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Mitochondrial impact in innate immunity activation in sporadic Parkinson's disease

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Resumo

A Doença de Parkinson (DP) é uma doença neurodegenerativa com uma etiologia fortemente associada com alterações metabólicas, nomeadamente com disfunção mitocondrial. A disfunção mitocondrial é considerada um dos eventos iniciais do processo neurodegenerativo na DP esporádica. Em condições normais, mitocôndrias disfuncionais são degradadas através da mitofagia, no entanto na DP esta via parece estar também comprometida, impedindo a remoção destes organelos disfuncionais. Uma rede mitocondrial fragmentada permite a exposição de padrões moleculares associados a danos (DAMPS), o que leva a uma ativação da imunidade inata. Neste estudo abordámos o "crosstalk" entre a disfunção mitocondrial e a ativação da imunidade inata em células citoplasmáticas híbridas (cíbridos), derivadas de teratocarcinoma humano (NT2) que contêm ADN mitocondrial de doentes com DP ou de indivíduos controlo. Para este propósito expusemos os cíbridos de controlo e de DP a lipolissacarídeo (LPS) de bactérias gram-negativas e/ou a leupeptina e cloreto de amónia, um par de compostos que bloqueia a autofagia, e consequentemente a mitofagia. O objetivo foi mostrar que a disfunção mitocondrial pode regular respostas da imunidade inata no contexto de DP. A este respeito, foi observado que cíbridos de DP têm o potencial membranar mitocondrial diminuído, o que se correlaciona com uma rede mitocondrial fragmentada que foi visualizada por microscopia, e a um aumento dos níveis de DRP1 fosforilada. Uma rede mitocondrial fragmentada expõe cardiolipin que funciona como um DAMP ativando a imunidade inata. Observámos que cíbridos de DP têm níveis basais de TLR3, NLRP3 e a sua proteína adaptadora ASC aumentados. No entanto, verificamos que o LPS e a leupeptina e o cloreto de amónia conseguem aumentar as respostas imunes. Resultados similares foram obtidos para a ativação da caspase 1, que está aumentada em condições basais nos cíbridos DP e que aumentou com ambos os tratamentos. Verificamos que os

cíbridos de DP também têm um fluxo autofágico de LC3 basal e de p62 comprometido, o que demonstra que a mitofagia também está comprometida. A acumulação de mitocôndrias disfuncionais aumenta as respostas da imunidade inata em cíbridos DP aumentando ainda mais a ativação do inflamossoma NLPR3. Finalmente, investigámos a oligomerização de α-sinucleína (α-sin) nas condições descritas. Detetámos que os cíbridos de DP têm uma acumulação de oligómeros de α-sin basal e que apena o tratamento com leupeptina e cloreto de amónia aumenta a sua acumulação. Em conclusão, nós provamos a disfunção mitocondrial está na base da ativação da imunidade inata em DP.

Palavras-chave: Doença de Parkinson; Disfunção Mitocondrial; Imunidade Inata

Summary

Parkinson Disease (PD) is a neurodegenerative disorder which etiology has been strongly associated with metabolic abnormalities namely mitochondrial dysfunction. Since mitochondria control a wide range of cellular processes the up-stream position of this organelle in the cascade of events leading to neurodegeneration in PD has been recognized. In healthy conditions, dysfunctional mitochondria are degraded through mitophagy however in PD mitochondrial degradation is impaired, preventing the clearance of these dysfunctional organelles. Without this clearance innate immunity responses will be activated, through the exposition of damage associated molecular patterns (DAMPs). In this study we addressed the crosstalk between mitochondrial dysfunction and the activation of innate immunity pathways, through the use of cytoplasmatic hybrids (cybrid) cells, derived from NT2 teratocarcinoma, repopulated with mitochondrial DNA from PD patients or age-matched control subjects. For this purpose, we exposed PD and control cybrids to lipopolysaccharide (LPS) from gram negative bacteria and/or to leupeptin plus ammonium chloride (NH_4Cl), a pair of compounds that blocks autophagy, and consequently, mitophagy. The goal was to provide evidence that mitochondrial dysfunction can regulate innate immune responses in a context of PD. In this regard, observed that PD cybrids have decreased mitochondrial membrane potential which correlates with a fragmented mitochondrial network visualized by microscopy and the increased levels of phosphorylated DRP1. Fragmented mitochondrial network exposes cardiolipin that functions as a DAMP activating innate immunity responses. We observed that PD cybrids have basal increased levels of TLR3, NLRP3 and its adaptor protein ASC and can further increment immune responses when exposed to LPS and leupeptin plus ammonium chloride. Similar results were obtained for caspase 1 activity, which is increased in PD

cybrids in basal conditions and increase with both treatments. PD cybrids also have basal impaired LC3 autophagic flux and p62 flux, which is specific for mitophagy. This accumulation of dysfunctional mitochondria upholds innate immunity responses in PD cybrids further increasing NLRP3 inflammasome activity. Finally, we investigated the oligomerization of α-synuclein (α-syn) in the described conditions. We detected that PD cybrids have basal accumulation of α-syn oligomers and only leupeptin and ammonium chloride treatment further increases its accumulation. In conclusion we prove that mitochondria are at the base of innate immunity activation in PD.

Keywords: Parkinson Disease; Mitochondrial Dysfunction; Innate Immunity

List of abbreviations

APCs, antigen presenting cells

ASC, apoptosis-associated speck-like protein containing a CARD

Cybrid, cytoplasmatic hybrids

DAMPs, damage associated molecular patterns

FBS, Fetal Bovine Serum

FCCP, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

IFN, interferon

IL, interleukin

IMM, inner mitochondrial membrane

iNOS, inducible nitric oxide synthase

LBs, Lewy Bodies

LC3, microtubule-associated light chain 3

LPS, lipopolysaccharide

MPT, mitochondrial permeability transition

mtDNA, mitochondrial DNA

MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide

NAO, 10-N-Nonyl acridine orange

NH_4Cl, ammonium chloride

NL, NH_4Cl plus leupeptin

NLRP3, leucine-rich repeat pyrin domain containing 3

NLRs, NOD-like receptors

NO, nitric oxide

OMM, outer mitochondrial membrane

PAMPs, pathogen associated molecular patterns

PD, Parkinson Disease

PGC-1α, proliferator-activated receptor gamma coactivator1-alpha

PINK1, PTEN-induced putative kinase 1

RLRs, RIG-I-like receptors

ROS, reactive oxygen species

SN, substantia nigra

SNpc, *substantia nigra pars compacta*

VAD, Val-Ala-Asp

α-syn, α-synuclein

1. Introduction

1.1. Parkinson Disease

Parkinson Disease (PD) was initially described by James Parkinson in 1817, as a neurological syndrome (Parkinson 2002; Goetz 2011). PD is the second most common neurodegenerative disease, being preceded by Alzheimer disease (Dawson 2007), with unknown etiology and where aging is the primary risk factor (Darden 2007). PD is typically a chronic and slowly progressive disorder with a mean duration of 15 years from clinical diagnosis until death (Dawson 2007). Clinically, PD is characterized by movement alterations, such as bradykinesia, resting tremor, muscular rigidity and postural instability (Gelb et al. 1999). These motor symptoms are usually sufficient to perform a clinical diagnosis of PD, however this pathology also has prodromal non-motor symptoms, such as constipation, depression, anxiety, urinary incontinence and sexual impotence (Khoo et al. 2013). PD brain pathology is characterized by progressive and selective loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) (Eriksen et al. 2009). This loss is accompanied by the presence of aggregates of ubiquitinated and phosphorylated alpha-synuclein (α-syn) that form the Lewy Bodies (LBs), oxidative stress and alterations in the complex I of the mitochondrial respiratory chain (Baba et al. 1998; Winklhofer and Haass 2010). Several evidences from autopsy studies showed that multiple processes are involved in dopaminergic cell death, including oxidative stress, mitochondrial dysfunction, neuroinflammation, excitotoxicity and accumulation of misfolded proteins due to proteasomal and autophagic impairment (Olanow 2007). In sporadic PD mitochondrial dysfunction is a striking feature. Data shows that mitochondrial deficits occur in PD patient's brains, platelets and lymphocytes (Bose and Beal 2016), playing a critical role

in the loss of dopaminergic neurons (Ryan et al. 2015). Furthermore, data suggests that mitochondrial dysfunction can be potentiated by defects in mitochondrial biogenesis caused by the dysregulation of transcription factors, such as peroxisome proliferator-activated receptor gamma coactivator1-alpha (PGC-1α) (Hu and Wang 2016), which levels are decreased in *post-mortem* brains of PD and in white blood cells (Bose and Beal 2016). Recent studies in *post-mortem* PD brain tissue showed that nigrostriatal axon terminals are dysfunctional, which can alter normal axonal transport. Also, the generation of reactive oxygen species (ROS) induces the damage of complex I and III, protein oxidation on mitochondria and in cytoplasmic proteins, leading to mitochondrial dysfunction (Poewe et al. 2017). Further, PD is also characterized by a general activation of neuroinflammation, a number of studies demonstrated the existence of activated microglia cells, accompanied with a 30% increase in the density of astrocytes and an increase in several pro-inflammatory molecules such as interleukin (IL) 6, 2 and 1β, TNF-α and β2-microglobulin in the SNpc (Hirsch and Hunot 2009) . Some studies point that the production of ROS by the activated microglia cells can lead to α-syn nitration, which facilitates its aggregation, and consequently cell death (Mosley et al. 2012).

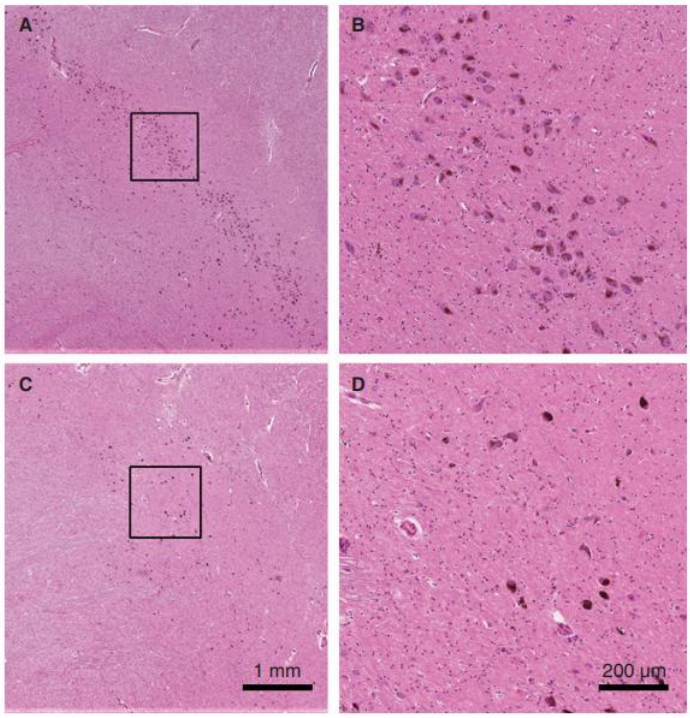


Figure 1. Coronal section at the level of the *substantia nigra pars compacta* (SNpc) in a control (A and B) and a PD brain (C and D) stained by haematoxylin and eosin. The squared areas in A and C are magnified in B and D, respectively. Dark brown cells are neuromelanin-containing dopaminergic neurons (adapted from Kouli et al. 2018).

1.2. Autophagy in Parkinson Disease

Autophagy, the pathway that allows the degradation of long-lived proteins, protein aggregates and damaged cytoplasmatic organelles through fusion of autophagic vesicles with lysosomes, is dysfunctional in PD (Arduíno et al. 2012). Autophagy efficiency is critical for the proper functioning of cells, particularly in neurons and other long-lived cells. The postmitotic nature, the extreme size and polarization of neurons indicates that the accumulation of aggregated proteins or damaged organelles may compromise their survival (Stavoe and Holzbaur 2019). Macroautophagy is activated as a compensatory route to remove the α-syn aggregates (Massey et al. 2006), and its oligomeric intermediates (Lee et al. 2004), considered to be the most toxic forms of α-syn (Outeiro

et al. 2008). Indeed, it was identified an abnormal presence of autophagosomes in dopaminergic SNpc neurons of *post-mortem* PD patients' brains. In agreement, Dehay et al. (2010) also observed an accumulation of autophagic vesicles in *post-mortem* PD nigra samples, where LBs strongly colocalized with the autophagy marker LC3, which suggests an autophagy induction, as an attempt to ameliorate α-syn pathology (Cook et al. 2012). Studies demonstrated that macroautophagy inhibition potentiates α-syn aggregation and toxicity in cellular and mouse models (Crews et al. 2010). Moreover, the induction of autophagy with different types of compounds results in an amelioration of the molecular traits of PD (Crews et al. 2010; Ghavami et al. 2014). The impairment of autophagy also prevents the clearance of other substrates, like dysfunctional mitochondria, thus preventing mitophagy (Arduíno et al. 2012), which is known to be impaired in PD. The genes *Pink1* and *Parkin* are often found mutated in PD. These genes encode the PTEN-induced putative kinase 1 (PINK1), a mitochondrial serine/threonine-protein kinase, and Parkin, an E3 ubiquitin ligase, respectively (Ding and Yin 2012). PINK1 is found in the intermembrane space of mitochondria or the outer mitochondrial membrane, being transported to the inner membrane of the mitochondria where it is degraded by PARL, but in damaged and depolarized mitochondria this transport does not happen and PINK1 accumulates in the outer membrane recruiting Parkin, which usually is found in the cytosol. Parkin in turn promotes the colocalization of mitochondria with LC3, thus allowing a selective and efficient signalling of damaged mitochondria to mitophagy. Parkin also recruits proteasomes to induce the rupture of the outer mitochondrial membrane and leads to the degradation of mitochondrial outer membrane and intermembrane space proteins (Ding and Yin 2012).

Mitochondrial dynamics is crucial to maintain mitochondrial function with fission and fusion processes being tightly regulated. The fusion of the mitochondria permits a

genetic complementation between two mitochondria (Hales 2004), which is regulated by Opa1, Mfn1, and Mfn2 (Santos et al. 2015). On the other hand, fission facilitates equal segregation into daughter cells during the cellular division allowing efficient distribution of mitochondria along microtubules tracks and even help isolating segments of damaged mitochondria to clearance, which is regulated by Drp1 and Fis1 (Arduíno et al. 2011; Santos et al. 2015). The lower basal mitochondrial content in the substantia nigra (SN) compared to other midbrain neurons creates a vulnerability to external and internal toxic stimuli that can affect mitochondrial fusion, fission and mitophagy, compromising the homeostasis of mitochondria during PD pathogenesis (Santos et al. 2015). Upon the compromise oxidative phosphorylation, OPA1 long isoform is cleaved into a short isoform and Drp1 is activated causing an excess in mitochondrial fragmentation (Santos et al. 2015). The impairment of mitophagy can also happen due to an alteration on the assembly of microtubules that prevents the transport of mitochondria towards lysosomes (Arduíno et al. 2013). These alterations happen at the level of the tubulin, through the decrease of NADH level increases the ratio of NAD+/NADH leading to the activation of cytosolic SIRT2. SIRT2 decreases the levels of acetylated tubulin, which prevents the retrograde transport of mitophagosomes into the lysosomes, causing their accumulation at the synapse (Esteves et al. 2018).

When dysfunctional mitochondria accumulate and degradation does not occur mitochondria may initiate cell death pathways, namely, apoptosis. Apoptosis may be initiate through mitochondrial permeability transition (MPT) mediated by the permeability transition pore, which is composed by a voltage-dependent anion channel in the outer membrane, the adenine nucleotide translocator in the inner membrane, and cyclophilin D in the matrix space. MPT turns the mitochondrial membrane permeable to solutes up to a molecular mass of about 1500 Da, which can lead to mitochondrial

depolarization, triggering the release of pro-apoptotic mitochondrial intermembrane space proteins, like cytochrome c, apoptosis-inducing factor, and Smac/Diablo into the cytosol (Kim et al. 2007).

1.3. Neuroinflammation in Parkinson Disease

Neuroinflammation is poised to play an important role in PD progression. Neuroinflammation is a mechanism associated with the restoration of normal structure and function of the brain by neutralization of an insult (Yang 2019). The activation of this mechanism that has been associated with PD, can be triggered by viral infection, exposure to heavy metals, organophosphate compounds, neurotoxins like MPTP, and certain pesticides (Tansey and Goldberg 2010). Neuroinflammation features are the presence of activated microglia and reactive astrocytes, increased production of cytokines and reactive oxygen and nitrogen species. One well known environmental trigger is lipopolysaccharide (LPS), an endotoxin present in the membrane of Gram-negative bacteria (Bing and Liu 2011). Recently α-syn was proposed as an antimicrobial peptide (AMP) (Park et al. 2016). Although mutations in α-syn are associated with PD, it may also be a vehicle for inducing an innate immune response in the brain. Recent studies showed that α-syn is capable of interacting with the plasma membranes and negatively charged phospholipid vesicles to form pores that change its permeability (Pacheco et al. 2012; Park et al. 2016). Since Gram-negative bacteria possess negatively charged phospholipids in their membrane, such as LPS, peptidoglycan, cardiolipin and phosphatidylglycerol, α-syn is able to interact with these disrupting the membrane of the bacteria and inhibiting cell growth (Park et al. 2016).

Dopaminergic neurons are selectively targeted in PD and a number of mechanisms have been proposed to underlie this selectivity. On one side, dying neurons release substances that are recognized by glial cells, activating them, such as dopamine, neuromelanin and α-syn (Herrera et al. 2015). Dominguez-Meijide and colleagues observed that the decrease in dopamine levels in early stages of PD promote neuroinflammation and disease progression via glial RAS exacerbation (Dominguez-Meijide et al. 2017). Neuromelanin is able to activate microglia cells leading to neuroinflammatory processes and dopaminergic neuron degeneration (Karlsson and Lindquist 2013; Viceconte et al. 2015). Extracellular and misfolded α-syn prompts microglia activation and pro-inflammatory molecules production (Zhang et al. 2005; Klegeris et al. 2008; Lema Tomé et al. 2013). Further, glial cells are recruited into damaged areas phagocytising dopaminergic neurons, and overexpressing various inflammatory markers such as HLA-DR of the human MHC II complex, complement receptor type 3 (CR3), CD68, CD23, ferritin, CD11a and CD54. These reactive glia also secrete various pro-inflammatory cytokines, IFN-c, TNF-α, IL-1β (Lee Mosley et al. 2006), that will induce the expression of CD23 in glial cells triggering the production of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) release. These will amplify the secretion of pro-inflammatory cytokines to dopaminergic neurons activating caspases (Hunot et al. 2003), like caspase 11, that can induce programmed cell death, also known as apoptosis (Furuya et al. 2004). Caspase 1 is the primary activator of pro-IL-1β and pro-IL-18 into their mature forms (Furuya et al. 2004; Liu et al. 2017). The NO released can also enhance oxidative stress by releasing free iron from ferritin and by interacting with superoxide to form peroxynitrite, which will cause more damage in proteins and various cell constituents (Hunot et al. 2003). For the exposed, the activation of microglia may lead to dopaminergic neuronal cell death in PD,

establishing a vicious cycle where the overactivation of neuroinflammation will lead to more damage to dopaminergic neurons, which will continue signalling the activation of microglial cells (Hunot et al. 2003).

Apart from activated microglia, neurons have been shown to express Toll-like receptors (TLRs) also present on the surface of antigen presenting cells (APCs) (Nguyen et al. 2002). These cells are rapidly activated upon the binding of pathogens to specific TLRs, activating the innate immunity and mounting inflammatory responses with the activation of leucine-rich repeat pyrin domain containing 3 (NLRP3) inflammasome and the release of pro-inflammatory cytokines such as TNF-α and IL-1β (Nguyen et al. 2002). Inflammatory cytokines are also released in peripheral tissues that can reach the brain due reduced function of efflux pumps in the blood-brain barrier in PD (Tansey and Goldberg 2010). Populations of CD4+ and CD8+ T cells can be recruited into the SNpc and modulate pro-inflammatory responses through the Fas/FasL pathway, which induces the release of TNF-α and IL-1β (Park et al. 2003; Brochard et al. 2009), facilitating neuronal death associated with PD.

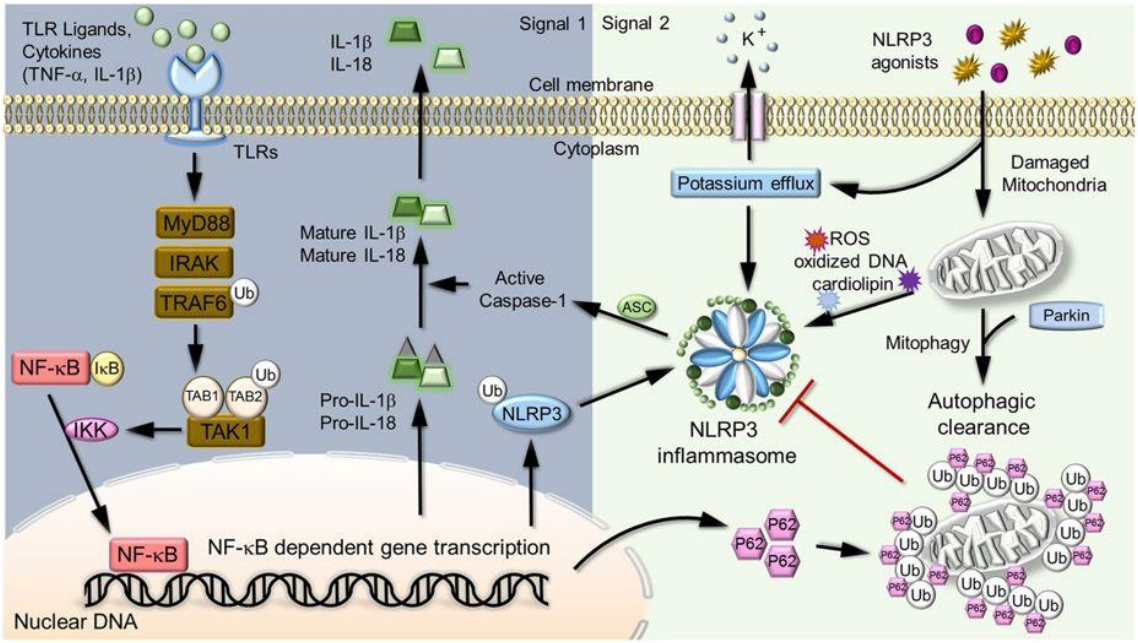


Figure 2. Regulation of innate immunity leads to the activation of the NLRP3 inflammasome. The binding of an extracellular stimulus, like LPS, to TLR activates the NF-κB signalling, which promotes its translocation to the nucleus, and transcription of the NF-κB-dependent genes *Pro-IL-1β, Pro-IL-18* and *NLRP3*. NLRP3 agonists activate the formation of the NLRP3 inflammasome, which leads to the secretion of mature IL-1β and IL18 through the activation of caspase 1. NF-κB also releases p62, which binds to mitochondrial poly-ubiquitin through Parkin, and clears the dysfunctional mitochondria (adapted from, Liu et al. 2017).

1.4. Mitochondrial control of Innate Immunity

Recent insights revealed that mitochondria are an important source of damage associated molecular patterns (DAMPs). A number of studies indicate that mitochondrial dysfunction and fragmentation may expose DAMPs, such as mitochondrial N-formyl peptides and mitochondrial DNA (mtDNA) (Zhang et al. 2010; Weinberg et al. 2015). This is due to the fact that mitochondria and bacteria have a common ancestral displaying some similarities, such as circular DNA, N-formylated proteins and are double-membrane structures, evidence used in support of the endosymbiotic theory. mtDNA is similar to bacterial DNA in that it contains CpG

motifs, which activate the Toll-like receptor 9 (West et al. 2006; Zhang et al. 2010). DAMPs activate RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and TLRs driving an hyperactivation of innate immune responses (Weinberg et al. 2015), such as the activation of inflammasomes, specifically the NLRP3 inflammasome. A variety of insults, resulting from cellular infection or stress, can promote mitochondrial dysfunction and activate the NLRP3 inflammasome (Gurung et al. 2015), however the molecular mechanisms underlying the contribution of mitochondria to the activation of the NLRP3 inflammasome have only recently been described. While initial studies showed that mitochondrial dysfunction and mitochondrial ROS production are required for NLRP3 inflammasome activation (Nakahira et al. 2011; Zhou et al. 2011), further evidence has shown that mtDNA translocation to the cytosol plays an active role in this process (Nakahira et al. 2011; Shimada et al. 2012), where it can directly bind to and activate the NRLP3 inflammasome (Shimada et al. 2012). This inflammasome is formed when NLRP3, which is usually associated with the endoplasmic reticulum membrane, is redistributed to nuclear and mitochondrial membranes upon activation. Another mitochondrial-dependent mechanism by which NLRP3 becomes activated consists in its interaction with cardiolipin. Cardiolipin is a non-bilayer-forming phospholipid present in the inner membrane of mitochondria of eukaryotic cells that undergoes oxidation, and might be released as a mitochondrial DAMP that binds to NLRP3 docking it to the mitochondria and activating the inflammasome (Iyer et al. 2013; Mohanty et al. 2019). The inflammasome then forms oligomers with apoptosis-associated speck-like protein containing a CARD (ASC) (Wilkins et al. 2017). Upon this oligomerization pro-caspase 1 is recruited into the complex formed by NLRP3 and ASC through the CARD domain of ASC, which acts as an adaptor protein (Walsh et al. 2014; Zahid et al. 2019). It will then promote the maturation of

pro-caspase 1 into caspase 1 and release of pro-inflammatory cytokines IL-1β and IL-18 (Weinberg et al. 2015). Despite the number of studies describing mitochondria as a source of DAMPs during inflammation in the periphery, the potential for mitochondrial DAMPs to trigger, or exacerbate, inflammation in the brain is now being explored. In recent studies, this possibility was tested by treating different brain cell types with mitochondrial components and measuring markers of inflammation afterwards. Neuronal and microglial cell lines exposed to mitochondrial lysates displayed increased markers of inflammation, with mtDNA being identified as the candidate DAMP responsible for the inflammatory changes (Wilkins et al. 2015). Moreover, mice injected with isolated mitochondria into the brain also revealed increased markers of inflammation such as an increase in TNF-α, NFκB phosphorylation and GFAP protein, and a decrease in Trem2 mRNA (Wilkins et al. 2016). Also, α-syn overproduction may trigger mitochondrial damage. The link between PD and α-syn was discovered upon observation that a mutation in the α-syn encoding gene *SNCA*, which leads to an A53T amino acid change (Stefanis 2012) causes PD. This mutation causes an increase in α-syn within mitochondria, which in turn down-regulates the activity of complex I activity and induces mitochondrial fragmentation and excessive mitophagy (Stefanis 2012).

1.5. Objective

Our main goal was to understand the crosstalk between mitochondrial dysfunction and the activation of innate immunity pathways in PD. To reach this objective we took advantage of cytoplasmatic hybrids (cybrids) cells, derived from the NT2 teratocarcinoma cell line and repopulated with mtDNA from PD patients or

age-matched control subjects, and exposed them to LPS and to the inhibitors of autophagy ammonium chloride (NH_4Cl) and leupeptin.

2. Materials and methods

2.1. Chemicals

Chemicals	Manufacturer
10-N-Nonyl acridine orange (NAO)	Enzo Lifesciences (Lausen, Switzerland); Cat. No. 08091739
Caspase 1 substrate	Sigma Chemical Co (St. Louis, MO, USA); Cat. No. SCP0066
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)	*Sigma Chemical Co (St. Louis, MO, USA); Cat. No. C2759*
3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT)	Sigma Chemical Co (St. Louis, MO, USA); Cat. No. M2128
Leupeptin	Sigma Chemical Co (St. Louis, MO, USA); Cat. No. L2023
Lipopolysaccharides (LPS)	026:B6; Sigma Chemical Co (St. Louis, MO, USA; Cat. No. L2654
MitoPY1	Sigma Chemical Co (St. Louis, MO, USA); Cat. No. SML0734
Oligomycin	Alfa Aesar (Karlsruhe, Germany); Cat. No. J60211

Tetramethylrhodamine, methyl ester (TMRM)	Molecular Probes (Eugene, OR, USA); Cat. No. T668

2.2. Human subjects

Sporadic PD and age-matched healthy individuals were all recruited after approval by the University of Kansas School of Medicine Institutional Review Board. Individuals in the PD group were followed regularly in a tertiary referral movement disorders clinic at the Kansas University Medical Center and met criteria commonly used to diagnose PD in clinical and research settings. None of the patients were believed to have alternative diagnoses, degeneration of related systems, drug-induced parkinsonism, or any other serious medical illness, including another neurodegenerative disease. The control subjects were participants of a longitudinal "normal aging/normal cognition" cohort that is characterized serially by the Brain Aging Project at the University of Kansas School of Medicine and have not been diagnosed with a neurodegenerative or pre-neurodegenerative disease condition. The age of the PD subjects who participated in this study was 64 + 12.8 years and for the control subjects was 74.3 + 5.5 years. After providing informed consent sporadic PD (n=9) and the respective age-matched control subjects (n=5) underwent a 10 mL phlebotomy using tubes containing acid–citrate–dextrose, as an anticoagulant, to provide the platelets needed for cell fusions.

2.3. Generation, maintenance and treatments of cybrid cell lines

To create the cybrid cell lines for this study, we used NT2 (Ntera2/ D1) cells, a teratocarcinoma cell line with neuronal characteristics (Stratagene, La Jolla, CA). These

cells were depleted of endogenous mtDNA (rho0 cells) via long-term ethidium bromide exposure. Rho0 cells lack intact mtDNA, do not possess a functional electron transport chain, and are auxotrophic for pyruvate and uridine. Consequently, platelet mitochondria from either PD or control subjects were isolated from the individual blood samples and were used to repopulate NT2 rho0 cells with mtDNA as previously described. Briefly, Rho0 cells were co-incubated in Polyethylene glycol (Merck Chemicals) with platelets from the human subjects, as previously described by our group. After fusion, selection is performed to remove the Rho0 cells that have not repopulated with platelet mtDNA. After selection, the resultant cybrid cells were switched to cybrid growth medium and were grown in 75 cm² tissue culture flasks maintained in a humidified incubator at 37 °C and 5% CO_2. Cybrids growth medium is Optimem, obtained from Gibco-Invitrogen (Life Technologies Ltd., UK) supplemented with 10% non-dialyzed Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution. Non-dialyzed FBS was obtained from Gibco-Invitrogen (Life Technologies Ltd., UK). Rho0 cell growth medium, Optimem, was supplemented with 10% non-dialyzed FBS, 200 μg/mL sodium pyruvate from Sigma (St. Louis, MO, USA), 100 μg/mL uridine from Sigma (St. Louis, MO, USA) and 1% penicillin/streptomycin solution. Prior to experiments, cell lines were maintained in the cybrid growth medium. For western blotting, cybrid cell lines were seeded in 6 well plated at a density of 0.25 × 10^6 cells/mL. For MTT and mitochondrial membrane potential experiments cells were plated in 48 well plates at a density of 100 000 cells/mL. For cardiolipin experiments, cybrid cell lines were grown on Ibidi 8 well plates at a density of 80 000 cells/mL. After 24 h the medium was refreshed, and experimental treatments were performed. Cybrids were exposed to 1μg/μl of LPS. Where indicated, 20 mM NH_4Cl and 100 μM leupeptin (Sigma, St. Louis, MO, USA) were added for 4 h to the culture medium. The

combination of NH_4Cl with leupeptin blocks all types of autophagy, as it reduces the activity of all lysosomal proteases by increasing the lysosomal lumen pH without affecting the activity of other intracellular proteolysis systems. For all experimental procedures, controls were performed in the absence of the described agents.

2.4. MTT cell viability assay

Cellular viability was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay. In viable cells, NAD(P)H-dependent oxidoreductase enzymes reduce the MTT reagent to formazan, an insoluble crystalline product with a purple colour that absorbs light at 570 nm. Following treatments described, 0.5 mg/ml of MTT was added to each well. Cybrid cell lines were then incubated at 37 ºC for 2 h. At the end of the incubation period the formazan precipitates were solubilized with 0.5 ml of acidic isopropanol (0.04 M HCl/Isopropanol). The absorbance was measured at 570 nm.

2.5. Mitochondrial membrane potential (ΔΨmit) analysis

Mitochondrial membrane potential (ΔΨmit) was detected using the fluorescent dye TMRM. After the described treatments, cybrid cell lines were loaded with 300 nM of TMRM, in the dark, at 37 ºC for 1 h. The fluorescence of TMRM (λexc = 540 nm; λem = 590 nm) was recorded using a Spectramax Plus 384 spectrofluorometer (Molecular Devices) during 3 min before (base line) and 5 min after mitochondrial depolarization with FCCP. Maximal mitochondrial depolarization (Δψm collapse) was performed by adding 1 μM FCCP (proton ionophore), which was always preceded by oligomycin (2 μg/ml) to prevent ATP synthase reversal. The dye retention was

determined by the difference between maximum fluorescence, after depolarization, and the basal value of fluorescence and expressed in relation to untreated condition. Since TMRM is a cell-permeant cationic dye, it is readily sequestered by functional mitochondria and a decrease of cellular retention of these dyes has been associated with a decrease in $\Delta\psi m$.

2.6. Immunostaining

For immunostaining assays, approximately 250 000 cells per well were seeded in 12 well plates containing glass coverslips. Following cell treatments, including a positive control with cells exposed to 5µM FCCP for 2h, cultures were fixed for 10 min at room temperature using 4% paraformaldehyde. Fixed cells were washed again with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), permeabilized with 0.2% Triton X-100 during 3 min and blocked with 3% BSA for 30 min. The permeabilized cells were incubated with primary antibody 1:200 anti-TOM20 (Cat. No. sc-11415) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), overnight in a wet chamber, at 4 ºC. Subsequently, cells were incubated 1h with secondary antibody (1:400 Alexa Fluor 488 from ThermoFisher Scientific (Cat. No. A11001) (Waltham, MA, USA). Finally, cells were washed in PBS, incubated for 5 min with Hoechst 33342 from Sigma Chemical Co (St. Louis, MO, USA; Cat. No. H1399) (15 mg/L in PBS, pH 7.4) in the dark, and visualized by confocal microscopy. Images were acquired on a Zeiss LSM 710 (40 × 1.4NA plan-apochromat oil immersion lens) and analyzed using ImageJ software. An ImageJ macro tool was used to analyze mitochondrial network as previously described (Valente et al. 2017).

2.7. 10-N-Nonyl acridine orange (NAO) staining

10-N-Nonyl acridine orange (NAO) binds to negatively charged phospholipids cardiolipin, phosphatidylinositol and phosphatidylserine, but with higher affinity to cardiolipin, and is largely independent of mitochondrial membrane potential. Cardiolipin distribution and fluorescence was measured using the NAO probe. After treatments, cybrid cell lines were washed with HBSS (5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $NaH_2PO_4.H_2O$, 5 mM Glucose, 5.36 mM Sodium Pyruvate, 5.36 mM Hepes, pH 7.2) and then loaded in the dark with 100 nM Cardiolipin in HBSS for 1 h. After a gentle wash, cells were kept in HBSS during image acquisition. Images were obtained using a Plan-Apochromat/1.4NA 63x lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software.

The images of cells stained with NAO were extracted to greyscale, inverted to show NAO-specific fluorescence as black pixels and thresholded to optimally resolve NAO staining. Background fluorescence and specific NAO fluorescence were determined. The final value for fluorescence intensity resulted from the subtraction of background fluorescence from specific fluorescence and the result was further divided by the number of cells from each acquired image.

2.8. Immunoblotting

To prepare cytosolic samples for TLR3, NLRP3, ASC western blot analyses, after incubations, cybrids cells were washed in ice-cold PBS and lysed in lysis buffer (10 mM HEPES; 3 mM $MgCl_2$; 1 mM EGTA; 10 mM NaCl, pH 7.5, supplemented with 2 mM DTT, 0.1 mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail)

supplemented with 2 mM sodium orthovanadate and 50 mM sodium fluoride and 0.1% Triton X-100. Cybrids were scraped on ice, transferred to the respective tubes and incubated on ice for 40 min. Afterwards, samples were centrifuged at 2 300×*g* for 10 min at 4 ºC and the resulting supernatant contains the cytosolic fraction. To prepare samples for LC3, p62 and α-syn protein levels determination the hypotonic lysis buffer (25 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA supplemented with 2 mM DTT, 0.1 mM PMSF and a 1:1000 dilution of a protease inhibitor cocktail) was supplemented with 1% Triton X-100. After 3 cycles of freezing and thawing, samples were centrifuged at 14 000×*rpm* for 10 min at 4 ºC and the resulting supernatant contains the cytosolic fraction. The total amount of all resulting cell lysates obtained were removed and stored at -80°C. Protein content was determined using BCA Protein Assay protein assay (Pierce; Cat. No. 23227). Samples were suspended in 6× sample buffer under reducing conditions (4× Tris.HCl/SDS, pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue). Depending on the protein molecular weight of interest, samples containing 35 μg of protein were loaded onto 10% SDS-PAGE gels for analysis of TLR3, NLRP3 and ASC and 15% SDS-PAGE gels for analysis of LC3 and p62. Specifically, for the analysis of α-syn oligomers, samples were not boiled and separated by electrophoresis in 10% SDS-PAGE gels. After transfer to PVDF membranes (Millipore, Billerica, MA, USA), non-specific binding was blocked by gently agitating the membranes in 3% BSA and 0.1% Tween-20 in Tris-Buffered Solution (TBS - 20mM Tris, 150 mM NaCl and 0.1% (w/v) Tween-20 ®, pH 7.6) for 1 h at room temperature. The blots were subsequently incubated with the respective primary antibodies overnight at 4°C with gentle agitation: 1:1000 anti-p62 (Cat. No. P0067) and 1:5000 anti-β-actin (Cat. No. A5441) both from Sigma Chemical Co (St. Louis, MO, USA); 1:750 anti-NLRP3 (Cat. No. MA5-23919) from ThermoFisher

Scientific (Waltham, MA, USA); 1:1000 anti-TLR3 (Cat. No. ab84911) from Abcam (Abcam, Cambridge, UK); 1:200 anti-ASC (Cat.No. sc-271054) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 1:100 anti-LC3B XP® (Cat. No. 3868) from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA) and 1:500 anti-α-synuclein (Cat. No. ABN 2265) from Sigma Chemical Co (St. Louis, MO, USA). After overnight incubation to membranes were washed 3 times with 0.1% Tween-20 in TBS for 5 min, then subsequently incubated with the respective secondary antibodies during 1 h at room temperature with gentle agitation with respective secondary antibodies. The blots were then washed 3 times with 0.1% Tween-20 in TBS for 5min and incubated with ECF substrate for 5 min. Blots were visualized using a ChemiDoc Imaging System and quantified using Image Lab Software (Bio-Rad, Hercules, CA, USA).

2.9. Caspase 1 Activity Assay

To assess the activity of caspase 1 it was used the ability of the protein to cleave the site VAD (Val-Ala-Asp) of a caspase 1 substrate, which emits fluorescence at 405 nm. The cytosolic samples for the caspase 1 assay activity were prepared using the same protocol as the samples for TLR3, NLRP3 and ASC described above. Protein content was determined using BCA Protein Assay protein assay (Pierce; Cat. No. 23227). Samples containing 40 μg of protein were incubated at 37 °C for 2 h in CHAPS Buffer, 2 mM DTT with 5 μg of Caspase 1 substrate (Cat. No. SCP0066). The absorbance was measured at 405 nm.

2.10. Data Analysis

All data are expressed as means ± standard error (SEM) of at least 3 independent experiments. Statistical analyses were performed using GraphPad Prism 8 for Windows (GraphPad Software, San Diego, CA, USA). To compare means between groups we used t-test analysis. p-values < 0.05 were considered significant.

3. Results

3.1. LPS and autophagic inhibitors have no additional effect in PD cybrids cell viability

We used the ability of cells to reduce MTT as a parameter of cell viability in order to establish a concentration of LPS and NH_4Cl plus leupeptin (NL) that could represent a minor damage to control and PD cybrid cell lines. We observed that 1 μg/mL LPS and 15 mM NH_4Cl/100 μM leupeptin did not induce a decrease in cell viability (Fig. 3). In accordance to previous results from our group, PD cell lines have, in basal conditions, a reduction in the cell viability (Esteves et al. 2008). Further PD cells treated with both LPS and NL presented significant reduction in cell viability when compared with untreated control cells.

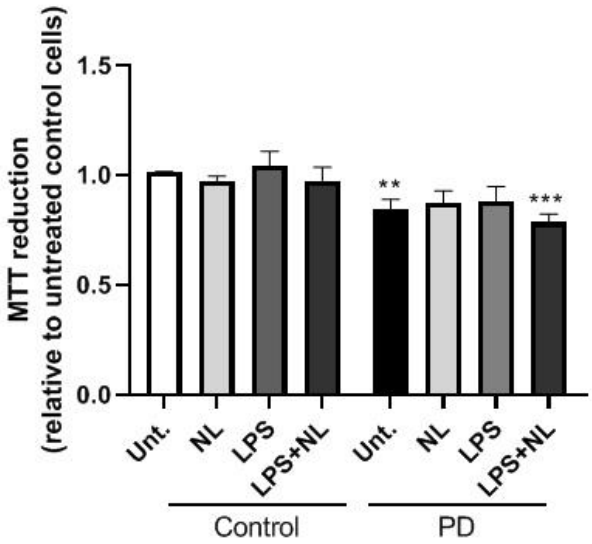


Figure 3. Effect of 1μg/mL LPS and 15mM NH_4Cl/100μM Leupeptin (NL) on cell proliferation of cybrid cells. Cells were exposed to LPS for 24 h and NL was added in the last 4 h of incubation to inhibit autophagy. MTT reduction was estimated spectrophotometrically at 570 nm, as described in Materials and Methods. Data represent the mean ± SEM and are expressed in comparison with untreated control cells (n=5). ** $p < 0.01$, *** $p < 0.001$, significantly different relative to untreated control cells.

3.2. LPS and autophagic inhibitors decrease mitochondrial potential in Control cybrids but have no additional effects in PD cybrids

The amount of TMRM taken up and retained by mitochondria is proportional to the magnitude of the mitochondrial membrane potential (Δψmit). In order to evaluate PD and control cellular metabolic state Δψmit was assessed by measuring the sequestration of the TMRM probe. In accordance to previous works (Esteves et al. 2008) it was verified that PD cells have a basal impairment of mitochondrial potential and LPS has no further effects on PD cells. In control cybrids both NL and LPS show a tendency to reduce Δψmit (Fig.4).

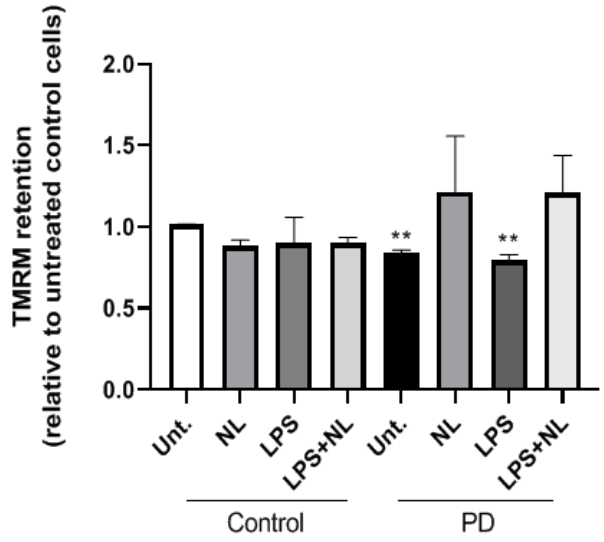


Figure 4. Effect of 1μg/mL LPS and 15mM NH_4Cl/100μM Leupeptin (NL) on of cybrid cells on mitochondrial membrane potential. Mitochondrial membrane potential was estimated using fluorescent cationic dye TMRM. Data represent the mean ± SEM and are expressed in comparison with untreated control cells (n=3). ** $p < 0.01$, significantly different relative to untreated Control cells.

3.3. LPS and autophagy inhibitors do not exacerbate mitochondrial defects in PD cybrids

Cardiolipin is a phospholipid localized and synthesized in the inner mitochondrial membrane (IMM) and its abundance is regulated by mitochondrial dynamics (Paradies et al. 2019). Cardiolipin exposition upon mitochondrial dysfunction fragmentation was evaluated using 10-N-Nonyl acridine orange (NAO). Live cell imaging acquired from PD and control cybrids showed that in the untreated PD cells occurs an increase in cardiolipin exposure which correlates with increased levels of p-DRP1 (Santos et al. 2015) and fragmented mitochondrial network from cells stained with TOM20. The exposure to LPS and NL results in further increase in cardiolipin exposure in both cell types.

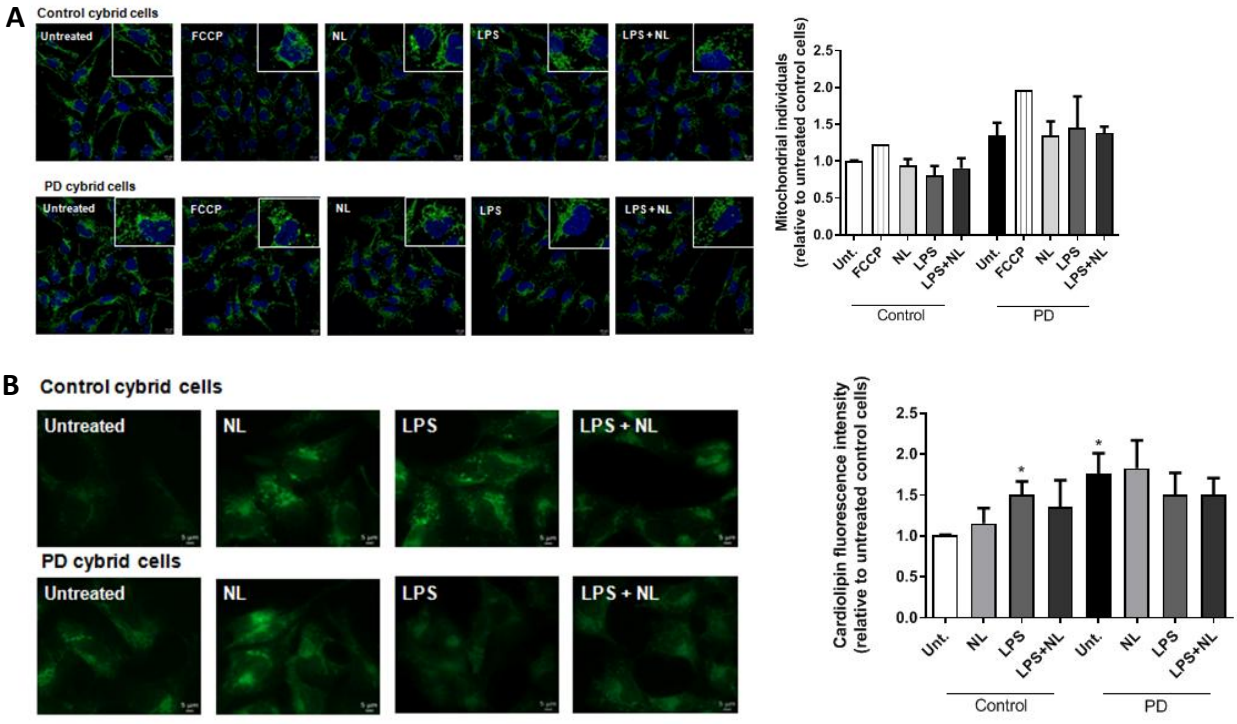
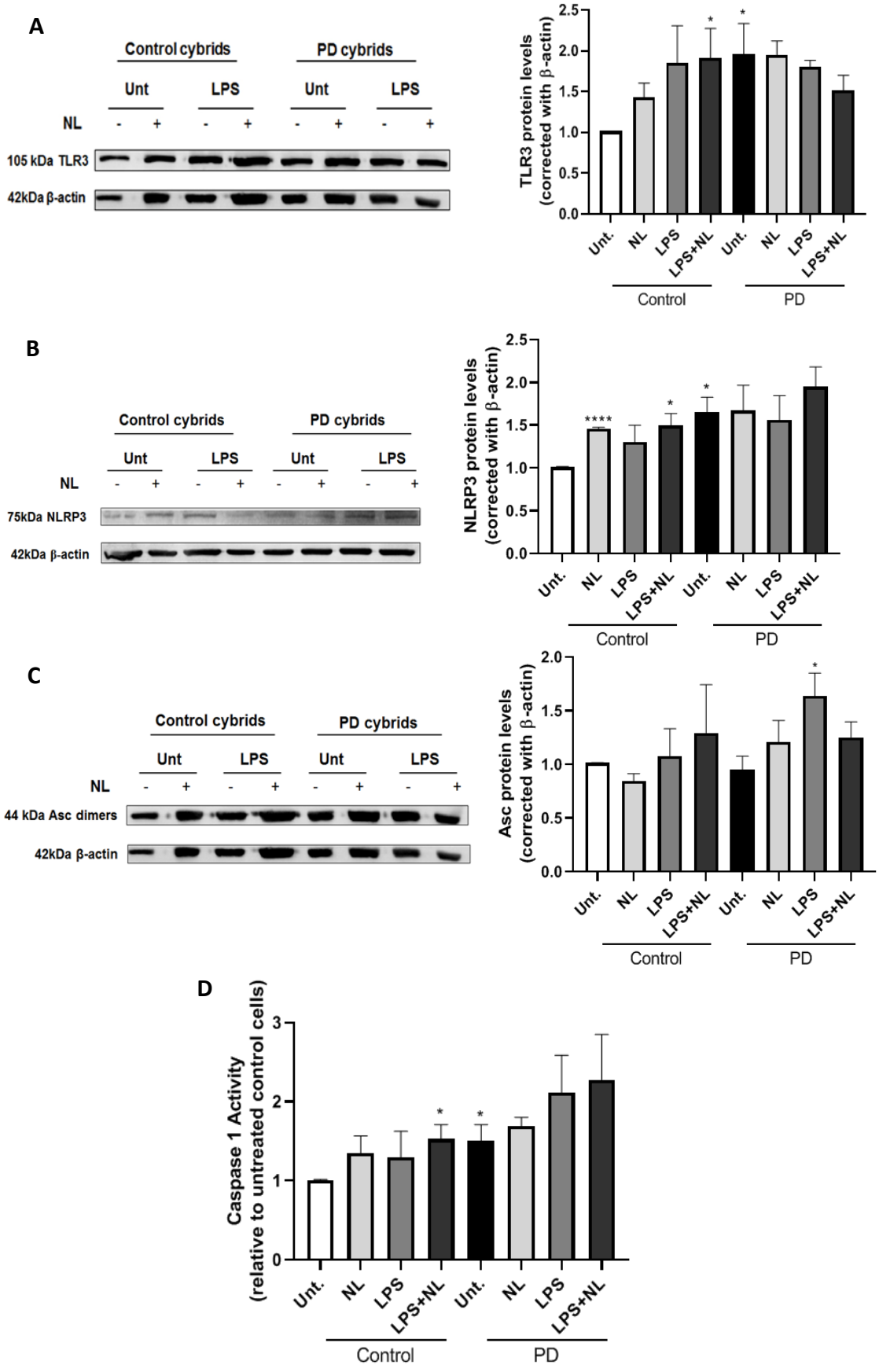


Figure 5. (A) Immunostaining of Control and PD cybrids with anti-TOM20 antibody after 1μg/mL LPS and 15mM NH_4Cl/100μM Leupeptin (NL) exposure (n=2). (B) Effect of LPS and NL on cardiolipin fluorescence intensity. Staining of living Control and PD cybrids cells with 10-N-Nonyl acridine orange (NAO) after LPS and NL exposure. Data represent the mean ± SEM and are expressed in comparison with untreated control cells (n=3). * $p < 0.05$, significantly different relative to untreated Control cells.

3.4. LPS and autophagy inhibitors activate innate immunity in Control cybrids but had no additive effect in PD cybrids

Evidence has emerged that suggests that mitochondria provide a platform for signalling pathways involved in innate immune response. Innate immunity activation converges in the formation of inflammasomes, multiprotein complexes that promotes the processing of precursor cytokines (pro-IL1β and pro-IL-18) through caspase 1 activation (Sandhir et al. 2017). The levels of TLR3, NLRP3 and ASC as well as Caspase 1 activity were evaluated to assess the activation of innate immunity and NLRP3 inflammasome activation (Fig. 6 A,B,C,D). Western blot analysis of TLR3, NLRP3 and its adaptor ASC showed that in the control cells the innate immunity responses are less activated in basal conditions than in PD cells and that the joint use of LPS and NL increases the activation of the inflammasome, especially in control cells.


A
Control cybrids
PD cybrids
Unt
LPS
Unt
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- + - + - + - +
105 kDa TLR3
42kDa β-actin
TLR3 protein levels (corrected with β-actin)
Unt.
NL
LPS
LPS+NL
Control
PD
B
75kDa NLRP3
NLRP3 protein levels (corrected with β-actin)
****
C
44 kDa Asc dimers
Asc protein levels (corrected with β-actin)
D
Caspase 1 Activity (relative to untreated control cells)

Figure 6. TLR3, NLRP3 and ASC protein levels, Caspase 1 activity assay after 1μg/mL LPS and 15mM NH_4Cl/100μM Leupeptin (NL) exposure, on control and PD cells. (A) Western Blot analysis of TLR3 using anti-TLR3 and anti-actin antibodies; Densitometric analysis of TLR3 corrected with actin (n=5). (B) Western Blot analysis of NLRP3 using anti-NLRP3 and anti-actin antibodies; Densitometric analysis of NLRP3 corrected with actin cells (n=3). (C) Western Blot analysis of ASC using anti-ASC and anti-actin antibodies; Densitometric analysis of ASC corrected with actin (n=3). (D) Caspase activity was measured spectrophotometrically at 405 nm, as described in Materials and Methods (n=3). Data represent the mean ± SEM and are expressed in comparison with untreated control cells. * $p < 0.05$, **** $p < 0.0001$, significantly different from the untreated Control cells.

3.5. LPS exposure induces a decrease in p62 and LC3II fluxes in Control cybrids

Mitochondrial degradation through mitophagy plays a key role in the regulation of inflammatory signalling, and mitochondrial danger signals such as mitochondrial DNA translocated into the cytosol or exposition of cardiolipin can lead to an overactivation of inflammatory responses (Cho et al. 2020). Our results show that autophagic flux evaluated by the levels of basal LC3II divided by LC3II after NL treatment and p62 flux, are decreased in PD cybrids, comparably to previous data from our group (Arduíno et al. 2012). LPS does not have an effect in autophagic flux in PD cybrids whereas it shows a tendency to decrease, both LC3 and p62 fluxes, in control cells.

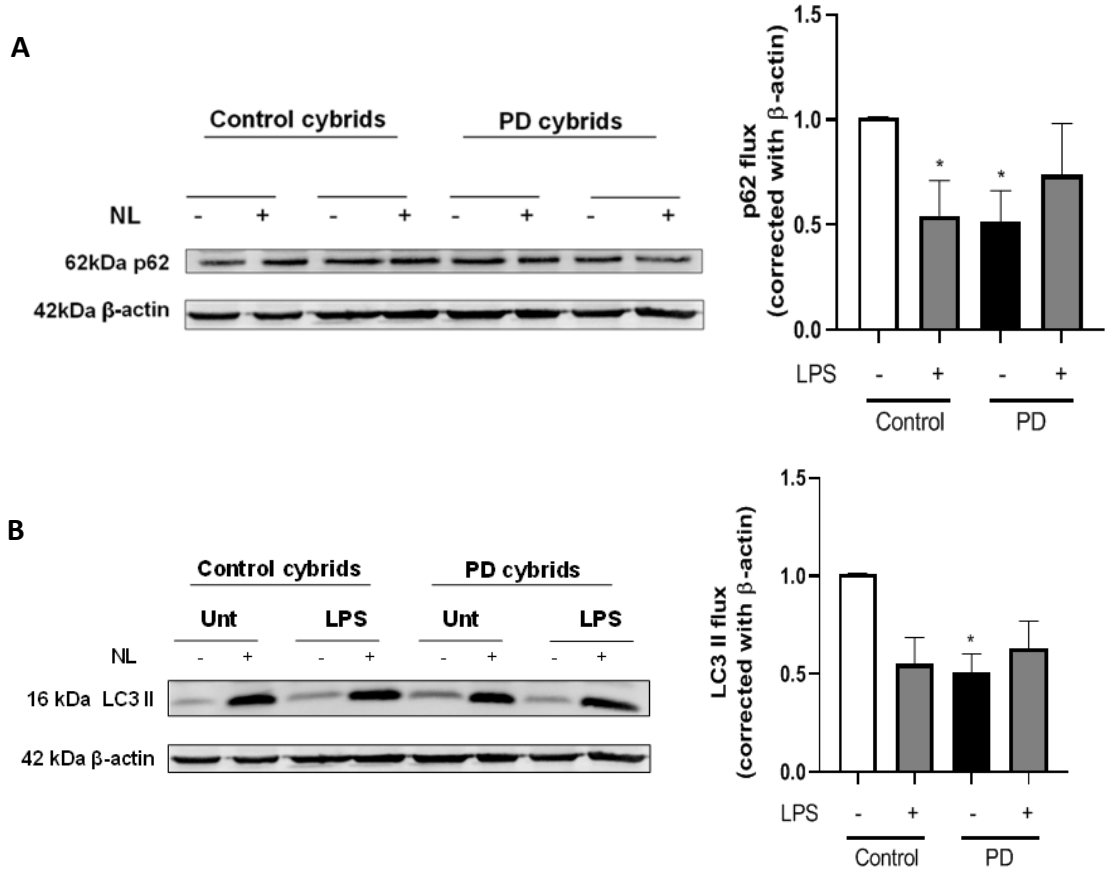

Figure 7. (A) Western Blot analysis of p62 using anti-p62 and anti-actin antibodies; Densitometric analysis of p62 flux corrected with actin (n=4). (B) Western Blot analysis of LC3 II using anti-LC3 and anti-actin antibodies; Densitometric analysis of LC3 II flux corrected with actin. (n=4). Data represent the mean ± SEM and are expressed in comparison with untreated control cells. * $p < 0.05$, significantly different from the untreated Control cells.

3.6. α-synuclein oligomers increase upon LPS and autophagy inhibitors in Control cybrids with no additional effect in PD cybrids

Accumulation of abnormal forms of α-syn is a striking feature of PD, but recent evidence suggests that much of the downstream neurodegeneration may result from inflammatory responses (Reish and Standaert 2015). In order to relate autophagy, inflammation and α-syn oligomerization, α-syn oligomers were determined by western blot in non-reducing and non-denaturing conditions. PD cybrids have higher levels of α-syn in basal conditions when compared to untreated control cells. When autophagic

flux is blocked, α-syn oligomers increase in both control and PD cybrids. Interestingly, LPS increases α-syn oligomerization in control cells but fails to induce further increase PD cells.

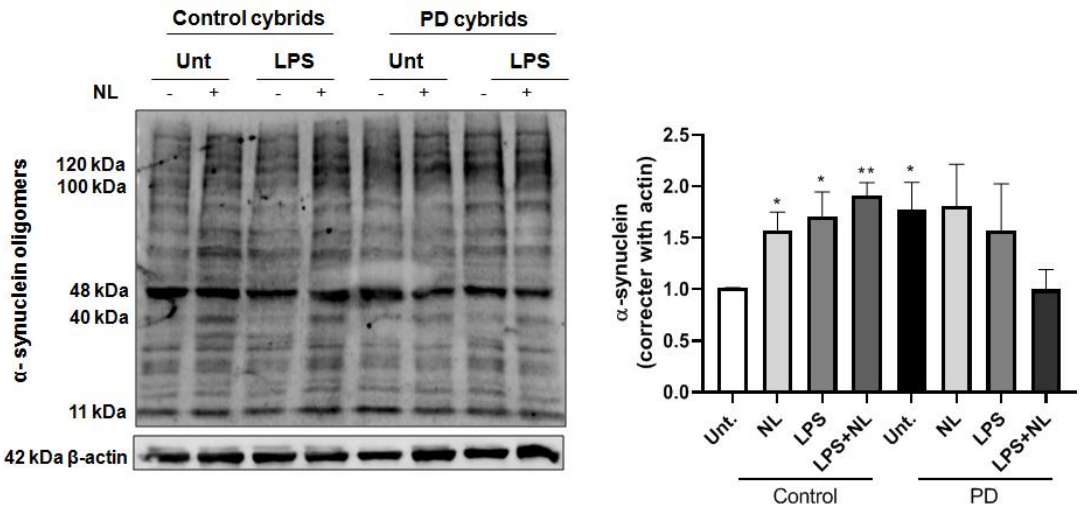

Figure 8. α-synuclein oligomers levels after 1μg/mL LPS and 15mM NH_4Cl/100μM Leupeptin (NL) exposure on control and PD cells. Western Blot analysis of α-synuclein using anti-α-synuclein and anti-actin antibodies; Densitometric analysis of α-synuclein oligomers corrected with actin (n=3). Data represent the mean ± SEM and are expressed in comparison with untreated control cells. * $p < 0.05$, ** $p < 0.01$, significantly different from the untreated Control cells.

4. Discussion

The mitochondrial cascade hypothesis for PD postulates that mitochondrial dysfunction precedes α-syn aggregation (Cardoso 2011), and neuroinflammation, all distinctive hallmarks of the disease. In this thesis we investigated the hypothesis of whether mitochondrial dysfunction can regulate innate immune responses in a context of PD. We provide evidence that PD cybrids have intrinsic mitochondrial dysfunction in agreement with previous work from our group (Esteves et al. 2010, 2014, 2018; Arduíno et al. 2012). Interestingly, recent hypothesis linked bacterial toxins produced by certain taxa with mitochondrial dysfunction (Silva et al. 2020). Mitochondria are evolutionary symbionts of early eukaryotic cells, having their origin in a proteobacterial lineage (Ku et al. 2015), which makes them natural targets of many microbial byproducts. In fact, a body of evidences has shown that bacterial endotoxins, such as LPS, cause mitochondrial dysfunction while causing neuroinflammation in specific brain regions (Noh et al. 2014). LPS systemic injection to wild-type animals induced higher concentration of cytokines and microglia activation in the frontal cortex, striatum and the hippocampus (Noh et al. 2014). Intracerebroventricular models and intrapallidal administration of LPS can be used to model PD, with the ability to induce microglial activation in the SNpc and subsequently, degeneration of dopaminergic neurons (Deng et al. 2020). Intranigral administration of 10 μg of LPS in rats induces a 50-80 % reduction in the number of tyrosine hydroxylase positive cells in the SN compared to shams at 4 weeks post-treatment (Bao et al. 2018). Also, it was previously shown in PD models that stereotaxic administration of LPS reduces the activity of mitochondrial complex I of the electron transport chain in both the SN and striatum (Choi et al. 2009). Recently, LPS was associated with excess mitochondrial fission through

phospho-DRP1, resulting in activation of inflammatory responses in macrophages (Kapetanovic et al. 2020). Our results corroborate these observations since we observe that although mitochondrial membrane potential is unaltered, treating control cybrids with 1 ug/µl of LPS results in mitochondrial network fragmentation and cardiolipin exposure. When adding NL to cells either alone or in conjugation with LPS, we observe a tendency for mitochondrial hyperpolarization which might be due to excess dysfunctional mitochondrial accumulation, which results in cardiolipin increased fluorescence. Cardiolipin is a phospholipid present in the IMM that is exposed to the outer mitochondrial membrane (OMM) upon oxidation by ROS, which are generated during mitochondrial dysfunction. Cardiolipin oxidation decreases its binding affinity for cytochrome c, an pro-apoptotic protein, causing it to be released triggering apoptosis signalling (Ott et al. 2007). Since cardiolipin is found only in the inner mitochondrial membrane and in the membranes of most bacteria, this lipid is considered a mitochondrial-derived DAMP recognized by NLRP3 (He et al. 2016). PD cybrids present higher levels of cardiolipin exposition in basal conditions as well as after exposure to NL but not after LPS which indicates that PD cybrids have basal activation of innate immunity pathways that are mainly governed by mitochondrial dysfunction. When LPS and NL are used in conjunction the fluorescence does not increase, which may indicate that PD cells are already engaged innate immune responses that may precede cell death, either by apoptosis or pyroptosis. Pyroptosis consists in the activation of inflammasomes that trigger the activation of caspase 1 which induces the maturation of pro-IL-1β and pro-IL-18 and in turn cleave gasdermin D to induce pore opening causing the rupture of the membrane (Jorgensen et al. 2017).

Although cybrids are neuronal-like cells, which are not commonly described as inflammation-responding cells, we observed that the exacerbation of the defects in

mitochondria will promote the activation of the innate immune responses. Apart from main-stream understanding of inflammatory responses in the brain, we propose that neurons, in this case, neuronal-like cells, are capable of sensing cellular insults and responding by activating innate immunity responses. Our results show that PD cybrids have basal increase levels of TLR3 and NLRP3 and caspase 1 activity.In support of our results that show overexpression of TLR3, evidence from more than a decade ago demonstrated that human neuronal cells express TLR3 and TLR8 and can mount innate immunity responses against double-stranded RNA Rabies virus and single-stranded RNA herpes simplex virus (Préhaud et al. 2005). Future work should be performed in order to address cytokine production in PD cybrids. Previous work from our group and others have shown that neurons also produced chemokines, inflammatory cytokines such as IL-6 and interferon (IFN) that may mediate innate immunity in the absence of glial cells (Kaul et al. 2012; Silva et al. 2020). Previously it was demonstrated that ASC is an essential regulator of the inflammasome (Mariathasan et al. 2004). The NLR proteins such as NLRP3, and the TRIM-family member pyrin do not associate directly with pro-caspase 1 and require the adapter protein ASC to recruit and activate caspase 1 (Agostini et al. 2004). ASC dimers were reported to mediate inflammatory cell death via caspase 1 activation in macrophages (Fernandes-Alnemri et al. 2007). Our results show that ASC dimers are only further recruited in PD cybrids exposed to LPS, in opposition to control cells. This fact may occur because PD cybrids are more prone to respond to inflammation and potentially activate inflammation-dependent cell death pathways in comparison to control cybrids.

The autophagic flux, measured with LC3II and p62, specific for mitotophagy, is already decreased in the PD cells. In control cells p62 levels were significantly decreased upon exposure to LPS and LC3II levels also showed a tendency to decrease.

However, in PD cells LPS does not further decrease the autophagic flux. By not clearing dysfunctional mitochondria that will accumulate, the signal for the activation of innate immunity will continue in PD cybrids, with additional release of cytochrome c, a pro-apoptotic factor (Kim et al. 2007; Ghavami et al. 2014).

We also verified that accumulation of α-syn in PD cybrids in basal conditions, corroborating previous work from our group (Arduíno et al. 2015). α-syn oligomers increase in both PD and control cells when the autophagic flux is stopped by NL. Unfolded α-syn is degraded through chaperone-mediated autophagy (CMA) (Cuervo et al. 2004), however this pathway can't clear aggregates of α-syn since it relies on the identification of binding sites (da Fonseca et al. 2015). Thus when aggregated α-syn has to be cleared by macroautophagy (Winslow et al. 2010; da Fonseca et al. 2015). By inhibiting the autophagy flux α-syn will continue to be produced but it will not be cleared leading to a bigger accumulation.

In this study we show that glial cells are not the only cells capable of activate innate immunity pathways. We also show evidences that dysfunctional mitochondria provide the base for innate immunity activation and, thus, further inflammatory stimulus may not have additional effects. Our results support a major contribution of mitochondrial dysfunction and inflammation in PD progression.

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