EXAMINING THE ROLE OF MITOCHONDRIA IN IMMUNE RESPONSES: INFLAMMATION AND MACROPHAGE POLARIZATION

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ABSTRACT

Mitochondria play a crucial role in immune responses. Mutations in mitochondrial DNA (mtDNA) are associated with dysfunctional mitochondria, causing a series of downstream effects including increased mitochondrial reactive oxygen species (mROS) and release of mitochondria-related toxins such as mitochondrial damage-associated molecular patterns (mtDAMPs). One example is mitochondrial DNA (mtDNA), which results in inflammatory responses. However, the direct relationship between mtDNA mutation and dysfunction in cytokine production has not been characterized. In this study, we focused on elucidating the relationship between complex V disruption, which elicits mitochondrial permeability transition pore (mPTP) opening, and cytokine production. We found a decrease in cytokine expression levels upon blocking mPTP opening. Furthermore, we found that mitochondria dysfunction and subsequent cytokine production are associated with the polarization state of macrophages, the resident immune cells of the body in charge of inflammation and regeneration. In addition, we studied the direct effects of foreign mitochondrial internalization via mitoception on repolarization of macrophages and we observed an increase in M2 marker levels upon addition and internalization of donor mitochondria to recipient macrophages, supporting a direct effect of mitochondria on macrophage polarization.

BIOGRAPHICAL SKETCH

Jiye was born in Seoul, Korea. She received her Bachelor of Science in Food and Nutrition from Yonsei University in 2020. At Yonsei, she focused on the molecular and biochemical side of nutrition, doing two undergraduate internships at the Molecular Nutrition & Physiology Lab of Korea University and the Molecular & Cellular Nutrition Lab of Yonsei University. She then joined the Division of Nutritional Sciences at Cornell University as a Master's student in the fall of 2020, where she investigated the role of mitochondria in immune responses.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
αKG	α-ketoglutarate
ALS	Amyotrophic Lateral Sclerosis
AMT	Artificial mitochondria transfer
ATP	Adenosine Triphosphate
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CSA	Cyclosporine A
DAMPs	Damage-associated molecular pattern molecules
DRP1	Dynamin-related protein 1
ETC	Electron transport chain
ER	Endoplasmic reticulum
EV	Extracellular vesicles
HD	Huntington's disease
IFN	Interferon
LPS	Lipopolysaccharide
MAVS	Mitochondrial antiviral signaling protein
mHTT	Mutant huntingtin
MPT	Mitochondrial permeability transition
mPTP	mitochondrial permeability transition pore
mt	Mitochondria
mtDAMP	Mitochondrial damage-associated molecular pattern molecules
mtDNA	Mitochondrial DNA
MFN1	Mitofusin 1
mtROS	Mitochondrial reactive oxygen species
nDNA	Nuclear DNA
NF- ϰB	Nuclear factor-kB
NO	Nitric oxide
OMM	Outer mitochondrial membrane

PAMPs	pathogen-associated molecular patterns
PET	Positron emission tomography
PRR	pattern-recognition receptors
RIG-1	Retinoic acid-inducible gene I
ROS	Reactive oxygen species
TGF-β	Transforming growth factor-β
TLRs	Toll like receptors
UCP2	Uncoupling protein 2

CHAPTER 1

INTRODUCTION

Mitochondria in Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder which is hallmarked by the expansion of CAG repeats in the *huntingtin* (HTT) gene. Mutant HTT (mHTT) leads to protein aggregates that cause neurotoxicity. mHTT has the potential to cause mitochondrial dysfunction through disturbing membrane potential, cellular respiration, or direct interaction with the organelle itself (Federico et al., 2012). In HD patients' brains, reduced respiratory chain activity, especially that of complex II has been observed (Kwong et al., 2006). mHTT has been observed to increase the activity of Dynamin-related protein 1 (DRP1), a mitochondrial fission protein, causing increased mitochondrial fragmentation leading to a degeneration of synapses (U. Shirendeb et al., 2011; U. P. Shirendeb et al., 2012). mHTT induced OXPHOS dysfunction and decreased mitochondrial biogenesis observed in HD mouse models further reinforce mitochondrial involvement in the HD pathogenesis (Huanzheng Li et al., 2019).

Mitochondrial DNA (mtDNA) mutations have been shown to have associations with multiple diseases. Regardless of the mechanism of pathogenesis, the final phenotypes that are shared in mitochondrial disorders are failure of mitochondrial function due to impaired respiratory chain activity. Many studies have shown that increased oxidative stress and cellular apoptosis are linked to pathogenesis and progression of neurodegenerative disorders like Alzheimer's disease, Huntington's disease, and Amyotrophic Lateral Sclerosis (ALS) (Federico et al., 2012). Decreased amount of mitochondrial DNA has also been observed in human HD cell cultures (Siddiqui et al.,

2012). mtDNA is highly susceptible to reactive oxygen species (ROS) damage, and damaged mtDNA is associated with respiratory chain activity-related pathogenesis in HD (Huanzheng Li et al., 2019). Impairment of mitochondrial function leads to increased production of ROS, which plays a crucial role in cell death; strong evidence suggests that mitochondrial dysfunction plays an important role in the HD pathogenesis (Browne, 2008; Browne & Beal, 2004; Damiano et al., 2010; Reddy et al., 2009). Significant reductions in the expression of subunits of the electron transport chain (ETC) have been reported to be affected, especially regarding mitochondrial complexes I, II, III, and IV activities (Brennan et al., 1985; Damiano et al., 2010; Fukui & Moraes, 2007; Howell, 1997; Jun et al., 1994; Mann et al., 1990; Parker et al., 1990).

Immune Response Mediated by Mitochondria

Impaired immune cell function is considered the basis for diverse chronic diseases, and changes in cell metabolism can be the main cause underlying this impairment. Mitochondria are central to cellular energy production and also play a significant role in the regulation of activation, differentiation, transcription, and survival of immune cells through the release of signals such as mtDNA or mitochondria-derived reactive oxygen species (mtROS) (Angajala et al., 2018). Four major means of mitochondrial involvement in immune cell function have been studied. The first is altering of metabolic pathways, with M1 and M2 state macrophages as an example, where M1 macrophages have been observed to have an impaired TCA cycle function and mainly rely on glycolysis for energy metabolism while M2 has a normal TCA cycle function, using the OXPHOS chain as the main source of energy production (Angajala et al., 2018). The second is by activating inflammatory responses. The third is the influence of mitochondrial fission and fusion on immune functions (Angajala et al., 2018). Lastly, the junction signaling between mitochondria and

endoplasmic reticulum (ER) influences the metabolism of immune cells (Angajala et al., 2018). Precise control of the metabolic pathways and inflammatory responses are the main topics of this work. Therefore, we will focus on the first two roles of mitochondria in immune cell regulation.

Mitochondria in altering immune cell metabolic pathways

Observations show that OXPHOS affects the activity of immune cells in macrophages. Macrophages can be categorized into two states, M1 (classically activated) and M2 (alternatively activated). The phenotypes of each state are controlled by the cytokine production of other immune cells. M1 macrophages produce nitric oxide (NO) and can be activated by lipopolysaccharide (LPS) or IFN-γ produced by Th1 cells. The M2 state is activated by IL-4 or IL-13 (He & Carter, 2015; Wynn et al., 2013). Mitochondrial uncoupling protein 2 (UCP2) is a factor known to be involved in macrophage polarization and is localized in the inner mitochondrial membrane. Increasing evidence points to UCP2 controlling mtROS and influencing the polarization of macrophages. UCP2 exhibits decreased expression in M1 macrophages, and blocking UCP2 leads to decreased IL-4 induced activation of M2 macrophages (De Simone et al., 2015). α-ketoglutarate (αKG) which is an intermediate product of the TCA cycle is another important factor in M2 macrophage activation. Low αKG /succinate ratio which indicates a decreased glutamine metabolism, increases M1 phenotypes and inhibition of glutamine-synthetase leads to skewing of M2 macrophages toward M1 phenotypes (P. S. Liu et al., 2017; Palmieri et al., 2017).

Mitochondria in inflammatory responses

Mitochondria have been shown to participate in a diverse range of innate immune pathways as signaling platforms, playing the role of central hubs in the innate immune system (West et al.,

2011). Initial recognition of microorganisms following infection is performed by pattern-recognition receptors (PRRs). PRRs can sense pathogen-associated molecular patterns (PAMPs) and the PRR ligation initiates multiple signaling pathways that culminate to activate nuclear factor-κB (NF- κB), which controls the expression of type I interferons (IFNs), pro-inflammatory cytokines, and chemokines (Hayden et al., 2006; Takeuchi & Akira, 2009; West et al., 2006). Recent evidence suggests that mitochondria participate in RIG-1-like receptor (RLR) signaling, sterile inflammation, and anti-bacterial immunity (West et al., 2011). Mitochondrial antiviral signaling protein (MAVS) is associated with the outer mitochondrial membrane (OMM) and plays an essential role in the activation of downstream NF- κB and IRF signaling pathways for type I IFN and pro-inflammatory cytokine production (Kawai et al., 2005; Meylan et al., 2005; Potter et al., 2008; Seth et al., 2005). During infection, as mitofusin 1 (MFN1) and MFN2 induce mitochondrial network fusion, a retinoic acid-inducible gene I (RIG-I) and MAVS-enriched mitochondria are recruited to the epicenter of viral replication to enhance MAVS signaling (West et al., 2011).

Huntington's disease and neuronal cells

Increasing studies have observed neuronal damage in neuroinflammatory diseases of the brain. Acute neuroinflammation occurs in meningitis and meningoencephalitis, and similar observations have been made in chronic autoimmune disorders of the brain as well (Aktas et al., 2007). Such inflammatory changes have been identified as key players in noninflammatory CNS disorders such as Alzheimer's disease and Huntington's Disease, which exhibit signs of immunological activation (Aktas et al., 2007). In the HD-affected brain, accumulation of reactive microglia in the direct vicinity of HD-positive pyramidal neurons has been observed (Aktas et al.,

2007). This abnormal microglia has been observed to be exacerbated in an age-dependent manner throughout the HD progression (Yang et al., 2017).

Microglial cells and inflammation

Microglial cells, present throughout the central nervous system (CNS) are the main cell type of the innate immune system of the CNS. Like macrophages, they too express toll-like receptors (TLRs), respond to the ligands and produce pro-inflammatory reactions (Rivest, 2003, 2006). Microglia, along with small neurons, astrocytes, and macrophages, are primarily observed in high density around circumventricular organs and highly vascularized regions of the CNS and show rapid response to circulating pathogens (Rivest, 2009). Such activation of microglia does not only occur due to pathogenic organisms but also during brain injury and chronic disease, with a decreased ability of microglial cells to clear toxic proteins as a potential mechanism behind neurodegenerative disease (Rivest, 2009). Regarding the mechanistic details behind the microglia-mediated inflammatory response, it has been suggested that activation of TLRs on macrophages and microglial cells in the circumventricular organs initiates TNF synthesis, which then activates NF- ϰB in microglial cells in the vicinity resulting in the transcription of pro-inflammatory genes (Nadeau & Rivest, 2000).

Activation of microglial cells in HD

Elevated levels of pro-inflammatory cytokines have been observed in both HD brains and plasma (Politis et al., 2015). Increased astrogliosis and microgliosis which are defense mechanisms to minimize damage after injuries in the CNS in post-mortem brains of HD patients along with the accumulation of microglia in HD brains were also observed (Sapp et al., 2001; Singhrao et al.,

1999). In vivo positron emission tomography (PET) demonstrated significant microglial activation in HD regions of the brain with increasing severity of pathology in severe HD cases and detected activation of microglia before the manifestation of HD. Taken together, these findings emphasize the crucial role of microglial activation crucial role in the pathogenesis of HD (Pavese et al., 2006; Politis et al., 2015; Tai et al., 2007). mHTT aggregates preferentially formed along with neuronal processes and axonal terminals which transformed microglia into activated states resulting in neurotoxicity due to the increased capacity of releasing toxins in the over-activated microglia (Hanisch & Kettenmann, 2007; He Li et al., 2000; Zhao et al., 2016). Expression of mHTT in microglia was also found to promote cell-autonomous pro-inflammatory expression of genes and showed enhanced neurotoxicity (Crotti & Glass, 2015; Yang et al., 2017).

Microglia and Macrophage in Brain Injury

Microglia are the resident immune cells of the CNS and share many similarities with macrophages. Along with their peripheral counterparts, microglia too, express TLRs (Yang et al., 2017). Microglia and macrophages act as some of the most potent modulators of the CNS regeneration and damage repair (Hanisch & Kettenmann, 2007). Microglia too can be activated into two phases (M1 and M2) which also are pro-inflammatory and anti-inflammatory, respectively. In normal conditions, the inactivated microglia show small cell bodies (Hanisch & Kettenmann, 2007). However, with adverse stimuli, they exhibit swollen shapes (Möller, 2010). Studies have shown that M1 microglia can be transformed into M2 microglia with cyclic adenosine monophosphate (cAMP) playing a crucial role in the process (Ghosh et al., 2016). Such transition has been observed to initiate remyelination in multiple sclerosis (MS) showing neuroprotective properties (Miron et al., 2013). Although the dichotomic view on classifying microglia and

macrophages into M1 and M2 states is now being considered overly simplified, it remains to be a useful concept in comprehending the functional roles of microglia and macrophages in brain-related pathogenesis.

Microglial functions in brain repair

The identified roles of microglia in brain repair are very diverse, but there are 5 major functions: neurogenesis, axonal regeneration, synaptic plasticity, white matter integrity, and angiogenesis and vascular repair (Hu et al., 2015). Similar to M2 state macrophages, M2 state microglia promote basal neurogenesis via the production of specific trophic factors which enhances neuroblast migration, the proliferation of neural precursor cells, and functional integration of nascent neurons into the existing circuitry (Butovsky et al., 2006; Choi et al., 2008; Nikolakopoulou et al., 2013; Yan et al., 2009). Microglia also play an important role in regulating synaptic plasticity. It has been observed that M1 microglia exhibit an adaptive role in synaptic plasticity regulation, yet more research is required to better understand the mechanism behind it (Hu et al., 2015). Regarding white matter integrity, a switch from M1 to M2 predominant phenotypes during initiation of remyelination of MS models has been observed (Miron et al., 2013).

Macrophage functions in brain repair

Macrophages are prominent cells in wounds displaying a multitude of functions from host defense, promotion and resolution of inflammation, and tissue restoration. Unlike microglia, macrophages exist beyond the brain. They are activated by damage-associated molecular pattern molecules (DAMPs) and pro-inflammatory mediators released due to injury (Zhang & Mosser, 2008). The function differs depending on the polarized state of macrophages. M2 macrophages

play a crucial role in axonal regeneration while M1 macrophages inhibit the growth of the neurite (Kitayama et al., 2011; Shechter et al., 2009). Proangiogenic factors have been shown to be produced by M2 macrophages which promote the vascular repair (Willenborg et al., 2012). It has been suggested by several studies that phenotypic alterations in macrophages play an essential role in the pathogenesis of wounds (Sindrilaru et al., 2011; Zamboni et al., 2006).

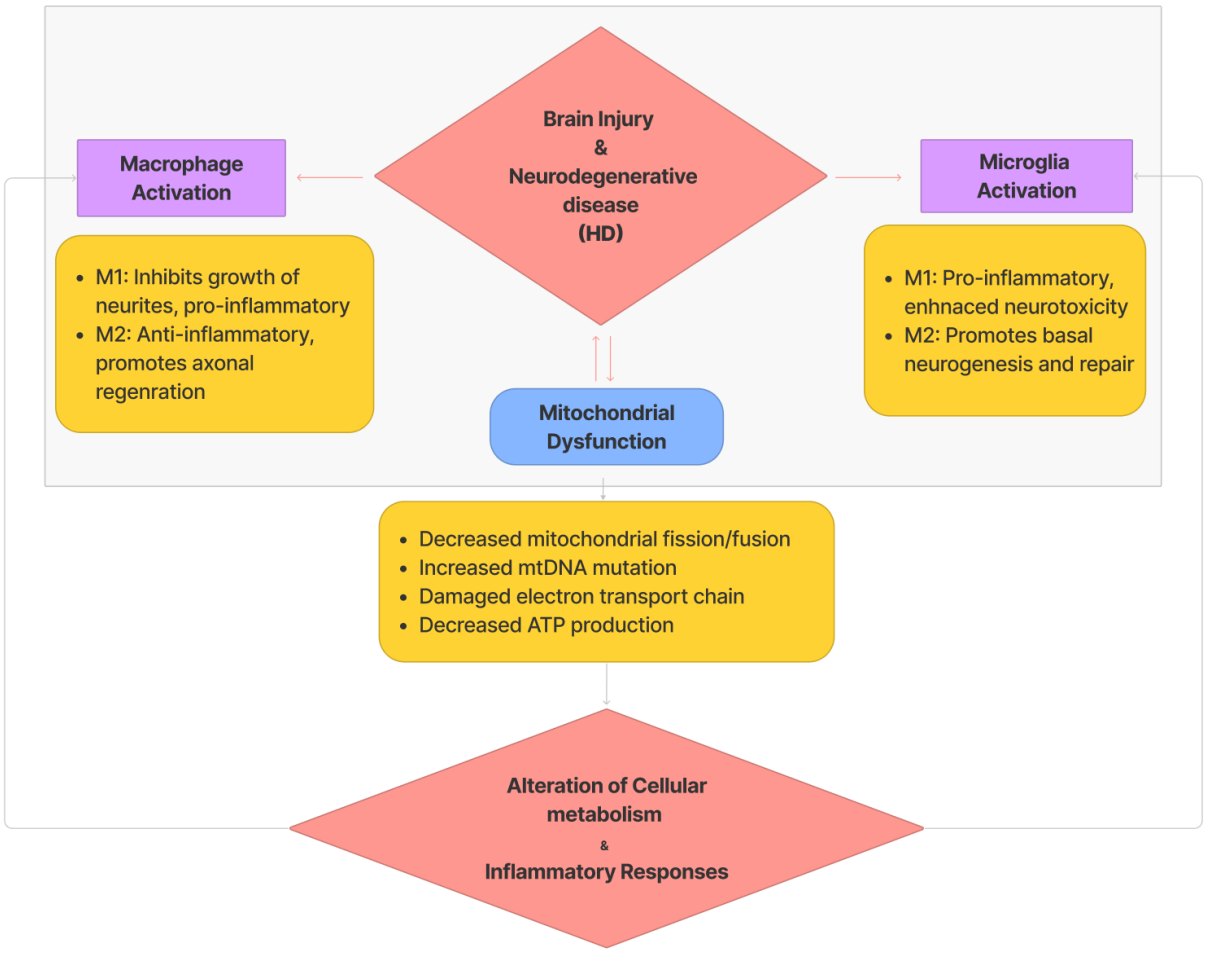

Figure 1. Relationships among Huntington's disease, microglia, macrophage, and mitochondria.

M1 and M2 refer to macrophage states.

Conclusion

The importance of mitochondrial dysfunction in HD pathogenesis and inflammation is evident. While the introduction mainly focuses on HD of the numerous neurodegenerative diseases, it shows sufficient importance of mitochondria's role in not just HD but the pathogenesis of neuronal and inflammatory diseases holistically (Fig. 1). However, despite the sufficient evidence pointing to the importance of mitochondria's role in not just HD but the pathogenesis of neuronal

and inflammatory diseases holistically, not much is known about the mechanism behind it. Therefore, we have decided to focus on the specific roles of mitochondria in inflammatory responses in neurons and macrophages in the following two chapters.

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CHAPTER 2

OLIGOMYCIN TREATMENT ON N2A CELLS CAUSES CYTOKINE PRODUCTION VIA MPTP OPENING

Abstract

Oligomycin leads to increased cytokine responses via inhibition of mitochondrial complex V. Regarding increased cytokine responses, mitochondrial permeability transition pore (mPTP) opening has also been shown to also be involved, with research showing that opening of mPTP elicits decreased ATP production and increased cytokine production. In this study, we examined the effects of mPTP opening on cytokine production following oligomycin treatment in the N2a neuronal cell line. We demonstrated a significant decrease in the expression levels of cytokines upon the mPTP opening inhibitor. We also have confirmed that the cGAS-STING inflammatory pathway (Guo et al., 2020) was not involved in our observations by showing that treatment of cGAS-STING inhibitor C-176 had no significant effects on oligomycin induced cytokine expression.

Introduction

The majority of cellular energy is produced in the form of ATP by mitochondria, which are organelles that play a crucial role in aerobic respiration through the oxidative phosphorylation system (OXPHOS). The mitochondrial and nuclear genomes both exert control over the mitochondrial OXPHOS system (Tuppen et al., 2010). The mitochondrial genome consists of 37 genes that encode 13 proteins, 22 transfer RNAs, and 2 ribosomal RNAs. The 13 mitochondrial-encoded proteins constitute the core subunits of the OXPHOS system's enzyme complexes (I, III, IV, and V) while the nuclear genome encodes genes necessary for mitochondrial DNA maintenance and function (Anderson et al., 1981). Mutations within either the nuclear DNA (nDNA) or mtDNA may lead to respiratory chain deficiency.

Mitochondria have been known to affect diverse pathologies with the damage to the membrane putting energy production at risk and thereby jeopardizing cell fate (Garbaisz et al., 2014). Mitochondrial permeability transition (MPT) which is the mechanism behind mitochondrial membrane depolarization is induced by the mitochondrial permeability transition pore (mPTP) opening (Zhong et al., 2007). mPTP is a non-specific inner mitochondrial membrane channel and the opening can be induced via overload of mitochondrial matrix calcium (Mukherjee et al., 2016). Once the mPTP is open, the mitochondrial electron transport chain is interrupted, deterring mitochondrial energy production and causing the production of reactive oxygen species (ROS), which leads to further decline of the membrane potential and a consequential further opening of the mPTPs (Duina et al., 1996; Springer et al., 2018).

ATP synthase, also known as complex V consists of the F1 domain located in the mitochondrial matrix and F0 which is situated in the inner mitochondrial membrane (Jonckheere et al., 2012). It phosphorylates ADP to ATP using the energy created by the proton electrochemical gradient (Jonckheere et al., 2012). ATP created through this pathway is the main source of energy in intracellular metabolic pathways (Schapira, 2006). Being the fifth multi-subunit of the OXPHOS, deficiencies or mutations in ATP synthase often give rise to pathologies.

Oligomycin is widely known as the inhibitor of mitochondrial complex V, and multiple studies have shown that oligomycin treatment leads to increased cytokine responses via inhibition of the mitochondrial complex V (Vaamonde-García et al., 2012). Regarding increased cytokine responses, mitochondrial permeability transition pore (mPTP) opening has also been shown to be involved, with research showing that opening of mPTP elicits decreased ATP production and increased cytokine production (Mukherjee et al., 2016). Yet, a research gap exists due to the lack of studies regarding the role of mPTP opening in cytokine production upon oligomycin treatment. Knowing that a large portion of current research focuses on mitochondrial damage-associated molecular patterns (mtDAMPs) concerning mitochondrial dysfunction-related inflammatory responses, examining the association between oligomycin treatment-induced cytokine release and mPTP opening may highlight a new mechanism that better explains the role of mitochondria in inflammation. With our interest in the role of mitochondrial DNA mutation on Huntington's disease and other neurodegenerative diseases, we decided upon the murine neuronal cell line N2a to test the effects of oligomycin on mPTP opening and consequential cytokine production. We hypothesized that mutation in complex V via oligomycin treatment leads to cytokine responses via mPTP opening.

Methods

Cell line

Mouse neuroblastoma cell line N2a cells were used. They were obtained from the Hu lab of Cornell University BMCB. They were thawed in media that consisted of 98% DMEM, 1% FBS, and 1% Pen Strep in a 10 cm plate, incubated at 37°C until they reached 80-90% confluency.

Hoechst/PI staining

Cell viability was checked via Hoechst/PI staining. The media was removed from the incubated cells that were treated with respective treatments. Hoechst and PI dyes were mixed into the medium at 0.5 μL /1 mL and 2 μL /1 mL concentrations. The cells were treated with the mixture and then incubated at 37°C for 15 minutes. Following incubation, the mixture was aspirated, a clean medium was added then the cells were imaged using Celigo for viability. The total cell count was based on the brightfield with red fluorescence accounting for dead cells and blue fluorescence for the live.

Inhibiting Mitochondrial Complex V

Inhibition of the mitochondrial complex V was conducted by treating the cells with 10 μM oligomycin (VWR International, 76303-196). DMSO (Fisher Scientific, BP2311) was used to dilute oligomycin to the desired concentration. Then 10 μM of oligomycin was treated to each of the wells with 0.01% DMSO as the negative control and 1 μM of LPS as the positive control. N2a cells showed high sensitivity to DMSO so the total concentration of DMSO in the medium was

kept at 0.01%. A 6 well plate (Corning) was used and seeded at the density of $4x10^5$ then were incubated at 37°C for 24 hrs before being collected by TriZol (Life sciences) for RNA extraction.

Inhibiting mPTP opening

To inhibit mPTP opening, cells were simultaneously treated with oligomycin 10 μM and cyclosporine A (CSA) 1 μM, seeded in a 6 well plate at the density of $4x10^5$ then were incubated at 37°C for 24 hrs pre-collection.

RNA isolation and cDNA synthesis

Total RNA was isolated using TriZol (Life sciences). Frozen samples were thawed on ice. 200 μL of chloroform was added per 1 mL of the TriZol sample. The mixture was then vortexed and incubated at room temperature (RT) for 5 minutes. Post RT incubation, the samples were centrifuged at 15000 RPM at 4°C for 15 minutes. The supernatants of each sample were collected and transferred to RNAase-free tubes to which equal amounts of isopropanol to the sample were added, inverted 6 times, then were incubated at RT for 15 minutes. The samples then were centrifuged at 15000 RPM for 30 minutes, and the pellets were obtained. 1mL of 75% ethanol was added per 1mL of TriZol, the samples were vortexed then were centrifuged at 15000 RPM for 15 minutes. This step was repeated twice, the supernatant was removed, and the pellet was dried to which 20 μL of dH_2O was added. After isolation, RNA concentration was determined using Nanodrop spectrophotometer (Thermo Fisher Scientific) and the relative purity of the extracted RNA was assessed via A260/280 ratio. The RNA was then reverse transcribed with a qScript™ cDNA Synthesis Kit (Quantabio).

Quantitative RT-qPCR and Data Analysis

Relative mRNA expression was determined by RT-qPCR using LightCycler® 480 SYBR Green I Master Mix on a LightCyler® 480 instrument (Roche). All reactions were performed in 6 μL volumes, including 30 ng of cDNA and 0.3 μL of forward and reverse primers each at the concentration of 10 μM specific to the mRNA of *TNF-α and IL-6*. Amplification was performed with a 7-minute denaturation step at 95°C, followed by 45 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 10 sec. The specificity of the PCR products was confirmed through the dissociation curve analysis performed using the default settings of the software. The $2^{-\Delta\Delta Ct}$ method was used to analyze data for each target gene. Oligonucleotide primers for *TNF-α*, *IL-6,* and *β-actin* were designed using NCBI primer BLAST and obtained from Integrated DNA Technologies (IDT).

Statistical Analysis

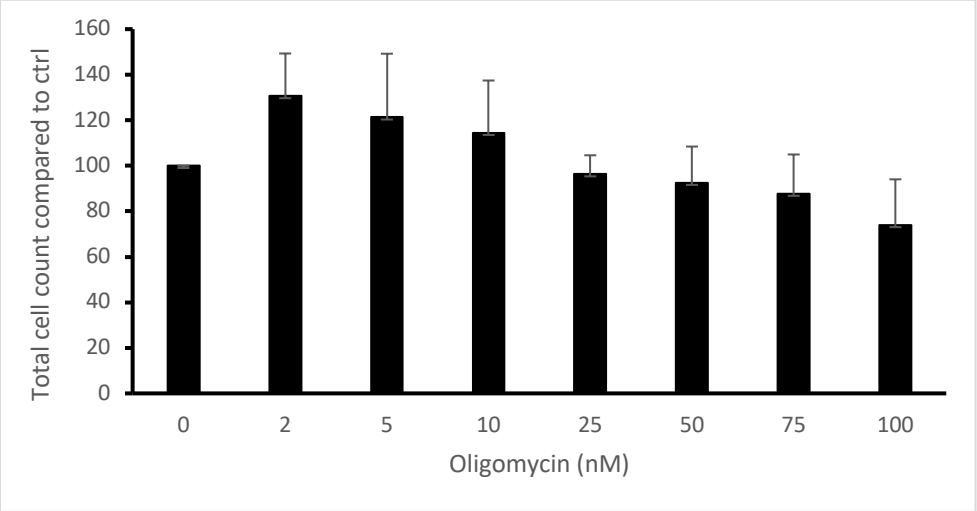
Statistical significance was determined via Graphpad Prism (ver. 6), obtaining a two-tailed p-value by conducting a student's T-test.

Results

Determination of Oligomycin concentration

The optimal concentration of oligomycin was determined by treating N2a cells with different concentrations of oligomycin and incubating them for 4 hrs and 24 hrs. Based on the literature search and Celigo imaging results, 10 nM of oligomycin was used as the final concentration because it had a less than 1% effect on cell viability upon treatment (Fig. 1).

A



B

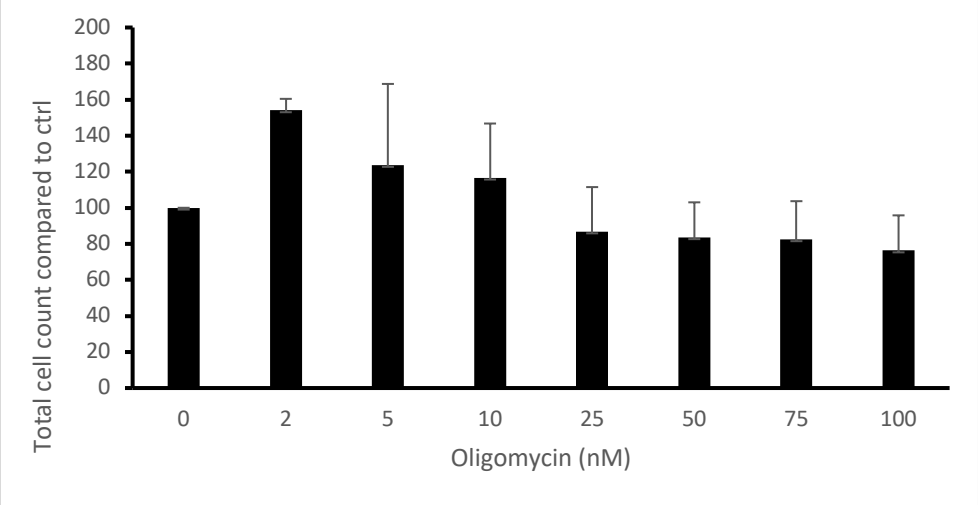


Figure 1. The effect of oligomycin concentration on N2a cell viability.

N2a cells were treated with oligomycin and incubated for 4 hrs (A) and 24 hrs (B). The bars show the mean of total cell count compared to that of the control and the error bars represent +1 STD for n = 3.

Oligomycin causes an increase in specific cytokine production

N2a cells were treated with DMSO as the negative control, LPS as the positive control, and oligomycin as the treatment. Compared to the DMSO-treated cells, oligomycin treated cells showed more than a two-fold increase compared to the negative control with respect to both *TNF-α* and *IL-6* expression levels (Fig. 2).

mPTP opening inhibitor CSA treatment decreases cytokine production

Upon confirmation of the effects of oligomycin treatment on cytokines, an additional treatment group was added. When N2a cells were treated with both oligomycin and 1 μM CSA (mPTP opening inhibitor), the production of *IL-6* decreased significantly to roughly the same level as the control (Fig. 2A). CSA treatment had no effect on *TNF-α* levels (Fig. 2B).

A B

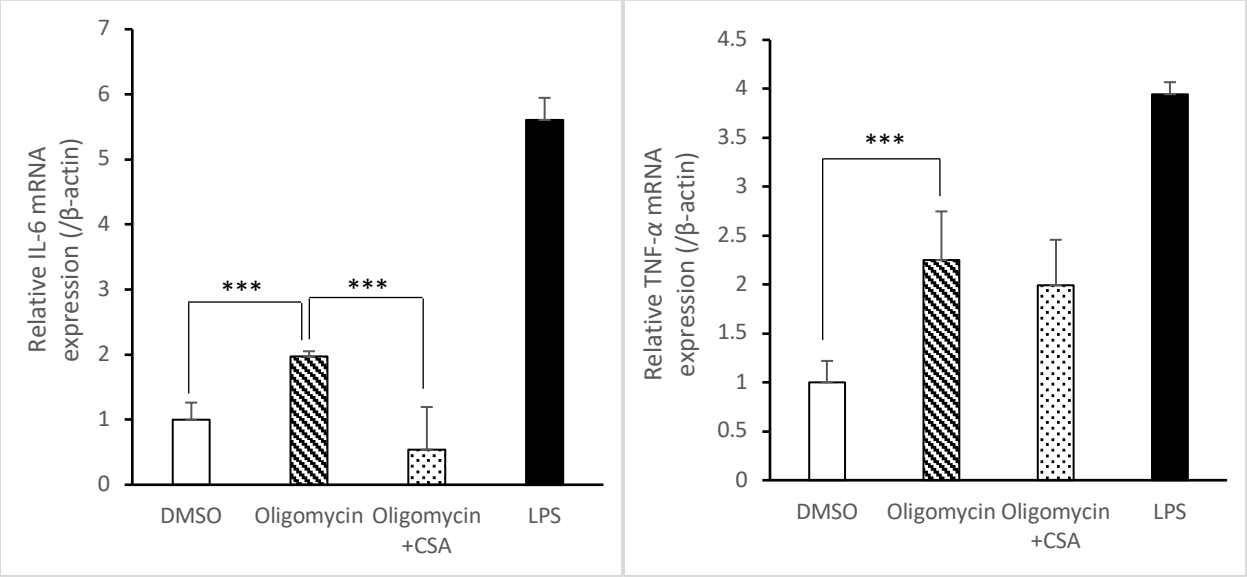


Figure 2. Effect of Oligomycin, LPS, and Oligomycin plus CSA on the expression of cytokines. RT-qPCR of *TNF-α* (A) and *IL-6* (B) expression in murine N2a cells upon treatment with DMSO (negative control), Oligomycin, Oligomycin + CSA, and LPS (positive control). The cells were incubated for 24 hrs post-treatment with oligomycin 10 μM, oligomycin 10 μM and CSA 1 μM, or LPS 1 μM. Fold changes are shown relative to the β-actin expression levels. Error bars represent +1 STD for n = 9. (*: p-value ≤ 0.05, **: p-value ≤ 0.01, ***: p-value ≤ 0.001).

Involvement of cGAS-STING pathway in oligomycin mediated cytokine production

To understand the involvement of the cGAS-STING pathway in oligomycin-mediated cytokine production, the cGAS-STING inhibitor C-176 was administered at the concentration of 1 μM. It did not lead to decreased cytokine production, but rather increased the levels of both *IL-6* and *TNF-α* production. The mRNA expression levels of both *IL-6* and *TNF-α* were increased upon treatment with oligomycin and C-176 (Fig. 3 A, B). However, the result might be due to the

increased DMSO administration to the cells which were 0.01% in the oligomycin treated samples but twice that in the oligomycin + C176 treated samples (Fig. 3).

A B

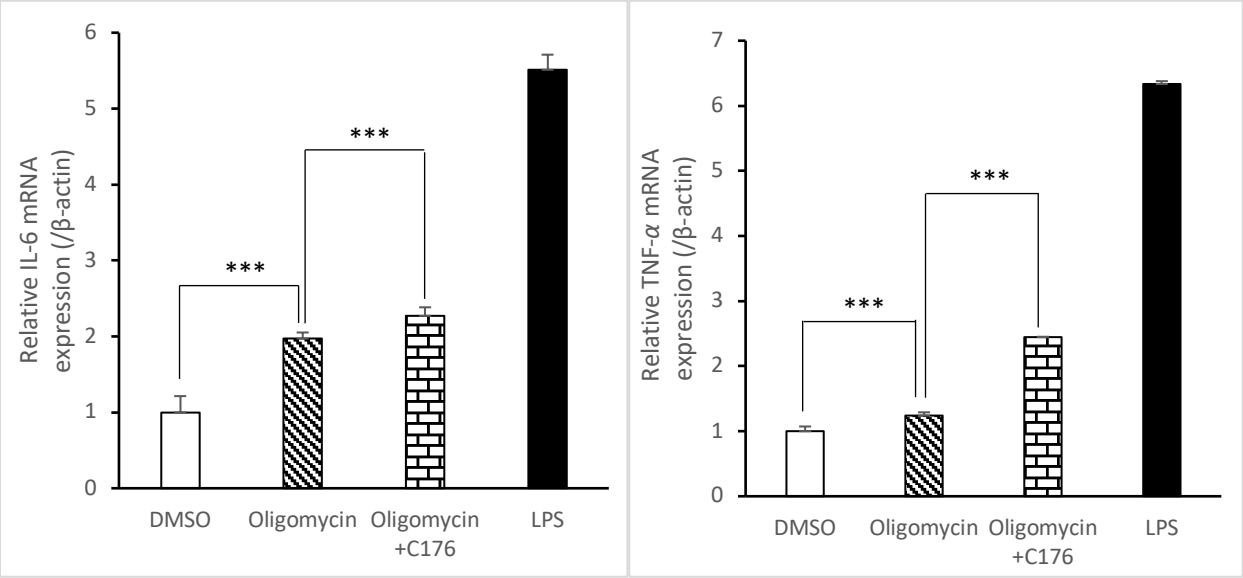


Figure 3. Effect of cGAS-STING inhibitor C176 on cytokine production.

RT-qPCR of *IL-6* (A) and *TNF-α* (B) expression in murine N2a cells upon treatment with DMSO (negative control), Oligomycin, Oligomycin + C176, and LPS (positive control). The cells were incubated for 24 hrs post treatment with oligomycin 10 μM, oligomycin 10 μM and C176 1 μM, or LPS 1 μM. Fold changes are shown relative to the β-actin expression levels. Error bars represent +1 STD for n = 9. (***: p-value ≤ 0.001)

Discussion

In the current study, we examined the role of mPTP opening in ATP synthase dysfunction-induced cytokine production. While a large number of studies look at cytokines as a cause of mPTP opening, we chose to examine this in a different way by assessing the effect of mPTP opening on

cytokine expression. With our novelty lying in our focus on mPTP opening as a reason behind oligomycin-induced cytokine production, we have demonstrated that upon mPTP opening inhibitor treatment, the effect of oligomycin on the expression level of *IL-6* has completely disappeared. This is consistent with the observations that have been made in studies testing the effects of CSA on cytokine production that also showed decreased expression of *IL-6* (Fellman et al., n.d.; Hamada et al., n.d.; Melgar et al., n.d.). We also showed that the production of cytokine observed in the experiments was from activation of NF- ϰB pathway, not cGAS-STING the other major mitochondrial dysfunction associated inflammatory pathway (Guo et al., 2020). The results of our experiments show no significant decrease in cytokine expression levels upon C-176 treatment which is consistent with the findings of previous studies (Han et al., 2021) and rather showed an increase in cytokine expression levels upon treatment with oligomycin and C-176 (Fig. 3). As a result, it was found that oligomycin-induced cytokine production was related to mPTP opening but not to the cGAS-STING inflammatory pathway. These observations provide support to our hypothesis that mPTP opening may play a role in complex V dysfunction-associated inflammatory responses via activation of the NF- ϰB instead of cGAS-STING. This implies that factors other than frequently observed free cytosolic mtDNA underlie the observed inflammation. Such findings could be translated into a novel approach to resolving inflammation by setting mPTP as the main target.

This study has some limitations. First, mPTP opening could have been measured upon oligomycin treatment and CSA treatment using a mitochondrial permeability transition pore detecting kit to reinforce mPTP's involvement in the decreased cytokine production. Additionally, an increased variety of cytokines could have been assayed for expression to suggest which

downstream pathways are involved. The release of cytosolic mtDNA could also have been assayed to clarify the mechanism behind the observed inflammatory reactions. Mutant cells with reduced mPTP expression could have also been used to verify the involvement of mPTP opening in the increased cytokine expression. In addition, the increased cytokine expression upon C176 treatment combined with oligomycin in Figure 3 could potentially be caused by an increase in the DMSO content in the cell culture. However, we did not further examine this observation because we concluded that cGAS-STING was not involved in the observed oligomycin-induced cytokine increase and thus decided to proceed with CSA as the treatment instead. Additionally, the level of *TNF-α* expression fluctuates for Figures 2 and 3 upon oligomycin treatment and we believe this is due to very low levels of *TNF-α* expression within the cell lines due to the nature of N2a cell lines not being very responsive when it comes to inflammatory responses since cGAS-STING and TLR9 are not expressed in this cell line.

Supplementary Material

Gene (mouse)	Forward primer	Reverser primer
β-actin	ATTGCCGACAGGATGCAGAA	GCTGATCCACATCTGCTGGAA
TNF-α	ATGGCCTCCCTCTCATCAGT	TTTGCTACGACGTGGGCTAC
IL-6	CCCCAATTTCCAATGCTCTCC	CGCACTAGGTTTGCCGAGTA

Table S1. RT-qPCR primers for N2a cells.

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CHAPTER 3

INTERNALIZATION OF MITOCHONDRIA VIA MITOCEPTION LEADS TO REPOLARIZATION OF M1 MACROPHAGES TO M2 STATE

Abstract

Many studies have examined the role of mitochondria in macrophage polarization. One of the major differences between M1 and M2 state macrophages is the energy synthesis mechanism, which cannot be understood without considering mitochondrial function. We examined the role of direct mitochondria addition in macrophage repolarization using mitoception as the method to deliver extracted mitochondria from HEK293 cells to recipient THP-1 cells. Considering the major differences in ATP synthesis mechanisms in M1 and M2 we believe that identifying the specific role and the importance of mitochondria in macrophage polarization will bring about novel methods of inducing anti-inflammatory responses and potential interventions for diverse diseases. In this study, we observed that mitoception of donor mitochondria into M1 state differentiated human THP-1 cells lead to an increased expression of an M2 marker, CD206.

Introduction

Macrophages play an important role in both innate and acquired immune responses by participating in antigen recognition, capture, clearance, and transport (S. Gordon, 1998). Being one of the first cells to interact with abnormal or foreign cells and their byproducts, macrophages secrete various signaling products such as chemokines, which differ depending on the type of receptors used and state and stage of differentiation (S. Gordon, 1998). Their phagocytic capacity, which is established by their polarization state, can determine whether they eliminate pathogens or present antigens to T and lymphocytes (S. Gordon, 1998).

Macrophages show high plasticity and can be polarized into different states depending on how they have been activated. The state of polarization is not fixed and is inseparable from inflammation (Murray, 2017). Interferon (IFN)- γ, which is produced by lymphocytes, has been identified to activate macrophages into classically activated macrophages (CAM, or M1) which are pro-inflammatory, have a stronger antigen-presenting capacity and phagocytic activities, and secrete more pro-inflammatory cytokines (Y. C. Liu et al., 2014; Nathan et al., 1983). In comparison, interleukin (IL)-4 and diverse stimuli such as lipopolysaccharide (LPS) and transforming growth factor- β (TGF- β) have been found to have the ability to alternatively activate macrophages into M2-like phenotypes which exhibit anti-inflammatory reactions and participate in tissue remodeling (Biswas & Mantovani, 2010; Siamon Gordon & Martinez, 2010; Y. C. Liu et al., 2014). One of the major phenotypic determinants between the two polarization states is the energy metabolism mechanisms of each state. While M1 macrophages are glycolytic, M2 macrophages require mitochondrial oxidative phosphorylation for development and activation (Huang et al., 2014; O'Neill & Pearce, 2016).

Many studies have shown the fundamental roles of macrophages in the immune system, especially regarding the regulation of macrophages' response to infections, inflammation, and tissue damage (Miller et al., 2018). Studies have shown that mitochondrial metabolism could be associated with macrophage polarization. In one study, the activation of M1 macrophages inhibits mitochondrial OXPHOS, preventing the repolarization of macrophages into the more anti-inflammatory M2 phenotype (Van den Bossche et al., 2016). It has also been shown that mitophagy leading to mitochondrial clearance plays a crucial role in macrophage activation, and evidence suggests that mitophagy is activated during the process of polarization of macrophages into the M1 state (Ramond et al., 2019).

However, a gap in research remains due to the lack of investigation on the role of direct mitochondria addition in macrophage repolarization. Also, while there are multiple ways of artificial mitochondria transfer (AMT) such as direct injection, co-incubation, and utilization of extracellular vesicles (EV)s, we saw the need to incorporate a more efficient method to introduce extracted mitochondria into recipient cells (Caicedo et al., 2017). Thus, we aim to use mitoception to introduce foreign mitochondria into the recipient cells to enhance internalization efficiency. Considering the major differences in ATP synthesis mechanisms in M1 and M2 we believe that identifying the specific role and the importance of mitochondria in macrophage polarization state will bring about possibilities of novel methods of inducing anti-inflammatory responses and potential intervention methods for diverse diseases. Based on this, we hypothesized that mitochondria play a direct role in macrophage repolarization.

Methods

Cell line

Human HEK293, THP-1, and murine RAW 264.7, NIH3T3 cells were used. All the cells were obtained from Cornell University. Human HEK293 and murine NIH3T3 cells were thawed in media that consisted of 98% DMEM, 1% FBS, and 1% Pen Strep in a 10cm plate, incubated at 37°C until they reached 80-90% confluency. Human THP-1 cells were thawed in media that consisted of rpmi 1640, 10% premium FBS, 1% Pen Strep, 1mM sodium pyruvate, 0.5 mM 2-ME, and 10 μM of hepes in a 10cm plate, incubated in 37°C until they reached 80-90% confluency. Murine RAW264.7 cells were thawed in media that consisted of DMEM, 10% FBS, 1% Pen Strep, hepes, and L-glutamine in a 10cm plate, incubated at 37°C until they reached 80-90% confluency. Fully cultured Rho zero cells were obtained from the Gu lab.

Extraction of Mitochondria

First, the donor cells HEK293 and NIH3T3 were prepared by being split into 15 cm plates and being incubated for 3 to 4 days until they reached an 80-90% confluency. They were then collected using Mitochondria Isolation Kit (Qproteome) following the manufacturer's protocol and the quantity of total protein in extracted mitochondria was measured using Pierce protein assay BCA kit (VWR International), according to the manufacturer's instructions.

Mitoception

Extracted mitochondria were added to the 6 well plates containing post-treatment THP-1 and RAW 264.7 cells that had been incubated for 24 hrs, respectively. The plates were centrifuged

at 1500g for 15 minutes at 4°C, were incubated for an hour at 37°C then were centrifuged again under the same conditions.

Polarization of macrophages

Human monocyte THP-1 cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA) at the concentration of 5 ng/ml as performed previously (Daigneault et al., 2010). They were then polarized into M1 and M2 states, using 2 μL /1 mL of IFN-γ and 1 μL /2 mL TNF-α for M1 and IL4 20 ng/mL for M2.

DNA extraction and Sanger sequencing

DNA was extracted using TriZol (Life sciences) according to the manufacturer's protocol and amplified. Then was purified using a Monarch PCR & DNA cleanup kit (New England Biolabs, Inc.) following the manufacturer's protocol, was quantified via nanodrop then primer was added then the samples were sent in for Sanger sequencing.

Flow cytometry

Recipient cells were collected and dyed with Hoechst at 0.5 μL /1 mL concentrations then incubated at 37°C for 15 minutes. They were then washed with PBS twice to prevent staining of donor mitochondria and then incubated until further use. Mitochondria were dyed using mitotracker deep red (Life technologies), immediately upon isolation. Mitotracker deep red was diluted to a 200 μM concentration working stock, treated to isolated mitochondria, and were incubated at 37°C for 10 minutes. Then the mitochondria were washed, collected, and added to

recipient cells via mitoception. The cells were then fixed with 2% PFA in PBS for 20 minutes at 4°C, then were taken to be imaged by flow cytometry.

RNA isolation and cDNA synthesis

Total RNA was isolated using TriZol (Life sciences). Frozen samples were thawed on ice. 200 µL of chloroform was added per 1 mL of the TriZol sample. The mixture was then vortexed and incubated at room temperature (RT) for 5 minutes. Post RT incubation, the samples were centrifuged at 15000 RPM at 4°C for 15 minutes. The supernatants of each sample were collected and transferred to RNAase-free tubes to which equal amounts of isopropanol to the sample were added, inverted 6 times, then were incubated at RT for 15 minutes. The samples then were centrifuged at 15000 RPM for 30 minutes, and the pellets were obtained. 1mL of 75% ethanol was added per 1mL of TriZol, the samples were vortexed then were centrifuged at 15000 RPM for 15 minutes. This step was repeated twice, the supernatant was removed, and the pellet was dried to which 20 µL of dH_2O was added. After isolation, RNA concentration was determined using Nanodrop spectrophotometer (Thermo Fisher Scientific) and the relative purity of the extracted RNA was assessed via A260/280 ratio. The RNA was then reverse transcribed with a qScript™ cDNA Synthesis Kit (Quantabio).

Quantitative RT-qPCR and Data Analysis

Relative mRNA expression was determined by RT-qPCR using LightCycler® 480 SYBR Green I Master Mix on a LightCyler® 480 instrument (Roche). All reactions were performed in 6 µL volumes, including 30 ng of cDNA and 0.3 µL of forward and reverse primers each at the concentration of 10 µM specific to the mRNA of M1 and M2 state marker genes. Amplification

was performed with a 7-minute denaturation step at 95°C, followed by 45 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 10 sec. A dissociation curve analysis was performed using the default settings of the software to confirm the specificity of the PCR products. The $2^{-\Delta\Delta Ct}$ method was used to analyze data for each target gene. Oligonucleotide primers for *CD163*, *CD206* (M2), and *iNOS* (M1) were designed using NCBI primer BLAST and obtained from Integrated DNA Technologies (IDT).

Statistical Analysis

Statistical significance was determined via Graphpad Prism (ver. 6), obtaining a two-tailed p-value by conducting a student's T-test.

Results

Mitoception shows high mitochondria internalization efficiency

Mitoception was assessed by using agarose gel electrophoresis and monitoring for internalized donor mtDNA in Rho zero cells by flow cytometry at different amounts of added mitochondria. Before flow cytometry analysis, an agarose gel was used to check the bands for the presence of the donor mitochondrial DNA within each sample (Fig. 1). While the control and rest of the samples showed a band corresponding to mtDNA, the band was not seen in sample 4 where no mitochondria were added and no mitoception was performed. These results suggest the mitoception was performed successfully for all mitochondria concentrations. When checked for mitoception's efficiency using flow cytometry for both human THP-1 and HEK293 and murine RAW 264.7 and NIH3T3 cell lines, high efficiency was observed (Fig. 2).

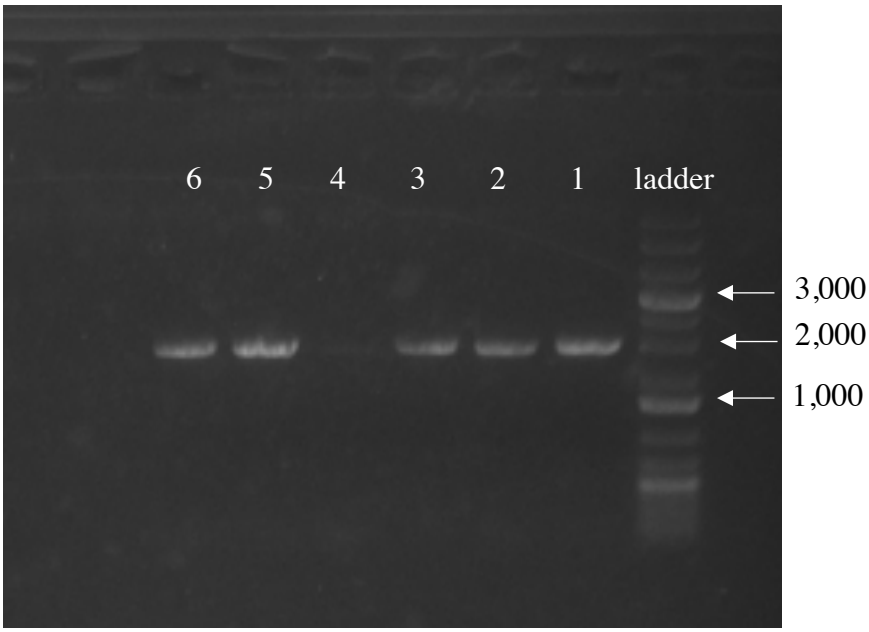
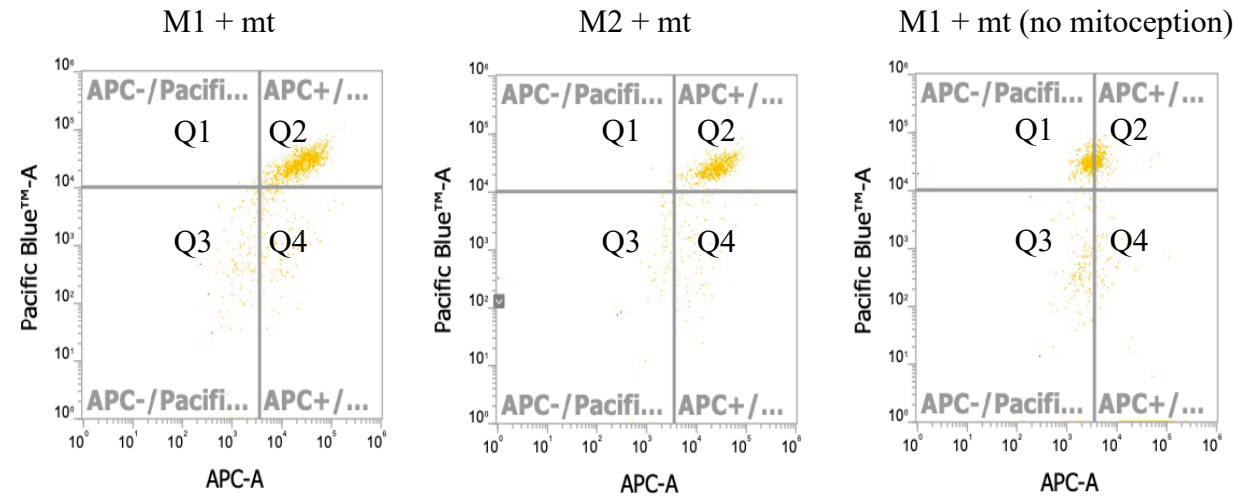


Figure 1. Electrophoresis analysis for mitochondrial DNA in Rho zero cells showing mitochondria internalization by mitoception.

Different amounts of donor mitochondria were added to Rho zero cells; 1: 100 µL, 2: 200 µL, 3: 300 µL, 4: 0 µL, 5: 400 µL, 6: 500 µL. A 10 kb PLUS DNA Ladder was used.

A



B

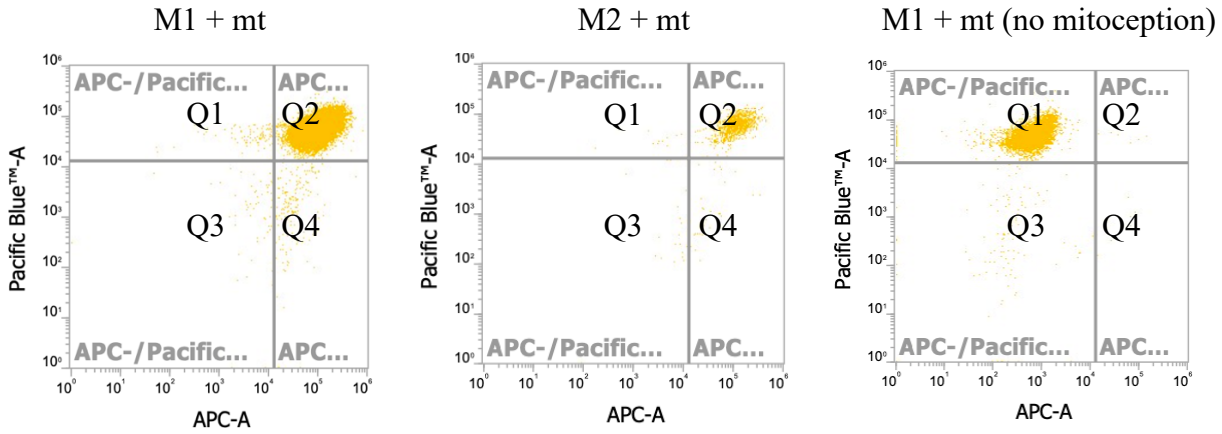


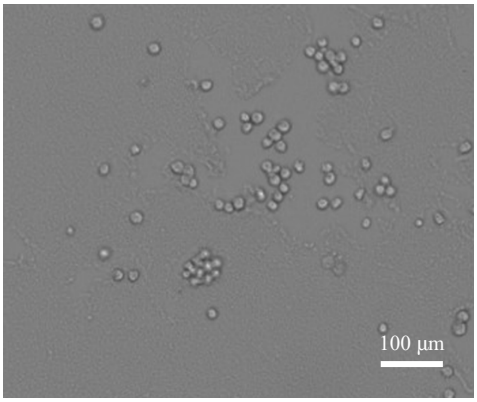
Figure 2. Flow cytometry results show mitochondrial internalization by mitoception.

Histograms depicting mitochondrial internalization post mitoception via flow cytometry. Q1, Q2: Hoechst-stained recipient cells, Q2, Q4: Mitotracker deep red stained isolated mitochondria, Q3: Debris. The histograms show M1 state macrophage + mitochondria, M2 + mitochondria, and just a mixture of mitochondria and M1 state macrophages without mitoception (from left to right) in each RAW 264.7 (A) and THP-1 (B) cells.

The addition of mitochondria leads to phenotypical changes

Upon internalization of mitochondria, phenotypical changes were observed after 48 hours. While undifferentiated THP-1 remains as suspended cells (Fig. 3 A), PMA differentiated M1 state THP-1 cells treated with 200 μL of extracted mitochondria showed an increase in adherence and spindle-like shapes (Fig. 3 C).

A



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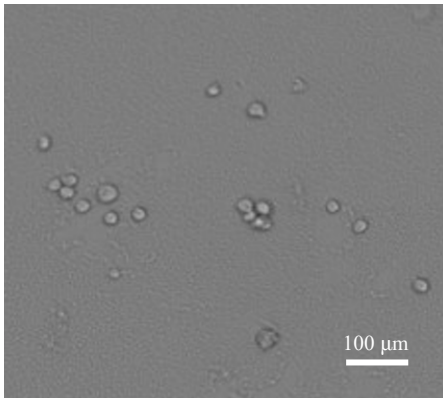


C

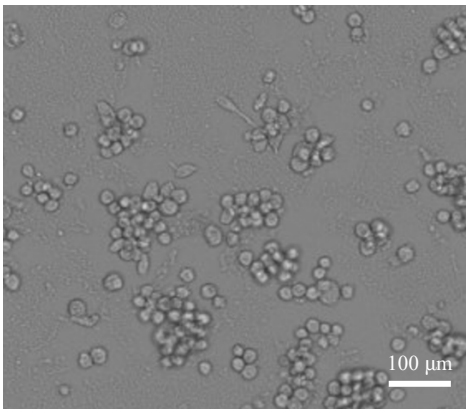


Figure 3. Phenotypical changes upon mitochondrial addition in THP-1 cells.

Imaging of human THP-1 monocytes was performed via an imaging cytometer. Undifferentiated THP-1 cells are suspension cells and thus are smaller in size in comparison to activated cells. (A)

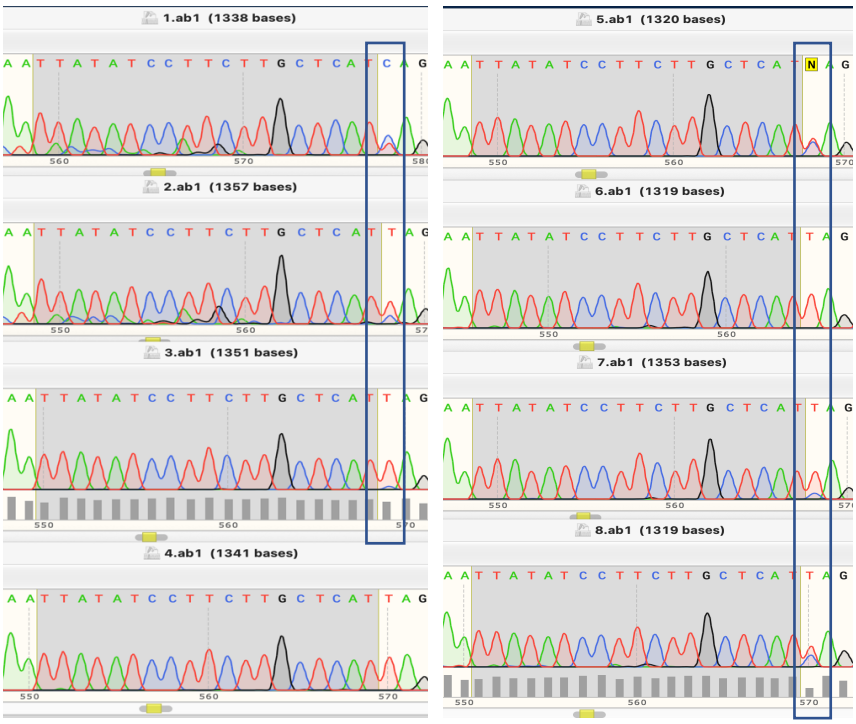
THP-1 cells differentiated with PMA become adherent cells upon polarization into M1 state. (B) Activated THP-1 cells with 200 μL of extracted mitochondria added on which mitoception was performed. (C) A change in morphology was observed showing spindle-like shapes.

Detection of polymorphic sites in respective donor and recipient cell lines.

Since the flow cytometry results showed an extremely high efficiency, the internalization of donor mitochondria and its efficiency was double-checked through Sanger sequencing. Polymorphic sites were checked after mitoception was performed to track the internalized mitochondria and to reinforce the internalization of donor mitochondria into recipient cells via mitoception. With Sanger sequencing, we noticed that Raw264.7 cells and their donor NIH3T3 cells had no polymorphic sites, thus they were excluded from the rest of the experiment.

However, THP-1 and HEK293 had 2 polymorphisms at 7778 bp and 9348 bp sites on the genome which were verified via Sanger sequencing (Fig. 4). The donor HEK293 cell line bears universal mutations that differ depending on the recipient, thus could be chosen as the marker to quantify mitochondria internalization efficiency.

A


1.ab1 (1338 bases)
2.ab1 (1357 bases)
3.ab1 (1351 bases)
4.ab1 (1341 bases)
5.ab1 (1320 bases)
6.ab1 (1319 bases)
7.ab1 (1353 bases)
8.ab1 (1319 bases)

B

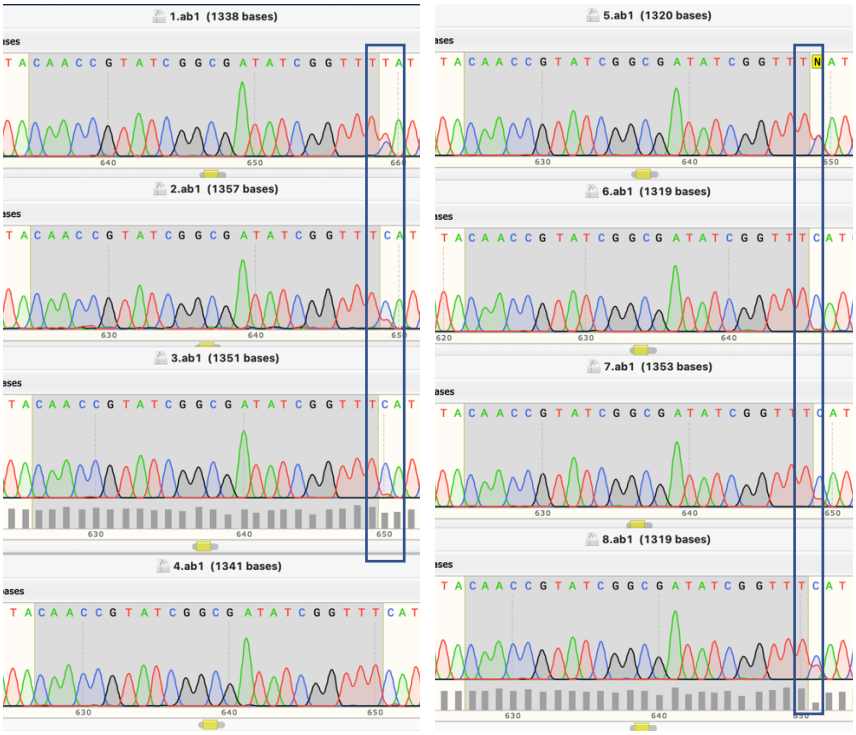

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Figure 4. THP-1 and HEK 293 cells have 2 polymorphic sites.

Results of Sanger sequencing checking for polymorphic sites at 7778 bp and 9348 bp sites of the genome post mitoception. Samples 1-8 had differing amounts of mitochondria added via mitoception (sample 1: 100 μL, 2: 150 μL, 3: 200 μL, 4: 0 μL, 5: 250 μL, 6: 300 μL, 7: 350 μL, 8: 400 μL) but sample 4 had no isolated mitochondria added to it. For the first donor mtDNA marker sequence TTATATCCTTCTTGCTCAT (site 7778), a polymorphism is observed for all the samples except for sample 4 which mitochondria were not added to. (A) The same is observed for the second donor mtDNA marker sequence CAACCGTATCGGCGATATCGGTTT (site 9348). (B)

The addition of donor mitochondria leads to increased M2 marker

We observed an increase in M2 markers *CD206* and *CD163* upon the addition of donor mitochondria into M1 state and non-differentiated THP-1 monocytes indicated as the control group in Figure 5. The observed increase in marker expression was determined to be statistically significant between the M1 group and M1 + mitochondria group with an increase in expression level from 1.25 to 1.65 for *CD163* and 0.03 to 4.96 in *CD206* expression (Fig. 5). Additionally, mitoception did not seem to lead to changes in M2 marker expression levels in non-differentiated THP-1 monocytes.

A B

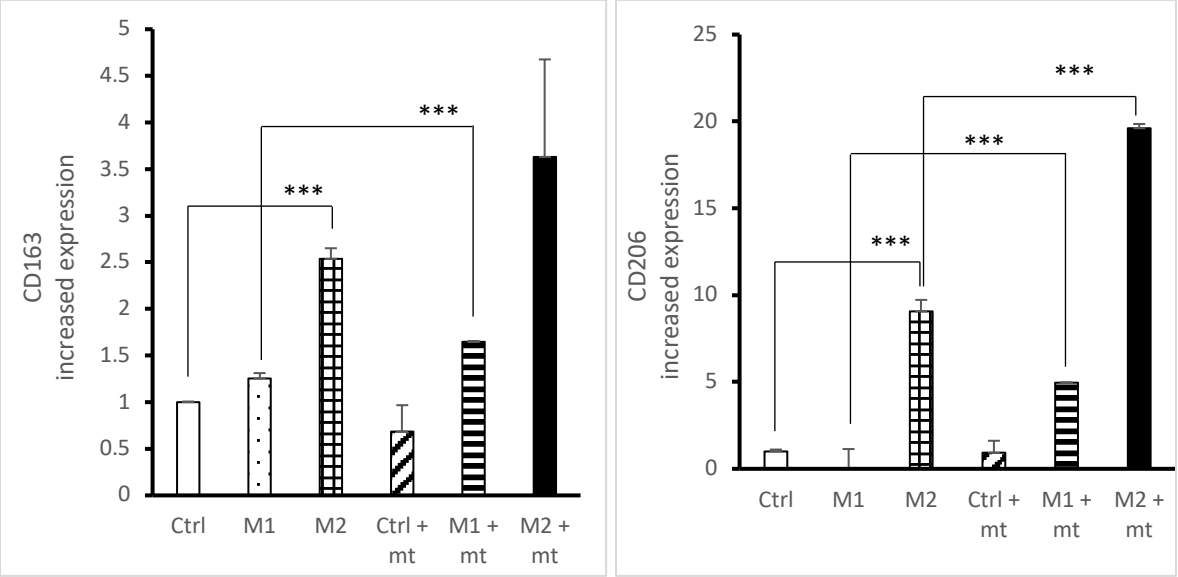

Figure 5. Change of M2 markers after mitochondria addition.

RT-qPCR of *CD163* (M2) and *CD206* (M2) expression in differentiated human THP-1 cells upon treatment with water (negative control), 2 μL/1 mL of IFN-γ, and 1 μL/2 mL TNF-α for M1 and IL4 20 ng/mL for M2, the addition of equal amounts of mitochondria to each water control, M1 and M2 polarized macrophages. The cells were incubated for 24 hrs post treatment. Fold changes are shown relative to the *β-actin* expression levels. Error bars represent +1 STD for n = 4. (***: p-value ≤ 0.001) A significant increase in *CD163* (A) and *CD206* (B) expression upon addition of extracted mitochondria is observed.

Discussion

In the present study, human monocyte THP-1 cells were activated into macrophages to check for the direct role of mitochondria on the macrophage polarization state. Here, with a novel approach regarding the incorporation of mitoception as a method to induce donor mitochondria

internalization and examining the effect of direct mitochondria addition on macrophage repolarization, we demonstrate that the addition of donor mitochondria does lead to a significantly increased expression of certain M2 markers. When donor mitochondria were added to the recipient cells that were differentiated and polarized into M1 state, this addition of mitochondria led to significantly increased expression of M2 markers, supporting our hypothesis that mitochondria play a crucial role in the macrophage polarization state via altering the energy generation mechanism. This observation further highlights the importance of the role of mitochondria in the macrophage polarization state. These findings can also be tested with microglial cell lines to see if similar results may be observed, and this could shine a light on a new potential treatment for neurodegenerative diseases.

However, this project can be improved in the following few ways. ATP production rate could have been checked after mitochondrial extraction and mitoception/repolarization via cellular metabolic flux assay (i.e., Agilent Seahorse) to ensure the quality of extracted mitochondria and check for their roles in metabolic shifts upon mitoception. Changes in growth rate and phenotype of Rho zero cells could have been assessed to ensure mitochondria internalization in post mitoception cells. Protein expression levels of each M2 marker could also have been verified via western blotting. The increased gene expression upon addition of mitochondria was limited to a few markers and no significant increase nor decrease was observed for the M1 marker (Fig. S1). This could have been more thoroughly investigated using more efficient primer sets, checked for changes in the levels of phagocytic activities, and assessed the efficiency of ATP production via cellular metabolic flux assay to ensure that the differentiated THP-1 monocytes were correctly polarized into M1 and M2 states upon respective treatments. The polarization state before and after

mitoception could have been further examined by comparing the changes in phagocytic activities. Centrifugation of the samples that did not have mitochondria added to them in Figure 5 could have ensured the change in expression levels of M2 markers were not due to the added stress.

Supplementary Materials

Gene (human)	**Forward primer**	**Reverser primer**
β-actin	ACTGAATTCACCCCCCACTGA	CCTCCATGATGCTGCTTACA
CD206 (M2)	TGATTACGAGCAGTGGAAGC	GTTCACCGTAAGCCCAATTT
CD163 (M2)	CTTGGGGTTGTTCTGTTGGC	CTCTTGAGGAAACTGCAAGCC
iNOS (M1)	AGCTTCTGCCTCAAGCCATT	TTTGTTACGGCTTCCAGCCT

Table S1. RT-qPCR primers for THP-1 cells.

Target (human)	**Forward primer**	**Reverser primer**
1	ATGCTAAAACTAATCGTCCCAA	AAGAGGAAAACCCGGTAAT
2	TTACCGAGAAAGCTCACAAGAA	CAGGGAGGTAGCGATGAGAG
Target (mouse)	**Forward primer**	**Reverser primer**
1	TATATGGCCTACCCATTCCAACT	AGGTTGAAGAAGGTAGATGGCATAT
2	GGATCTAACCATAGCTTTATGCCCATT	TTATTGAGAATGGTAGACGTGCAGAGC

Table S2. Sanger sequencing primers for murine and human cell lines.

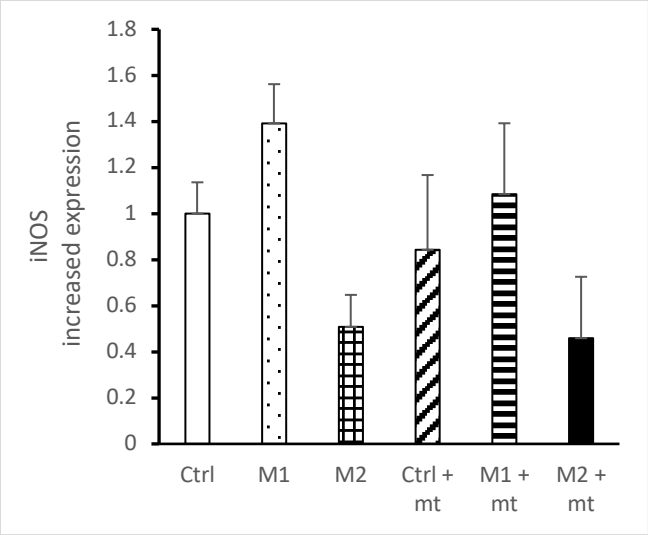

Figure S1. Change in M1 marker after mitochondria addition.

RT-qPCR of *iNOS* (M1) expression in differentiated human THP-1 cells upon treatment with water (negative control), 2 μL/1 mL of IFN-γ, and 1 μL/2 mL TNF-α for M1 and IL4 20 ng/mL for M2, the addition of equal amounts of mitochondria to each water control, M1 and M2 polarized macrophages. The cells were incubated for 24 hrs post treatment. Fold changes are shown relative to the β-actin expression levels. Error bars represent +1 STD for n = 3. Not much change was observed between the CTRL to M1 (p-value = 0.057) and M1 to M1 + mt (p-value = 0.2207) groups.

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CHAPTER 4

FUTURE DIRECTIONS

Mitochondria's direct and indirect role in the activation of immune responses has been examined by many. Through the series of experiments conducted, we further clarified the connections between mitochondria and immune responses. Mitochondria have been identified as a core of multiple pathologies and a change in MPT induced by mPTP opening leading to a disruption in the ETC has been studied as one of the main contributors to mitochondrial dysfunction (Duina et al., 1996; Garbaisz et al., 2014; Springer et al., 2018; Zhong et al., 2007). Despite knowing that mPTP opening leads to decreased ATP production and increased cytokine production, not enough investigation has been done on the association between decreased ATP production and cytokine production (Mukherjee et al., 2016). In chapter 2, we demonstrated that mPTP opening is involved in oligomycin-induced complex V dysfunction-associated cytokine (especially *IL-6*) production. In addition, these experiments demonstrated that complex V mutation and its membranes are involved in inflammatory responses in murine N2a cell lines. Figure 2 of chapter 2 shows that inhibition of mPTP opening significantly suppresses the inflammatory reaction which was a result of complex V mutation via oligomycin treatment. Regarding this project with murine N2a cell line, the experiment can be expanded both to murine and human microglial cell lines which are the resident macrophages of the CNS to get a stronger cytokine reaction upon treatment. This would be more in line with the second project since it examined the role of mitochondria in macrophage repolarization.

Macrophage's role in multiple immune responses has been clearly studied which differs depending on differentiation and polarization state and can either be pro or anti-inflammatory, displaying different degrees of varying features including phagocytic and antigen-presenting capacities (Gordon, 1998; Liu et al., 2014; Nathan et al., 1983). While it has been established that one of the major phenotypical differences between M1 and M2 macrophages is in their energy metabolism mechanism and that mitochondrial metabolism may play an important role in macrophage polarization state (Huang et al., 2014; O'Neill & Pearce, 2016; Ramond et al., 2019a; Van den Bossche et al., 2016), mitochondria's direct role in repolarization of macrophages via shifting the metabolic mechanism was unanswered. Findings reported in chapter 3 indicate that the addition of mitochondria led to the repolarization of M1 state macrophages into M2 state in the differentiated human THP-1 cell line. However there remain many gaps to be filled, and many weaknesses to be addressed within the current study. The experiments were limited to cell lines thus we do not know how translatable the results are to clinical studies. Results of project 2 could be further developed and reinforced by checking the quality of extracted mitochondria using cellular metabolic flux assay (i.e., Agilent Seahorse) before internalization to ensure that the observed changes can be attributed to addition of mitochondria and tracking the changes in phagocytic activity levels before and after mitoception to use as an additional marker for M1 and M2 polarization. A more thorough investigation of the post mitoception repolarized states could be conducted. Given time and opportunity further research investigating the observations of the current study could be expanded to *in vivo* experiments to examine whether oligomycin still shows inflammatory responses through mPTP opening and mitochondria addition increases M2 phenotypes in M1 state macrophages. These then could potentially be utilized to seek therapeutic methods for HD, suppressing the inflammatory responses in the HD brain by focusing on inhibiting

mPTP opening to directly decrease cytokine production while inducing repolarization of microglia to promote rebuilding of the damaged CNS.

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