 amino acids (70). This protein is highly expressed in the heart, testis, brain, and skeletal muscle (70). A wide variety of cytosolic and outer mitochondrial membrane proteins are ubiquitinated by Parkin (71, 72). Usually, Parkin is activated by conditions such as Mitochondria depolarization or epidermal growth factor signaling (73-76). Some conditions that de-repress its activity include heat treatment, N-terminal deletions, and point mutations (88-91). When Parkin is activated, it forms multiple types of ubiquitin chains including K63, K48, K11, and K6 linkages (77). The proper function of Parkin is very dependent on the integrity of its structure and is tightly controlled by multiple mechanisms of autoinhibition that require proper folding of the protein (78). Pathogenic mutations in Parkin can either cause improper folding of the protein structure, interference with binding of substrates, or directly affect the enzyme catalysis (78).

1.3.2.2 PINK1, Parkin, and Mitophagy.

A mechanism by which mitochondrial stability is maintained in cells is mitophagy. Mitophagy is the process by which mitochondria are selectively sequestered and delivered for lysosomal degradation through autophagy (49). Mitophagy could be upregulated in response to mitochondrial injury, cellular differentiation, or stress-induced metabolism adaptation (79). Defects in mitophagy result in the accumulation of damaged mitochondria in the cytosol and ultimately neuronal death. Proteins implicated in PD including α-syn, Parkin, PINK1, Protein deglycase (DJ-1), LRRK2, and glucocerebrosidase (GBA) also play a role in mitophagy (80).

Mitophagy could be divided into 2 pathways: Parkin-dependent and Parkin-independent mitophagy (81, 82).

In the Parkin-dependent pathway, PINK1 detects mitochondrial dysfunction and activates Parkin, who specifically ubiquitinates damaged mitochondria (59, 83-86). PINK1's mode of action is dependent on its ability to sense damaged mitochondria. Under normal conditions, PINK1 is targeted to the mitochondria where it is imported through the Translocase of the Outer Membrane (TOM) complex of the Outer Mitochondrial Membrane (OMM) (Fig.1a). Once PINK1 is in the intermembrane space, it is immediately translocated by the Translocase of the Inner Membrane (TIM) complex of the Inner Mitochondrial Membrane (IMM) (Fig. 1a) (87). There, PINK1 is cleaved by Mitochondrial Process Peptides (MPP) (87), then it is cleaved once more by an inner membrane protease, Presenilin Associated Rhomboid-Like (PARL) (88, 89) (Fig.1a). This last cleavage results in an N-terminal deleted form of PINK1 (90). Once cleaved, PINK1 is rapidly identified by the N-degron pathway (a proteolytic system wherein single N-terminal amino acid of protein, referred to as N-degron, is recognized (91)) and targeted for protein degradation by the ubiquitin-proteasome system (Fig. 1a) (92). This cycle is constantly repeated, which explains how the levels of PINK1 under normal/healthy conditions are very low (93).

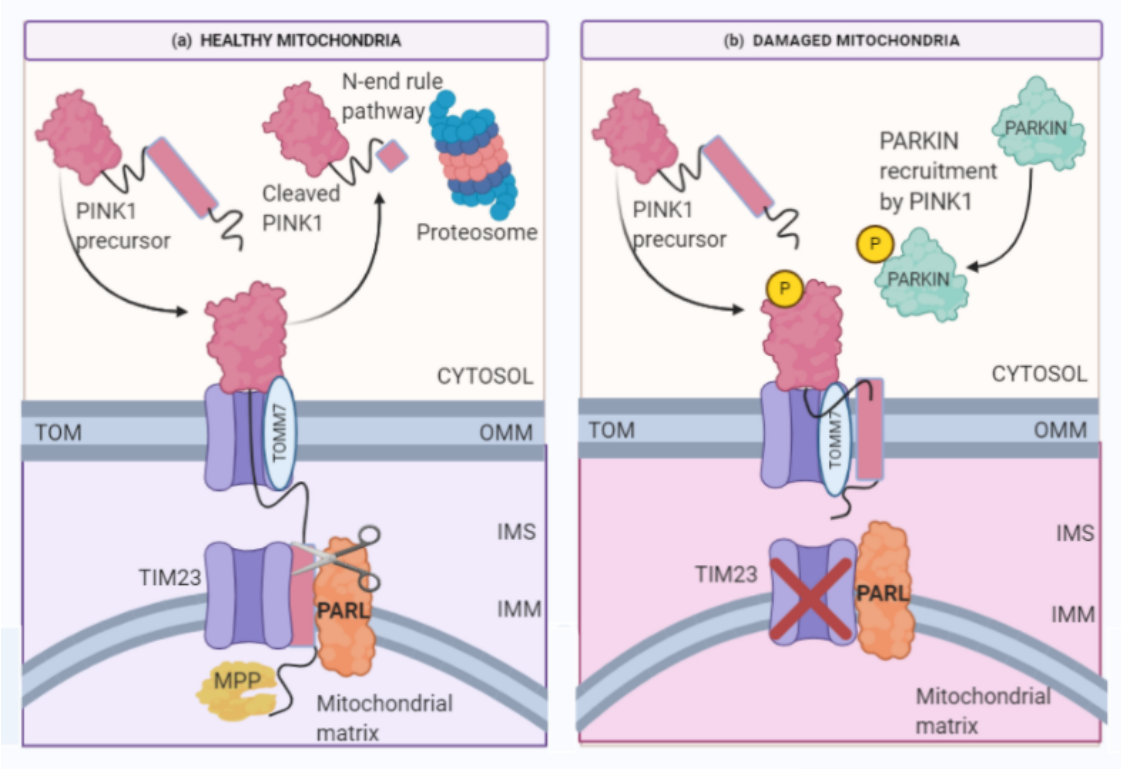

Figure 1-PINK1 activation pathway.

Under healthy condition (a) PINK1 is constantly targeted to mitochondrial by its Mitochondria-targeted sequence (89). PINK1 is imported to the mitochondria is through the TIM complex driven by the mitochondrial membrane potential (89). MPP and PARL cleave PINK1's mitochondrial-targeted sequence and transmembrane domain (89). PINK1 is then identified by the N-degron type 2 E3 ubiquitin ligase that then targets the kinase for proteasomal degradation (89). (b) Under stress (depolarizing agents, OXPHOS inhibitors, genetic or environmental stresses, unfolded proteins) mitochondrial membrane gets depolarized resulting in the accumulation of PINK1 at the OMM where it activates Parkin and recruits the machinery for mitophagy. (89) *Source of image: Camberly Hernandez Paredes*

Under mitochondrial stress such as depolarizing agents, OXPHOS inhibitors, genetic and environmental stress, or even misfolded proteins, the mitochondrial membrane potential is lost resulting in inhibition of TIM complex, preventing PINK1 from being imported to the inner mitochondria (Fig. 1b) (94-96). The exact mechanism responsible for PINK1 import has not yet been determined. Nevertheless, TIM complex inhibition results in the accumulation of PINK1 in the OMM preventing its processing by MPP and PARL in the inner mitochondria membrane (Fig. 1b) (93). PINK1 rapidly recruits and phosphorylates Parkin at its Ser65 to activate Parkin's E3 ubiquitin ligase activity (97, 98). Ubiquitin is also phosphorylated by PINK1 at its Ser65, resulting in maximal Parkin activation (99-101).

The ubiquitination of different mitochondrial proteins by Parkin recruits the mitophagy machinery. Parkin ubiquitylates numerous outer mitochondrial membrane (OMM) proteins, which in turn recruits other proteins to mitochondria to initiate mitophagy (71, 84, 85, 102). Two adapter proteins have been identified to be essential for mitophagy NDP52 and Optineurin (OPTN). Knocking out these genes results in the inhibition of mitophagy (103). The recruitment of these adaptor proteins requires PINK1 activation, but not Parkin activation (Fig 2) (104).-

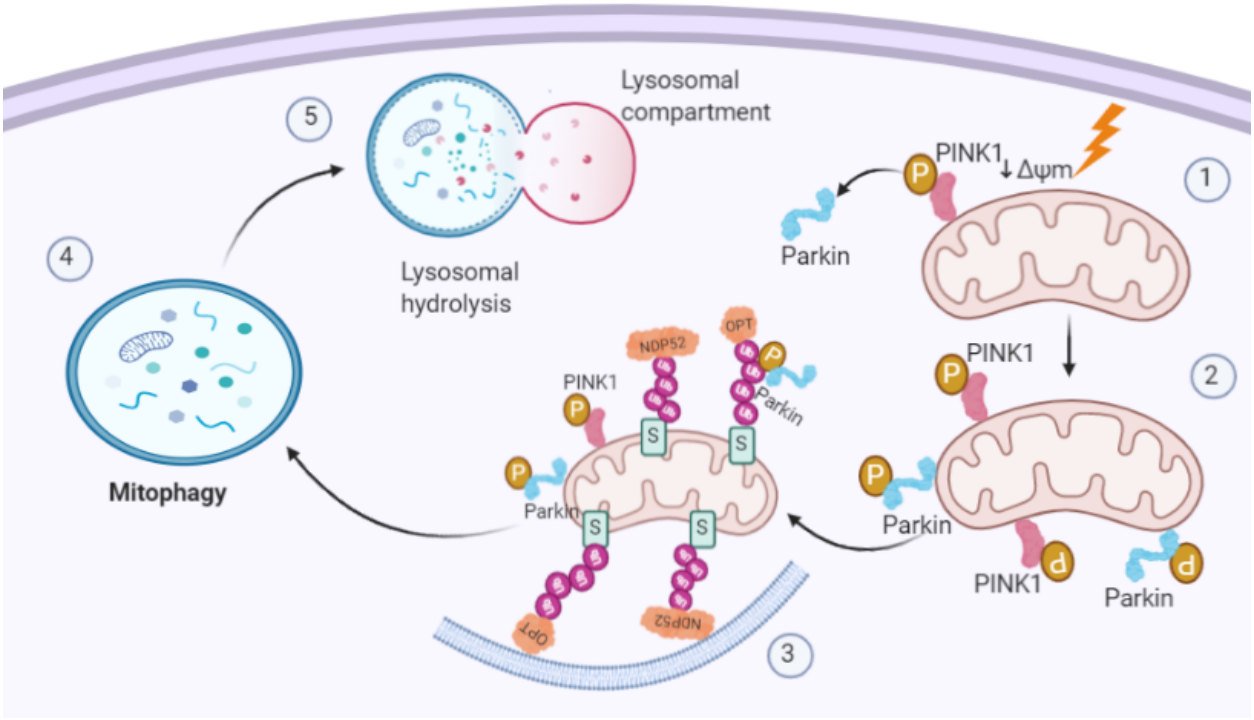

Figure 2-The Mitophagy pathway

(1) Under mitochondrial stress, loss of mitochondrial membrane potential prevents the import of PINK1 into the mitochondria which result in its activation (94-96). (2) Activated PINK1 recruits and activates Parkin via phosphorylation (97, 98). (3) Active Parkin ubiquitinates many protein substrates resulting in the activation of autophagy (67, 80, 81, 98). (4) Once the mitophagosome is completed, (5) mitophagy is completed by fusing the mitophagosome to the lysosomal compartment resulting in the proteolytic degradation of the damaged mitochondria. *Source of the image: Camberly Hernandez Paredes*.

The mechanism by which Parkin is recruited to damaged mitochondria is unknown. However, Parkin can recognize active PINK1 even when targeted to another organelle like the peroxisome (94). Catalytically inactive mutants of Parkin are unable to accumulate following mitochondrial depolarization, suggesting that Parkin ubiquitin ligase activity is required for its recruitment (59,

105). Activation of Parkin increases the amount of mitochondrially conjugated ubiquitin, which is then phosphorylated by PINK1 resulting in further Parkin recruitment (78). This creates a positive feedback loop that explains how low basal levels of PINK1 in the cytosol can recruit high levels of Parkin, as well as how the catalytic activity of both PINK1 and Parkin are needed for Parkin's recruitment (78). This model has been further confirmed by bypassing Parkin activity using tetra-ubiquitin chains artificially targeted to mitochondria (106), and by mimicking PINK1 activity using a phosphor-mimetic chain for S65 tetraubiquitin (107). In cultured cells, PINK1/Parkin pathway can be activated using uncouplers like Carbonyl Cyanide m-Chlorophenyl hydrazone (CCCP), which induce massive mitochondria depolarization. This depolarization results in increased recruitment of Parkin to the mitochondrial followed by clearance of Mitochondria (78). This suggests that this pathway acts as a mitochondrial quality control system.

Nevertheless, whether PINK1 and Parkin cause PD through dysfunctional mitophagy is not clear, since PINK1-independent and Parkin-independent pathways of mitophagy exist (108-110) and intact mitophagy is seen in S65A Parkin mutant that causes PD (111). Moreover, *PINK1 KO* and *Parkin KO* models have been shown to lack any motor impairment (112). This suggests that PINK1 and Parkin cause PD through a mechanism other than mitophagy.

1.3.2.3 Mitochondrial-Derived Vesicles

Besides mitophagy, another mitochondria quality control pathway lies in the generation of mitochondrial-derived vesicles (MDVs) (113). These small vesicular structures carry mitochondrial proteins and lipids from the mitochondria to late endosomes/lysosomes or peroxisomes (113). MDVs size range between 70 to 150 nm and they do not rely on mitochondrial fission machinery proteins like the GTPase dynamin-related protein 1 Drp1 (114, 115) (who has

been shown to facilitate mitophagy by fragmenting mitochondria to ease autophagosome engulfment (116)) suggesting that the mitophagy machinery is not required for to start MDV's formation. MDVs are generated through the selective incorporation of protein cargoes from the outer, inner, and matrix contents of the mitochondria (114). Although a family of these MDV's are targeted to the peroxisomes, whether they play an important function in other processes is to be determine (114). Cargoes destined to the lysosomes are enriched with oxidized proteins and the nature of the mitochondrial stress determines the cargo carried in the MDVs (115). For example, xanthine oxidase/ xanthine, an enzyme that generates ROS, results in MDVs carrying mainly Voltage-dependent anion Channels (VDAC) whereas Antimycin A, an autophagy inhibitor, resulted in MDVs containing complex III subunit (115). A major regulator of MDVs biogenesis is Sortin Nexin 9 (Snx9) (66). Snx9 is a dynamin-binding partner essential for clathrin-mediated endocytosis (117). Other molecular partners required for MDVs formation and fusion with the late endosomal compartment include Rab9 and Rab7 (66). Depending on the stress applied to the cell, MDVs will contain different cargos and will be regulated differently by PINK1 and Parkin (66). Indeed, under oxidative stress, MDVs will contain cargos such as TOM20 and their formation is positively regulated by PINK1 and Parkin. In this context, PINK1 and Parkin-dependent MDVs will be generated as an immediate response to mitochondrial oxidative stress (114, 115, 118) by sending oxidized and damaged proteins to the lysosomal compartment for degradation. This is a faster and more localized mitochondrial control quality system that maintain mitochondria stability until the mitophagy machinery is recruited (118, 119). Supporting this idea are kinetic analysis using Antimycin A showing that MDVs generation starts at the early stages of ROS production, whereas complete depolarization is needed for mitophagy (119). Moreover, MDVs

formation does not require activation of the mitophagy machinery suggesting that MDVs are an immediate response to oxidative stress (119). The PINK1 and Parkin-dependent MDVs pathway formation remain unknown; however, it is believed to rely on import channels (113). Local protein aggregation at the import channels due to mitochondrial stress may halt the import process of oxidized lipids resulting in altered membrane curvature (120-122). An example of such lipids is cardiolipin which oxidation to phosphatic acid increases membrane curvature (120). Protein aggregation results in chaperone saturation and PINK1 accumulation at the failed import channel (113). Parkin activation results in the recruitment of proteins necessary for vesicle formation and budding (113). Many of these proteins have not been identified yet. Interestingly, Parkin has been shown to be required for trafficking of TOM 20 positive MDVs to the lysosome with the help of Tollip (a ubiquitin-binding protein that interacts with several Toll-like receptors signaling cascade components) (123). Parkin interacts with many of the players required for receptor-mediated endocytosis linking Parkin to the vesicle budding and trafficking machinery (71, 72, 85, 86, 102, 124-126).

Under heat stress conditions or after infection, another type of MDVs will be generated containing mitochondrial proteins such as oxoglutarate dehydrogenase (OGDH) (127, 128). The generation of this type of MDV is negatively regulated by PINK1 and Parkin. Indeed, after Parkin is phosphorylated by PINK1 it ubiquitinates several proteins including Snx9 (127). This ubiquitination is rapidly recognized by the proteasome, resulting in Snx9 degradation and inhibition of MDV formation (127). These vesicles are related to the presentation of mitochondrial antigens on major histocompatibility complex (MHC) molecules at the surface of APC.

1.3.2.4 Mitochondrial Antigen Presentation (MitAP).

Although mitochondria represent an essential organelle for cell survival, the mechanism responsible for the presentation of mitochondrial antigens remained largely unknown until very recently (127). Unlike the antigens from other cell compartments (nucleus, cytoplasm), mitochondrial antigens are not presented by the classical MHC presentation pathways. Indeed, while common endogenous antigens are processed by the proteasome, mitochondrial antigens are processed by the lysosomal compartment after being delivered by MDVs (127). MitAP is independent of autophagy and relies fully on MDVs (127). Cells capable of performing MitAP include APC and Neurons (66, 128). MitAP is triggered by LPS (a bacterial endotoxin found in gram-negative bacteria), gram-negative bacteria, and Heat shock treatment (Fig 3) (127). PINK1 and Parkin are major negative regulators of MitAP (Fig 3) (66). Indeed, in the absence of PINK1 and Parkin, Snx9 is recruited to the mitochondria and along with Rab9, where it induces the generation of MDVs (Fig 3) (127). These MDVs containing mitochondrial antigen are then transported to the late endosomal compartment where the Rab7 GTPase promotes the MDVs/late endosomes fusion (Fig 3) (66).

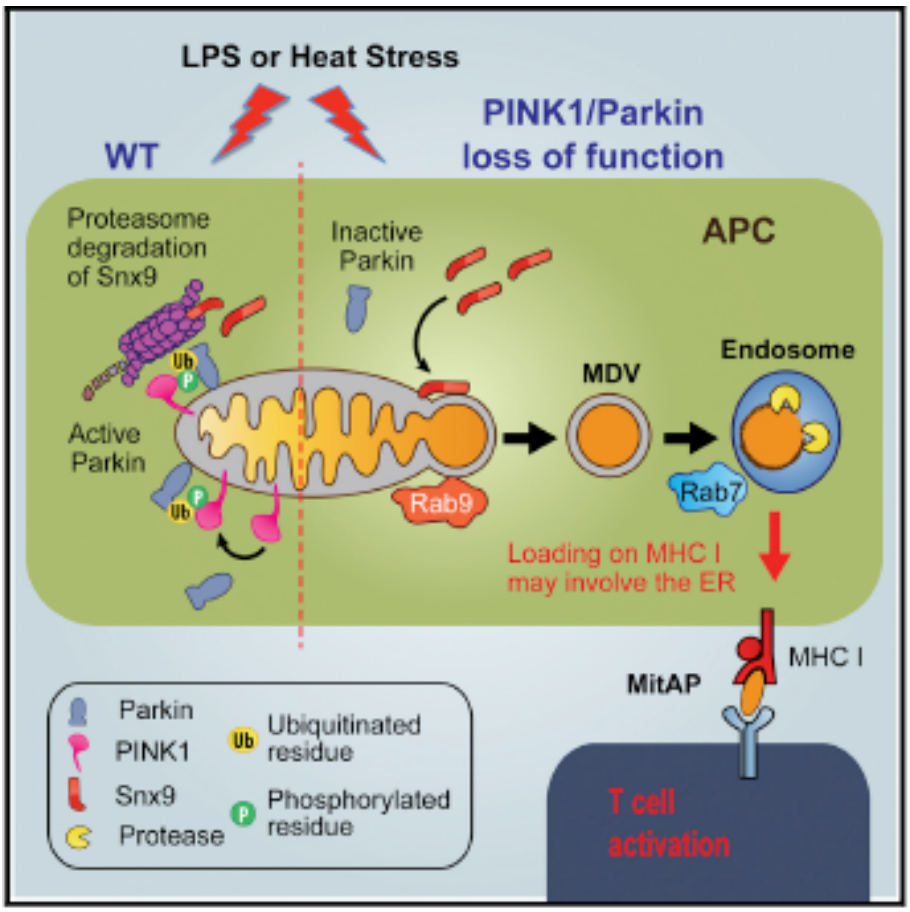

Figure 3-The MitAP pathway

Under stress conditions (gram-negative bacterial infection, LPS, or Heat Stress) PINK1 phosphorylates Parkin which in terms induces the proteasome-dependent degradation of Snx9 (127). When PINK1 or Parkin are absent, Snx9 is not targeted for degradation (127). Snx9 and Rab9 are recruited to the mitochondria resulting in the formation of MDVs (127). MDVs will then fuse to the endosomes with the help of Rab7 (127). These MDVs contain mitochondrial antigens are then processed by lysosomal hydrolytic enzymes and the resulting peptides are loaded on

MHC molecules (127). The MHC/peptide complex is then transported to the cell surface for the activation of mitochondrial-specific T cells (127). Source of Image: *Matheoud et al, 2016.*

1.3.2.5 PINK1 and Parkin and their role in peripheral immunity

One of the mitochondrial antigens that have been shown to be presented through MitAP is OGDH (66, 128). Interestingly, OGDH has previously been identified as an autoantigen for Primary Biliary Cholangitis (PBC) (an autoimmune liver disease characterized by biliary destruction, progressive cholestasis, and potentially liver cirrhosis (129), (130), which highlights the importance of MitAP in the development of autoimmune diseases. Mitochondrial-specific CD8+ cytotoxic T-cells have been shown to infiltrate the brain and cause DN loss in *PINK1 KO* mice infected with *Citrobacter Rodentiun* (a gram-negative bacteria) (128). Furthermore, Parkinson's-like symptoms were observed in this model as early as four months post-infection, a phenotype that was reversed using L-DOPA treatment (a treatment usually used to treat PD) (128). In support of these observations, fine motor deficits and selective loss of DA neurons in the SN were observed previously in *Parkin KO* mice after LPS treatment (131).

Further proof that PINK1 plays a role in adaptive immunity is its role in the control of the Protein Kinase B (PKB) activity, an essential kinase for the conversion of T cells into inducible regulatory T-Cell (iTReg) (132). Indeed, *PINK1*-null iTRegs have reduced the capacity to suppress lymphocyte proliferation (132). The inability to repress CD4+ and CD8+T cells could increase infiltration into the brain like it was observed in the infected PINK1 KO mice (128) and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- treated mice (6). These data correlate with what was found in PD patients in which impaired Treg cell functions (133) and abnormal T-cell population in the periphery was observed (14, 133-135).

PINK1 and Parkin also play a role in the regulation of cytokine production. Indeed, in the absence of these proteins and when mitochondrial stress is applied, high levels of pro-inflammatory cytokines are found in mice serum (136). Moreover, in PINK1 and Parkin absence, exhaustive exercise (mitochondrial stress) results in the activation of cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathways (an inflammatory pathway that upon cytosolic double-stranded DNA recognition results in the secretion of type 1 interferons) (136). PINK1 loss has also been shown to induce increased production of pro-inflammatory cytokines in the striatum after peripheral stimulation with LPS (137) and in microglia after LPS/IFN-γ stimulation (138). Moreover, elevated mRNA levels of TNF-α, IL1β, and IL-6 were observed in *PINK1 KO* organotypic cultures slices compared to those of WT slices (139). PINK1 is also suggested to interact with key components of the TLR signaling pathway, with consequent effects on NFκB activity (140, 141). Similarly, peritoneal macrophages from Parkin KO mice express increased levels of TNF-α, IL-1β, and Inducible nitric oxide synthase (iNOS) mRNA after LPS induction (142). Parkin has also been shown to target TNF-α receptor (TNFR) associated factor 2 (TRAF2) and 6 (TRAF6) to proteasomal degradation (143), two essential mediators of cytokine signaling by regulating c-Jun N-terminal kinase (JNK) and NFκB signaling (143). In tissues of PD patients, an inverse relationship between the expression of TRAF6 and Parkin has been shown (143). Furthermore, Parkin has also been shown to decrease pro-inflammatory mediators through regulation of Mitogen-activated protein kinases (MAPK) and NFκB activation in BV-2 microglial cells (144). Whether the activation of these pro-inflammatory substances is beneficial or deleterious is still to be determined. However, a dysregulation of the cytokine context could have

a major impact on T cell polarization creating a link between mitochondrial instability and adaptive immunity.

1.3.2.6 Mitochondrial DNA

Mitochondria are the only other cellular organelle harboring their own genome. mtDNA is a compact (16569 bp) circular, double-stranded genome, comprised of 37 genes of which 13 encode for the subunit of the oxidative phosphorylation (OXPHOS) system (this system is composed of multimeric complexes, coenzyme Q and cytochrome *c,* forming the mitochondrial respiratory chain (I–IV) used for ATP synthesis) (145, 146) In contrast with nuclear DNA (nDNA), mtDNA exist as multiple copies within the mitochondria depending on the energy requirement of the cell (147). Moreover, mtDNA is more susceptible to accumulate errors due to a lack of histone protection and its proximity to damaging products from OXPHOS complexes (e.g. ROS) (148). Furthermore, mtDNA is believed to have a higher mutational rate due to the low fidelity of the mtDNA polymerase gamma (POLG1) (149). High levels of mtDNA deletions and mutations were observed in the SN of PD patients (150). Recent studies have reported lower mtDNA copy number values between PD patients and controls in SN (151-153), suggesting leakage of this important mitochondrial component. A potential source of mtDNA leakage in PD through the activation of NOD-, LRR- and pyrin domain-containing protein 3 (NL3RP) inflammasome. The NLRP3 inflammasome is a cytosolic complex that can initiate an inflammatory response upon recognition of a variety of pathogen-associated molecular patterns (PAMPs) and damage-associated molecule patterns (DAMPs) including LPS, bacterial toxins, particulate materials, amyloid-b, and prion protein fibrils (154-158). NL3RP inflammasome activation by LPS and ATP

activates the mitochondrial membrane permeability transition (MPT) causing leakage of mtDNA into the cytosol (159). MtDNA contains a significant number of unmethylated CpG DNA repeats like the bacterial genome, a PAMP that potently activates the production of pro-inflammatory cytokines through TLR9 or the cGAS/STING pathways (136, 160-162), ultimately resulting in an immune response.

1.4 . Peripheral immunity and its role in PD

Genetic, and environmental factors in PD that exacerbate the disease have a common ground, that is, they activate inflammation. Typical chronic inflammation hallmarks like a high level of pro-inflammatory cytokines, chemokines, reactive oxygen species (ROS), abnormal T-cell behavior, are observed in sporadic and familial cases of PD (163-165). Innate immunity is the mechanism that is activated as soon as any foreign particle/pathogen or danger signal (PAMPs and DAMPs) appears. Functions of this innate system include removal of any foreign substances by phagocytosis, recruitment of immune cells to the site of infection, cytokine production, activation of cascade complement, and antigen presentation for activation of the adaptive immunity (166). Innate immune activity is regulated by the activation of receptors including TLRs, Nod Like Receptors (NLRs), and RIG-I Like receptors (RLRs) (167). Innate immune receptors initiate biological processes through the recognition of PAMPs as well as endogenously produced DAMPs. Cytokines and chemokines secretion is the result of these receptors activation (168). Cytokines are the key immunomodulator/immunoregulator of adaptive immunity as they regulate the polarization of lymphocytes (166).

T-cell infiltration (6, 169) and abnormal T-cell populations (14, 133-135) have been found in PD patients. Cytokines, chemokines, and other products of the peripheral immune system could also modulate the action, differentiation, and survival of neuronal cells (166).

Innate immunity	Adaptive immunity
• Depends on recognition of conserved molecular patterns found in many microorganisms (PAMP, DAMP) (168) • Fast first-line defense and includes the skin, mucous membranes, and other barriers to infection; lysozyme in tears, stomach acid, other antibacterial molecules, and numerous other factors belong to innate immunity (170). • Functions include removal of foreign substance by phagocytosis, recruitment of immune cell to the site of infection, cytokine production, activation of cascade complement, and antigen presentation for activation of adaptive immunity (171). • Mediated by macrophages, DCs, natural killer cells, complement and cytokines (170).	• Protection mechanism from an infectious disease agent due to clinical or subclinical infection with that agent or by deliberate immunization against that agent with products from it (170). • It is characterized by specificity, immunological memory, and self/non-self-recognition (170). • Response involves the clonal selection of lymphocytes that respond to a specific antigen (170). • Mediated by B and T cells following exposure to a specific antigen (170).

Table 1-Comparison between Innate and adaptive immunity.

1.4.1 Peripheral Innate immunity in PD

Many studies pertaining to neuroinflammation in PD focus on microglia undermining the role of the peripheral innate immune system. However, neuroinflammation is a global process that

encompasses the brain and involves players from both the CNS and the periphery (172). Dendritic cells (DCs) and macrophages are innate immune cells that similarly to microglia have antigen-presenting and cytokine production abilities (173).

1.4.2 Antigen Presentation

The adaptive immune response is based on T- lymphocytes recognition of antigen presented by APCs (172). There are two classical pathways of antigen presentation. The major histocompatibility complex (MHC) class I (endogenous pathways) presents peptides derived from endogenous molecules processed in the cytosol by the proteasome and loaded on MHC-I molecules in the ER (174). On the other hand, MHC class II (exogenous pathways) present peptides derived from exogenous proteins processed in the lysosome (175). Exogenous proteins can also be presented on MHC class I molecules through "cross-presentation" (175). Therefore, APCs display small peptides derived from processed antigens bound to MHC-I and II molecules (172). Naïve T-cells then recognize peptides presented on the MHC molecules and with the adequate cytokine context become effector T-Cells. Autoimmunity happens in an environment where regulatory mechanisms fail to control T-cell responses directed against the self-antigens (176). Autoimmunity is greatly controlled in the thymus where T-cells recognizing self-antigens presented by APCs are destroyed, a process called central tolerance (177). However, central tolerance cannot eliminate all self-reactive lymphocytes mainly because not all self-antigens are presented at the primary site of lymphocyte development (177). In the periphery, APCs play a major role in peripheral tolerance as the presentation of self-antigens to T-cells induce T-cell anergy or regulatory T-cells (Treg) (178, 179). Therefore, APCs play a crucial role in the

mechanism of tolerance and the properties of these cells and their local environment are essential to induce tolerance or immunity.

Professional APCs such as macrophages (180), monocytes (180), B cells (181), and microglia (182) express high levels of MHC-II and are very efficient at presenting antigens on MHC molecules (181).

1.4.3 Antigen-presenting cells: Dendritic Cells.

Dendritic cells, compared to other types of APCs, have superior abilities to sense, process, and present antigens, migrate to lymph nodes, and prime naïve T cells (181). DCs are bone marrow-derived cells playing a major role in immunosurveillance (172). They accumulate in inflamed tissues where they can locally present antigens to effector T-cells (172, 183). Studies have detected higher levels of DCs in the CSF (184) as well as in the CNS parenchyma of PD patients (185) compared to healthy donors. Under pathological conditions, DCs cannot always be distinguished as peripheral (186, 187) or resident microglia-derived (188) since microglia is such a heterogeneous population expressing many of the same markers as peripheral DCs (185). Inflammatory stimuli promote the migration and maturation of tissue-derived DCs to the draining lymph nodes (189). A current hypothesis of the role of DCs in PD pathology suggests that chronic neuroinflammation could attract DCs to the CNS (172). Data supporting this hypothesis have shown reduced levels of blood DCs in PD patients compared to healthy controls and the lower amount of DCs in patients correlates with higher motor impairment (190). DCs at the site of neuroinflammation might mature and migrate to the cervical lymph nodes where the presentation of autoantigens to T-cells and B cells may occur triggering an autoimmune response.

These studies suggest that DCs might contribute to the pathology of PD by establishing autoimmune lymphocytes and reinforce the growing concept that autoimmunity might contribute to PD.

A typical model used widely to study DC in vitro is Bone marrow-derived dendritic cells. After GM-CSF induction, these cells are able to process live intact Gram-negative bacteria for peptide presentation by MHC-I and MHC-II (191). Different TLR receptor ligands treatment results in different patterns of cytokine secretion by BMDCs suggesting potential primming for divergent immune responses (192). Co-culture experiments of BMDCs and Lymph-nodes cells have shown CD4+CD25+Foxp3+ T-cell expansion (a population of cells with similar markers as those found in regulatory T cells (Tregs) (193)). Moreover, co-culture experiments of BMDCs with naïve T-cells and vitamin C treatment resulted in preferred differentiation of Th1 cells (193). These studies suggest that under different conditions and cell population BMDCs have the ability to increase the differentiation of specific T-cell population ex-vivo.

1.4.4 Antigen-presenting cells: Macrophages

Macrophages are antigen-presenting cells that originate from a progenitor that migrates from the bone marrow to the peripheral blood (194). After migration to different tissues, macrophages differentiate after exposure to different local growth factors, pro-inflammatory cytokines, and microbial products(194). Their function includes phagocytosis (debris, infectious pathogens, and death cells), antigen presentation, and cytokine production (194). In PD patients, peripheral blood macrophages have been shown to produce lower TNF-α compare to healthy control (195).

Several monocyte-linage cell lines are available to study the behavior of the macrophages including the murine macrophage-like RAW 264.7 cell line. Unlike Bone marrow-derived macrophages (BMM), these tumors-derived cell-line has genetic alteration that allow them to grow continuously (196). This offer great advantages since additional mutation introduces to this cell line could be maintained to perform further studies. In terms of functionality, RAW 264.7 cells have been found to mimic BMM very closely in terms of cell receptors and response to microbial ligands that initiate cellular activation via TLR3 and TLR4 (196). However, this is only true depending on the number of times these cells are kept in culture (196). Furthermore, LPS treatment in RAW 264.7 results in the up-regulation of cell surface markers know to be expressed on dendritic cells and involved in antigen presentation and T-cell activation (197). RAW 264.7 could express a wide range of cytokines and chemokines except for IL-1β. RAW 264.7 macrophages lack the apoptosis-associated speck-like protein containing a CARD domain (ASC) and therefore are deficient in the processing and release of IL-1β (198, 199). BMM and RAW 264.7 both mediate chemotaxis and phagocytic activity through histamine H_4-receptor (200).

1.4.5 TLRs Pathways.

The interplay between inflammation and neuronal dysfunction is complex (201). In the CNS, innate immune responses are mainly mediated by resident immune cells such as microglia, astrocytes, and macrophages. These cells express TLRs that detect PAMPs and DAMPs followed by the activation inflammatory processes (202). TLRs are type I transmembrane protein with ectodomain containing Leucine-rich repeat that mediates the recognition of PAMPs derived from pathogens and DAMPs from dying or injured cells (202-205). The TLRs also express a Toll-interlukin1 receptor (TIR) domain at the C-terminal in addition to the leucine-rich repeat at the N terminal (203). Thirteen types of TLRs have been identified to which 1-9 are conserved in both mouse and human. TLR 1, 2, 4, 5, 6, and 10 are expressed on the cell surface and their main objective is sensing the extracellular milieu whereas TLR 3,7, 8, 9. 11, 12, and 13 are expressed in the endosome and they sense endogenous components such as viral RNA and DNA (203, 206, 207).

TLRs at the cell surface mainly recognize microbial membrane components such as lipids, lipoproteins, and proteins. TLRs (exogenous and endogenous) are activated as a homodimer or heterodimer (TLR1 and TLR6) (208). There are two TLR-mediated signaling pathways, the Myeloid differentiation primary response protein 88 dependent (MyD88) pathways and the TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathway (208). In the MyD88 dependent pathway, after TLR engagement, MyD88 along with kinases from the interleukin-1 receptor-associated kinase (IRAK) family forms the Myddosome (Fig. 4) (209). IRAK 4 activates IRAK1 (209), which is phosphorylated and later associates with the RING-domain of the E3

ubiquitin ligase TRAF6 (210, 211). TRAF6 polyubiquitinates itself and the transforming growth factor-β-activated kinase-1 (TAK1) (a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family) forming a complex to activate TRAF6 (Fig. 4). TAK1 then activates two different pathways that lead to the activation of the IκB kinase (IKK) complex: NFκB pathways and the MAPK pathways. TAK1, together with TAK1 binding proteins form a complex with TRAF6, which leads to the phosphorylation of IκB causing the activation of the IKK complex (Fig. 4) (210, 211). The IKK complex phosphorylates IκBα, the inhibitory protein of NFκB, which is then degraded causing NFκB to translocate to the nucleus, resulting in proinflammatory genes expression. TAK1 also activates the MAPK family members which regulate inflammatory responses (Fig. 4) (207). In the TRIF-dependent pathway, TRIF interacts with TRAF6 and TRAF3. TRAF3 binds the receptor-interacting protein kinase 1 (RIP-1) forming a complex that activates TAK1 (Fig. 4). This results in the activation of NF-κB, which induces the expression of inflammatory cytokines (Fig. 4). TRAF3 also recruits IKK-related kinase TANK-binding kinase 1 (TBK1) and IKK1 along with NF-kappa-B essential modulator (NEMO) for Interferon regulatory factor 3 (IRF3) phosphorylation Fig. 4). IRF3 forms a dimmer and translocates to the nucleus from the cytoplasm, resulting in the induction of type I IFN genes (Fig. 4). Based on studies using MyD88-deficient macrophages, the Myd88-dependent pathway was shown to be responsible for proinflammatory cytokine expression, while the TRIF-dependent pathway results in Type 1 interferons and interferon-inducible genes (207, 212).

1.4.5.1 TLR4 pathways

The first TLR to be characterized was TLR4 (208). TLR4 is a homodimer (213) that can be activated by several PAMPs including LPS, fusion protein from the respiratory syncytial virus (RSV), and the envelope protein from mouse mammary tumor virus (MMTV). Endogenous proteins that have been shown to directly or indirectly interact with TLR4 include heat shock proteins and hyaluronic acid and β-defensin 2 (214-216). TLR4 can activate both the MyD88 dependent and TRIF dependent pathways (Fig. 4). TLR4 signaling is negatively regulated by various mechanisms to prevent or terminate excessive immune responses that could be detrimental (208). Excessive immune responses have been associated with autoimmunity and inflammatory diseases (217). The MyD88- dependent pathway is suppressed by ST2825, SOCS1, and Cbl-b while SARM and TAG are suppressors of the TRIF-dependent pathway (218, 219). TLR4 has been implicated in the development of autoimmune diseases including Multiple sclerosis, Systemic Lupus Erythematosus, Systemic Sclerosis, Psoriasis, Sjogren's Syndrome, and Autoimmune Diabetes (220). In PD, TLR4 is upregulated (221) and its absence prevents striatal dopaminergic neuron loss and inflammasome activation in MPTP treated mice (222). Similar results were observed in *TLR4 KO* mice treated with rotenone (221). Moreover, picomolar concentrations of oligomeric α-syn have been shown to sensitize the production of proinflammatory cytokines by the TLR4 pathway in both microglia and astrocytes (223). Inhibition of the TLR4 pathway has been shown to attenuate inflammatory mediators in rat models of PD (224, 225). It has been suggested that mitochondrial dysfunction contributes to TLR4 activation by the production of endogenous high-affinity TLR-4 ligands (226). Interestingly, LPS-induced superoxide production in microglia has

been shown to be dependent on TLR4 activation which suggests an important role of TLR4 in PD pathogenesis through the production of proinflammatory cytokines (227).

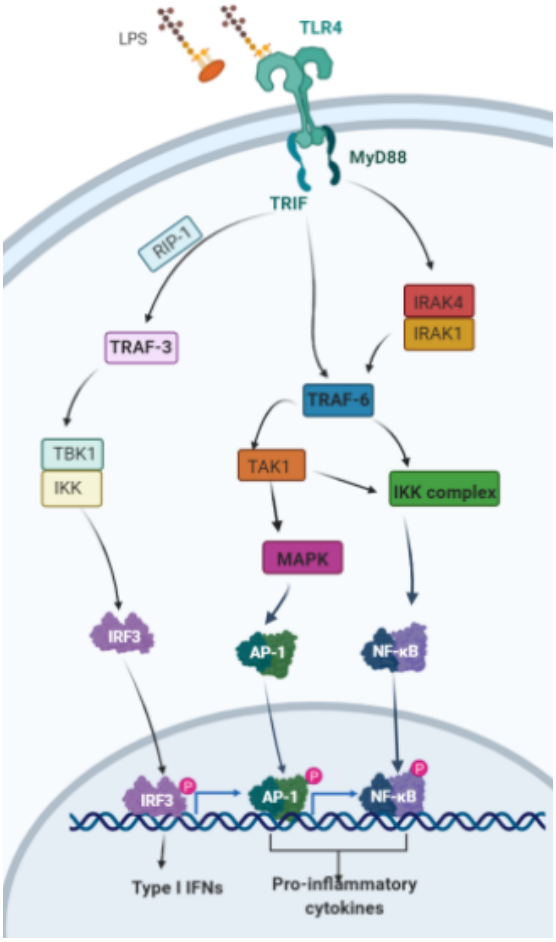

Figure 4-An overview of the TLR4 and TLR9 pathways

(a) LPS activates TLR4 homodimer formation and activates the MyD88-dependent signaling pathway that recruits the MyD88 complex and its downstream kinases, the IRAK1/IRAK4 complex. TRAF6 is then recruited to activate IKKs complex and TAK1 complex, which leads to the nuclear translocation of NF-κB and activation of MAPK. MAPK then activates AP-1 which translocate to the nucleus and result in the expression of proinflammatory cytokines. In the MyD88-independent pathways, after LPS ligation, TLR4 forms a complex that activates TRIF which

interacts with TRAF3 and TRAF6. Activation of TRAF6 and RIP1 leads to the nuclear translocation of NF-κB, while TRAF3 induces activation of TBK1/IKKi with subsequent nuclear translocation of IRF3. Source of Image: *Camberly Hernandez Paredes*

1.4.5.2.1 Lipopolysaccharides (LPS)

The pathogen-associated molecular pattern LPS is the most abundant component within the cell wall of Gram-Negative bacteria (228). LPS activates TLR4 resulting in the activation of the NF-κB pathway (228). LPS is extensively used as a model of inflammation as it mimics the inflammatory response produced by gram-negative bacteria (228). Inflammatory effects in various cell types resulting from LPS activation of TLR4 pathways include secretion of inflammatory cytokines such as TNF-α, IL-1β, or IL-6 (228).

Prolonged cytokines production has been shown to weaken the integrity of the intestinal barrier and mucosal layers resulting in bacterial infiltration and an increased concentration of LPS in the plasma (229). Several studies have shown an increase in intestinal permeability (230) and changes in the intestinal epithelial barrier in PD Patients (231).

Moreover, high inflammatory conditions (cytokines and chemokines) and circulating levels of LPS affect the permeability of the BBB. Thus, gut-induced inflammation may increase the leakage of immune cells in the CNS (232). Cells that infiltrate the brain include T-cells (169), and mast cells (233). As a result, additional release of inflammatory and neurotoxic molecules in the brain contributes to chronic inflammation and neuronal death (234, 235).

1.4.5.3 STING-Pathway

Another cytokine-producing pathway activated by DAMPS is cyclic GMP-AMP (cGAMP) synthase (cGAS)-STING Pathways. cGAS is a cytosolic DNA sensor that activates innate immune responses through the production of a second messenger cGAMP (236). In the absence of DNA, cGAS exists in an autoinhibited state (237-241) and is activated by double-stranded DNA regardless of the DNA sequence, however, oxidized DNA leads to greater induction of interferons (242). cGAMP functions as a secondary messenger that binds to the endoplasmic reticulum membrane adaptor STING, resulting in a conformational change and the activation of STING (243-245). STING traffics from the ER to the ER-Golgi intermediate compartment and the Golgi apparatus (Fig. 5). Active STING activates the kinase TBK1 that phosphorylates IRF3 which dimerizes and enters the nucleus. STING also activates the Kinase IKK which phosphorylate IκB family of NF-kB inhibitors and prompts their degradation (243). Then, NF-κB enters the nucleus and together with IRF3 induce the expression of cytokines such as TNFs, IL-1β, and IL-6 (Fig 5) (246).

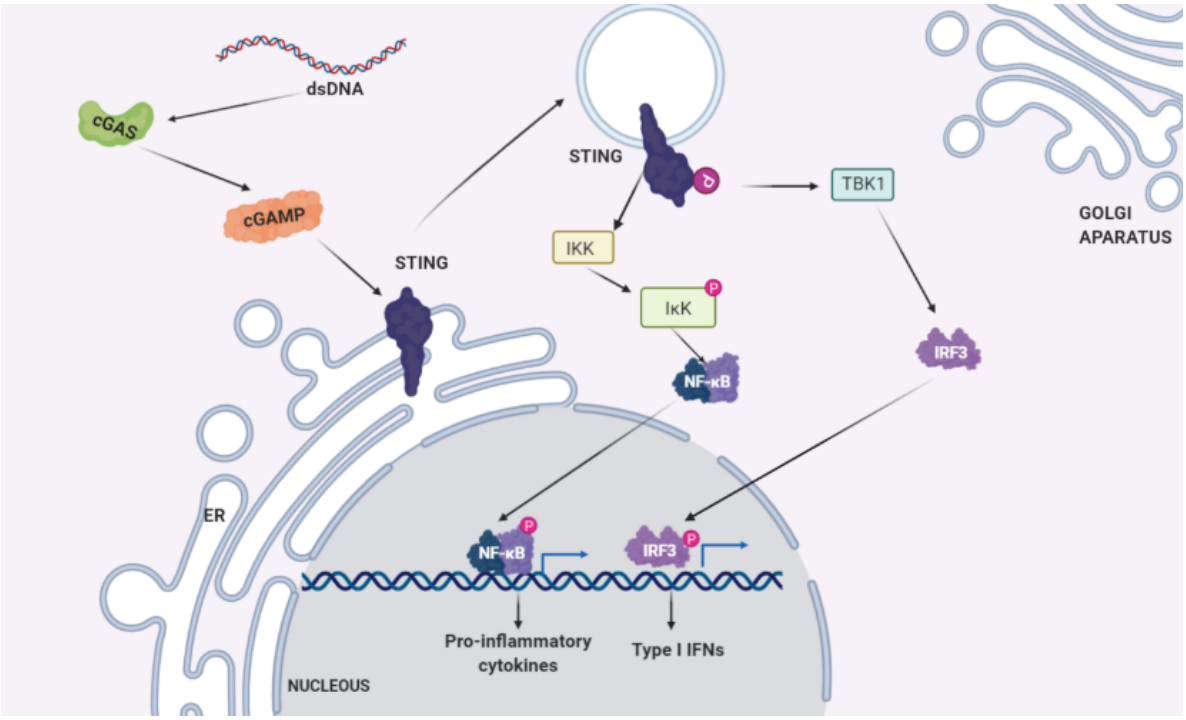

Figure 5-STING Pathway overview.

cGAS in the cytosol senses dsDNA initiating its activation. A secondary messenger cGAMP is produced which later binds and phosphorylates STING (159). STING translocates from the ER to the ER-Golgi intermediate compartment (166-168). Active STING activates IKK which then phosphorylates IκB that is in a complex with NF-kB (166). Once IkB is phosphorylated, it releases NF-kB that then translocates to the nucleus and activates pro-inflammatory cytokines expression. Active STING also activates TBK1 which results in the phosphorylation of IRF3. IRF3 then dimerizes and enters the nucleus resulting in the activation of Type 1 interferons (246). *Source of Image: Camberly Hernandez Paredes*

The association of STING with PD was proposed in an elegant experiment where both *PINK1 KO and Parkin KO* mice were crossed with the mutator mice resulting in the aberrant activation of innate immune signaling (136). Knocking out *STING* in these mice, resulted in a mitigation of the

inflammatory phenotype, DN degeneration, and motor impairment (136). Interestingly, contradicted effects have been demonstrated in *PINK1/ Parkin* Drosophila models, where knocking-out STING resulted in no effect in the amelioration of the phenotypic signature. This suggests that the role phenotype observed in *PINK 1 KO* and *Parkin KO* murine models might not universally depend on cGAS-STING activation (247). Similarly, another PD-related gene associated with neuroinflammation, *LRRK2*, seems to depend on the cGAS-STING pathway to induce inflammation. Indeed, *LRRK2 KO* macrophages under mitochondrial stress resulted in elevated basal levels of type I IFN (237) whereas double LRRK2/cGAS KO resulted in lowered type I IFN levels (248).

1.4.6 Cytokine dysregulation in PD

Both genetic and environmental factors, which ultimately result in a dysregulation of innate immune pathways and acute inflammation via cytokine secretion, have been shown to be associated with PD (4). Proinflammatory substances shown to be elevated in the striatum of PD patients include TNF-α, epidermal growth factor, transforming growth factor-alpha and beta, and IL-1β, 6, and 2. TNF-α, IL-1β, and IFN-γ have been detected in the SN (2), CSF (5), and serum (4, 249, 250) of PD patients. Expression of cytokines receptors, like the Tumor necrosis receptor 1 (TNF-R1) (a receptor that may induce apoptosis upon binding of TNF-α), has been shown to be elevated in the SN of PD patients (251). Proinflammatory cytokines have been shown to increase the level of MHC molecules expression in glia (252, 253) and neurons (254-256). DA neurons are particularly sensitive to inflammation and express higher levels of MHC molecules at their surface in inflammatory conditions compared to other types of neurons (257). Glia and lymphocytes will

in turn release cytokines, chemokines, and ROS creating a pro-inflammatory vicious cycle (258, 259).

1.4.7 Adaptive immunity in PD.

A constant interplay between innate and adaptive immunity exists and it is necessary to maintain an organism's homeostasis. In PD, the innate immune system is triggered by environmental and genetic factors that result in the activation of APC in the periphery and the CNS (172). Ultimately, antigen presentation, secretion of cytokines, chemokines, and expression of genes required for immune regulation may result in the engagement of adaptive immunity (7). T-cell population dysregulation and T-cell infiltration are some of the adaptive responses observed in PD (260). Descriptions of different T-cell populations are shown in Table 2.

Table 2-Types of T-cells.

Types of T -cells	Description	Cytokines required for differentiation	Cytokine produced	Ref.
CD4+Th2	• Orchestrate protective type 2 immune responses. • Facilitate tissue repair. • Contribute to chronic inflammatory diseases.	IL-4	Il-4 Il-13 Il-5	(261, 262)
CD4+Th17	• Defend against gut pathogens at mucosal barriers.	Il-1 β Il-6 Il-23 TGF-β	Il-17a Il-21 Il-17F Il-22	(261, 263)
CD4+Th1	• Produce IFN-γ.	Il-12 Il-18	IFN-γ Il-2	(261)
Treg	• Suppress T cell responses.	TGF-β Retinoic acid	Il-10 Il-35	(264, 265)
CD4+T_{FH}	• Support B cell.	Il-6 Il-21	Il-4 Il-21	(265, 266)
CD8+ Tc	• Kill virus-infected cells.	-	-	(267)

1.4.7.2 T-cell activation

The initial step for T-cell activation requires the interaction between the T-cell receptor (TCR) and CD4/CD8 coreceptors expressed at the surface of T-cells with peptide-MHC-II complexes presented by APCs. MHC class I is restricted to $CD8^+$cytotoxic T-cells while MHC class II is restricted to $CD4^+$ T-cells (268).

The second step for T-cell activation is co-stimulation. In this step pathogenic product, small stimuli and even broken cells byproducts can stimulate the B7 surface receptors (these receptors regulate immune responses via 'costimulatory' or 'coinhibitory' signals (269)) in APCs (270). These receptors are recognized by the CD28 receptor on T-cells. This recognition allows T-cells to respond to an antigen (270). Without this signal, T-cells become anergic, making it more difficult to activate in the future (270).

The third step depends on the cytokine milieu that will drive lineage-specific differentiation (proinflammatory (Th1, Th17) or anti-inflammatory (Th2, Treg) (Fig. 6) (271, 272).

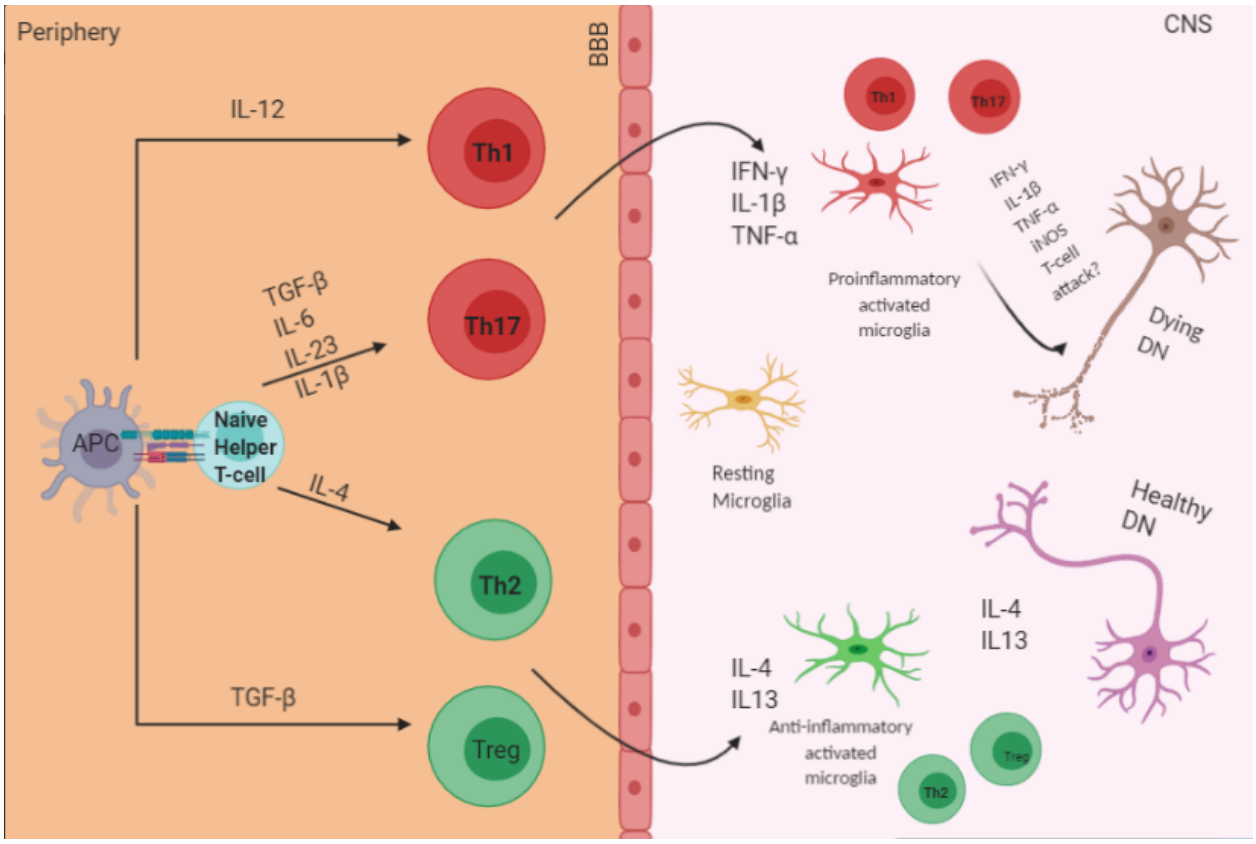

Figure 6-APCs activate Naïve Helper T cells through the interaction of MHC receptor and the TCRs in the periphery.

Naïve T-cells polarize into different sub-populations according to the cytokine context produced by APCs (260). Activated T-cells have the ability to cross the BBB, and once in the CNS, they interact with resident microglia that will again polarize these cells. The proinflammatory context will induce the differentiation of T-cells into Th1 and Th17 subtypes resulting in the production of other proinflammatory molecules including TNF-α, IL-1β, IL-6, inducible nitric oxide synthase (iNOS), and IL-17 (260). The anti-inflammatory context will favor T-cell polarization into Th2 helper or T-regs that produce IL-4, IL-5, IL-10, transcription growth factor β (TGF-β), and neuronal

growth factors such as Brain-derived neurotrophic factor (BDNF) (260). *Source of Image: Camberly Hernandez Paredes.*

In PD, a decrease in the total T-cell population is observed (7). T-cell population abnormalities are commonly observed as well (Table 3) (251). T-cell clones expressing specific α and β chains of the T-cell receptor (TCRαβ^{+}Tcells) as well as T-cell populations expressing γ and δ chains of the T-cell receptor (TCRγδ^{+}T cells) have been found in elevated frequencies in the CSF of PD patients (14). These subsets of cells are thought to play a possible role in Th17 inflammation in the CNS (273-275). In recent studies, two regions from the α-syn were described as immunogenic as specific T-cells for these peptides were found in patients with PD (15).In support of this finding, *in vivo* studies using MPTP mice have shown that α-syn drains to the cervical lymph nodes where it could be identified and processed by APCs that would later activate specific T-cells (276). This supports a role for T cells in PD through autoimmunity.

Dysregulated T-cell population in PD.	**Source**
Decreased $CD4^+$ helper T cell subtypes.	(134, 135, 277-279)
Decreased number of naïve T-cells.	(134, 135, 277-279)
Relative increased of proinflammatory subtypes Th1 and Th17.	(134,266,)
Decreased Tregs.	(267)
Increased frequency of $CD4^+$ T cells expressing Fas.	(134)
Increased IFN-γ producing Th1 cells in the periphery.	(134)
Decreased IL-4 producing Th2 in the periphery.	(134)
Decreased $CD4^+CD25^+$ T cells in the periphery.	(134)

Table 3-Abnormalities in T Cell population observed in PD (251).

$CD8^+$ T-cells have also been found in the SN of parkinsonian patients (280). Moreover, in the MPTP murine model of PD, $CD4^+$ and $CD8^+$ T-cells have both been observed in the SN and striatum (276, 281-283). More recently, mitochondrial-specific $CD8^+$ T-cells have been found in the brain of infected *PINK KO* mice (128). These results are again in favor of the hypothesis supporting the implication of autoimmunity in PD pathogenesis.

1.4.8 Th17 T cells.

T-cell polarization into Th17 cells requires a specific inflammatory context provided by APCs that produce IL-6, IL-1b, IL-23, and TGF-β. Th17 differentiation is regulated by several transcription factors including signal transducer and activator of transcription 3 (STAT3), retinoic acid receptor-related orphan receptor-gamma-t (RORγt), and aryl hydrocarbon receptor, (AHR) (284). IL-17, IL-8, IL-21, IL-22, IL-26, TNF-a, granulocyte-monocyte colony stimulatory factor (GM-CSF), CCL20, and IL-10, are some of the cytokines produced by Th17 cells that allow the recruitment of

neutrophils at inflammatory sites (285, 286). Th17 lineage function is the clearance of specific types of pathogens that require a massive inflammatory response and are not adequately dealt with by Th1 or Th2 cells (287). Some of the pathogens that can trigger a strong Th17 response include the Gram-positive *Propionibacterium acnes*; the Gram-negative: *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Bacteroides*, and *Borrelia*; the acid-fast *Mycobacterium tuberculosis*; and some fungi-like *Pneumocystis carinii* and *Candida albicans* (287). Th-17 cells have a strong pro-inflammatory property and they have been implicated in the development of several autoimmune diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), psoriasis, and inflammatory bowel disease (IBD) (288). Interestingly, studies have shown that Th17 cells could be divided into two types non-pathogenic and pathogenic cells. These two cell types differ in the production of cytokines when activated as well as in the cytokine milieu present during polarization. For instance, non- pathogenic Th17 cells arise from the treatment of naïve CD4+ T-cell with TGF-β1 and IL-6 and they are able to produce IL-10 (289). In contrast, pathogenic Th17 cells require the presence of TGF-β1, IL-6, and IL-23. In EAE, the murine model of Multiple sclerosis (MS) (an autoimmune disease where immune cells attacked the myelin covering the neuronal axons in the brain), induced Th17 cells with TGF-β1, and IL-6 were transferred to WT mice, do not trigger EAE. In contrast, Th17 cells polarized in the presence of TGF-β1, IL-6, and IL-23 resulted in severe EAE symptoms (289). Th17 cells have been shown to be increased in PD patients compared to healthy controls (290). Moreover, Th17 lymphocytes present in the blood of PD patients induce neuronal cell death in a human iPSC-derived DN. In MPTP mice models, Th17 cells have been shown to cross the BBB and invade the SN where they exacerbate neuronal loss (286).

Another subset of T-cells with the ability to secret IL-17 after exposure to the Th17 polarizing cytokine milieu are $CD8^+$T- cells (Tc17) (291). Although the functional and phenotypic characteristics of IL-17-secreting $CD8^+$T cells have not been systematically investigated (291)

1.5 The microbiome and PD

Besides the motor impairment, PD also has several non-motor symptoms including hyposmia, constipation, gastroparesis, and defecation dysfunction (292-294). Gastrointestinal (GI) dysfunction is a well-recognized hallmark of PD and the majority of the symptoms appear in the pre-stage of PD (295, 296). GI dysfunction is believed to be an important contributor to PD pathogenesis.

A healthy and stable microbiome community is essential for the proper homeostatic balance of barrier integrity, function, metabolism, and immunity of the gut; and a vital part of regulating the Gut-Brain Axis (297). Thus, the role of gut microbiota and microbial metabolites in PD pathogenesis is increasingly being investigated. Evidence exists for a bidirectional interaction between the Gut Microbiome (GM) and the CNS, typically known as the Microbiota-Gut-Brain-Axis (MGBA). Gut microbial dysbiosis has been associated with PD.

Altered Bacteria	Consequences	source
Prevalence of *Helicobacter pylori (H. Pylori).*	-Decreases L-dopa absorption,	(34), (298)
Increase *Lactobacillus.* Reduce *Clostridium coccoide.* Decrease *Bacteroides fragilis.* Decrease *Prevotella.*	-Lower mucus synthesis -Increase permeability in the gut.	(299)
Increase *Ralstonia.* Decrease *Blautia.* Decrease *Coprococcus.* Decrease *Faecalibacterium.* Decrease *Roseburia.*	-Bacterial population with anti-inflammatory properties	(168)
Increase *Enterobacteriaceae.* Decrease *Prevotellaceae.* Increase *Bifidobacterium.* Decrease *Enterococcaceae.* Decrease *Lactobacillaceae.* Decrease *Faecalibacterium prausnitzii.*	- Increase the severity of postural instability and gait abnormality	*(*300*)*

Table 4-Evidence of abnormal bacterial population and its Consequences in PD

Initial reports of dysbiosis of gut microbiota were reported years ago when a high prevalence of *Helicobacter pylori (H. Pylori)* was observed in PD patients (298). Several studies have associated bacteria population dysregulation to PD pathology. (Table 4)

1.5.5 Gram-Negative Bacteria: Enteropathogenic Escherichia coli (EPEC)

EPEC is a gram-negative bacterial which causes watery persistent diarrhea (301). This bacterium adheres to human intestinal epithelial cells and weakens the host cytoskeleton (301). Its mechanism of action begins extracellularly where it directly injects virulent factors through the host plasma membrane (301). Effects of EPEC virulent protein include reorganization of actin cytoskeleton resulting in pedestal formation, disruption of tight junctions, inhibition of phagocytosis, and induction of immune response (301). EPEC has been shown to upregulate the expression of TLR2 and TLR4 in rat intestinal epithelial cell18 (IEC18) (302).

1.6 A novel model of PD: intestinal infection of PINK1 deficient mice.

Because PD is a multifactorial disease, it is complicated to find appropriate animal models that comprise all the factors resulting in the disease. MPTP (28, 29), and rotenone (30, 31) are widely used across the field, however, they lack very important elements that highly impact the severity and progression of the disease. Only certain strains of mice are affected by MPTP treatment which causes difficulty when working with KO/KI PD-related mutations (303, 304). Many of the genetic mice models of PD do not show any phenotype including *PINK1 KO* and *Parkin KO* (112). However, *Parkin KO* mice were shown to have Parkinson-like phenotype when crossed with a mutator mouse (a mice strain that carries a proof-reading deficient version of PolgA (the nucleus-encoded catalytic subunit of mtDNA polymerase), resulting in an accumulation of mitochondrial

DNA mutation, premature aging, and reduce life spam without any neurodegenerative phenotype) (136, 305). This study suggests that mice models lacking phenotype probably required an initial trigger or extra environmental/molecular factors to develop any symptoms. Indeed, this theory was reinforced when with *PINK1 KO* mice. PINK1 controls mitochondrial homeostasis through the activation of mitophagy. It is also a major repressor of MitAP (66). When *PINK1 KO* mice (that do not show any phenotype when kept in germ-free conditions) are infected with the gram-negative bacteria *C. rodentium* (a mouse intestinal pathogen that is used as a model of human EPEC infection), the phenotype observed closely mimics that of PD (306). Parkinson's-like symptoms are detected as early as four months after infections and reversed by L-Dopa (128). An interesting feature of this model is the presence of mitochondrial-specific CD8+T-cells, which links mitochondrial dysfunction, inflammation, and probably the gut microbiota since it demonstrates that bacterial infection is enough trigger to induce an autoimmune response that results in PD-like pathogenicity. CD8+T-cells observed were able to attack dopaminergic neurons in vitro, providing a mechanism by which DNs are lost during PD. Another factor often observed in PD and that is well mimicked by these models was the CD8+T-cell infiltration in the brain (128). Because of the novelty of this model, many of the other hallmarks of the disease remain to be investigated. For instance, α-syn aggregation and Lewis body formation or cognitive impairment are yet to be determined. However, this model demonstrates that a combination of genetic and environmental factors is needed for the development of the PD. In comparison to the MPTP model (a widely used model that uses 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a trigger for PD), this model offers a much slower disease progression, which would provide investigators the chance to determine

molecular and phenotypic changes that could later be used as early biomarkers of the disease. Most importantly, this model had opened the door to develop new models of PD since a similar strategy could be used with the other asymptomatic mice models of PD

Chapter 2 -Rational and Hypothesis

PD is a complex and multifactorial aged associated disease (307). Mitochondrial dysfunction, genetic, and environmental factors all play an important role in the pathogenesis of PD (307). However, how all these factors interact or are interconnected is not fully understood. Interestingly, genetic defects associated with familial PD are primarily found in genes whose main function is to maintain mitochondrial homeostasis (*PINK1, Parkin, LRRK2*, etc.) (62, 65, 308). Recently *Matheoud, et al. 2016* connected mitochondrial dysfunction to immunity with the discovery of MitAP. This novel pathway of antigen presentation is regulated by PD-related genes *PINK1* and *Parkin* (66). In the absence of PINK1, gram-negative bacteria can induce MitAP, ultimately resulting in the generation of autoreactive mitochondrial-specific T-cells (66, 128). In addition to their important role in MitAP and mitophagy, recent evidence indicates that PINK1 and Parkin regulate innate immune responses (136, 139). Loss of function of these proteins in PD alters the production of pro-inflammatory cytokines(136), a process likely to have major consequences on the adaptive arm of the immune system, especially on T cell polarization. Indeed, the differentiation of T-cells into various T cell subsets is regulated by the release of cytokines by a variety of cells including APCs (271, 272). Even though APC orchestrate the T-cell response, these cells are poorly studied in PD. Moreover, the exact mechanism driving cytokine production dysregulation in the absence of PINK1 and Parkin is not well known. It was suggested that in the absence of these proteins and in the presence of stress, mtDNA is released from the mitochondria and stimulates pro-inflammatory cytokine production through the activation of the cGAS/STING pathway (160-162). However, the mechanism underlying mtDNA release into the

cytoplasm is not known. It has been shown that in lupus, mtDNA can exit the mitochondria through the formation of MDVs (309). Therefore, we hypothesize that the MDV/MitAP pathway promotes pro-inflammatory cytokine production in PINK1 deficient APC and that T-cell polarization is then strongly affected.

The hypothesis that autoimmune mechanisms are involved in the etiology of PD is gaining increasing support in the scientific community (310). Autoantibodies against antigens from proteins relevant to PD, such as α-synuclein (311), as well as T cells against this protein have been identified in PD patients (16, 312). Analysis of GWAS data also found an association between PD and several autoimmune diseases, including type 1 diabetes, Crohn disease, ulcerative colitis, and others (313). Stemming from this new PD murine model observations, we hypothesize that the frequency of mitochondrial-specific T cells is higher in PD patients compared to healthy individuals. A summary of the aims from our study are described in Figure 7.

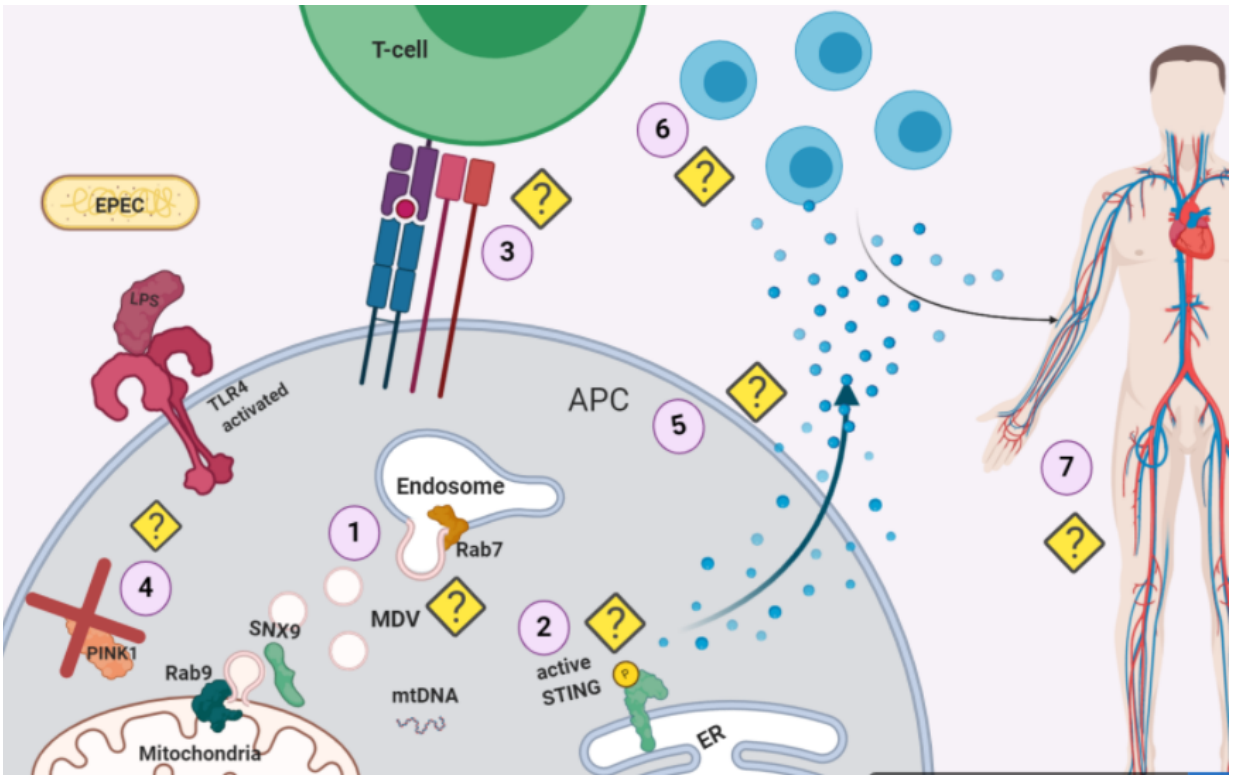

Figure 7-Hypothesis and rationale.

In this study we aimed to answer the following questions: (1) Is there a relationship between MitAP activation and cytokine secretion? (2,3) Does STING plays a role in mitochondrial antigen presentation and in proinflammatory cytokine secretion? (4,5) What is the consequence of the absence of PINK1 in cytokine secretion? (5,6) What consequences does an increased mitochondrial antigen presentation and increased proinflammatory cytokine secretion by APCs have in T-cells? (7) Can mitochondrial antigen specific T-cells be detected in PD patients? If they are detectable, what is their T-cell profile?

Chapter 3 -Materials and Methods

2.1 Animals and murine cells

C57BL/6J, Pink1-/- and littermate control mice were handled in strict accordance with good animal practice as defined by the Canadian Council on Animal care and according to protocols approved by the Comité de déontologie animale of the Université de Montréal, the McGill University animal care facility and CRCHUM animal care facility.

2.2 Raw macrophages

The RAW 264.7 macrophage cell line (ATCC), was cultured in DMEM complete (Gibco, 10569-010) (DMEM with 10% Fetal Bovine Serum (FBS) (Wisent, 080150) , 1% penicillin-streptomycin solution (Wisent Bioproducts 450-200-EL)). These cell lines express glycoprotein B (gB) from herpes simplex virus 1 (HSV1) targeted to the mitochondrial matrix (66). For the MitAP assay, a β-galactosidase-inducible HSV gB/Kb-restricted HSV-2.3.2E2 CD8+ T cell hybridoma (2E2) was used. This cell line is a CD8+ T cell hybridoma line that recognizes the $gB_{498–505}$ peptide loaded on MHC class I molecules to monitor antigen presentation (66). Hybridoma was maintained in RPMI complete (RPMI (GIBCO, 61870-036) with 10% FBS and 1% penicillin-streptomycin solution (Wisent Bioproducts 450-200-EL)). The list of cell lines used are described in Table 5.

Name	Description

Mito-gB	This cell line expresses glycoprotein B gB from HSV1 targeted to the mitochondrial matrix
SCR	This cell line is derived from Mito-gB with a scrambled shRNA.
Snx9 KD	This cell line is derived from Mito-gB with an shRNA targeted to Snx9 to decrease its expression.
Rab7 KD	This cell line is derived from Mito-gB with an shRNA targeted to Rab7 to decrease its expression.
Rab9 KD	This cell line is derived from Mito-gB with an shRNA targeted to Rab9 to decrease its expression.
Parkin+	This cell line is derived from Mito-gB, with increase expression of Parkin.
STING KO	This cell line is derived from Mito-gB with a STING knock out using CRISPER Cas9 Technology.

Table 5-Raw macrophages cell lines used.

2.3 Preparation of EPEC Bacterial cultures for in vitro experiments

Enteropathogenic Escherichia coli (strain E2348/69, provided by Samantha Gruenheid) were prepared in overnight cultures of LB broth and incubated at 37 °C, 5% CO_2 while shaking. EPEC was diluted to a final concentration of 1x10^5 CFU/µl in RPM+10% fetal bovine serum (FBS) before being used to infects cells at Multiplicity of infection (MOI) 1 (which is commonly defined as the ratio of infectious virions to cells in a culture).

2.4 Raw Macrophages (MDV/MitAP deficient cells, STING KO cells) treatments (EPEC/LPS) and RNA extraction.

Snx9 KD, Rab9 KD, Rab7 KD, Parkin+, and STING KO Mito-gb RAW cells were counted using the hemocytometer. For EPEC infection, 1 million cells per well were added in a 24 wells plate (SARSTEDT) in DMEM with 10% FBS for ELISAs. 2 million cells per well were plated for qPCRs in a 12 wells plate. Cells were left overnight for proper attachment. The next day, old media was removed and new media DMEM with10% FBS was added. EPEC was diluted to a final concentration of 1x10^5 CFU/µl in DMEM with 10% FBS before being used to infect cells at MOI 1. Cells were incubated at 37 °C, 5% CO_2 for 4hr (for qPCRS), or 6 hrs (For ELISA). Supernatants were left on ice for 10 min and centrifuged at full speed for 10 min to remove dead bacteria. For LPS stimulation, 1 million cells per well were added in a 24 wells plate for ELISA and 2 million cells per well were plated for qPCRs in a 12 wells plate, 16h before LPS addition. Then, old media was removed and new media containing DMEM complete with 50 ng/ml of LPS was added for 4hr (for qPCRs) and 6 hrs (For ELISA). IL-6 ELISA and IL-6, IL-1b, IL-23, and TGFb. For qPCRs, cells were lysed with Lysis Solution from Aura total RNA Mini Kit. RNA was purified using Aura Total RNA Mini Kit (BioRad-7326820).

2.5 BMDCs/Raw Macrophages ELISA and RT-qPCRs.

After BMDC infection, IL-6, IL-23, and IL-1b cytokine concentration were assessed in the supernatant using ELISA. (Mouse IL-6 DuoSet ELISA (R&D systems, DY406-05), Mouse IL-23 DuoSet ELISA (R&D systems, DY1887-05, Mouse IlL-1 beta/IL-1 F2 DuoSet ELISA (R&D systems, DY401-05)).

For qPCRs, extracted RNA was normalized to a final concentration of 50 ng/ul using NanoDrop® ND-1000 UV-Vis Spectrophotometer. cDNA was synthesized using iScript™

Select cDNA Synthesis Kit (BioRad-1708896) from 100 ng of RNA. The Primers used for qPCR are listed on Table 6. The cycle was set up as suggested by the kit. Thermocycler used for this reaction was Biometra T-Gradient. For qPCR reaction, SsoFast™ EvaGreen® Supermix (BioRad-1725201) was used and the Rotor-Gene® Q by QIAGEN was used to run the reaction. The cycle setup was as follows: Enzyme activation: 95 °C for 30 sec, denaturing: 95°C for 5 sec and annealing/extension: 62 °C for 10 sec.

Cytokine	Forward sequence	Reverse sequence
IL-1b	GGGCCTCAAAGGAAAGAATC	TACCAGTTGGGAACTCTGC
IL-6	AGTTGCCTTCTTGGGACTGA	GGCACTAAGGGCTCAGTCAG
IL-23	CCCCGTATCCAGTGTGAAGA	AAGTTGCTCCATGGGGCTAT
TGF-β	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC
IL-12	CCAGGTGTCTTAGCCAGTCC	GCAGTGCAGGAATAATGTTTCA
GAPDH	CAACTCCCACTCTTCCACCT	GAGTTGGGATAGGGCCTCTC

Table 6 List of primers for qPCR

2.6 Knocking out STING using CRISPER Cas9

The protocol used for the creation of this cell line was established at the Laboratorie of Michel Desjardins. This protocol was divided into 3 steps: CRISPER Cas9 electroporation, Cleavage assay, and Single-cell culture.

2.6.1 CRISPER Cas9 Electroporation.

For this step of the protocol, the guide RNAs are designed, synthesized and loaded into the CRISPER Cas9 enzyme, then loaded enzyme are transfected to cells using electroporation.

Three full-length guide RNA (gRNA) were designed using Invitrogen GeneArt Precission gRNA Synthesis Kit (A29377). The primer used for guide RNA synthesis (Guide RNA Invitro transcription TMEM 173 T1) of STING are described in Table 7. 5. 10^5 cells were transfected with 400 ng of gRNA, 2 µg of TrueCut Cas9 enzyme (Invitrogen, A36498), and 6 ul of R buffer (Invitrogen, MPK1025) were transfected to Mito-gb cells using Neon transfection system. Cells were electroporated at a voltage of 1680V with a width of 20 ms and 1 pulse (Neon Transfection systems, Thermo Fisher, MPK10025). Electroporated cells were transferred to a warm 24 wells plate containing 2 ml of DMEM with 10% FBS. Once cells reached about 80%-90% confluency, half of the cells were scraped off the plate for the Cleavage assay, the other half was used for single-cell culture (a method to isolate single-cell clones to create a homogenous population of cells).

Name	Forward Primer	Reverse primer
Guide RNA Invitro transcription TMEM 173 T1	TAATACGACTCACTATAGG TACCCAATGTAGTAT	TTCTAGCTCTAAAACGGCTATG CGTACACTGGAG
GCD SET 1 TMEM 173-T1	CCCCAGAAGCATAGCTGTG GAT	TCTTCCCACACCACCCACCT

Table 7-List of primers for CRISPER Cas9

2.6.2 Cleavage essay

This assay was divided into 3 steps: Step 1 is cell lysis and DNA extraction; Step 2 is DNA amplification and Step 3 Denature- reannealing of amplicon and cleavage. GeneArt® Genomic Cleavage Detection Kit (Life Technologies, A24372) was used for the digestion assay

2.6.2.1 Cell Lysis and DNA Extraction.

To lyse the cells, 50 µl of cell lysis buffer with 2 µl of protein degrader (provided by the GeneArt® Genomic Cleavage Detection Kit) were added to 1.5 X 10^6 cells. Cells were resuspended and transferred to PCR tubes. Samples were run in the thermocycler using the following cycle: 68 °C for 15 min, 95 °C for 10 min, then 4 °C on hold.

2.6.2.2 PCR amplification

For PCR amplification 2 µl of cells lysate, 1 µl of Forward, 1 µl Reverse primer (we used GCD SET 1 TMEM 173-T1 primers for this (Table 7), 25 µl of ampliTaq Gold 360 Master Mix and 22 µl of water were added. Then the DNA was amplified using the following cycle.

Stage	Temperature	Time	Cycle
Enzyme activation	95 °C	10 min	1X
Denaturing	95 °C	30 sec	40X
Annealing	62.5 °C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	7 min	1X
Hold	4 °C	hold	1X

Table 8-Condition for DNA amplification for Digestion assay.

2.6.2.3 Denaturation, re-annealing reaction, and cleavage.

3 µl of the PCR product was added to 1 µl of 10X Reaction Buffer and 8 µl of water in a PCR tube. PCR was centrifuged and the following program was used.

Stage	Temperature	Time	Cycle
1	95 °C	5 min	-
2	95 °C-85 °C	-	-2°C/sec
3	85 °C-25 °C	-	-0.1 °C/sec
4	4 °C	-	Hold*

Table 9-Condition for Denaturation and re-annealing reaction.

After the denaturation and the re-annealing reaction, a heteroduplex DNA is formed containing the insertion, deletion, or mismatched nucleotides. The detection enzyme subsequently recognizes the mismatches resulting from the insertion/deletion of nucleotides by the DNA repair mechanism (Non-homologous end joining) and cleaves it. Therefore, 2 bands could be detected in an agarose gel. 1 µl of detection enzyme (from GeneArt® Genomic Cleavage Detection Kit) was added to the sample, mixed, and incubated in a water bath for 37 °C for 1 hour. Immediately after the digestion steps, 10 µl of the digested product was loaded in 2% Agarose gel to check if the transfected cells have successfully mutated.

2.6.3 Single-cell culture

Cells were scraped and counted using the hemocytometer. Cells were diluted in DMEM complete to a concentration of 4 cells per 1 ml. 200 µl of the dilution was added per well in 96 well plates flat bottom. Wells were checked for single colonies periodically until colonies appeared (colonies usually appeared 15 days after plating). Wells that contained 1 colony were resuspended, split, and transferred to two 24 well plates. Once the cells were 70% confluent, one plate was kept for Western blot analysis and the other for DNA Sequencing DNA purification.

2.6.4 Western blotting

Total cell lysates were collected in lamelli lysis buffer containing Benzonase nuclease (1 ul) (Sigma-Aldrich, E1014-25KU). Quantification was performed using Nanodroper and 60 µg of proteins were used for immunoblotting. Proteins were separated on mini-Protean precast gels

(BioRad) at 200 V for 40 min and transferred electrophoretically at 100 V for 90 min onto an activated PVDF membrane. Membranes were blocked in PBS with 0.4% Tween20 (PBST) plus 5% milk for 30 min at room temperature. Then membranes were incubated OVN at 4C with primary antibodies (Sigma-Aldrich, MABF213, 1:500) and anti-GAPDH (Millipore, MAB374, dilution: 1:2000) as a loading control), diluted in PBS and 1% bovine serum albumin. The next day the membranes were washed 3 times in Phosphate-Buffered Saline-Tween (PBS-T) 0.05%, incubated in secondary antibody diluted in PBS-T plus 5% milk, (Goat-Anti-Rabbit-HRP: Jackson Labs, catalog number 111-035-144, 1:5000; Goat-Anti-Mouse-HRP: Jackson Labs, 115-035-003, 1:5000) for 30 min at room temperature. After washing the membranes 3 more times in PBS-T, they were developed using Clarity Western ECL substrate (BioRad, 1705060).

2.6.5 DNA sequencing

Selected colonies that revealed the KO in the Western blot. were grown and DNA was purified using the same purification method used for Digestion assay. A further step of DNA purification was applied using MinElute PCR Purification Kit (Qiagen, 28004). Samples were then sent for sequencing to the IRIC Genomics Platform.

2.7 MitAP Assay

The mitochondrial antigen presentation assay is a cocultured assay where the levels of mitochondrial antigen presentation can be assessed. Our laboratory has a line of mouse Raw Macrophages (RAW 264.7) that express gB from HSV1 targeted to the mitochondrial matrix. In theory, after 8h of LPS stimulation, these cells are fixed and a β-galactosidase-inducible HSV gB/Kb-restricted HSV-2.3.2E2 CD8+ T cell hybridoma (2E2) is used to determine the level of gB loaded in MHC class 1 receptors. The 2E2 cells are incubated for 16h at 37°C. After

activation by gB recognition, the 2E2 T cells would express IL-2 (an indicator to T-cell activation) and the reported gene β-galactosidase. Following lysis, β-galactosidase hydrolyze CPRG converting the yellow-orange CPRG substrate into the red chromophore chlorophenol red (314). This results in a dark red solution that can be measured at 595 nm.

In 24 wells plate, 1 million Mito-gb cells were plated. The next day, cells were treated with 50 ng/ml of LPS for 6 hr. Cells were then fixed with 1% PFA for 10 min at RT and washed with RPMI, 10% FBS, and 0.1M glycine. Finally, 10^5 2E2 T cells were added for 16 hr. Cells were lysed and incubated with chlorophenol red-beta-D-galactopyranoside (CPRG) solution at 37°C. The β-galactosidase activity was measured at 595 nm after the addition of the CPRG substrate.

2.8 Bone marrow-derived dendritic cells (BMDC) generation

The tibia and femur were dissected from PINK1 -/- and WT mice. Bones were placed at %70 ethanol for 1 min then washed with sterile PBS. Using a 25 mm sterile syringe and RPMI complete bones were punctured, and cells were removed from bones. Cells were filtered with a 40 µm filter in a 50 ml tube, then centrifuged for 5 min at 1400 rpm. The supernatants were removed, and cells were resuspended in 2 ml of Ammonium-Chloride-Potassium (ACK) buffer (this buffer was used to lyse any red blood cells present in the population of bone marrow cells extracted) for 1 min. 10 ml of RPMI complete was added and cells were centrifuged for 5 min at 1400 rpm. The supernatant was removed, and cells were split in fresh RPMI complete with GM-CSF enriched medium (1:20, previously produced using J558L cells and titrated). Cells were incubated at 37 °C, 5% CO_2 for 7 days. GM-CSF was added on day 4.

2.9 BMDC EPEC infection and RNA extraction.

The protocol used for infection of BMDCs and Raw macrophages was the same established by *Matheoud et Cannon 2019 (*127*).* Two million BMDCs were plated in 24 well TC-plate (SARSTEDT) in 1 ml of RPM with 10% FBS. EPEC was diluted to a final concentration of 1x10^5 CFU/µl in RPMI with 10% FBS before being used to infect cells at MOI 1. Cells were incubated at 37 °C, 5% CO_2 for 4hr. Supernatants were collected, and 10% Penicillin-Streptomycin Solution (Wisent Bioproducts 450-200-EL) was added to neutralize bacteria. Supernatants were left on ice for 10 min and centrifuged at full speed for 10 min to remove dead bacteria. Cells were lysed with Lysis Solution from Aura total RNA Mini Kit. RNA was purified using Aura Total RNA Mini Kit (BioRad-7326820).

2.10 T-cell polarization

Because we have shown previously that there was a dysregulation of proinflammatory cytokines, then we wanted the investigate the consequences of this dysregulation in T-cell populations. We designed an experiment where BMDCs from PINK1 KO mice would be activated with LPS (since EPEC infection after a couple of days could result in cell death) and co-cultured with naïve T-cells. The LPS treatment would active a constant cytokine secretion that would create a milieu that could induce Naïve T-cell differentiation. We hypothesize that the cytokines induced by LPS would result in a biased differentiation of naïve T-cell into Th17 cells. As a control for this experiment, we used Dynabead Mouse T-activator CD3/CD28 cells to check activation and a cocktail that induced Th17 cells to determine if the phenotype observed would be comparable. Finally, T-cell activation was determined using Flow cytometry analysis.

Spleens from C57BL/6J were collected and naïve CD4 T cells were purified using a $CD4^+CD62L$ cell isolation kit (Miltnyi Biotec). Cells were counted and resuspended RPMI

complete at a concentration of 2 million cells per ml. 500 µl of the cell solution was placed as triplicated in a 48 wells plate. Dynabeads Mouse T-activator CD3/CD28 (Gibco) were added to T-cells (1 bead per 2 cells). WT and PINK1 -/- BMDC were added in a 1:1 ratio in RPMI complete with LPS (50 ng/ml, InvivoGen, tlrl-eblps) at day 0, day 3, and day 5.

As a positive control, a Th17 inducer cocktail was used (human TGF-β (R&D systems, 240-B) at a concentration of 3 ng/ml, recombinant mouse IL-23 (R&D systems, 1887-ML-010) at a concentration 20 ng/ml, recombinant mouse IL-6 at 20 ng/ml (R&D systems, 406-ML), mouse anti-INF-γ antibody (InvivoMab BE0055) at a concentration 10 µg/ml). On days 3 and 5, human IL-2 (R&D systems, 202-IL at a concentration of 50 U/ml) and recombinant mouse IL-23 (R&D systems, 1887-ML-010) were added at a concentration of 10 ng/ml.

The negative control consisted of the CD4 T-cell stimulation with anti-CD3/anti-CD28 beads (Dynabeads Mouse T-activator CD3/CD28 (Gibco, 11456D) 1 bead per 2 cells). Cells were ready for staining on day 7.

2.11 Murine FACS analysis

Cells from the BMDC/ T-cell co-culture were stained using the same protocol as for PBMC. For the FcR blocking, we used anti-CD16/CD32 (1:50, BD, 553142). The antibodies used are described in Table 10.

Antibody	Dilution	Source	location
APC-H7 Rat Anti-mouse CD4	1:25	BD Pharmingen, 560659	Extracellular
BV786 Anti- mouse CD3e	1:100	BD Horizon, 564379	Extracellular
PERCP-CY5.5 Rat Anti-mouse TNF-α	1:50,	BD Pharmingen, 560659	Intracellular
BV421 Rat Anti-mouse GM-CSF	1:25	BD Horizon, 564747	Intracellular

APC Rat Anti-Mouse IFN-γ	1:25	BD Pharmingen, 554413	Intracellular
PE Rat Anti-Mouse IL-17A	1:25	BD Pharmingen,559502	Intracellular

Table 10-List of antibodies used for murine cells FACS staining.

2.12 Peripheral blood mononuclear cell (PBMCs) mitochondrial antigen stimulation

The goal of this protocol was to determine if there were any mitochondrial specific T-cells in the PBMCs of PD Patients. In theory, mitochondrial protein-coated beads would be phagocytosed by the APC population in the PBMCs and mitochondrial antigen would be presented to T-cells. If there is any T-cell activation in this population it suggests mitochondrial antigen-specificity. A diagram of this protocol is shown below (Fig. 8). This protocol is divided into 4 parts, Beads preparation, PBMCs isolation, PBMCs stimulation, and flow cytometry analysis.

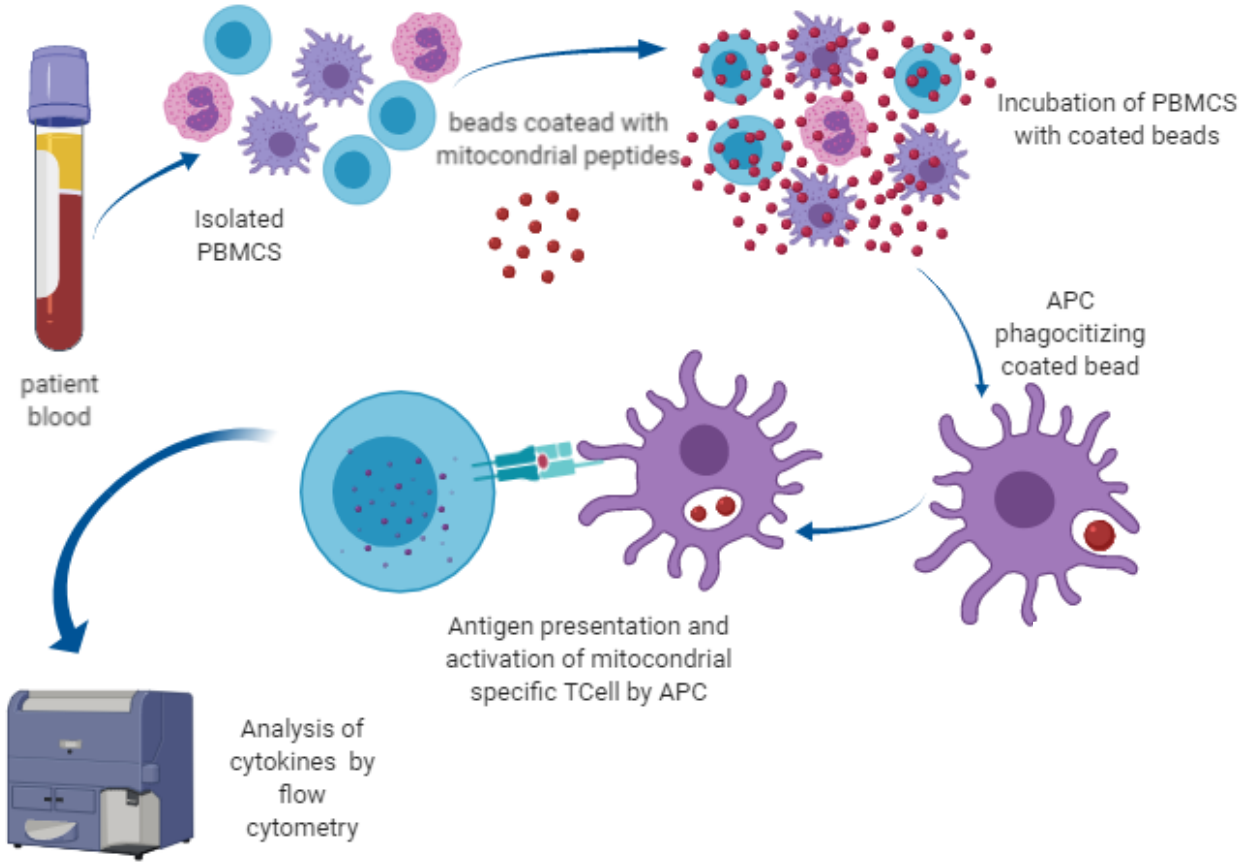

Figure 8-Mitochondrial antigen stimulation protocol

PBMCs were isolated from PD patients and age/sex-matched healthy control. PBMCs were then stimulated for 5 days with latex beads coated with mitochondrial proteins or Bovine Serum Albumin (BSA) (as negative control). CD3/ CD28 beads were used as a positive control. Cells were then stained and analyzed by flow cytometry. *Source: Camberly Hernandez Paredes.*

2.12.1 Bead's preparation

Beads (Eastapore white beads microspheres, K010, 39469081, Sigma-Aldrich) were prepared a day before receiving human samples. Beads were mixed well and vortexed before usage. 50 µl of beads were placed in a 1.5 ml microtube tube, vortexed, and centrifuged at 1400 rpm for 5 min. The supernatant was removed, and 1 ml of PBS was added to the beads in order to wash them. Beads were pipetted up and down until uniformly spread in the tube. In order to homogenize the

beads suspension, beads were sonicated for 10 min, then vortexed and centrifuged at 1400 rpm for 5 min. The supernatant was removed, and beads were resuspended in 50 µl of mitochondria purified proteins (for this experiment we used purified mitochondria from human placenta, 1mg/ml), human recombinant OGDH (BioSource, MBS969587, 1mg/ml), and BSA (BioShop, 9048-46-8, 1mg/ml). Beads were vortexed, sonicated for 10 min and incubated overnight at 4 °C with rotation.

2.12.2 Human PBMCs

Human blood samples were processed according to the Centre de recherche du CHUM. Patients and age/sex-matched healthy donors with a mean age of ~60 were recruited at the Andre-Barbeau Movement Disorder Unit of CHUM in collaboration with Dr. Michel Panisset (CHUM, Neurologist, Movement disorder clinic), Drs. Arbour, and Dr. Prat. 50 ml of blood will be collected from patients with informed consent. A description of the basic information about the patients used in this study are listed on Table 11. In this study, we wanted to prevent any biased by the clinical state of the patient, thus our experimental procedure was blinded until the end of the collection. Once our lab has enough patients to have significant results, then our focus would switch to the clinical profile of each patient.

Donor	Condition	Sex	Age
AA-SC-1052	PD	Male	62
PD-55	Control	Male	62
LF-SC-1227	PD	Female	63
DG-045	Control	Female	61
DM001P	PD	Male	~70
DM002C	Control	Male	~70
DM003C	Control	Male	~70
DM005P	PD	Male	72
DM006C	Control	Female	72
DM007P	PD	Female	70
DM008P	PD	Male	74
DM009C	Control	Male	55-60
DM0010P	PD	Male	55-60
DM0011C	Control	Female	56

Table 11-List of blood donors for mitochondrial activation experiment.

2.12.3 PBMC Isolation

Two tubes of blood (about 20 ml of blood) were transferred in 50 ml conical tubes, each tube was rinsed with 7 ml of PBS-EDTA 2mM and poured in the 50 ml tubes already containing the blood. Tubes were completed with PBS EDTA 2mM until they had a volume of around 34-35 ml. Thirteen ml of Ficoll-Paque Plus (VWR International, CA95021-205L) were pipetted at the bottom of the tube very carefully and slowly in order to form a distinct layer underneath the blood. Samples were centrifuged at 800 g for 30 min at room temperature without brake

(acceleration 5, deceleration 1). The top yellow layer (PBS and Serum) was removed and the middle band containing the PBMCs was transferred to a fresh 50 ml canonical tube. Cells were washed with 45 ml of PBS-EDTA 2 mM and centrifuged at 500 g for 15 min (acceleration 9, deceleration). The supernatant was discarded, the pellet was resuspended, and all tubes were pooled together. PBMCs were washed with 45 ml of PBS-EDTA 2 mM and centrifuged at 200 g for 10 min (acceleration 9, deceleration 9) to remove all platelets. Cells were resuspended in 20 ml of RPMI (GIBCO, 61870-036) (GIBCO, 61870-036) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Wisent Bioproducts 450-200-EL).

2.12.4 PBMCs mitochondrial antigen stimulation

Isolated PBMCs were diluted at a concentration of 2 million cells/ml in RPMI complete media. Coated beads and CD3/ CD28 beads (Dynabeads, 11131D) were added (1:500 dilution) together with recombinant human IL-2 (20 U/ml). Cells were incubated at 37 °C, 5% CO_2 for 5 days. Cells were then transferred in a round bottom 96 well plate for Flow cytometry staining.

2.12.5 Human FACS staining.

PBMCs were stimulated during the last 4 hrs of incubation with protein secretion inhibitors (Protein Transport Inhibitor (Containing Monensin) Golgi Stop, BD, 554724, 1:1000; Protein Transport Inhibitor (Containing Brefeldin A Golgi Plug, BD, 555029, 1:1000) and non-specific T-cell activation drugs (Phorbol 12-myristate 13-acetate, PMA, Sigma, 79346-1MG, 1mg/ml 1:20000 and Ionomycin calcium salt from *Streptomyces conglobatus*, Sigma-Aldrich, I0634-1MG, 1mg/ml 1:1000) in order to activate the cells and to accumulate the related cytokines inside the cell. Cells were first stained with Fixable Viability Stain (BD Horizon, 564997, 1:3000) and their Fc receptors were blocked using FcX Fc Receptor Blocking Solution (1:50, BioLegend 422301). Cells were incubated for 5 min at RT in the dark. The surface markers were

stained by adding the appropriate antibodies (refer to Table IX) for 20min at 4°C with agitation in the dark. Cells were fixed and permeabilized using a kit from BD (Fixation/Permeabilization solution kit, 554714). The cytofix/cytoperm solution was added for 20 min at 4°C with agitation in the dark. Cells were blocked using FcX Fc Receptor Blocking Solution (1:50) diluted in the perm/wash buffer (15 min at 4°C with agitation in the dark). Cells were stained for intracellular cytokines (refer to Table 13) overnight at 4°C with agitation in the dark. Cells were analyzed using BD LSRFortessa. Final data analysis was done using Flowjo.

Antibody	**Dilution**	**Source**	**Location**
PerCP/Cyanine5.5 anti-human CD3	1:50	BioLegend, 344807	Extracellular
BUV737 Mouse anti-Human CD4	1:10	BD, 564305	Extracellular
APC-H7 Mouse anti-Human CD8	1:25	BD, 560179	Extracellular
APC anti-human GM-CSF	1:10,	BioLegend, 502310	Intracellular
PE anti-human IFN-γ	1:10	BioLegend, 506507	Intracellular
BV421 anti-human IL-17A	1:10	BioLegend, 512322	Intracellular
Alexa Fluor 488 anti-human TNF-α	1:10	BioLegend, 502915	Intracellular

Table 12-List of antibodies used in PBMCs for FACS staining.

2.13 Statistical analysis

p values were calculated using Prism (GraphPad Software Inc., San Diego, CA). Differences between the two groups were tested with a Man Whitneys test, and the differences between three or more groups were tested by one-way or two-way ANOVA.

Chapter 4 -Results

3.1 The MDV/MitAP pathways might play a role in cytokine production.

PINK1 KO DCs have been previously shown to have high levels of MitAP and MDVs (66). Previous studies have implicated PINK1 in cytokine production regulation through the inhibition of mtDNA release from the mitochondria (a strong stimulator of pro-inflammatory cytokine production) (315). Furthermore, it has been shown that MDVs can carry mtDNA (309) and activate the innate immune system. Therefore, we hypothesize that the MDV/MitAP pathway regulates pro-inflammatory cytokine production in raw macrophages. Rab9, Rab7, and Snx9 have been shown previously to regulate MDV generation and fusion with lysosomes in RAW 264.7 macrophages (66). To determine the role of MitAP in the regulation of cytokines production, different macrophage cell lines with reduced levels of Rab9, Rab7, Snx9, and a cell line in which Parkin was re-introduced (APC express low levels of Parkin), were tested for their ability to produce cytokines upon TLR4 stimulation. In this experiment a scrambled shRNA control (SCR) was used, this is a cell line of macrophages with scramble small hairpin RNA (66). We decided to use these cell lines because they have been previously used by *Matheoud et al 2016* to confirm that levels of MitAP in Snx9 KD (Knock Down), Rab9 KD, and Parkin+, were lower compared to SCR control and PINK1 KD when treated with LPS (refer to *Matheoud et al 2016*). *Matheoud, Cannon et al 2019* also used them to confirmed that MitAP levels were lower on Snx9 KD and Parkin+ compared to SCR control after 8hrs of EPEC infection (refer to *Matheoud, Cannon et al 2019* Fig. 1) (128). Thus, we assessed the levels of Il-6 produced in SCR, Rab9 KD, Rab7 KD, Snx9 KD, and Parkin+ treated with EPEC for 6hrs. The protocol used

for this experiment was established by *Matheoud et Cannon 2019*, thus the treatment used should not affect cell survival (128). We used EPEC because is a gram-negative bacteria found in the human gut that mainly activates the TLR4 pathways (301). We found that IL-6 secretion was decreased in cells in which MitAP was affected (Rab9 KD, Rab7 KD, Snx9 KD, and Parkin+) compared to SCR control cells (Fig. 9a). To determine whether this decrease in cytokine secretion was due to a lower gene expression we decided to perform quantitative PCR. Raw macrophages with different Knockdowns cells were stimulated with EPEC for 4 hrs, and the levels of mRNA were measured. Similar to their cytokine profiles, the mRNA level for IL-6, IL-1β, IL-23, and TGF-b (Fig. 9b) were decreased. (The fold change observed is treated KDs to nontreated KDs sample normalized using GAPDH as a house keeping gene). These results suggest that the MDV/MitAP pathway could play an important role in the production of proinflammatory cytokines in APC.

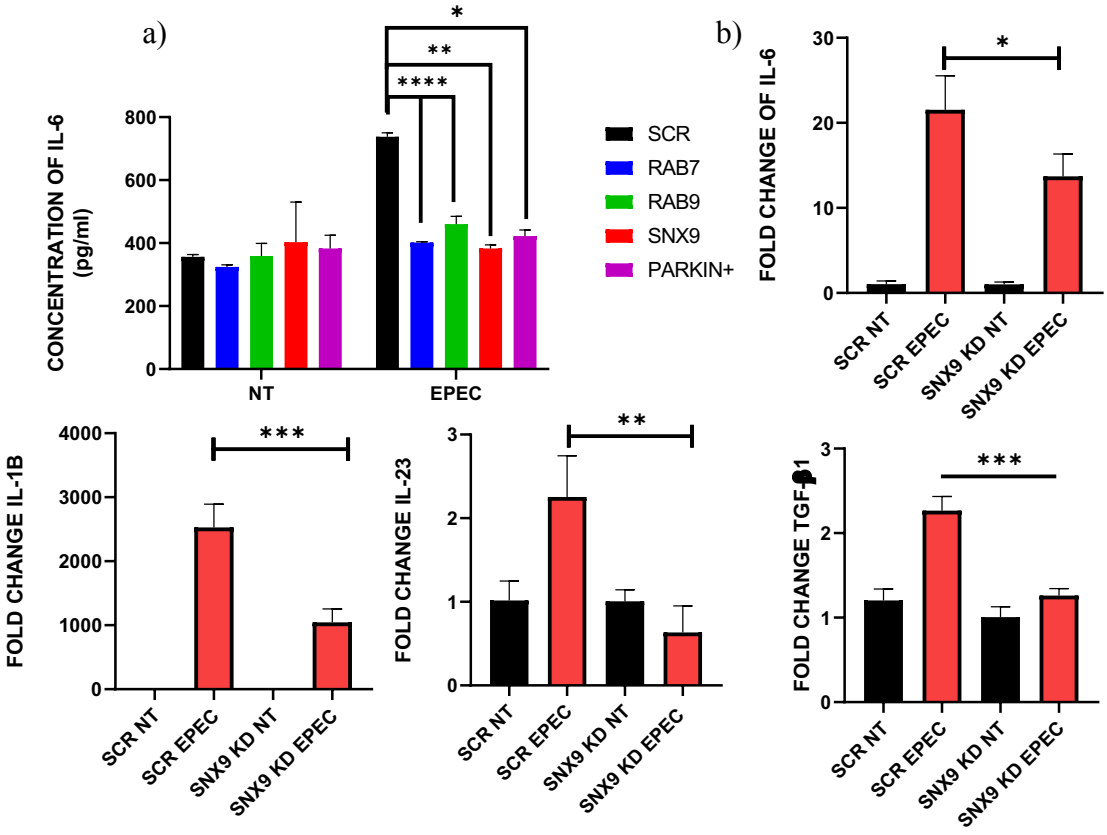

Figure 9-MitAP regulates cytokine production in APC.

(A). Measuremtn of IL-6 secretion in SCR, Rab7 KD, Rab9 KD, Snx9 KD and Parkin+ cells infected with EPEC (MOI 1) for 6h. (b) The expression of different cytokines was assessed by RT-qPCR in SCR and Snx9 KD infected with EPEC for 4hrs. Statistics: one-way ANOVA. ****p<0.0001., ***p<0.001, **p<0.01, *p<0.01. Data is represented as means +SD.

3.2 The cGAS/STING pathway regulates MitAP and cytokine production.

Our results suggest that the MDV/MitAP pathway influence some innate immune pathway responsible for cytokine secretion. One major DAMP that activates immune pathways is mtDNA. This DAMP was found to be in high levels in *Parkin KO* mice serum (136). Mitochondrial DNA can potentially activate the TLR9 pathways (162), and the STING pathway.

Sliter et al. have previously shown that knocking out *STING* in *PINK1* and *Parkin* deficient mice could mitigate the over-inflammation observed after mtDNA mutation or exhausting exercise (136). As MDVs can carry mtDNA and allow their release from the mitochondria (309), we hypothesize that MDVs are the shuttle for mtDNA to exit the mitochondria and stimulate the cGAS/STING pathway. Thus, we decided to determine if the STING Pathways plays any role in MitAP, since it has been shown to alleviate PINK1 KO-induced inflammation. We decided to develop a STING KO cell line in our Mito-gb RAW 264.7 macrophages using CRISPER Cas9 technology. We used our Mito-gb RAW 264.7 because they have already the technology to easily measure MitAP levels. We designed and amplified Cas9 guide RNA targeting STING. Then, we transfected Mito-gb cells with the gRNA. Then, we did a cleavage assay to determine if the transfection was successful. In the cleavage assay, the locus where the mutation is introducing is amplified using PCR. The PCR product is denatured and reannealed creating heteroduplex DNA with mismatch strands. The mismatch strand forms a bubble that then is recognized by a digesting enzyme. The primers are designed to amplify the DNA loci targeted to cover a region of about 600 base pairs(bp). The mutation site is located ~200 base pairs away from the 5' end of the strand. Thus, if a mutation is introduced, two bands of different sizes would show in the agarose gel (Fig. 10a). Once we confirmed that the cells had introduced a mutation, a single-cell culture was started to isolate cells with a mutation and create a homozygous population. Only wells with a single colony of cells were sent for sequencing. The sequencing data showed a 25 bp deletion followed by a frameshift mutation that resulted in premature stop codon 7 codons away from the site of deletion (Fig 10c). The expression of STING was also assessed by western blot (Fig. 10b).

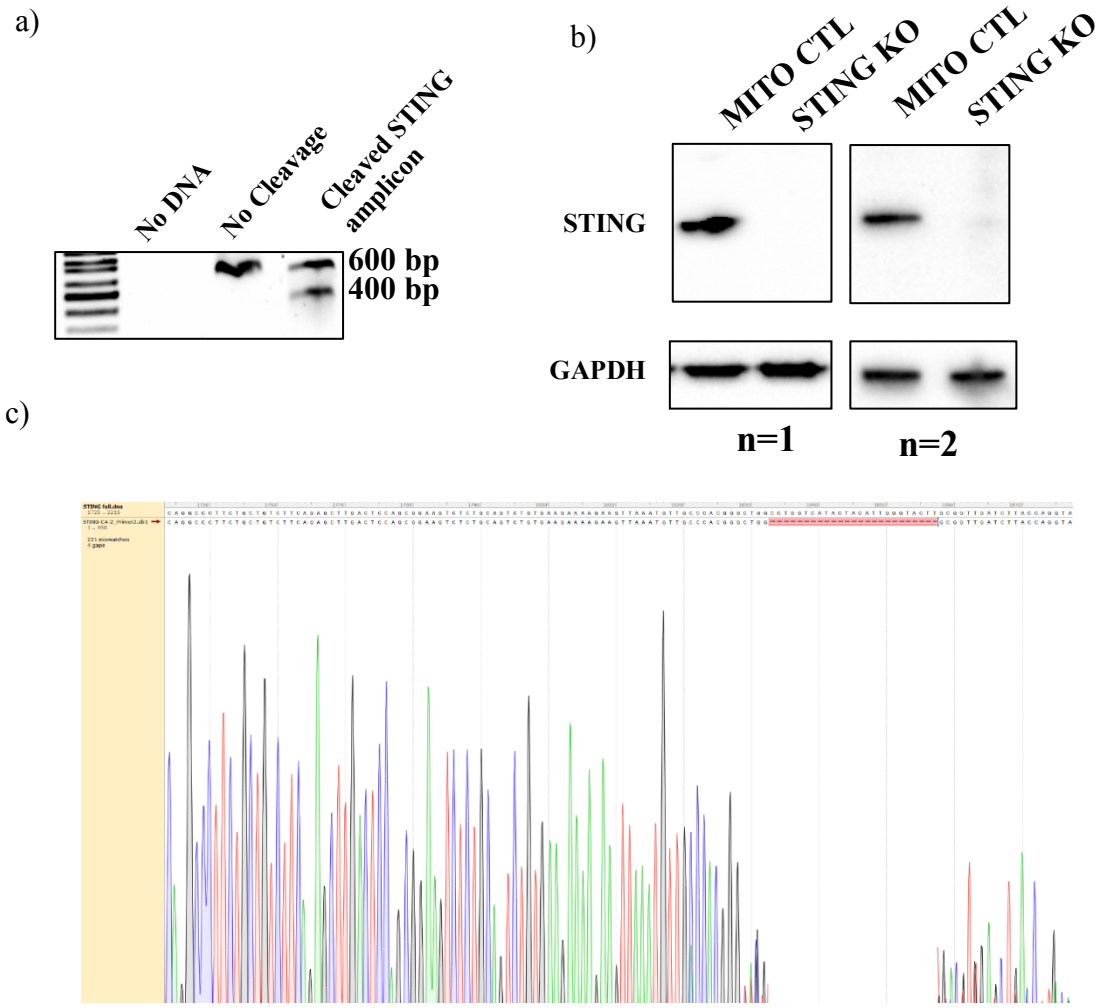

Figure 10-CRISPER Cas9 for STING KO in Raw Macrophages.

After the transfection of Mito-gB cells, DNA was purified, amplified, and digested to determine if the mutation was introduced. (a) The positive amplicon shows 2 bands one at 600 bp which is the size of the undigested amplicon, and one at 400 bp showing the digested bp. (b) The production of STING was determined using western blot in raw macrophages (n=3 times). (c) Sanger sequencing shows a 25 bp deletion that resulted in a frameshift.

Then we assessed the levels of MitAP in the absence of STING using the gB-specific CD8-T cell hybridoma. To monitor the levels of MitAP (recognition of gB protein by 2E2 T-cell hybridomas) in the *STING KO* cells line, we treated *STING KO* cells and control cells with LPS.

To our surprise, we found that the levels of MitAP were strongly inhibited in the absence of STING (Fig 11a) after 6 hrs of LPS treatment. To determine whether there is an issue with cell viability in the MitAP assay a peptide control is also run along with each sample. In this control, we saturate all the surface MHC class 1 receptors with the gB peptides on the raw macrophages, as a result, peptides on the MHC class 1 receptor are replaced by the gB peptide and then recognized by the 2E2 hybridoma. Thus, any difference amongst treatment is an indication that the treatment could be toxic to cells or it affects the expression of MHC class 1 receptors (127). Figure 11b shows no statistically significant difference between MitAP levels between the raw Mito control nontreated, STING KO no treated, Mito LPS treated, and STING KO LPS treated samples. This suggest that the treatment used in this experimental set up did not affect MHC-1 expression or cell viability. We also assessed the levels of cytokine production After LPS treatment for 4 hrs, we found that the levels of mRNA expression IL-6, IL-1β, IL-23, and TGF-β cytokines were decreased in *STING KO* compare to the SCR control (Fig 11c). These results suggest that the cGAS/STING pathway regulates MitAP and cytokine production.

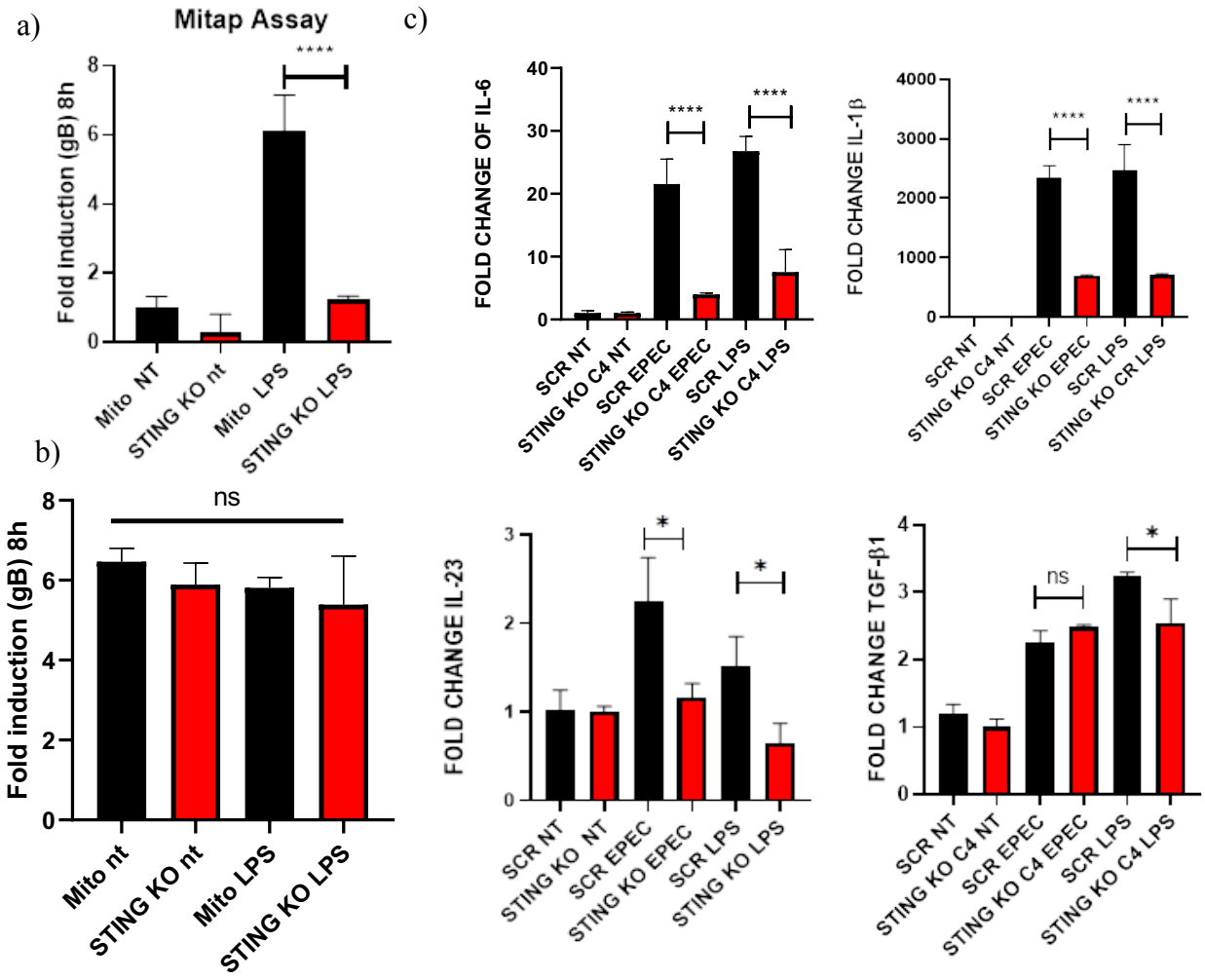

Figure 11-STING KO regulates MitAP and cytokine production after EPEC and LPS infection

(a) Lower levels of MitAP (presentation of gb) in STING KO cells compared to Mito control treated with LPS (b) To determine if the treatment used would have an impact on the viability of the cells or in the expression of MHC class 1 we used a peptide control. This control shows no statistical difference between the Mito and STING KO cells. (c) Decrease expression of Il-6, IL-1β, and IL-23 (but not TGF-β) is observed in STING KO cells infected with EPEC or treated with LPS (4 hrs treatments) compare to SCR control. Statistics: One way ANOVA (MitAP

peptide control) two-way ANOVA (MitAP assay and qPCR). ****p<0.0001., ***p<0.001, **p<0.01, *p<0.01. Data are represented as means +SD.

3.3 Pro-inflammatory Cytokines production is increased in vivo in PINK1 KO BMDCs infected with EPEC.

In PD patients, increase circulating levels of proinflammatory cytokines are observed in serum, CSF, and post-modern brain (3-5) Moreover, PD-related genes such as PINK1 and Parkin have been shown to regulate the levels of cytokine production in mice sera (136). However, the cells responsible for the secretion of these cytokines are still unknown. In this study, we previously used raw macrophages with different mutations to try to determine the relation between Mitochondrial antigen presentation and cytokine production. Unfortunately, for this part of the study, we had difficulties generating a PINK1 KO cell line of raw macrophages since PINK1 is not easily detectable by Western blot in this cell line. Furthermore, the cell line of raw macrophages PINK1 KD used by *Matheoud et al 2016* was lost. However, in the periphery, a major producer of cytokines are DCs. An alteration in the cytokine profile of DCs would have major consequences in the polarization of T cells. *Matheoud et al 2019* have previously shown that PINK1 KO BMDCs have a higher level of MitAP when treated with HS, LPS, or EPEC. Therefore, we decided to focus this part of our study on BMDCs. We started by checking the levels secretion of cytokine in *PINK1 KO* BMDCs compared to WT BMDCs after TLR4 stimulation. IL-6, IL-1β, and IL-23 secretion was higher in *PINK1KO* BMDCs compare to WT after 6hrs of EPEC infection (Fig 12a). In parallel, PINK1 KO BMDCs and WT BMDCs were infected with EPEC for 4hr, and RNA was extracted for qPCR. There was an increase in the expression of IL-6, IL-1β, and IL-23 in EPEC infected PINK1 KO BMDCs compared to WT EPEC infect BMDCs (Fig 12b). Our qPCR result suggests that not only the secretion but also the

production of these cytokines was altered. Moreover, these data also suggest that the dysregulation of cytokine expression is specific to a certain set of cytokines since IL-12 and TGF-β expression levels are not altered (Fig 12b).

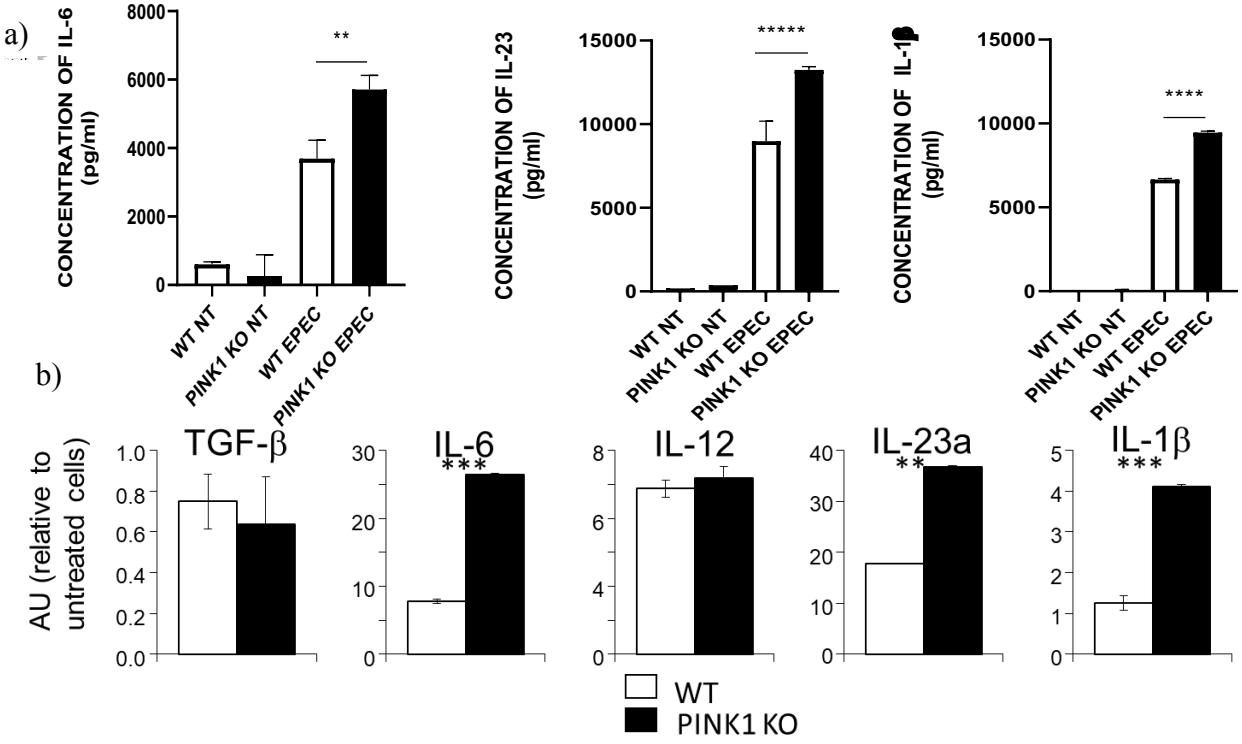

Figure 12-EPEC infection induces overproduction of proinflammatory cytokines in PINK1 KO BMDCs

PINK1 KO or WT BMDC were infected with EPEC (MOI 1) for either 4h (for qPCR) or 6h (for ELISA) (a) IL-6, IL-23 and IL-1β, secretion level was assessed by ELISA (n=3) (Statistics: one-way ANOVA. ****p<0.0001., ***p<0.001, **p<0.01, *p<0.01. Data is represented as means +SD). (b) The expression of different proinflammatory cytokine TGF-β, IL-6, IL-23, and IL-1β cytokines and anti-inflammatory IL-12 were assessed by qPCR Data is representative of 3 independent experiments. Statistics: Mann Whitney test. **p<0.01; ***p<0.001. Data are represented as means +SD**.**

3.4 PINK1 deficiency in BMDCs and the related cytokine production dysregulation, promote Il-17 production.

T-cell differentiation is dependent on the cytokine milieu established by APCs (271). Proinflammatory milieu results in differentiation of T-cells into either Th1 or Th17 while anti-inflammatory milieu results in the anti-inflammatory subsets Th2 and Treg (271). Th17 cells develop in the presence of TGF-β and pro-inflammatory cytokines such as IL-23, IL6, and IL-1β (284). While Th2 differentiation is still dependent on IL-6 (similar to Th17) the main difference in their polarization is the need for endogenous IL-4 for their proper differentiation (262). In *PINK KO* BMDCs infected with EPEC, we have shown that some proinflammatory cytokines levels are increased. Interestingly, the proinflammatory cytokines that are upregulated in *PINK1 KO* BMDCs after TLR4 stimulation are required for Th17 differentiation (IL-6, IL-1□, and IL-23). Thus, we hypothesize that a potential effect of this dysregulation is an increased production of IL-17 by Th17 cells. To test this hypothesis, we decided to purify WT- CD4+ T-Cells and co-culture them with pre-activated (LPS) *PINK1* KO and WT BMDCs. BMDCs were treated with LPS to induce cytokine secretion. LPS is a glycolipid present in the outer membrane of all gram-negative bacteria that is also a strong activator of the TLR4 pathway (316). We did not use EPEC for this assay since bacteria could overgrow and induce cell death. As a positive control, WT CD4+ T-cells were treated with a cocktail that induces Th17 differentiation. Cells were stained with antibodies allowing the assessment of Th1 and Th17 polarization. We found that the frequency of IL17 + CD4+ T- cells was higher in T-cells co-cultured with *PINK1 KO* BMDCs compared to those co-cultured with WT BMDCs (Fig 13a, 13b). This suggests that the PINK1 dysregulation of the BMDCs cytokine profile results in increase production of IL-17.

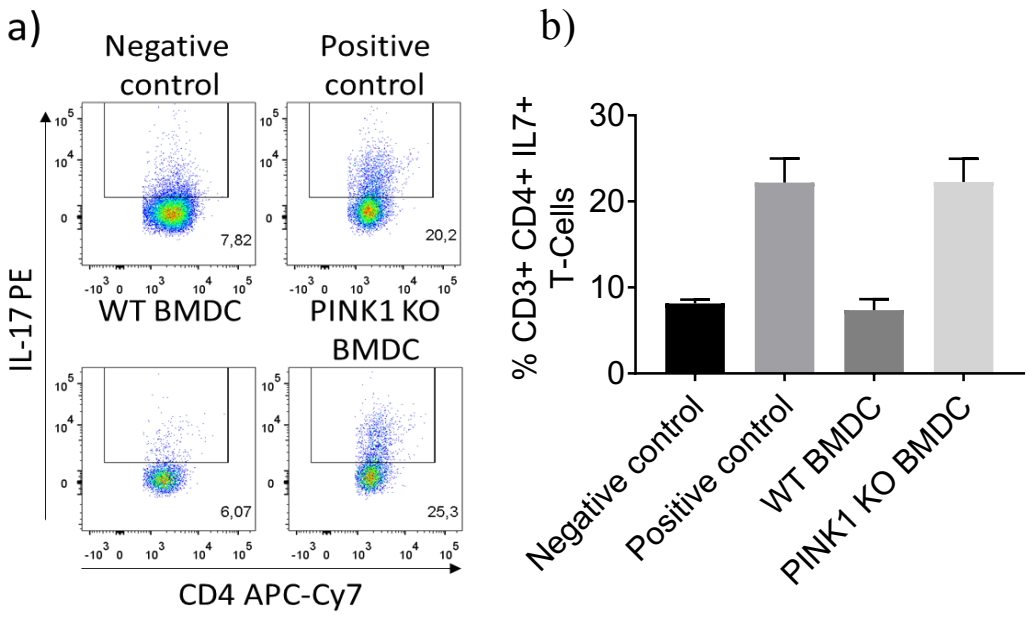

Figure 13 The absence of PINK1 in APC skews CD4 T cell polarization towards Th17 cells.

Naïve CD4+ T cells were purified from WT mice and cultured for 7 days in the presence of anti-CD3/anti-CD28 microbeads (negative control), a cocktail of pro-Th17 differentiation cytokines (positive control) or LPS treated (50ng/ml) WT or PINK1 KO BMDC. Cells were then stained with live dead, anti-CD3, anti-CD4, and anti-IL17 and analyzed by Flow Cytometry. Doublets were excluded using FSC-H, FSC-W, SSC-H; dead cells were excluded using the lived dead staining. (a) Dot plot show percentages of CD3+ CD4+ IL17+ cells. (b) The histogram summarizes the results from triplicates. The experimental procedure was replicated 3 independent times. Statistics: One-way ANOVA. *p≤0.05; **p≤0.001; ****p≤0.0001. All data represented as means +/- SD.

3.5 Identification and characterization of mitochondrial antigen-specific T-cells in PD Patients.

Mitochondria dysfunction and inflammation have long been described as hallmarks of PD. The recent discovery of MitAP and the mitochondrial-related autoimmune response in a murine PD

model provides a link between mitochondria and immunity. Most importantly it strongly reinforces the theory implicating autoimmunity in PD with a very solid mechanistic basis (128). Although MitAP was described in PD-related genes deficiency models, autoimmunity is thought to be implicated in idiopathic PD as autoreactive T cells have been found in PD patients' blood (15, 16). Autoimmune-associated Th17 cells are increased in PD patients and seem to be implicated in DN death (15), however, their antigen-specificity is still unknown. Thus, we wondered if there was a connection between this linage of T-cells and mitochondrial antigen presentation. We decided to measure MitAP activity in PD patients through the characterization of mitochondrial antigen-specific T-cells and determine if they produce IL-17. In order to specifically and efficiently re-stimulate these T-cells to increase their number and easily identify them, we designed an assay using mitochondrial antigens coated on latex beads. Because antigen-mediated endocytosis is a poor inducer of antigen presentation, the use of particulate antigens (mitochondrial-proteins coated beads) will strongly potentiate antigen presentation by APC present in PBMC, as beads will be internalized by phagocytosis, a highly efficient pathway for antigen presentation. Therefore, we fed PBMC from PD patients and sex/age-matched healthy donors with latex beads coated with highly purified mitochondrial proteins. APC will phagocyte the beads, process mitochondrial antigens into peptides that will be loaded on MHC-I and II molecules. Mitochondrial antigen-specific T cells will recognize the MHC/peptide complex and will get activated. The frequency and the T cell phenotype (T-cell subset) will be easily analyzed by FACS. Cells were incubated with the latex beads for 5 days and then stained with antibodies that allow the identification of Th1, Th17, and cytotoxic Tc17 cells. A Fluorescence minus one control (FMO) was also included. This control is important to set up gating and assess the background level for the Th17 subset (317). Using this approach, we

observed that 5 out of the 7 PD patients tested so far have detectable circulating IL17+ mitochondrial -specific $CD4^{+}$T-cells, compared to none of the healthy controls (Fig 14a, 14b). Not only we observed the exclusive presence of mitochondrial-specific T-cells in PD patients, but their phenotype was exclusively IL-17 secreting T-cells. To determine if there was a difference in the ability of activation between PD patients and healthy donors, we stimulated PBMCs with anti-CD3/anti-CD28 beads. No statistical difference was observed between PD patients and healthy donors (Fig 14a 14b). Moreover, the autoreactive T-cells also produce GM-CSF and INF-γ, markers of Th17 high pathogenicity (284). Indeed, mitochondrial- antigen-specific T-cells that are double positive for IL17+/GM-CSF+ and for IL17+/IFNg+, are specifically present in PD patients (Fig 14c and 14d). No statistical difference was observed between PD patients and healthy donors after anti-CD3/anti-CD28 beads incubation (Fig 14d). These results suggest the presence of mitochondrial antigen-specific Th17 cells in PD, with some level of pathogenicity.

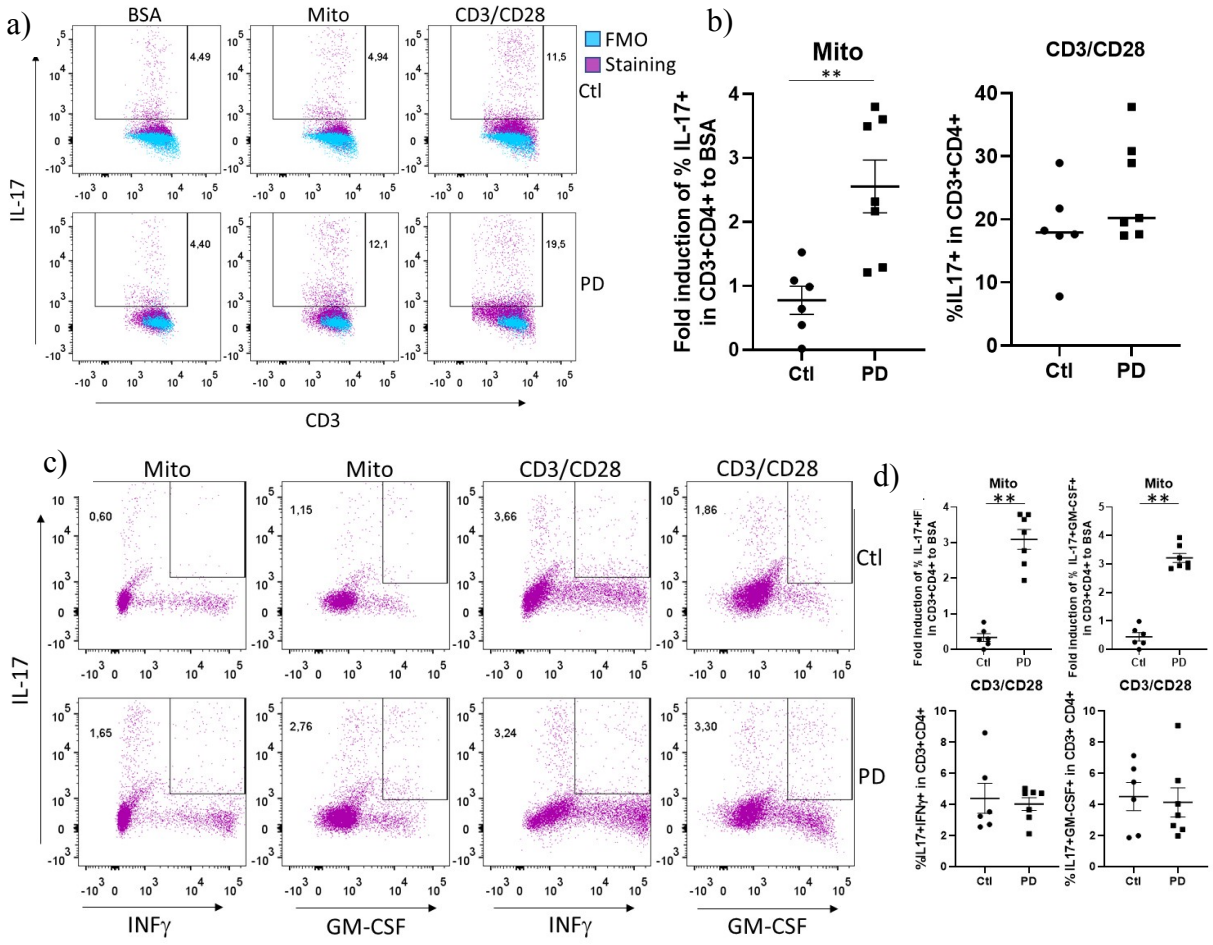

Figure 14-Identification and characterization of mitochondrial antigen-specific T cells in PD patients.

(a,c) PBMC from PD patients and sex/age-matched healthy donors (Ctl) were stimulated for 5 days with mitochondrial proteins (Mito) coated latex beads, with control beads (BSA coated) or with anti-CD3/anti-CD28 beads in the presence of 20U of IL-2. Cells were then stained with live dead, anti-CD3, anti-CD4, anti-IFNγ, anti-IL17, and anti-GM-CSF antibodies and analyzed by flow cytometry. Doublets were excluded using FSC-H, FSC-W, SSC-H, and SSC-W; dead cells were excluded using the live dead staining. b) The dot plots show the percentage of IL-17+ cells in the CD3+CD4+ population for a PD patient and a healthy donor (Ctl) after mitochondrial proteins stimulation (Mito), BSA stimulation, or an anti-CD3/anti-CD28 stimulation; the histogram summarizes the results obtained for the 7 PD patients and the 6 controls. d) The dot

plots show the percentage of IL-17+IFNg+ and IL-17+GM-CSF+ cells in the CD3+CD4+ population for a PD patient and a healthy donor after mitochondrial proteins stimulation and an anti-CD3/anti-CD28 stimulation. The histograms summarise the results obtained for the 7 PD patients and the 6 controls. Statistics: Mann-Whitney. **$p \leq 0.001$. All data represented as means +/- SD.

Chapter 5- Discussion

This study aimed to determine the role of mitochondrial antigen presentation and the related autoimmune mechanisms in PD pathogenesis. Several conclusions could be drawn from the data obtained suggesting the role of MitAP in PD. In this study, we show that: 1) The MDV/MitAP pathways seem to have a role in the regulation of cytokine production in raw macrophages; potentially through the cGAS/STING pathway, 2) in a PD murine model *PINK1 KO* BMDCs have a dysregulated cytokine profile that could potentially induce T-cell polarization toward a Th17 subset, and lastly, 3) PD patients have a higher incidence of autoreactive and potentially pathogenic mitochondrial antigen-specific T-cells.

4.1 MitAP regulates the secretion of pro-inflammatory cytokines by APC.

Previous studies have found that in the absence of PINK1, mtDNA was released from the mitochondria and activates the cGAS/STING pathway resulting in the production of pro-inflammatory cytokines (136). PINK1 has also been shown to regulates the MDV/MitAP pathway. Furthermore, MDVs were shown to contain mtDNA suggesting that this DAMP could potentially activate other molecular pathways that could induce cytokine secretion. We decided to assess whether the MDV/MitAP pathway was implicated in cytokine production by allowing the release of mtDNA from the mitochondria. Using raw macrophages deficient for genes implicated in the MDV/MitAP pathway (Snx9 KD, Rab7 KD, Rab 5 KD, Parkin +) we assessed the role of MDVs/MitAP in cytokine production. *Matheoud et al. 2016* have previously shown that these cell lines have

low levels of mitochondrial antigen presentation (127). Mirroring these results, we found that these cells produce lower levels of IL-6 secretion. To further confirm our results, we decided to focus on the Snx9 KD cell line and showed that there is a decrease in the level of expression of IL-6, IL-1β, IL-23, and TGF-β when compared to the SCR control. In this assay, we decided to used qPCR versus other methods of cytokine quantification (for example, FACS) because we were interested in determining the effect of these genes (Snx9, Rab7, Rab9, and Parkin) in the expression of cytokines, and not just cytokine production. Our result suggests that the MDV/MitAP pathway regulates the secretion of cytokines since, in the absence of MDV, (which results in inhibition of MitAP), there is lower production and expression of cytokines. Unfortunately, classic macrophages inflammatory cytokines were not measured in this assay including TNF-α and Il-1β. A limitation of this study is the cell lines used. Raw macrophages do not secrete all proinflammatory cytokines. For instance, RAW 264.7 macrophages lack the apoptosis-associated speck-like protein containing a CARD domain (ASC) and therefore are deficient in the processing and release of IL-1β (198, 199). Thus, utilizing a cell line that secretes all the cytokines would further validate the results observed in RAW 264.7 macrophages. Moreover, TNF-α secretion levels and anti-inflammatory cytokines levels must also be assessed to better understand if the result observed is specific to pro-inflammatory cytokines. Another important setback from this experiment is that we did not assess the integrity of the molecular pathway that results in cytokine secretion. Snx9, Rab7, and Rab9 are ubiquitous proteins that have many other roles in the cell besides MDV formation. Thus determining whether MDV deficiency is indeed responsible for the low levels of cytokines is necessary (318, 319). For instance, to have stronger proof of the link between cytokine and MitAP it will be necessary to

rule out any effects that the downregulation of these trafficking proteins (Snx9 KD, Rab9 KD, and Rab7 KD) would have in the trafficking of cytokines, or in the internalization/trafficking of the activated receptor including TLR4 and STING after activation. For instance, the TLR4 receptors has two signaling pathways, the Tir domain-containing adaptor protein (TIRAP) and MyD88 pathways that are activated upon encounter with LPS which results in the activation of the NF-KB pathway (320). The secondary pathway requires the internalization of the LPS- TLR4 complex into the endosomal network ultimately reaching the Golgi-like structure (320). This translocation triggers TRIF-related adaptor molecule (TRAM) and TRIF activation, which results in the activation of IRFF-3, a regulator of Type I interferons (320). The internalization LPS-TLR4 complex has been shown to involve dynamin and clathrin (320). Interestingly, Snx9 is a dynamin-binding partner essential for clathrin-mediated endocytosis (113), however, whether Snx9 plays a role in the TLR4 internalization is still to be determined. Therefore, whether the result observed in this experiment are due to reduce MDV formation or reduce internalization of the TLR4 is unknown. Moreover, whether these cell lines (Snx9 KD, Rab7 KD, Rab9 KD, and Parkin +) have defective TLR4 expression due to these missing genes is unknown. This is an important factor since TLR4 activation would greatly impact cytokines expression and MitAP activation in Raw macrophages. Snx9 have also been implicated in the nuclear import of SMAD2 and SMAD 3 (the primary mediator for TGFβ) (321), suggesting that Snx9 could affect the transcription of certain cytokine by facilitating the nuclear import of certain transcription factors. Indeed, our qPCR results show a decrease in the expression of TGFβ which coincides with the role of Snx9 in the TGF-β pathway. Nevertheless, our assay does not show a direct link between MDV formation MitAP, and cytokine secretion; thus, the result observe could be an epiphenomenon. Furthermore, an important issue

that needs to be addressed in this experiment is the viability of the cells at the time of assessing their cytokines secretion/production, since prolonged bacterial infection could result in cellular death. Although an established protocol was used in this study for EPEC/LPS infection it would be important to utilize another method to determine cell viability (like trypan blue) after each treatment. Nevertheless, clearly determining the mechanism by which MitAP affects cytokine expression/secretion and how often PD patients have uncontrolled MitAP could open a novel therapy target and help develop new treatments for PD patients.

4.2 STING regulates MitAP and secretion of pro-inflammatory cytokine by APC.

Sliter et al 2018 have previously shown that *Parkin KO*:mutator mice have increased cytokines secretion levels. This was reversed by knocking down *STING,* suggesting that the inflammation observed in the absence of Parkin is through the activation through STING(136). We decided to use our Mito-Gb raw macrophages to generate a STING KO using CRISPER Cas9 technology. These cell lines are stable, easy to use and protocol have already been optimized to generate KO in our laboratory. Moreover, the Mito-Gb system makes it extremely easy to assay for Mitochondrial antigen presentation. To determine the presence of STING Western blot and sequencing was used (Fig. 10). Unfortunately, we only sequence the region targeted by Cas9, thus, any other mutation introduced by off-targets effects are were not assessed. STING function was not assessed in this experiment either, thus whether the mutation observed in our assays has further repercussions is unknown. An important follow-up experiment to determine the integrity of these STING KO macrophages would be to use other stimulators of STING for instance cGAMP to

check if there is a response/activation of the STING pathway. Moreover, in this experiment we mainly used LPS as a stimulator, thus we could use other PAMPs/DAMPs to determine normal cell function. Furthermore, it would be important to determine the integrity and expression levels of the TLR4 since that was the pathway targeted by LPS.

Most importantly, our results suggest that STING plays a bigger role than just pro-inflammatory cytokine secretion regulation in raw macrophages since we observed inhibition of MitAP when STING is absent. This raises the query of whether STING controls adaptive immunity not only by providing appropriate cytokine milieu for T-cell differentiation but also by controlling the levels of antigen presentation in raw macrophages. An important experiment to determine the role of STING in antigen presentation would be to create a *STING* KO cell line with gB protein targeted to the nucleus and to the cytoplasm. These cells lines could help determine whether the decrease in mitochondrial antigen presentation observed in our study is specific to mitochondrial proteins or if it is just a general inhibition of antigen presentation. The expression of IL-6, IL-1β, and IL-23 was decreased in the absence of STING compared to SCR control. This is expected since *Sliter et al 2018* showed decreased secretion of proinflammatory cytokines (Il-6, IL-1β) in its *Parkin KO*/*STING KO* mice (136). Even though our peptide control gave us an idea about the viability and MHC class 1 expression and toxicity of our treatments, our experiment lacks a proper cell viability test. This raises the question of whether the result observed is due to decrease expression of cytokines or simply cell death by LPS. Furthermore, another important experiment that needs to be done to draw a better conclusion is determine the integrity of the ER compartment in the STING KO raw macrophages since STING is localized in the ER. To verify ER stability, it would be important to measure the levels of activation of the proteins involved in the unfolded protein

response (UPR, a pathway that is activated when the ER is under stress condition). The levels of phosphorylation of inositol-requiring protein 1(IRE1), and PRKR-like endoplasmic reticulum kinase (PERK) are usually used to assess UPR activation and thus ER stability (322).

Our results also suggest that STING activation happens regardless of the presence of exogenous DNA since cells are only treated with LPS. Thus, the DNA recognized by STING is endogenous. mtDNA is a potential source of endogenous DNA. In endothelial cells, LPS has been shown to activate the pore-forming protein Gasdermin D, which forms mitochondrial pores and induces mtDNA release into the cytosol. This increase of mtDNA in the cytosol ultimately resulted in the activation of the STING pathway (323). Moreover, depletion mitophagy/autophagy proteins result in the accumulation of damaged mitochondria and mtDNA release in LPS treated bone marrow-derived macrophages (159). PINK1 and Parkin are the main regulators of mitophagy, which suggest that the absence of these proteins might result in increased mtDNA release, thus giving the possible source of STING activation in *PINK1* and *Parkin* deficient cells. An obvious limitation of our study is we do not have a clear link between MitAP, STING, and cytokine secretion. STING has been shown to achieve maximal activation after its translocation to the ER/Golgi intermediate (324). Both STING signaling and degradation requires ER exit (325). Interestingly, post-Golgi STING vesicles are sorted to Rab7-positive endolysosomes, suggesting that a possible mechanism control by Rab-7 might be the regulation of STING degradation. Bafilomycin-A (inhibitor of lysosome acidification) has been shown to strongly block STING degradation and enhance STING activation when combined with its inducer cGAMP (325). Furthermore, bafilomycin-A has also been shown to be a MitAP inhibitor. Bafilomycin-A inhibits the vacuolar pathway of antigen presentation on which MitAP depends (66). An interesting

experiment that would determine whether the MitAP and cytokine secretion are part of the same pathway would be to treat cells with Bafilomycin-A. This experiment should result in increased level of cytokines secretion due to block degradation of activated STING (325) with low levels of MitAP, suggesting that these pathways are independent of the other. The next query emanating from these studies is whether this *invivo* experiment could be reproducible *exvivo* using STING KO BMDCs for instance. Because *Sliter et al 2018* have already shown attenuation of PINK1 inflammation when STING is absent (136), it is highly probable that similar results to the ones observed in raw macrophages could be observed in primary cell lines.

TLR9 is another potential receptor for mtDNA. Interestingly, *PINK1* expression has been shown to attenuate mtDNA release resulting in decreased activation of TLR9 in lung type II alveolar epithelial cells (AECII) (315). In this scenario, MDV- might be carriers of mtDNA that and fuse with the late endosomes where TLR9 is expressed, resulting in an inflammatory response. Thus, in the future, it will be interested to determine the role of TLR9 in the MitAP-mediated cytokine production. Overall, our results suggest that STING links innate and adaptive immunity by regulating MitAP.

4.3 PINK1 KO BMDCs have a dysregulated cytokine profile.

PINK1 and Parkin have previously been shown to regulate mitophagy (80) and to repress MitAP(127). Recently, they were also implicated in the regulation of cytokine production. However, the origin of the cells overproducing pro-inflammatory cytokines is unknown. In this study, we showed that *PINK1 KO* BMDCs produce increased levels of IL-6, IL-1β, and IL-23. It also

suggests that this dysregulation is specific to pro-inflammatory cytokines since qPCRs for il-10 and Il-12 levels show no change after infection.

A main problem with the PINK1 KO murine model is that there is no phenotype in these mice under sterile conditions (112). However, our results are in accordance with the literature since previous studies have shown that PINK1 KO mice treated with LPS have increased levels of proinflammatory cytokines in the striatum (137). Recently, *Matheoud, Cannon et al 2019* shown that intestinal infection with *Citrobacter rodentium*- a mouse intestinal bacteria used to model Human EPEC infection, result in PD-like symptoms after several infections, suggesting that bacterial infection plays an important role as a trigger of PD (128). Our study shows another possible mechanism by which bacterial infection/product (LPS) induces uncontrolled proinflammatory cytokine secretion which contributes to the pathophysiology of the PD.

The *PINK1 KO* model of PD could be a useful tool to further determine the origin of cells overproducing proinflammatory cytokines. It would be interesting to purify DC from different compartments and compare their cytokine profile. Interestingly, in WT mice, the intestinal mucosa is particularly conducive to the induction of helper T cells producing type 2 (IL-4, IL-5, and IL-10) and type 3 (transforming growth factor β (TGF-β)) cytokines (326). Thus, it will be interesting to determine if the intestinal mucosa in *PINK1 KO* mice, in particular, intestinal DCs, present this dysregulation. For instance, purifying intestinal DC from *C. Rodentum* infected *PINK1 KO* mice and determining their cytokine profile could help us determine the APC population that is affected by the bacterial infection as well as the consequences of this infection. Moreover, it will also help us connect the role of the microbiota in the development of PD.

4.4 PINK1 KO BMDCs increase the differentiation of IL-17 producing T-cells.

One important consequence of the cytokine dysregulation observed in APC is T-cell polarization. Interestingly, the secreted cytokines that are dysregulated in the *PINK1 KO* BMDC are, in part, the main cytokines required to induce Th17 differentiation. Preliminary data from our lab (data not shown) showed an increased incidence of Th17 cells in *PINK1 KO* mouse infected with *C. rodentium* suggesting a skewed Th17 differentiation (data not shown). In this study, an increased frequency of IL17+ T- cell was observed when CD4+ T-cells were coculture with *PINK1 KO* BMDC compared to those cocultured with WT BMDC. Our result provides *in vitro* evidence that the cytokine dysregulation of APC like BMDCs, can result in an increased polarization of IL-17 producing CD4+ T-cells. *Sommer et al 2018* recently shown that there is an increased incidence of Th17 cells in PBMC of PD patients and that these cells can cause neuronal death in iPSC-derived neurons (290). Our results provide a mechanism that causes a possible increase in the Th17 cell population observed in PD patients and suggest that the cells responsible for this dysregulated polarization are in part APCs. Although our study does not provide a direct explanation of why some early non-motor symptoms appear (constipation, hyposmia), other studies have shown that peripheral immunity might play a part in the severity of these symptoms. For instance, CD4+ cells can infiltrate the colonic mucosal tissue (327). This is important because in PD patients with constipation a positive correlation between the severity of the colonic involvement and the Th17 and Treg cell proportions was observed (327). This suggests that there is a connection between autoimmunity and some of the non-motor symptoms of PD. Our studies also support the Gut-Brain axis models of PD, since it shows a mechanism by which a bacterially

derived substance (LPS) could activate the secretion of pro-inflammatory cytokines resulting in an autoimmune response (possible polarization of Th17 cells) that ultimately reaches the CNS.

The main limitation of this study is that other linages of T-cells were not check in parallel. For instance, it is unknown whether the PINK1 KO BMDCs cytokine dysregulation induces the differentiation of other subsets of CD4+ T-cells. Furthermore, we did not show whether the Th17 cells observed are preferably polarized by the PINK1 KO cytokine milieu. This control is essential to reduce any bias in our results. To draw a stronger conclusion of whether these IL-17 producing cells are indeed Th17 cells it will be necessary to do qPCRs to determine the level of expression of the transcription factors characteristically express by Th17 cells like RORγT, STAT3, or AHR (284). The pathogenicity of these IL17+CD4+ T-cells was undetermined. In fact, in this experiment we did check for markers of pathogenicity like INF-γ and GM-CSF, however, our results were inconclusive since it only showed a tendency for pathogenic activation (Data not shown). Another important pathogenicity factor that needed to be checked in this experiment is the presence of IL-10 in the newly polarized Th17 cells since Th17 cells producing IL-10 have been shown to have low pathogenicity (289).

Another limitation of this experiment was that the specificity of these Th17 cells was not assessed. *Matheoud, Cannon et al 2019* have previously shown that PINK1 KO BMDCs have a higher level of MitAP after LPS treatment (128). However, this does not directly mean that the Th17 cells observed in our experiment are mitochondrial antigen-specific. Thus, in future experiments, the specificity of these cells could be assessed in infected PINK1 KO mice. On the other hand, an advantage of this protocol is that this experimental set up could be expanded to

other PD related genes that have previously shown to increase pro-inflammatory cytokines like LRRK2, Parkin, etc. (136) to see if this Th17 cell dysregulation is observed and could potentially be used as a biomarker of the disease. Overall, these experiments provide further evidence that PD has an autoimmune component.

4.5 PD patients have a higher incidence of mitochondrial specific T-cells.

Mitochondrial dysfunction is a hallmark of PD. Several of the genes that have been identified to cause familiar cases of PD (*PINK1, Parkin, LRRK2,* etc.) have been implicated in mitochondrial quality control and inflammation (66, 328). The discovery of MitAP provided a link between these two PD hallmarks. Furthermore, despite some evidence that suggested the implication of autoimmunity in the disease (autoantibodies (311, 329, 330), autoreactive T-cells(15)), MitAP is the first mechanism that formally implicates autoimmunity in PD pathogenesis. Altogether, this evidence prompts us to assess mitochondrial autoimmunity in PD patients. Not only we observed the presence of mitochondrial antigen-specific T-cells in PD patients, but we found that these T-cells have an autoimmune-related phenotype. Indeed, they produce IL17 and co-secrete GM-CSF and IFNg, a phenotype associated with highly pathogenic Th17 cells. Because pathogenic Th17 cells are essential for the development of other autoimmune disease like MS or IBD, we can speculate that PD has an autoimmune component that could partially lie on the MitAP pathway.

The mechanism by which these Th17 cells are increased in PD could be speculated based on our previous study. We demonstrated in murine models lacking PINK1 (murine models with mitochondrial instability) an increased differentiation of IL-17 producing cells. Thus, we can

propose a similar mechanism in PD patients (since mitochondrial instability is a common hallmark of the disease). We can speculate that a combination of genetic factors (mutations that increase mitochondrial instability) and environmental factors (increase gram-negative bacterial infection/endotoxins or increase mtDNA leakage) would result in overactivation of certain immune pathways in particular the TLR4 and STING Pathways. Consequently, an increased mitochondrial antigen presentation and cytokine secretion would occur, providing a cytokine milieu ideal for Th17 differentiation. Thus, in our PD patient PBMCs, we can detect these mitochondrial antigen specific Th17 cells. Interestingly, combining both of our results we can propose that maybe inhibitors of TLR4 and STING could potentially be used as treatments to slow down the pathogenesis of PD.

The role of these autoreactive T-cells in patients is still unknown. However, in murine models, DN can present mitochondrial antigens on MHC-I molecules under inflammatory conditions and can be targeted and killed by mitochondrial-specific T-cells (128). IPSC-derived DN also died when exposed to Th17 cells from PD patients (290). Thus, we can speculate that these mitochondrial-specific Th17 cells found in patients, could cross the BBB and attack/destroy DN neurons presenting mitochondrial antigens resulting in PD.

One might argue that the T-cell activation observed in this study is due to an increased presentation of beads specific to the APCs in PD patients however if that was the case, the same amount and behavior of presentation and thus T-cell activation would be observed amongst the BSA control beads and the Mito covered beads. An important experimental control that needed to be considered is the quantity of APCs (DCs and Macrophages) and Th0 cells in PBMCs. When

designing the experiment this factor was not considered, thus it might have an impact on the results observed. By quantifying the proportion of APCs in each blood sample one can remove ambiguities including a lower number of antigen-presenting cells versus T-cells in each sample. Especially since it has been shown before that idiopathic PD patients have a lower number of DCs in the periphery compared to healthy control (190).

Although the cells identified in this study were not cytotoxic T-cells, Th17 cells from PD patients have been shown to induce neuronal cell death in IPSC-derived dopaminergic neurons, suggesting a certain level of pathogenicity on Th17 cells (290). In this study, Th17 cells pathogenicity could be further characterized by the addition of IL-10 staining. Indeed, IL-10 secreting Th17 cells have been shown to be non-pathogenic (289), therefore, this marker will enable us to better discriminate pathogenic Th17 cells in patients. A co-culture between DN and these mitochondrial antigen-specific Th17 cells could give us a better idea of the level of cytotoxicity of these cells. Furthermore, it is important to include other T-cells linages, to reduce the biases of our results. The impact of the increased population of Th17 could be further measure in the patient sera, since an increase of Th17 cells would mean an increase of the level of circulating Il-17 in the serum, reinforcing the result observed in our study.

Another important limitation of this assay is mitochondrial antigens purity. Indeed, these proteins were extracted from highly purified mitochondria but cannot exclude the presence of contaminants coming from other cell compartments. In the future, these results need to be confirmed with recombinant mitochondrial proteins (such as OGDH). However, the use of such

an approach will need a higher number of patients as mitochondrial antigen-specific T-cells could have different antigen specificities according to patients.

It is important to understand that the approach used in this study to measure T-cell specificity is a novel technique, meaning that the result observed need to be further confirm using an already established protocol to determine T-cells specificities such as cell-proliferation assays, ELISpot/FluoroSpot, or flow-cytometry based assays (331). However, similar approaches to our antigen covered latex beads technique have been used to determine T-cell specificity. Previously, covalently bound protein antigens to superparamagnetic micro-beads were used in PBMCs to determine their specificity to cytomegalovirus (an enveloped DNA virus (332)) and tetanus-diphtheria (331). This approached shows antigen specificity and high sensitivity, suggesting a bead-based assay to determine antigen specificity is a plausible method. In the future, the same assay could be repeated to assess other mitochondrial proteins which dysfunction has been previously linked to PD. For instance, in MPTP models, ETC proteins inhibition result in the pathophysiological symptoms observed. In drosophila lacking PINK1 and Parkin, a slower turnover of ETC complexes is observed. This could be due to dysfunctional mitophagy that results in a switch to another mitochondrial quality control system, such as MDV. Thus, repeating this assay with ETC proteins like NADH-ubiquinone reductase (complex I of the ETC), CoQH2-cytochrome c reductase (complex III of the ETC), and cytochrome c oxidase (complex IV of the ETC) could be interesting (333).

In general, mitochondrial specific T-cells were present in the 5 out of the 7 patients tested suggesting that MitAP could potentially be active in a high proportion of PD Patients (Fig 14). An

interesting assumption that could be drawn from our result is that mitochondrial antigen-specific T-cells are not unique to PINK1/Parkin mutated patients (early-onset PD patients) suggest its potential as a biomarker. This was shown in our study since we decided to collect our data as a blinded study to prevent biased of our result. In the future, it would be very interesting to determine what is the clinical state of each patient and to investigate the differences, if any, is present in the 2 patients that do not show mitochondrial antigen-specific T-cells. These are studies that need to be done in the future.

One of the biggest difficulties when encountering PD is the lack of appropriate biomarkers to early diagnose the disease (334). Motor impairment in PD appears when nearly 60% of the DN population is irreversibly lost. This complicates treatment since the only available treatment is to ease the symptoms without stopping neurodegeneration. Furthermore, it is difficult to differentiate between PD and other parkinsonism's. Over the year several prodromal signs and biomarkers of PD have been proposed such as hyposmia or loss of olfaction. Olfactory impairment is one of the most common and best-characterized non-motor features of PD with a prevalence of 50%–90% (335). Hyposmia might help with the identification of pre-motor PD due to the early pathologic involvement of olfactory pathways (335). mtDNA copy number has also been propose as a biomarker for PD since it has been reported that lower copy number of mtDNA copy numbers are observed in the periphery and affected brain tissue of PD patients (336). A problem with this method is that other neurodegenerative diseases like Alzheimer's also have lower mtDNA copy number observed in the periphery. Five miRNA (miR-195 was up-regulated, and miR-185, miR-15b, miR-221, and miR-181a were down-regulated) were identified in the serum of PD patients that could precisely distinguish PD patients from healthy individuals (337).

Unfortunately, identifying a successful biomarker depends inevitably on fully understanding the pathophysiology underlying the disease. Despite advances in the understanding of pathogenesis and pathophysiology underlying PD, neurologists still have not found a reliable marker for the proper and early diagnosis of the disease (334). Mitochondrial autoreactive T- cells could be used and combined with other biomarkers in order to potentialize the diagnosis of asymptomatic patients. Indeed, the potential of mitochondrial specific T-cells as a biomarker in PD has been shown in infected PINK1 KO mice. In this model, mice infected with *C. Rodentum,* have been shown to develop Parkinson like symptoms as early as 4-month post-infection (128). However mitochondrial T-cells were detected early after infection and months before symptoms development and DN generation appeared (128). This suggests that T-cell analysis could be used in patients with an increased incidence of other common and early symptoms of PD, like constipation, to draw an early diagnosis for said patient. Therefore, mitochondrial specific T-cells could be used as an early pre-symptomatic biomarker of the disease.

Chapter 6 -Conclusion

In conclusion in this study, we demonstrated that MitAP might play an important role in the pathology of PD. To begin with, we demonstrated a new role for the MDV/MitAP pathway in the regulation of cytokine production. Then, we showed that STING plays a role not just in the production of cytokines but also in Mitochondrial antigen presentation. In mice models, we showed that in *PINK1* KO BMDCs higher levels of production of the proinflammatory cytokines including IL-6, IL-1β, and IL-23. Furthermore, we showed that one of the consequences of this cytokine dysregulation is a skewed polarization of Th17 cells *invitro*. Finally, we showed that mitochondrial specific T-cells are present in PBMCs of PD Patients. Our study provides evidence that MitAP is active in PD patients supporting the theory that PD has a base in autoimmunity. This discovery has a great impact in the field of Parkinson's disease since the lack of appropriate biomarker to early diagnose the diseases is one of the biggest problems in PD diagnosis (334) Motor impairment in PD appears when nearly 60% of the DN population is irreversibly lost (334). This complicates treatment since the only available drugs are used to ease the symptoms and not to stop the neurodegeneration. Although mitochondrial antigen-specific autoreactive T-cells are still not fully understood they offer great potential as biomarkers as well as a new therapeutic avenue. If the levels of mitochondrial antigen autoreactive T-cells differ at the various stages of the disease it could help identify PD at early stages even before a significant neuronal loss is observed. Furthermore, the implication of the immune system in this model suggests that immunosuppressant and anti-inflammatory drugs could have the potential to act as a preventive treatment of the disease. In fact, commonly used immunosuppressant treatment (corticosteroids

and inosine monophosphate dehydrogenase inhibitors) have been associated with a reduced risk of PD (338). Our study also suggested that autoimmune patients have a higher risk of developing PD, however, this could only be possible if the autoimmune disease is rooted in a mechanism that results in mitochondrial dysfunction. For instance, recent studies have suggested a role of mitochondrial dysfunction in IBD (a chronic inflammatory disorder) (339). Interestingly, IBD patients have been shown to have an increased number of Th17 cells (340). Most importantly, it has been shown that there is a significant association between IBD and later occurrence of PD (341). Although many aspects of mitochondrial antigen presentation remained to be determined, our study provides further understanding of the connection between mitochondria and autoimmunity in the pathophysiology of PD.

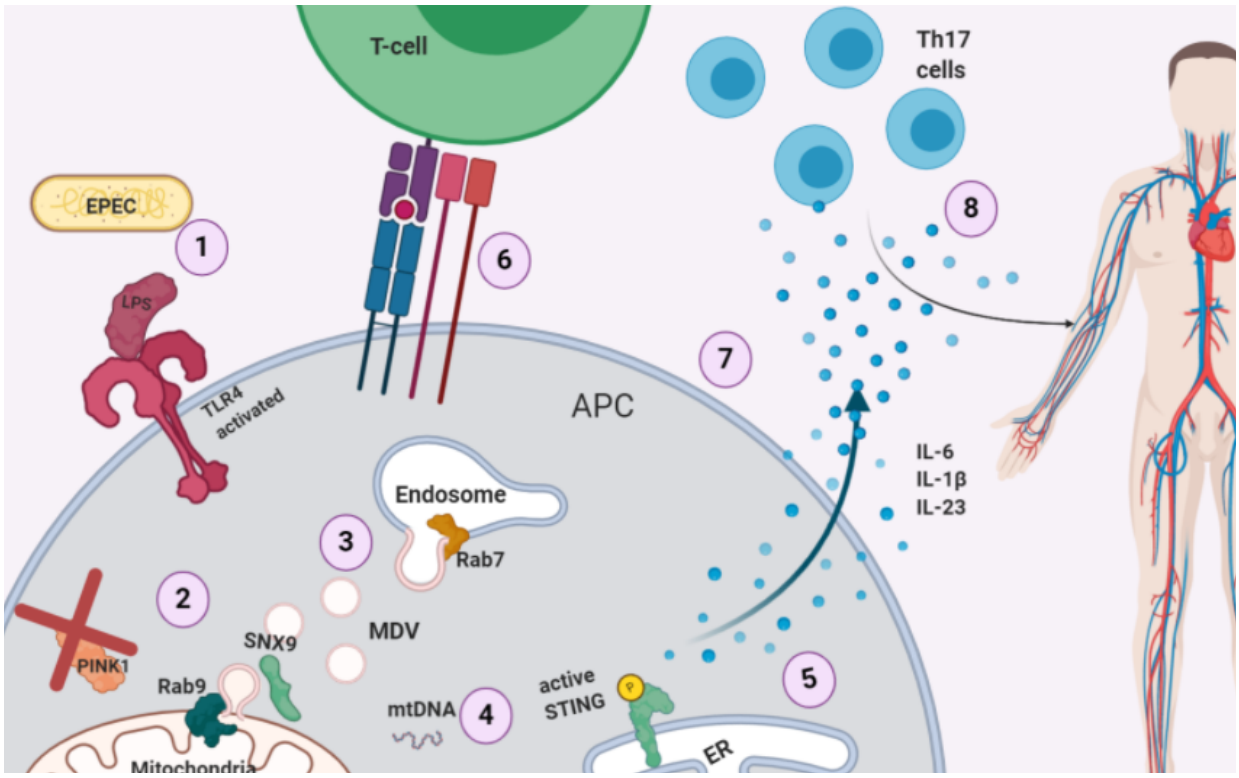

Figure 15 Summary of results.

(1) TLR4 activation by LPS (from gram-negative bacteria like EPEC) induces mitochondrial instability resulting in the activation of PINK1. (2) In the absence of PINK1, (3) active SNX9, RAB9, and RAB7 result in MDVs formation and fusion with the endosome and increased cytokine secretion. (4) Mitochondrial instability may also increase mtDNA leakage and (5) activates the STING pathway. STING activation induces cytokine secretion as well as mitochondrial antigen presentation. (6) Mitochondrial antigens in MHC molecules are recognized by naïve T-cells. After co-stimulation, (7) the proinflammatory cytokines IL-6, IL-23, and Il-1β induce their differentiation into Th17 cells. (8) As a result, this mitochondrial antigen-specific could be identified the periphery of PD patients after re-stimulation with mitochondrial antigens.

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