

A Bioenergetics Study of Mitochondrial Function in Bone Marrow Derived Macrophages

By

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Sahar Abdulrahman Alkhodair

In loving memory of

MUM & SAMAR

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In the name of Allah most gracious, most merciful.

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Summary

Mitochondria are organelles involved in the generation of Adenosine Triphosphate (ATP), storage of intracellular calcium ions, regulation of the apoptotic pathway as well as numerous cell signalling pathways. Moreover, mitochondria are an important source of reactive oxygen species (ROS), which are molecules containing oxygen that can readily oxidize other molecules. Immune activation of macrophages with Lipopolysaccharides (LPS) is known to initiate a metabolic switching from oxidative phosphorylation to glycolysis; however, the bioenergetic mechanisms that underlie such events are not fully known. In this study, the effect of LPS treatment of mouse immortalized Bone Marrow-derived Macrophages (iBMDMs) and primary BMDMs on electron transport chain activities, oxygen consumption rates, ROS production and the production of inflammatory cytokines, including IL-1 β and TNF were investigated.

The data from electron transfer complexes assays and oxygen respiration assays indicated that the stimulation of iBMDMs and BMDMs by LPS significantly decreased the activities of complexes I and II and citrate synthase up to 80% in a time-dependent manner, which may underlie time-dependent decreases in observed oxygen respiration. Importantly, this study showed that the LPS-related inhibition of complex II was reversible and that of complex I was time-dependent but irreversible. In activated macrophages, itaconate is one of the most highly induced metabolites. In this study, itaconate was found to be a time-dependent and reversible inhibitor of complex I and II activities in iBMDMs and BMDMs. The type of itaconate-induced inhibition of complex II was competitive and the K_i values calculated to be 0.014 mM and 0.039 mM in iBMDMs and BMDMs, respectively. These studies suggest that the itaconate concentrations that are known to exist in LPS-activated macrophages are sufficient to reversibly inhibit complex I and II activities, but that the mechanism for LPS-induced irreversible inhibition of complex I activity remains unknown.

Metabolic control analysis (MCA) was used to investigate the spread of control among complex I and complex II/III over oxygen consumption in iBMDMs and BMDMs. The results provide information on the control possessed by the complexes and the level by which the complexes must be inhibited before deleterious effect are imposed on mitochondrial respiration. The results show that complex I possessed the highest level of control of the complexes examined over OCRs in iBMDMs and BMDMs. In iBMDMs the complex I and II/III energy thresholds were high at ~60 and ~80%, respectively. However, in BMDMs a lower inhibition thresholds of 40% and 60% were found for complexes I and II/III, respectively. These results suggest that the 40% and 60% decrease in complex I specific activities, observed at 4h and 24h following LPS-activation in BMDMs, are sufficient to cause the corresponding decreases in mitochondrial respiration. Similarly the 50% and 80% decreases in complex II/III activities at 4h and 24h following LPS-activation will also overcome inhibition thresholds and reduce respiration rates in BMDMs.

As previous studies hypothesised that mitochondrial ROS are a driving force for cytokine secretion in LPS-activated macrophages, experiments were performed to assay both parameters during ETC complex titrations. While ROS levels were found to be 2-fold higher in iBMDMs compared to BMDMs, LPS-activation increased ROS only in BMDMs. Both rotenone and antimycin were found to increase ROS by ~1.4-fold only at concentrations >20nM, which also exceeded the inhibition thresholds. However, at lower concentrations of 5 nM which do not exceed inhibition thresholds, IL-1 β secretion was found to be significantly reduced, especially in inflammasome-activated (LPS + ATP) conditions in BMDMs. Overall, these results suggest that slight perturbations of complex I or II/III activities are sufficient to regulate respiratory flux through the ETC and subsequently decrease cytokine secretion from LPS-activated macrophages. This may be the mechanism in which LPS-activated macrophages decrease complex I and II/III activities and feedback regulate the secretion of pro-inflammatory cytokines.

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Abbreviations

$\Delta\Psi_m$	Mitochondrial Membrane Potential.
Δp	Proton Electrochemical Potential Gradient.
ΔpH	pH gradient.
AA	Antimycin A.
ACPA	Anti-Citrullinated Protein Antibody.
ADP	Adenosine Diphosphate.
AMPK	AMP-activated protein kinase.
ANOVA	Analysis of Variance.
Anti-TNF	Anti-tumour necrosis factor.
APC	Antigen-presenting cells.
ATP	Adenosine Triphosphate
BMDMs	Bone Marrow-Derived Macrophages.
BSA	Bovine Serum Albumin.
CAC	Citric acid cycle.
CoQ	Coenzyme Q.
CS	Citric synthase.
DCPIP	Dichlorophenol-indophenol.
DHE	Dihydroethidium.
DNA	Deoxyribonucleic Acid.
DNCB	1-chloro-2,4-dinitrobenzene.
DNTB	5,5'-Dithiobis (2-nitrobenzoic acid).
ECAR	Extracellular Acidification Rate.
ER	Endoplasmic Reticulum.
ETC	Electron Transport Chain.

FAO	Fatty Acid Oxidation.
FCC	Fux Control Coefficient.
FCCP	Carbonyl cyanide-p-trifluoromethoxy phenylhydrazone.
FADH	Flavin Adenine Dinucleotide.
HIF-1 α	Hypoxia- inducible factor 1 alpha.
iBMDMs	Immortalized Bone Marrow-derived Macrophages.
IDH	Isocitrate dehydrogenase.
I-3-P	Indole-3-pyruvic acid.
IMM	Inner Mitochondrial Membrane.
IMS	Intermembrane Space.
IL-6	Interleukin-6.
IL-10	Interleukin-10.
ICAM-1	Intercellular adhesion molecule-1.
ICAM-2	Intercellular adhesion molecule-2.
ITA	Itaconate.
KCN	Potassium Cyanide.
α -KG	α -keto- glutarate.
LPS	Lipopolysaccharides.
LTA	Lipoteichoic acid.
MARCO	Macrophage receptor with collagenous structure.
MAPK	Mitogen-activated protein kinase.
MCA	Metabolic Control Analysis.
M-CSF	Macrophage Colony-Stimulating Factor.
MHC	Major histocompatibility complex.
MRC	Mitochondrial Respiratory Chain.

MTX	Methotrexate.
6-MP	Mercaptopurine.
mtDNA	Mitochondrial DNA.
mtROS	Mitochondria-derived ROS.
N	The number of replicates of an experimental condition.
NADH	Nicotinamide adenine dinucleotide.
NK	Natural killer.
NO	Nitric oxide.
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3.
O ₂	Oxygen.
O ₂ ^{•-}	Superoxide Anion.
•OH	Hydroxyl radicals.
OCR	Oxygen Consumption Rate.
OMM	Outer Mitochondrial Membrane.
OSCP	Oligomycin Sensitivity-Conferring Protein.
OXPHOS	Oxidative Phosphorylation.
PAMPs	Pathogen-associated Molecular Patterns.
PBS	Phosphate Buffered Saline.
Pi	inorganic Phosphate.
PHDs	Prolyl Hydroxylases.
PTP	Permeability Transition Pore.
PRRs	Pathogen Recognition Receptors.
Prx	Peroxiredoxin.
RET	Reverse Electron Transport.
ROS	Reactive Oxygen Species.

Rot	Rotenone.
SDH	Succinate Dehydrogenase.
SEM	Standard Error of the Mean.
SOD	Superoxide Dismutase.
SLP	Substrate-level Phosphorylation.
SUCL	Succinate-CoA ligase.
TCA	Tricarboxylic Acid.
6-TG	Tioguanin.
TIM	Translocase of the Inner Membrane.
TNB	Thionitrobenzoic Acid.
TNF	Tumour necrosis factor.
TOM	Translocase of the Outer Membrane.
TPN	2-Aminoethylglycine-carbonylmethylene-thymine.
TTFA	Thenoyltrifluoroacetone.

CHAPTER 1

Introduction

1.1 Immunometabolism

Immunometabolism is an emerging field of investigation at the interface between metabolic and immunologic processes (Mathis and Shoelson, 2011). This means, within the immune cells there are distinct metabolic pathways that change, reshape, and adapt according to the environment. It is suggested that metabolism and the immunological state are inextricably linked, from early notifications that inflammatory cytokines were induced in obese adipose tissue and that these cytokines contributed to metabolic disease (Makowski et al., 2020).

There are strong link between glucose, fatty acid and proteins fuel and metabolic pathways. The energy and all-important biosynthetic intermediates are provided by the glycolytic pathway, pentose-phosphate pathway, tricarboxylic acid (TCA) cycle , fatty acid oxidation and fatty acid synthesis and amino acid metabolism as well as the mitochondrial respiratory chain. The intermediates are elemental for cellular proliferation, the immunological profile of a cell and the modulation of cellular signalling (O'Neill et al., 2016).

During the glycolysis process, glucose enters the cells via glucose transporters and it is converted into pyruvate (Bolaños et al., 2010, CH, 2008). In normoxic conditions of homeostasis, pyruvate obtained from glycolysis is broken down into acetyl coenzyme A (acetyl CoA) and carbon dioxide. Acetyl CoA enters the tricarboxylic acid (TCA) cycle, which then produces NADH to fuel OXPHOS, which in turn produces large amounts of ATP. On the other hand, under hypoxic conditions, cells obtain ATP just from the breakdown of glucose to pyruvate, which is then converted into lactate and NAD^+ and lactate is released in the extracellular milieu. This is known as Warburg effect . Glycolysis is an inefficient way to produce ATP (2xATP/ molecule of glucose) (Figure 1.1) as

compared to OXPHOS (36xATP/molecule of glucose). However, under normoxic conditions, many activated immune cells switch their metabolism to glycolysis as a preferred way of metabolism. This is known as aerobic glycolysis, and it is adopted by activated immune cells to facilitate cellular biosynthetic pathways (Lunt and Vander Heiden, 2011, Jones and Bianchi, 2015). Many factors such as nutrient and oxygen availability and cytokine stimulation can upregulate immune cell metabolism and functions (Loftus and Finlay, 2016). There are some studies high light the Mitochondrial function contributes to macrophage metabolism and function for example the study that done by Mill et al., find that succinate oxidation is an important regulator of inflammatory signalling opens up a number of new therapeutic opportunities (Mills et al., 2016). Also, the study that done by Dawling et al., the main finding in this study that Arg-2 facilitates oxidative respiration via its impact on CII activity at the ETC (Dowling et al., 2021). Moreover the study that done by Billingham et al., this study report that Inhibitors of the mitochondrial ETC complex I, II, III, IV and V have been shown to prevent NLRP3 inflammasome activation lead to decrease ATP levels and increase the risk of oxidative stress (Billingham et al., 2022). Multiple studies have discovered that metabolic control points are present in mitochondria that influence macrophage function. However, the mechanisms that underlie these events still require resolution.

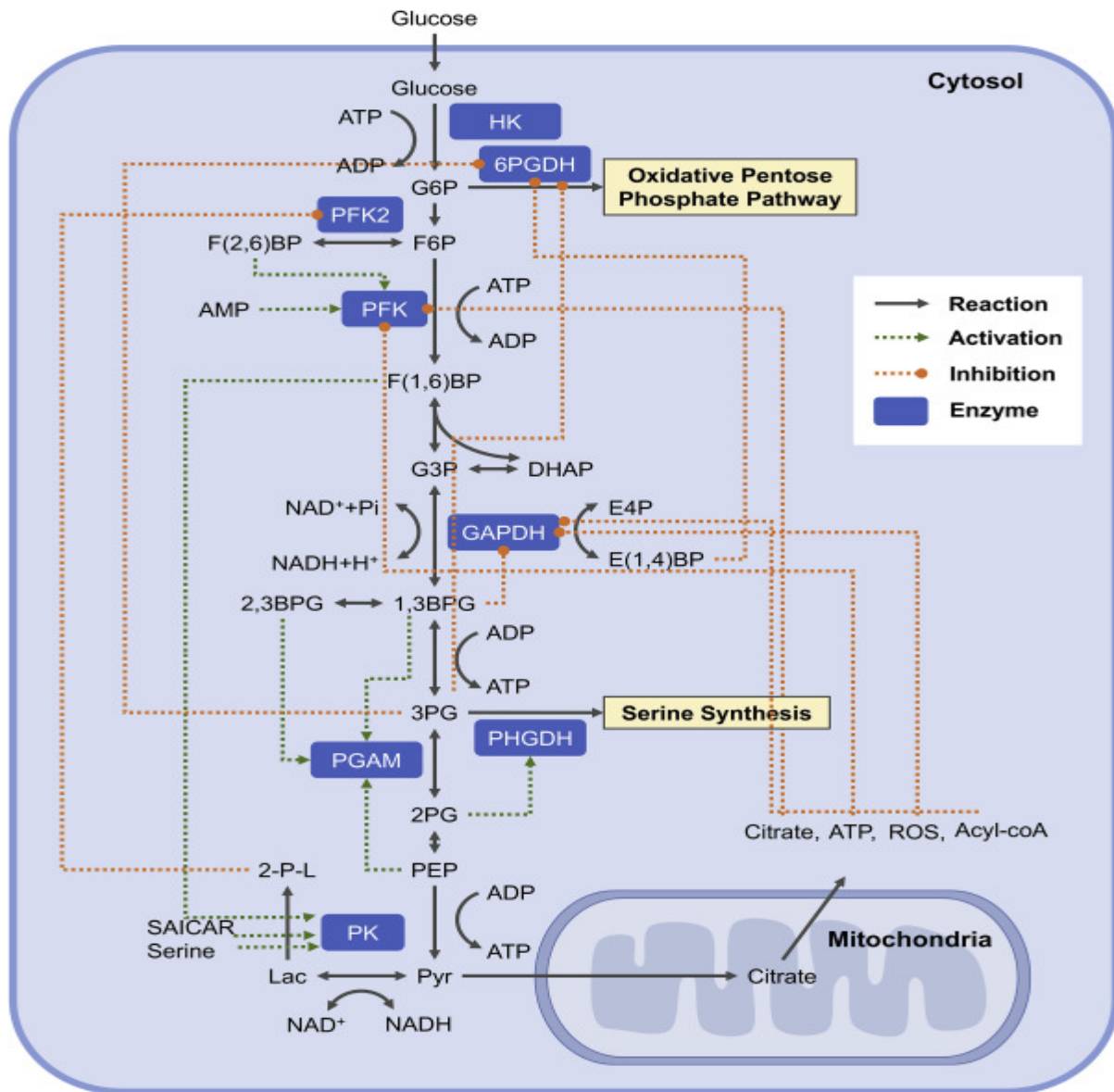


Figure 1.1. Glycolysis overview

Overview of glycolysis and new mechanisms of feedforward and feedback regulation. The pathway of glycolysis begins with the uptake of glucose and ends with the production of lactate and CO₂ in the mitochondria (Locasale, 2018).

1.2 Innate immune system

The Immune System can be schematically divided into two categories, innate and adaptive immunity, depending on the speed and specificity of the response (Figure 1.2) (Parkin and Cohen, 2001). Usually, innate immunity has been known as a relatively unspecific first line of defence against invading pathogen (Beutler, 2004).

Innate immune cells comprise professional phagocytes include monocytes, macrophages and neutrophils, which kill bacteria and fungi by phagocytosis; eosinophils, basophils and mast cells which attack worms and parasites by releasing toxic mediators; and natural killer (NK) cells which kill infected and malignant host cells.

The innate immune system is a phylogenetically conserved non-specific defence line which are able to detect pathogen-associated molecular patterns (PAMPs) through a series of germline-encoded receptors, called pathogen recognition receptors (PRRs), leading to their activation (Medzhitov and Janeway Jr, 2000, Iwasaki and Medzhitov, 2015, Hato and Dagher, 2015).

1.2.1 Toll-like Receptors

The TLRs are a family of structurally-related receptors (PRRs), play crucial roles in PAMPs, which leads to an appropriate host immune response upon microbial invasion (Medzhitov et al., 1997) . Toll was initially discovered for its role in the establishment of dorso-ventral polarity during embryonic development in *Drosophila*, however, also importantly in antifungal responses (Lemaitre et al., 1996).

TLRs are type I transmembrane proteins with ectodomains containing leucine-rich which repeats responsible for the recognition of the PAMPs. A transmembrane domain connects the ectodomain to an intracellular domain required for downstream signal transduction, termed Toll–interleukin 1 (IL-1) receptor (TIR). Markedly, the TIR domain is shared by TLRs and the IL-1 receptor, and mediates the recruitment of TIR domain-containing adaptors like Myeloid differentiation primary response gene (88) (MyD88) and TIR-domain-containing adapter-inducing interferon- β (Leifer et al., 2004).

13 mammalian TLRs have been identified, 10 of that are expressed in humans (TLR1–10). Each of them is described by a certain degree of specificity in recognizing related molecular structures (Medzhitov et al., 1997). TLR2 recognizes an enormous number of lipopeptides and peptidoglycan, in heterodimeric association with TLR1 or TLR6 (Takeuchi et al., 2000, Takeuchi et al., 2001). TLR3 recognizes double-stranded RNA (dsRNA) which is produced from several viruses during replication (Alexopoulou et al., 2001), while TLR4 is known to identify Gram-negative bacteria-derived lipopolysaccharide (LPS) (Poltorak et al., 1998). TLR5 is strongly stimulated by flagellin, the major constituent of bacterial flagella (Hayashi et al., 2001). TLR7 and TLR8 recognize single stranded RNA viruses (Diebold et al., 2004, Heil et al., 2004). TLR9 recognizes unmethylated CpG characteristic of bacteria and some DNA viruses (Leifer et al., 2004).

1.2.2 Adaptive immune system

The adaptive immune system is composed of T and B lymphocytes which have an ability to recognise specific antigens via large repertoire of antigen-specific recognition receptors. The adaptive immune system is primed by the innate immune system. Usually, the adaptive immune response takes days to be fully activated and to initiate effective responses against

invading pathogens (Dunkelberger and Song, 2010, Hoebe et al., 2004). T and B cells could respond to a massive range of antigens and the specificity of the adaptive immune response as a result of somatic hypermutation and genomic recombination of receptor genes. Moreover, these cells have the ability to develop immunological memory which can initiate a faster response to re-infection and prevent chronic disease (Janeway Jr et al., 2001).

The main role of B cells is to produce antibodies. B cells inhibit the binding of microbes to tissues in addition to enhancing the ability of innate immune cells to phagocytise pathogens, through opsonisation process (Beum et al., 2011). The importance of T cell is their ability to recognise antigens. The antigen must be presented through MHC on the surface of an Antigen Presenting Cell (APC). MHC I is found on the surface of all nucleated cells and invades CD8 T cells that can recognize and kill virus-infected cells. In contrast, MHC-II molecules are found only on the surface of antigen-presenting cells and are required for antigen presentation to CD4 T cells (Wülfing et al., 2002, Robinson and Delvig, 2002). CD4 is directly involved in the main CD8 immune response and may help "programme" the differentiation of effector and memory CD8 cells after an antigen response (Kitchen et al., 2005).

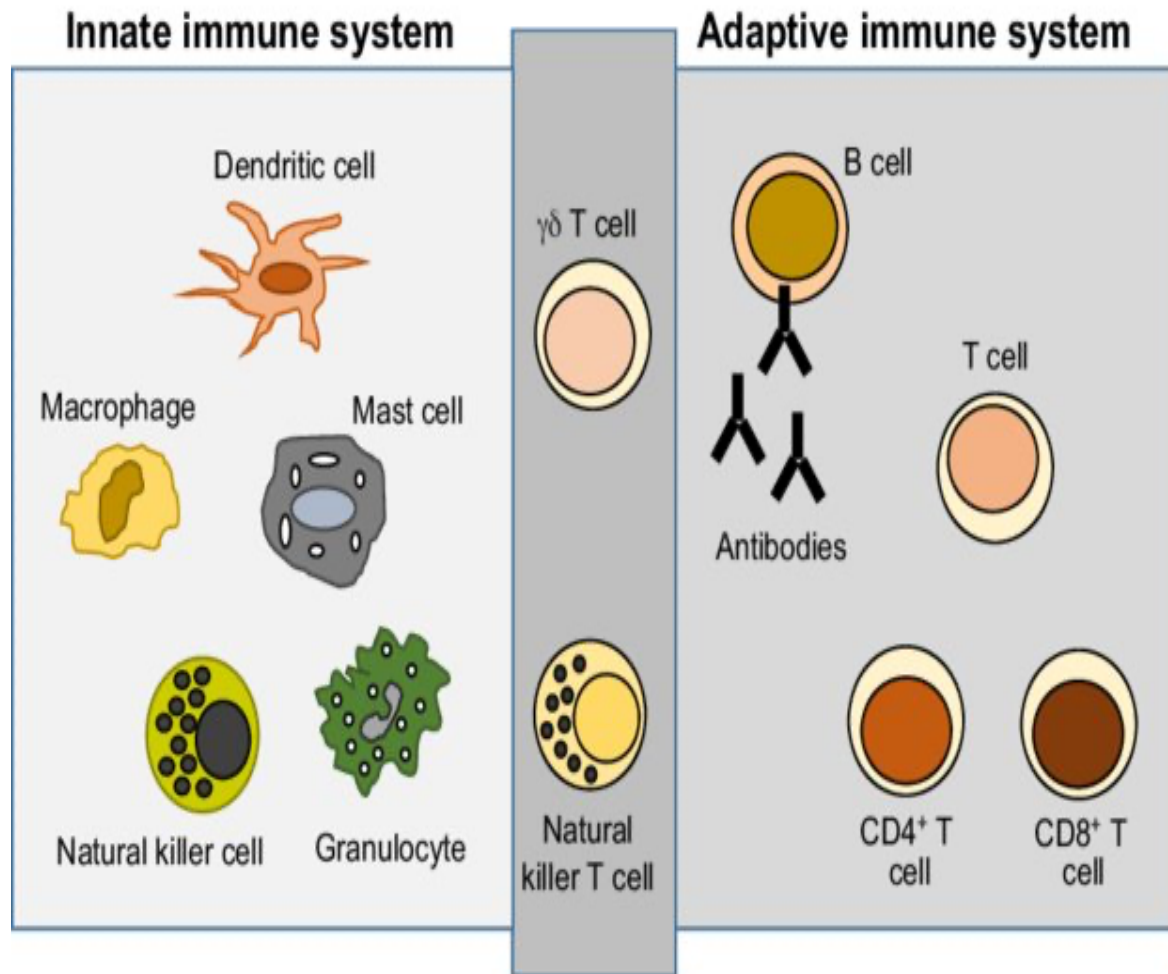


Figure 1.2. Cells of the innate and adaptive immune systems.

When foreign targets are detected, the innate immune system responds quickly, usually within minutes to hours. It consists of a broad mix of cells, including granulocytes, macrophages, dendritic cells, and natural killer cells, as well as a number of soluble substances and proteins. The adaptive or acquired immune system, which provides particular, long-lasting immunological responses, is the second branch of the immune system. The adaptive and innate immune systems are intertwined; dendritic cells, for example, are critical adaptive immune system cell activators (Sharpe and Mount, 2015).

1.3 Cytokines

Cytokines are classified as a category of proteins with low molecular weight (~5–20 kDa). They play important roles in signalling; modulating the complex functions of the immunologic system in regulating non-inflammatory phagocytosis and maintaining M2 macrophages (Silva et al., 2019, McDERMOTT, 2001, Coppack, 2001). Cytokines can be classified based on the nature of the immune response into three classes: cytokines involved in acute inflammation such as IL-6, IL-11 and IL-8, cytokines involved in chronic inflammation such as IFNs, TNF- β and IL-2, and cytokines involved in both acute and chronic inflammation such as IL-1, TNF- α (Feghali and Wright, 1997). Moreover, each cytokine has a specific role depending on the cell type and location (**Table 1.1**) (Turner et al., 2014).

Cytokines are classified as class I or class II cytokines depending on the structural homology of their receptors. Many of interleukins (ILs), CSFs, and interferons (IFNs) belong to one of these two classifications. Moreover, many cytokines mediate their effects through the Janus kinase signalling and transcriptional activator (JAK-STAT) pathways.

The other three major cytokine families include the IL-1 family of many such cytokines (including IL-1 α , IL-1 β , IL-1ra, and IL-18), the TNF family, and the TGF- β superfamily.

Members of the IL-1 and TNF families activate nuclear factor- κ B (NF- κ B) and mitogen-activated protein (MAP) kinase signalling pathways, and members of the TGF- β superfamily activate Smad family signalling proteins (Sma and Mad proteins from *Caenorhabditis elegans* and *Drosophila*, respectively) (Tedgui and Mallat, 2006).

Tumour necrosis factor (TNF) is a 17 kDa protein composed of 157 amino acids, a solution homotrimer produced primarily by activated macrophages, T lymphocytes, and natural killer (NK) cells (Maloy and Hughes, 2013). TNF- α is a powerful paracrine and endocrine mediator of inflammatory and immune function. It is also known to regulate the growth and differentiation of a wide variety of cell types. TNF has a significant impact on bone remodelling. Upregulating the expression of colony-stimulating factor 1 receptor (cfms) directly regulates the bone marrow level of osteoclast precursor cells and activates osteoclasts by enhancing the NF- κ B receptor activator (RANK) signalling mechanism. It also plays an important role in combating infection. The release of TNF by macrophages appears to be necessary for the formation and maintenance of granulomas. It plays an important role in defence against the invasion of intracellular parasites (Fitzgerald et al., 2001).

The cytokine interleukin-1 (IL-1) α and β are endogenous febrile proteins induced by bacterial endotoxin. The two forms bind to the same receptor, IL-1 receptor type 1, and exhibit the same biological activity but they have distinct biological roles. IL-1 β is released from multiple cell types, including activated macrophages, monocytes, and hypothalamic cells, and can stimulate its own expression (Stevens et al., 2010). The cytokine interleukin-1 β (IL-1 β) is an important mediator of the inflammatory response. Essential for host response and resistance to pathogens, exacerbates damage during chronic diseases and acute tissue damage (Lopez-Castejon and Brough, 2011).

LPS treatment promotes the production of NLRP3 and pro-IL-1 and stimulates NF-B signalling via toll-like receptor TLR- 4 (signal 1). Caspase-1 is triggered by extracellular ATP, which also activates inflammasomes via P₂X₇ receptors (signal 2) and causes the

release of IL- β . The macrophage P_2X_7 receptor detects extracellular ATP generated by harmed cells or bacteria, which results in a loss of potassium ions and activates the NLRP3 inflammasome (Gombault et al., 2013, Binderman et al., 2017, Huang et al., 2021).

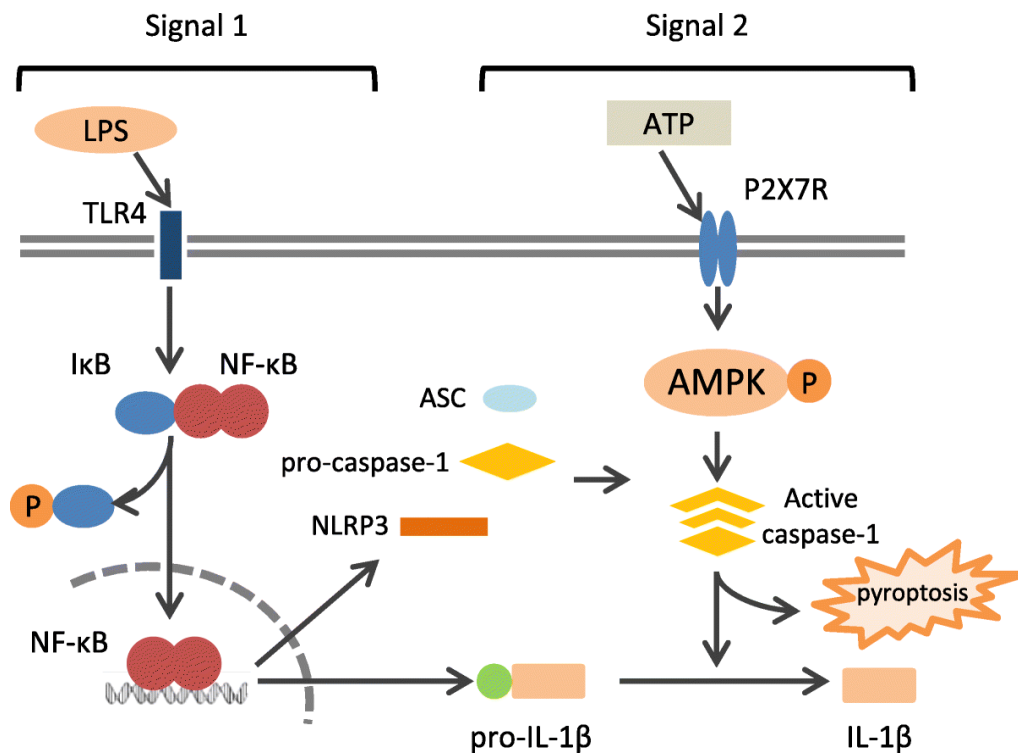


Figure 1.3. LPS/ATP signalling pathway.

Treatment with LPS activates NF-κB signalling via toll-like receptor TLR- 4 and induces the expression of NLRP3 and pro-IL-1β. Extracellular ATP activates inflammasomes via P₂X₇ receptors and caspase-1, which leads to the secretion of IL-1β (Maruyama et al., 2019).

Cytokine	Primary Cell Sources	Key Functions in Inflammation
IL-1	Macrophages Endothelial cells	Synthesis of acute phase proteins by hepatocytes; Local and systemic inflammatory effects
IL-2	Activated T cells Th1 cells	Proliferation of T cells, B cells; Proliferation and activation of NK cells
IL-6	Macrophages Th2 cells Endothelial cells Adipocytes Myocytes Osteoblasts	Synthesis of acute phase proteins by hepatocytes; Proliferation of B cells; Down-regulation of IL-1 and TNF production; Activation of immune cells, osteoclasts, endothelial cells; Hypothalamic Pituitary Axis—fever & hormone release
IL-10	Macrophages Th2 cells	Resolution of inflammation; Inhibition of Th1 inflammatory cytokine synthesis; Inhibition of activated macrophages and dendritic cells
IL-12	Macrophages Dendritic cells	Promotion of Th1 differentiation; Stimulation of IFN- γ production by T cells, NK cells
TNF- α	Macrophages T cells NK cells Lymphoid cells Endothelial cells Adipocytes Cardiac myocytes Fibroblasts Neuronal cells	Synthesis of acute phase proteins by hepatocytes; Recruitment and activation of neutrophils and monocytes at sites of infection; Stimulation of CRP release from liver; Activation of NF- κ B pathway; Induction of insulin resistance
TGF- β	Macrophages T cells	Resolution of inflammation; Limit production of IL-2, IFN- γ , and TNF; Inhibition of proliferation/activation of B cells, T cells, macrophages.
IFN- γ	Th1 cells NK cells	Activation of macrophages; Suppression of Th2 cell activity; Promotion of leukocyte migration

Abbreviations: IFN, interferon; IL, interleukin; NK, natural killer; NF- κ B, nuclear factor-kappaB; Th, T helper;

Table1.1. Selected cytokines

Some cell sources and examples of principal functions in inflammation (Foster and Samman, 2012).

1.4 Macrophages

In the 19th century, macrophages were first discovered by Ilya Metchnikoff (Cooper and Alder, 2006, Epelman et al., 2014, Gordon, 2008). In the 1960s, Van Furth suggested that all macrophages originate from either bone marrow progenitors that mature to peripheral blood monocytes or from tissue macrophage intermediates that may replicate and mature in tissues (van Furth and Cohn, 1968, van Furth et al., 1972). Macrophages are divided into osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue) and Kupffer cells (liver), based on their anatomical location and functional phenotype (Gordon and Taylor, 2005).

Macrophages play critical roles in host defence against microbial infection. They surround and digest cellular fragments, foreign particles, microbes, and cancer cells in a degradation process called phagocytosis, using lysosomal compartments filled with degradative enzymes. Macrophages bind with pathogens or antigens through phagocytic receptors which start the cytoskeleton rearrangements and membrane re-arrangements. There are several different microbicidal processes, including oxidative or nitrosative stress producing reactive oxygen/nitrogen species that harm the pathogens or proteases that release cytotoxic peptides (Aderem and Underhill, 1999, Underhill and Ozinsky, 2002).

Macrophages have incredibly important functions in the body. They patrol for invading pathogens, initiate innate immune responses, remodel and repair tissues following an infection or inflammation, and help to resolve the inflammatory response. Monocytes that are recruited to the site of infection may also differentiate into macrophages, given the right conditions and presence of macrophage colony stimulating factor (M-CSF). If a macrophage is activated, cells may release pro-inflammatory cytokines IL-1, IL-6 and

TNF. Other cytokines released include IL-8 to recruit and attract neutrophils, and IL-12 to activate Natural killer cells (NK), and initiate the differentiation of T helper 1 cells (TH1).

1.4.1 Macrophages in host defence

Macrophages play critical functions in host defence against microbial infection. They are present in all tissues where they act as sentinels responding to pathogens. Moreover, microbial infiltration of tissues triggers the release of a variety of chemotactic agents that alert the macrophages to the infection. Pathogens and macrophages are connected by phagocytic receptors (Ernst, 1998), that initiate the cytoskeleton rearrangements and membrane re-arrangements, which initiate the phagocytosis-mediated internalisation of pathogens. The internalised microbes remain in a phagosome, that fuses with lysosomes and is converted into a phagolysosome where the pathogen is killed by a variety of microbicidal mechanisms such as oxidative or nitrosative stress involving reactive oxygen/nitrogen species and by proteases and cytotoxic peptides (Aderem and Underhill, 1999, Underhill and Ozinsky, 2002, Greenberg and Grinstein, 2002). Microbial peptides released from dead pathogens are processed via the action of hydrolases in the phagolysosomes. Consequently, the microbial peptides are processed and expressed as immunogenic epitopes in association with the major histocompatibility complex (MHC)-II, thus facilitating the activation of an adaptive immune response, involving T cell responses (Savina and Amigorena, 2007) (section 1.2.2 Adaptive immune system).

Nevertheless, macrophages have limited capacity to clear bacteria, and they may no longer be able to control bacteria in the airway if there is a high inoculum, as suggested from studies of mice infection (Dockrell et al., 2003, Knapp et al., 2003).

Furthermore, macrophages do not only induce apoptosis of inflammatory cells (such as neutrophils) but also remove apoptotic cells by a process called efferocytosis, resulting in the down-regulation of pro-inflammatory cytokines and resolution of lung inflammation (Dockrell et al., 2001, Marriott et al., 2006).

1.4.2 Macrophages Phagocytosis

Phagocytosis is an ancient process for tissue balance, and there are many types of cells can perform this process. However, just specialized cells termed professional phagocytes perform this process with high efficiency such as dendritic cells, macrophages, and neutrophils (Cassidy-Hanley, 2012, Zumerle et al., 2019).

Macrophages use phagocytosis as a strategy to control inflammation through the removal of senescent cells which are produced either by programmed cell death or pathogen induced apoptosis. Also, dendritic cells also use phagocytosis to ingest particles, including micro-organism, as a source of antigens for presentation to T-cells. In addition, it is essential for embryonic development and tissue remodelling. However, higher organisms use this process to bolster host defence (Cardelli, 2001).

Phagocytosis is a complex process that is started by the interaction of specific receptors on the surface of macrophages with ligands on the surface of microbes or other particles (Pommier et al., 1983). It leads to the polymerisation of actin at the site of ingestion which triggers internalisation of microbes, cells, or other particles through an actin-based mechanism. Afterwards, the actin surrounding the endosome containing the internalised particle is shed from the phagosome which matures by a sequence of fusion and fission events. This process involves the endocytic pathway and culminates in the formation of a

mature phagolysosome, that enhances killing of microbes by different mechanisms (Zhang et al., 2002, Castellano et al., 2001).

Phagocytic cells can engulf both non-opsonic microbes via engaging related PRRs with microbial surface antigens, or they can initiate opsonic phagocytosis via deposition of proteins, for example antibodies or complement on microbes, which are recognised by cell surface receptors of phagocytes such as macrophages (Underhill & Ozinsky, 2002). Lipoteichoic acid (LTA) of non-opsonised pneumococci is recognised by scavenger receptors SR-AI/II or MARCO (macrophage receptor with collagenous structure) of macrophages. Mice deficient in scavenger receptors SR-AI/II or MARCO have reduced bacterial clearance capacity and increased mortality because they increase cytokine responses and inflammation in the lung following pneumococcal infection. However, anti-pneumococcal immunoglobulin G (IgG) mediates opsonisation and potentially enhances the Fc-gamma receptor (FcγR)-associated internalisation of pneumococci in macrophages. Interestingly, serum complement proteins also opsonise microbes or particles in an antibody dependent or independent manner. For instance, complement protein iC3b mediates the interaction of opsonised-pneumococci with CR3 (complement receptor 3), that increases pneumococcal phagocytosis by macrophages (Arredouani et al., 2006, Arredouani et al., 2004, Ali et al., 2003).

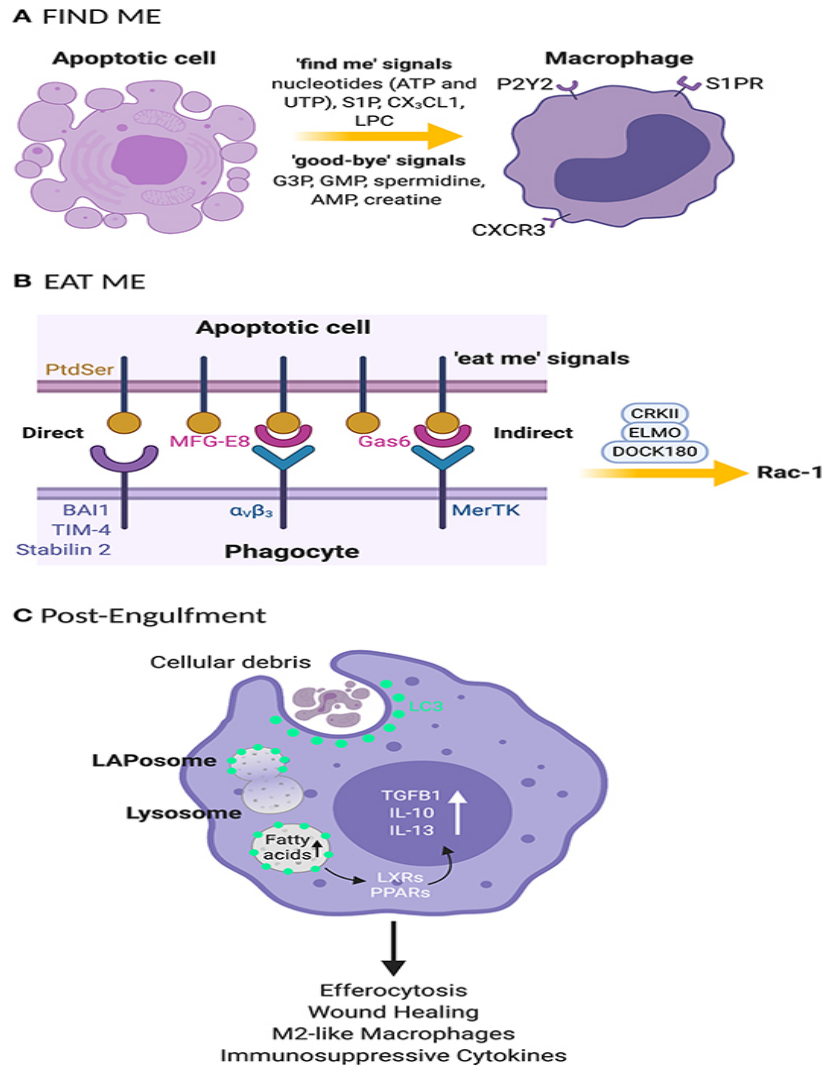


Figure 1.4. Phagocytosis

Apoptotic cell clearance by phagocytes is based on "find me" and "eat me" signals that signal their internalization. (Asare et al., 2020)

1.5 The Warburg Effect and metabolic reprogramming in Activated Macrophages

A metabolomics screen and microarray analysis in macrophages activated by LPS have confirmed that activated macrophages have increased hexokinase activity along with increased glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities (Ó Maoldomhnaigh et al., 2021), that lead to an increase in glycolysis and the pentose phosphate pathway in these cells (Williams and O'Neill, 2018b). Significantly, the switch in the metabolic state directly impacts on the inflammatory state of the cell. Inhibition of glycolysis by 2-deoxyglucose (2-DG) decreases the production of IL-1 in response to LPS stimulation but has no effect on TNF or IL-6 expression (Williams and O'Neill, 2018b).

Macrophages can be categorized mainly into two groups: M1 LPS or classically activated macrophages and M2 (IL-4-alternatively activated) macrophages. Inflammatory mediators, such as nitric oxide (NO), reactive oxygen species (ROS) and inflammatory cytokines are produced from M1 macrophages because they are more pro-inflammatory. M1 macrophages play an important role of the first line of defence against bacterial infections. Classically activated macrophages obtain energy via glycolysis during acute inflammation or anti-bacterial defence. In M1 macrophages, the tricarboxylic acid (TCA) cycle intermediate succinate regulates hypoxia-inducible factor-1 α (HIF-1 α) , which is responsible for driving the sustained production of the pro-inflammatory cytokine IL-1 β . On the other hand, M2 macrophages are anti-parasitic and also are important in secreting growth factors to assist in tissue repair and wound healing. M2 macrophages obtain their energy mainly through oxidative phosphorylation. M1 and M2 macrophages are thought to have different metabolic profiles because M1 macrophages are mainly found in hypoxic

environments and depend on glycolysis for their ATP production, produced through HIF-1 α (Galván-Peña and O'Neill, 2014b, Mills and O'Neill, 2014, Mills and O'Neill, 2016, Williams and O'Neill, 2018b).

In M2 macrophages, increased amino-sugar and nucleotide sugar metabolites are present, characterized by high amounts of UDP-N-acetyl-alpha-D- glucosamine (UDP-GlcNAc) and a corresponding increase in enzymes producing these metabolites (O'Neill, 2015). These results were consistent with previous studies indicating that highly glycosylated lectin/mannose receptors are markers for M2 macrophages (O'Neill, 2015).

Glycolysis is a biological process that breaks down glucose sugar that the cell has absorbed. It is important in energy metabolism because it rapidly produces ATP and other substrates that mitochondria can use to generate more ATP via oxidative phosphorylation (Chang et al., 2013). The glycolytic pathway provides the cell with necessary metabolic intermediates for the manufacture of amino and fatty acids, as well as ribose for nucleotide synthesis, in addition to providing energy. It can be quickly induced, which is perhaps needed for the rapid activation that occurs in M1 macrophages during infection. The decrease in ETC activity and oxygen respiration rates in M1 macrophages is thought to increase production of ROS, and also increase NADPH by the pentose phosphate pathway, which is required for the NADPH oxidase and the respiratory burst (Galván-Peña and O'Neill, 2014a). For M2 macrophages, severe activation is less of an issue, as their main function is in wound healing and anti-parasitic defence. M2 macrophages also do not generate ROS and therefore have a fully functional respiratory redox chain, allowing for oxidation of fatty acids. β -oxidation of fatty acids has, in fact, been shown to be anti-inflammatory, possibly because of a decrease in the production of prostaglandins (Williams and O'Neill, 2018b).

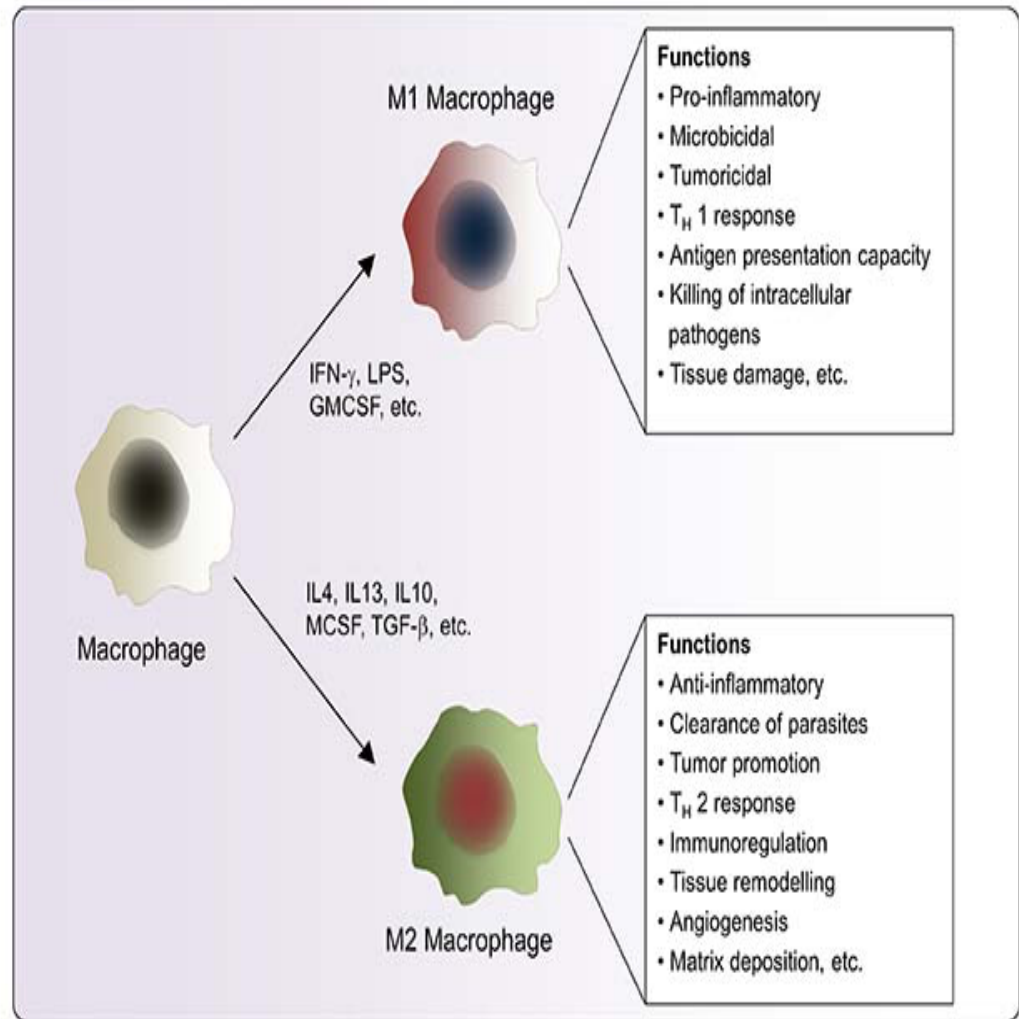


Figure 1.5. M1 and M2 macrophages functions.

M1 and M2 macrophages have different phenotypes and release different cytokines (Saqib et al., 2018).

Multiple changes in bioenergetic pathways in activated immune cells is known as metabolic reprogramming. Some of the main metabolic cascades that are modulated are glycolysis, oxidative phosphorylation, the pentose phosphate pathway, fatty acid oxidation and amino acid metabolism (Vijayan et al., 2019).

Switching from OXPHOS to aerobic glycolysis is known as the Warburg effect (Palsson-McDermott and O'Neill, 2013). The increase in glycolysis allows for a rapid increase in ATP production. This increase in ATP is required for metabolic processes such as biosynthesis and needed to maintain the mitochondrial membrane potential, which drops in LPS-activated cells. This allows the macrophages to stay bioenergetically functional during the host defence response (McGettrick and O'Neill, 2013). The increase in the pentose phosphate pathway can produce intermediates for biosynthesis, such as purines and pyrimidines. However, the shift in metabolism has a more specific consequence, which concerns changes in Tricarboxylic Acid cycle (TCA cycle) intermediates like succinate and citrate. This relates to “Warburg metabolism” as it is now understood (McGettrick and O'Neill, 2013).

Resting macrophages use an intact TCA cycle and oxidative phosphorylation (OXPHOS) to generate ATP. Nevertheless, in response to lipopolysaccharide (LPS) and other pro-inflammatory stimuli such as IFN- γ , TNF- α (M1) they undergo glycolytic reprogramming to generate ATP and lactate, while the Krebs cycle is broken, leading to accumulation of succinate and citrate, which act as signals to alter immune function. TCA cycle fragmentation in activated macrophages is characterized by citrate and succinate accumulation. Succinate that has accumulated in the mitochondrial matrix can be transferred to the cytosol via the dicarboxylate carrier SLC25A10, the mitochondrial citrate carrier (CIC), where it functions as a metabolic signal to promote the accumulation of HIF-

1 α and enhance inflammatory responses via metabolic repurposing in activated macrophages (Mills and O'Neill, 2014).

Accompanying this remodelling is a downregulation of OXPHOS and ATP production by the electron transport chain (ETC). In IL-4-activated macrophages (M2 macrophages), the TCA cycle and oxidative phosphorylation are complete and fatty acid oxidation (FAO) is used (Ryan and O'Neill, 2017, Krstic et al., 2017).

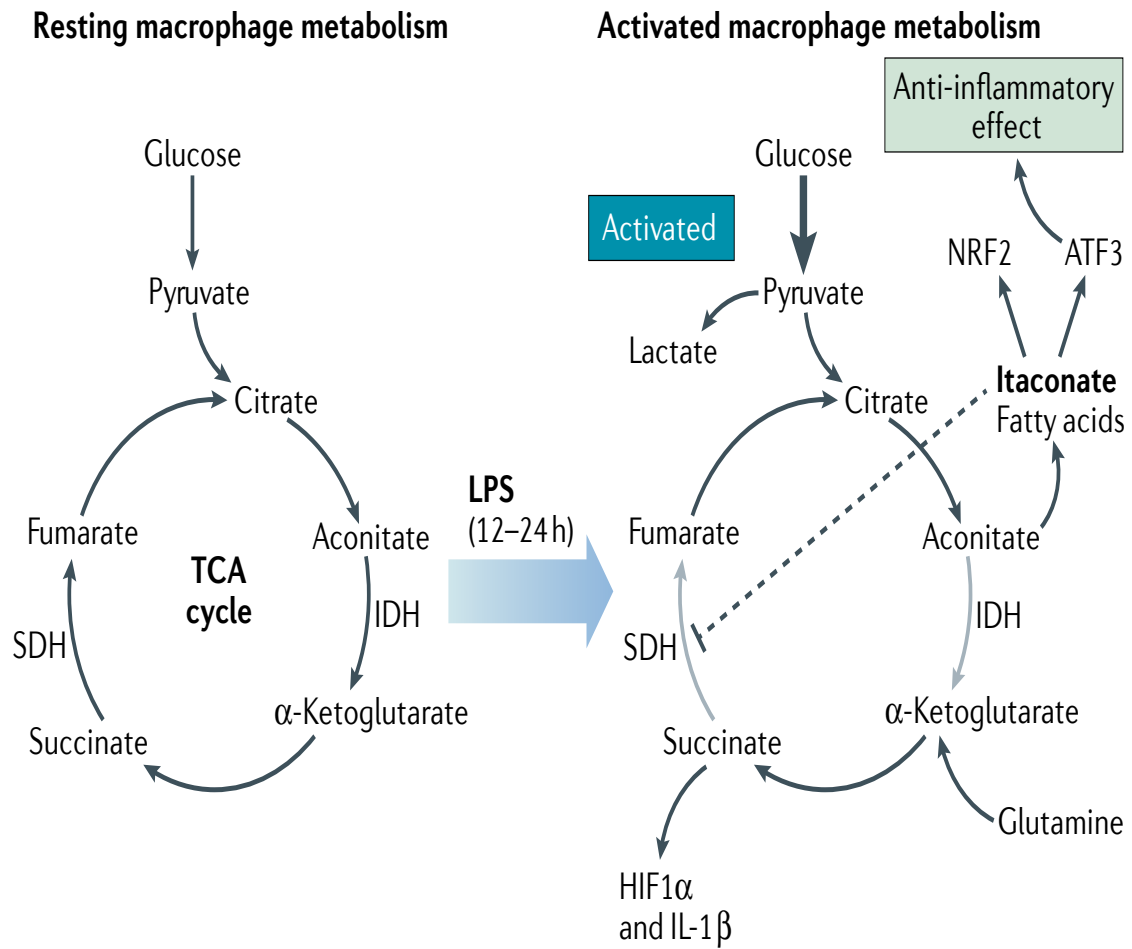


Figure 1.6. Resting and activating macrophages.

Activation of macrophages by LPS leads to increase glycolytic flux and the transition towards the tricarboxylic acid (TCA) cycle with high production of itaconate (O'Neill and Artyomov, 2019).

Many of metabolites with significant inflammatory signalling functions are generated by The TCA cycle. These metabolites accumulate in M1 macrophages possible because of the presence of some break points in the TCA cycle (Mills et al., 2017). There are two suggested breaks in the TCA cycle, one which occurs at the enzyme Succinate Dehydrogenase (SDH) leading to an accumulation of succinate, while other one occurs after citrate. SDH converts succinate to fumarate also serves as complex II of the electron transport chain (ETC). In LPS-activated macrophages, succinate acts as a signal leading to HIF-1 α activation under normoxia (Krstic et al., 2017).

The mechanism by which this succinate-driven inflammation occurs is thought to involve at least three parallel ways. The first way, through the stabilization of the transcription factor hypoxia-inducible factor-1 α (HIF-1 α), thereby supporting IL-1 β expression by inflammatory macrophages (Mills and O'Neill, 2016). The second way, enhanced mitochondrial membrane potential and succinate oxidation by SDH together encourage the generation of mitochondrial ROS that drive IL-1 β and inflammation via reverse electron transfer (RET). The third way, occurs when succinate is released from inflammatory macrophages and is recycled by the succinate receptor GPR91, generating a feedforward loop that increases HIF-1 α dependent IL- 1 β secretion (Van den Bossche et al., 2017). In addition, the increase in succinate leads to the inhibition of prolyl hydroxylases (PHDs). Succinate inhibits PHD activity by product inhibition. It prevents the decarboxylation of α -ketoglutarate (α -KG) to succinate, an essential co-reaction in the hydroxylation of targets by PHDs (McGettrick and O'Neill, 2013). Succinylation is also increased in the presence of LPS, a post-translational modification in that a succinyl group is added to a lysine residue of a protein. Increased succinylation of various proteins has been observed, including malate dehydrogenase, GAPDH, glutamate carrier 1, L-lactate dehydrogenase A chain, and transaldolase (McGettrick and O'Neill, 2013).

Macrophages are usually found in inflamed places, which are characterized by low oxygen levels (hypoxia) and glycolysis. HIF-1 α also plays an essential role in the response to pathological stress as well as environmental adaptation (Galván-Peña and O'Neill, 2014b, Mills and O'Neill, 2014). HIF-1 α has two isoforms, 1 and 2, in macrophage polarization. HIF-1 α has been associated with classical macrophage activation, while HIF-2 α has been linked to an M2 phenotype. A main mediator regulated by HIF-1 α is the M1 marker iNOS. Under hypoxic conditions, nitric oxide production through iNOS is HIF-1 α -dependent so implicating HIF-1 α in bacterial clearance. Moreover, HIF-1 α / macrophages have impaired capacity to clear both Gram-positive and Gram-negative bacteria. HIF-2 α controls IL-1 β production, which is associated with an M1 phenotype rather than M2 (Mills and O'Neill, 2014, Van den Bossche et al., 2017).

A second breakpoint in the TCA cycle occurs at isocitrate dehydrogenase (IDH), allows for the withdrawal of citrate from the cycle. This proves important for lipid biosynthesis in macrophages and to produce both pro- and anti-inflammatory mediators (Williams and O'Neill, 2018b). IDH expression is suppressed in inflammatory macrophages and leads to citrate accumulation. Citrate is used for lipogenesis and for the production of the proinflammatory mediators such as NO (Van den Bossche et al., 2017). Citrate can be redirected to itaconate by the enzyme encoded by immunoresponsive gene 1 (Irg1), which can inhibit SDH in response to LPS and thereby links citrate and succinate accumulation (Van den Bossche et al., 2017).

Itaconate has been recently indicated to be made by macrophages in response to LPS and certain infections and is generated by the mitochondria-associated enzyme cis-aconitate decarboxylase (Luan and Medzhitov, 2016). Itaconate is supposed to have anti-bacterial

function due to its ability to inhibit isocitrate lyase, a bacterial glyoxylate shunt enzyme and due to its bactericidal effect when added at supraphysiological concentrations to macrophage-free *S. enterica*, *M. tuberculosis*, and *L. pneumonophila* cultures. An effect of itaconate on mammalian enzymes was reported in the context of SDH inhibition (Lampropoulou et al., 2016).

1.6 Mitochondria

The mitochondrion is an organelle in the cytoplasm of cells that is known as power house of the eukaryotic cell (Andersson et al., 2003, Groen et al., 1982, Halestrap et al., 2000). Mitochondria differ in number and volume according to cell type and the requirement on how much energy the cell needs to produce. Also, the number of mitochondria in cells differs between different species (Prasai, 2017).

Mitochondria have many functions which are essential for cell survival. The TCA takes place in the mitochondrion. This cycle involves the oxidation of pyruvate, which comes from glucose, to form the molecule Acetyl-CoA. Acetyl-CoA is a source of carbons for the TCA cycle and is in turn oxidized for energy production (Johannsen and Ravussin, 2009, Weissig et al., 2004, Bouchier-Hayes et al., 2005). A main function of mitochondria in eukaryotes is oxidative phosphorylation (OXPHOS) whereby reducing equivalent products of TCA cycle metabolism (NADH, FADH₂), are oxidised by electron transfer complexes I and II, respectively, with the concomitant reduction of molecular oxygen to H₂O. During this process a protonmotive force generated by the transport of protons through complexes I, III and IV is used to drive ATP synthesis by complex V (ATP Synthase) in the mitochondrial inner membrane.

Additionally, mitochondria play a key role in some of the signalling pathways for apoptosis (Collins et al., 2002, Chan, 2006). When apoptosis occurs, the mitochondrial permeability transition pore (PTP) opens in conjunction with a depolarization of the mitochondria membrane potential ($\Delta\Psi_m$), release of pro-apoptotic factors from the inter-membrane space into the cytosol and inhibition of oxidative phosphorylation (Ly et al., 2003, Collins et al., 2002).

1.6.1 Mitochondrial Structure

Mitochondria are composed of two membranes, a mitochondrial outer membrane (MOM) and a mitochondrial inner membrane (MIM) that form the intermembrane space (IMS) and matrix compartments. These two membranes are made of phospholipid layers and proteins. The two mitochondrial membranes vary considerably in their structure and composition (Walther and Rapaport, 2009b). Mitochondria have their own DNA called mitochondrial DNA or mtDNA which is organized into discrete nucleoids in the matrix (Lecrenier et al., 1997).

A main function of the MOM is to regulate the exchange of molecules between the mitochondria and cytosol and other organelles, such as the endoplasmic reticulum (ER). Also, the MOM plays an important role in dynamic behaviour and morphology of the mitochondria. The MOM is normally considered to be permeable to small molecules (<1000 Da) because of the presence of many porins, and the voltage-dependent anion channels (VDAC) (Eskes et al., 2000). VDAC plays a key role in the increase of mitochondrial membrane permeability and mitochondria-mediated apoptosis (Tsujimoto and Shimizu, 2002). On the other hand, large proteins are transported through translocase

of the outer membrane (TOM) (Walther and Rapaport, 2009a). TOM is associated with MIM through translocase of the inner membrane (TIM), to aid translocation of certain proteins into the matrix.

The MIM has a larger surface area than the MOM. Usually, the MIM is depicted as forming multiple in-folds which form the cristae. Cristae have a variety of mitochondrial enzymes systems which are membrane bound (van der Laan et al., 2016). The inner membrane has much more restricted permeability, much like the plasma membrane of a cell. The inner membrane is also loaded with proteins involved in electron transport and ATP synthesis.

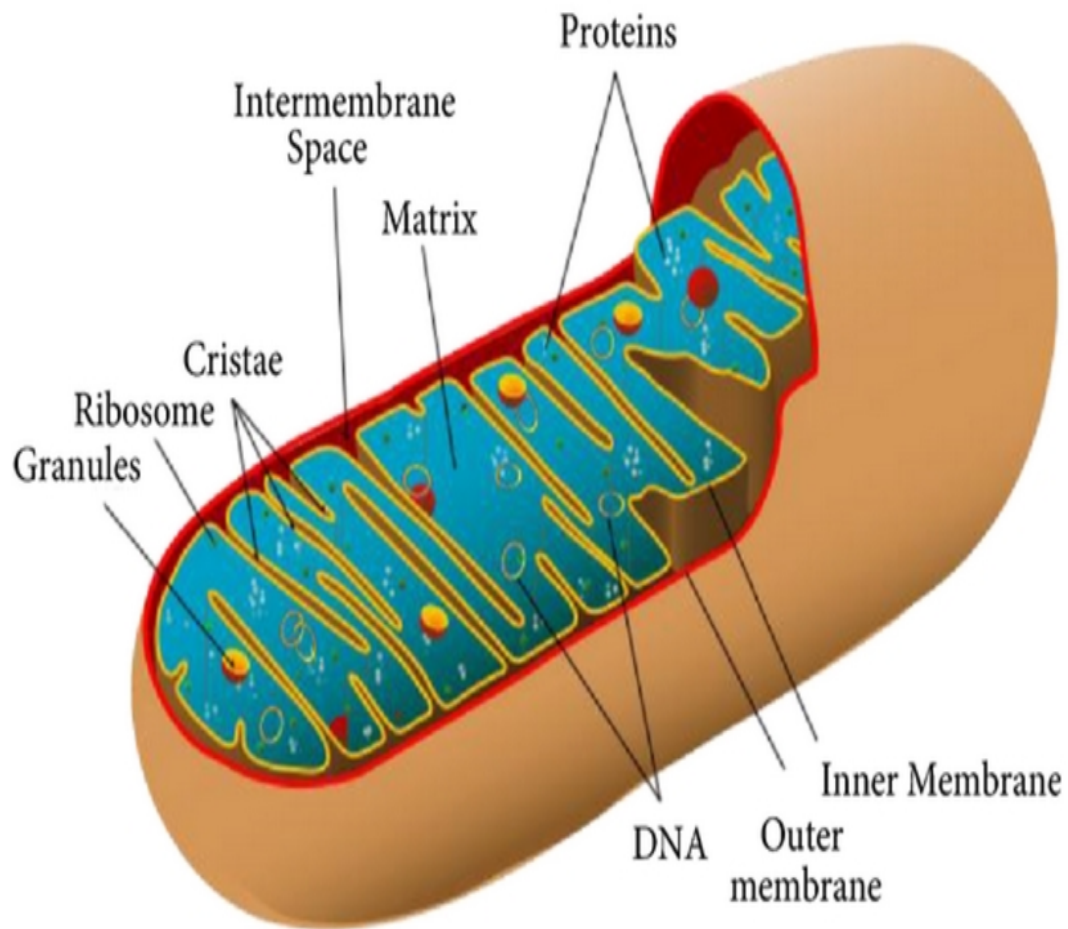


Figure 1.7. An overview of the structure of the mitochondria

A simple design of the basic structure of mitochondria showing the double membranes structure including the semi permeable smooth mitochondrial outer membrane (MOM) and the impermeable folded mitochondrial inner membrane (MIM). The intermembrane space (IMS) and the matrix are also shown (Petersen, 2013).

1.7 Oxidative Phosphorylation

Mitochondrial ATP synthase also referred to as complex V, EC (3.6.3.14) or F₁-F₀ATP synthase, is found in the MIM, associated with The Electron Transport Chain (ETC) (Wittig and Schägger, 2008). The main function of complex V is to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) in the mitochondrial matrix coupled to a reverse flow of protons back into the matrix the final step of OXPHOS (Jonckheere et al., 2012, Capaldi et al., 1994, Futai and Kanazawa, 1983).

The enzyme has a complex structure consisting of two main proteins F₁ and F₀ with multiple subunits, which are connected *via* a narrow stalk. F₁ is a soluble portion founded in mitochondrial matrix, which is responsible for catalysis. F₁ is composed of five subunits, which α and β subunits have three copies of each, while γ , δ ϵ subunits have one of each (Futai and Kanazawa, 1983). F₀ is bound to the IMM, which forms a H⁺-channel. F₀ is the hydrophobic complex composed of eight copies of c-ring and one copy of each subunit a, b, c, d, F₆ and the oligomycin sensitivity-conferring protein (OSCP) (Watt et al., 2010).

The OXPHOS process allows use of electrons which have passed through the electron transport chain in the MIM with translocation of H⁺ into the IMS to create the protonmotive force (made up of the pH gradient and mitochondrial membrane potential)(Koopman et al., 2013). The protonmotive force drives the synthesis of adenosine triphosphate (ATP) from adenoside diphosphate (ADP) and phosphate (Pi) through ATP synthase. Electrons from NADH and FADH₂, which come from the TCA cycle and Fatty acid and amino acid metabolism, are used as substrates for the ETC (Nolfi-Donagan et al., 2020).

1.8 Mitochondrial Membrane potential

Mitochondrial Membrane potential ($\Delta\Psi_M$) has an important role in ATP generation, Ca^{2+} uptake and storage, and the generation and detoxification of reactive oxygen species (ROS) (Nicholls, 2004). When electrons pass through the respiratory chain, the difference in reduction potential pumps protons across the MIM, generating a proton electrochemical potential gradient (Δp). The Δp consists of a Mitochondrial Membrane potential ($\Delta\Psi_M$) of ~120–180 mV and slight pH gradient and is the main bioenergetics factor that controls the mitochondrial ATP synthesis (Logan et al., 2016). $\Delta\Psi_M$ is regulated by leakage of H^+ and e^- transfer through the ETC (Nicholls and Budd, 2000).

1.9 The Electron Transport Chain

The mitochondrial ETC comprises four protein complexes (I, II, III and IV) and two mobile electron carriers, ubiquinone and cytochrome c. The first and largest complex is complex I, also known as NADH: ubiquinone oxidoreductase (CI; EC 1.6.5.3), a multi-subunit integral membrane complex of the mitochondrial electron transport chain (Janssen et al., 2006). This enzyme catalyses the oxidation of NADH to NAD^+ and the reduction of ubiquinone to ubiquinol while facilitating the transfer of four H^+ from the matrix to the IMS:



CI consists of 45 different subunits, 7 of which are encoded by the mitochondrial genome together having a molecular mass of close to 1 MDa (Koopman et al., 2007). It is an L-shaped complex with one arm in the plane of the inner mitochondrial membrane and the other lying adjacent to it, protruding into the mitochondrial matrix (Carroll et al., 2003).

Mitochondrial complex II (Succinate: ubiquinone oxidoreductase, CII or EC 1.3.5.1) cannot pump protons directly. It consists of four subunits: flavoprotein (Fp) and iron-sulfur protein (Ip), which are hydrophilic proteins, and two transmembrane proteins (CybL and Cybex). Complex II is a key membrane complex in the TCA cycle oxidising succinate to fumarate and reducing ubiquinone (Q) in the respiratory chain (Sun et al., 2005, Quinlan et al., 2012).

Complex III (CIII), which is also known as Cytochrome bc_1 or ubiquinol-cytochrome *c* oxidoreductase (EC 1.10.2.2), consists of 11 different polypeptide subunits with the total mass of a monomer is ~240 kD (Iwata et al., 1998). The primary function of CIII is oxidises ubiquinol to ubiquinone that lead to the pumping two protons from the matrix to the intermembrane space. Then e^- transfer between ubiquinol and cytochrome *c* via cytochromes *b* and c_1 of complex III results in the reduction of cytochrome *c* (Raha and Robinson, 2000). Ten of these subunits are encoded by nuclear DNA, and one, cytochrome *b*, is encoded by the mitochondrial genome (Fernandez-Vizarra et al., 2007). Cytochrome *C* is a small, mobile electron carrier whose reduction by $CoQH_2$ is mediated by CIII. During this “Q cycle”, $CoQH_2$ undergoes two cycles of reoxidation, which reduces two cytochrome *c* proteins and the translocation of 4 protons in total across the inner mitochondrial membrane (Barel et al., 2008).

Complex IV (CIV; EC 1.9.3.1), or cytochrome *C* oxidase, is the terminal enzyme in the ETC where molecular oxygen is reduced to the water (Capaldi, 1990). CIV is the large transmembrane protein complex consisting of 13 subunits; three of them are mitochondrially encoded subunits which represent almost 60% of the total mass of the complex and play an extremely important role in proton pumping and the transfer of electrons to the oxygen (Suthammarak et al., 2009).

Ubiquinone (also known as coenzyme Q and coenzyme Q₁₀) is a lipid-soluble molecule in all eukaryotic species and probably every cell (Wang and Hekimi, 2016, Olson and Rudney, 1983). It is located in the mitochondrial inner membrane, where it plays a role in electron transfer. Two main functions are related to this compound. As an electron transporter in the mitochondrial respiratory chain and as a fat-soluble antioxidant. Ubiquinone also acts as a cofactor in the functions of uncoupling proteins and as a modulator of mitochondrial permeability transition. (Dallner and Sindelar, 2000).

The most common form found in humans and animals is ubiquinone-10 which consists of a quinone head and ten isoprenoid units in the side chain. Ubiquinone functions as an electron transfer molecule in the mitochondrial inner membrane, transferring electrons from Complex I and Complex II to Complex III (Kawamukai, 2002). Ubiquinone exists in three central redox states: ubiquinone (UQ, oxidized), ubiquinol (UQH₂, reduced), and ubisemiquinone (partially reduced) (Ernster and Dallner, 1995).

Cytochrome c is a small, highly conserved water-soluble protein (~12 kDa) loosely bound to the mitochondrial inner membrane (Garrido et al., 2006). It exists in two forms, oxidised and reduced. The main function of cytochrome c is as an electron carrier in the electron transport chain where it transfers electrons from complex III to complex IV. It also plays an important role in apoptosis. Release of cytochrome c and other apoptosis-inducing factors from mitochondria via the permeable transition pore (PTP) initiates the process of cell death by apoptosis (Jiang and Wang, 2004).

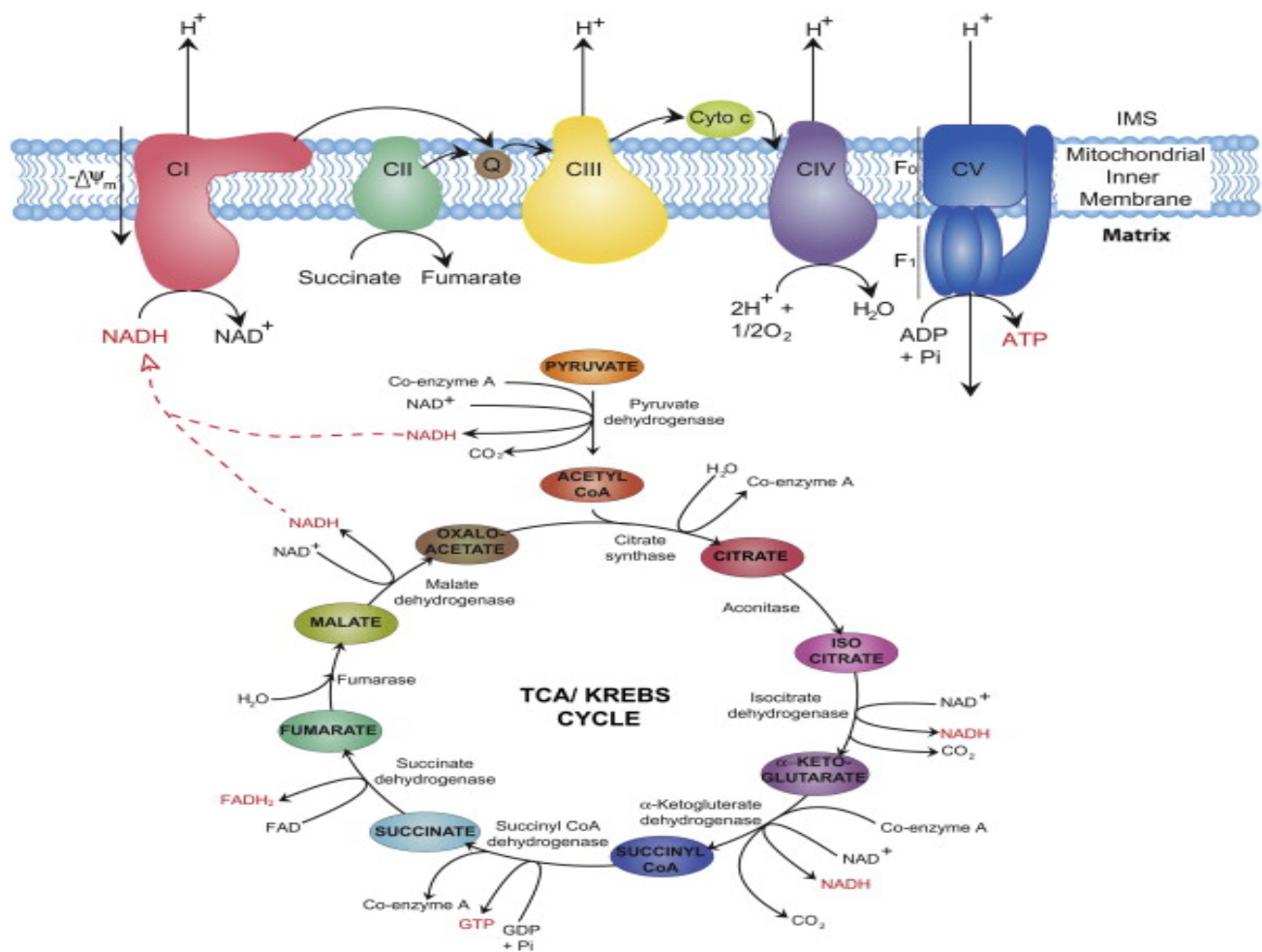


Figure 1.8. A summary of oxidative phosphorylation and the TCA cycle.

The electron transport chain comprises four complexes, complex I, II, III and IV, and two-electron carriers: ubiquinone and cytochrome c. Electrons donated by NADH and FADH₂ are passed through the complexes, and H⁺ are pumped into the intermembrane space (IMS) to create a pH gradient across the membrane and then used by complex V to create ATP (Osellame et al., 2012).

1.10 Metabolic control analysis

Metabolic control analysis (MCA) is one way to address the complexity of dynamic changes of species in a complex metabolic system and also can be used to examine the spread of control between components of the system, for example the respiratory chain complexes on mitochondrial respiration. In the 1960s, the rate-limiting step theory was the only approach to explain how enzymes in metabolic pathways were controlled. According to this idea, the rate of a complete reaction pathway is determined by the slowest step in the chain. This limitation in the theory did not take into account the potential that control of a reaction pathway's rate could be distributed across multiple phases or control points. MCA has two principal methods for analysis distribution of flux control in metabolic pathways, the bottom-up MCA approach and top-down MCA approach.

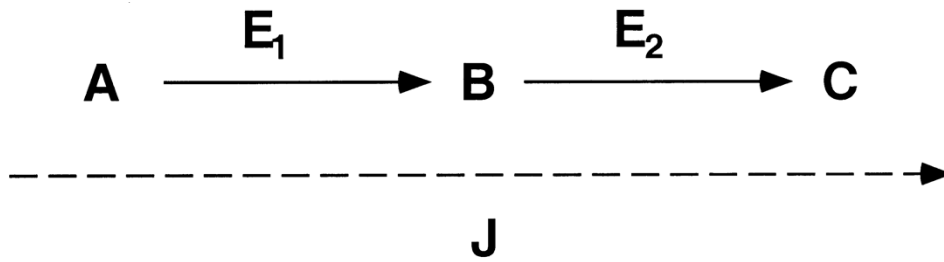
Bottom-up MCA uses irreversible inhibitors to titrate the activity of complexes of interest to identify their effects on the flux of a system. Bottom-up MCA could be used to determine two parameters of control, the flux control coefficient and the inhibition threshold of a particular step in the pathway.

The flux control coefficient (FCC) is defined as the degree of control exerted by the step on the entire rate of the pathway. The inhibition threshold gives information on the amount of inhibition of a particular action that can occur before an effect is seen on the overall pathway.

The first application of bottom-up MCA to mitochondrial oxidative phosphorylation was to determine the flux control coefficients of the various components of mitochondrial oxidative phosphorylation over respiration in isolated mitochondria. For instance, for the

electron transport chain complexes, adenine nucleotide carrier, proton leak, phosphate carrier and pyruvate carrier possess control over oxidative phosphorylation. Also, the level of control possessed by the electron transport chain complexes (complexes I - IV) over mitochondrial function, e.g., mitochondrial oxygen consumption and ATP synthesis can be investigated using MCA by calculate a flux control coefficient value and inhibition threshold for each of the complexes of interest (Groen et al., 1982, Tager et al., 1983).

Figure 1.9 shows activity such as an enzyme (E1) controls the overall flux (J), another enzyme activity (E2) or the size of a metabolite pool (B) only if changing the activity of E1 alters the magnitude of J, the activity of E2 or the size of B. Control is quite distinct from regulation, which implies a specific endpoint that is physiologically relevant, and system components with substantial control may not regulate the system in vivo.



$$\text{Control Coefficient} = C_p^V = \lim_{\delta P \rightarrow 0} \frac{\delta V/V}{\delta P/P} = \frac{\partial V}{\partial P} \cdot \frac{P}{V} = \frac{\partial \ln[V]}{\partial \ln P}$$

$$\text{Elasticity Coefficient} = \epsilon_S^E = \frac{\partial v_E}{\partial S} \cdot \frac{S}{v} = \frac{\partial \ln v_E}{\partial \ln S}$$

$$\text{Summation Theorem} = \sum_{i=1}^n C_i^J = 1$$

$$\text{Connectivity Theorem} = \sum_{i=1}^n C_i^J \epsilon_S^i = 0$$

Figure 1.9. Application of MCA to a simple metabolic pathway.

The upper section shows a simple metabolic pathway comprising the metabolite pools A, B and C interconnected by the enzymes E1 and E2, while J is the overall flux through the pathway. In the lower section, some of the equations used in MCA are shown, in this study we are used the first and third equations only (Murphy, 2001).

However, in a top-down elasticity analysis, the metabolic system is grouped into several reaction blocks that supply or consume common intermediates. By calculating the elasticities of these blocks to the common intermediate, the overall flux control coefficients of the blocks can be calculated (BROWN et al., 1990).

Figure 1.10 shows that, when applying the top-down approach to mitochondrial oxidative phosphorylation, the system is usually divided into three blocks connected by membrane potential. The Δp_H component of Δp is generally considered negligible relative to the far larger and more quickly measured membrane potential. Membrane potentials are generated by one reaction block and consumed by two other blocks (substrate transport, metabolism and the respiratory chain), proton leaks through the mitochondrial inner membrane, and ATP synthesis, transport, and turnover reactions.

The elasticities of the three subsystems to their common intermediate are determined from appropriate uncoupler or inhibitor titrations and the connectivity theorem is used to calculate the flux control coefficients of the three blocks over respiration, ATP synthesis or the proton leaks (BROWN et al., 1990, HAFNER et al., 1990).

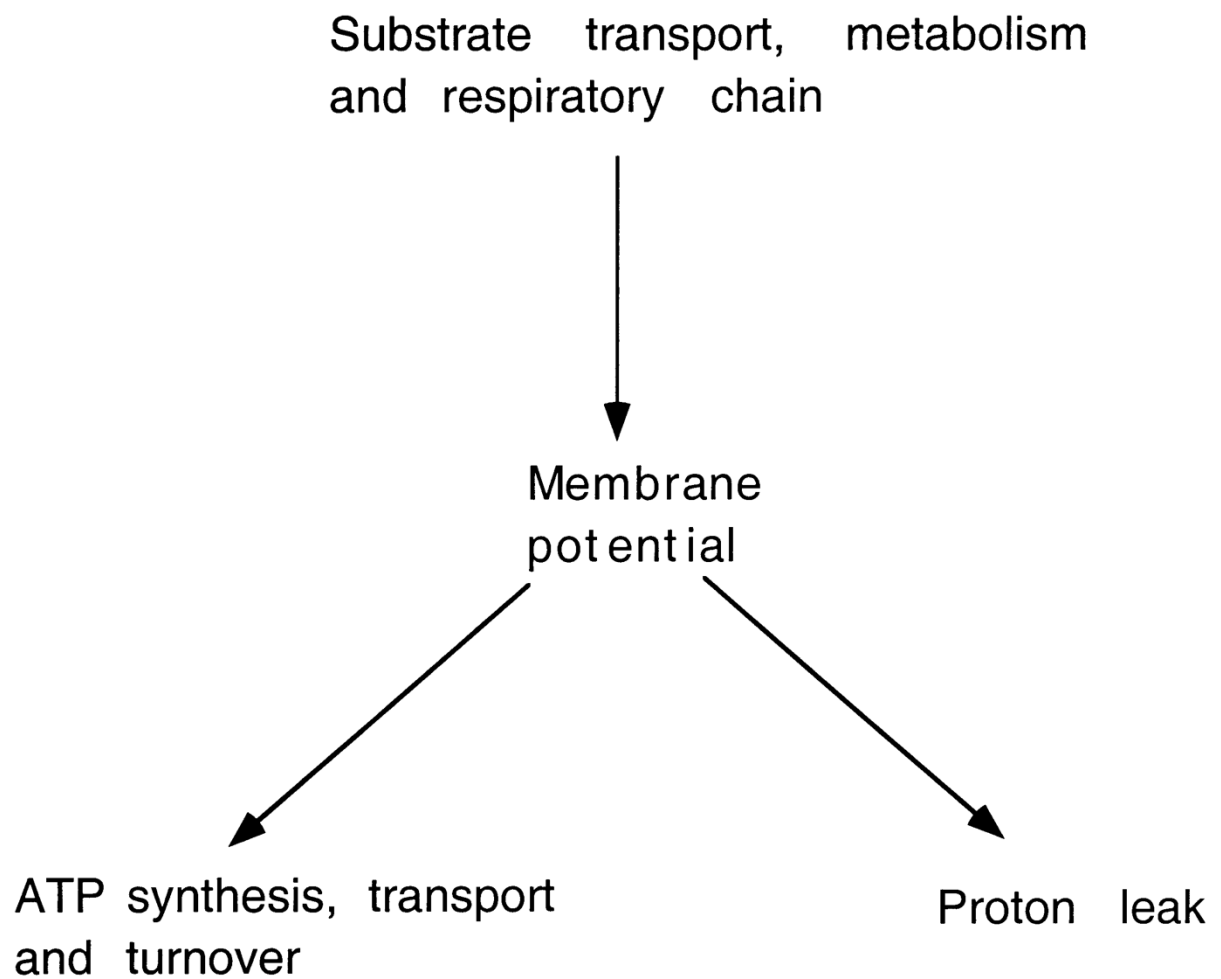


Figure 1.10. Top-down analysis of oxidative phosphorylation.

The top-down approach to mitochondrial oxidative phosphorylation divides the system into three blocks that are connected by the membrane potential (Murphy, 2001).

1.11 Reactive Oxygen Species (ROS)

In most mammalian cells mitochondria are an important source of reactive oxygen species (ROS), which are molecules derived from oxygen (O_2) that can readily oxidise other molecules. The most common forms of ROS are superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$). The majority of $O_2^{\bullet -}$ generation occurs at the electron transport chain (ETC) under physiological conditions as 1-2% of electrons leak out of the ETC during e- transfer and before reaching Complex IV (Brand, 2016). $O_2^{\bullet -}$ is produced by the one-electron reduction of O_2 at complexes I and III. However, some studies suggest that CIII only produced ROS when artificially inhibited (Adam-Vizi and Chinopoulos, 2006). CI production of ROS occurs by two mechanisms. The first one, when the matrix NADH/ NAD^+ ratio is high, leads to a reduced flavin mononucleotide site on CI, which will produce $O_2^{\bullet -}$ (Votyakova and Reynolds, 2001). When electrons from ubiquinol are returned to respiratory complex I and reduce NAD^+ to NADH, reverse electron transport (RET) is produced. Significant ROS production occurs during this procedure (Scialò et al., 2017). The second mechanism is when electron supply reduces the CoQ pool in the presence of high proton-motive force, pushing the electrons back from $CoQH_2$ into CI, reducing NAD^+ to NADH at the Flavin mononucleotide site, which is known as reverse electron transport (RET) (Chance and Hollunger, 1961a). CIII production of ROS occurs when it is supplied with $CoQH_2$ and when the Qi site is inhibited by antimycin A from the reaction O_2 with a ubisemiquinone bound to the Qo site (Zhang et al., 1998). However, the Qo site ubisemiquinone is not stabilised and $O_2^{\bullet -}$ production by CIII is low, so not physiologically relevant in the absence of antimycin A (Forman and Azzi, 1997). When $O_2^{\bullet -}$ accumulate, cell damage occur through oxidation of iron-sulfur clusters (Fridovich, 1997).

The most unstable form of ROS is $\bullet\text{OH}$, which is generated via the Fenton reaction from $\text{O}_2\bullet$ and H_2O_2 . This molecule can cause significant damage through the oxidation of proteins, lipids and DNA (Dizdaroglu and Jaruga, 2012). Superoxide is converted to H_2O_2 by superoxide dismutase (SODs). ROS have many critical physiological functions in signal transduction and autophagy processes, and maintain cell function if the production and clearance are kept well balanced. The cell has both enzymatic and non- enzymatic antioxidants to keep the levels balanced, which is essential in protecting cells from damage.

1.12 Antioxidant Systems in Mitochondria

Mitochondria have been considered a major contributor to cellular oxidative damage by generating reactive oxygen species. Cells are protected against oxidative damage by multiple enzymatic mechanisms and by antioxidant molecules. These antioxidants inhibit cellular damage mainly through their free radical scavenging properties. Antioxidants act as radical scavengers, hydrogen donors, electron donors and enzyme inhibitors. There are two main H_2O_2 scavengers in mitochondria, namely, the glutathione (GSH) and thioredoxin (Trx) systems (Aon et al., 2012). The main enzymes of these pathways are glutathione reductase (GR) and thioredoxin reductase (TR), respectively. NADPH is the primary electron donor of the large-capacity GSH (GSH and GSSG) and the Trx (Trx_{red} and Trx_{ox}) systems responsible for scavenging H_2O_2 (see Fig. 1.11).

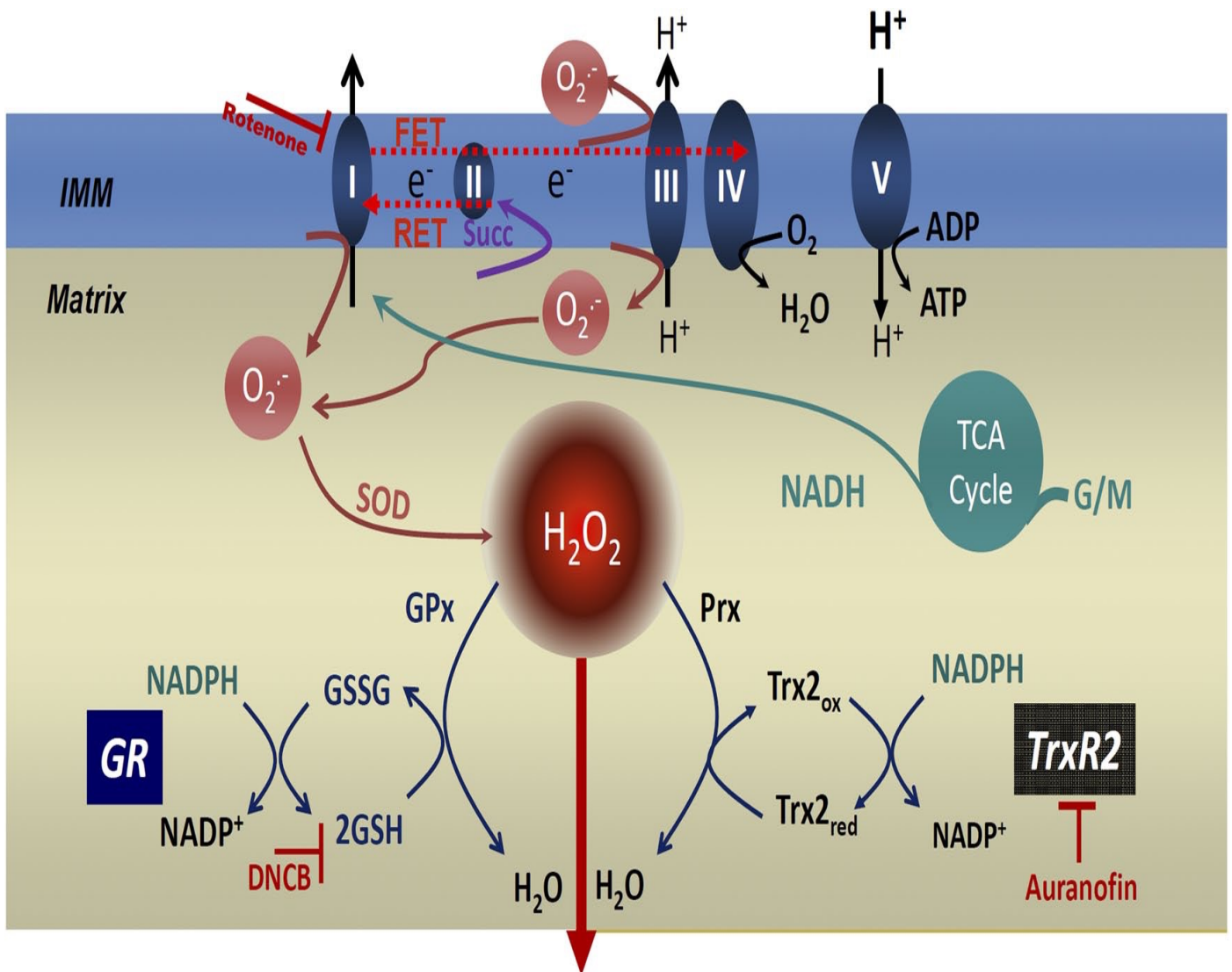


Figure 1.11. The production and scavenging of mitochondrial ROS.

The scheme shows the antioxidant systems in mitochondria: the GSH and Trx systems as the major H_2O_2 scavengers. Complexes I and III can produce superoxide anions via reverse electron transport (RET) or forward electron transport (FET). Superoxide dismutase (SOD) can convert superoxide to hydrogen peroxide. The hydrogen peroxide produced can be scavenged by both the GSH and Trx systems (Aon et al., 2012).

1.13 Aims of research

Mitochondria are organelles involved in the generation of ATP, storage of intracellular calcium ions, regulation of the apoptotic pathway as well as numerous cell signalling pathways. Immune activation of macrophages with LPS is known to initiate a metabolic switching from oxidative phosphorylation to glycolysis, however, the bioenergetic mechanisms that underlie such events are not fully understood.

Aim 1: To study the effects of LPS-activation of mouse iBMDMs and primary BMDMs on mitochondrial electron transport chain activities and elucidate how the metabolite, itaconate, regulates these activities.

Aim 2: To undertake a metabolic control analysis study of mitochondrial function in activated and un-activated macrophages. This includes ETC titrations and assaying basal and maximal oxygen respiration rates. Calculating the flux control coefficients of the ETC complexes in activated and un-activated conditions are an important objective in this study. The results will provide additional insight into the mechanisms by which mitochondria control the immune response in macrophages.

Aim 3: To investigate the effect of inhibition of complex I, II, and III activities on ROS production in LPS-activated iBMDMs and BMDMs, and to correlate mitochondrial ROS production and oxygen consumption with proinflammatory cytokine secretion.

These aims will enable further delineation of mitochondrial bioenergetics in LPS-activated macrophages and elucidation of the relationship between mitochondrial ETC complex activities, reactive oxygen species and pro-inflammatory cytokine secretion.

CHAPTER 2

Materials and General Methods

2.1 Materials and Reagents

ATP, antimycin A, ascorbic acid, bovine serum albumin, calcium chloride, cytochrome c, decylubiquinone, dipotassium hydrogen phosphate (monobasic), D-glucose, DMSO, ethanol, EDTA, , FCCP, fetal bovine serum, ferricyanide, magnesium chloride, magnesium sulphate, methanol, NADH, penicillin-streptomycin solution, phosphate buffered saline, poly-D-lysine, potassium cyanide, potassium EDTA, potassium phosphate, rotenone, chloride, sodium pyruvate, succinate, sucrose, , trypsin-EDTA, Itaconate, tetramethyl ethylenediamine, Trizma base, diethyl succinate, dimethyl succinate (Kosher),dimethyl fumarate, vigabatrin, phenformin hydrochloride, D-serine, azathioprine, 6-thioguanine, hydrocortisone, prednisolone, progesterone, corticosterone, γ -Amino-n-butyric acid, ivermectin, methotrexate and D- α -hydroxyglutaric acid were all purchased from Sigma Aldrich, C.o., Poole, Dorset, U.K.

Thionicotinamid-TPN was obtained from P-L-Biochemicals Inc, Milwaukee,Wis.53205.

Carboxymethoxylamind hemihydrochloride,98% was obtained from ACROS ORGANICS, Japan.

6-mercaptopurine and 6-methylnicotinamide were purchased from fluorochem Ltd,Unit 14, Graphite Way, Hadfield, Sk13 1QH, UK.

Coomassie (Bradford) Protein Kit and Trypan blue solution were obtained from Thermo Scientific, UK.

LPS and Auranofin were obtained from Enzo Life Sciences, Inc.

DMEM (Dulbecco's Modified Eagle Medium), high glucose (4500 mg/L) and RPMI 1640 (1x), L- GLUTAMINE were obtained from Thermo Fisher.

XF assay buffer, Seahorse cell culture plates, utility plates and calibrant solution were obtained from Agilent.

Enzyme Linked Immunosorbent Assay (ELISA) quantification of supernatant cytokine content was carried out using ELISA MAX from BioLegend for IL-1 β and DuoSet for TNF α .

2.2 Pipetting

A set of Gilson Pipetteman Classic TM was used to pipette volumes from 200 μ l to 5 μ l.

2.3 Preparation of Solution and pH measurements

All reagents were weighed on a Mettler College Model analytical balance for weights above 5 g, while a Mettler K7t top-loading balance was used for weights below 5g. All aqueous solutions were prepared using deionized water from a Millipore Elix advantage ten water purification system. All solutions were adjusted to the required pH using a Corning pH meter, Model 240, which was calibrated daily using standard buffer solution of pH 4.0, 7.0 and 10.0.

2.4 Buffers

Krebs buffer: 3 mM KCl, 140 mM NaCl, 25 mM Tris-HCl and 2 mM MgCl₂. 10 mM glucose, pH 7.4.

Complex I buffer: 25 mM KH₂PO₄, 10 mM MgCl₂, pH 7.4.

Complex II buffer: 25 mM KH₂PO₄, pH 7.5.

Complex II/III buffer: 100 mM KH₂PO₄, 0.3 mM K-EDTA, pH 7.4.

Complex IV buffer: 100 mM KH₂PO₄, pH 7.0.

Citrate synthase buffer: 200mM Tris with Triton X-100 (0.2% (vol/vol)), pH 8.0.

STE (Sucrose-Tris- EDTA) buffer: 320 mM Sucrose, 10 mM Tris, 1 mM EDTA, pH 8.

ELISA Buffers:

10X PBS

NaCl: 80g, KCl: 2g, Na₂HPO₄: 14.4g, KH₂PO₄: 2.4g and ddH₂O: 800ml

Dissolve well, then adjust pH to 7.4. Bring up the volume to 1 L with ddH₂O ,then sterilize by autoclaving.

1X Wash Buffer

10X PBS: 100 ml, ddH₂O: 900ml and Tween-20: 50ul

1X Blocking Buffer

10X PBS: 10ml, ddH₂O: 90ml and BSA: 1g.Dissolve well.

Stop Solution

0.5M H₂SO₄

2.5 Cell Culture Cell

Cell culture was done in Class II HEPA-filtered biosafety cabinets. Cells were split every 48 h. Cells were counted using Trypan Blue dye with a Countess Automated Cell Counter (Life Technology).

2.5.1 iBMDMs Culture

DMEM GlutaMAX media supplemented with 10% v/v foetal bovine serum (FBS) and 1% v/v penicillin-streptomycin was used for cell culture. iBMDMs were cultured in this media and kept in HEPA-filtered humidified incubator at 37°C in 5% CO₂ (Thermo Fisher Scientific).

2.5.2 BMDMs Culture

Femur and tibia bones were isolated from a mouse, the bone marrow flushed out from each mouse into a separate falcon tubes containing 5 mL of DMEM media by inserting the top of the syringe filled with DMEM into the larger orifice and flushed through. This was spun down at 1500 rpm for 5 minutes. The supernatant was decanted. 3 mL of ACL (Ammonium-Chloride) Lysing Buffer was added for 2 min to remove all the Erythrocytes, carefully pipetting up and down twice with P1000 Gilson pipette before leaving for 3 min. The reaction was stopped by adding 7 mL of DMEM. A cell strainer was placed on top of fresh falcon and transfer solution through into fresh falcon. This was spun @ 1500 rpm for 5 min and the supernatant removed. The pellet was re-suspended in 24 mL of DMEM media and 6 mL of 20% L929 media and 10 mL plated of each into 10 cm dishes.

On day 7, cells were scraped, counted and seeded at 1×10^6 / mL in DMEM 10% FBS, 5% L929-conditioned medium one day prior to experiments.

2.5.3 Mitochondrial Isolation

Mitochondrial isolation was performed using differential centrifugation. A two-step centrifugation was carried out, firstly, a low-speed spin to remove intact cells, cell and tissue debris, and nuclei from whole-cell extracts. The second high-speed spin was to concentrate mitochondria and separate them from other organelles. A pellet of $2-4 \times 10^7$ BMDMs were generated by centrifugation at 1500 rpm for 5 minutes. The supernatant was carefully removed and discarded. The pellet was resuspended in 1 mL STE (Sucrose-Tris-EDTA) buffer and vortexed and topped up with 1 mL STE buffer. The low speed spin ($1000 \times g$) was carried out for 10 minutes. After that, the supernatant was transferred to a

fresh 2 ml Eppendorf tube and centrifuged at high speed (12,000 x g) for 15 min. The supernatant was transferred to a fresh tube and stored at -80 °C for further experiments. The brown pellet is the pure Mitochondria.

2.5.4 Culture of L929 cells and generation of L929-conditioned medium

The L929 cell line is a murine fibroblast cell line. L929 cells produce the M-CSF required for macrophage differentiation. L929-conditioned medium was generated by seeding 4.7×10^5 L929 cells in a 75 cm² culture flask in 55 mL of medium (DMEM GlutaMAX I, 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin). Cells were left to become over-confluent for seven days. The supernatant was then collected and sterile-filtered using a 0.45 µM filter. The filtered supernatant was pooled, aliquoted and frozen at - 80°C.

2.6 Centrifugation

An Eppendorf Centrifuge 5810 R was used for centrifugation of large volumes. During cell culture work, iBMDMs were spun at 1200 rpm, and BMDMs were spun at 1500 rpm. A bench-top Eppendorf Centrifuge 5415 R was used centrifugation volumes less than 2 mL and for sample preparations for enzymatic assays.

2.7 Spectrophotometry

Two different Spectrophotometers were used for different assays. Absorbance and fluorescence assays were read using 96-well plate format with a SpectraMax M3 plate reader SoftMax Pro 6.2.1 software. Clear 96-well plates were used for absorbance readings,

and black 96- well plates were used for fluorescence readings. For kinetic readings, plates were read overtime at 37°C. Plates were shaken before reads and in between reads when running kinetic assays. A Shimadzu UV-VIS Spectrophotometer UV-2600 with different attachments was used to assay the activities of different ETC complexes.

2.8 Protein Estimation- Bradford Assay

The Bradford assay was used to estimate protein concentration. The principle of this assay is based on the binding of Coomassie Blue G250 dye to proteins in an acidic medium, resulting in a colour change from brown to blue at 595 nm. A stock of bovine serum albumin (2 mg/mL) was used to create a set of standards whose concentration is known (0-2000 µg/mL). A 96-well plate was used by pipetting 250 µL Bradford reagent into the wells and 5 µL sample/standard, while blank wells consisted of adding 5 µL dH₂O, in triplicate and then incubated for 10 min in the dark at room temperature. **Figure 2.1** showed that A BSA standard curve was created daily for the Bradford assay.

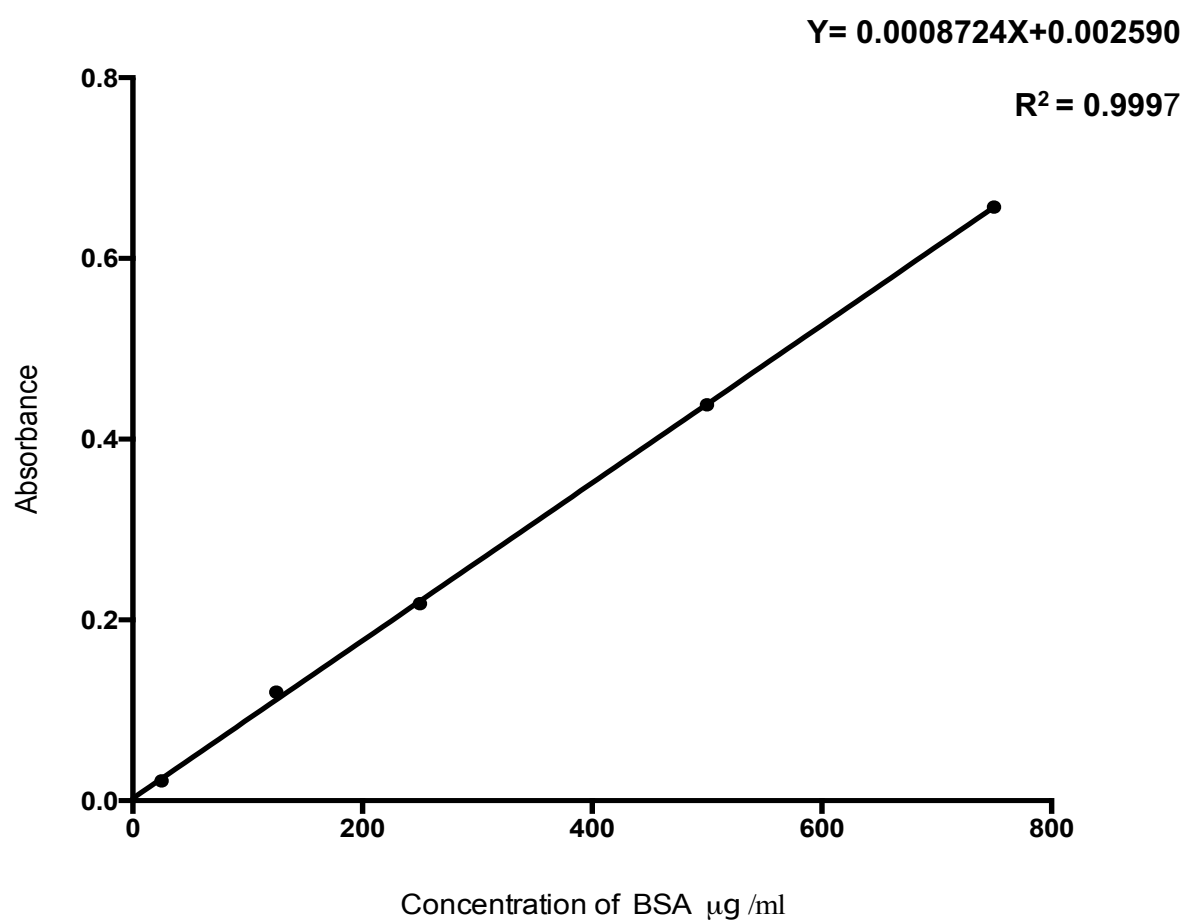


Figure 2.1: Bradford Standard Curve

A representative standard curve for a Bradford protein estimation generated for each experiment, where the slope is defined by the equation $y = mx + c$ and the R^2 value estimates goodness of fit of data linear regression. Data is presented as mean $\pm$ SD.

2.9 Electron Transport Chain Complex Assays

iBMDMs and BMDMs were prepared and protein concentration was determined as described in section 2.8 using Bradford assay. Samples were then freeze-fractured three times in liquid nitrogen before the activity of complex I, complex II, complex II/III complex and IV activities to compare them between iBMDMs $\pm$ 4h ,24 h LPS. Experiments were carried out at 37°C and Complex I, II, II/III and Citrate synthase rates were expressed as pmol/min/mg and complex IV as k/min/mg.

2.9.1 Complex I Assay

The complex I (NADH-CoQ reductase) activity was based on a protocol described by Darley-Usmar (1987). The assay measures the oxidation of NADH to NAD⁺ with a concomitant decrease in absorbance at 340 nm at 37°C. The reaction mixture contained assay buffer, 0.2 mM NADH, 2.5 mg fatty acid free-BSA, 1 mM KCN and 0.05 mg/mL protein for iBMDMs and BMDMs cells lysate while 0.01 mg/mL protein for mitochondria isolated from BMDMs. The assay was started, and activity was measured for 2-3 minutes to make sure there is stable baseline. Decylubiquinone (50 μ M) was added to each cuvette to start the reaction in the spectrophotometer, and the reaction rate was followed for 6-9 minutes. Rotenone (10 μ M) was later added to obtain the rotenone sensitive rates, and the reaction was further followed for 5 minutes. The rotenone sensitive rates were subtracted from the decylubiquinone rates, and the Beer-Lambert Law ($A = \epsilon \times c \times L$) was used to obtain the specific activity of the enzyme. The specific activity was expressed as nmol/min/mg of protein using the equation below:

$$\text{Specific activity (nmol/min/mg)} = \Delta \text{Absorbance/min} \times 1000 \times \text{samples volume (mL)} \times [\text{protein}] \text{ mg/mL}$$

2.9.2 Complex II Assay

To a 1 mL cuvette, add 645 μ L of potassium phosphate buffer (0.5 M, pH 7.5), 1 mg /ml BSA, 0.3 mM KCN, 20 mM succinate, the 0.2 mg/mL iBMDMs, 0.1 mg/mL BMDMs cell lysate and 0.002 mg/ml protein for mitochondria isolated from BMDMs follow by 75 μ M of DCPIP. Mix by inverting the cuvette and incubate inside the spectrophotometer at 37 °C for 10 min. Read the baseline activity at 600 nm for the last 2 minutes. Start the reaction by adding DQ (50 μ M) , mix by inversion and follow the decrease of absorbance at 600 nm for 3 minutes. The specific activity was expressed as nmol/min/mg of protein.

2.9.3 Complex II/III Assay

Complex II/III activity was measured using a method based on that King (1967). The reaction follows the reduction of cytochrome c using succinate as the electron donor, measured at $\lambda=550$ nm at 37°C. The reaction mixture contained assay buffer, 20 mM succinate, 1 mM KCN and 0.1 mg/mL protein iBMDMs, BMDMs cell lysate and 0.01 mg/ml protein for mitochondria isolated from BMDMs. The reaction was started by the addition of 100 μ M oxidised cytochrome c in final volume of 1000 μ L. The resulting increase in absorbance was measured for 6-9 min. Antimycin A (1 μ M) was added to inhibit the reaction and the antimycin A sensitive rates were followed for 5 min. The antimycin A sensitive rates were subtract from the initial rates and the specific activity of the enzyme was calculated using the Beer-Lambert Law ($A=\epsilon \times c \times L$) and the specific activity was expressed as nmol/min/mg of protein.

2.9.4 Complex IV Assay

The protocol used to measure complex IV activity was based on that of Wharton and Tzagaloff (1967). The assay follows the oxidation of reduced cytochrome c by complex IV (cytochrome c oxidase) and is measured as a decrease in absorbance at $\lambda=550$ nm at 37°C. The reaction mixture contained 50 μ M reduced cytochrome c and dH₂O with a final volume of 1 ml in the cuvette. The reaction was started by adding 0.2 mg/mL iBMDMs, BMDMs cell lysate and 0.001 mg/ml protein for mitochondria isolated from BMDMs to the cuvette and the reaction was followed for 10 minutes. The activity is non-linear and is therefore expressed as a zero order-rate constant (k/min/mg).

2.9.4.1 Preparation of reduced Cytochrome c:

Oxidised cytochrome c (25 mg/ 2.5 mL) was reduced by the addition of a few crystals of ascorbic acid. A colour change from a deep red to a brighter pink was observed. Ascorbic acid was removed by passing this solution through a PD₁₀ gel filtration column. Prior to the addition of this solution, the column had been rinsed with 50 mL 1:10 (v/v) dilution of potassium phosphate buffer (100 mM), pH 7.0. Oxidised cytochrome c was added to the column at a maximum volume of 2.5 mL. The column was eluted with 50 mL 1:10 (v/v) dilution of assay buffer. The reduced cytochrome c was obtained by collecting the darkest band of eluent from the column. The concentration of reduced cytochrome c was determined on a Cary UV spectrophotometer using 10 μ l of mM ferricyanide to oxidise cytochrome c.

2.9.5 Citrate Synthase

Citrate synthase is an enzyme in the TCA cycle. It is localized in the mitochondrial matrix and is often used as a quantitative marker enzyme for the content of mitochondria within cell or tissue samples. Citrate synthase enzyme activity was measured

spectrophotometrically by a coloured coupled reaction using a method adapted from that originally described by (Srere, 1969). The activity of citrate synthase was determined by monitoring the rate of production of thionitrobenzoic acid (TNB) at a wavelength of 412 nm. iBMDMs and BMDMs cells lysate (0.033 mg/ml) and pure mitochondria (0.003 mg/ml) were incubated in a 1 ml cuvette with tris buffer (200 mM, pH 8.1) and the following reaction components were added; 5,5' dithiobis-(2-nitrobenzoic acid (DNTB) (100 μ M), acetyl coenzyme A (300 μ M) and Triton X-100 (0.1%). A blank rate was measured for 2 min before oxaloacetate (500 μ M) was added to initiate the reaction and an increase in absorbance was measured for 3 min at 412 nm.

2.10 Measurement of cellular reactive oxygen species (ROS)

ROS was measured using dihydroethidium, a fluorescent dye which binds specifically to superoxide and hydrogen peroxide. DHE, a non-selective fluorescent dye for reactive oxygen species, was performed in iBMDMs and BMDMs. DHE is oxidised to 2-hydroxyethidium by superoxide with an excitation wavelength of 500-530 nm and emission wavelength of 590-620 nm,] allowing it to be measured fluorometrically. iBMDMs and BMDMs were loaded with 20 μ M DHE in PBS + 10 mM glucose, after which increasing concentrations of inhibitors were added. Cells were incubated with inhibitors for 30 min before spinning down and washing twice with PBS. Fluorescence was analysed using the PE channel after gating on iBMDMs and BMDMs on BD FACS Canto.

2.11 Assaying Metabolic Activity

2.11.1 Examination of iBMDMs and BMDMs oxygen consumption rates

A Seahorse XFe96 flux analyser was used for the measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). These measurements were used as an indicator of oxidative phosphorylation rates and glycolysis, respectively. A day prior to the beginning of the experiment, a Seahorse cartridge was hydrated using a seahorse calibration buffer in a CO₂ incubator at 37°C. Also day prior to the beginning of the experiment, the cell culture plate was coated with poly-d lysine, and iBMDM were seeded at a density of 50,000 cells/well in a Seahorse 96 well cell plate. BMDM were seeded at a density of 100,000 cells/well in a Seahorse 96 well cell plate. The plates were placed in a CO₂ incubator for 24 h prior to the initiation of the experiment.

On the day of the experiment, Seahorse media was supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine and warmed to 37°C. The inhibitors in Seahorse media were added to designated ports of the Seahorse cartridge plate. The Seahorse cartridge was then calibrated in an analyser, and a cell plate was placed in the Seahorse machine.

To determine the rate of OXPHOS for MCA (i.e. cellular respiration measured as OCR), we used rotenone titration (0-1000 nM), TTFA (0-200 µM) or antimycin A (0-1000 nM) as the ATP synthase inhibitor. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential as the electron transport chain accelerator, rotenone as the electron transport chain complex I inhibitor and antimycin A as the electron transport chain complex III inhibitors. Basal rates of OXPHOS can be determined by the amount of oxygen being consumed over time.

To determine the rate of oxidative phosphorylation for Cell Mito Stress (i.e. cellular respiration measured as OCR), we used oligomycin as the ATP synthase inhibitor. Basal rates of OXPHOS can be determined by the amount of oxygen being consumed over time. This allows you to calculate the actual amount of OXPHOS.

Oligomycin inhibits ATP synthase, but FCCP cleaves it, widening the proton gradient across the inner mitochondrial membrane and maximizing OXPHOS. Antimycin A and rotenone block the electron transport chain, allowing the calculation of residual oxygen consumption. This allows you to calculate the actual amount of OXPHOS. **Figure 2.2** showed The oxygen consumption rate (OCR).

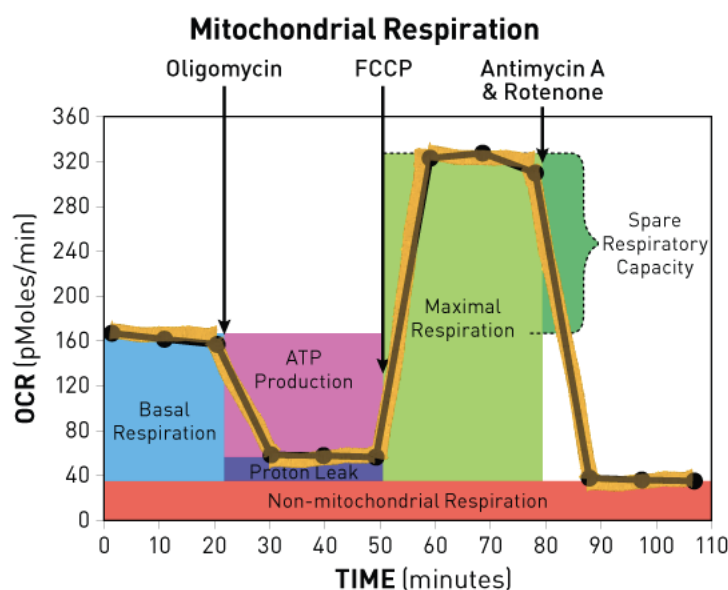


Figure 2.2 The Seahorse XF Cell Mito Stress Test Profile

Oligomycin, FCCP, and rotenone/antimycin A are serially injected to measure ATP-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. Proton leak and spare respiratory capacity (SRC) are then calculated using basal respiration and these parameters. Basal rates of OXPHOS can be determined by the amount of oxygen being consumed over time. While Oligomycin inhibits ATP synthase, FCCP uncouples it which will dissipate the gradient of protons across the inner mitochondrial membrane and thus maximising OXPHOS. Antimycin A and Rotenone inhibit the Electron transport chain thus any residual oxygen consumption can be discarded. This allows us to calculate the actual rates of OXPHOS. Figure adapted from Seahorse Bioscience.

2.11.2 Measurement of respiratory chain complex activities

Following analysis of oxygen consumption in iBMDMs and BMDMs, the cells were freeze-fractured three times using liquid nitrogen and were frozen at -80°C . The samples were assayed for the appropriate complex activity within a week of storage. The rotenone-treated iBMDMs and BMDMs samples were examined for complex I activity (see Section 2.9.1). TTFA treated samples were examined for complex II activity (see Section 2.9.2). The antimycin A-treated samples were examined for complex II/III activity (see Section 2.9.3).

2.11.3 Determination of Flux Control Coefficients

Flux control coefficients can be used to monitor changes in the spread of control in a pathway. Flux control coefficients provide information on the control that the respiratory chain complexes possess over the entire flux of iBMDMs and BMDMs oxygen consumption. The flux control coefficients for complex I and complex II/III were determined by using the results from titrations of iBMDMs and BMDMs oxygen consumption and complex activity with the appropriate inhibitors at low concentrations of the inhibitors according to Equation :

$$\text{FCC} = [(\text{dOCR}/\text{d}(\text{inhibitor}))]/[\text{dVc}/\text{d}(\text{inhibitor})]$$

For oxygen consumption iBMDMs and BMDMs, where FCC is the flux control coefficient of the complex of interest, $\text{dVc}/\text{d}(\text{inhibitor})$ is the rate of change of complex activity and

dOCR/d (inhibitor) is the rate of change of iBMDMs and BMDMs oxygen consumption (entire flux) at low inhibitor concentrations. Values of $R^2 > 0.9$ were required for the linear regression of the initial oxygen consumption rates and complex activities.

2.11.4 Determination of Inhibition Threshold Levels

The inhibition thresholds provide information on the level by which the activity of a complex can be inhibited before a detrimental effect is seen on oxygen consumption rates in iBMDMs and BMDMs. To determine the inhibition threshold levels, the percentage inhibition of complex activity (titrated with the appropriate inhibitor) was plotted against the percentage of basal and maximal oxygen consumption rates in iBMDMs and BMDMs. Inhibition thresholds were investigated by fitting curves to the plots by hand, and approximate thresholds were determined by extrapolating a straight line through the initial points and another line through the rapidly decreasing values.

In addition, the inhibition thresholds for complex I and complex II/III were plotting against Extracellular acidification rate (ECAR). this indicates the % inhibition for complex I and complex II/III in macrophages that can be tolerated before the cells increase their glycolytic flux to compensate for inhibition of mitochondrial respiration.

2.12 Detection of secreted cytokines by ELISA

High binding flat-bottom 96 well plates were coated with 50 μ L per well of capture antibody diluted in 1xPBS buffer and incubated overnight at 4° C. Plates were washed three times the following day in ELISA wash buffer (0.05% Tween 20 in PBS). After each wash step, plates were dried by banging them upside down on tissue. 200 μ L of ELISA diluent was added to each well to block any remaining binding sites, and the plate was incubated

for 1 hour at room temperature.

Standards were made using a serial dilution method in ELISA diluent. Frozen supernatant samples were thawed and vortexed. The plate was washed three times, and the standards and samples were added in triplicate, including ELISA diluent as a blank. For some cytokines (e.g. TNF- α), the samples were diluted to ensure they were within the range of the standard curve. The plates were incubated at 4° C overnight.

Plates were washed three times, and 50 μ L of detection antibody diluted in 1% BSA/PBS was added to each well. Plates were then incubated at room temperature for one hour. Plates were washed three times. 50 μ L of HRP diluted in 1% BSA/PBS was added to each well. Plates were incubated in the dark for 20 minutes at room temperature. Plates were then washed seven times. 50 μ L of TMB substrate reagent was added to each well. The colorimetric reaction was allowed to develop until the lowest standards of the standard curve turned light blue. The reaction was stopped with 25 μ L of “stop solution” (0.5 M H₂SO₄). The plate was read using a microplate reader set to a wavelength of 450 nm. Microsoft Excel software was used to generate a standard curve from which the cytokine concentration of the samples was determined.

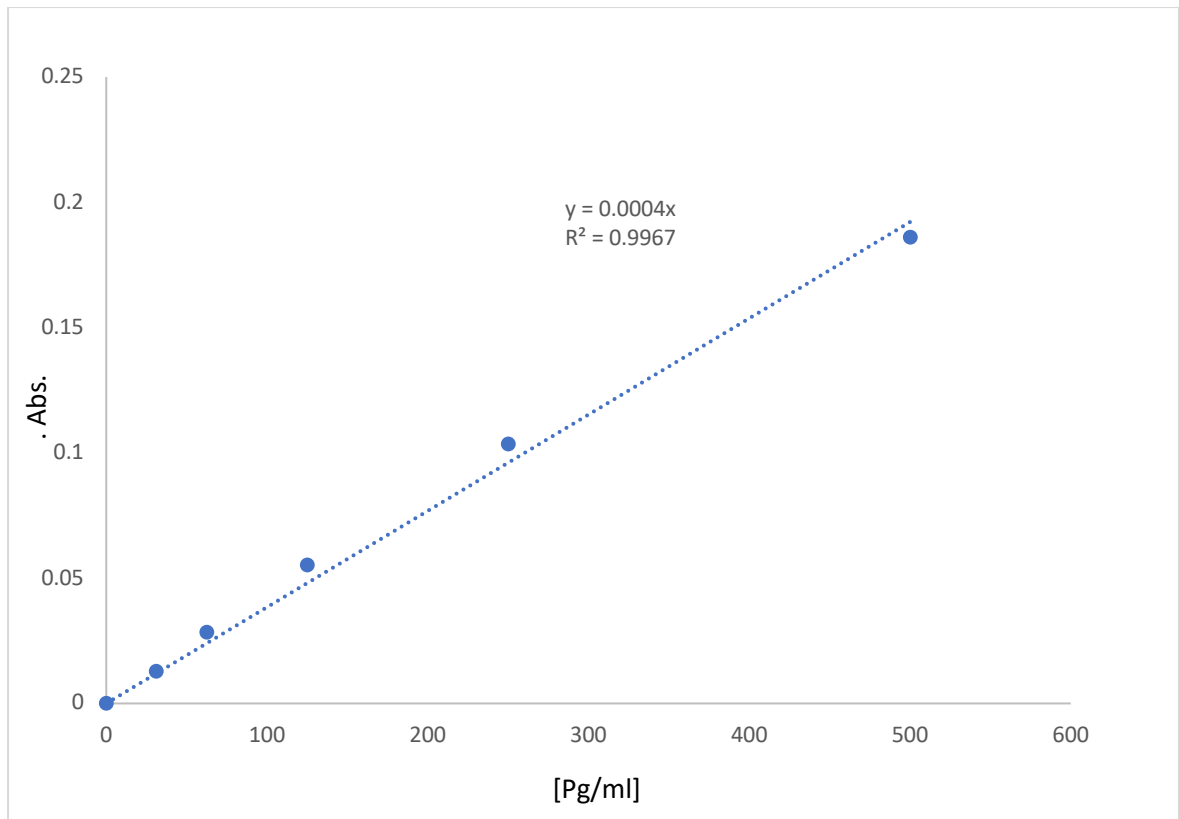


Figure 2.3. TNF- α Standard Curve. A representative standard curve for TNF- α estimation generated for each experiment, where the slope is defined by the equation $y = mx + c$ and the R^2 value estimates goodness of fit of data linear regression. Data is presented as mean $\pm$ SD.

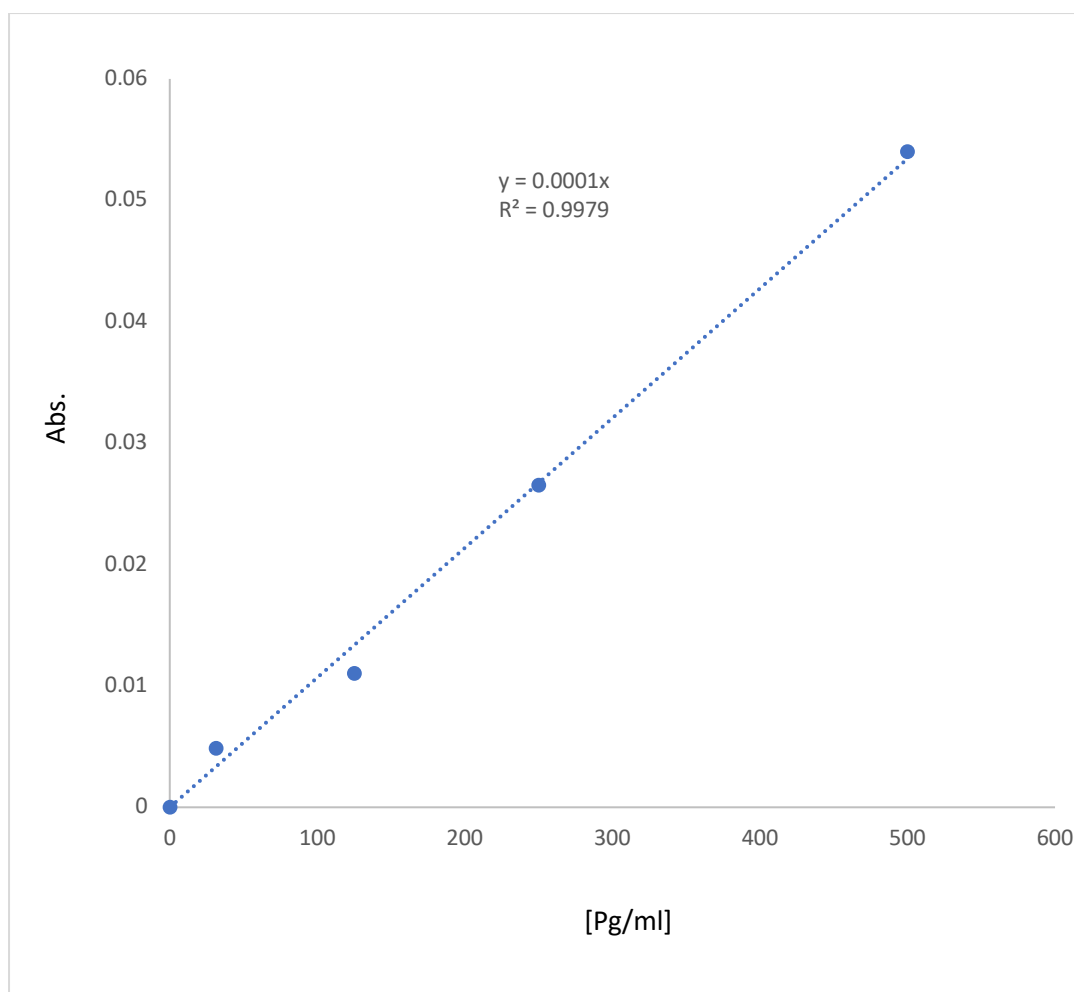


Figure 2.4. IL-1 β Standard Curve. A representative standard curve for IL-1 β estimation generated for each experiment, where the slope is defined by the equation $y = mx + c$ and the R^2 value estimates goodness of fit of data linear regression. Data is presented as mean $\pm$ SD.

2.14 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 9. All data sets were tested for Gaussian distribution and equal variance within the GraphPad Prism software. All data are presented as mean $\pm$ SEM unless otherwise stated. A P- value of 0.05 or less was deemed to be statistically significant. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

CHAPTER 3

CHARACTERISATION OF THE EFFECT OF LPS- ACTIVATION AND ITACONATE ON MITOCHONDRIAL ETC ACTIVITIES IN MACROPHAGES

3.1 Introduction

Most cellular ATP is generated in the mitochondrion. Protons are pumped out across the mitochondrial inner membrane through complexes I, III, and IV by the energy that is released as the electrons flow along the ETC (Johannsen and Ravussin, 2009). The TCA cycle produces several intermediate metabolites, such as citrate, fumarate, α -ketoglutarate, and succinate, metabolites which are known to affect several signalling pathways that regulate cellular functions.

In 1546, succinic acid was discovered by a German chemist called Georgius Agricola. The dehydrogenation of succinate to fumarate by succinate dehydrogenase (SDH) was described by Albert Szent-Györgyi in 1937. Succinate was identified as an important intermediate in the TCA cycle by Sir Hans Krebs (Christos, 2013, Tretter et al., 2016). Substrate-level phosphorylation (SLP) is a process of the conversion of succinyl-CoA and ADP (or GDP) to CoASH, succinate and ATP (or GTP) by Succinate-CoA ligase (SUCL), also known as succinyl coenzyme A synthetase, or succinate thiokinase (Johnson et al., 1998).

In 2013, the O'Neill group found that the TCA cycle plays an important role in pro-inflammatory macrophages, and that LPS stimulation of primary mouse macrophages leads to elevated intracellular succinate levels, and that glutamine is a precursor to succinic acid in these cells (Tannahill et al., 2013, Palsson-McDermott and O'Neill, 2013). Succinic acid stabilizes HIF-1 α by inhibiting procollagen-hydroxylase activity and increases IL-1 β expression levels through upregulation of GLUT1, lactate dehydrogenase (LDH), and other enzymes involved in glycolytic energy production. This was found to bring about an increase in glycolytic activity. After this role of succinic acid in pro-inflammatory

macrophages was discovered, mitochondrial metabolites were found to play an important role in ROS production in ischemic reperfusion (Chouchani et al., 2014). Here, the use of stable isotope-labelled glucose and fatty acids enabled TCA cycle intermediates under hypoxic-ischemic conditions to be followed. The results showed a reduced carbon influx and reversal of SDH activity (Chouchani et al., 2014). This has been found in vivo to cause ROS production during reperfusion, suggesting that succinic acid accumulation is a major cause of ischemic injury (Chouchani et al., 2014). Blocking succinate accumulation reduces damage in rat stroke models and provides evidence of the role of succinic acid in ischemic reperfusion injury. The other roles for succinic acid are beginning to emerge, both in macrophages and other cell types. Enzymes such as SDH, pyruvate dehydrogenase complex, and isocitrate dehydrogenase isoform 2 (IDH2) have been shown to be lysine-succinylated, regulating the immune response following TLR activation and subsequent inflammatory cytokine production (Park et al., 2013, Zhou et al., 2016a, Rubic et al., 2008).

In 1836, Jean Louis Lassaigne and Samuel Baup discovered itaconic acid through the thermal decomposition of citric acid. In 1840, Gustav Crasso presented that a product of the thermal decomposition of cis-aconitate is itaconate (ITA) (O'Neill and Artyomov, 2019, Godfrey and Kornberg, 2020). Itaconic acid, a compound used in the synthesis of polymers, is known to have antibacterial properties and inhibit the enzyme isocitrate lyase of some species of bacteria. Itaconate inhibits SDH activity and subsequently regulates succinate metabolism (McFadden and Purohit, 1977).

Recently, itaconic acid was found to be produced from cis-aconitate acid and dramatically increased in LPS-stimulated mouse macrophages (Strelko et al., 2011).

Following this discovery, further studies delved into the production and regulation of itaconic acid in mammalian macrophages (Michelucci et al., 2013). Using metabolomic stable isotope labelling techniques, the production of itaconic acid from cis-aconitate acid was confirmed, and its presence in stimulated primary mouse and human macrophages was determined. In addition, it was found that the synthesis of itaconic acid production in mammalian cells is catalysed by the immune-responsive gene 1 (Irg1) protein. Finally, the antibacterial properties of itaconic acid were confirmed in vitro.

Figure 3.1 show that the metabolism of itaconate is associated with the TCA cycle. Several enzymes and chemicals are involved in the TCA cycle to generate reducing equivalents used by the process of Oxidative Phosphorylation to produce ATP. Itaconate is synthesized via the following steps: firstly, citrate is transformed to cis-aconitate by aconitate hydratase. Then, cis-aconitate is catalysed by cis-aconitate decarboxylase (CAD), encoded by *Acod1*, which is responsible for the generation of itaconate in macrophages. Eight active sites critical for CAD function and crucial amino acids that make up the active centre (Michelucci et al., 2013). The synthesis of itaconate is effectively enhanced by LPS through activation of *Acod1*. Silencing *Acod1* significantly decreases itaconate levels in LPS stimulated RAW264.7 cells (a mouse macrophage cell line) (Chen et al., 2019).

Pyruvate dehydrogenase is in part associated with the biosynthesis of itaconate. Pyruvate dehydrogenase kinase 1 will increase the phosphorylation of pyruvate dehydrogenase and consequently suppresses its activity. LPS can inhibit the activity of pyruvate dehydrogenase kinase1, which results in more conversion of pyruvate to acetyl-CoA via the activation of pyruvate dehydrogenase. Acetyl-CoA is a vital precursor for the production of citrate, and sufficient citrate is vital for the synthesis of itaconate. To eliminate itaconate in LPS-

induced RAW264.7 cells, itaconate is first converted to itaconyl-CoA. Then, itaconyl-CoA is catabolized through citramalyl-CoA lyase, a reaction conserved and localized inside the mitochondria, to provide pyruvate and acetyl-CoA. The latter may be reused within the TCA cycle. All of these reactions illustrate the metabolic cycle in macrophages. Whether or not other metabolic routes of itaconate exist still needs in additional exploration (Godfrey and Kornberg, 2020).

In macrophages, itaconate synthesis is catalysed by means of the immune-responsive gene 1 (IRG1) protein, which mediates the decarboxylation of cis-aconitate to itaconate. Metabolomic and fluxomic analysis of LPS-stimulated macrophages demonstrated reduced isocitrate dehydrogenase-1 (IDH1) expression and elevated IRG1 expression, resulting in a diversion of citrate from the TCA cycle towards itaconate production (Strelko et al., 2011). In 2011, Itaconate was first detected to be present in mammalian cells (Cordes and Metallo, 2021).

Via an interpolated metabolomics and transcriptomics analysis combined with network integration, Jha and his group identified the rewiring of the TCA cycle during pro-inflammatory macrophage polarization. They discovered two "metabolic breakpoints" that contributed to the accumulation of itaconic acid and succinic acid. With the use of [$^{13}\text{C}_6$] glucose and [$^{13}\text{C}_5$] glutamine, it was observed that the carbon flux between citric acid and α -ketoglutaric acid (α -KG) was blocked and citric acid accumulated. Blocking this flow is associated with the downregulation of isocitrate dehydrogenase isoform₁ (IDH₁) mRNA in inflammatory macrophages (Jha et al., 2015).

This increase in citrate can play multiple roles in the TCA cycle and it was shown that mitochondrial citrate carriers increase under LPS stimulation, leading to an increase in nitric oxide and ROS from citric acid metabolized to acetyl-CoA (Infantino et al., 2011). Itaconic acid production also results from a series of reactions immediately downstream of citric acid production. Increasing citric acid can increase itaconic acid production. All three of these compounds are critical mediators of pro-inflammatory macrophage activity, emphasizing citric acid as a key pro-inflammatory metabolite.

Studies further suggested that inefficient SDH activity leading to the accumulation of succinic acid pools due to excessive glutamine uptake (Jha et al., 2015). An increase in malic acid, via the aspartate arginosuccinate shunt, which is the interface between the intracellular TCA cycle and the urea cycle, was also observed. The combination of increased cycle activity of citric acid (from glucose) and the urea cycle (from glutamine) underscores the use of multiple cellular energy sources for the same pro-inflammatory cellular function (Jha et al., 2015). In the same studies, macrophages have been found to modify their metabolism under pro-inflammatory conditions to produce several pro-inflammatory mediator molecules, including itaconic acid and succinic acid. These studies highlight the important role of these metabolites in pro-inflammatory macrophages and consolidate the TCA cycle as a major player in macrophage metabolism during the inflammatory process (Jha et al., 2015).

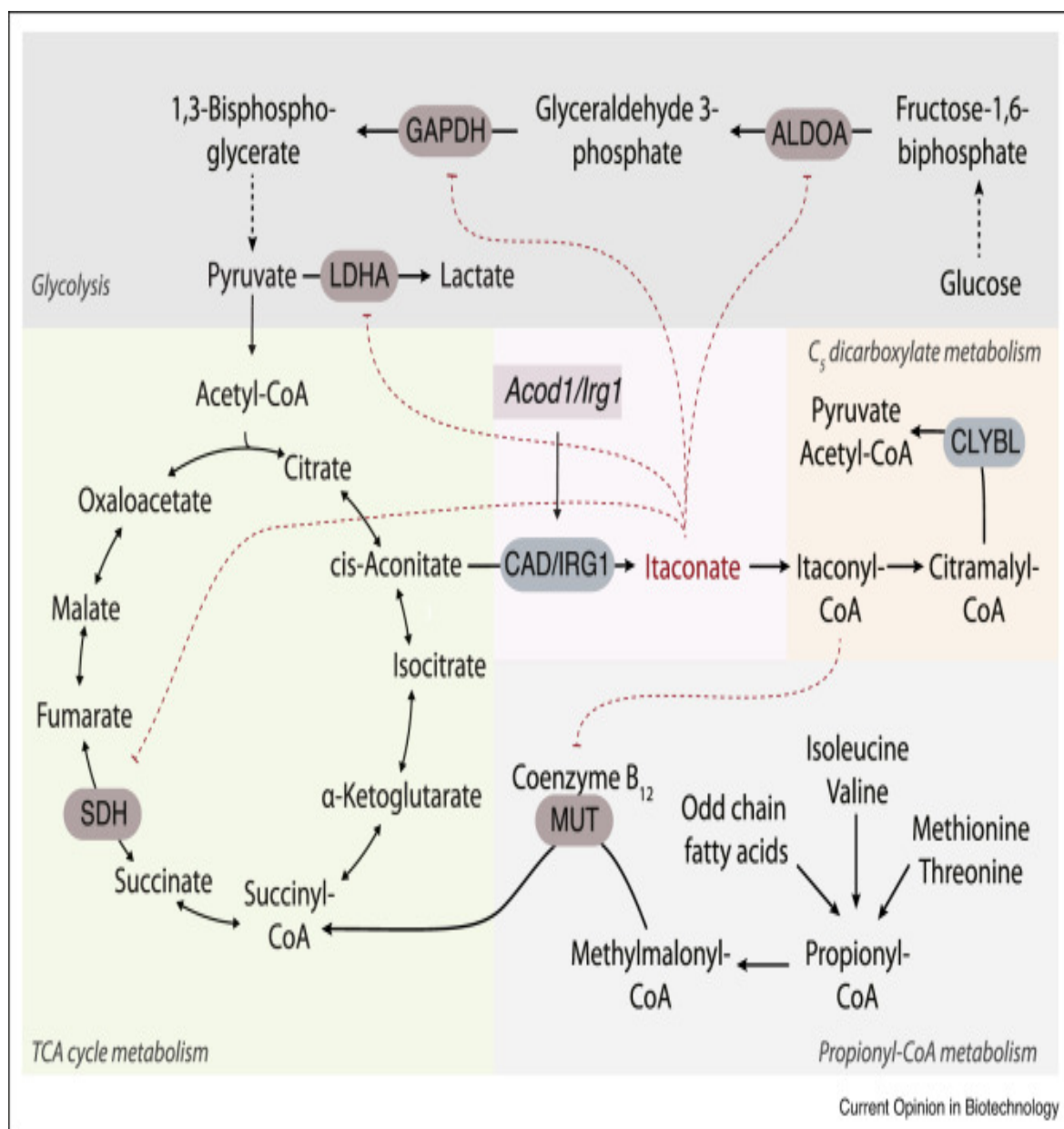


Figure 3.1: Itaconate regulates a number of metabolic control points. In mammalian cells, itaconate metabolism affects glucose, glutamine, branched-chain amino acids (BCAA), CoA species, and fatty acid metabolism (Cordes and Metallo, 2021).

From the studies described above, it is clear that LPS-activation of macrophages results in a change in mitochondrial metabolism. While a number of control points have been

identified it still remains unclear what happens to mitochondrial ETC activities in macrophages following LPS-activation and if itaconate can influence electron transport through the ETC.

3.1.2 Aims of the Chapter

- To Compare the specific activities of the mitochondrial ETC complexes, CI, CII, CII/III, CIV and citric synthase in iBMDMs and BMDMs following LPS treatment for 4h and 24h.
- To study possible reversibility effects of ETC function following LPS treatment in iBMDMs and BMDMs.
- To characterise the itaconate inhibition profiles for CI and CII activities iBMDMs and BMDMs.
- To investigate the time-dependence and reversibility of itaconate inhibition on ETC activities in iBMDMs and BMDMs.

3.2 Results

3.2.1 Mitochondrial electron transport chain complex activities in LPS-activated iBMDMs and BMDMs.

To examine the activities of the respiratory complexes in iBMDMs and BMDMs $\pm$ LPS for 4 hours and 24 hours, samples were assayed for complex I, complex II, complex II/III, complex IV and citrate synthase activities.

Figure 3.2 (A) shows that complex I activity was lower in iBMDMs + 4 hours LPS (100 ng/ml) by $\sim 50\%$ (+ LPS = 19.53 ± 0.41 pmol/min/mg, - LPS = 39.60 ± 1.13 pmol/min/mg). Figure 3.2 (B) shows that complex I activity was significantly lower in iBMDMs + 24h LPS (100 ng/ml) by $\sim 75\%$, (+ LPS = 14.45 ± 0.90 pmol/min/mg; - LPS = 46.51 ± 1.4 pmol/min/mg).

Figure 3.3 (A) shows that complex II activity was significantly lower in iBMDMs + 4 hours LPS (100 ng/ml) by $\sim 20\%$, (+ LPS = 17.04 ± 0.27 pmol/min/mg; - LPS = 21.62 ± 0.24 pmol/min/mg). Figure 3.3 (B) indicates that complex II activity was significantly reduced in iBMDMs + 24 hours LPS (100ng/ml) by $\sim 70\%$, (+ LPS = 7.39 ± 0.46 pmol/min/mg; - LPS = 23.34 ± 0.58 pmol/min/mg).

However, **Figure 3.4** (A) and (B) show that there was no significant different in complex II/III activity between iBMDMs $\pm$ 4 hours LPS (100 ng/ml), or $\pm$ 24 hours LPS (100 ng/ml).

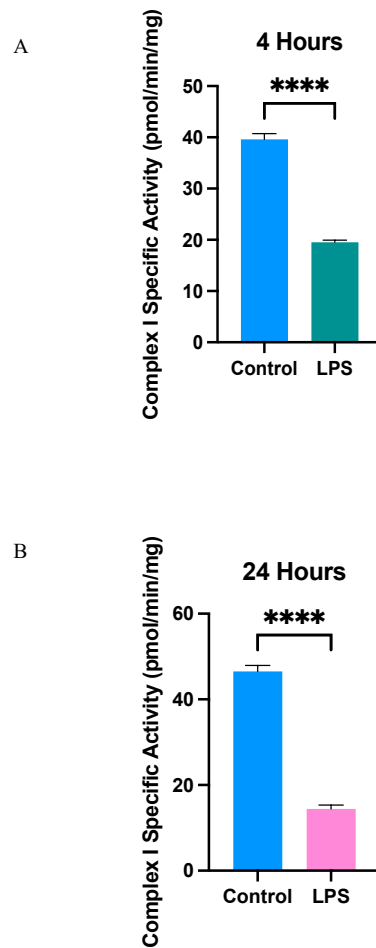


Figure 3.2. Complex I specific activity is decreased in LPS-activated iBMDMs.

iBMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.1. Samples were freeze fractured three times and complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex I in iBMDMs $\pm$ LPS 4 h and 24 h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were significantly different to each other are shown with **** for $p \leq 0.0001$.

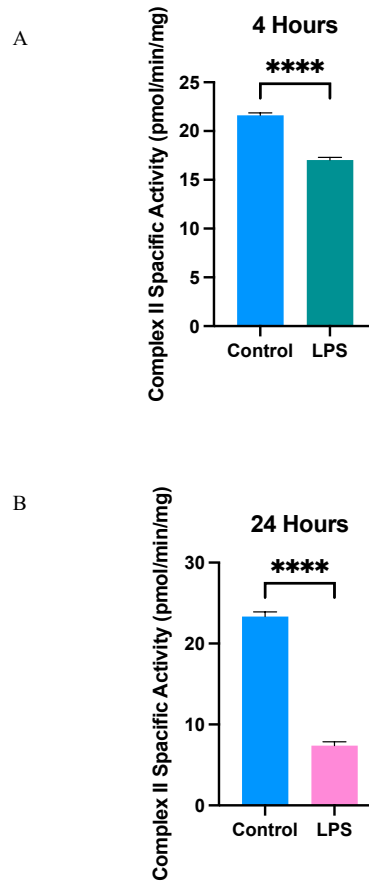


Figure 3.3. Complex II specific activity is decreased in LPS-activated iBMDMs.

iBMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.2. Samples were freeze fractured three times and complex II activity was measured with a spectrophotometric assay using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600$ nm at 37°C. The specific activity of complex II in iBMDMs $\pm$ LPS 4h and 24h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were significantly different to each other are shown with **** for $p \leq 0.0001$.

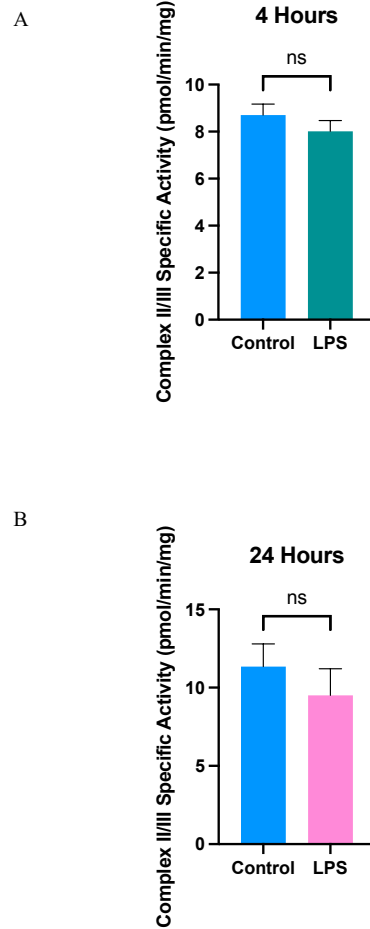


Figure 3.4. Complex II/III specific activity is unchanged in LPS-activated iBMDMs.

iBMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24h (B) as described in Section 2.9.3. Samples were freeze fractured three times and complex II/III activity was measured with spectrophotometric assay following the reduction of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex II/III in iBMDMs $\pm$ LPS 4h and 24h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were non-significantly different to each other.

Figure 3.5 (A) and (B) illustrates that there was no significant different in complex IV activity between iBMDMs $\pm$ 4 hours LPS (100ng/ml) or $\pm$ 24 hours LPS (100 ng/ml).

Figure 3.6 (A) and (B) show that citrate synthase activity was significantly decreased in iBMDMs + 4 hours LPS (100 ng/ml) by ~ 60 %, (+ LPS = 9.06 ± 2.2 ; - LPS = 22.59 ± 1.48). CS activity was significantly reduced in iBMDM + 24 hours LPS (100 ng/ml) by ~ 90 %, (+ LPS = 2.24 ± 0.27 ; 22.59 ± 1.48).

To examine the reversibility of complex I activity in LPS-treated iBMDMs, 4 samples were prepared, two as control and two treated with LPS. iBMDMs were incubated with 100 ng/ml for 4 hours and 24 hours. Then, one control and one of treated cells were assayed as usual, while control 2 and sample 2 were sonicated and homogenised 20 times. After that, the samples were centrifuged at 15000 rpm for 10 minutes. The supernatant was carefully removed and discarded. The pellet was resuspended in complex I assay buffer and vortexed and assayed in section 2.9.1). Complex I activity was significantly lower in iBMDMs + 4 hours LPS (100 ng/ml) by ~ 50 % (control 1 and LPS 1) while complex I activity was significantly lower in iBMDMs + 4 hours LPS (100 ng/ml) by ~ 40 % (control 2 and LPS 2). Complex I activity was significantly lower in iBMDMs + 24 hours LPS (100 ng/ml) by ~ 75 % (control 1 and LPS 1) while complex I activity was significantly lower in iBMDMs + 24 hours LPS (100 ng/ml) by ~ 70 % (control 2 and LPS 2). **Figure 3.7** (A) and (B) show that LPS treatment of iBMDMs results in irreversible inhibition of complex I activity.

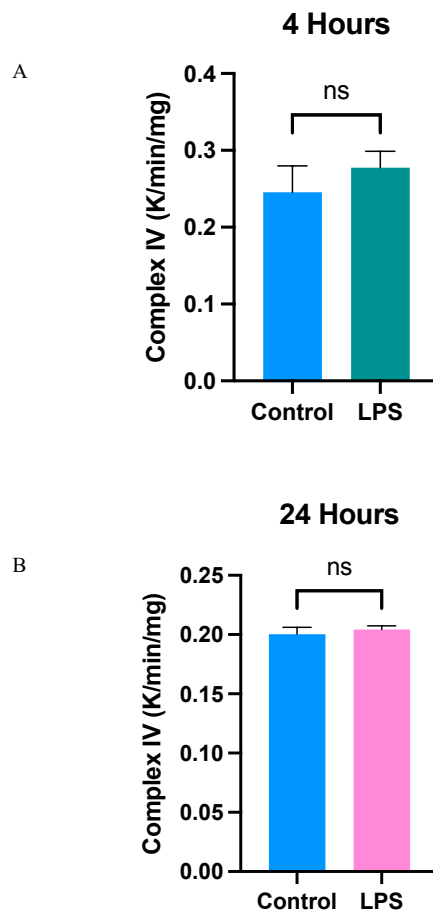


Figure 3.5. Complex IV specific activity is unchanged in LPS-activated iBMDMs.

iBMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.4. Samples were freeze fractured three times and complex IV activity was measured with a spectrophotometric assay following the oxidation of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex IV in iBMDMs $\pm$ LPS 4h and 24h LPS is expressed as K/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were non-significantly different to each other.

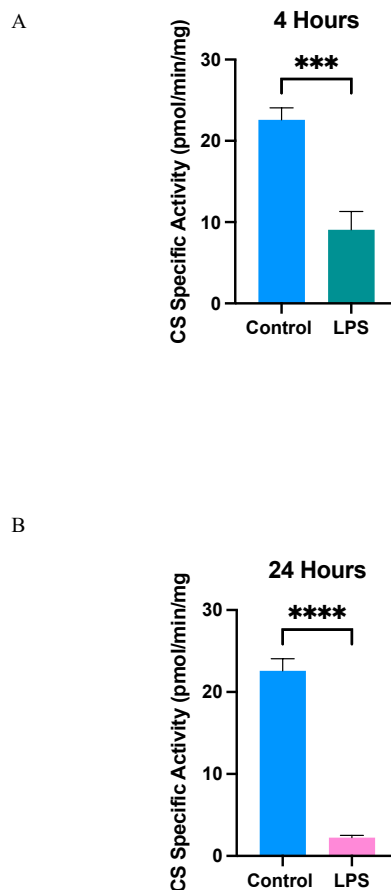


Figure 3.6. Citrate Synthase specific activity is decreased in LPS-activated iBMDMs.

iBMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.5 Samples were freeze fractured three times and CS activity was measured with a spectrophotometric assay determined by monitoring the rate of production of dithionitrobenzoic acid at $\lambda = 412$ nm at 37°C. The specific activity of CS in iBMDMs $\pm$ LPS 4h and 24h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were significantly different to each other are shown with *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

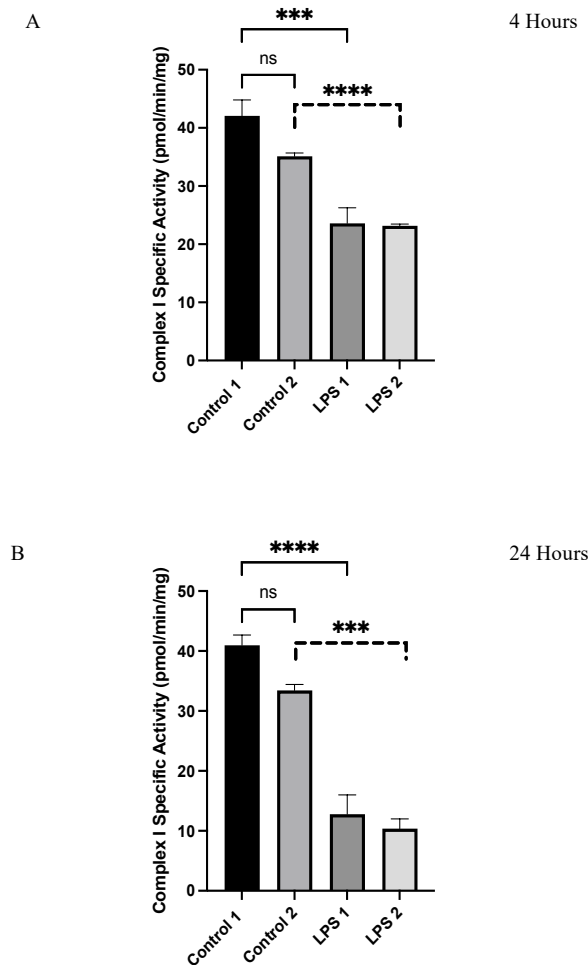


Figure 3.7. Inhibition of Complex I activity in LPS-activated iBMDMs is irreversible. iBMDMs were incubated with 100 ng/ml LPS for 4 hours (A) and 24 hours (B). The Control 2 and LPS 2 samples were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 10 minutes. The supernatant was carefully removed and discarded. All 4 samples were resuspended in complex I assay buffer, vortexed and assayed for complex I specific activity (described in section 2.9.1). An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

To examine the type of inhibition of complex II activity in LPS-treated iBMDMs, 4 samples were prepared two as control and two treated with LPS. iBMDMs were incubated with 100 ng/ml for 4 hours and 24 hours. Then, control 1 and LPS1 treated cells were assayed for complex II activity, while the other samples were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 10 min. The supernatant was carefully removed and discarded. The pellet was resuspended in complex II assay buffer and vortexed and assayed for complex II assay (described in section 2.9.2). **Figures 3.8 (A) and (B)** showed that, LPS had reversible effects with iBMDMs in complex II activity.

Because primary BMDMs are more sensitive than iBMDMs, we compared the different in mitochondrial electron transport chain complex activities in LPS-activated iBMDMs and BMDMs. **Figure 3.9 (A)** shows that complex I activity was lower in BMDMs + 4 hours LPS (100 ng/ml) by ~ 61 %, (+ LPS = 12.71 ± 1.11 pmol/min/mg; - LPS = 21.21 ± 1.25 pmol/min/mg). **Figure 3.9 (B)** shows that complex I activity was significantly lower BMDMs + 24 hours LPS (100 ng/ml) by ~ 47%, (+ LPS = 10.65 ± 0.44 pmol/min/mg; - LPS = 22.47 ± 0.70 pmol/min/mg).

Figure 3.10 (A) shows that complex II activity was significantly reduced in BMDMs + 4 hours LPS (100 ng/ml) by ~ 30 %, (+ LPS = 15.89 ± 1.09 pmol/min/mg; - LPS = 22.61 ± 1.47 pmol/min/mg). **Figure 3.10 (B)** indicates that complex II activity was significantly reduced in BMDMs + 24 hours LPS (100 ng/ml) by ~ 80%, (+ LPS = 5.50 ± 0.62 pmol/min/mg; - LPS = 25.78 ± 1.724 pmol/min/mg). In addition, **Figures 3.11 (A)** shows that complex II/III activity was significantly lower in BMDMs + 4 hours LPS (100 ng/ml) by ~ 50 %, (+ LPS = 4.6 ± 0.18 pmol/min/mg; - LPS = 8.21 ± 0.34 pmol/min/mg). **Figure**

3.11 (B) indicates that complex II/III activity was significantly reduced in BMDMs + 24 hours LPS (100 ng/ml) by ~ 78 %, (+ LPS = 1.87 ± 0.27 pmol/min/mg; - LPS = 8.21 ± 0.35 pmol/min/mg).

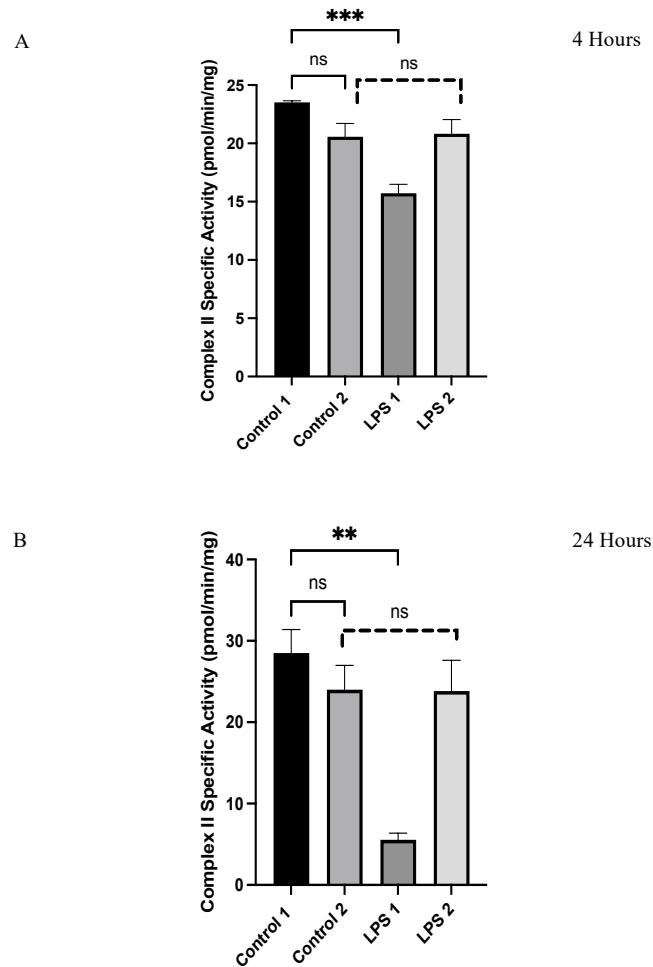


Figure 3.8: Inhibition of Complex II activity in LPS-activated iBMDMs is reversible
iBMDMs were incubated with 100 ng/ml LPS for 4 hours (A) and 24 hours (B). The Control 2 and LPS 2 samples were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 10 minutes. The supernatant was carefully removed and discarded. All 4 samples were resuspended in complex II assay buffer, vortexed and assayed for complex II specific activity (described in section 2.9.1). An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with ** for $p \leq 0.01$, *** for $p \leq 0.001$.

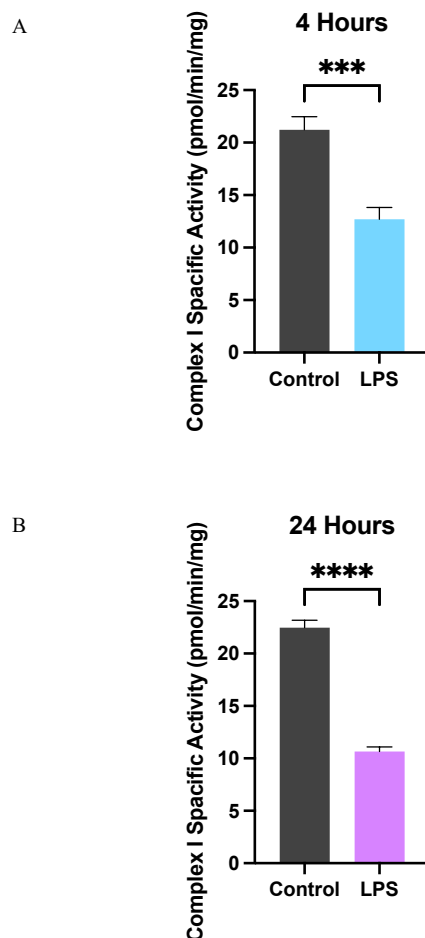


Figure 3.9. Complex I specific activity is decreased in LPS-activated BMDMs.

BMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.1. Samples were freeze fractured three times and complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex I in BMDMs $\pm$ LPS 4 h and 24 h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

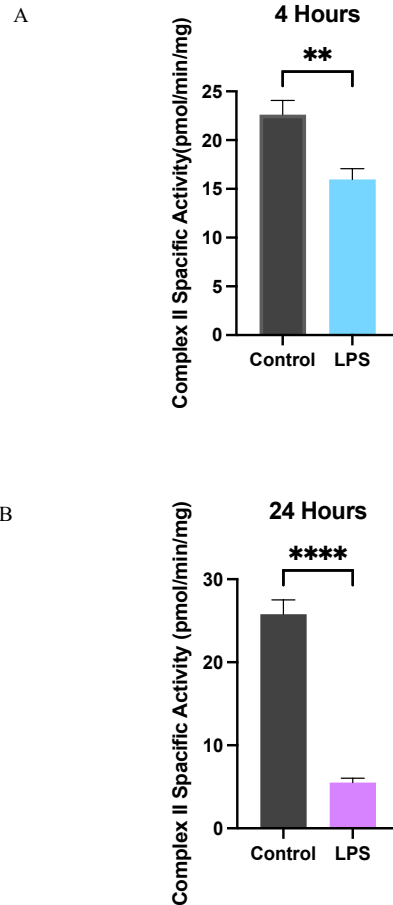


Figure 3.10. Complex II specific activity is decreased in LPS-activated BMDMs.

BMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.2. Samples were freeze fractured three times and complex II activity was measured with a spectrophotometric assay using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600$ nm at 37°C. The specific activity of complex II in BMDMs $\pm$ LPS 4h and 24h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with ** for $p \leq 0.01$, and **** for $p \leq 0.0001$.

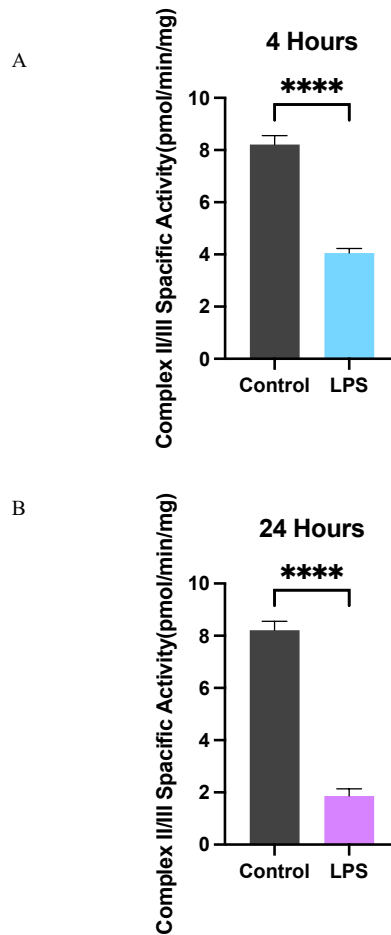


Figure 3.11. Complex II/III specific activity is decreased in LPS-activated BMDMs.

BMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24h (B) as described in Section 2.9.3 Samples were freeze fractured three times and complex II/III activity was measured with spectrophotometric assay following the reduction of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex II/III in BMDMs $\pm$ LPS 4h and 24h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values.

Samples were significantly different to each other with **** for $p \leq 0.0001$.

Figure 3.12 (A) and (B) illustrates that there was no significant difference in complex IV activity between BMDMs $\pm$ 4 hours LPS (100ng/ml) or $\pm$ 24 hours LPS (100 ng/ml).

Figure 3.13 (A) and (B) show that citrate synthase (CS) activity was significantly decreased in BMDMs + 4 hours LPS (100 ng/ml) by $\sim 30\%$, (+ LPS = 2.93 ± 0.14 ; - LPS = 4.37 ± 0.37). CS activity was extremely significantly lower BMDM + 24 hours LPS (100 ng/ml) by $\sim 90\%$, (+lps = 1.84 ± 0.07 ; -lps = 22.59 ± 1.48).

To examine the reversibility of LPS in complex I activity, 4 samples were prepared, two as control and two treated with LPS. BMDMs were incubated with 100 ng/ml for 4 hours and 24 hours. Then, control1 and LPS1 cells were assayed for CI activity as usual while the control 2 and sample 2 were sonicated and homogenised 20 times. After that, the samples were centrifuged at 15000 rpm for 10 min. The supernatant was carefully removed and discarded. The pellet was resuspended in complex I assay buffer, vortexed and assayed for complex I assay (described in section 2.9.1). Complex I activity was significantly lower in BMDMs + 4 hours LPS (100 ng/ml) $\sim 37\%$ (control 1 and LPS 1), however there was no significant decrease in complex I activity in BMDMs + 4 hours LPS (100 ng/ml) and control (control 2 and LPS 2).

Complex I activity was significantly lower in BMDMs + 24 hours LPS (100 ng/ml) by $\sim 50\%$ (control 1 and LPS 1). While, Complex I activity was significantly lower in BMDMs + 24 hours LPS (100 ng/ml) by $\sim 40\%$ (control 2 and LPS 2) there was no significant decrease in complex I activity between the two controls. **Figures 3.14** (A) and (B) show that the inhibition of complex I activity in 24 h LPS-treated BMDMs was irreversible.

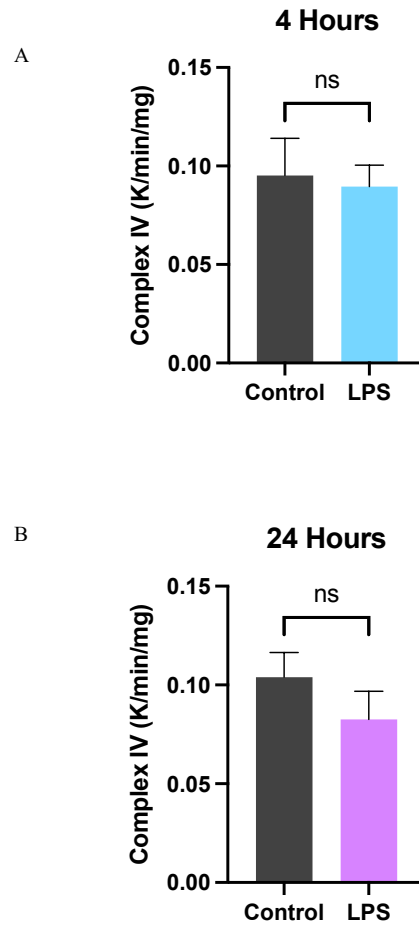


Figure 3.12. Complex IV specific activity is unchanged in LPS-activated BMDMs.

BMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.4 Samples were freeze fractured three times and complex IV activity was measured with a spectrophotometric assay following the oxidation of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex IV in in BMDMs $\pm$ LPS 4h and 24h LPS is expressed as K/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were non-significantly different to each other.

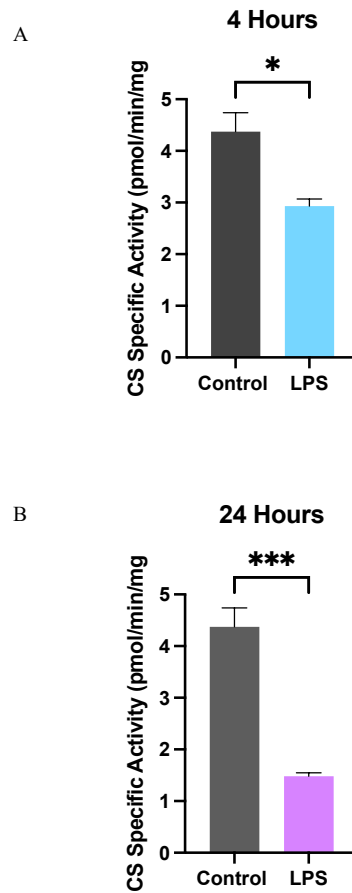


Figure 3.13. Citrate Synthase specific activity is decreased in LPS-activated BMDMs.

BMDMs were incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.5. Samples were freeze fractured three times and CS activity was measured with a spectrophotometric assay determined by monitoring the rate of production of dithionitrobenzoic acid at $\lambda = 412$ nm at 37°C. The specific activity of CS in BMDMs $\pm$ LPS 4h and 24h LPS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were significantly different to each other are shown with * for $p \leq 0.05$, *** for $p \leq 0.001$.

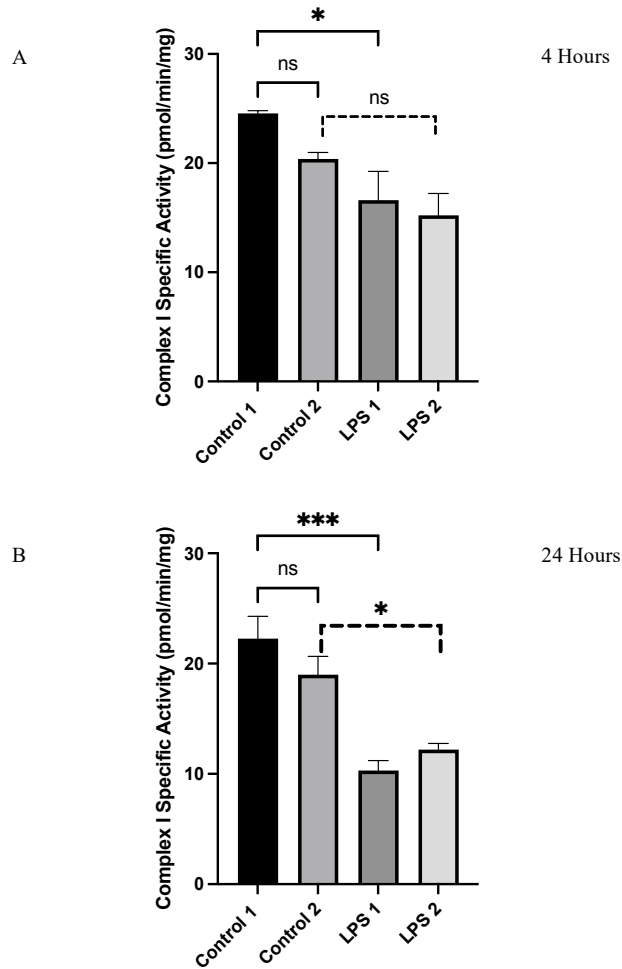


Figure 3.14. Inhibition of Complex I activity in LPS-activated BMDMs is irreversible BMDMs were incubated with 100 ng/ml LPS for 4 hours (A) and 24 hours (B). The Control 2 and LPS 2 samples were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 10 minutes. The supernatant was carefully removed and discarded. All 4 samples were resuspended in complex I assay buffer, vortexed and assayed for complex I specific activity (described in section 2.9.1). An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with * for $p \leq 0.05$, *** for $p \leq 0.001$.

To examine the reversibility of LPS in complex II activity, 4 samples were prepared, two as control and two treated with LPS. BMDMs were incubated with 100 ng/ml for 4 hours and 24 hours. Then, control 1 and LPS 1 cells were assayed for CII activity as usual while the other samples were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 10 minutes. The supernatant was carefully removed and discarded. The pellet was resuspended in complex II assay buffer, vortexed and assayed for complex II activity (described in section 2.9.2). **Figures 3.15** (A) and (B) showed that the inhibition of complex II activity in LPS-treated BMDMs was reversible.

Figure 3.16 (A) shows there was no significant different in complex I activity in mitochondria isolated from BMDMs $\pm$ 4 hours LPS (100 ng/ml), (- LPS= 291.2 ± 28.89 pmol/min/mg; + LPS= 194.2 ± 38.76 pmol/min/mg). However, Figure 3.16 (B) shows that complex I activity was significantly lower in mitochondria isolated from BMDMs + 24h LPS (100 ng/ml) by $\sim 65\%$, (- LPS = 100.9 ± 37.17 pmol/min/mg; + LPS= 291.2 ± 28.89 pmol/min/mg).

Moreover, **Figure 3.17** (A) shows there was no significant different in complex II activity between isolated mitochondria from BMDMs $\pm$ 4 hours LPS (100 ng/ml), (- LPS= 583.9 ± 91 pmol/min/mg; + LPS = 469 ± 61.36 pmol/min/mg). Figure 3.17 (B) indicates that complex II activity was significantly lower in isolated mitochondria from BMDMs + 24h LPS (100ng/ml) by $\sim 65\%$, (+ LPS = 209 ± 26.86 pmol/min/mg; - LPS= 583.9 ± 91 pmol/min/mg).

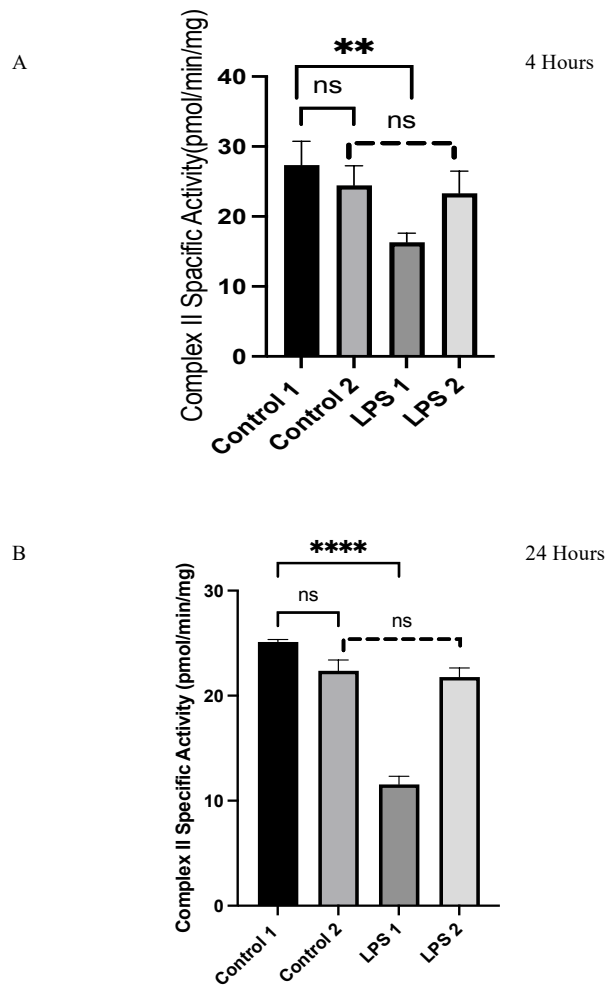


Figure 3.15. Inhibition of Complex II activity in LPS-activated BMDMs is reversible
 BMDMs were incubated with 100 ng/ml LPS for 4 hours (A) and 24 hours (B). The Control 2 and LPS 2 samples were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 10 minutes. The supernatant was carefully removed and discarded. All 4 samples were resuspended in complex II assay buffer, vortexed and assayed for complex II specific activity (described in section 2.9.1). An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with ** for $p \leq 0.01$, **** for $p \leq 0.0001$.

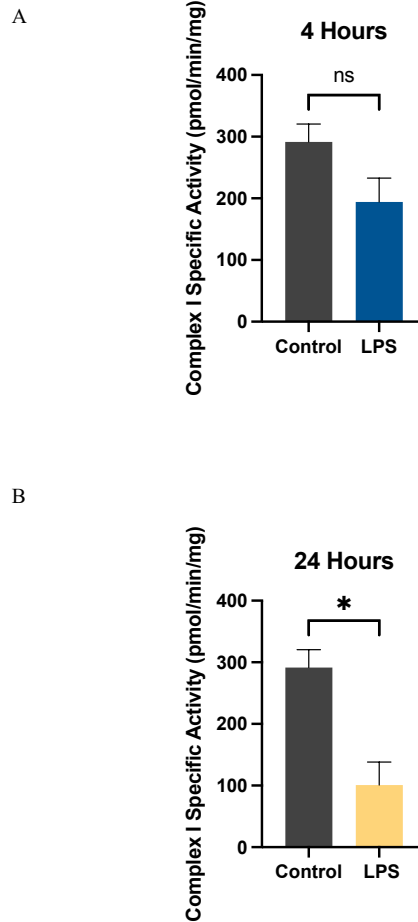


Figure 3.16. Complex I specific activity is decreased in Mitochondria isolated from LPS-activated BMDMs in a time-dependent manner.

Isolated mitochondria were extracted from BMDMs incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.1 Samples were freeze fractured three times and complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex I is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were significantly different to each other are shown with * for $p \leq 0.05$.

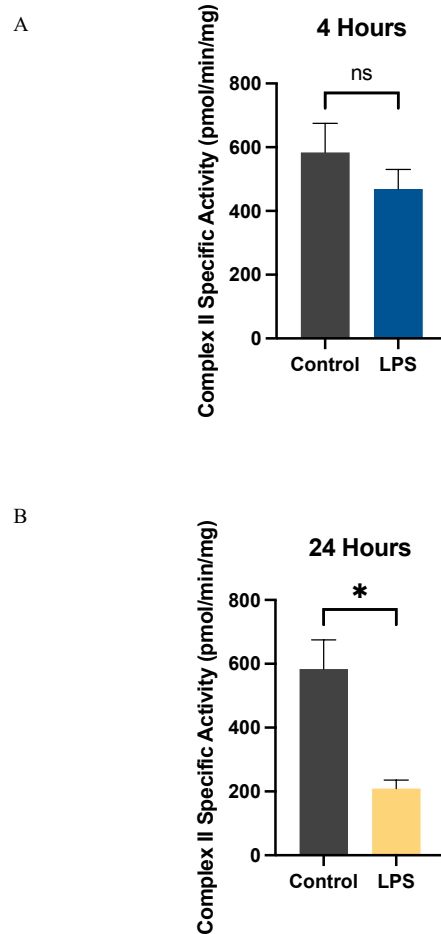


Figure 3.17. Complex II specific activity is decreased in Mitochondria isolated from LPS-activated BMDMs in a time-dependent manner.

Isolated mitochondria were extracted from BMDMs incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.2. Samples were freeze fractured three times and complex II activity was measured with a spectrophotometric assay using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600$ nm at 37°C. The specific activity of complex II is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with * for $p \leq 0.05$.

Figure 3.18 (A) shows that complex II/III activity was significantly lower in mitochondria isolated from BMDMs + 4 hours LPS (100 ng/ml) by ~ 55%, (+ LPS = 42.94 ± 2.00 pmol/min/mg; - LPS = 87.09 ± 3.60 pmol/min/mg). Figure 3.18 (B) indicates that complex II/III activity was significantly reduced by ~ 75% in mitochondria + 24 hours LPS (100 ng/ml), (+ LPS = 22.35 ± 3.034 pmol/min/mg; - LPS = 87.09 ± 3.596 pmol/min/mg).

However, **Figure 3.19** (A) and (B) illustration that there was no significant different in complex IV activity between mitochondria isolated from BMDMs $\pm$ 4 hours LPS (100ng/ml), or $\pm$ 24 hours LPS.

Figure 3.20 (A) and (B) show that citrate synthase (CS) activity was significantly decreased in mitochondria isolated from BMDMs + 4 hours LPS (100 ng/ml) by ~ 28 %, (+ LPS = 18.86 ± 0.80 ; - LPS = 25.90 ± 1.89). Moreover, CS activity was extremely significantly lowered in mitochondria isolated from BMDMs + 24 hours LPS (100 ng/ml) by ~ 50 %, (+ LPS = 13.31 ± 1.64 ; - LPS = 25.90 ± 1.89).

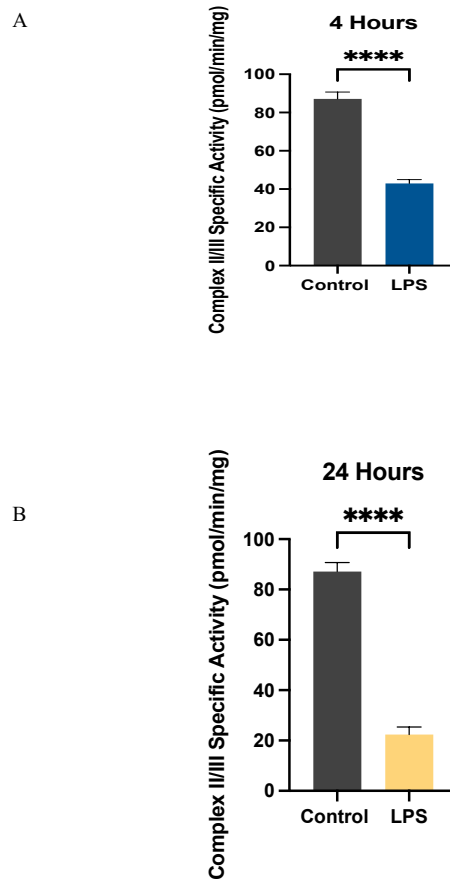
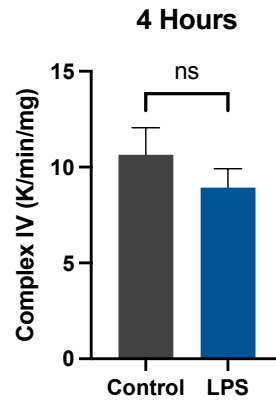


Figure 3.18. Complex II/III specific activity is decreased in Mitochondria isolated from LPS-activated BMDMs in a time-dependent manner

Isolated mitochondria were extracted from BMDMs incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.1 Samples were freeze fractured three times and complex II activity was measured with a spectrophotometric assay following the reduction of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex II/III is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were significantly different to each other are shown with **** for $p \leq 0.0001$.

A



B

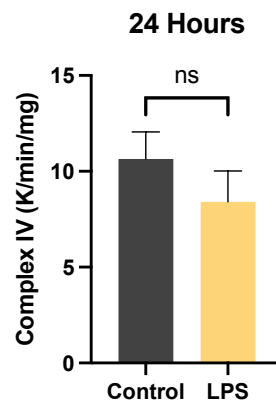


Figure 3.19. Complex IV specific activity is unchanged in isolated mitochondria from LPS-activated BMDMs.

Isolated mitochondria were extracted from BMDMs incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.4. Samples were freeze fractured three times and complex IV activity was measured with a spectrophotometric assay following the oxidation of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex IV is expressed as K/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were non-significantly different to each other.

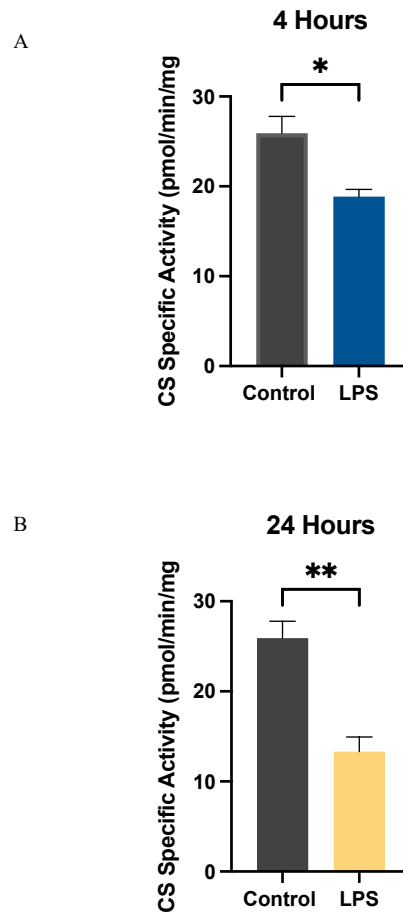


Figure 3.20. Citrate Synthase specific activity is decreased in isolated mitochondria from LPS-activated BMDMs.

Isolated mitochondria were extracted from BMDMs incubated with 100 ng/ml LPS for 4 h (A) and 24 h (B) as described in Section 2.9.5 Samples were freeze fractured three times and CS activity was measured with a spectrophotometric assay determined by monitoring the rate of production of dithionitrobenzoic acid at $\lambda = 412$ nm at 37°C. The specific activity of CS is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were significantly different to each other are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$.

3.2.2 IC₅₀ for complex I inhibition by itaconate in iBMDMs and BMDMs

Dose-response experiments were carried out on iBMDMs and BMDMs to investigate the inhibition of complex I and II activities by itaconate. To calculate IC₅₀, the cells were incubated with a different ITA concentration (0, 1, 3, 5, 10 and 20 mM) for 10 minutes. Then, complex I activity was measured as described in Section 2.9.1. **Figure 3.21 (A)** showed that the IC₅₀ for ITA inhibition of complex I activity in iBMDMs was 4.95 mM. **Figure 3.21 (B)** showed that the IC₅₀ for ITA inhibition of complex I activity in BMDMs was 3.48 mM.

3.3.3 IC₅₀ for complex II inhibition by itaconate in iBMDMs and BMDMs

The dose-response curves were used to determine the itaconate concentrations that induced 50% inhibition (IC₅₀) of complex II activity. The cells were treated for 10 minutes with different ITA concentrations (0, 1, 3, 5, 10, 20, 30 and 40 mM). Then, as described in section 2.9.2, complex II activity was assessed. **Figure 3.22 (A)** showed that the IC₅₀ for ITA inhibition of complex II activity in iBMDMs was 6.94mM. **Figure 3.22 (B)** showed that the IC₅₀ for ITA inhibition of complex II activity in BMDMs was 7.51 mM. **Figure 3.23 (A)** showed that the IC₅₀ for ITA in LPS-activated iBMDMs over 4 hours was 4.08 mM. **Figure 3.23 (B)** showed that the IC₅₀ for ITA in LPS-activated iBMDMs over 24 hours was 3.06 mM.

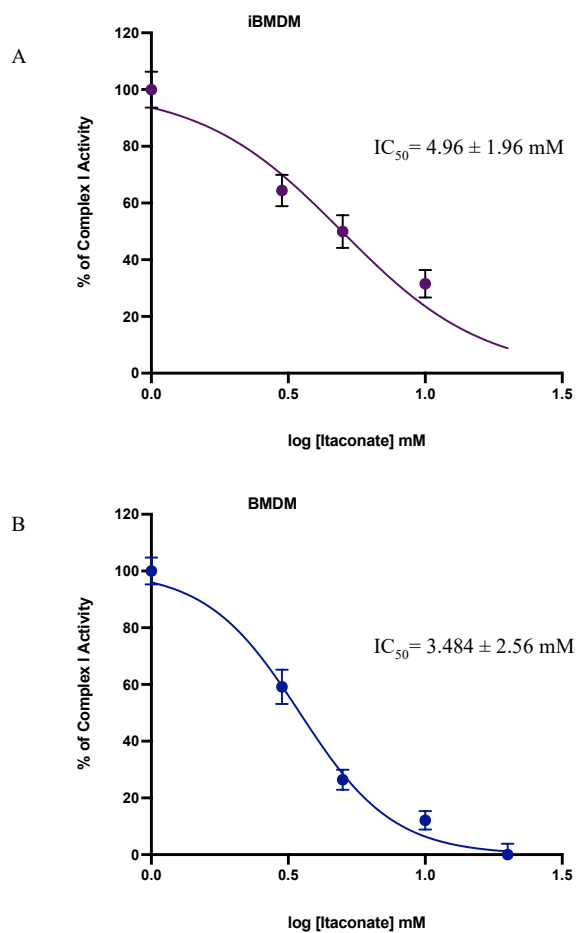


Figure 3.21. Itaconate inhibits Complex I activity in iBMDMs and BMDMs in a dose-dependent manner.

iBMDMs (A) and BMDMs (B) were incubated with a range of itaconate concentrations (0,1,3,5,10 and 20 mM) for 10 minutes. Complex I activity was assayed as described in Section 2.9.1 and the IC_{50} values were calculated.

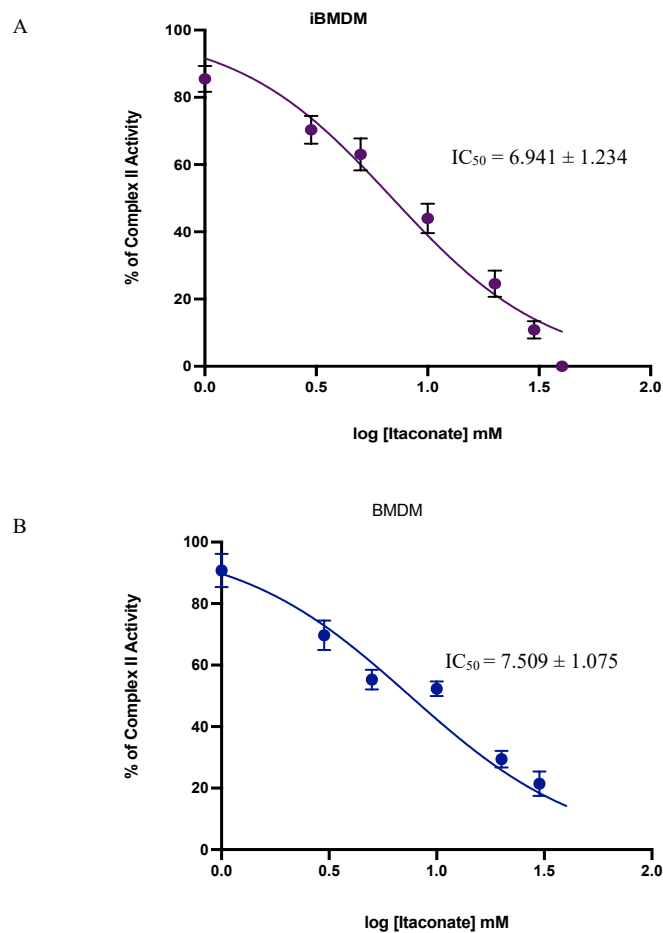


Figure 3.22. Itaconate inhibits Complex II activity in iBMDMs and BMDMs in a dose-dependent manner.

iBMDMs (A) and BMDMs (B) were incubated with a range of itaconate concentrations (0,1,3,5,10 and 20 mM) for 10 minutes. Complex II activity was assayed as described in Section 2.9.2. and the IC_{50} values were calculated.

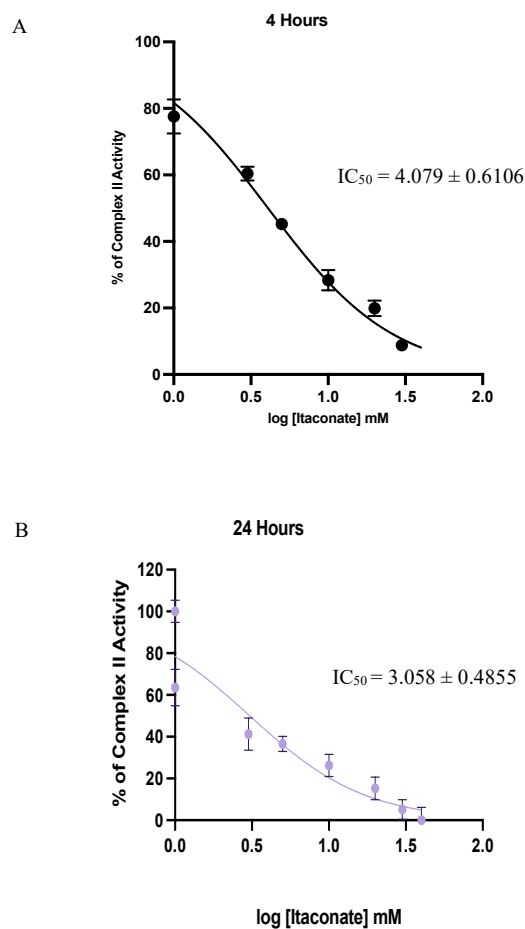


Figure 3.23. Itaconate inhibits Complex II activity in LPS-activated iBMDMs in a dose-dependent manner.

iBMDMs were activated with LPS for 4 hours (A) and 24 hours (B). The cells were incubated with a different itaconate concentration (0,1,3,5,10,20 ,30 and 40 mM) for 10 minutes. Then, complex II activity was measured as described at section 2.9.2 and the IC_{50} values calculated.

3.2.4. Itaconate is a competitive inhibitor of Complex II activity in macrophages.

A 5x5 experiment in which itaconate concentrations were added to lysed macrophages over a range of succinate substrate concentrations was performed. The purpose of this experiment was to examine the type of inhibition of SDH by itaconate and to calculate the K_i value. Michaelis–Menten saturation curves show the effect of itaconate on complex II activities (**Figure 3.24 (A)** and **Figure 3.25 (A)**). Lineweaver–Burk plots show itaconate causes competitive inhibition of Complex II activity in iBMDMs and BMDMs (**Figure 3.24 (B)** & **Figure 3.25 (B)**, respectively). The secondary plots where the slopes of the lines in the Lineweaver-Burk plots are plotted against their respective itaconate concentrations are shown in **Figures 3.24 (C)** and **3.25 (C)**. The $K_i = 0.014$ mM for itaconate inhibition of complex II activity in iBMDMs (**Figure 3.24 (C)**) and $K_i = 0.039$ mM for itaconate inhibition of complex II activity in BMDMs (**Figure 3.25 (C)**)

3.2.5 Itaconate is a time-dependent inhibitor of complex I activity in macrophages

Itaconate displayed a time-dependent inhibition of complex I activity in iBMDMs. While there was no effect on complex I activity at 20 mM ITA, initially (**Figure 3.26 (A)**), it was significantly decreased by ~ 30 % at 30 min preincubation (**Figure 3.26 (B)**) and by ~ 70 % at 60 min preincubation (**Figure 3.26 (C)**). A similar effect was observed in BMDMs with complex I activity decreased by ~ 15 % at 30 min preincubation (**Figure 3.27 (B)**) and by ~ 50 % at 60 min preincubation (**Figure 3.27 (C)**).

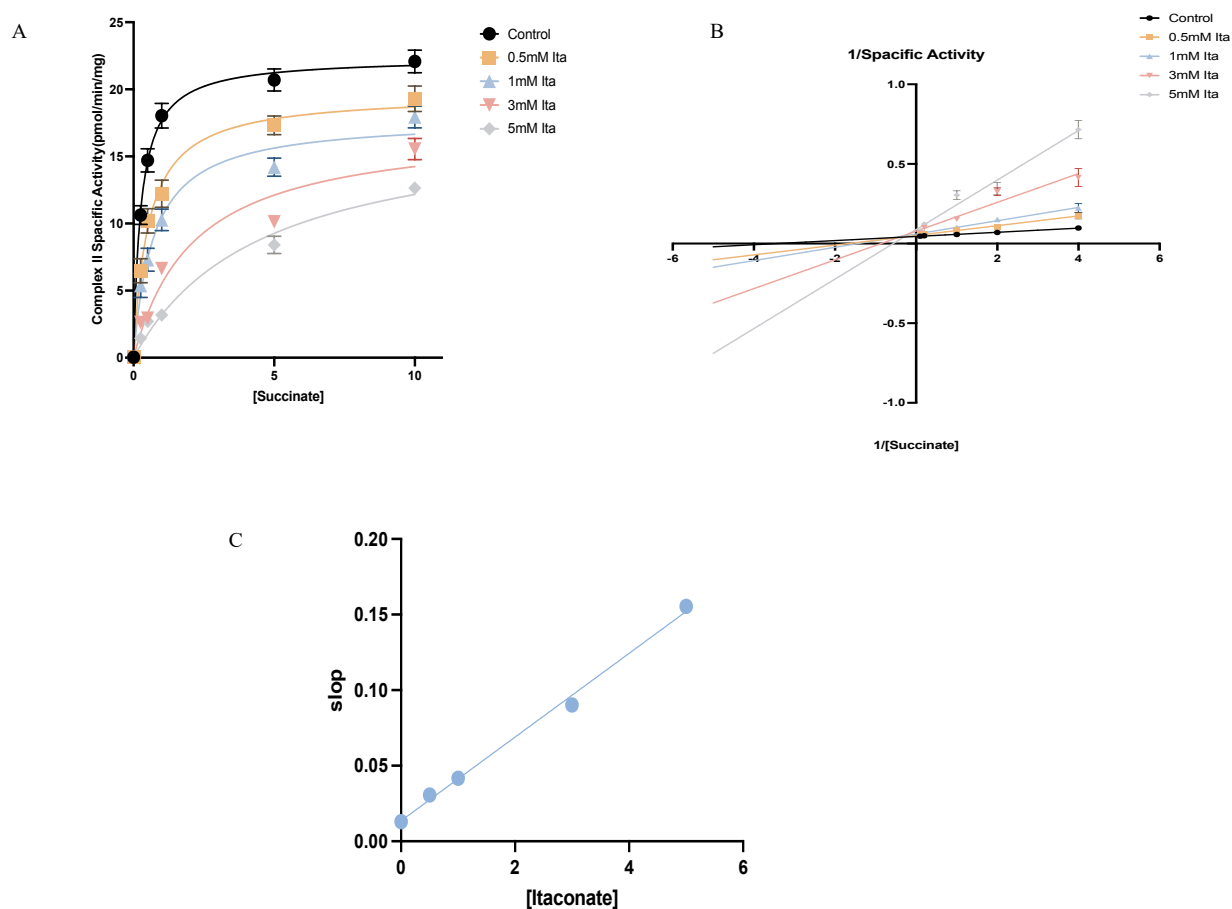


Figure 3.24. Itaconate induces competitive inhibition of Complex II activity in iBMDMs.

(A) Michaelis-Menten curves for complex II activity in iBMDMs was assayed by varying succinate concentrations in the presence of different itaconate concentrations (B) The reciprocal Lineweaver-Burk plot was plotted and indicated competitive inhibition (C) The secondary plot of the line slopes in the Lineweaver-Burk plot against the itaconate concentration indicate a K_i of 0.014 mM.

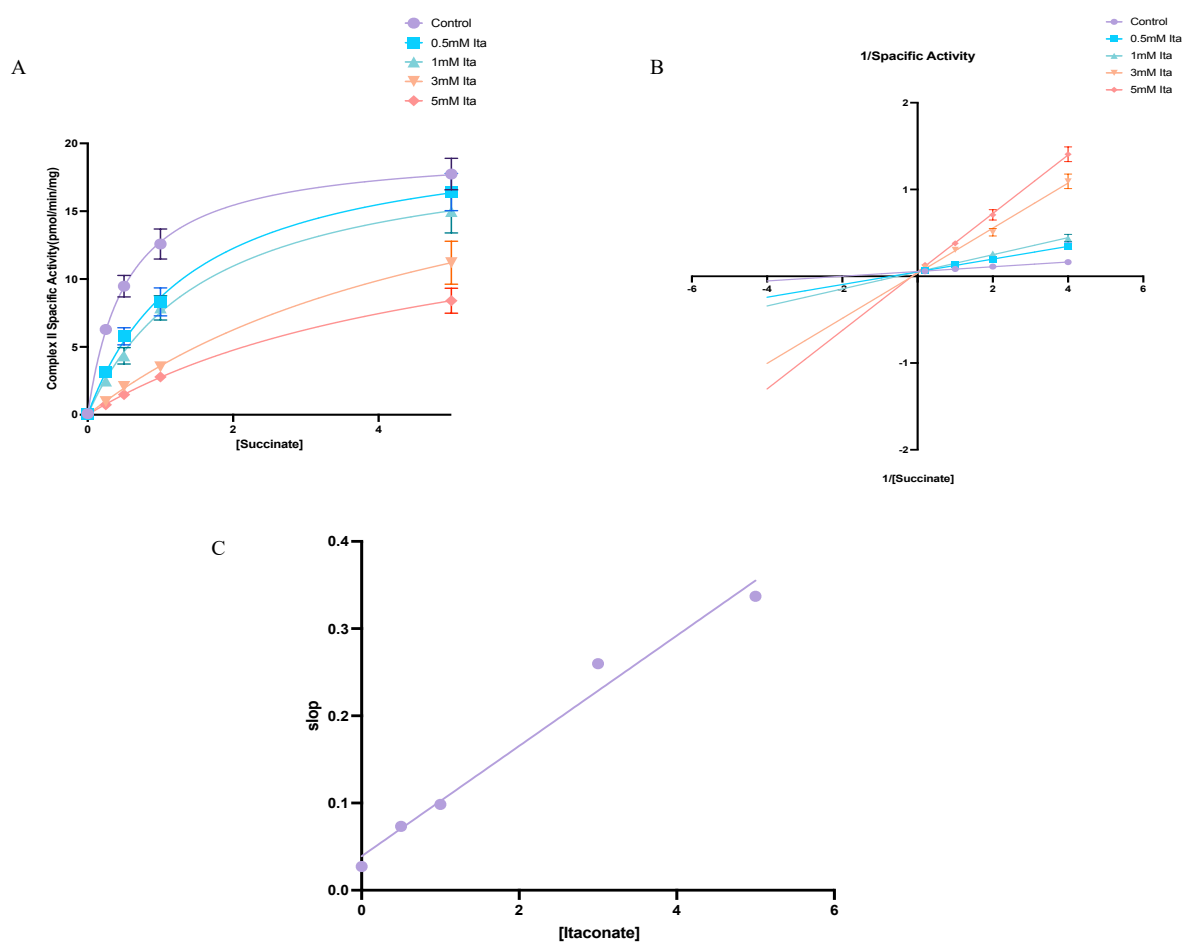


Figure 3.25: Itaconate induces competitive inhibition of Complex II activity in BMDMs.

(A) Michaelis-Menten curves for complex II activity in BMDMs was assayed by varying succinate concentrations in the presence of different itaconate concentrations (B) The reciprocal Lineweaver–Burk plot was plotted and indicated competitive inhibition (C) The secondary plot of the line slopes in the Lineweaver-Burk plot against the itaconate concentration indicate a K_i of 0.039 mM.

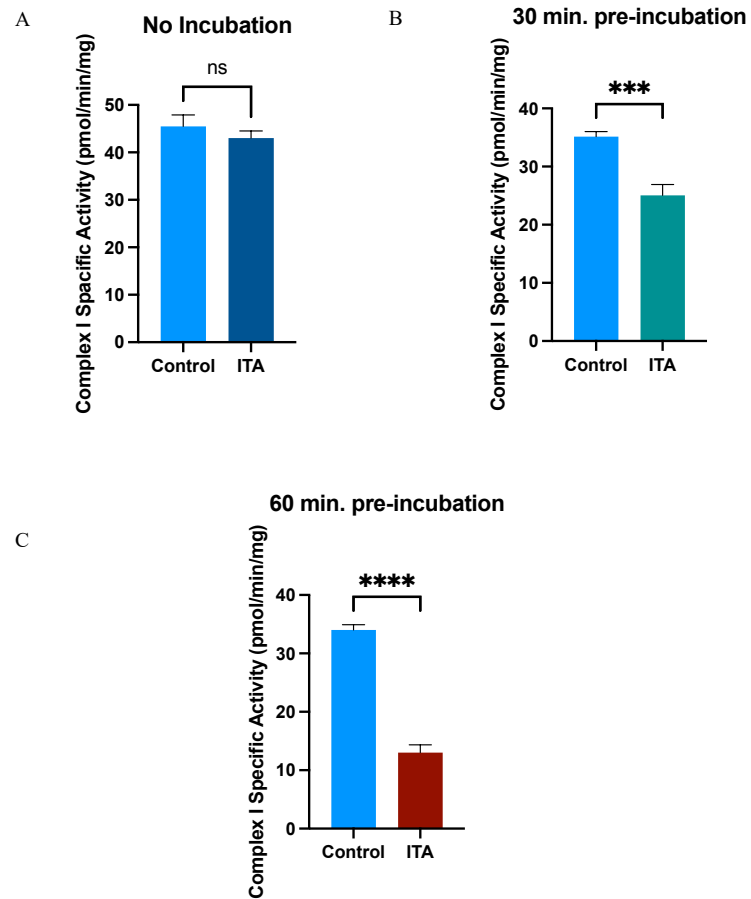


Figure 3.26: Inhibition of complex I activity in iBMDMs by itaconate is time-dependent.

iBMDMs were frozen fractured three times and incubated with 20 mM itaconate for 0, 30 and 60 min. Complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex I in iBMDMs is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$. (A) No incubation. (B) 30 min incubation. (C) 60 min incubation.

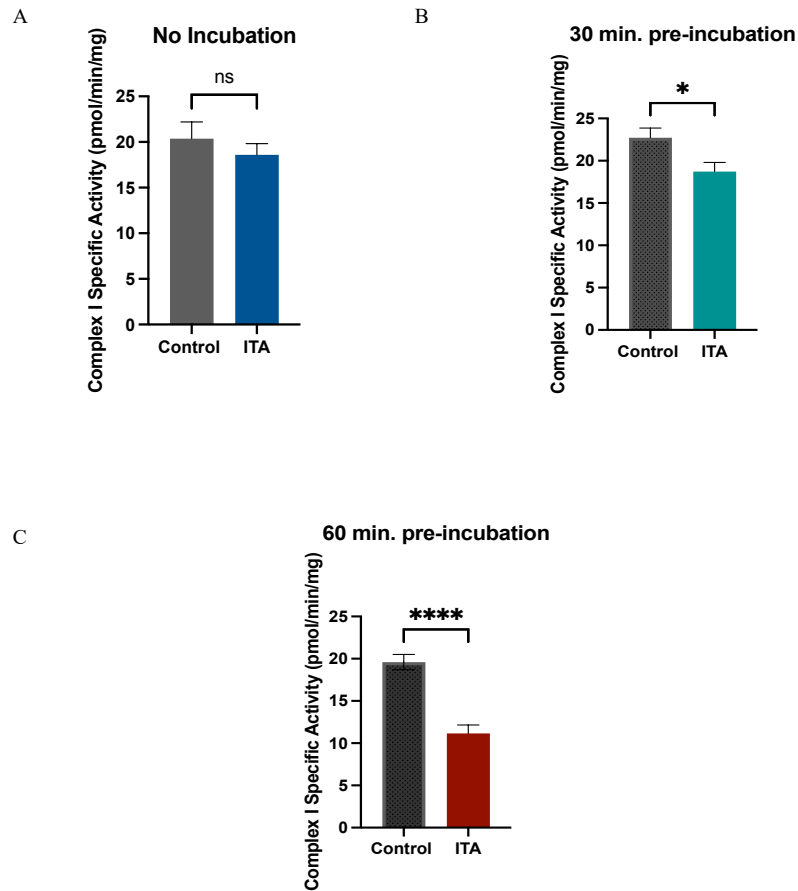


Figure 3.27: Inhibition of complex I activity in BMDMs by itaconate is time-dependent.

BMDMs were frozen fractured three times and incubated with 20 mM itaconate for (A) 0 min (B) 30 min and (C) 60 min. Complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex I in BMDMs is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with * for $p \leq 0.05$, and **** for $p \leq 0.0001$.

3.2.6 Itaconate inhibition of complex I and complex II activities in iBMDMs and BMDMs is reversible.

Figures 3.28 (A) and **3.29 (A)** showed that when 30 mM Itaconate was incubated with iBMDMs for 60 min and BMDMs for 20 minutes, complex I activity was significantly decreased by ~ 66 % in iBMDMs (**Figure 3.28 (A)**) and by ~ 25 % in BMDMs (**Figure 3.29 (A)**). To examine the reversibility of itaconate in complex I activity, iBMDMs were incubated with 30 mM ITA for 60 minutes and BMDMs with 30 mM ITA for 20 minutes, then the cells were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 5 min. The supernatant was carefully removed and discarded. The pellet was resuspended in complex I assay buffer, vortexed and assayed for complex I assay (described in section 2.9.1). **Figures 3.28 (B)** and **3.29 (B)** showed that the complex I inhibition by itaconate was reversed in both iBMDMs and BMDMs, respectively.

Figures 3.30 (A) and **3.31 (A)** showed that when 20 mM Itaconate was incubated with iBMDMs for 20 minutes and 30 mM Itaconate was incubated with BMDMs for 30 minutes, complex II activity was significantly decreased by ~ 50 % and ~ 88 %, respectively. To examine the reversibility of itaconate in complex II, iBMDMs were incubated the with 20 mM ITA for 20 minutes and 30 mM ITA with BMDMs for 30 minutes. Then the cells were sonicated and homogenised 20 times. After that, the cells were centrifuged at 15000 rpm for 5 min. The supernatant was carefully removed and discarded. The pellet was resuspended in complex II assay buffer, vortexed and complex II assayed (describe in section 2.9.2). **Figures 3.30 (B)** and **3.31 (B)** show that inhibition of complex II activity by itaconate is reversible in iBMDMs and BMDMs.

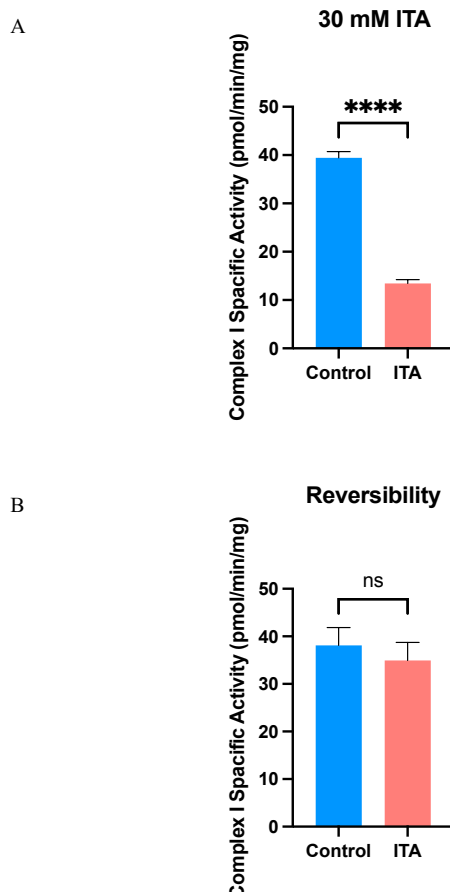


Figure 3.28: Inhibition of complex I activity in iBMDMs by itaconate is reversible.

iBMDMs were frozen fractured three times and incubated with 30 mM itaconate for 60 min, as described in Section 2.9.1. (A) Complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. In (B) cells incubated with 30 mM itaconate were sonicated, centrifuged at 15000 g for 10 min and the pellet resuspended in Complex I buffer and assayed for specific activity. The specific activity of complex I in iBMDMs is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with **** for $p \leq 0.0001$.

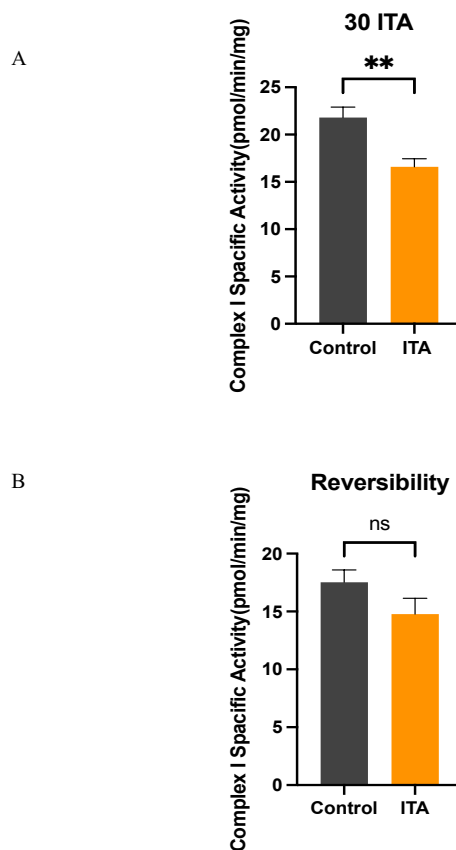


Figure 3.29: Inhibition of complex I activity in BMDMs by itaconate is reversible.

BMDMs were frozen fractured three times and incubated with 30 mM itaconate for 20 min. (A) Complex I activity was measured with a spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. In (B) cells incubated with 30 mM itaconate were sonicated, centrifuged at 15000 g for 10 min and the pellet resuspended in Complex I buffer and assayed for specific activity. The specific activity of complex I in BMDMs is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples that were highly significantly different to each other are shown with ** for $p \leq 0.01$.

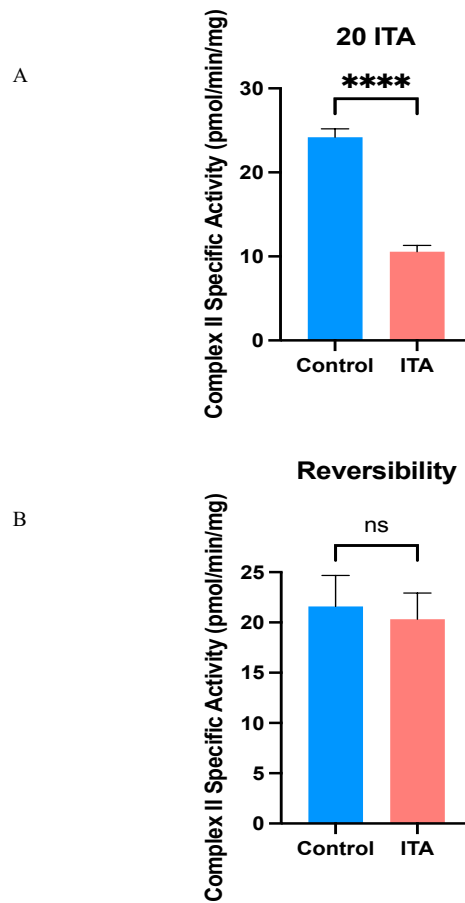


Figure 3.30: Inhibition of Complex II specific activity by itaconate in iBMDMs is reversible.

iBMDMs were incubated with 20 mM ITA for 20 min. as described in Section 2.9.2. Samples were freeze fractured three times and (A) Complex II activity was measured with a spectrophotometric assay using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600$ nm at 37°C. In (B) cells were incubated with 20 mM itaconate were sonicated, centrifuged at 15000 g for 10 min and the pellet resuspended in Complex II buffer and assayed for specific activity. The specific activity of complex II in iBMDMs is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with **** for $p \leq 0.0001$.

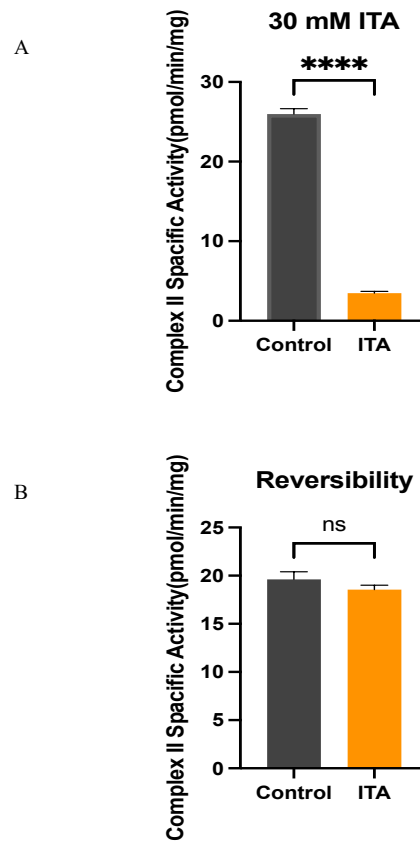


Figure 3.31: Itaconate inhibition of Complex II specific activity in BMDMs is reversible.

BMDMs were incubated with 30 mM ITA for 30 min. as described in Section 2.9.2 samples were freeze fractured three times and (A) Complex II activity was measured with a spectrophotometric assay using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600$ nm at 37°C. In (B) cells were incubated with 30 mM itaconate were sonicated, centrifuged at 15000 g for 10 min and the pellet resuspended in Complex II buffer and assayed for specific activity. The specific activity of complex II in BMDMs is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with **** for $p \leq 0.0001$.

3.3.7 Effects of Itaconate in iBMDMs and BMDMs on complex II/III and complex IV activities.

Itaconate was incubated with iBMDMs and BMDMs for 30 minutes, followed by complex II/III assay (described in section 2.9.3). **Figure 3.32** (A) shows that complex II/III activity was significantly reduced in iBMDMs treated with 30 mM itaconate by ~ 91 % (0.8318 ± 0.1023 pmol/min/mg and 8.707 ± 0.4604 pmol/min/mg for control). **Figure 3.32** (B) shows that complex II/III activity was significantly reduced in BMDMs treated with 30 mM itaconate ~ 66 % (2.220 ± 0.2380 pmol/min/mg and 6.502 ± 0.3805 pmol/min/mg for control).

However, when itaconate was incubated with iBMDMs and BMDMs for 30 minutes, followed by complex IV assay (described in section 2.9.4), no inhibition was observed. **Figures 3.33** (A) illustrate that there was no significant difference in complex IV between iBMDMs $\pm$ 40 mM ITA (0.1328 ± 0.01120 pmol/min/mg and 0.1297 ± 0.00933 pmol/min/mg). Also, **figures 3.33** (B) there was no significant difference in complex IV between iBMDMs $\pm$ 40 mM ITA (0.1039 ± 0.01249 pmol/min/mg and 0.8235 ± 0.01460 pmol/min/mg).

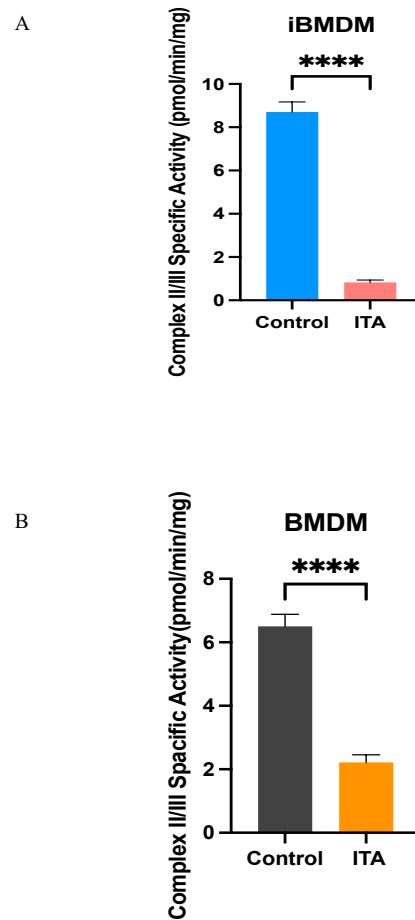


Figure 3.32: Itaconate decreases Complex II/III specific activity in iBMDMs and BMDMs

(A) iBMDMs and (B) BMDMs were incubated with 30 mM ITA for 30 min as described in Section 2.9.3. Samples were freeze fractured three times and complex II/III activity was measured with spectrophotometric assay following the reduction of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex II/III in iBMDMs and BMDMs is expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were non-significantly different to each other are shown with **** for $p \leq 0.0001$.

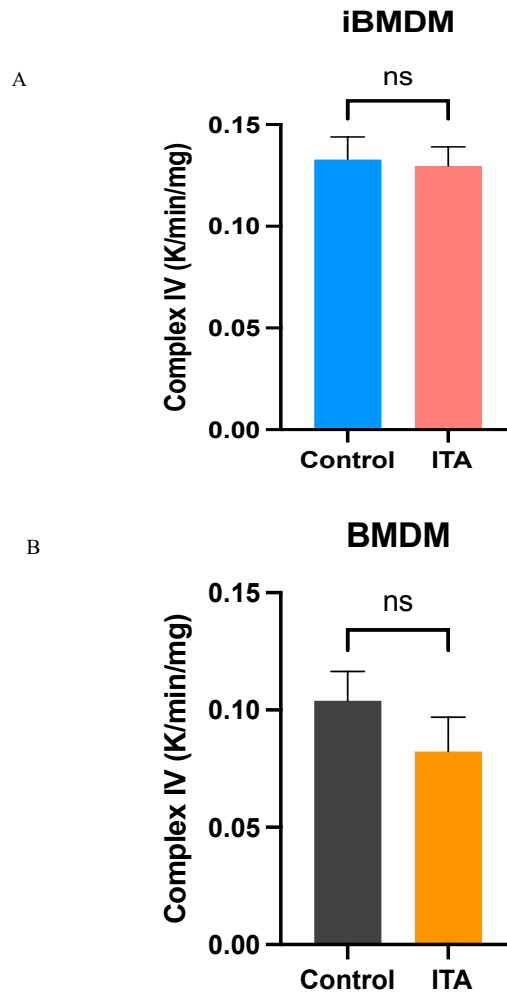


Figure 3.33: Complex IV specific activity was unchanged in ITA-treated iBMDMs and BMDMs.

(A) iBMDMs and (B) BMDMs were incubated with 40 mM ITA for 10 min as described in Section 2.9.4. Samples were frozen fractured three times, and complex IV activity was measured with spectrophotometric assay following the oxidation of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex IV is expressed as K/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values and differences were non-significant.

3.2.8 Effect of some immuno-regulatory molecules on complex I and complex II activities in BMDMs.

The effects of a series of immuno-regulatory molecules on complex II activity were tested following a 30 min pre-incubation period (**Figure 3.34**). BMDMs were pre-treated with 100 μ M of Vigabatrin, Aminooxy acetic acid, MTX, 6-TG, Methylnicotinamide, 6-MP, Azathioprine, Phenformin hydrochloride, Ivermectin, TPN, Hydrocortisone, Progesterone, Corticosterone and Prednisolone. In addition, BMDMs treated with 10 mM D-serine, 1-amino-n-Butyric acid, NA-3-hydroxy butyrate, Dimethyl fumarate, Dimethyl succinate and Diethyl fumarate. BMDMs were treated with 50 μ M MCC950 and 1 μ M of Hydroxyglutaric acid and indole-3-pyruvate. Complex II activity was significantly decreased in BMDMs by Ivermectin (~ 40 %) and by Hydroxyglutaric acid (~ 30 %). Moreover, there was more significant decrease in complex II activity in BMDMs treated with the inflammasome inhibitor, MCC950 (~ 50 %) and the indole derivative, indol-3-pyruvate (~ 60 %) .

The effects on complex I activity in BMDMs were tested (**Figure 3.35**) with 100 μ M Ivermectin, 100 μ M 6-TG , 50 μ M MCC950, 1 μ M Hydroxyglutaric acid and I3P, all with a 30 min preincubation. Complex I activity in BMDM treated was significantly decreased with Ivermectin (~ 40 %) and I3P (~ 20 %).

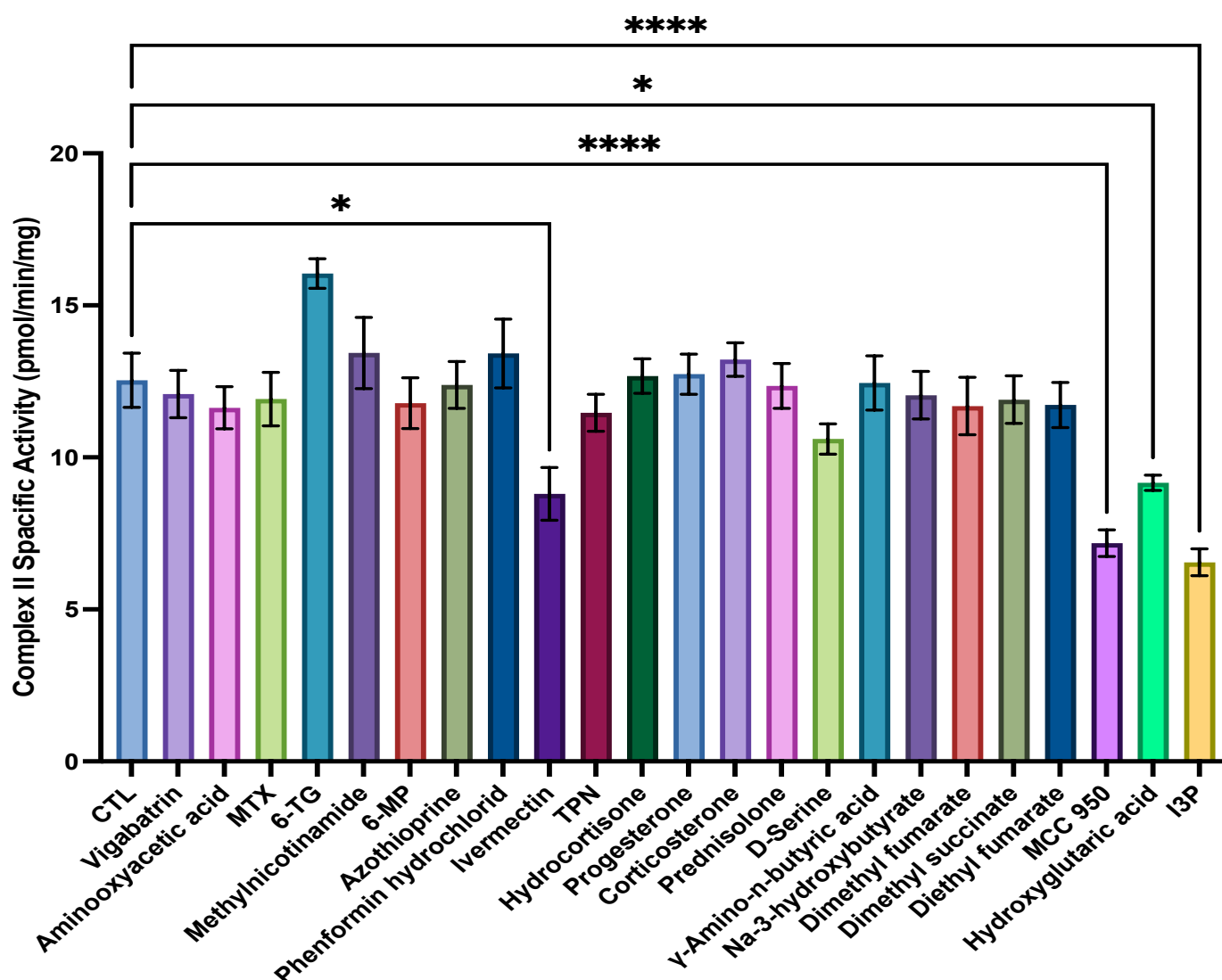


Figure 3.34: The effects of some immuno-regulatory molecules on complex II activity in BMDMs.

Samples were freeze fractured three times, and complex II activity was measured spectrophotometrically using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600$ nm at 37°C. The specific activity of complex II in BMDMs $\pm$ drugs for 30 min pre-incubation, expressed as pmol/min/mg $\pm$ SEM, n=4. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with * for $p \leq 0.05$, and **** for $p \leq 0.0001$. The final concentration of Vigabatrin, Aminoxy acetic acid, MTX (Methotrexate), 6-TG (6-thioguanidine), Methylnicotinamide, 6-MP, Azathioprine, Phenformin hydrochloride, Ivermectin, TPN, Hydrocortisone, Progesterone, Corticosterone and Prednisolone was 100 μ M. The final concentration of D-serine, 1-amino-n-Butyric acid, NA-3-hydroxybutyrate, Dimethyl fumarate, Dimethyl succinate and Diethyl fumarate was 10 mM. The final concentration of MCC 950 was 50 μ M. The final concentration of Hydroxyglutaric acid and I3P was 1 μ M.

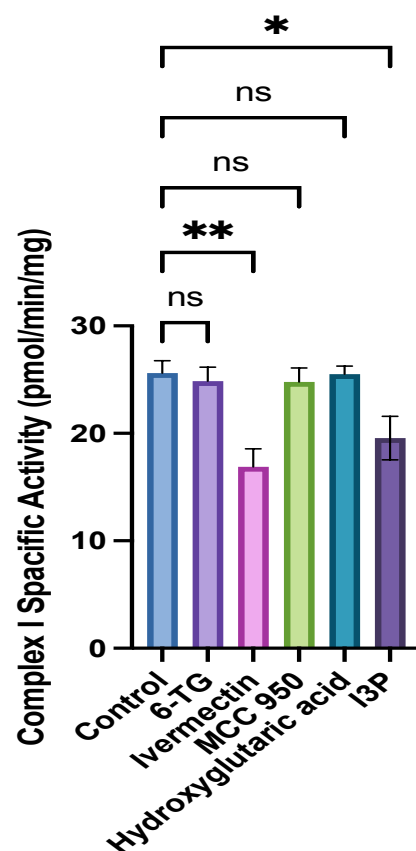


Figure 3.35: The effects of some immuno-regulatory molecules on complex I activity in BMDMs

Samples were frozen fractured three times and complex I activity was measured with spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex for 30 min pre-incubation, expressed as pmol/min/mg $\pm$ SEM, n=3. An unpaired t-test was performed on values. Samples were highly significantly different to each other are shown with * for $p \leq 0.05$, ** for $p \leq 0.01$. The final concentration of 6-TG and Ivermectin was 100 μ M. The final concentration of MCC 950 was 50 100 μ M. The final concentration of Hydroxyglutaric acid and I3P as 1 μ M.

3.3 Discussion

As previously stated, most catabolic processes supply electrons in the form of the reducing equivalents NADH and FADH₂ to the mitochondrial ETC. The type of the fuels that feed mitochondrial metabolism affects the intramitochondrial NADH/FADH₂ ratio, and the respiratory chain adapts to these fuel source changes, especially during PRR-mediated macrophage activation. Furthermore, alterations in the ETC have been observed in activated murine macrophages, according to two recent investigations. The mitochondrion, a bioenergetic organelle at the centre of metabolic pathways, not only contributes to energy production, biosynthesis, or cellular redox maintenance but also acts as a signalling centre for several innate immunological signalling pathways. (Stanley et al., 2014) Several studies have highlighted the potential importance of ETC respiratory complexes in macrophage activation. In mitochondria isolated from BMDMs, assessment of the activity of CI, CII, CII/III and CIV by quantitative spectrophotometry revealed differences and also indicated diminished CI-mediated respiration in response to *E. coli* (Garaude et al., 2016). Due to a decrease in the abundance of assembled CI, the ETC architecture was changed as soon as viable bacteria were found. There is a lot of knowledge about the metabolic pathways and reprogramming involved in inflammation that has been produced as a result of the usage of LPS to activate macrophages (O'Neill and Pearce, 2016).

In this study, we compared the specific activities for complex I, II, II/III, IV and citrate synthase in iBMDMs, BMDMs and isolated mitochondria from BMDMs after activation with 100 ng/ml LPS for 4 hours and 24 hours. We found that, complex I, II activities and citrate synthase were significantly decreased in a time-dependent manner in iBMDMs $\pm$ 4 hours and 24 hours LPS. However, there no significant change in complex II/III and IV activities. In BMDMs, complex I, II, II/III activities and citrate synthase were significantly

decreased in a time-dependent manner when cells were activated with LPS for 4 hours and 24 hours. Complex IV activity was unchanged.

Moreover, there were no significant decreases in the activities of complex I and II when assayed in isolated mitochondria from BMDMs activated with LPS for 4 hours. However, the activities of complex I and II was significantly decreased when activated the pure mitochondria with LPS for 24 hours. The activities of complex II/III and CS were also significantly decreased in isolated mitochondria from BMDMs following LPS activation.

Systemic injection of LPS causes region-specific neuroinflammation and oxidative phosphorylation and complex II, III dysfunction in the mouse brain, according to Noh et al (Noh et al., 2014). In rats with endotoxin-*induced* intestinal damage, Zhou et al. discovered a substantial decrease in mitochondria respiratory chain activity (Zhou et al., 2016b). Sun et al. found that LPS causes apoptosis in human umbilical vein endothelial cells via lowering the activities of mitochondria respiratory chain complexes (I, II, III, IV, and V) (Sun et al., 2016). We hypothesized that LPS activation of iBMDMs and BMDMs induced metabolic changes that disrupt the function of mitochondria ETC complexes, resulting in a decrease in oxidative phosphorylation. The results show that complex I and II activities are significantly decreased in a time-dependent manner and importantly, whereas complex II inhibition is reversible the inhibition of complex I is irreversible. The nature of this irreversible inhibition of complex I activity is unknown at present but may be nitric oxide or ROS-related, or even involve effects on ETC super-complex formation in mitochondria following response to LPS activation.

The ability of activated macrophages to kill tumour cells was discovered in the 1980s, and this NO-dependent activity was linked to a set of metabolic changes in both the target

tumour cells and the macrophages, including inhibition of mitochondrial aconitase, complex I and complex II, and the loss of intracellular iron (Hibbs Jr et al., 1987, Hibbs Jr et al., 1988, Stuehr and Nathan, 1989). Nitric oxide (NO) may have a normal role in limiting mitochondrial O₂ consumption by inhibiting cytochrome c oxidase. The fact that the apparent K_m for O₂ in whole cells is larger than that of isolated mitochondria could be explained by NO inhibiting cytochrome oxidase. Although NO inhibition of cytochrome oxidase is easily reversible, it can have significant consequences for the cell in pathophysiological disease states (Sharpe and Cooper, 1998, Brown, 1995, Brown and Cooper, 1994). Further work is required to examine the nature of complex I inhibition in macrophage mitochondria.

The fact that citrate synthase activity was reduced by 70% and 90% in BMDMs and iBMDMs, respectively, following LPS-activation reveals a novel aspect to metabolism in activated macrophages. Citrate synthase is an important metabolic control point in the Krebs Cycle, responsible for converting oxaloacetate and acetyl CoA to citrate, and its reduction suggests that LPS induced macrophages inhibit mitochondrial metabolism at multiple control points. In mitochondria extracted from LPS-activated BMDMs citrate synthase was only decreased by approximately 50 % at 24h. This suggests that some of the 70% inhibition of citrate synthase in BMDM cells may have been reversed during the homogenisation and centrifugation procedures used for mitochondria extraction.

Itaconate is known to regulate succinate levels, inflammatory cytokine production, mitochondrial respiration, and electron transport chain directionality. Itaconate was found to inhibit complex I and II activities that may underlie the observed inhibition of respiration in iBMDMs or BMDMs activated with LPS. It was suggested that itaconic acid at millimolar concentrations affects mitochondrial functions and viability of neurons

(Umbrasas et al., 2021). In iBMDMs and BMDMs, itaconate was found to reversibly inhibit complex II activity with IC₅₀s in the low millimolar range. In LPS-activated macrophages any remaining complex II activity was further inhibited by itaconate with similar IC₅₀s. Itaconate concentrations have been reported to reach higher concentration of approximately 5 mM in LPS-activated macrophages and would be expected to inhibit complex I and II activities in situ. However, the K_i value for itaconate inhibition of complex II activity was found to be in the micromolar range at 14 μM and 39 μM for iBMDMs and BMDMs, respectively. The type of itaconate-induced inhibition of complex II was found to be competitive. The approximate 100 fold difference in IC₅₀:K_i ratio can be explained by the high concentrations of succinate substrate that were used in the complex II assay (Section 2.9.2). As can be seen in the relationship between IC₅₀ and K_i for a competitive inhibitor, $IC_{50} = K_i(1+[S]/K_m)$, a succinate assay concentration of 20 mM and a low K_m of approx. 0.20 mM will result in an approximate 100 fold difference in IC₅₀:K_i ratio. The novel finding of a low K_i value of 39 μM for itaconate on complex II in resting macrophage (BMDMs) suggests that itaconate at much lower concentration than previously thought will inhibit complex II activity (Mills et al 2018).

The type of itaconate-induced inhibition of complex I was found to be time-dependent and reversible in resting macrophages. The reversible nature of complex I inhibition is intriguing as it was found to be irreversible in LPS-activated macrophages. This suggests something other than itaconate is responsible for causing irreversible inhibition of complex I activity in activated macrophages.

Succinate build-up is thought to enhance ROS generation in mitochondria and the cell when the respiratory chain complex II is inhibited (Mills et al., 2016). In that study experiments

showed increased mitochondrial oxidation of succinate via succinate dehydrogenase (complex II) and an elevation of mitochondrial membrane potential combine to drive mitochondrial ROS production. Further studies by the same group showed that itaconate is anti-inflammatory as its inhibition of complex II prevents reverse electron transfer (RET) from ubiquinol to complex I and reduces proinflammatory ROS (Mills et al., 2018; Hooftman et al., 2020). These studies also suggested that itaconate has additional anti-inflammatory properties such as Nrf2 activation and NLRP3 regulation. The inhibitor of complex II malonate has previously been found to greatly reduce the rate of H₂O₂ generation by mitochondria (Zhang et al., 2020, Belosludtsev et al., 2020, Williams and O'Neill, 2018a). While itaconate has been known for decades as a competitive inhibitor of SDH due to its structural similarity to succinate (Ackermann and Potter, 1949), the potential physiological role of itaconate as an endogenous inhibitor of SDH remains unexplored. SDH inhibition prevents the oxidation of succinate to fumarate, thereby preventing the generation of mitochondrial complex reactive oxygen species (mtROS) (Langston et al., 2017, Peace and O'Neill, 2022, Fischer et al., 2021).

Due to the observation that complex I, II and II/III activities were found to be decreased in LPS-activated macrophages and that ETC activities are known to be inhibited by a wide-range of molecules, a series of compounds (especially those used clinically for anti-inflammatory properties) were tested for inhibitory properties of several compounds on complex I and II activities. Ivermectin was found to decrease mitochondrial complex I and II activities. Ivermectin is a member of the ivermectin's (AVM) family of 16-membered macrocyclic lactone compounds discovered in the Japanese Kitasato Institute in 1967. Ivermectin is an antiparasitic drug that was first licensed in humans in 1987 to treat onchocerciasis, often known as river blindness, which is caused by the blackfly-transmitted

parasite *Onchocerca volvulus* in low-income populations in the tropics (Crump, 2017). Further work is required to investigate the mechanism of inhibition of complex I and II and the possibility that ivermectin may modulate macrophage function through effects on the mitochondrial ETC. Some evidence in the literature suggests that 8-hydroxy-2'-deoxyguanosine (8-OHdG), a common oxidative DNA damage marker may be affected by ivermectin appears as a result of oxidative stress produced by mitochondrial malfunction (Wang et al., 2018).

Other inhibitors of complex II activity were found to be MCC950 and hydroxyglutarate. 2-Hydroxyglutarate is structurally similar to α -ketoglutarate (α -KG), a tricarboxylic acid (TCA) cycle intermediate; it is made by converting the ketone group of α -KG to a hydroxyl group. While L-2-hydroxyglutarate activates HIF-1 α in LPS-activated macrophages (Williams et al., 2022) its enantiomer D-2-hydroxyglutarate increases in concentration and is thought to exert anti-inflammatory effects following TLR4 activation (de Goede et al., 2022). MCC950, a diarylsulfonylurea-containing molecule, has been identified as one of the most effective and highly selective small molecule inhibitors of both canonical and non-canonical NLRP3 inflammasome activation. It is thought to act by inhibiting apoptosis-associated speck-like protein containing a CARD (ASC) oligomerization (Coll et al., 2015, Zeng et al., 2021).

Indole-3-pyruvate is a tryptophan metabolite, which is rarely studied in the context of immunity. It is not produced by the human body (though it has been claimed to be produced with the help of commensal bacteria), and its impact on innate immune function is mainly unknown. The immunomodulatory effects of I3P have been studied in a UVB-induced inflammatory model. I3P is structurally similar to α -KG, an endogenous metabolite that

boosts prolyl hydroxylase activity, allowing for more HIF-1 α hydroxylation and degradation (McGettrick et al., 2016). In this study HIF-1 α protein levels were found to be reduced by I3P. Because HIF-1 α is a transcription factor, it will cause the expression of various HIF-1 α target genes to decrease, including GLUT1, VEGF, LDHA, and IL-1 β (McGettrick et al., 2016). This chapter also reports that indole-3-pyruvate also inhibits both complex I and II activities in BMDMs. Further studies will need to investigate the type of inhibition of complexes I and II, the potency of inhibition as well as time-dependence and reversibility.

CHAPTER 4

METABOLIC CONTROL ANALYSIS OF MITOCHONDRIAL BIOENERGETICS IN IBMDMs AND BMDMs

4.1 Introduction

The metabolism and functional phenotype of macrophages are interdependent. Recent studies have noticed that infectious stimuli alter the metabolic phenotype of macrophages or dendritic cells, and a switch in metabolism to glycolysis from mitochondrial oxidative phosphorylation (OXPHOS) is essential for innate immunity (Tannahill et al., 2013). Different stimuli cause macrophage polarisation, such as LPS or IFN- which mediate classical M1 activation, while IL-4 with IL-13 promote alternative M2 activation (Mantovani et al., 2004).

Metabolic Control Analysis (MCA), described in Chapter 1, can be used to investigate the spread of control over the entire flux of a system, such as the control that each of the respiratory chain complexes exerts on overall oxygen consumption in a cell type. Flux control coefficients and thresholds can change depending on tissue source, age of animals, energy state and experimental control conditions (Lenaz et al., 2000). MCA investigations on isolated synaptic and non-synaptic rat brain mitochondria and crude mitochondrial pellet samples have previously been conducted. When isolated non-synaptic mitochondria were compared to synaptic mitochondria, the spread of control between the respiratory chain complexes over synaptosomal oxygen intake and ATP generation was different, with complex I exerting a high flux control coefficient and low energy threshold (Davey, Canevari, & Clark, 1997; Davey & Clark, 1996; Davey, Peuchen, & Clark, 1998). Complex I activity was shown to be 36% lower in synaptic mitochondria than in non-synaptic mitochondria in a previous study, which may help to explain why synaptic and synaptosomal mitochondria had lower thresholds than non-synaptic mitochondria (Almeida et al., 1995).

4.1.1 ETC Inhibitors

A range of tight-binding small molecules have been effectively used as specific inhibitors of ETC complexes with varying consequences on mitochondrial biogenesis, cell viability and proliferation (Figure 4.1).

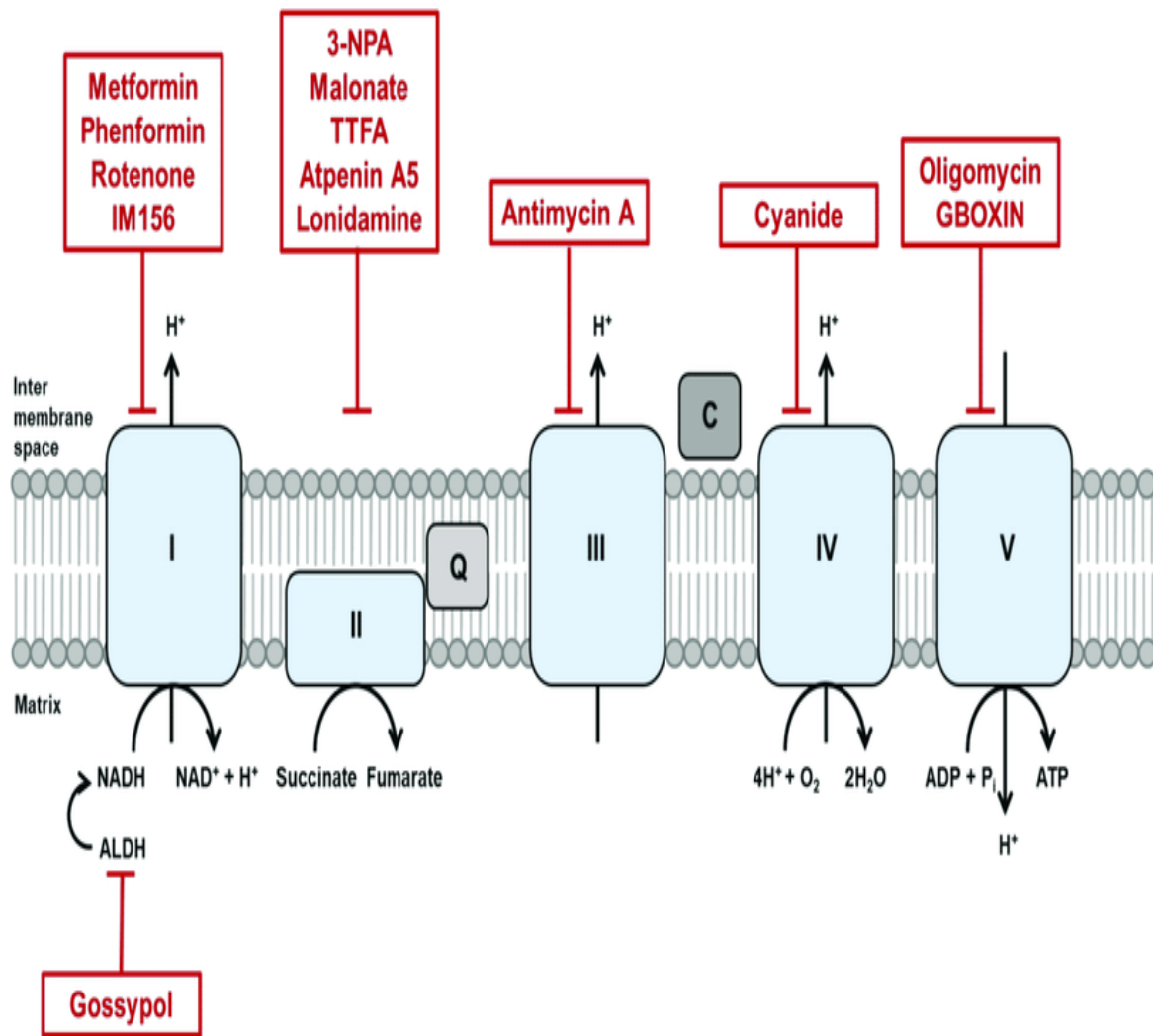


Figure 4.1: Typical inhibitors of mitochondrial ETC complexes and oxidative phosphorylation. OXPHOS generates ATP by moving electrons along the electron transport chain, a sequence of transmembrane complexes in the mitochondrial inner membrane (ETC). Electrons travel through complex I, complex II, coenzyme Q (Q), complex III, cytochrome c (C), and complex IV, with O₂ serving as an electron acceptor at the end (Kim and Cheong, 2020). Classical inhibitors of ETC complexes such as rotenone, antimycin A and potassium cyanide (KCN) have been used in MCA to study the control that individual complexes have over oxygen respiration and ATP synthesis.

4.1.1.1 Rotenone

Rotenone (Rot) is a naturally occurring lipophilic compound. It is mainly derived from the roots and stems of plants belonging to the Fabaceae family (Lonchocarpus and Derris species) (Heinz et al., 2017; Palmer, Horgan, Tisdale, Singer, & Beinert, 1968). Rotenone is a toxic compound that potently inhibits complex I of the mitochondrial respiratory chain, preventing NADH oxidation, and subsequently causing increased production of reactive oxygen species. It inhibits the transfer of electrons from the iron-sulfur centres of complex I to ubiquinone via binding to the ubiquinone binding site of complex I (Fato et al., 2009; Huang, Lin, Hung, Chuang, & Wu, 2018).

4.1.1.2 Thenoyltrifluoroacetone

Thenoyltrifluoroacetone (TTFA) is a chemical compound widely used to inhibit mitochondrial complex II. It is a well-known inhibitor of succinate oxidation (Ingledew & Ohnishi, 1977; Ulvik, 1975; J.-G. Zhang & Fariss, 2002). It binds to the ubiquinone binding site of Complex II, thereby blocking electron transfer and causing electron slip and superoxide formation (Kruspig, Valter, Skender, Zhivotovsky, & Gogvadze, 2016; Mehta & Shaha, 2004).

4.1.1.3 Antimycin A

Antimycin A (AA) is a chemical compound produced by *Streptomyces kitazawensis* (Nakayama, Okamoto, & Harada, 1956). It contains a formamide and salicylate component, formamidosalicylic acid, connected by an amide group to a dilactone ring. Acyl and alkyl side chains on the dilactone ring determine the nomenclature of AA (**Figure 4.1 (C)**) (Rühle & Leister, 2016). Nowadays, at least 20 different AA structures are known (Xu et al., 2011). AA inhibits the oxidation of ubiquinol in the electron transport chain, which blocks the mitochondrial electron transfer between cytochrome b and c by binding to the Q_i site of cytochrome reductase in the mitochondrial complex III (Alexandre & Lehninger, 1984; Xia

et al., 1997). Due to the inhibition of electron transport, which causes a breakdown of the proton gradient across the mitochondrial inner membrane, a loss of the mitochondrial membrane potential ($\Delta\Psi_m$) occurs (Balaban, Nemoto, & Finkel, 2005; Campo, Kinnally, & Tedeschi, 1992). A further consequence of AA inhibition of CIII is electron leakage and increased superoxide formation.

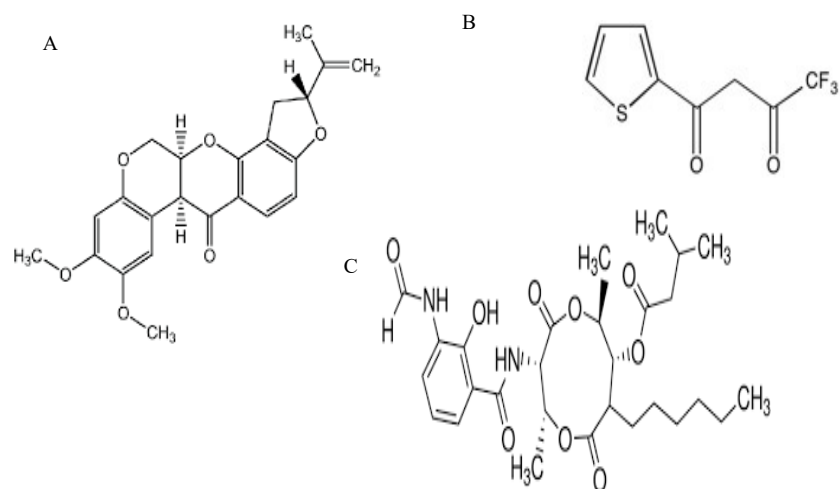


Figure 4.2. Chemical structures of ETC inhibitors. (A) Rotenone (B) TTFA (C) Antimycin A.

4.1.2 Aims of Chapter

- To investigate the spread of control of the respiratory chain complexes I, II and III over oxygen consumption in iBMDMs by determining their flux control coefficients and inhibition thresholds.
- To investigate the spread of control of the respiratory chain complexes I, II and III over oxygen consumption in BMDMs by determining their flux control coefficients and inhibition thresholds.
- To investigate the effect of LPS-activation on mitochondrial respiration in BMDMs over extended time-periods .
- To investigate the effect of the anti-inflammatory cytokine, IL-10, on mitochondrial bioenergetics in LPS-activated BMDMs.

4.2 Results

4.2.1 Metabolic control analysis of complex I in iBMDMs

In order to calculate the flux control coefficient and inhibition threshold for complex I over oxygen respiration in iBMDMs, a range of rotenone concentrations were used to titrate complex I activity while measuring OCR rates (**Figure 4.3 (A)**). Rotenone decreased the iBMDM basal and maximal oxygen consumption rates (OCR) and complex I activity in a dose-dependent manner (**Figure 4.3 (B) and (C)**). The OCR in iBMDMs fell more quickly than that of complex I specific activity (**Figures 4.3 (B) and 4.4**, respectively). Complex I activity decreased linearly to ~ 80% over the rotenone concentration range of 0 - 1000 nM; however, oxygen consumption remained at between ~ 80 and 95 % (**Figure 4.5**). The flux control coefficient was calculated using Equation 4.1. The rate of iBMDMs oxygen consumption at lower concentrations of rotenone was divided by the rate of complex I activity at lower concentrations of the inhibitor (see inset graph of **Figure 4.5 (A)**). The slope of the lower concentrations of iBMDMs oxygen consumption was -3.56. The slope of the lower concentrations of complex I activity was -15.61, giving a flux control coefficient for complex I of 0.23. The lower concentrations of iBMDMs maximal oxygen consumption slope were - 6.23. The slope of the lower concentrations of complex I activity was -15.61, giving the flux control coefficient for complex I 0.40 (see inset graph of **Figure 4.5 (B)**). The threshold level of complex I inhibition was determined from a curve created by expressing the rate of iBMDMs oxygen consumption as a function of the level of inhibition of complex I activity (**Figure 4.6**). The complex I inhibition threshold was found to be ~ 60 % for basal oxygen consumption and ~ 60 % for maximal oxygen consumption. **Figure 4.7** showed the complex I inhibition threshold for Extracellular acidification rate (ECAR) in iBMDMs is ~ 60 %.

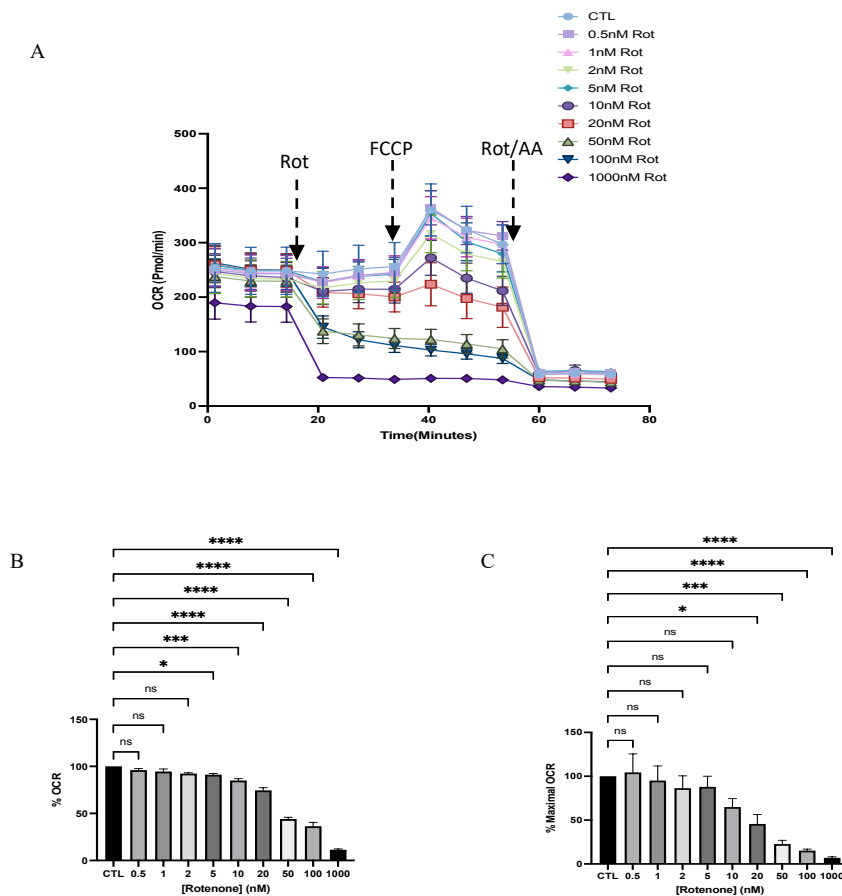


Figure 4.3. Rotenone titration of Oxygen Consumption Rate (OCR) in iBMDMs.

(A) iBMDMs were plated at 0.5×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. A range of rotenone concentrations (0.5nM-1 μM) were used to inhibit complex I activity and the Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. Following injection of the mitochondrial uncoupler, FCCP (1 μM), the Maximal Oxygen consumption rate (OCR) was measured. Following injection of rotenone + antimycin A (1 μM each), the non-mitochondrial OCR was measured. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) The oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. Results are expressed as mean $\pm$ SEM, $n=4$. (C) The Maximal Oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=4$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

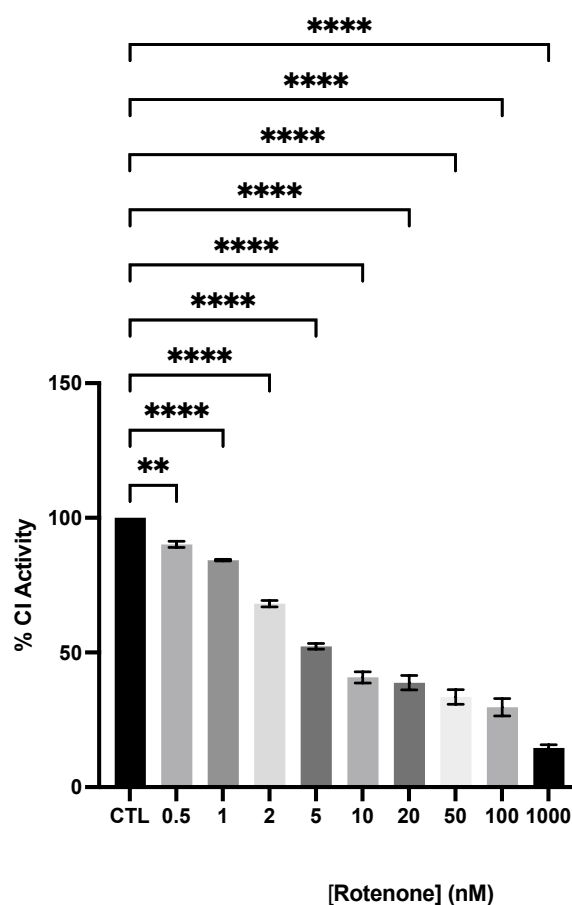


Figure 4.4. Rotenone titration of Complex I specific activity in iBMDMs.

iBMDMs were incubated at different concentrations of Rotenone (0.5 nM – 1 μ M) for 20 min. Samples were centrifuged, and pellets were resuspended in the assay buffer. Complex I activity was measured with spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex I in iBMDMs was expressed as percentages of their control value, and one-way ANOVA was performed the statistical analysis. Then results are expressed as mean $\pm$ SEM., n=3. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

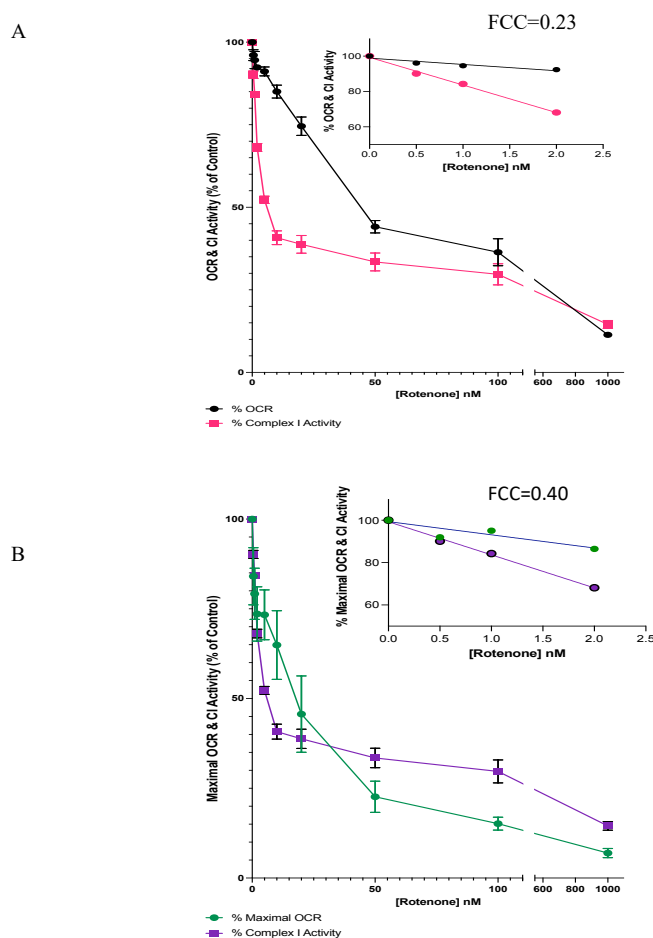


Figure 4.5. Calculation of the Flux Control Coefficients for complex I over basal and maximal oxygen consumption rates in iBMDMs.

(A) The OCRs and complex I activities produced in Figs 4.3 & 4.4, respectively, were expressed as percentages of their control values and plotted against their respective rotenone concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex I and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low rotenone inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.4478$ for the OCR line and $R^2 = 0.9849$ for the complex I activity line. (B) The maximal OCRs and complex I activities produced in Figs 4.3 & 4.4, respectively, were expressed as percentages of their control values and plotted against their respective rotenone concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex I and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low rotenone inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.05395$ for oxygen consumption and $R^2 = 0.9849$ for complex I activity.

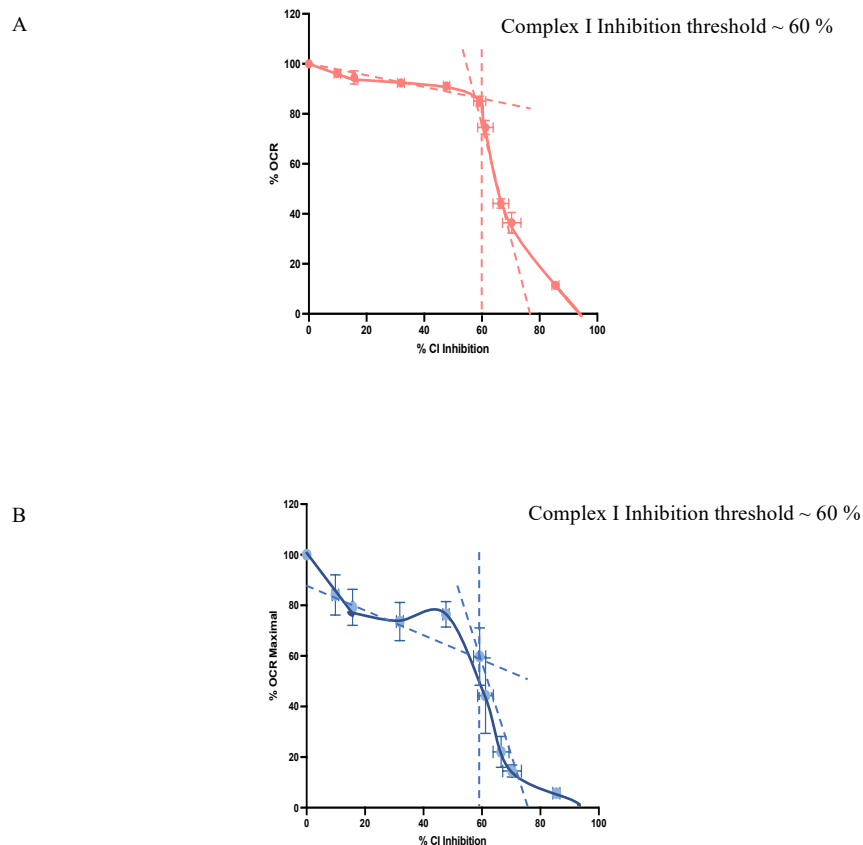


Figure 4.6. High complex I inhibition thresholds for basal and maximal OCRs in iBMDMs.

(A) The oxygen consumption results from Figure 4.5 (A) were plotted against inhibition of complex I activity (as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM. The curve was fitted by hand.

(B) The maximal oxygen consumption was plotted against inhibition of complex I activity (as a percentage of control) (Figure 4.5(B)). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM. The curve was fitted by hand.

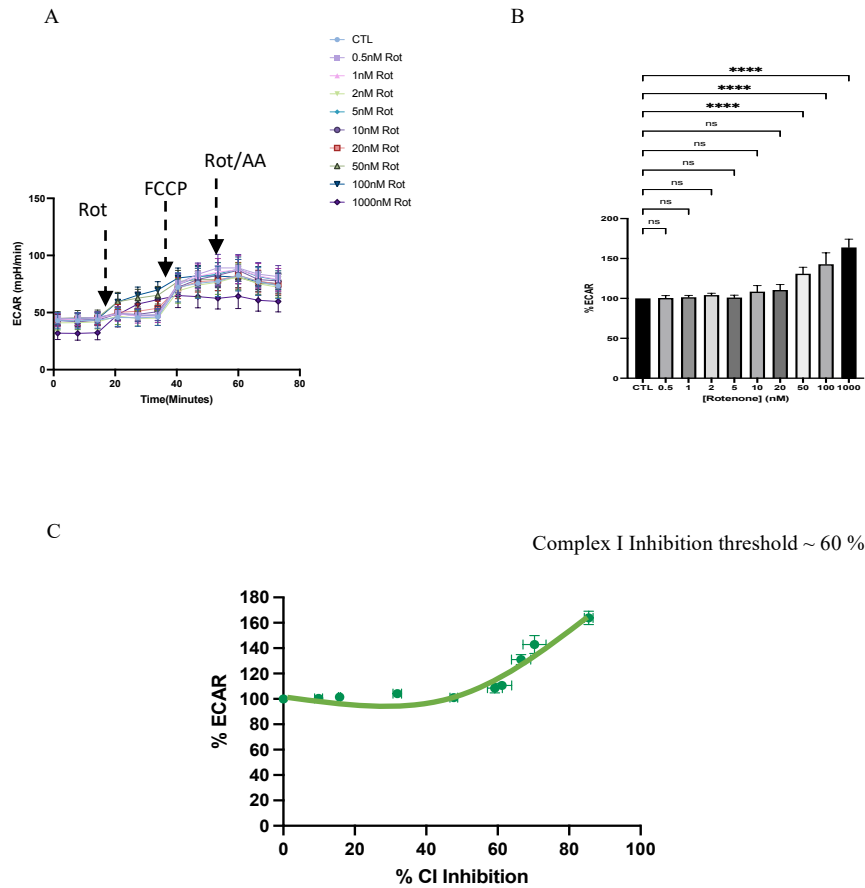


Figure 4.7. High complex I inhibition threshold for Extracellular acidification rate (ECAR) in iBMDMs.

(A) Extracellular acidification rates (ECAR) were simultaneously measured with OCR values (Fig 4.3) in the presence of rotenone titration. The data are presented as mean $\pm$ S.E.M., n=4. (B) Extracellular acidification rates (ECAR) were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. Then results are expressed as mean $\pm$ SEM., n=4. (C) ECAR was plotted against the inhibition of complex I activity (Fig 4.4), as a percentage of control. Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM (error bars). The curve was fitted by hand. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.2.2 Metabolic control analysis of complex II in iBMDMs

TTFA decreased the rate of iBMDMs maximal OCR but not the basal OCR (**Figure 4.8**). Complex II activity decreased linearly to ~ 30 % over the TTFA concentration range of 0 - 200 μ M (**Figure 4.9**). **Figures 4.10** and 4.11 showed that there were no threshold for complex II inhibitor in iBMDMs for basal or maximal OCR or ECAR.

4.2.3 Metabolic control analysis of complex II/III in iBMDMs

Antimycin A was used to investigate the level of control possessed by complex II/III over iBMDMs oxygen consumption. Increasing concentrations of antimycin A (0-1000 nM) reduced the OCR in iBMDMs and complex II/III activity. The OCR remained between ~ 90 and 100%, while complex II/III activity decreased linearly to ~ 90 % over the antimycin A concentration range of 0 - 1000 nM (**Figures 4.12 (B)** and 4.13). The slope of the lower concentrations of iBMDMs oxygen consumption, up to 1000 nM antimycin A, was -3.674 and the slope of the lower concentrations of complex II/III activity was -16.98 (see inset graph of **Figure 4.14 (A)**), giving a flux control coefficient for complex II/III of 0.216 when antimycin A was used to titrate out complex II/III activity. The slope of the lower concentrations of iBMDMs maximal oxygen consumption was - 4.230, and the slope of the lower concentrations of complex II/III activity was -16.98, giving the flux control coefficient for complex II/III 0.249 (see inset graph of **Figure 4.14 (B)**). The threshold level of complex II/III inhibition was determined from a curve created by expressing the rate of iBMDMs oxygen consumption as a function of the level of inhibition of complex II/III activity (**Figure 4.15**). The approximate complex II/III inhibition threshold was found to be ~ 80 % for basal and maximal oxygen consumption. **Figure 4.16** showed that high complex II/III inhibition threshold for Extracellular acidification rate (ECAR) in iBMDMs ~ 80 %.

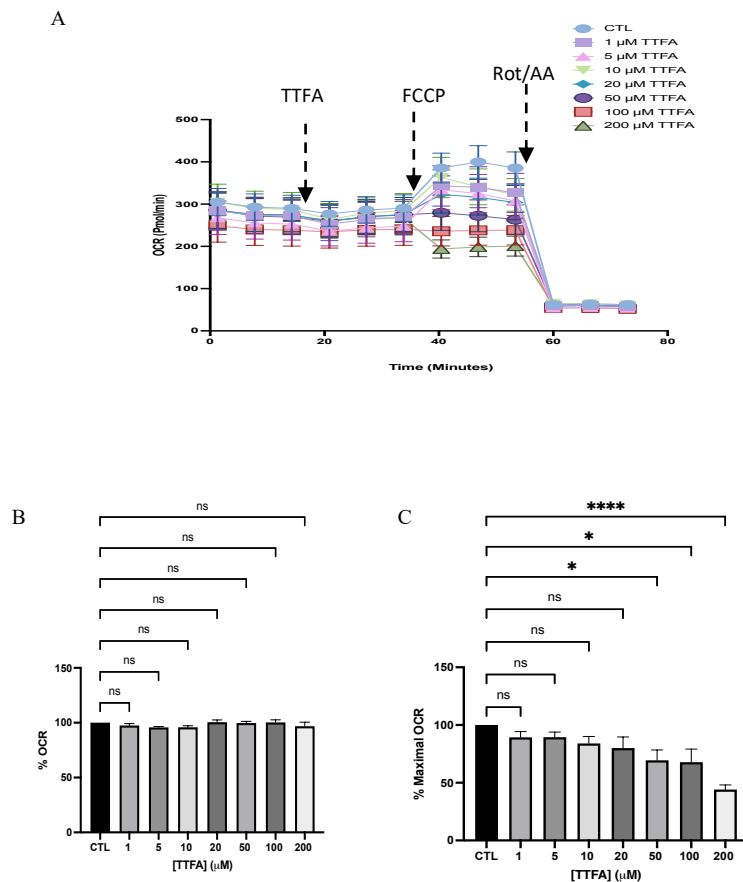


Figure 4.8. TTFA titration of Oxygen Consumption Rate (OCR) in iBMDMs.

(A) iBMDMs were plated at 0.5×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. A range of TTFA concentrations ($1\mu\text{M}$ - $200\mu\text{M}$) were used to inhibit complex II activity and the Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. Following injection of the mitochondrial uncoupler, FCCP ($1\mu\text{M}$), the Maximal Oxygen consumption rate (OCR) was measured. Following injection of rotenone + antimycin A ($1\mu\text{M}$ each), the non-mitochondrial OCR was measured. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) The oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. Results are expressed as mean $\pm$ SEM, $n=4$. (C) The Maximal Oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=4$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

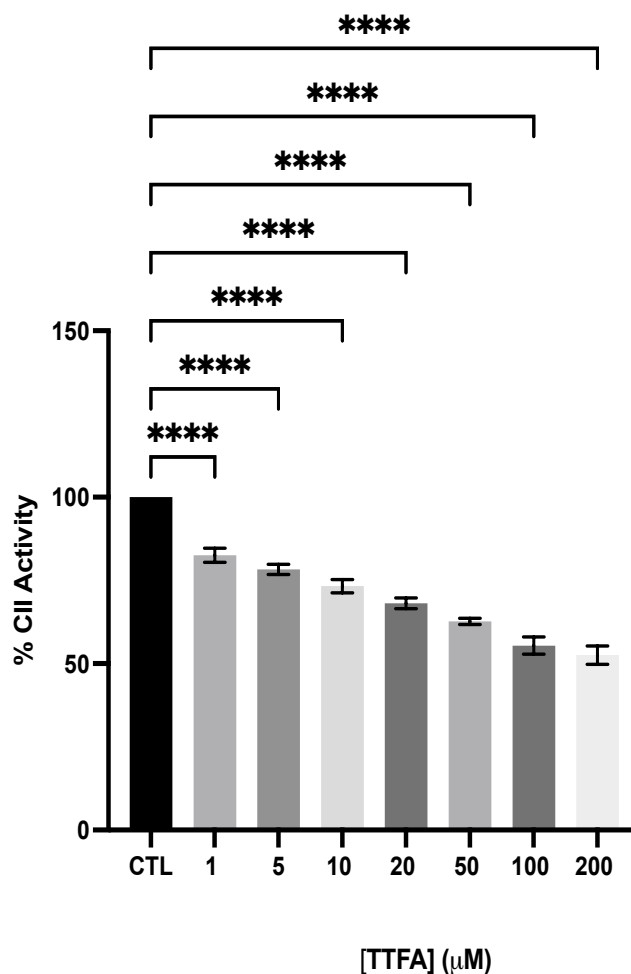


Figure 4.9. TTFA titration of Complex II specific activity in iBMDMs.

iBMDMs were incubated at different concentrations of TTFA (1-200 μM) for 20 min. Samples were centrifuged, and pellets were resuspended in the assay buffer. Complex II activity was measured with spectrophotometric assay using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600\text{ nm}$ at 37°C . The specific activity of complex II in iBMDMs was expressed as percentages of their control value, and one-way ANOVA was performed the statistical analysis. Then results are expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

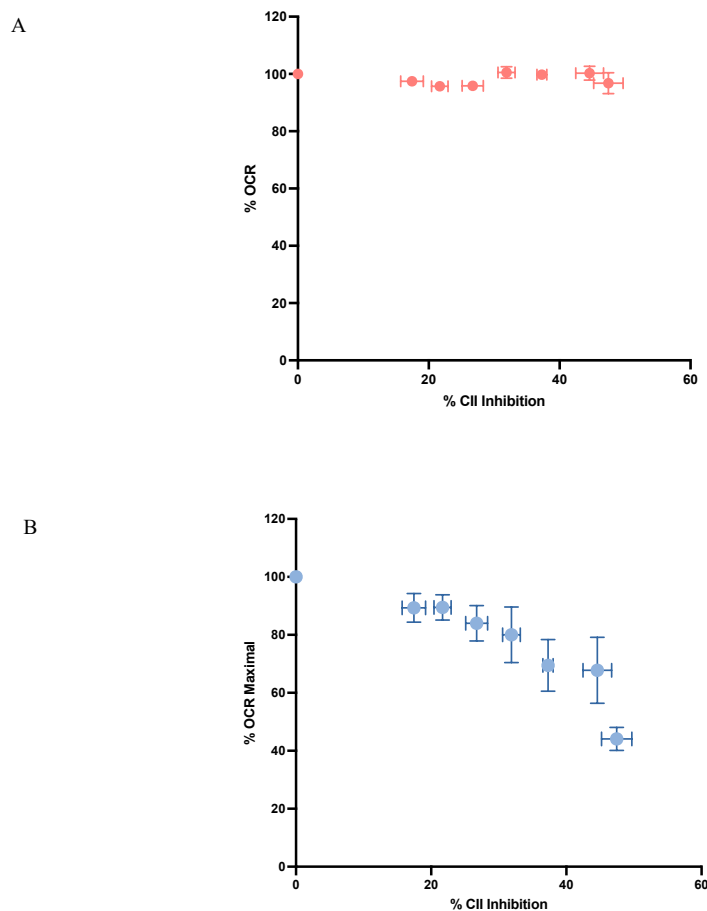


Figure 4.10. No presence of a Complex II inhibition thresholds for basal OCR in iBMDMs.

The oxygen consumption Figure 4.8 were plotted against inhibition of complex II activity (as a percentage of control). Experiments were performed on four individual preparations for Seahorse assay and three individual preparations for complex II activity, and the results are expressed as mean $\pm$ SEM. (B) The maximal oxygen consumption was plotted against inhibition of complex II activity (as a percentage of control) (Figure 4.9). Experiments were performed on four individual preparations for Seahorse assay and three individual preparations for complex II activity, and the results are expressed as mean $\pm$ SEM.

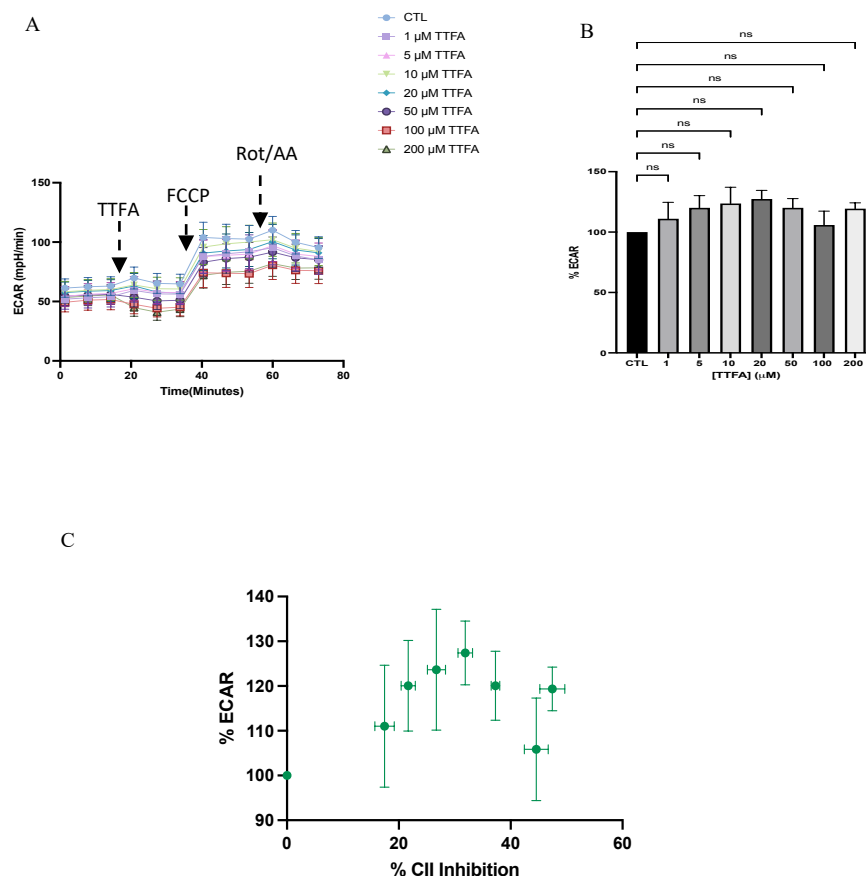


Figure 4.11. No complex II inhibition threshold for Extracellular acidification rate (ECAR) in iBMDMs.

(A) Extracellular acidification rates (ECAR) were simultaneously measured with OCR values (Fig 4.8) in the presence of TTFA titration. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) Extracellular acidification rates (ECAR) were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. Then results are expressed as mean $\pm$ SEM., $n=4$. (C) ECAR was plotted against the inhibition of complex II activity (Fig 4.9), as a percentage of control. Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM (error bars).

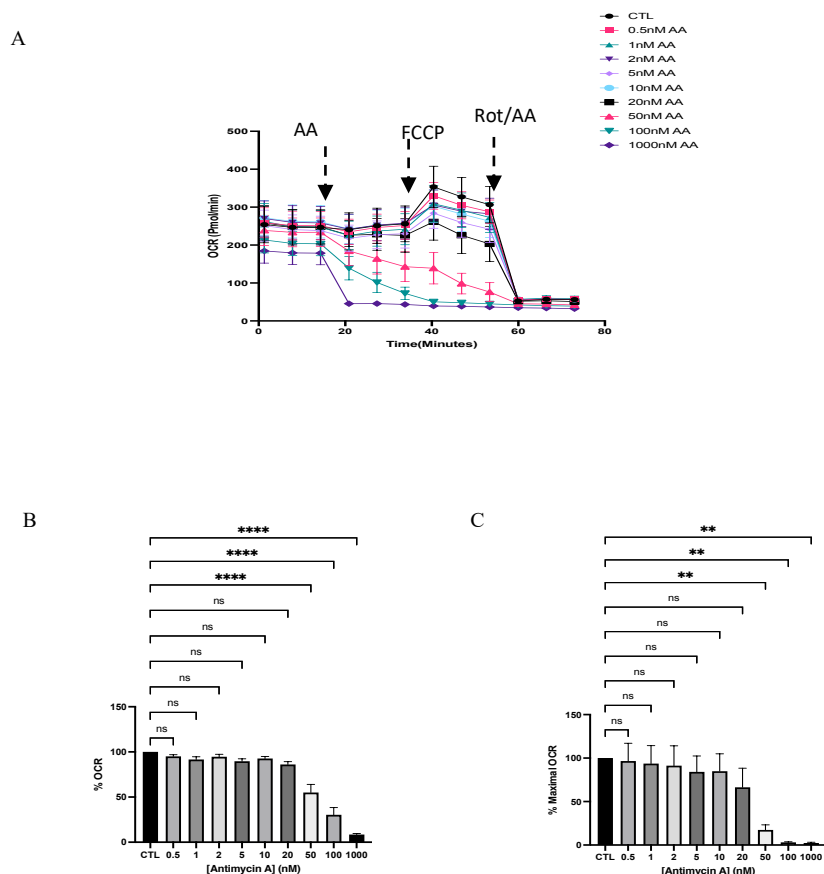


Figure 4.12. Antimycin A titration of Oxygen Consumption Rate (OCR) in iBMDMs.

(A) iBMDMs were plated at 0.5×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. A range of antimycin A concentrations (0.5nM-1 μM) were used to inhibit complex II-III activities and the Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. Following injection of the mitochondrial uncoupler, FCCP (1 μM), the Maximal Oxygen consumption rate (OCR) was measured. Following injection of rotenone + antimycin A (1 μM each), the non-mitochondrial OCR was measured. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) The oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. Results are expressed as mean $\pm$ SEM, $n=4$. (C) The Maximal Oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=4$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

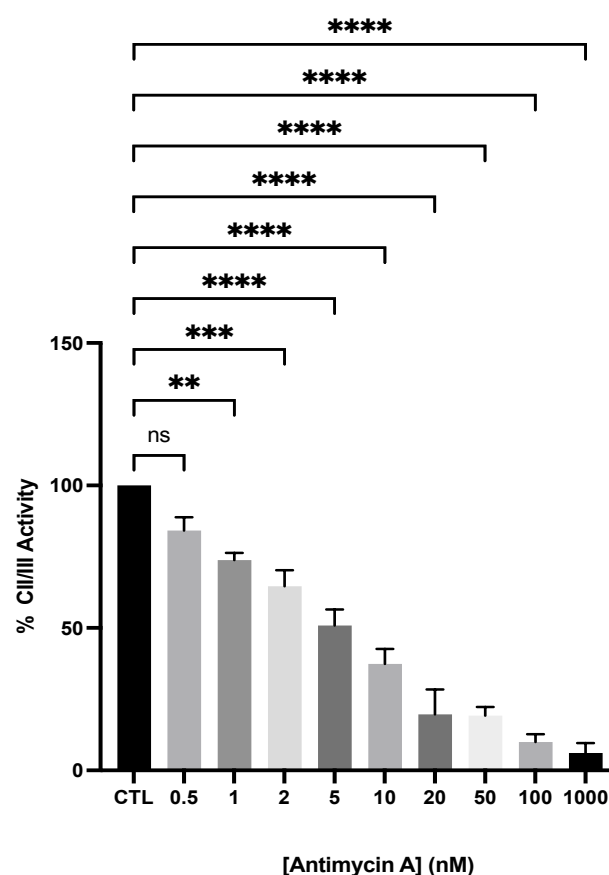


Figure 4.13. Antimycin A titration of Complex II/III specific activity in iBMDMs.

iBMDMs were incubated at different concentrations of Antimycin A (0.5-1 μ M) for 20 min. Samples were centrifuged, and pellets were resuspended in the assay buffer. Complex II/III activity was measured with spectrophotometric assay following the reduction of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex II/III in iBMDMs was expressed as percentages of their control value, and one-way ANOVA was performed the statistical analysis. The results are expressed as mean $\pm$ SEM., n=3. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$

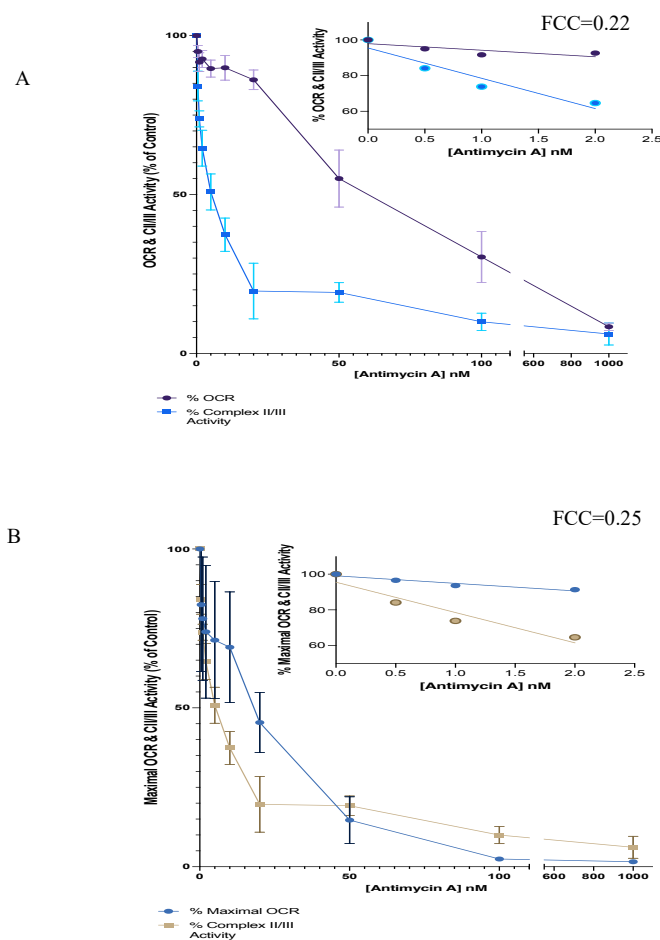


Figure 4.14. Calculation of the Flux Control Coefficients for complex II/III over basal and maximal oxygen consumption rates in iBMDMs.

(A) The OCRs and complex II/III activities produced in Figs 4.12 & 4.13, respectively, were expressed as percentages of their control values and plotted against their respective antimycin A concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex II/III and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low antimycin A inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.2831$ for oxygen consumption and $R^2 = 0.7772$ for complex II/III activity. (B) The maximal OCRs and complex II/III activities produced in Figs 4.12 & 4.13, respectively, were expressed as percentages of their control values and plotted against their respective antimycin A concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex II/III and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low antimycin A inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.009414$ for oxygen consumption and $R^2 = 0.7772$ for complex II/III activity.

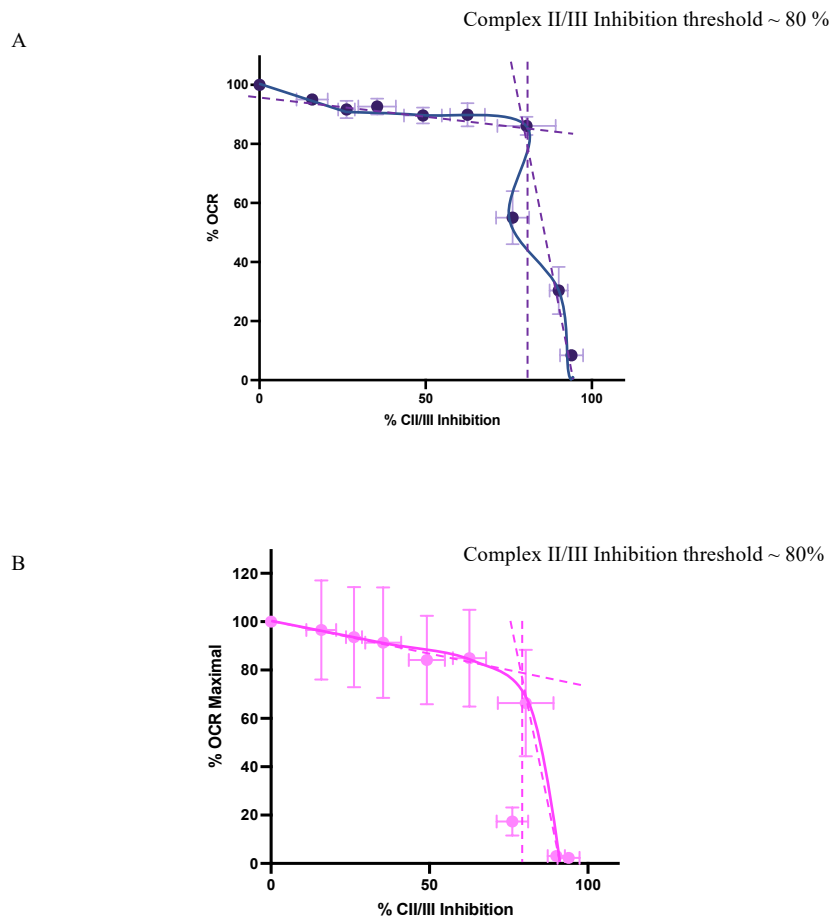


Figure 4.15. High complex II/III inhibition thresholds for basal and maximal OCRs in iBMDMs.

(A) The oxygen consumption results from Figure 4.14 (A) were plotted against inhibition of complex II/III activity (as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II/III activity, and results are expressed as mean $\pm$ SEM(error bars). The curve was fitted by hand. (B) The maximal oxygen consumption was plotted against inhibition of complex II/III activity (as a percentage of control) (Figure 4.14 (B)). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II/III activity and results are expressed as mean $\pm$ SEM(error bars). The curve was fitted by hand.

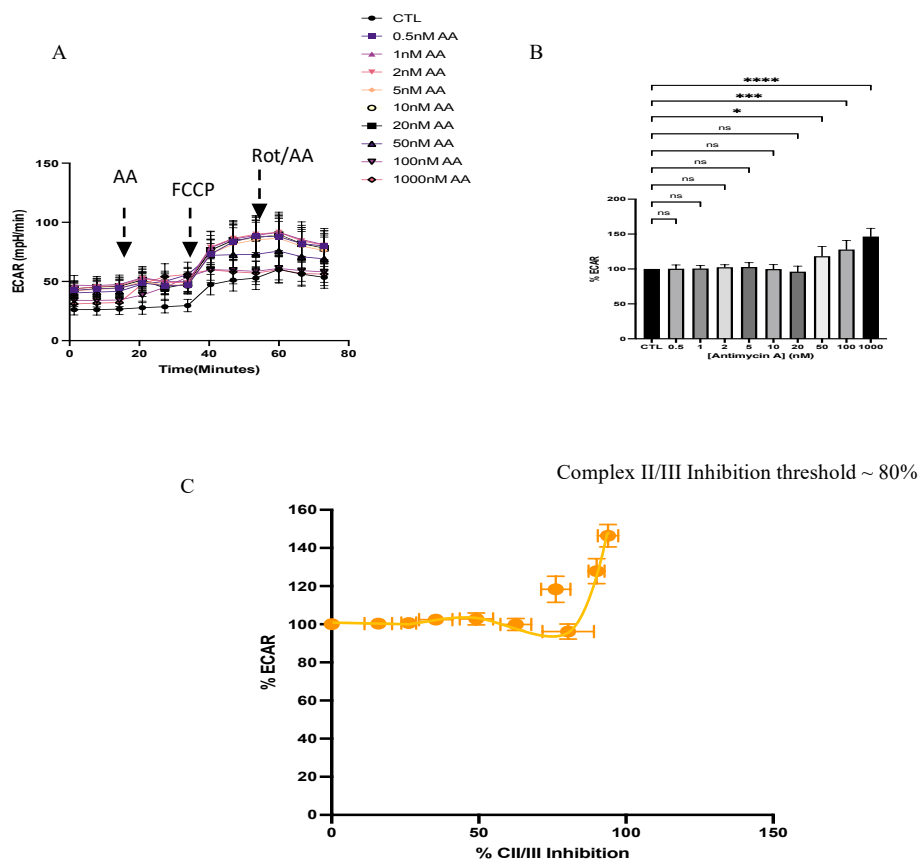


Figure 4.16. High complex II/III inhibition threshold for Extracellular acidification rate (ECAR) in iBMDMs.

(A) Extracellular acidification rates (ECAR) were simultaneously measured with OCR values (Fig 4.12) in the presence of antimycin A titration. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) Extracellular acidification rates (ECAR) were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. Then results are expressed as mean $\pm$ SEM., $n=4$. (C) ECAR was plotted against the inhibition of complex II/III activity (Fig 4.14), as a percentage of control. Experiments were performed on four individual preparations for Seahorse assay and three individual preparations for complex II/III activity, and the results are expressed as mean $\pm$ SEM (error bars). The curve was fitted by hand. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.2.4 Metabolic control analysis of complex I in BMDMs

The flux control coefficient and inhibition threshold were determined for complex I in BMDMs. Rotenone decreased the basal and maximal OCRs and complex I activity in a concentration-dependent manner in BMDMs (**Figure 4.17**). Complex I activity (**Figure 4.18**) decreased linearly to ~ 70 % over the rotenone concentration range of 0 - 1000 nM, however, OCR remained between ~ 75 and 85 %. The flux control coefficient was calculated using Equation 4.1. The rate of OCR at lower concentrations of rotenone was divided by the rate of complex I activity at lower concentrations of the inhibitor (see inset graph of **Figure 4.19 (A)**). The slope of the lower concentrations of OCR in BMDMs was -0.80 and the slope at the lower concentrations of complex I activity was -2.48, giving the flux control coefficient for complex I 0.33. The slope of the lower concentrations of maximal OCR in BMDMs was -2.93 and the slope at the lower concentrations of complex I activity was -2.48, giving the flux control coefficient for complex I 1.0 (see inset graph of **Figure 4.19 (B)**).

The threshold level of complex I inhibition was determined from a curve created by expressing the rate of OCR as a function of the level of inhibition of complex I activity (**Figure 4.20**). The complex I inhibition threshold was found to be ~ 38 %, for basal OCR and ~ 33 %, for maximal OCR. These complex I OCR thresholds are significantly lower, by ~20%, than those found for complex I in iBMDMs. **Figure 4.21** shows the complex I inhibition threshold for ECAR in BMDMs is over 50 %.

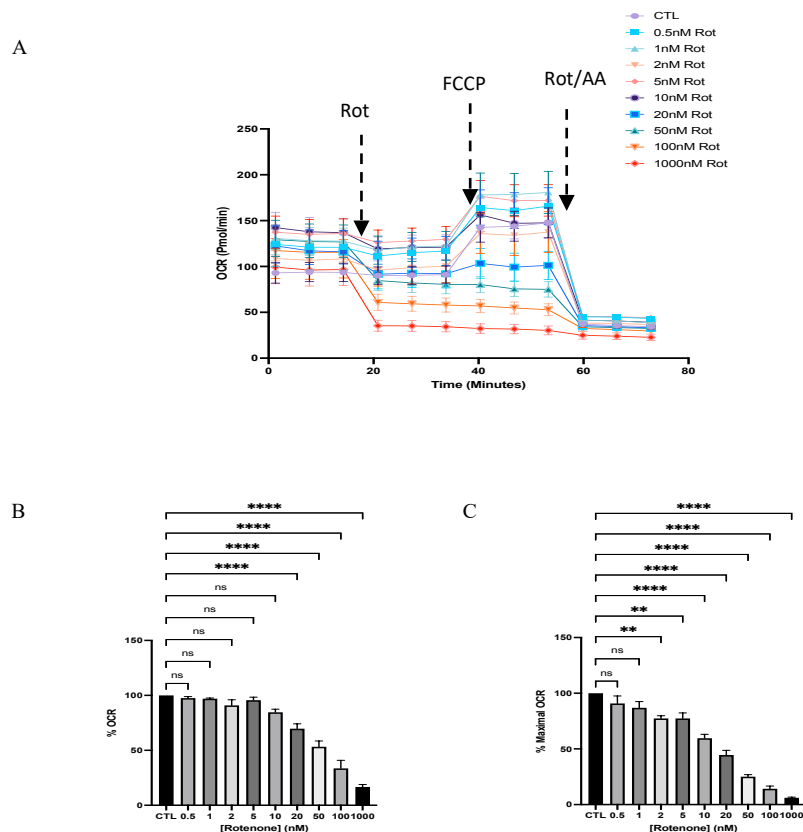


Figure 4.17. Rotenone titration of Oxygen Consumption Rate (OCR) in BMDMs.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. A range of rotenone concentrations (0.5nM-1 μM) were used to inhibit complex I activity and the Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. Following injection of the mitochondrial uncoupler, FCCP (1 μM), the Maximal Oxygen consumption rate (OCR) was measured. Following injection of rotenone + antimycin A (1 μM each), the non-mitochondrial OCR was measured. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) The oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. Results are expressed as mean $\pm$ SEM, $n=4$. (C) The Maximal Oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=4$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

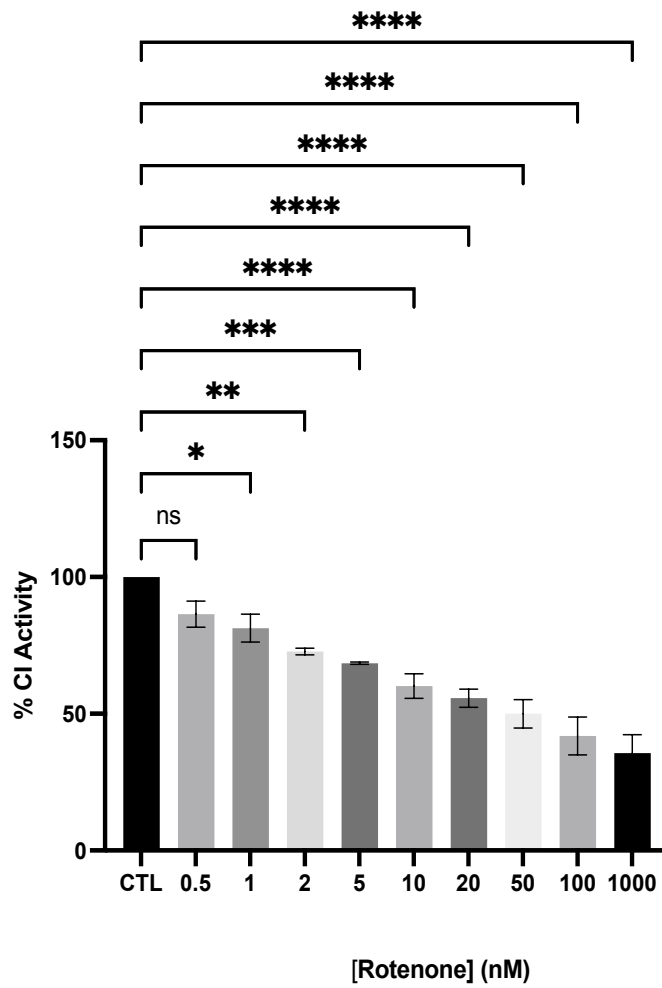


Figure 4.18. Rotenone titration of Complex I specific activity in BMDMs.

BMDMs were incubated at different concentrations of Rotenone (0.5 nM – 1 μ M) for 20 min. Samples were centrifuged, and pellets were resuspended in the assay buffer. Complex I activity was measured with spectrophotometric assay following the oxidation of NADH at $\lambda=340$ nm at 37°C. The specific activity of complex I in BMDMs was expressed as percentages of their control value, and one-way ANOVA was performed the statistical analysis. Then results are expressed as mean $\pm$ SEM., n=3. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

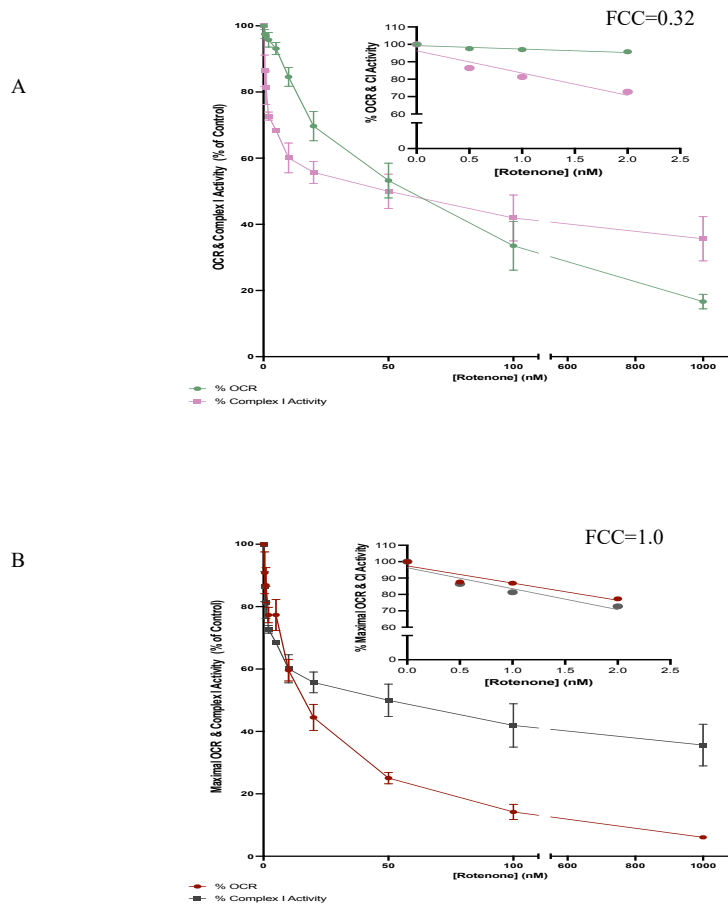


Figure 4.19. Calculation of the Flux Control Coefficients for complex I over basal and maximal oxygen consumption rates in BMDMs.

(A) The OCRs and complex I activities produced in Figs 4.17 & 4.18, respectively, were expressed as percentages of their control values and plotted against their respective rotenone concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex I and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low rotenone inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.3189$ for oxygen consumption and $R^2 = 0.7263$ for complex I activity. activity line. (B) The maximal OCRs and complex I activities produced in Figs 4.17 & 4.18, respectively, were expressed as percentages of their control values and plotted against their respective rotenone concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex I and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low rotenone inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.4958$ for oxygen consumption and $R^2 = 0.7263$ for complex I activity.

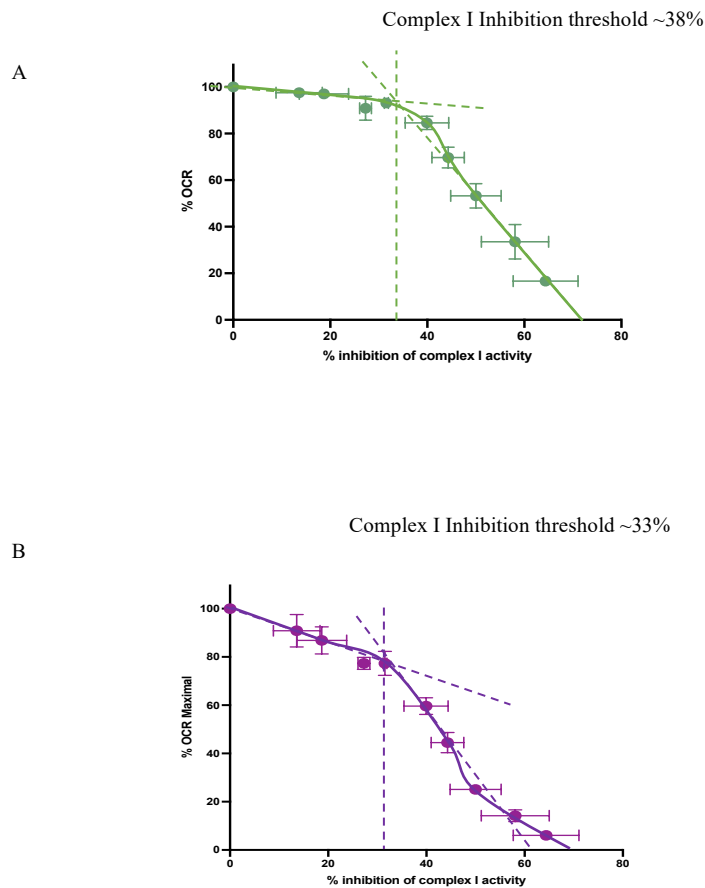


Figure 4.20. Low complex I inhibition thresholds for basal and maximal OCRs in BMDMs.

(A) The basal oxygen consumption rates from Figure 4.17 (B) were plotted against inhibition of complex I activity (Fig. 4.18, as a % of the control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM. The curve was fitted by hand.

(B) The maximal oxygen consumption rates from Figure 4.17 (C) were plotted against inhibition of complex I activity (Fig 4.18, as a % of the control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM. The curve was fitted by hand.

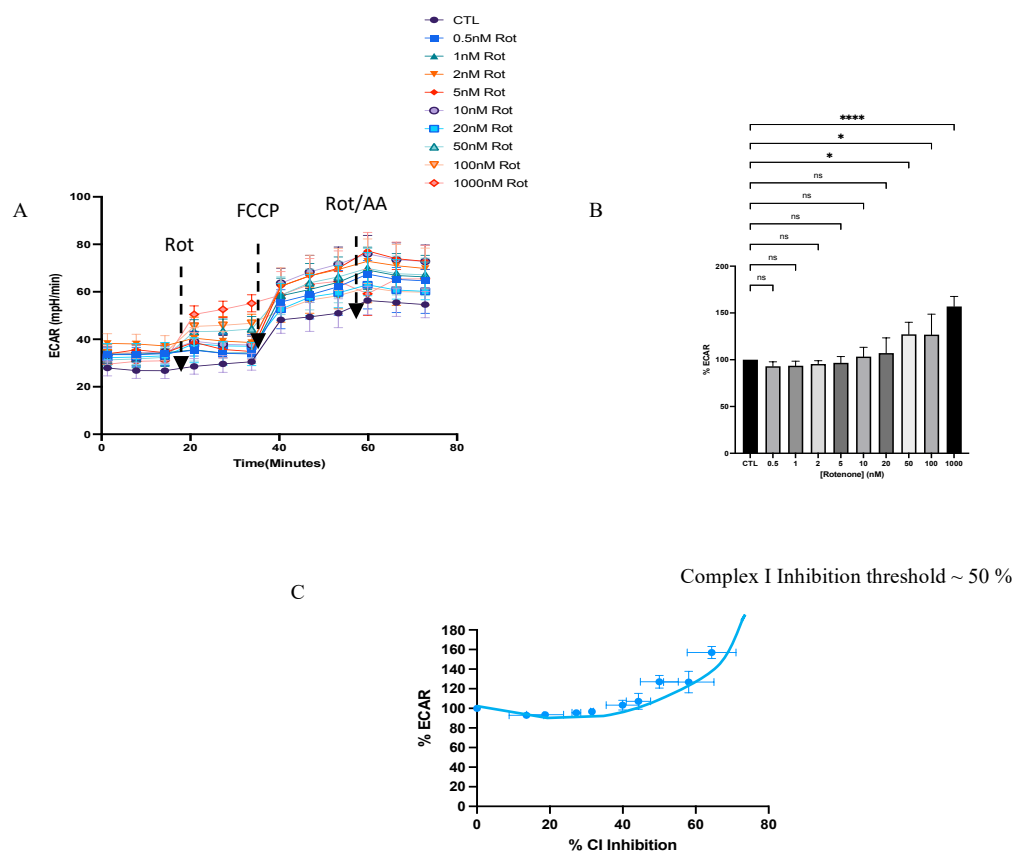


Figure 4.21. High complex I inhibition threshold for Extracellular acidification rate (ECAR) in BMDMs.

(A) Extracellular acidification rates (ECAR) were simultaneously measured with OCR values (Fig 4.17) in the presence of rotenone titration. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) Extracellular acidification rates (ECAR) were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. Then results are expressed as mean $\pm$ SEM., $n=4$. (C) ECAR was plotted against the inhibition of complex I activity (Fig 4.18), as a percentage of control. Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM (error bars). The curve was fitted by hand. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.2.5 Metabolic control analysis of complex II in BMDMs

TTFA decreased the rate of maximal OCR but not the basal OCR in BMDMs (**Figure 4.22**). Complex II activity was decreased linearly to ~ 30 % over the TTFA concentration range of 0 - 200 μ M (**Figure 4.23**). Figures 4.24 and 4.25 showed that there were no threshold for complex II inhibitor in BMDMs for basal or maximal OCRs or ECAR.

4.2.6 Metabolic control analysis of complex II/III in BMDMs

As with iBMDMs, increasing concentrations of antimycin A (0-1000 nM) reduced the rate of BMDMs OCR and complex II/III activity (**Figures 4.26**). OCR remained between ~ 80-90 %; however, complex II/III activity decreased linearly to ~ 70 % over the antimycin A concentration range of 0 - 1000 nM (**Figures 4.26 (B)** and 4.27). The slope at the lower concentrations of BMDMs OCR, was -3.12 and the slope at the lower concentrations of complex II/III activity was -24.95 (see inset graph of **Figure 4.28 (A)**), giving a flux control coefficient for complex II/III of 0.13 when antimycin A was used to titrate out complex II/III activity. The slope at the lower concentrations of maximal OCR was -4.23, and the slope at the lower concentrations of complex II/III activity was -24.95, giving a flux control coefficient for complex II/III of 0.07 (see inset graph of **Figure 4.28 (B)**).

The threshold level of complex II/III inhibition was determined from a curve created by expressing the rate of OCR as a function of the level of inhibition of complex II/III activity (**Figure 4.29**). The complex II/III inhibition threshold was found to be ~ 58% for basal and maximal OCRs. **Figure 4.30** showed a high complex II/III inhibition threshold for ECAR in BMDMs of ~ 63%.

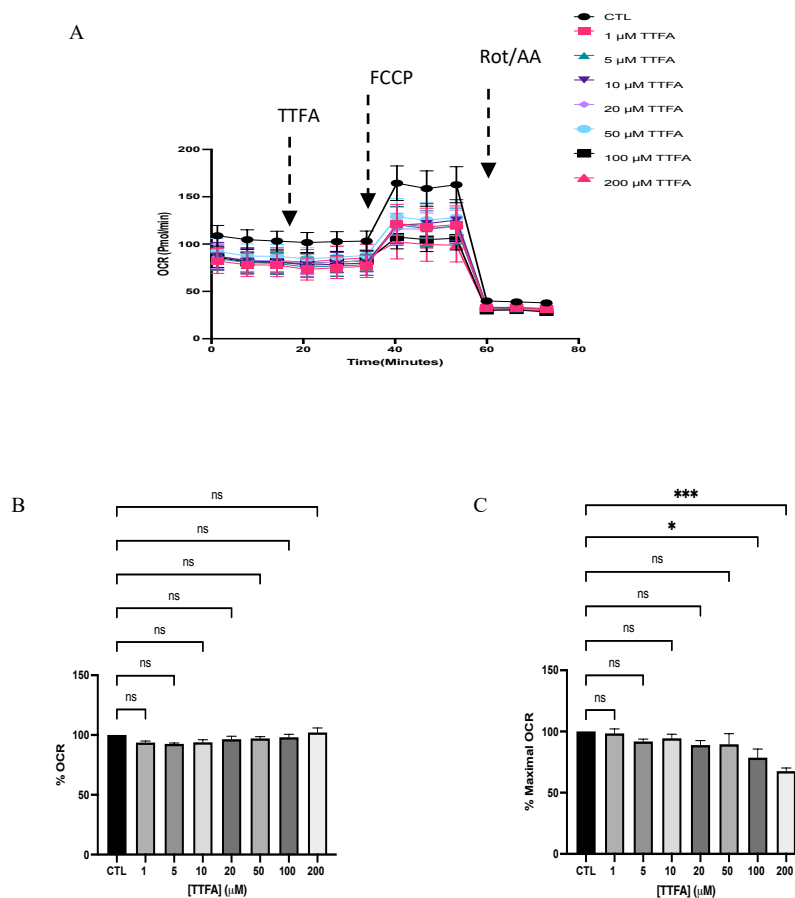


Figure 4.22. No presence of a Complex II inhibition thresholds for basal OCR in BMDMs.

The oxygen consumption rates in Fig 4.22 (A) were plotted against inhibition of complex II activity (Fig. 4.23 as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II activity, and the results are expressed as mean $\pm$ SEM. (B) The maximal oxygen consumption rates in Fig. 4.22 (C) were plotted against inhibition of complex II activity (Fig. 4.23 as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II activity, and the results are expressed as mean $\pm$ SEM.

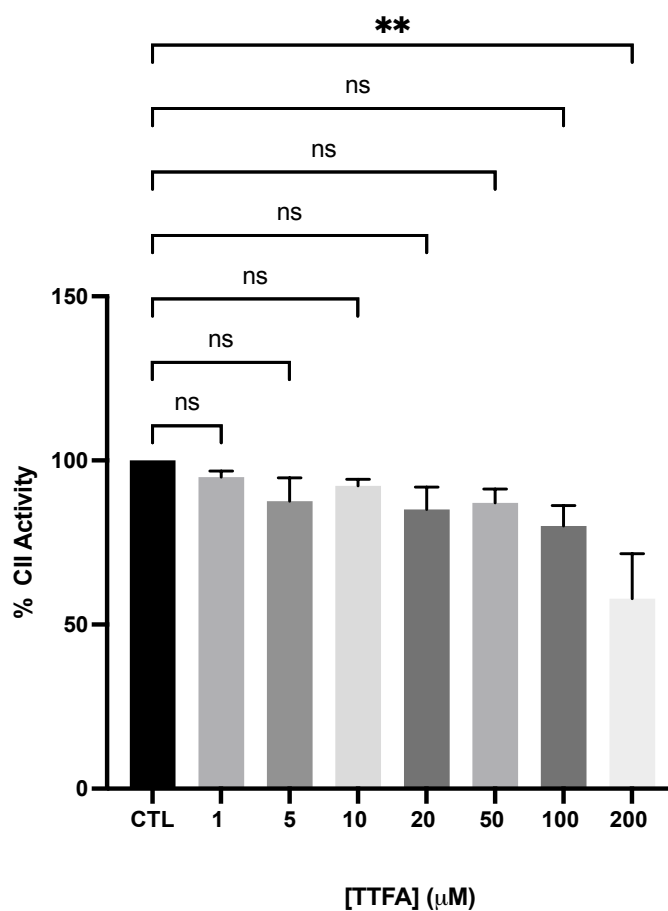


Figure 4.23. TTFA titration of Complex II specific activity in BMDMs.

BMDMs were incubated at different concentrations of TTFA (1-200 μM) for 20 min. Samples were centrifuged, and pellets were resuspended in the assay buffer. Complex II activity was measured with spectrophotometric assay using succinate as the substrate and DCPIP as the electron acceptor at $\lambda=600$ nm at 37°C . The specific activity of complex II in BMDMs was expressed as percentages of their control value, and one-way ANOVA was performed the statistical analysis. Then results are expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

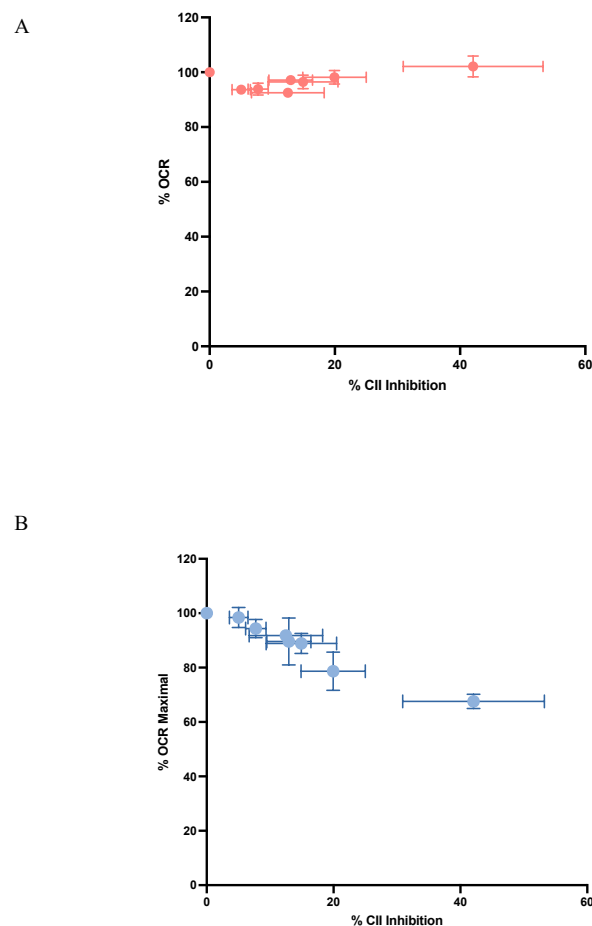


Figure 4.24. No presence of a Complex II inhibition thresholds for basal OCR in BMDMs.

The oxygen consumption rates in Fig 4.22 (A) were plotted against inhibition of complex II activity (Fig. 4.23 as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II activity, and the results are expressed as mean $\pm$ SEM. (B) The maximal oxygen consumption rates in Fig. 4.22 (C) were plotted against inhibition of complex II activity (Fig. 4.23 as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II activity, and the results are expressed as mean $\pm$ SEM.

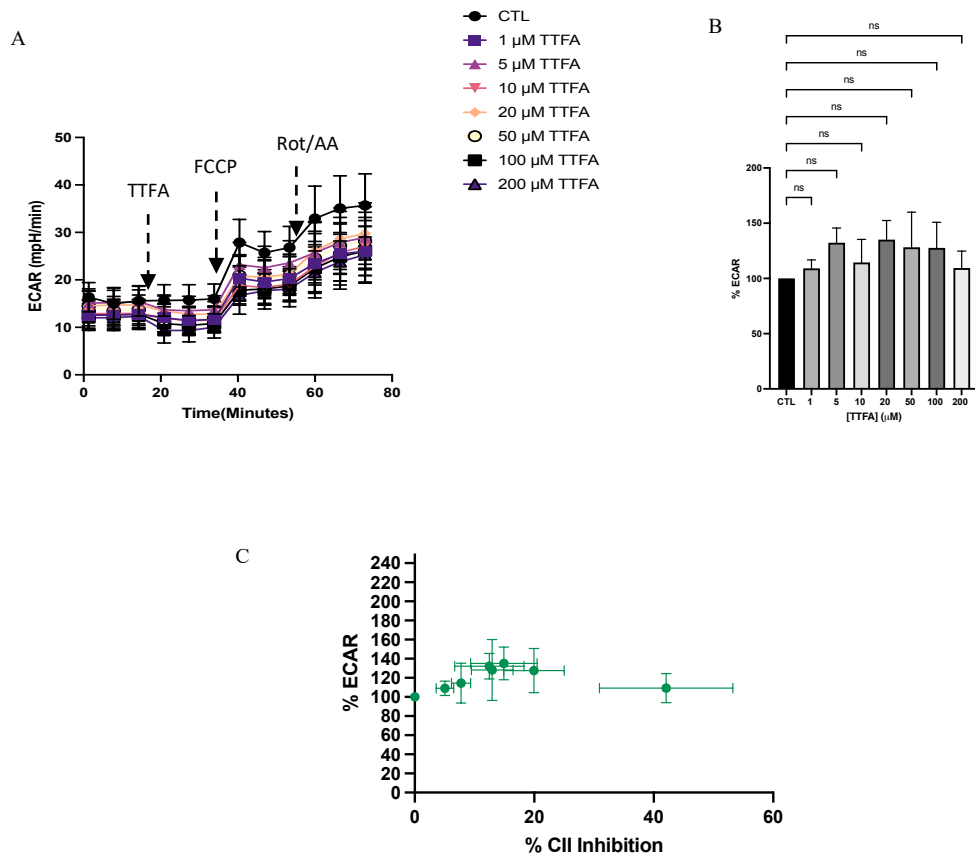


Figure 4.25. No complex II inhibition threshold for Extracellular acidification rate (ECAR) in BMDMs.

(A) Extracellular acidification rates (ECAR) were simultaneously measured with OCR values (Fig 4.22) in the presence of TTFA titration. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) Extracellular acidification rates (ECAR) were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. Then results are expressed as mean $\pm$ SEM., $n=4$. (C) ECAR was plotted against the inhibition of complex II activity (Fig 4.23), as a percentage of control. Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex I activity, and the results are expressed as mean $\pm$ SEM (error bars).

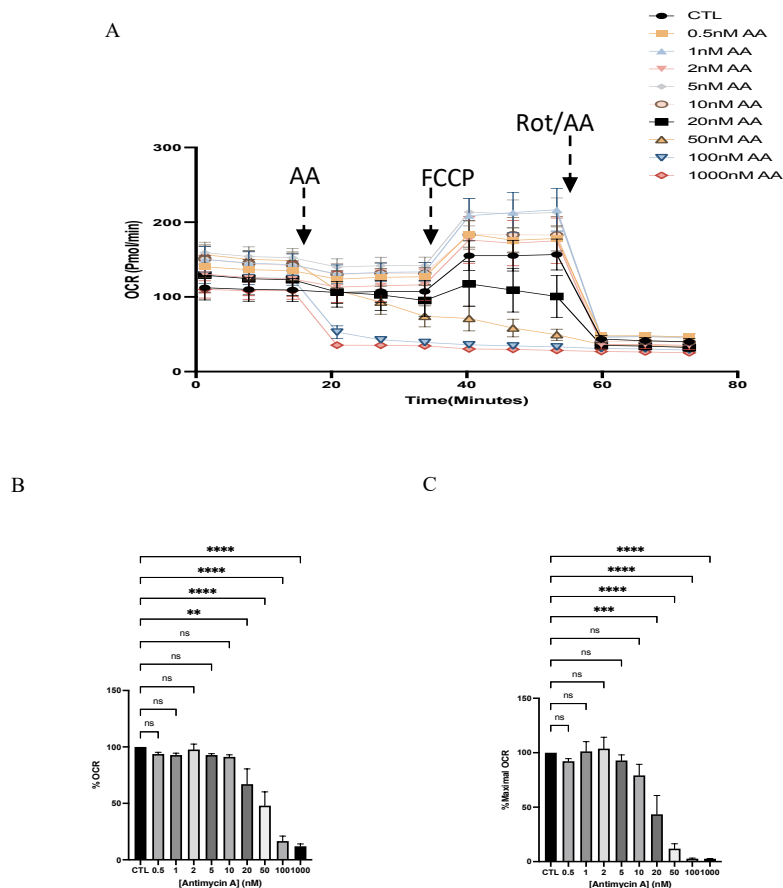


Figure 4.26. Antimycin A titration of Oxygen Consumption Rate (OCR) in BMDMs. (A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. A range of antimycin A concentrations (0.5nM–1 μM) were used to inhibit complex II-III activities and the Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. Following injection of the mitochondrial uncoupler, FCCP (1 μM), the Maximal Oxygen consumption rate (OCR) was measured. Following injection of rotenone + antimycin A (1 μM each), the non-mitochondrial OCR was measured. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) The oxygen consumption rate (OCR) was expressed as percentages of the control value and statistical analysis was performed by one-way ANOVA. Results are expressed as mean $\pm$ SEM, $n=4$. (C) The maximal OCR was expressed as a percentage of the control value and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=4$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

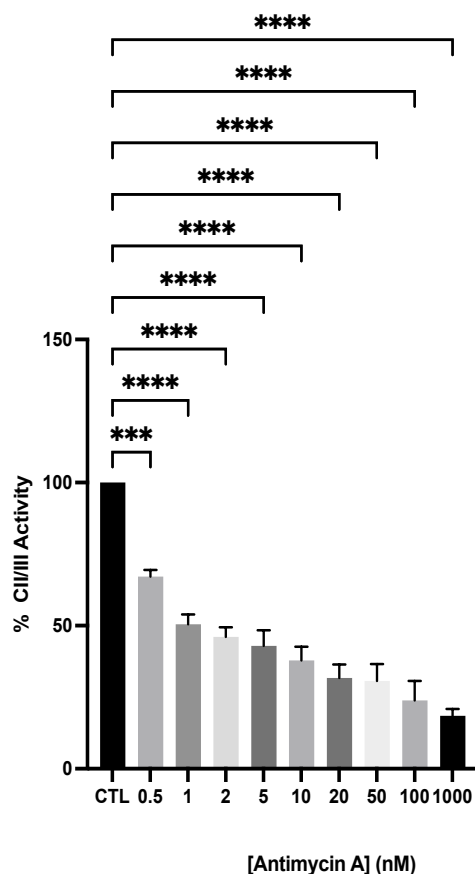


Figure 4.27. Antimycin A titration of Complex II/III specific activity in BMDMs.

BMDMs were incubated at different concentrations of Antimycin A (0.5-1 μ M) for 20 min. Samples were centrifuged, and pellets were resuspended in the assay buffer. Complex II/III activity was measured with spectrophotometric assay following the reduction of cytochrome C at $\lambda=550$ nm at 37°C. The specific activity of complex II/III in BMDMs was expressed as percentages of their control value, and one-way ANOVA was performed the statistical analysis. The results are expressed as mean $\pm$ SEM., n=3. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

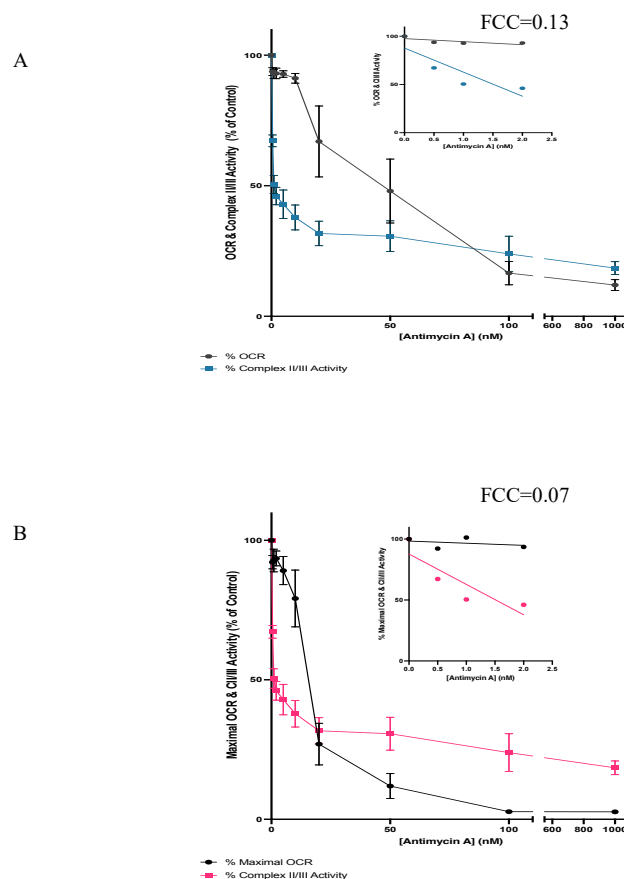


Figure 4.28. Calculation of the Flux Control Coefficients for complex II/III over basal and maximal oxygen consumption rates in BMDMs.

(A) The OCRs and complex II/III activities produced in Figs 4.26 & 4.27, respectively, were expressed as percentages of their control values and plotted against their respective antimycin A concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex II/III and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low antimycin A inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.3321$ for oxygen consumption and $R^2 = 0.7344$ for complex II/III activity. (B) The maximal OCRs and complex II/III activities produced in Figs 4.26 & 4.27, respectively, were expressed as percentages of their control values and plotted against their respective antimycin A concentration. Experiments were performed on four individual preparations for seahorse and on three individual preparations for complex II/III and results are expressed as mean $\pm$ SEM. The insert shows the expanded rates at low antimycin A inhibitor concentrations and the slopes were used to calculate the FCC as described in Section 4.2.3. $R^2 = 0.01747$ for oxygen consumption and $R^2 = 0.7344$ for complex II/III activity.

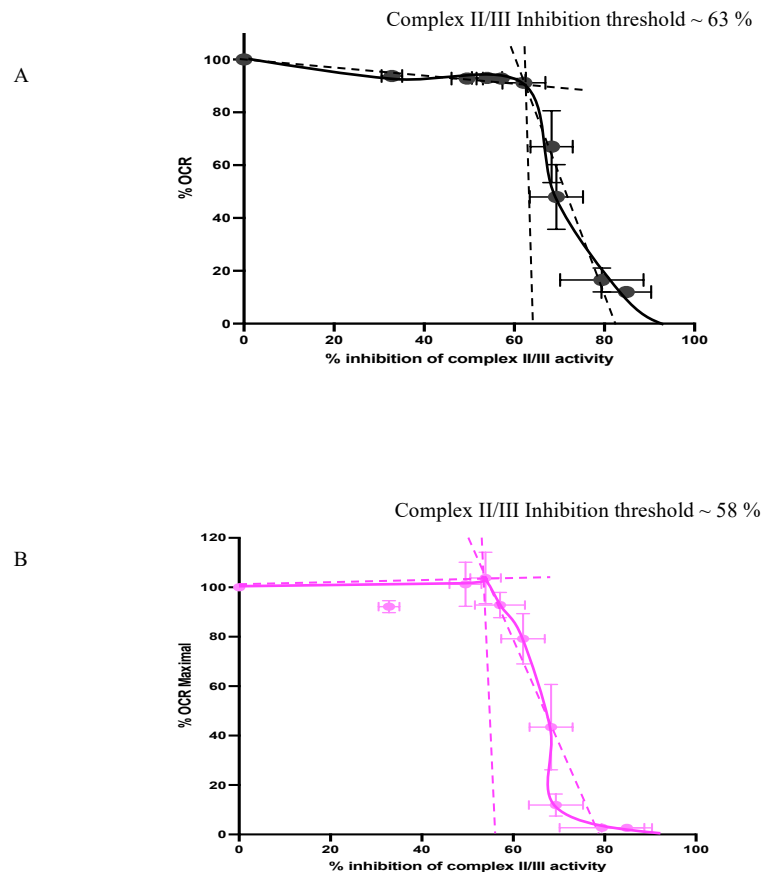


Figure 4.29. High complex II/III inhibition thresholds for basal and maximal OCRs in BMDMs.

(A) The basal OCRs from Figure 4.26 (B) were plotted against inhibition of complex II/III activity (Fig. 4.27 as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II/III activity, and results are expressed as mean $\pm$ SEM (error bars). The curve was fitted by hand. (B) The maximal OCRs from Fig. 4.26 (C) were plotted against inhibition of complex II/III activity (Fig. 4.27 as a percentage of control). Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II/III activity and results are expressed as mean $\pm$ SEM (error bars). The curve was fitted by hand.

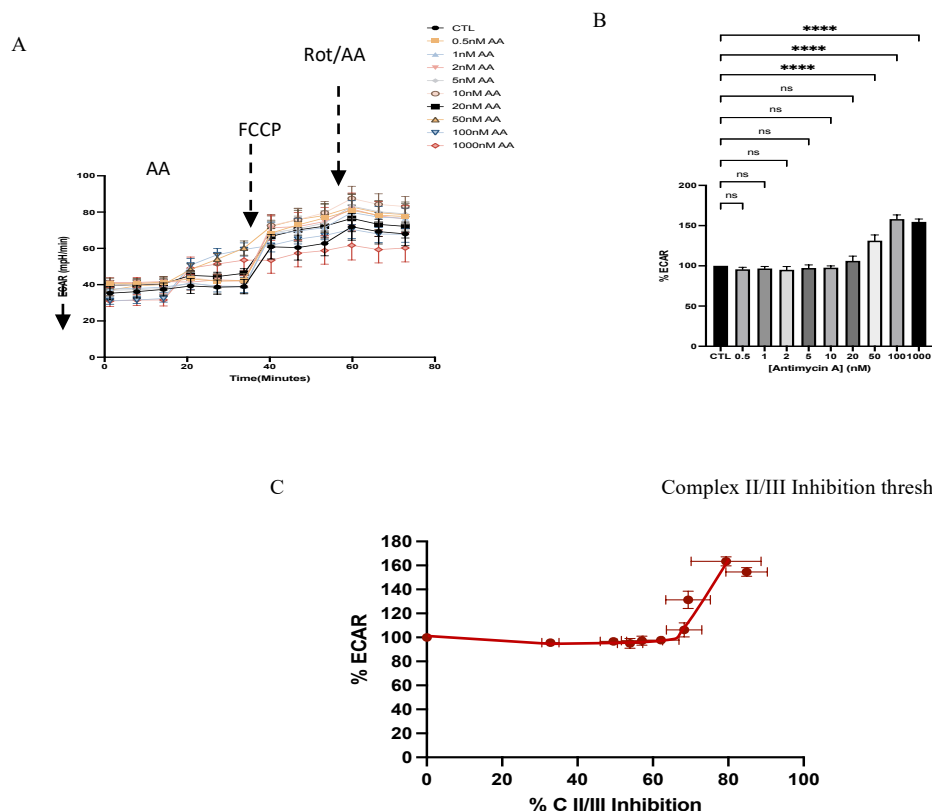


Figure 4.30. High complex II/III inhibition threshold for Extracellular acidification rate (ECAR) in BMDMs.

(A) Extracellular acidification rates (ECAR) were simultaneously measured with OCR values (Fig 4.26) in the presence of antimycin A titration. The data are presented as mean $\pm$ S.E.M., $n=4$. (B) Extracellular acidification rates (ECAR) were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. Then results are expressed as mean $\pm$ SEM., $n=4$. (C) ECAR was plotted against the inhibition of complex II/III activity (Fig 4.27), as a percentage of control. Experiments were performed on four individual preparations for seahorse assay and three individual preparations for complex II/III activity, and the results are expressed as mean $\pm$ SEM (error bars). The curve was fitted by hand.

4.2.7 Bioenergetics time course profile of OCR and ECAR in LPS-activated BMDMs

To examine if the Warburg effect (i.e., increased glycolysis and decreased oxidative phosphorylation) occurs in activated BMDMs, the kinetics of the metabolic response BMDMs stimulation with LPS (100 ng/ml) for different time points were analysed. The time course assay (**Figure 4.31(A)**) shows a rapid decrease in both basal and maximal OCRs following stimulation with LPS (100 ng/ml) for 2, 4, 6, 8, 12 and 24 h compared with the unstimulated control.

The basal oxygen OCR decreased to ~ 80-90 % (**Figure 4.31(B)**). Moreover, the maximal OCR was significantly reduced between ~ 90-95 % (**Figure 4.31 (C)**). There was sharp decrease in % OCR between 4 h and 6 h post-incubation with LPS. The changes in OCR correlated with a rapid increase in ECAR (**Figure 4.32 (B)**) up to 6 and 8 h post LPS-activation. A biphasic response was observed when ECAR decreased at 12 h and 24 h post LPS-activation.

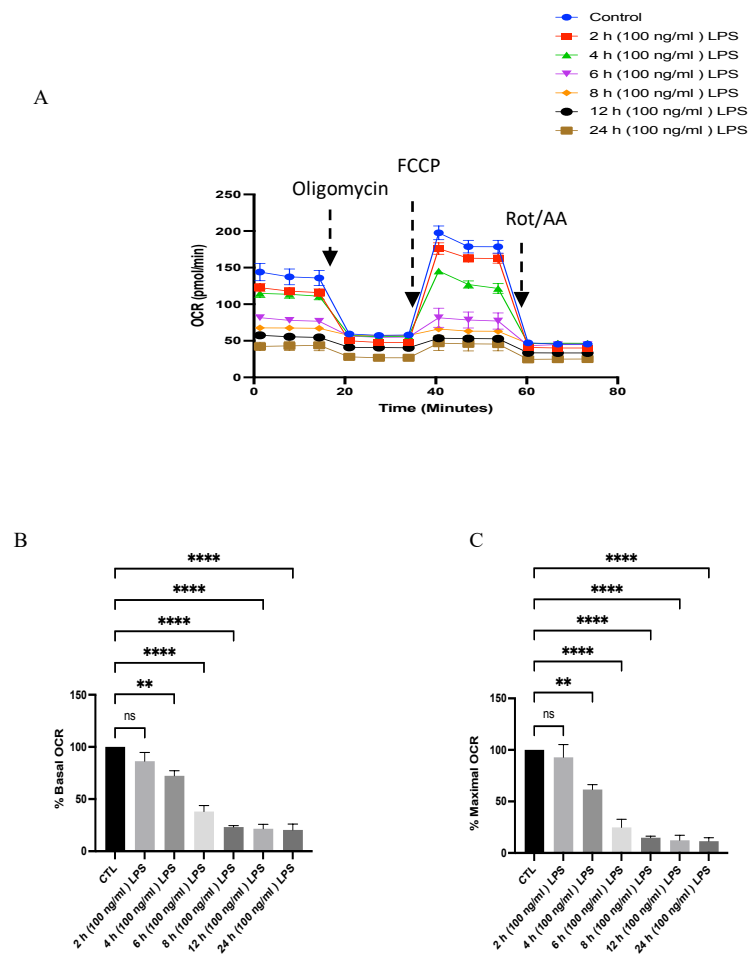


Figure 4.31. LPS activation of BMDMs decreases basal and maximal OCRs in BMDMs in a time-dependent manner.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight and treated with LPS (100 ng/ml) for 2,4,6,8,12 and 24h time periods. The OCR was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) The OCR was expressed as a percentages of their control values, and a one-way ANOVA performed statistical analysis. The results were expressed as the mean $\pm$ SEM., $n=3$. (C) The maximal OCRs were expressed as percentages of their control value, and one-way ANOVA performed statistical analysis; then, results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

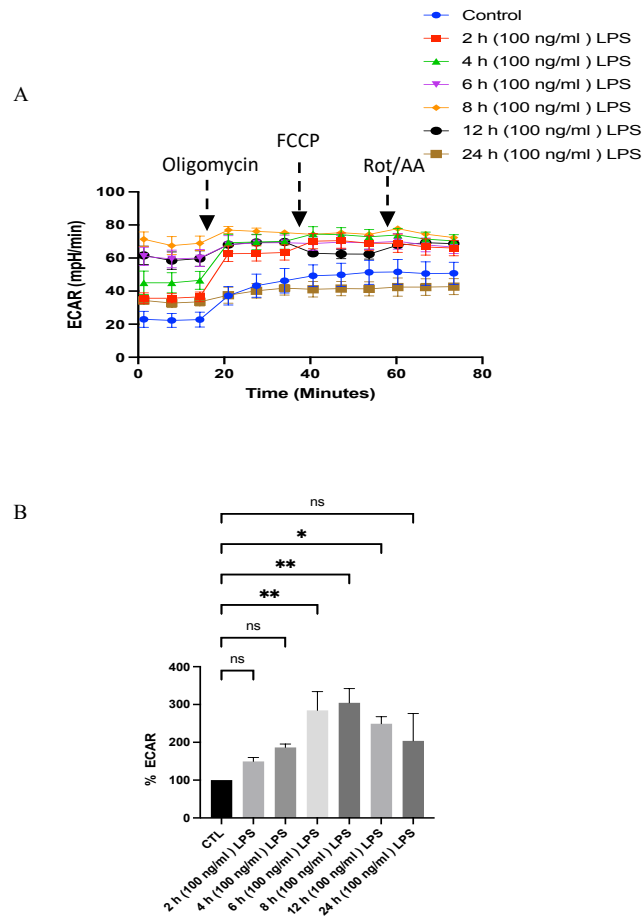


Figure 4.32. LPS activation of BMDMs increases ECAR in BMDMs in a biphasic time-dependent manner.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight and treated with LPS (100 ng/ml) for 2,4,6,8,12 and 24h time periods. The ECAR was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) The ECAR was expressed as a percentages of their control values, and a one-way ANOVA performed statistical analysis. The results were expressed as the mean $\pm$ SEM., $n=3$. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.2.8 A continuous bioenergetics profile in BMDMs during ETC titrations and LPS-activation over 4 hours.

To further characterise the immediate bioenergetic effects during LPS-activation, BMDMs were incubated with LPS (100ng/ml) and OCR and ECAR measured over 4 hours. The basal OCRs at 0 min, 18 min and 36 min were not changed (**Figure 4.33** (A),(B) and (C)) while OCRs at 2 and 4 h were significantly decreased by 15 % and 25 %, respectively (**Figure 4.33** (D) and (E)). However, LPS-activation produced a statistically significant increase in ECAR at 18 min, 36 min, 2 h and 4 h, indicating a rapid increase in glycolysis (**Figures 4.34** (A), (B), (C), (D) and (E)).

In response to a rotenone (0 - 100 nM) titration the OCR decreased by ~ 80 % in BMDMs over 4 h (**Figure 4.35**). This correlated with increase in ECAR, almost doubling after 2 h (**Figure 4.36**). As for complex I, when the experiment was run for 4 hours with the complex III inhibitor antimycin A (0-100 nM), the OCR decreased by ~ 95 % at the highest concentration (**Figures 4.37**). Interestingly the 10 nM and 20 nM antimycin A concentrations induced a delayed decrease in OCR after 18 min following which further OCR decreases were observed. **Figure 4.38** showed rapid increases in the ECAR, especially at the higher concentrations of antimycin.

The rotenone and antimycin A titrations were repeated in BMDMs activated with LPS over a 4 hour time course. **Figure 4.39** showed that the OCRs rates were totally inhibited when at higher concentrations of rotenone over 4 hours, which correlated with a significant increase in ECAR (**Figure 4.40**). **Figures 4.41** and **4.42** indicate that treatment of LPS-activated BMDMs also significantly decrease OCR and increase ECAR, especially at concentrations greater than 10 nM.

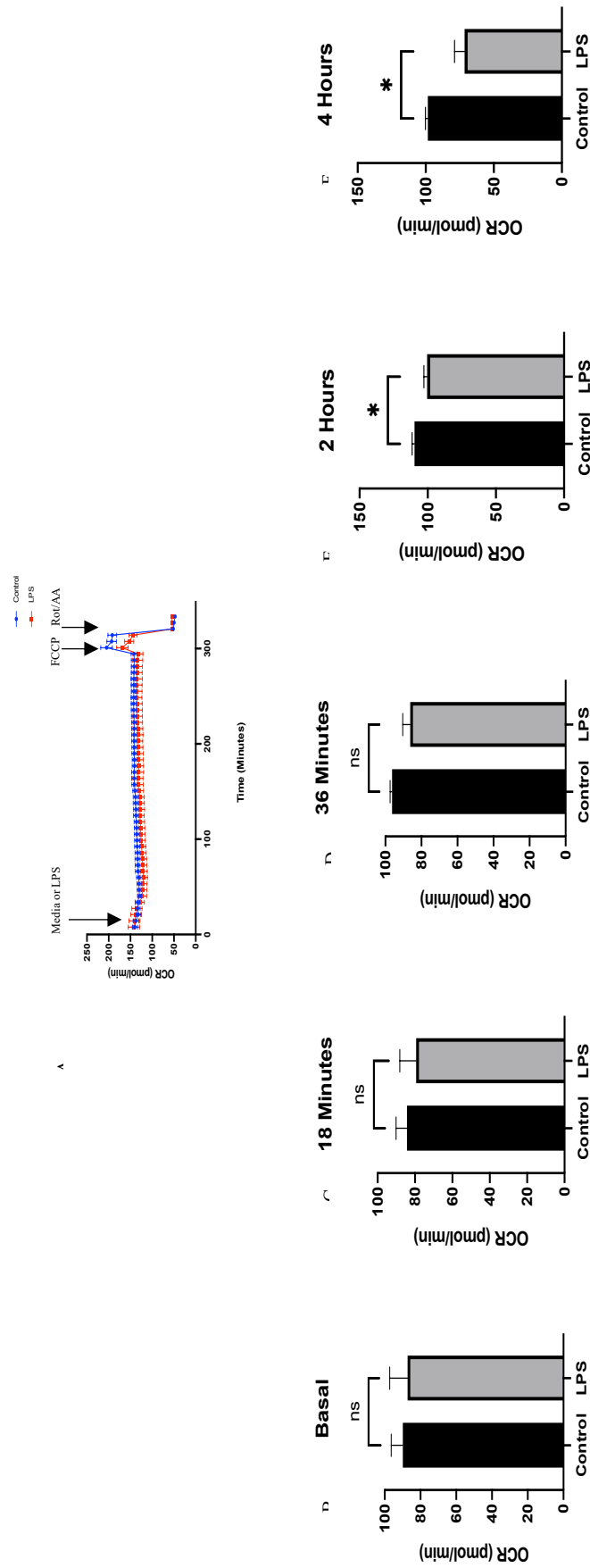


Figure 4.33. Oxygen consumption rates decrease in LPS-activated BMDMs in a time-dependent manner over 4 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D), (E) and (F) show the OCR values at Basal (0h) pre-LPS treatment and 18 min, 36 min, 2 h and 4 h post LPS (100 ng/ml) treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

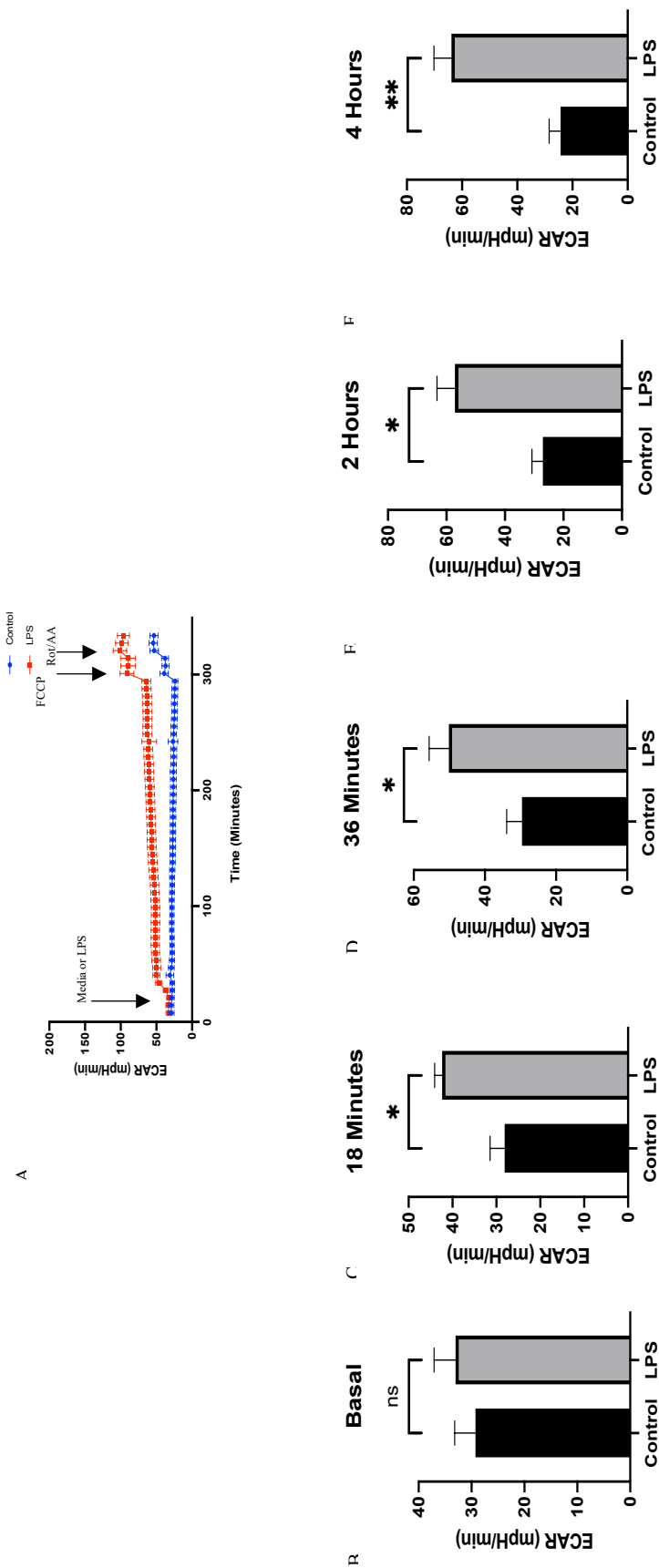


Figure 4.34. Extracellular acidification rates (ECAR) increased in LPS-activated BMDMs in a biphasic time-dependent manner over 4 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D), (E) and (F) show the ECAR values at Basal (0h) pre-LPS treatment and 18 min, 36 min, 2 h and 4 h post LPS (100 ng/ml) treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM, $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

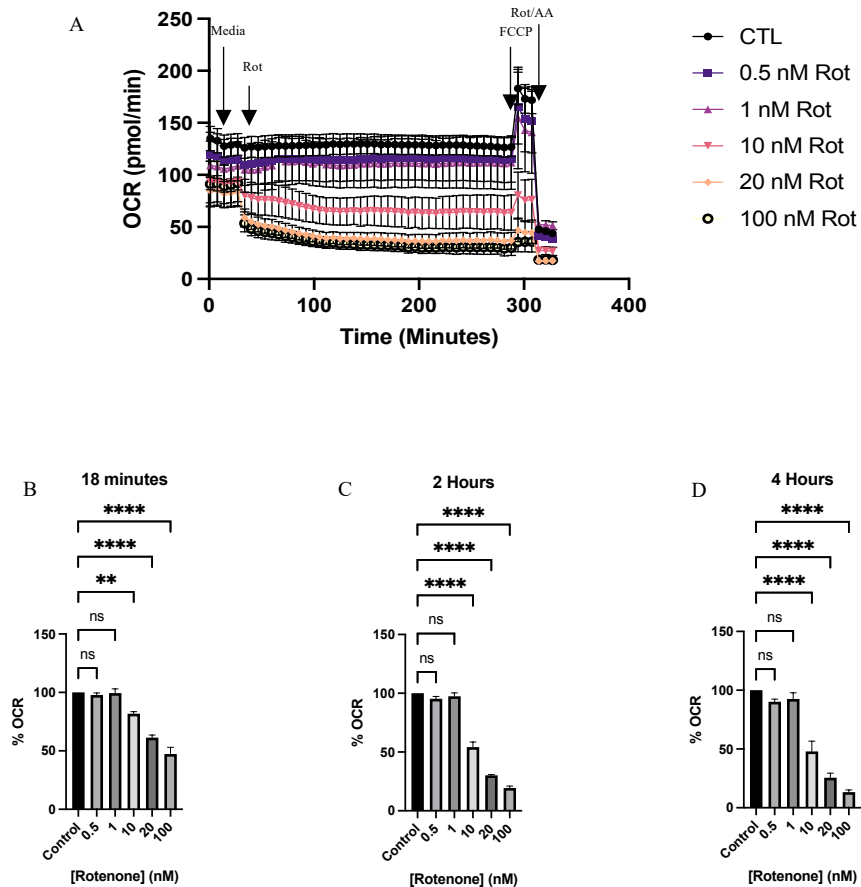


Figure 4.35. Rotenone titration of basal oxygen consumption rate in BMDMs over 4 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of a rotenone titration (0.5-100nM) over 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the OCR values at 18 min, 2 h and 4 h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

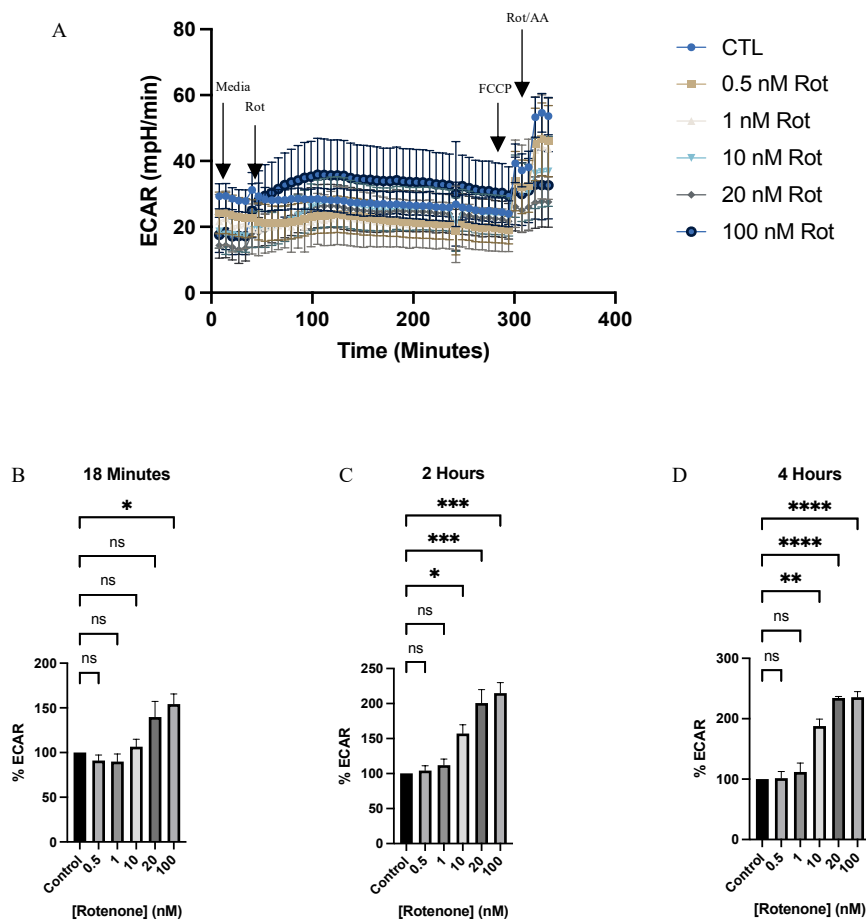


Figure 4.36. Rotenone titration of the extracellular acidification rate in BMDMs over 4 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of a rotenone titration (0.5-100nM) over 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the ECAR values at 18 min, 2 h and 4 h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

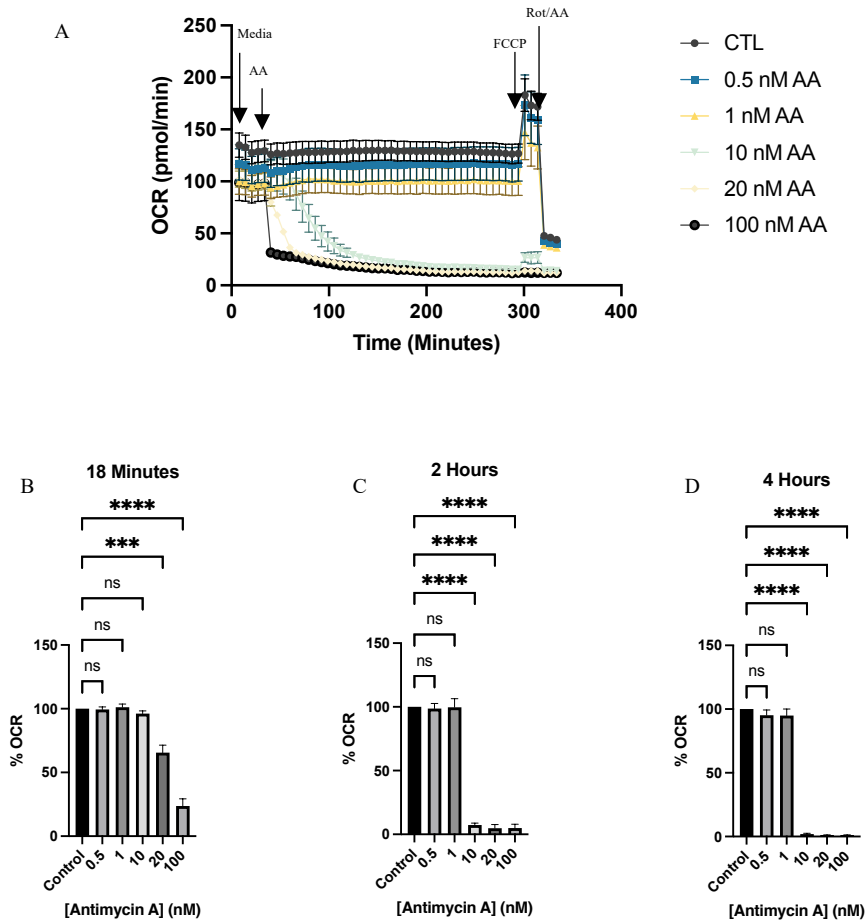


Figure 4.37. Antimycin A titration of basal oxygen consumption rate in BMDMs over 4 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of a antimycin A titration (0.5-100nM) over 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the OCR values at 18 min, 2 h and 4 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

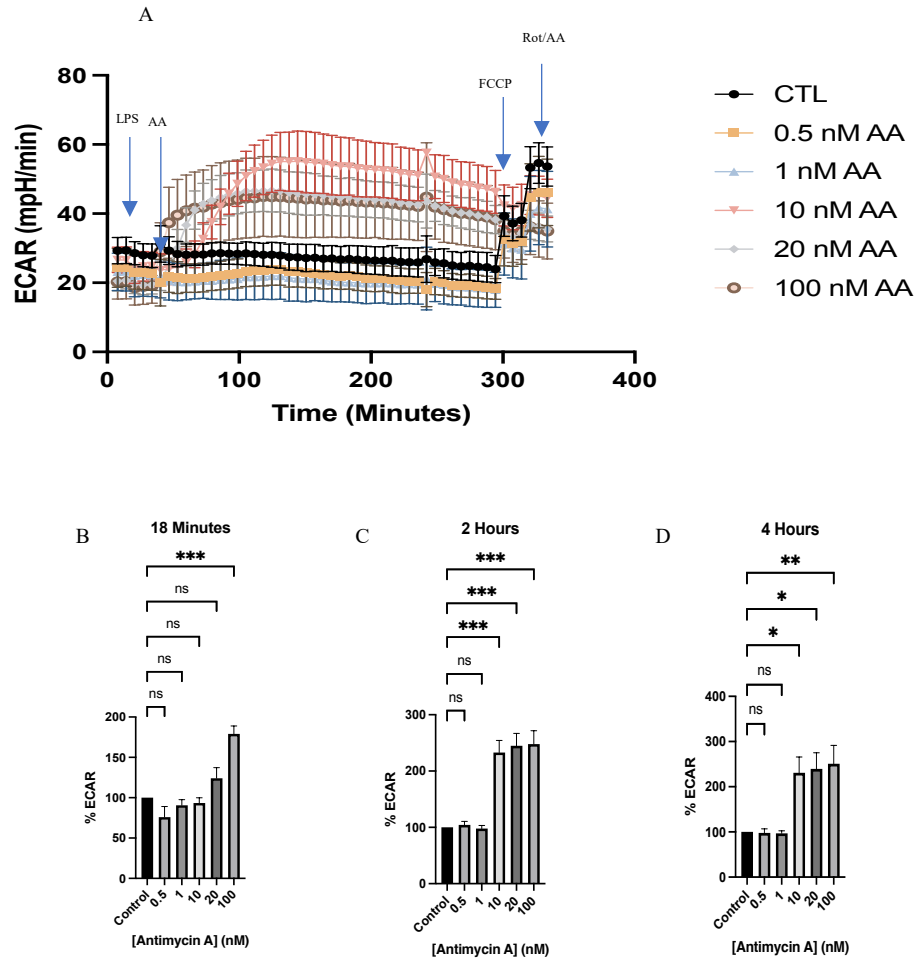


Figure 4.38. Antimycin A titration of the extracellular acidification rate in BMDMs over 4 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of a antimycin A titration (0.5-100nM) over 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the ECAR values at 18 min, 2 h and 4 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

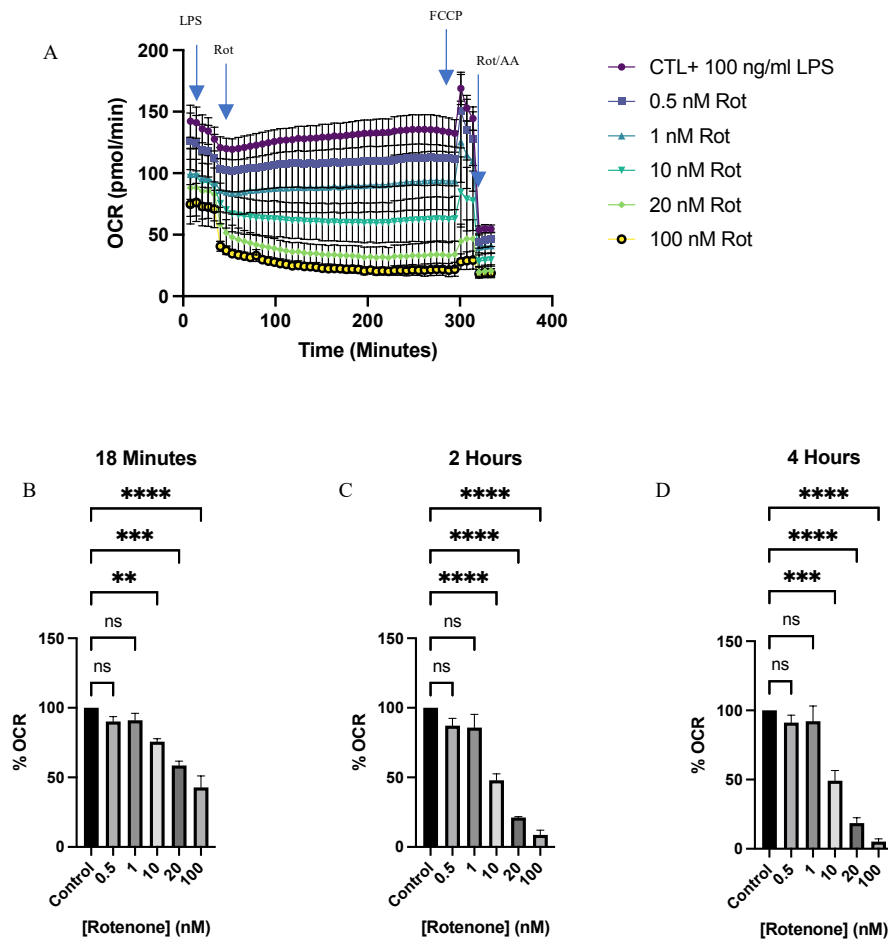


Figure 4.39. Rotenone titration of basal oxygen consumption rate in LPS activated BMDMs over a 4 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by a rotenone titration (0.5-100nM) after 18 min for 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the OCR values at 18 min, 2 h and 4 h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

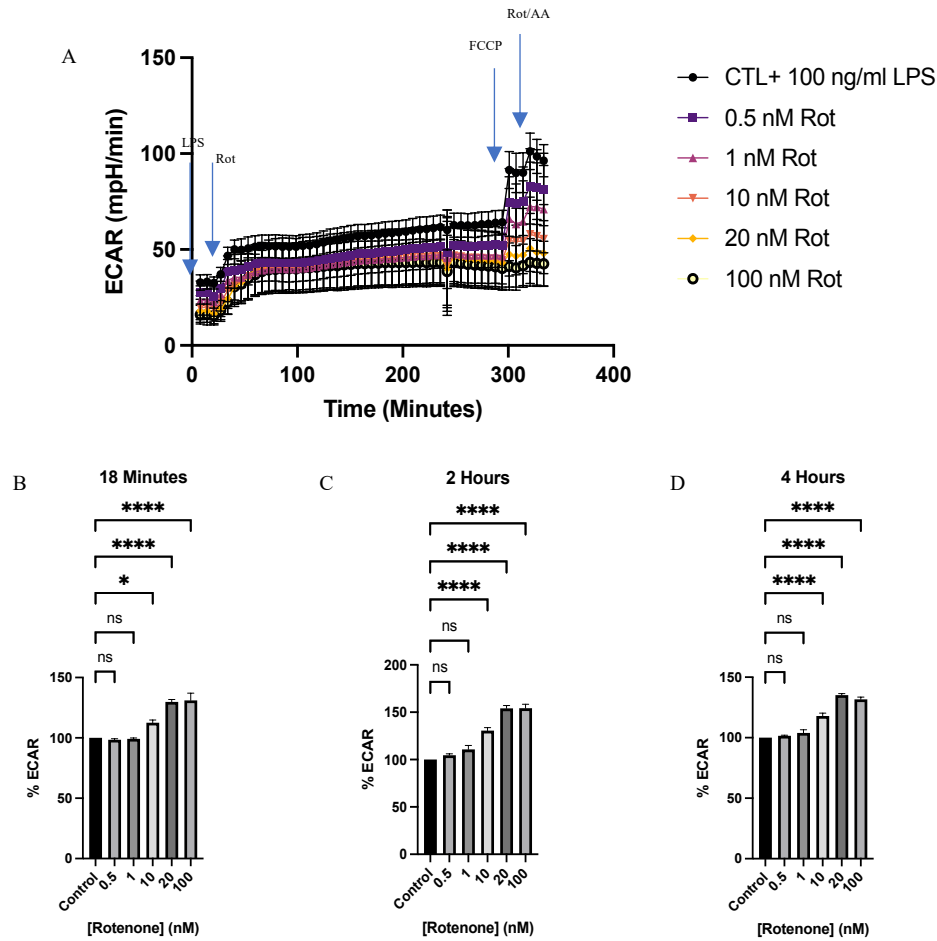


Figure 4.40. Rotenone titration of the basal extracellular acidification rate in LPS activated BMDMs over a 4 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by a rotenone titration (0.5-100nM) after 18 min for 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the ECAR values at 18 min, 2 h and 4 h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

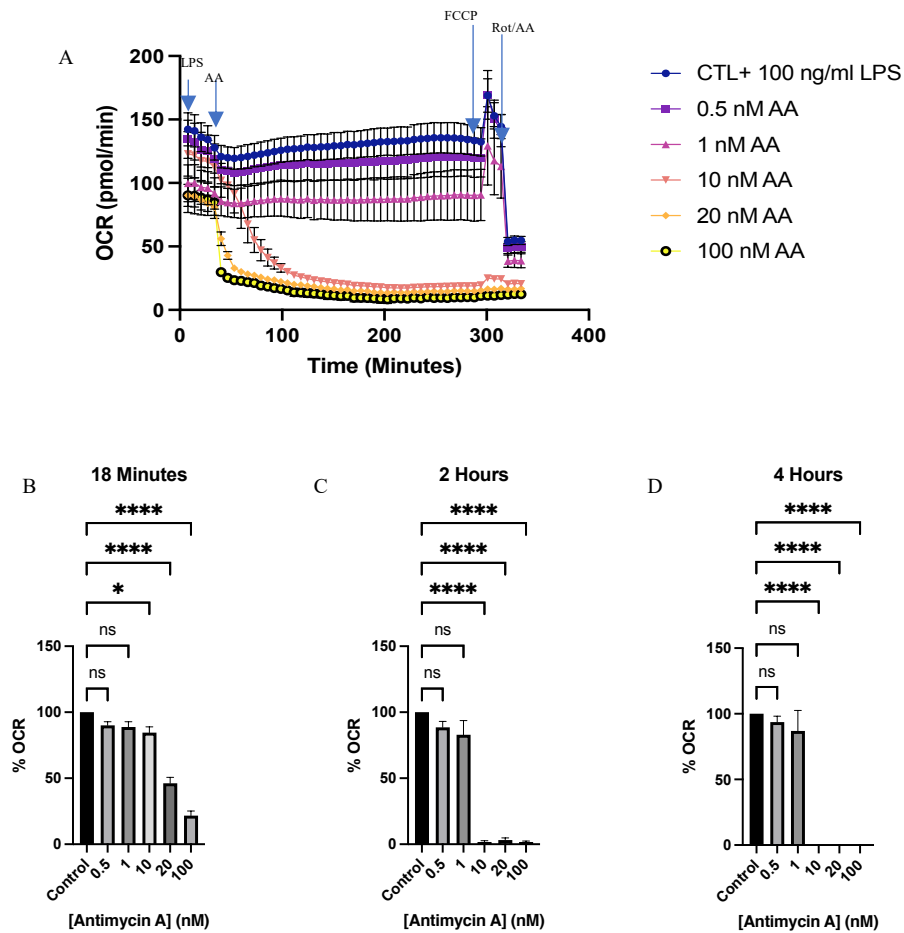


Figure 4.41. Antimycin A titration of basal oxygen consumption rate in LPS activated BMDMs over a 4 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by an antimycin A titration (0.5-100 nM) after 18 min for 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the OCR values at 18 min, 2 h and 4 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

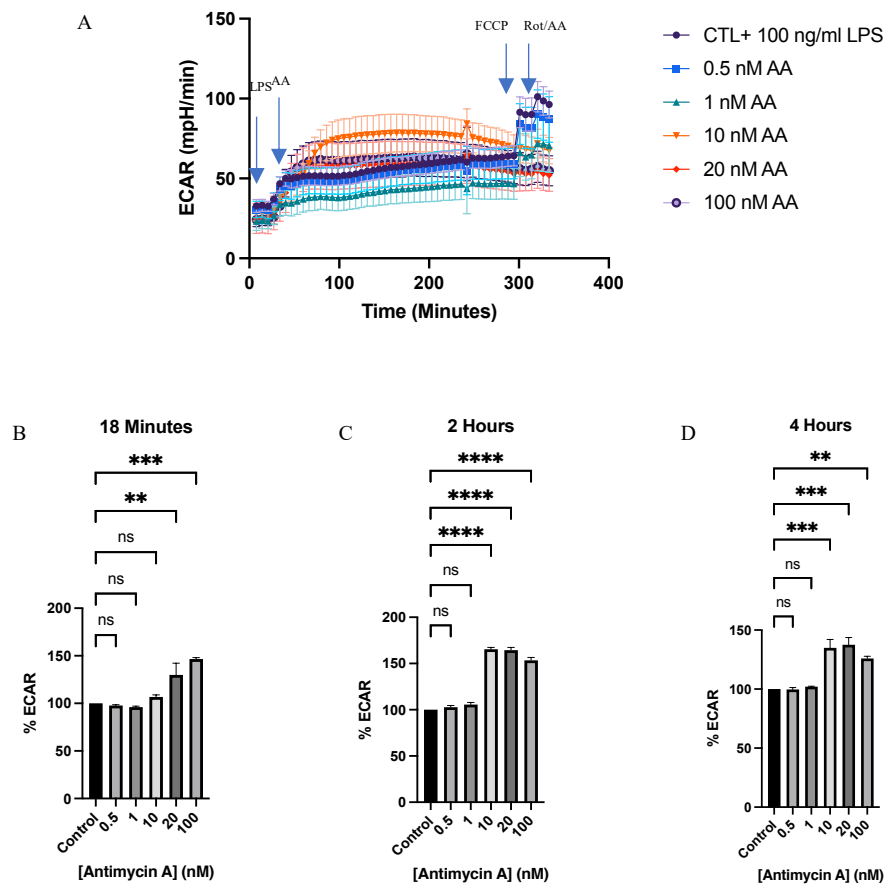


Figure 4.42. Antimycin A titration of the basal extracellular acidification rate in LPS activated BMDMs over a 4 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by a n antimycin A titration (0.5-100nM) after 18 min for 4 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the ECAR values at 18 min, 2 h and 4 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.2.9 A continuous bioenergetics profile in BMDMs during ETC titrations and LPS-activation over 6 hours.

Due to the observation of a biphasic decrease in OCR between 4-6h post LPS-activation (**Figure 3.31**) the bioenergetic effects LPS-activation were measured over a 6 h period. The basal OCRs at 0 min, 18 min and 36 min were not changed (**Figure 4.43** (A),(B) and (C)) while OCRs at 2h, 4h and 6h were significantly decreased by 15 %, 25 % and 60% respectively (**Figure 4.43** (D), (E) and (F)). However, LPS-activation produced a statistically significant increase in ECAR at 18 min, 36 min, 2 h, 4h and 6 h, indicating a rapid increase in glycolysis (**Figures 4.44** (A), (B), (C), (D), (E) and (F)).

In response to a rotenone (0 - 100 nM) titration the OCR decreased by ~ 95 % in BMDMs over 6 h (**Figure 4.45**). This correlated with increase in ECAR, almost doubling after 2 h (**Figure 4.46**). As seen for complex I, when the experiment was run for 6h with the complex III inhibitor antimycin A (0-100 nM), the OCR decreased by ~ 98 % at the highest concentration (**Figure 4.47**). Interestingly the 10 nM and 20 nM antimycin A concentrations induced a delayed decrease in OCR after 18 min following which further OCR decreases were observed. Figure 4.48 showed rapid increases in the ECAR, especially at the higher concentrations of antimycin.

The rotenone and antimycin A titrations were repeated in BMDMs activated with LPS over a 4 h time course. **Figure 4.49** showed that the OCRs rates were totally inhibited when at higher concentrations of rotenone over 6 h, which correlated with a significant increase in ECAR (**Figure 4.50**). **Figures 4.51** and **4.52** indicate that treatment of LPS-activated BMDMs also significantly decrease OCR and increase ECAR, especially at concentrations greater than 10 nM.

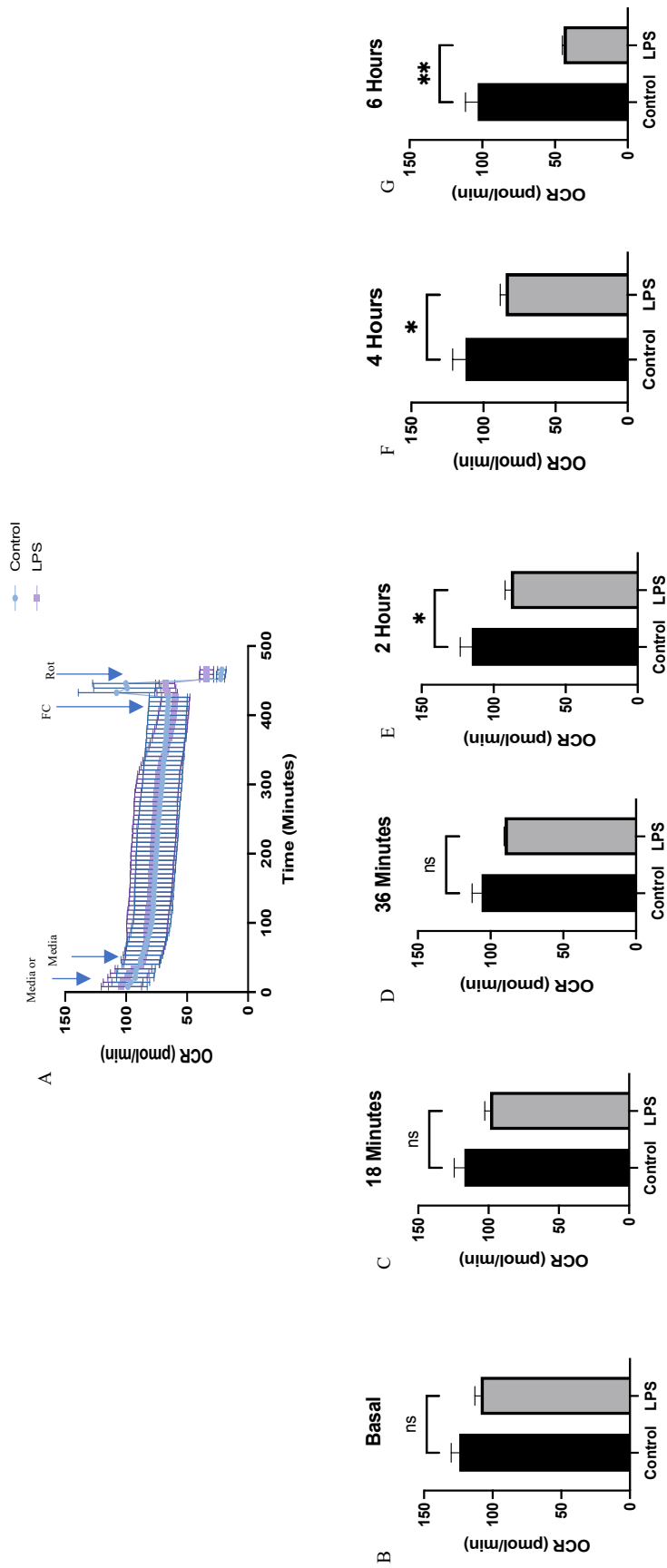


Figure 4.43. Oxygen consumption rates decrease in LPS-activated BMDMs in a time-dependent manner over 6 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D), (E), (F) and (G) show the OCR values at Basal (0h) pre-LPS treatment and 18 min, 36 min, 2 h, 4 h and 6 h post LPS (100 ng/ml) treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM, $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

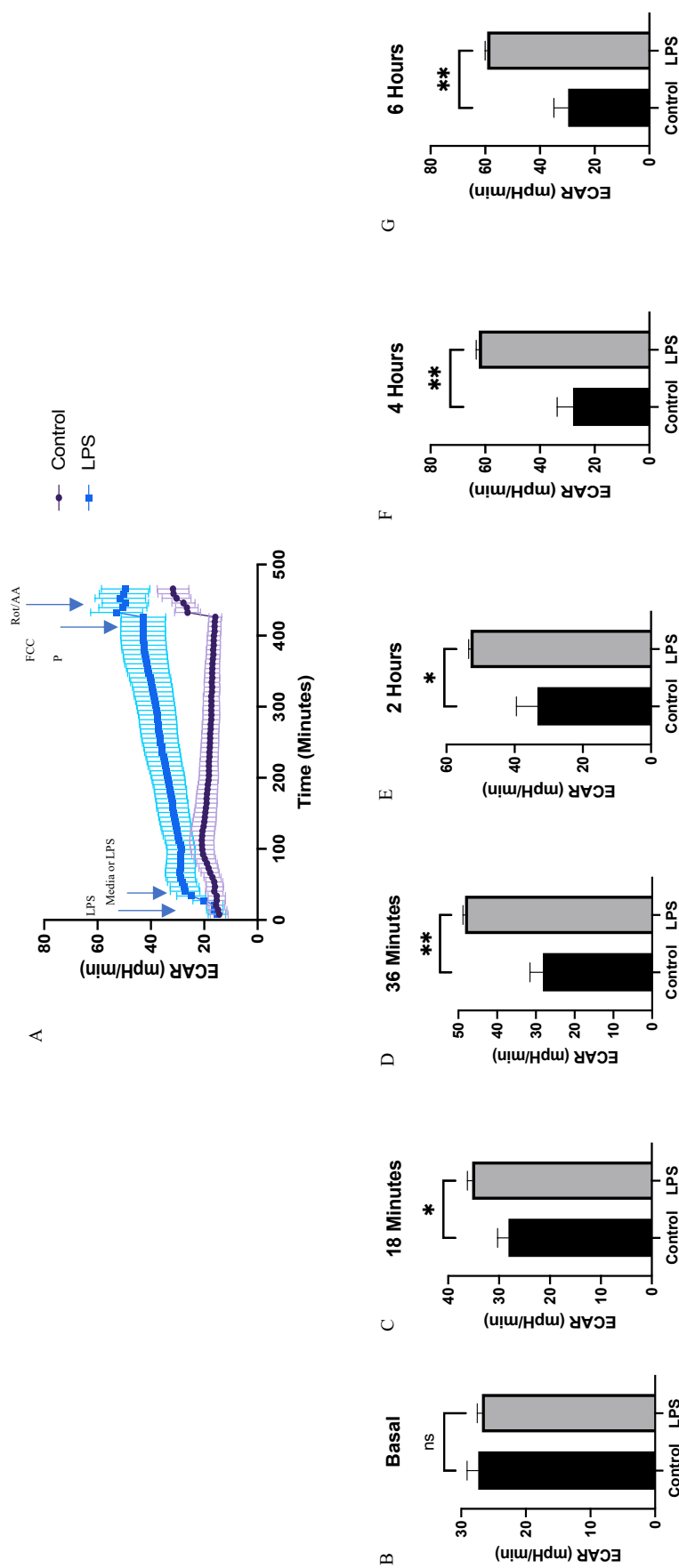


Figure 4.44. Extracellular acidification rates (ECAR) increased in LPS-activated BMDMs in a biphasic time-dependent manner over 6 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D), (E), (F) and (G) show the ECAR values at Basal (0h) pre-LPS treatment and 18 min, 36 min, 2 h, 4 h and 6 h post LPS (100 ng/ml) treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

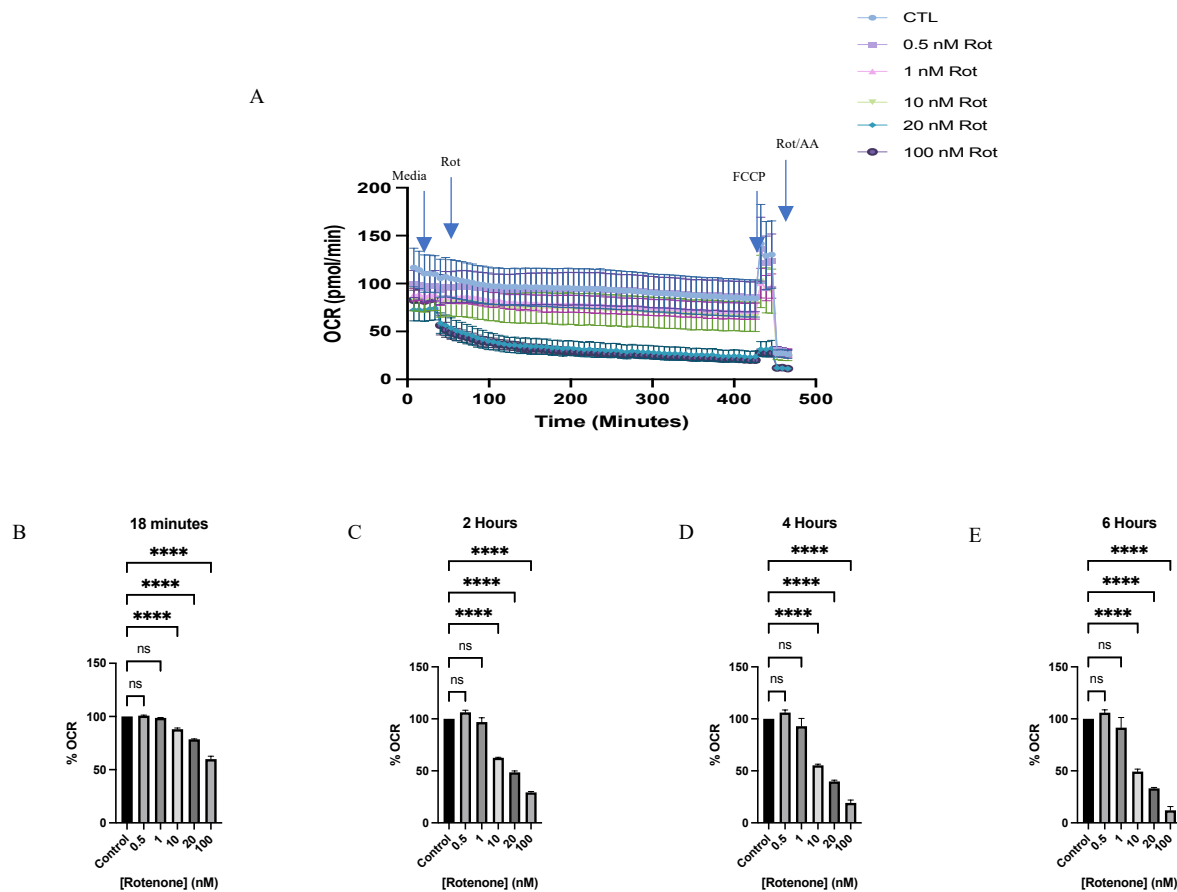


Figure 4.45. Rotenone titration of basal oxygen consumption rate in BMDMs over 6 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of a rotenone titration (0.5-100nM) over 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D) and (E) show the OCR values at 18 min, 2 h, 4 h and 6 h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

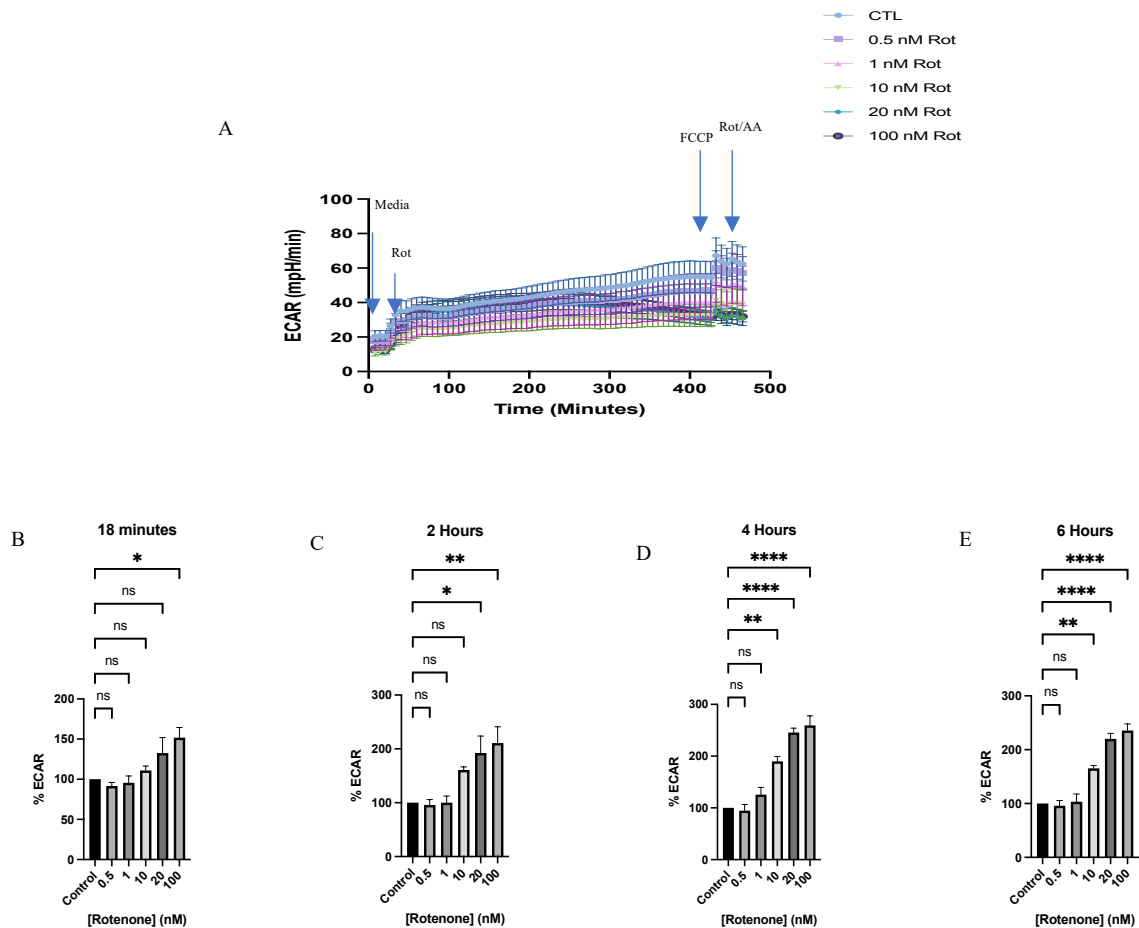


Figure 4.46. Rotenone titration of the extracellular acidification rate in BMDMs over 6 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of a rotenone titration (0.5-100nM) over 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D) and (E) show the ECAR values at 18 min, 2 h, 4 h and 6 h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

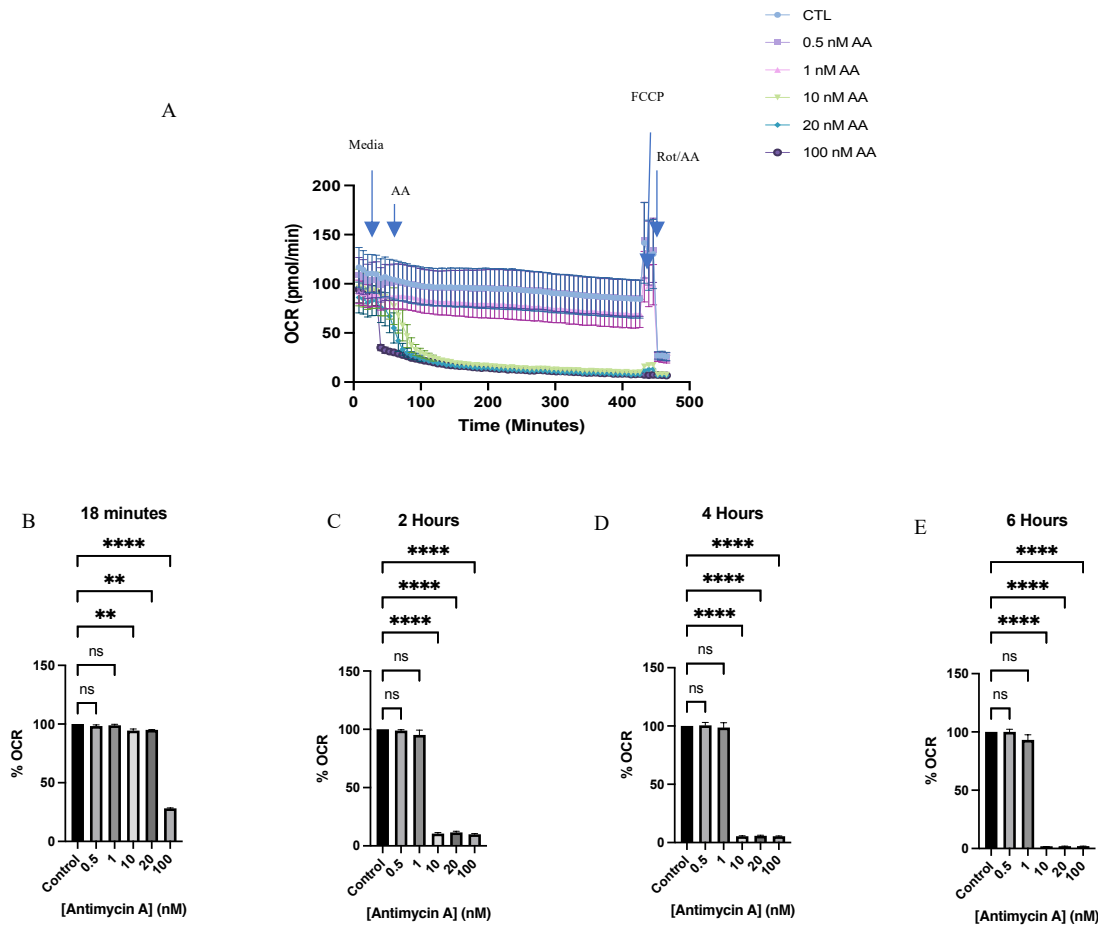


Figure 4.47. Antimycin A titration of basal oxygen consumption rate in BMDMs over 6 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of a antimycin A titration (0.5-100nM) over 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D) and (E) show the OCR values at 18 min, 2 h, 4 h and 6 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

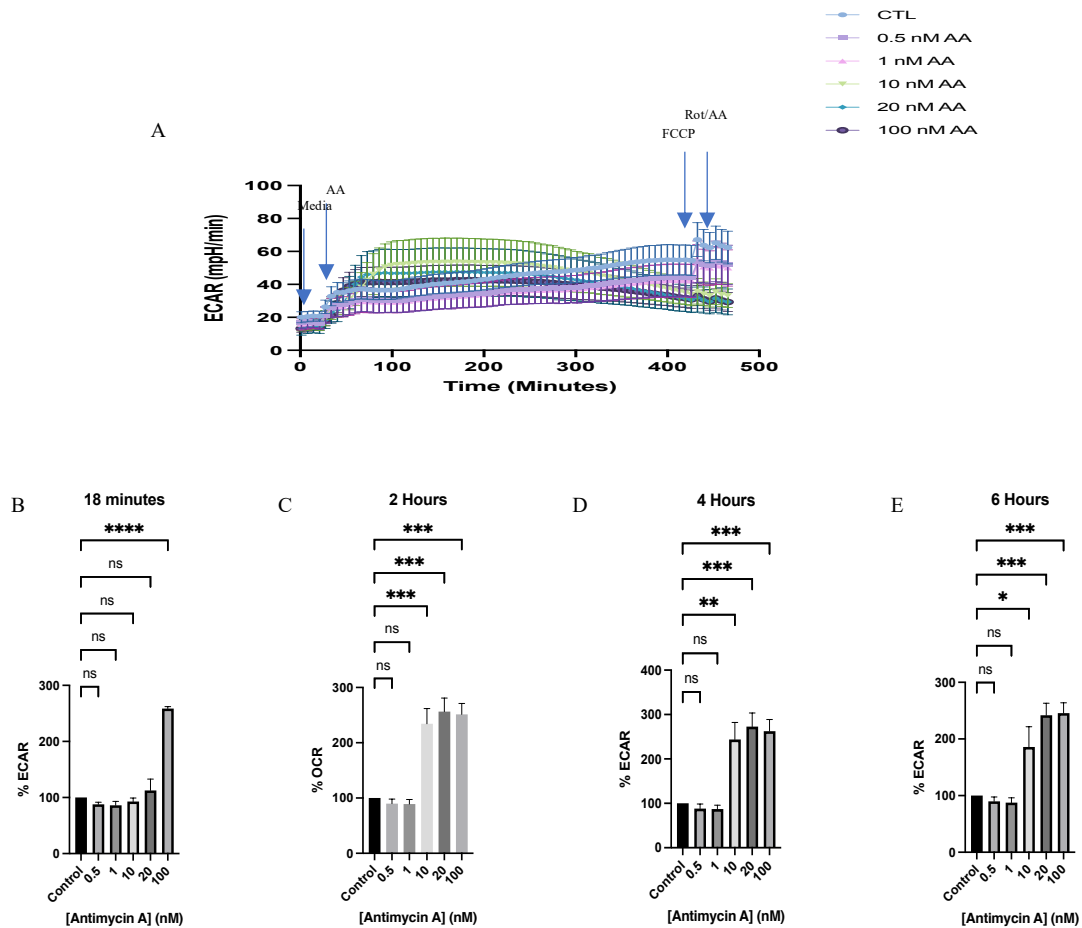


Figure 4.48. Antimycin A titration of the extracellular acidification rate in BMDMs over 6 h.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of a antimycin A titration (0.5-100nM) over 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C) and (D) show the ECAR values at 18 min, 2 h, 4 h and 6 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

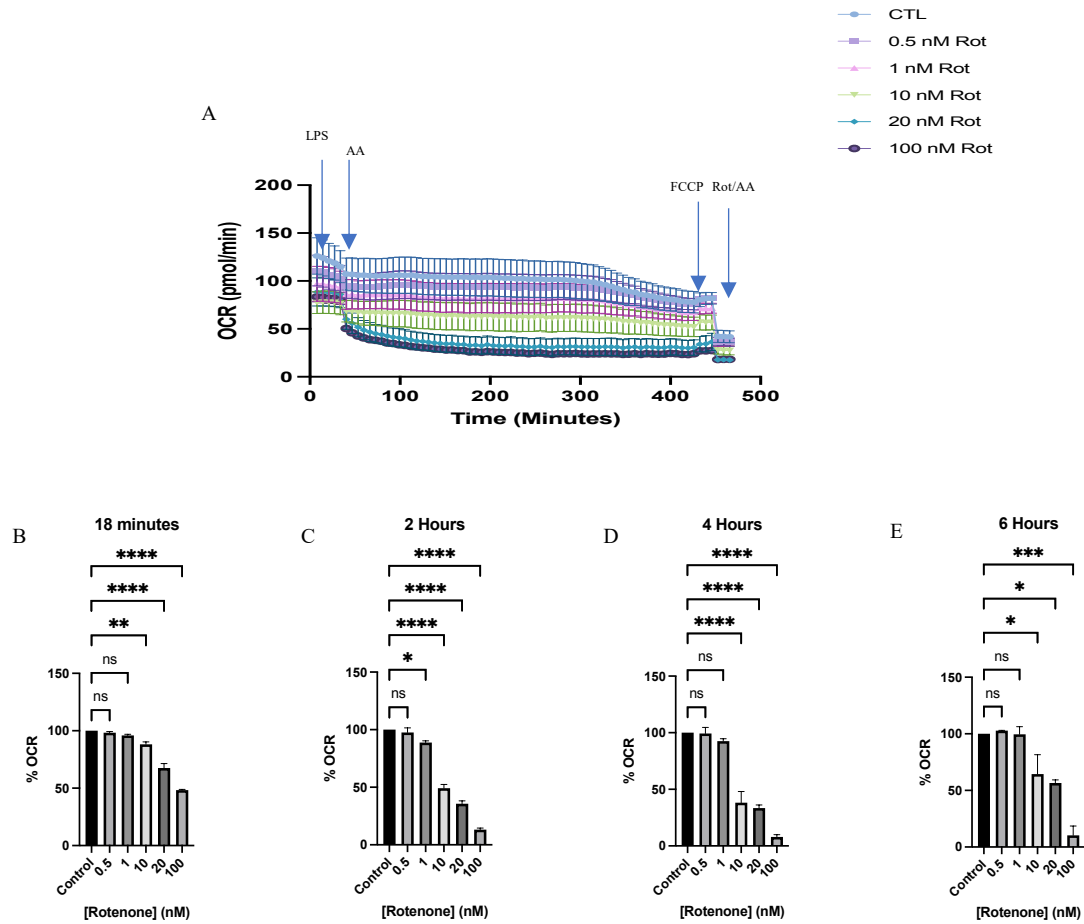


Figure 4.49. Rotenone titration of basal oxygen consumption rate in LPS activated BMDMs over a 6 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by a rotenone titration (0.5-100nM) after 18 min for 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D) and (E) show the OCR values at 18 min, 2 h, 4 h and 6 h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

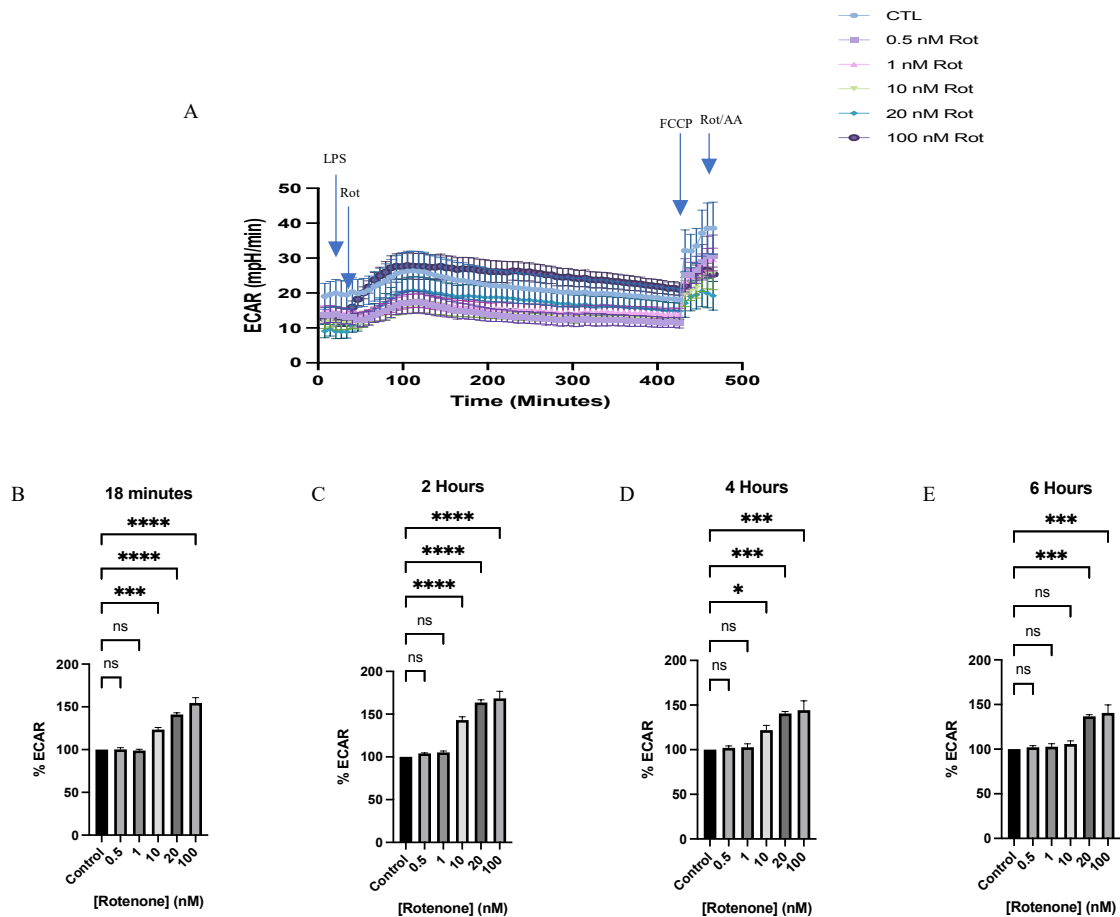


Figure 4.50. Rotenone titration of the basal extracellular acidification rate in LPS activated BMDMs over a 6 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by a rotenone titration (0.5-100nM) after 18 min for 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D) and (E) show the ECAR values at 18 min, 2 h, 4 h and 46h post rotenone treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

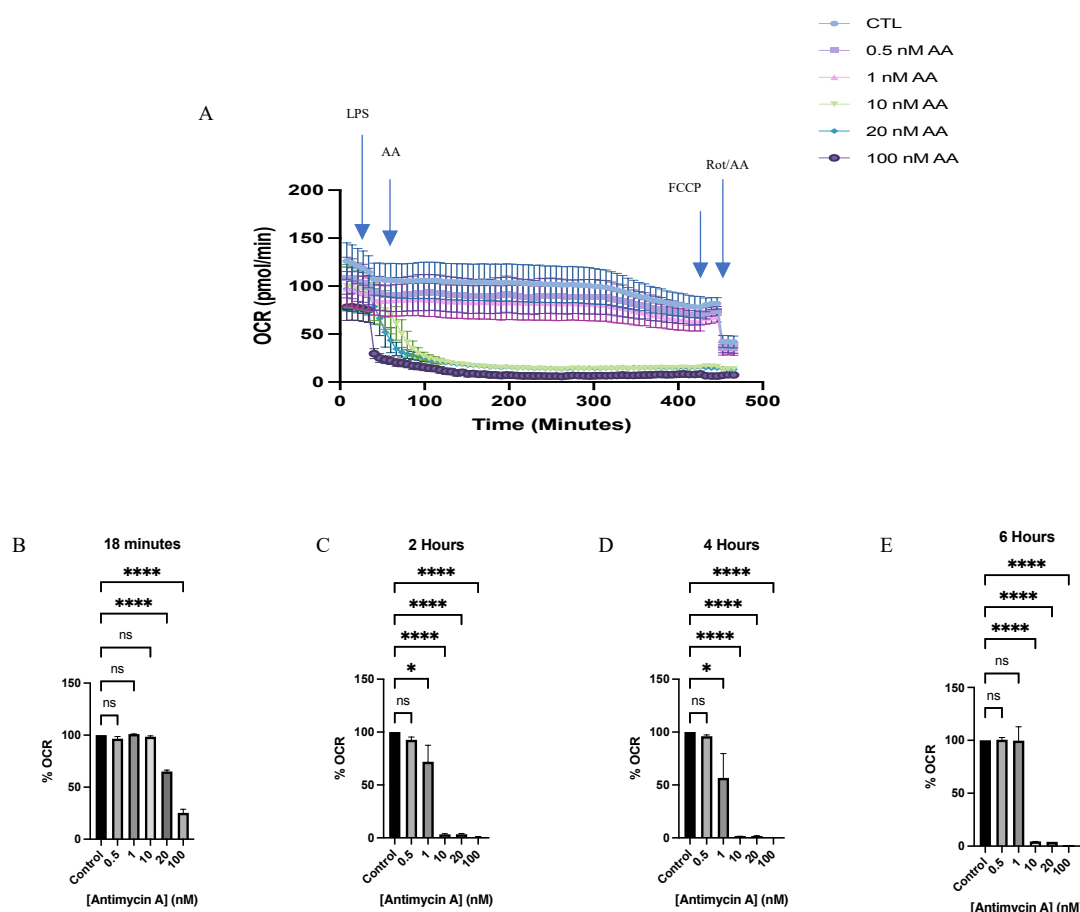


Figure 4.51. Antimycin A titration of basal oxygen consumption rate in LPS activated BMDMs over a 6 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by an antimycin A titration (0.5-100 nM) after 18 min for 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D) and (E) show the OCR values at 18 min, 2 h, 4 h and 6 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

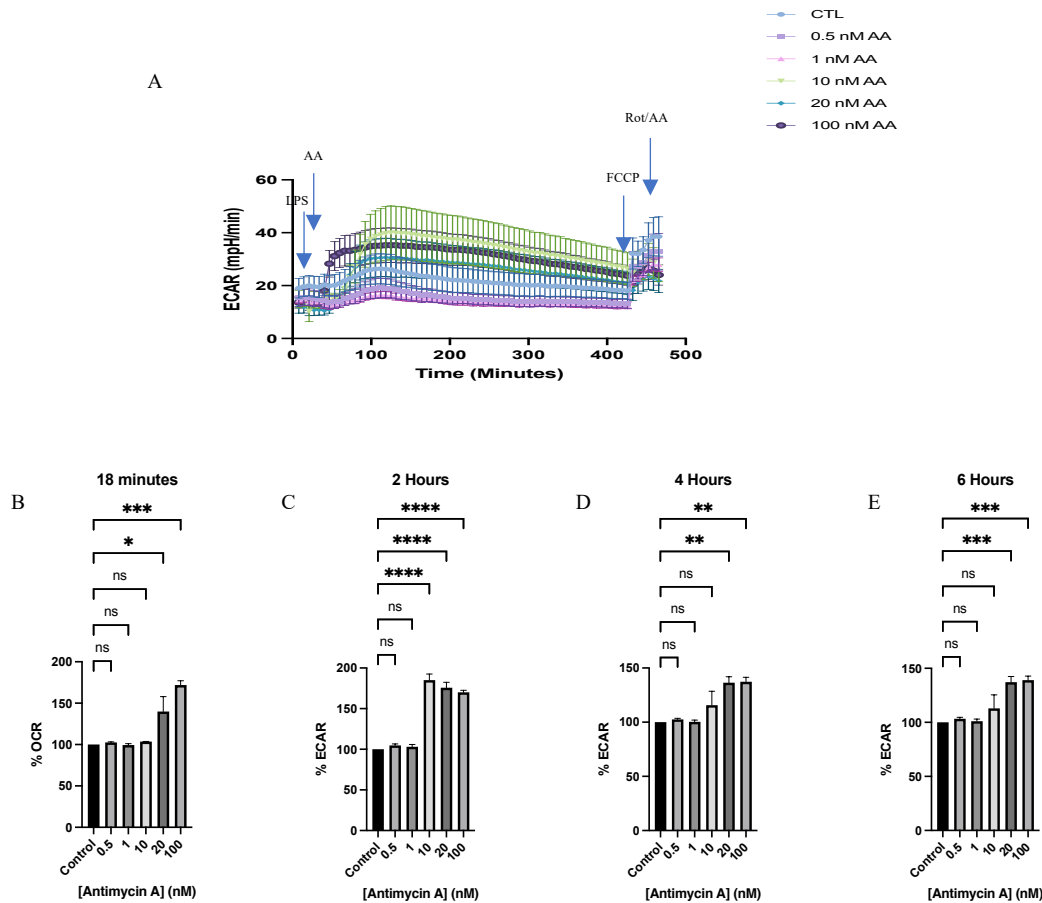


Figure 4.52. Antimycin A titration of the basal extracellular acidification rate in LPS activated BMDMs over a 6 h time period.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and incubated at 37°C overnight. Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser in the presence of LPS (100 ng/ml), followed by an antimycin A titration (0.5-100nM) after 18 min for 6 h. The data are presented as mean $\pm$ S.E.M., $n=3$. (B), (C), (D) and (E) show the ECAR values at 18 min, 2 h, 4 h and 6 h post antimycin A treatment, respectively. Results were expressed as percentages of their control value and a one-way ANOVA was performed statistical analysis. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

4.2.10 Effects of IL-10 on bioenergetic profiles in LPS-activated BMDMs in the absence and presence of rotenone and antimycin A titrations.

Figure 4.53 (A + B) showed that there was no differences in basal OCRs between BMDMs that were incubated with IL-10 (50 ng/ml) for 20min, followed by LPS (100 ng/ml) for 24 h. However, **Figure 4.53** (C), shows a small significant increase in maximal OCR in the IL-10 treated BMDMs. There were no significant differences in the extracellular acidification rates (**Figure 4.54**). Increasing concentrations of rotenone (0-1000 nM) reduced the OCRs in BMDMs activated by LPS (100 ng/ml). The basal and maximal OCRs decreased by ~ 40% at 5 nM (**Figure 4.55**), while the ECAR was not changed (**Figure 4.56**).

Rotenone (0 - 1000 nM) decreased the rates of BMDMs challenged with IL-10 (50 ng/ml) for 25 min followed by LPS (100 ng/ml) basal and maximal OCRs. The basal OCR decreased by ~ 35 % at 5 nM (**Figure 4.57** (B)) and maximal OCR decreased by ~ 50 % at 5 nM (**Figure 4.57** (C)). The ECAR was significantly increased (**Figure 4.58**), suggesting IL-10 treatment enables LPS-activated BMDMs to increase glycolytic capacity. **Figure 4.59** showed that there was a significant decreased in OCRs with increasing concentrations of antimycin A (0-1000 nM) in BMDMs that were challenged with LPS (100 ng/ml). The basal OCR decreased by ~ 20 % at 20 nM (**Figure 4.59** (B)) and maximal OCR decreased by ~ 80 % at 5 nM (**Figure 4.59** (C)). The ECAR was not significantly increased (**Figure 4.60**). Antimycin A (0 - 1000 nM) decreased the rates of BMDMs challenged with IL-10 (50 ng/ml) for 20 min, followed by LPS (100 ng/ml) basal and maximal oxygen consumption. The basal OCR was decreased by ~ 20 % at 20 nM (**Figure 4.61** (B)) and maximal OCR decreased by ~ 50 % at 20 nM (**Figure 4.61** (C)). The

ECAR was significantly increased (**Figure 4.62**), again suggesting IL-10 treatment increases glycolytic reserve in BMDMs.

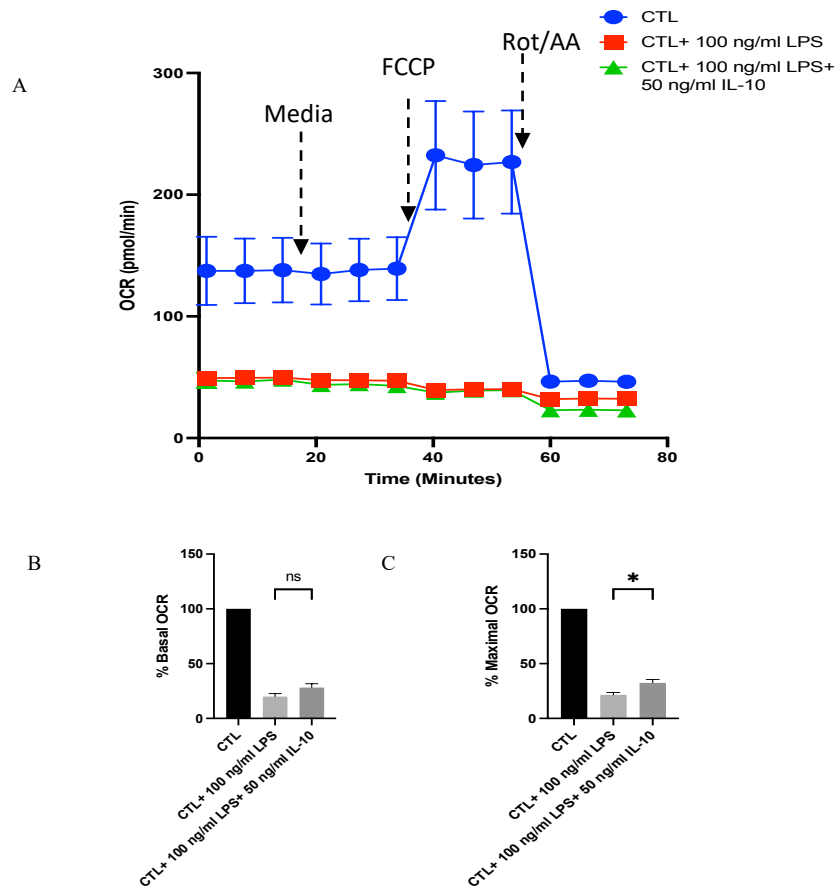


Figure 4.53. IL-10 does not prevent decrease in OCR in LPS-activated BMDMs.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and were incubated $\pm$ 100 ng/ml LPS for 24 h, in the presence or absence of IL-10 (50 ng/ml). Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) Basal OCR was expressed as percentages of their control value, and one-way ANOVA performed statistical analysis; then, results were expressed as mean $\pm$ SEM., $n=3$. (C) Maximal OCR was expressed as percentages of their control value, and one-way ANOVA performed statistical analysis; then, results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

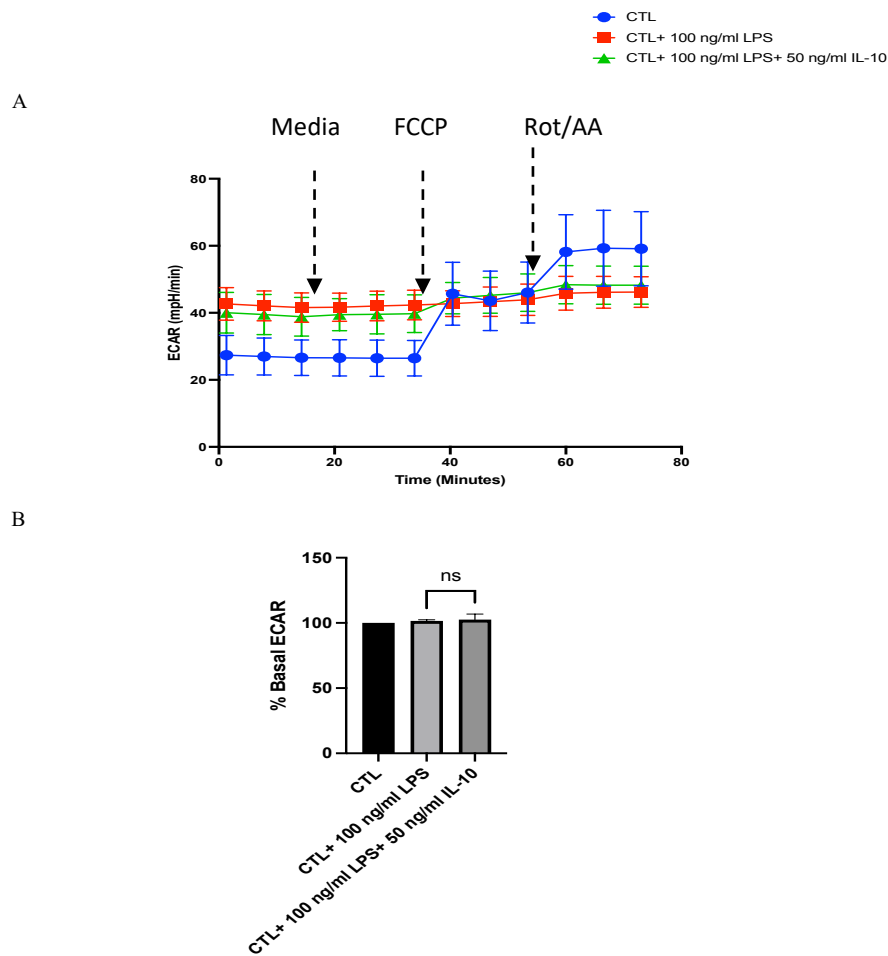


Figure 4.54. IL-10 does not prevent decrease in Extracellular Acidification Rate (ECAR) in LPS-activated BMDMs.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and were incubated $\pm$ 100 ng/ml LPS for 24 h, in the presence or absence of IL-10 (50 ng/ml). Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) Extracellular acidification rates (ECAR) were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results were expressed as mean $\pm$ SEM., $n=3$. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

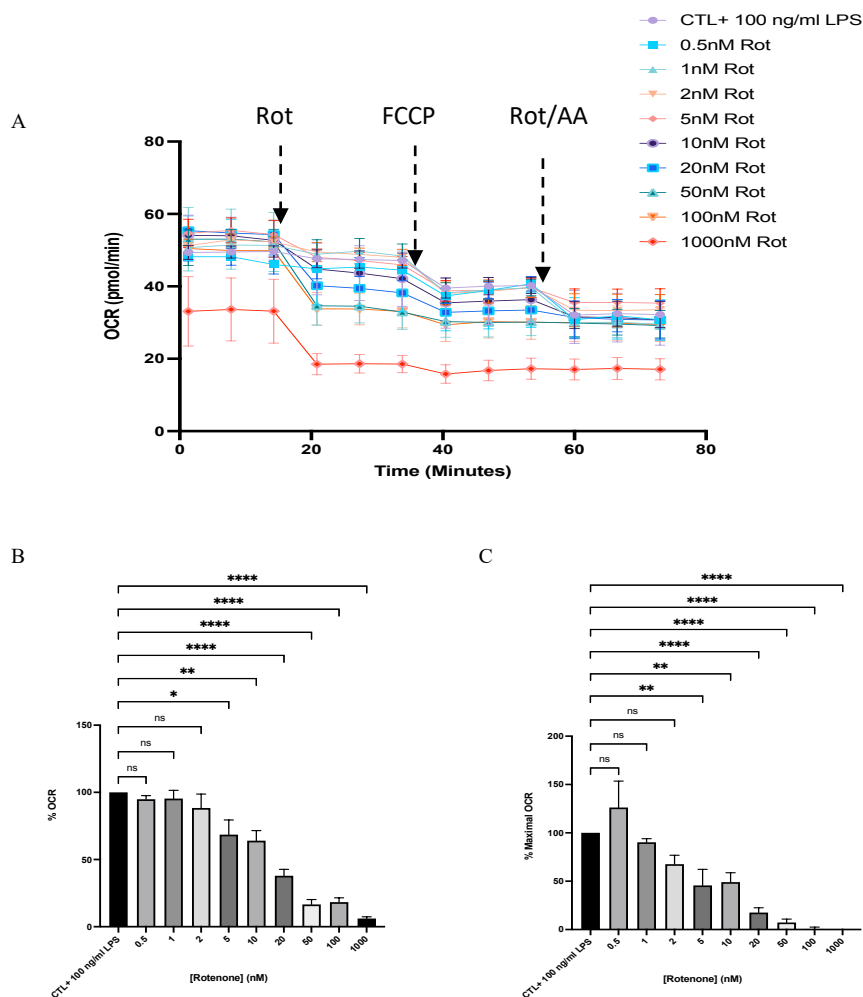


Figure 4.55. Rotenone titration of Oxygen Consumption Rate (OCR) in LPS - activated BMDMs.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and were incubated with 100 ng/ml LPS for 24 h, followed by a rotenone titration (0.5 nM – 1 μ M) and the Oxygen Consumption Rate (OCR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=3$. (C) The Maximal OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results were expressed as mean $\pm$ SEM., $n=3$.

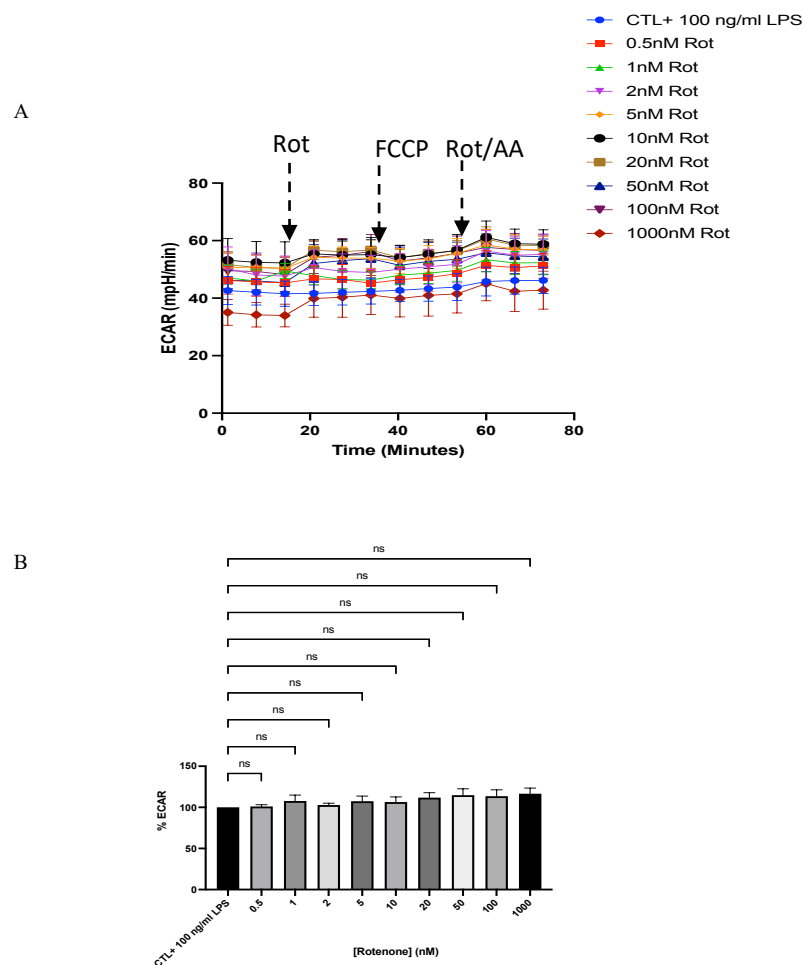


Figure 4.56. Rotenone titration of for Extracellular Acidification Rate (ECAR) in LPS - activated BMDMs.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and were incubated with 100 ng/ml LPS for 24 h followed by a rotenone titration (0.5 nM – 1 μ M) and the extracellular acidification rates (ECAR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) ECAR were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA; then, results were expressed as mean $\pm$ SEM., $n=3$.

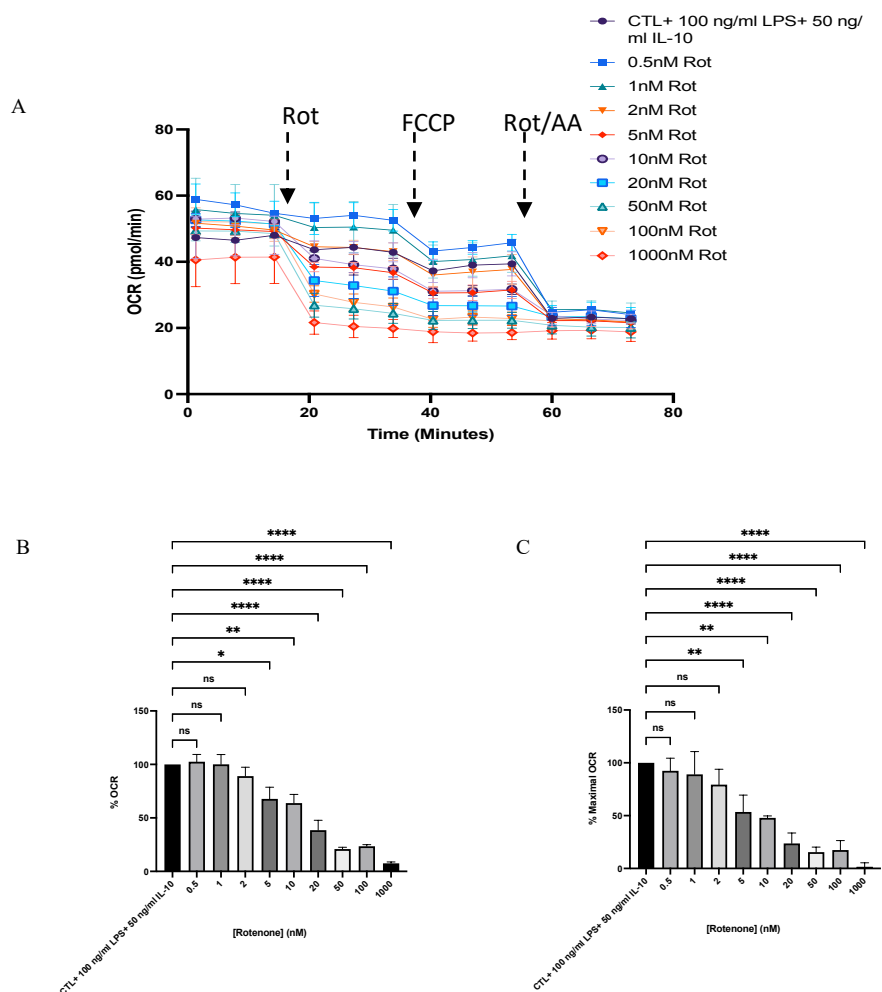
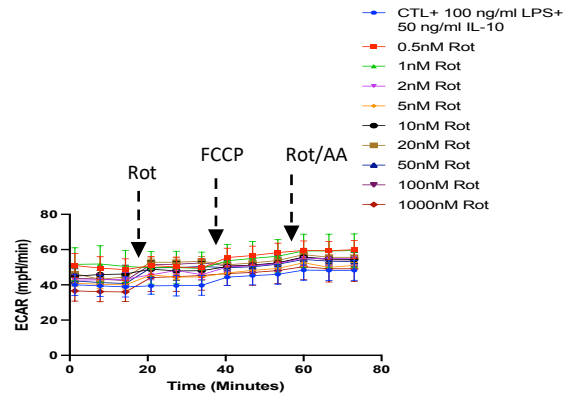


Figure 4.57. Rotenone titration of OCR LPS – activated BMDMs ± IL-10.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate at 37°C . BMDMs were incubated with 50 ng/ml IL-10 for 20 min, followed by 100 ng/ml LPS for 24 h, followed by a rotenone titration (0.5 nM – 1 μM). Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=3$. (C) The Maximal OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results were expressed as mean $\pm$ SEM., $n=3$.

A



B

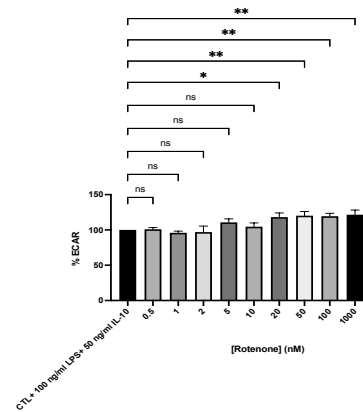


Figure 4.58. Rotenone titration of ECAR in LPS – activated BMDMs ± IL-10.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate at 37°C overnight. BMDMs were incubated with 50 ng/ml IL-10 for 20 min, followed by 100 ng/ml LPS for 24 h, followed by a rotenone titration (0.5 nM – 1 μM). The Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) ECAR were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results were expressed as mean $\pm$ SEM., $n=3$.

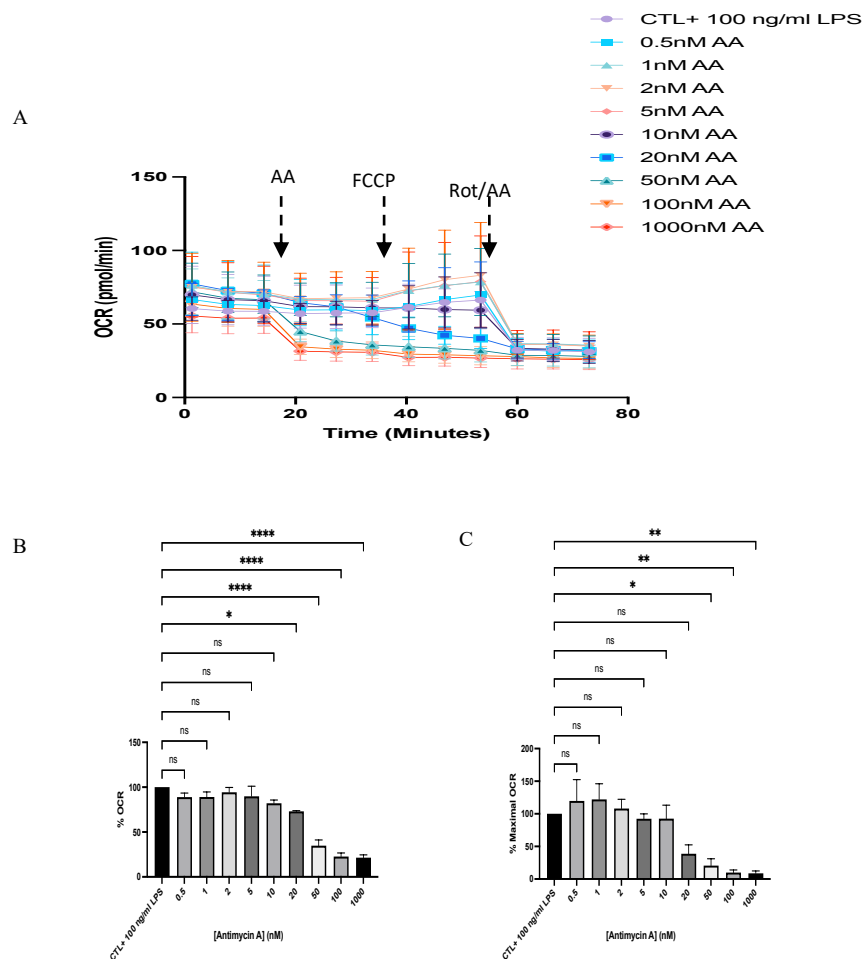


Figure 4.59. Antimycin A titration of Oxygen Consumption Rate (OCR) in LPS - activated BMDMs.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and were incubated with 100 ng/ml LPS for 24 h, followed by a antimycin A titration (0.5 nM – 1 μ M) and the Oxygen Consumption Rate (OCR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=3$. (C) The Maximal OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results were expressed as mean $\pm$ SEM., $n=3$.

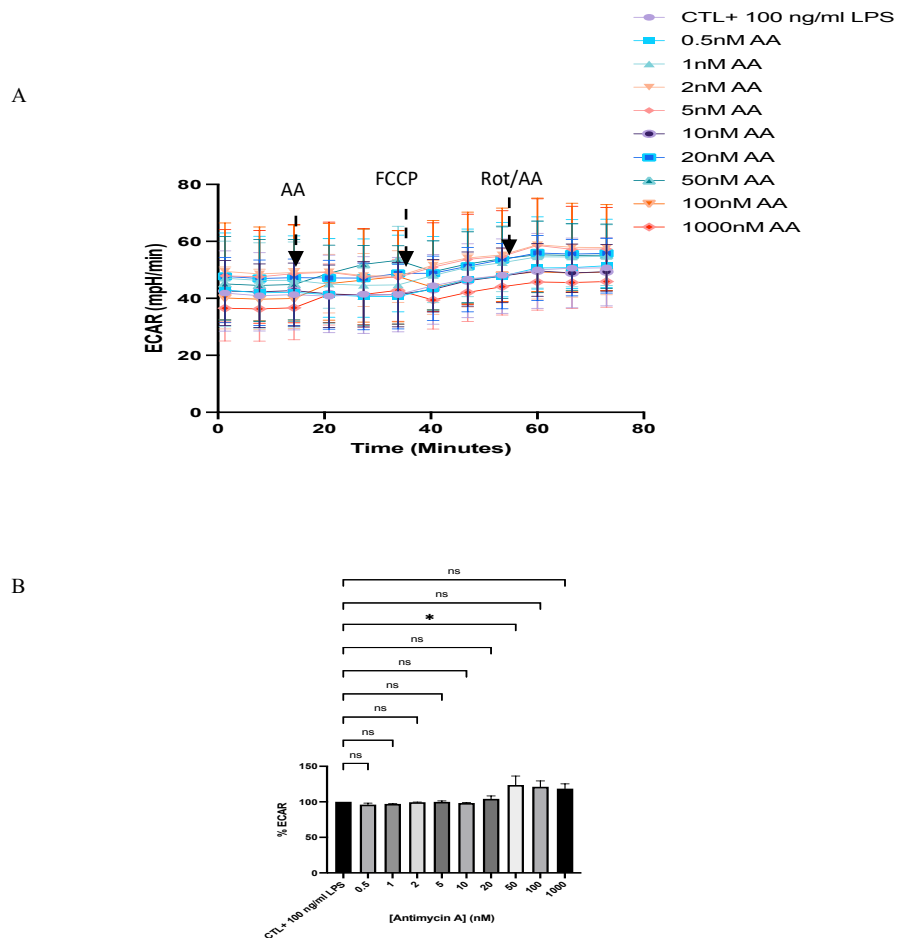


Figure 4.60. Antimycin A titration of for Extracellular Acidification Rate (ECAR) in LPS - activated BMDMs.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate and were incubated with 100 ng/ml LPS for 24 h followed by a antimycin A titration (0.5 nM – 1 μ M) and the Extracellular acidification rates (ECAR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., n=3. (B) ECAR were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA; then, results were expressed as mean $\pm$ SEM., n=3.

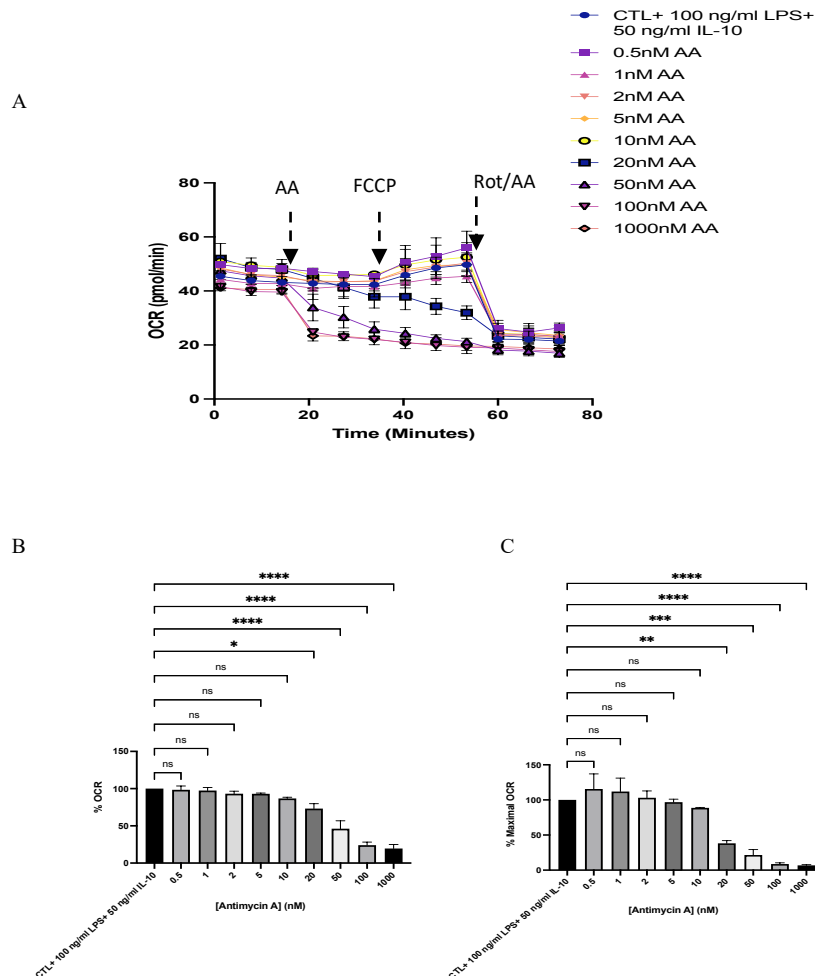
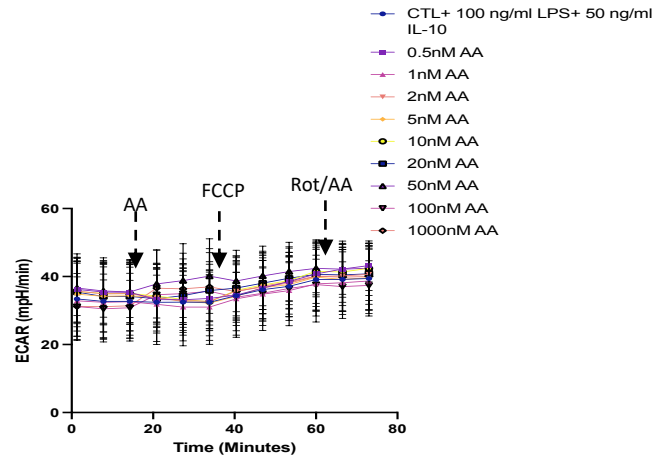


Figure 4.61. Antimycin A titration of OCR LPS – activated BMDMs ± IL-10.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate at 37°C . BMDMs were incubated with 50 ng/ml IL-10 50 ng/ml for 20 min, followed by 100 ng/ml LPS for 24 h, followed by an antimycin A titration (0.5 nM – 1 μM). Oxygen consumption rate (OCR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results are expressed as mean $\pm$ SEM., $n=3$. (C) The Maximal OCR was expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results were expressed as mean $\pm$ SEM., $n=3$.

A



B

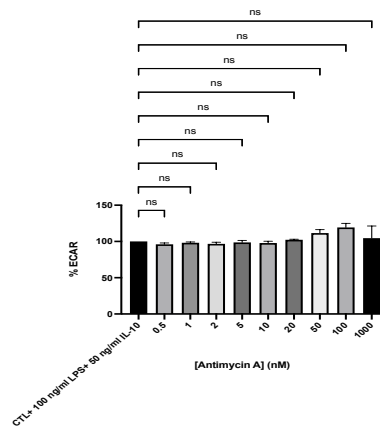


Figure 4.62. Antimycin A titration of ECR in LPS – activated BMDMs ± IL-10.

(A) BMDMs were plated at 1×10^5 cells per well in a 96-well plate at 37°C overnight. BMDMs were incubated with 50 ng/ml IL-10 for 20 min, followed by 100 ng/ml LPS for 24 h, followed by an antimycin A titration (0.5 nM – 1 μM). The Extracellular acidification rate (ECAR) was measured using a SeahorseXFe96 analyser. The data are presented as mean $\pm$ S.E.M., $n=3$. (B) ECAR were expressed as percentages of their control value, and statistical analysis was performed by one-way ANOVA. The results were expressed as mean $\pm$ SEM., $n=3$.

4.3 Discussion

There are many parameters that can affect the spread of control among the components of oxidative phosphorylation, such as ageing, differing experimental conditions, tissue variation and alterations in the energy state of the mitochondria (discussed in Chapter 1) (Rossignol et al., 1999, Villani and Attardi, 2000, Cascante et al., 2002).

In order to investigate the spread of control of the respiratory chain components in iBMDMs and BMDMs cells, we determine the control (flux control coefficients) possessed by the complexes of interest over oxygen consumption and the levels of inhibition of complex activity that could be tolerated before deleterious effects were seen on oxygen consumption (inhibition thresholds) or ECAR by using MCA. The results of this Metabolic Control Analysis study are summarised in (**Table 4.1**) demonstrate that complex I was the major controlling step of the electron transport chain complexes in iBMDMs and BMDMs. The flux control coefficient value for the basal oxygen consumption for complex I in iBMDMs (0.23) was higher than those for complex II/III (0.22) also the flux control coefficient value for the maximal oxygen consumption for complex I in iBMDMs (0.40) was higher than those for complex II/III (0.25). As in iBMDMs, the flux control coefficient value for basal oxygen consumption for complex I in BMDMs (0.32) was higher than those for complex II/III (0.13) and the flux control coefficient value for the maximal oxygen consumption for complex I in BMDMs (1.00) was higher than those for complex II/III (0.07). In iBMDMs, the complex I inhibition threshold level for both basal and maximal oxygen consumption (~ 60 %) was lower than those for complex II/III (~80%). However, the complex I inhibition threshold level (~38 %) was considerably lower than those for complex II/III (~ 58 %) in BMDMs. Immortalised BMDMs (iBMDMs) have lower flux

control coefficients and higher inhibition thresholds compared to primary BMDMs, and may not be a good cell culture model for studying immunometabolism.

Respiratory chain complex	iBMDMs Flux control coefficient	iBMDMs Inhibition threshold	iBMDMs Inhibition threshold (ECAR)	BMDMs Flux control coefficient	BMDMs Inhibition threshold	BMDMs Inhibition threshold (ECAR)
Complex I (Basal OCR)	0.23	~ 60 %	~ 60 %	0.32	~ 38 %,	~ 50 %
Complex I (Maximal OCR)	0.40	~ 60 %	---	1.00	~ 33 %,	---
Complex II/III (Basal OCR)	0.22	~ 80 %	~ 80 %	0.13	~ 58%	~ 60%
Complex II/III (Maximal OCR)	0.25	~ 80 %	---	0.07	~ 60%	---

Table 4.1: Flux control coefficients and inhibition thresholds for complex I and complex II/III for basal and maximal OCRs in iBMDMs and BMDMs.

Flux control coefficients and inhibition thresholds were determined using the results shown in Figures 4.5, 4.6, 4.7, 4.14, 4.15, 4.16, 4.20, 4.21, 4.30 and 4.33, as explained in “Experimental Procedures”.

An essential signature of most immune cells activation is metabolic reprogramming and mitochondrial metabolism is a pivotal part of immunometabolism that is linked to immune regulation (Loftus & Finlay, 2016). Altered metabolism in activated murine macrophages is akin to the altered metabolic function first observed in tumour cells by Otto van Warburg (Warburg, 1925), which is described in chapter 1.

Murine macrophages decrease oxidative phosphorylation and increase glycolysis when stimulated with lipopolysaccharide (LPS) (Tannahill et al., 2013; Van den Bossche, Baardman, & de Winther, 2015; Van den Bossche et al., 2016) and changes in immune cell metabolic function affects their ability to mount an effective immune response (Diskin & Pålsson-McDermott, 2018; Ogawa, Yoshida, & Mizuguchi, 1994).

In this study we found interesting results, BMDMs $\pm$ LPS (100 ng/ml) produced statistically significant decreases in both basal and maximal mitochondrial oxygen consumption over a discontinuous 2-24 h time period (**Figures 4.31 & 4.32**), as well as increases in ECAR, reflecting increased glycolytic flux. Interestingly, at the 4-6h post LPS-activation time points OCR decreased most significantly and this correlated with a significant increase in ECAR. To investigate these observations further, a continuous measurement of OCR and ECAR experiments was performed in LPS-activated BMDMs over 4 h (**Figures 4.33 & 4.42**) and 6 h (**Figures 4.43 & 4.51**) time periods, also during complex I and III activity titrations. In this case, while significant decreases in OCR were only observed from 2h onwards, an immediate increase in ECAR was observed at the 18 min time-point. This indicates that LPS-activated BMDMs immediately increase ECAR and from then onwards continue to increase ECAR, but a slower rate than at the 18 min time point. The results from these experiments also show that a more significant decrease in OCR during the 4-6

hour period following LPS-activation, however this did not correlate with any extra burst in ECAR during the same time period. Similar results were obtained when the Seahorse assay was run for 4 or 6 hours during rotenone and antimycin A titrations of complex I and III activities, respectively. However, LPS-activated BMDMs were not able to increase ECARs, at higher concentrations of rotenone and antimycin A, to the same extent that was observed in untreated BMDMs. This may be related to the decrease in citrate synthase activity observed in the previous chapter following LPS-activations. A decrease in citrate synthase activity will decrease the TCA cycle activity and reduce intracellular production of CO₂. This may indicate that acidification of the medium, which normally reflects lactate release due to increased glycolytic flux, was produced by an alternative CO₂ generating mechanism such as anaerobic respiration. These results are consistent with the current literature (Van den Bossche et al., 2015, Tannahill et al., 2013), although there are differences in OCR and ECAR. This may be due to the type or concentration of LPS used or may be potentially have been influenced by macrophage plasticity, with the cells adapting to the different media changes required both prior to and during the assay.

It is well-known that the mitochondrial ETC component cytochrome oxidase (Complex IV) reduces molecular oxygen (mitochondrial consumed oxygen) in water by adding two pairs of electrons. The source of these electrons are the NADH and FADH₂ reducing equivalents that gained electrons from substrate oxidation, particularly during the TCA cycle. Afterwards, the electrons pass through the electron carriers of the mitochondrial electron transport chain (ETC), coupled with pumping out protons from the mitochondrial matrix to the intermembrane space, creating a proton gradient or proton motive force (Δp). Then, this force drives protons back into the matrix through ATP synthase (complex v), which generates energy to synthesise ATP from ADP and Pi (Conley, 2016; Lesnefsky & Hoppel, 2006; Nolfi-Donagan, Braganza, & Shiva, 2020). The results in this chapter show LPS-

activation of BMDMs influences the above process in a time-dependent manner, resulting in decreases in OCR and increases in ECAR. There seems to be a biphasic response in which rapid increases in glycolytic flux (albeit those correlated with ECAR) are followed by decreases in mitochondrial respiration. This suggests that LPS affects macrophage metabolism opposite to the way in which the Warburg effect is thought to work, in that the classical argument is for a primary mitochondrial dysfunction followed by an increase in glycolysis to maintain cellular ATP levels (Conley, 2016; Lesnefsky & Hoppel, 2006; Nolfi-Donagan, Braganza, & Shiva, 2020). The rapid increase in glycolytic flux following LPS treatment has also been characterised by Tan and Kagan (2019). This team proposed that a supramolecular organising centre, known as the myddosome, drives the rapid induction of glycolysis. They identified the kinase TBK1 as the myddosome component that promotes glycolysis in activated macrophages. Certainly, some of the experiments in this chapter could be revisited in future work, whereby TBK1 inhibitors should be tested for their effects on OCR and ECAR over continuous 6 hour time courses, during LPS activation (Tan and Kagan, 2019).

Non-mitochondrial consumed oxygen has many physiological roles apart from the oxidation of non-respirational derived substrates. For instance, non-mitochondrial consumed oxygen can be utilised by NADPH oxidase and iNOS to generate reactive oxygen and nitrogen species individually. These species are well recognised immune components in the first line of microbicidal defence. Furthermore, macrophage consumed non-mitochondrial oxygen can be either reduced to oxygen anion (e.g. superoxide, mtROS) by single electron leakage from the mitochondrial respiration chain or used to produce NO by the inducible nitric oxide synthase (iNOS) at later points of infection. NO negatively

affects mitochondrial OXPHOS by nitrosylating irons-sulfur-rich proteins in complex I of ETC (Clementi, Brown, Feelisch, & Moncada, 1998).

Metabolic changes are intricately linked with the switch to a pro-inflammatory phenotype in macrophages, including the critical Warburg-like shift from oxidative metabolism to glycolytic ATP generation. NO is known to modulate ETC activity through nitrosation of Complex I and competitive inhibition of O₂-consuming Complex IV (Chouchani et al., 2013; Cleeter et al., 1994) and is responsible for inhibiting oxidative phosphorylation in both activated macrophages and dendritic cells (Everts et al., 2012; Van den Bossche et al., 2016). In accordance with these studies, Bailey et al., (2019) observed a NO-dependent inhibition of Complex I and mitochondrial respiration and found that NO modulates levels of the essential TCA cycle metabolites citrate and succinate, as well as the inflammatory mediator itaconate. Future experiments could investigate the possibility that LPS activation modulates effects on OCR and ECAR through nitric oxide synthase and the generation of NO. Future work will need to repeating some of the experiments in this chapter in the presence of NOS inhibitors and investigating if the rapid initial increase in ECAR is still present and if the time-dependent decrease in OCR and complex I specific activities are still observed.

Previous studies have shown the importance of glycolysis in the inflammatory response of macrophages. Interleukin 10 (IL-10) is an important anti-inflammatory cytokine that can be produced by activated immune cells. Since activated STAT3 is present in mitochondria and has been shown to be required for optimal electron transport chain function, STAT3 mediated IL-10 signalling may have a direct impact on mitochondrial function improvement. In a recent study TLR4 activation was found to induce a signalling cascade recruiting TRAF6 and TBK-1, while TBK-1 phosphorylated STAT3

on S727 (Balic et al., 2020). The showed that STAT3 Ser727 phosphorylation is critical for LPS-induced glycolytic reprogramming, production of the central immune response metabolite succinate and inflammatory cytokine production in a model of LPS-induced inflammation. Future work will need to investigate the possible involvement of STAT3 in the mechanism that leads to reduction of ETC activities observed in activated macrophages by measuring STAT3 using ELISA kit.

In addition it is increasingly recognized that mammalian target of rapamycin (mTOR) functions as a central regulator of cell metabolism (Wegrzyn et al., 2009, Sengupta et al., 2010, Ip et al., 2017, Dowling et al., 2021). The anti-inflammatory cytokine, IL-10, has also been shown to regulate macrophage preserve oxidative phosphorylation through either a suppression of NO or via suppression of mTOR. Recently, Dowling et al. (2021) showed that arginase-2 localises to the mitochondrion and is critical for IL-10-induced modulation of mitochondrial dynamics and oxidative respiration. Arginase-2 is essential for IL-10-mediated downregulation of the inflammatory mediator's succinate, hypoxia inducible factor 1 α (HIF-1 α) and IL-1 β in vitro (Dowling et al., 2021). As these findings suggest IL-10 may regulate ETC function in activated macrophages, the effects of IL-10 addition on OCR and ECAR during complex I and complex III titration in macrophages were characterised (Dowling et al., 2021).

IL-10 was found not to affect basal OCR and ECAR in BMDMs challenged with LPS for 24 h however, results suggested an increased cellular ability to regulate glycolytic flux in response to high inhibitor concentrations, compared to un-activated cells. These experiments required further analysis, such as an investigation into how mitochondrial protein levels change in the presence of IL-10 and subsequent changes on complex I and III specific activities.

CHAPTER 5

CHARACTERISATION OF THE EFFECTS OF MITOCHONDRIAL ETC INHIBITION ON REACTIVE OXYGEN SPECIES PRODUCTION AND CYTOKINE RELEASE IN IBMDMs AND BMDMs

5.1 Introduction

In eukaryotic cells, mitochondria are organelles with multiple functions that are important for producing cellular energy in the form of ATP. Mitochondria are highly metabolic and essential for cell survival, but by-products produced by mitochondria during ATP production can also be toxic to cells. Reactive oxygen species (ROS), which produce ROS during respiratory activity, act as a redox signalling molecule. This signalling is essential for cell homeostasis maintained at low levels of ROS. However, excess ROS is detrimental to the physiological function of the cell (Lambeth and Neish, 2014, Nohl et al., 2003).

Electrons are shuttled from NADH and FADH₂ via the four complexes I-IV of the ETC, generating a proton gradient which drives ATP production. Mitochondrial-derived ROS (mtROS) are mainly produced as O₂^{•-} by complexes I and III. Complex I produces O₂^{•-} in the mitochondrial matrix, where it is converted to H₂O₂ by SOD₂ (Fridovich, 1997, West et al., 2011b, Murphy, 2009).

Complex II can also generate matrix mtROS. Two different mechanisms are described as follows: firstly, complex II donates electrons to other ETC complexes where mtROS production occurs. Here, for example, mtROS generation occurs during a reverse electron transfer (RET) through complex I. A large amount of matrix mtROS is formed in complex I (Zhao et al., 2019, OHNISHI, 1987, Muller et al., 2008, Votyakova and Reynolds, 2001, Moreno-Sánchez et al., 2013). Electrons may also be used to reduce quinone which may leak electron at complex III, thus generating mtROS.

Secondly, complex II produces matrix mtROS directly at the matrix-localized FAD binding site however this exact mechanism is not fully understood (Quinlan et al., 2012, Bonke et al., 2015, Dröse, 2013).

Complex III not only releases electrons and produces $O_2^{\bullet -}$ into the matrix but also directly into the intermembrane space (IMS) (Murphy, 2009, Muller et al., 2004). From there, it reaches the cytosol via VDAC or is converted to H_2O_2 by SOD_1 localized to IMS and reaches the cytosol via diffusion or aquaporin. Because of the high efficiency of the matrix-localized antioxidant defence system, matrix-produced ROS cannot escape from intact mitochondria. This has been confirmed in both isolated mitochondria and cells (Tormos et al., 2011, Zhao et al., 2020, Roca and Ramakrishnan, 2013). Membrane-disrupting damage to mitochondria or the opening of mitochondrial permeability transition pores (mPTPs) is required for the release of matrix mtROS into the cytosol (Hos et al., 2017).

It has been postulated that mitochondrial-derived signals activate the NLRP3 inflammasome. The first evidence to suggest a direct role of mitochondrial signalling in the induction of the NLRP3 inflammasome comes from experiments in which macrophages were treated with mitochondrial ROS-producing agents. In vitro, macrophages treated with mitochondrial complex I and II inhibitors (rotenone and antimycin A) induced potent mitochondrial ROS and NLRP3 activation (Zhou et al., 2011, Gurung et al., 2015). In addition to mitochondrial-derived ROS, some disruption of mitochondrial homeostasis has been shown to activate the NLRP3 inflammasome.

Infection or aseptic damage leading to Ca^{2+} overload, NAD^+ reduction, cardiolipin externalization to the adventitia, and mitochondrial DNA (mtDNA) release has been shown to promote NLRP3 inflammasome activation (Iyer et al., 2013).

Secretion of inflammatory cytokines from macrophages regulate the inflammatory response. Inflammatory cytokines are small proteins that act as signalling molecules and help regulate the immune response and directly induce the destruction of pathogens. The major cytokines induced by pro-inflammatory macrophages include interleukin 1 beta (IL-1 β), tumour necrosis factor alpha (TNF- α), and IL-6, which are induced by different signalling cascades such as nuclear factor NF-kappa-B (NFB) and mitogen-activated protein kinase (MAPK) (Dale et al., 2008, Arango Duque and Descoteaux, 2014, Jayaraman et al., 2013, Shi et al., 2009).

While some studies have suggested a link between mitochondrial ROS generation and activation of the NLRP3 inflammasome there still remains confusion as to the mechanism(s) that may underlie such a phenomenon. Other studies have concluded that mitochondrial ROS do not activate the NLRP3 inflammasome. The previous results in this thesis show that LPS-activation of iBMDMs and BMDMs can induce mitochondrial dysfunction and that ETC activities can be reduced or indeed control metabolic function in these cells. How these events correlate with mitochondrial ROS and cytokine release remain unresolved. Therefore, this chapter focusses on the titration of complex I and III activities in macrophages with rotenone and antimycin A, known inducers of ROS, and if such events correlate with pro-inflammatory cytokine release.

5.1.2 Aims of the chapter

- To investigate ROS production in iBMDMs and BMDMs activated with LPS
- To investigate ROS production in iBMDMs and BMDMs where ETC complex activities are titrated with specific inhibitors
- To examine the effect of ETC complex titrations on pro-inflammatory cytokine release in iBMDMs and BMDMs. .

5.2 Results

5.2.1 LPS-activation increases the ROS production in BMDMs but not iBMDMs.

iBMDMs produced approximately a 2-fold higher amount of ROS than BMDMs (**Figure 5.1**). There was no significant increase in iBMDMs challenged by LPS for 4 hours and 24 hours (**Figure 5.1 (A)**). In BMDMs, LPS-activation for 4 hours increased ROS production by ~ 30-40 %, while over a 24 h LPS activation ROS increased 2-fold (**Figure 5.1 (B)**).

5.2.2 Complex I inhibition increases ROS production in iBMDMs and BMDMs.

iBMDMs and BMDMs were loaded with 20 μ M DHE in PBS + 10 mM glucose, after which increasing concentrations of inhibitors were added. Cells were incubated with inhibitors for 30 minutes before spinning down and washing twice with PBS. **Figure 5.2** shows that rotenone dose-dependently (0-1000 nM) increased ROS production in iBMDMs by ~ 80 % and BMDMs by ~ 50 % at the high concentration of rotenone.

5.2.3 Complex II inhibition does not affect the ROS production in iBMDMs and BMDMs. TTFA (0 - 200 μ M) did not affect the ROS production in iBMDMs and BMDMs (**Figure 5.3 (A)** and **(B)**).

5.2.4 Complex II/III inhibition increases ROS production in iBMDMs and BMDMs.

iBMDMs and BMDMs were loaded with 20 μ M DHE in PBS + 10 mM glucose, after which increasing concentrations of inhibitors were added. Cells were incubated with inhibitors for 30 minutes before spinning down and washing twice with PBS. **Figure 5.4** shows that antimycin A (0-1000 nM) increases ROS production in iBMDMs and BMDMs

by ~ 50 % at a high concentration of antimycin A. The ROS production was higher in iBMDMs than BMDMs.

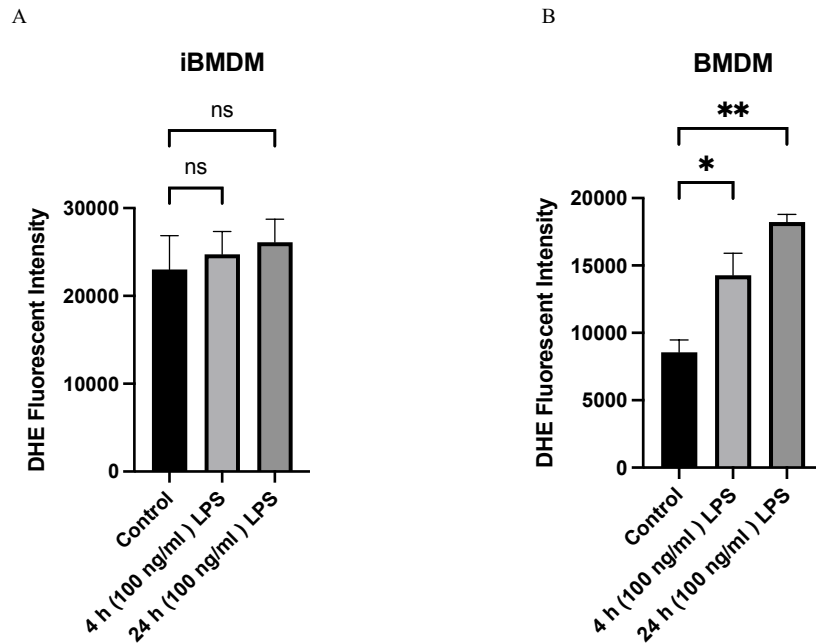


Figure 5.1. LPS-activation increases ROS production in BMDMs but not iBMDMs.

iBMDMs were seeded at 1×10^5 cell/mL, and BMDMs at 2×10^5 cells/mL in DMEM with 200 μ L per well in a U-shape 96-well plate and incubated with LPS (100ng/ml) for 4 h or 24 h. iBMDMs and BMDMs were loaded with 20 μ M DHE in PBS + 10 mM glucose. Cells were incubated with inhibitors for 30 minutes before spinning down and washing twice with PBS. Fluorescence was analysed using the PE channel after gating on iBMDMs and BMDMs on BD FACS Canto. Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

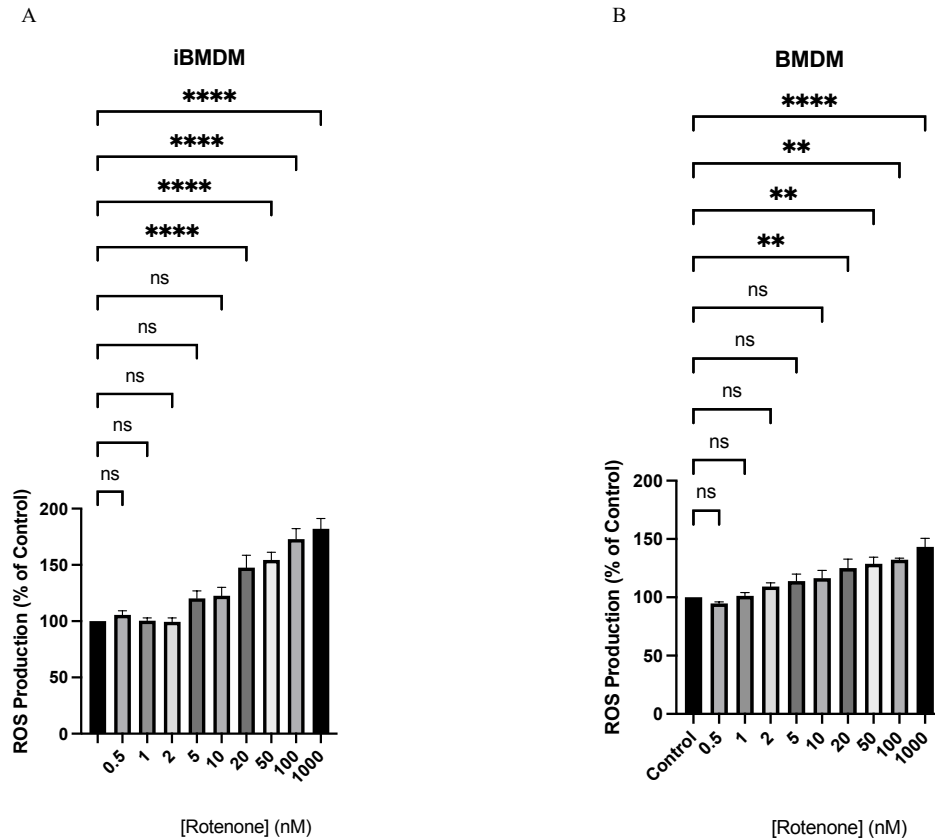


Figure 5.2. Complex I inhibition increases ROS production in iBMDMs and BMDMs.

iBMDMs were seeded at 1×10^5 cell/mL, and BMDMs at 2×10^5 cells/mL in DMEM with 200 μ L per well in a U-shape 96-well plate. iBMDMs and BMDMs were loaded with 20 μ M DHE in PBS + 10 mM glucose, after which increasing concentrations of rotenone (0.5nM-1 μ M) were added for 30 minutes before spinning down and washing twice with PBS. Fluorescence was analysed using the PE channel after gating on iBMDMs and BMDMs on BD FACS Canto. Data are presented as mean $\pm$ SEM, n=4. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

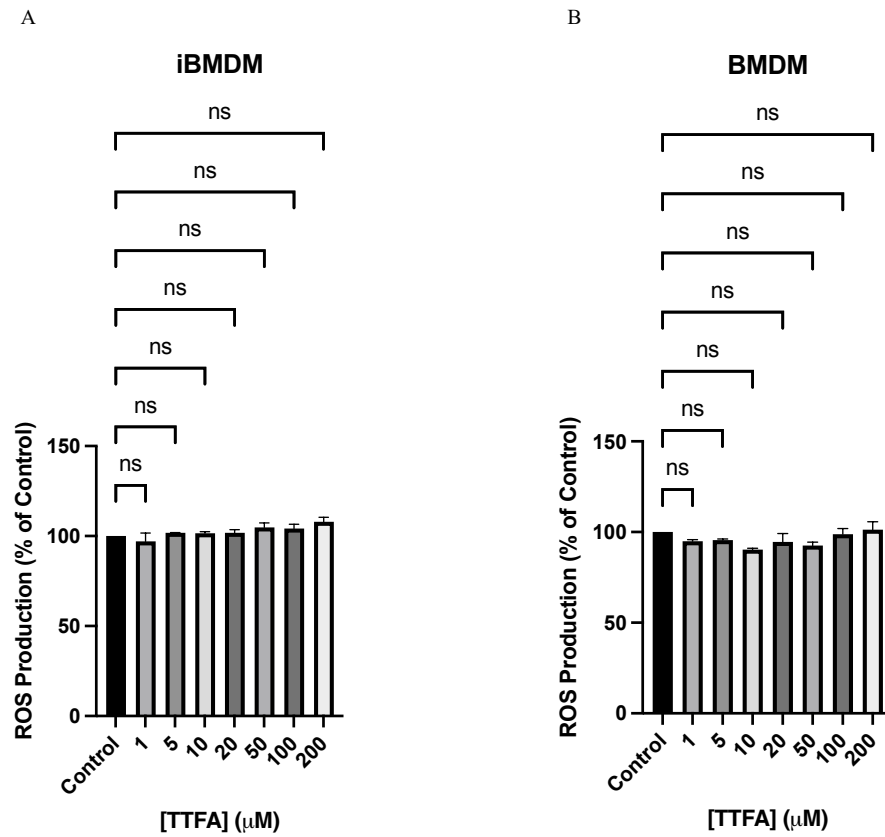


Figure 5.3. Complex II inhibition does not affect the ROS production in iBMDMs and BMDMs.

iBMDMs were seeded at 1×10^5 cell/mL, and BMDMs at 2×10^5 cells/mL in DMEM with 200 μ L per well in a U-shape 96-well plate. iBMDMs and BMDMs were loaded with 20 μ M DHE in PBS + 10 mM glucose, after which increasing concentrations of TTFA (1 μ M-200 μ M) were added for 30 minutes before spinning down and washing twice with PBS. Fluorescence was analysed using the PE channel after gating on iBMDMs and BMDMs on BD FACS Canto. Data are presented as mean $\pm$ SEM, $n=4$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

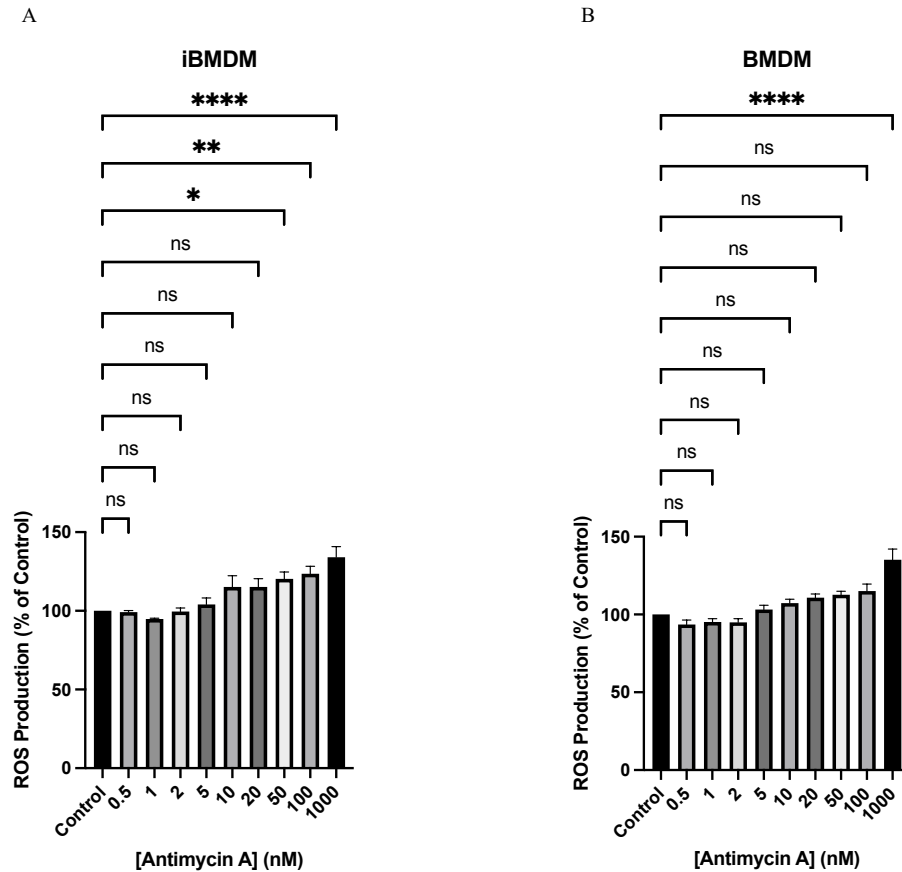


Figure 5.4. Complex III inhibition increases ROS production in iBMDMs and BMDMs.

iBMDMs were seeded at 1×10^5 cell/mL, and BMDMs at 2×10^5 cells/mL in DMEM with 200 μ L per well in a U-shape 96-well plate. iBMDMs and BMDMs were loaded with 20 μ M DHE in PBS + 10 mM glucose, after which increasing concentrations of antimycin A (0.5nM-1 μ M) were added for 30 minutes before spinning down and washing twice with PBS. Fluorescence was analysed using the PE channel after gating on iBMDMs and BMDMs on BD FACS Canto. Data are presented as mean $\pm$ SEM, n=4. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.2.5 Complex I inhibition decreases TNF- α secretion in LPS-activated iBMDMs.

Rotenone dose-dependently (0-1000 nM) inhibited LPS- induced TNF- α secretion in iBMDMs. **Figure 5.5** showed that in iBMDMs treated with different concentrations of rotenone for 30 minutes, then stimulated with 100 ng/ml LPS for 3 h, followed by 5mM ATP for one 1h, TNF- α secretion was decreased up to ~ 60 %.

5.2.6 Complex II inhibitor does not affect TNF- α secretion in iBMDMs LPS-activated.

Figure 5.6 shows that TNF- α secretion from iBMDMs was significantly decrease by ~ 15 % at the highest concentrations of complex II inhibitor, TTFA (0 - 200 μ M), when cells were LPS-activated for 3 h and treated with 5 mM ATP for 1h.

5.2.7 Complex III inhibition decreases TNF- α secretion in LPS-activated iBMDMs.

Antimycin A (0-1000 nM) inhibits TNF- α secretion in LPS-activated iBMDMs. **Figure 5.7** shows that in iBMDMs treated with different concentrations of antimycin A for 30 minutes then stimulated with 100 ng/ml LPS for 4 h and 5 mM ATP for 1 h, TNF- α secretion was decreased by ~ 60 % at the highest antimycin A concentrations.

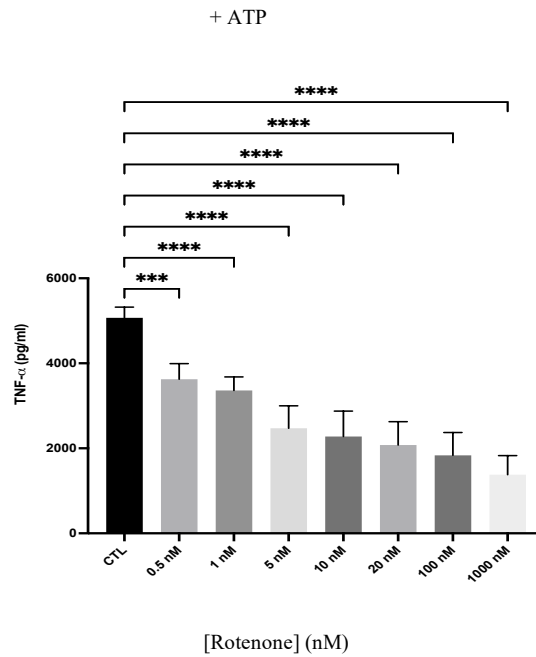


Figure 5.5. Complex I inhibition decreases TNF- α secretion in LPS-activated iBMDMs.

iBMDMs were seeded at 2.5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of rotenone (0.5nM-1 μ M) for 30 min before stimulation with LPS (100ng/mL) for three hours, followed by ATP (5mM) for one hour. ELISA was used for analysis of supernatants for TNF- α . Data are presented as mean $\pm$ SEM, n=3. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

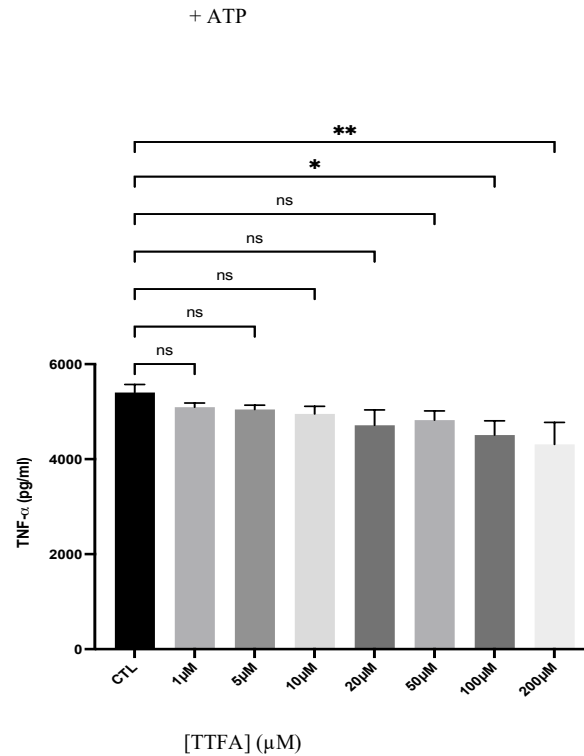


Figure 5.6. Complex II inhibition does not affect TNF- α secretion in LPS-activated iBMDMs.

iBMDMs were seeded at 2.5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of TTFA (1 μ M-200 μ M) for 30 min before stimulation with LPS (100ng/mL) for three hours, followed by ATP (5mM) for one hour. ELISA was used for analysis of supernatants for TNF- α . Data are presented as mean $\pm$ SEM, n=3. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

+ ATP

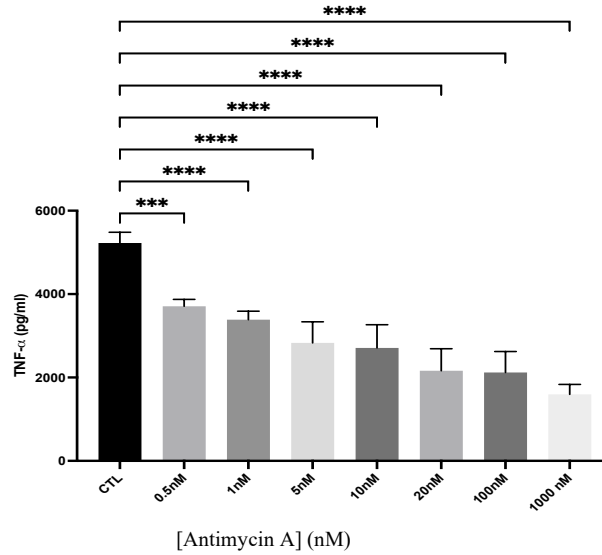


Figure 5.7. Complex III inhibition decreases TNF- α secretion in LPS-activated iBMDMs.

iBMDMs were seeded at 2.5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of antimycin A (0.5nM-1 μ M) for 30 min before stimulation with LPS (100ng/mL) for three hours, followed by ATP (5mM) for one hour. ELISA was used for analysis of supernatants for TNF- α . Data are presented as mean $\pm$ SEM, n=3. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.2.8 Complex I affects the inflammasome activated IL-1 β secretion in iBMDMs

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Rotenone (0-1000 nM) inhibits LPS- induced IL-1 β secretion in iBMDMs. **Figure 5.8 (A)** showed that in iBMDMs treated with different concentration of rotenone for 30 minutes then stimulated with 100 ng/ml LPS for 4 h, IL-1 β secretion was decreased to ~ 75 % over the rotenone concentration range of 0 - 1000 nM. **Figure 5.8 (B)** shows a 10-fold increase in IL-1 β secretion following inflammasome activation (LPS+ATP). In iBMDMs treated with different concentration of rotenone for 30 min, then stimulated with 100 ng/ml LPS for 3 h followed by 5mM ATP (inflammasome activation conditions) for 1 h, IL-1 β secretion was decreased to ~ 75 % over the rotenone concentration range of 0 - 1000 nM.

5.3.9 Complex II inhibitor does not affect IL-1 β secretion in iBMDMs and BMDMs primed with LPS $\pm$ ATP.

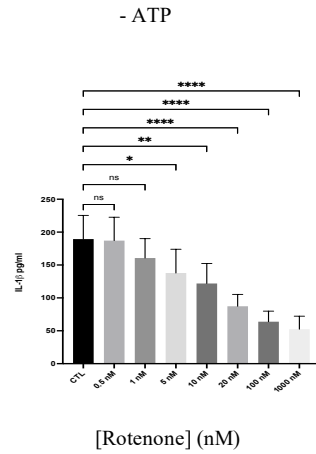
TTFA did not affect the IL-1 β secretion in BMDMs in the absence or presence of inflammasome activation conditions (**Figure 5.9 (A)** and **(B)**).

5.3.10 Complex III affects the inflammasome activated IL-1 β secretion in iBMDMs

Antimycin A (0-1000 nM) inhibits IL-1 β secretion in LPS-activated iBMDMs. **Figure 5.10 (A)** shows that in iBMDMs treated with different concentration of antimycin A for 30 min, then stimulated with 100 ng/ml LPS for 4 h, IL-1 β secretion was decreased to ~ 70 % over the antimycin A concentration range of 0-1000 nM. **Figure 5.10 (B)** shows a 10-fold increase in IL-1 β secretion following inflammasome activation (LPS+ATP). In iBMDMs treated with different concentration of antimycin A for 30 min, then stimulated with 100 ng/ml LPS for 3 h, followed by 5 mM ATP (inflammasome activation conditions)

for 1 h, IL-1 β secretion was decreased ~ 80 % over the antimycin A concentration range of 0-1000 nM.

A



B

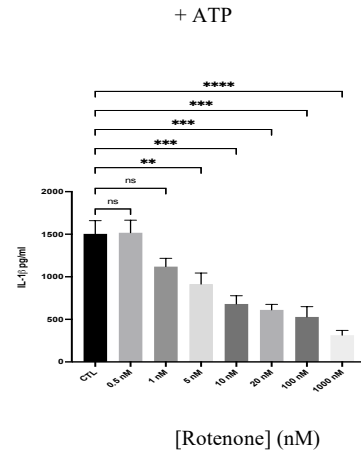
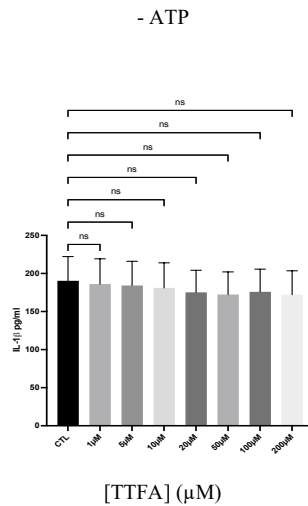


Figure 5.8. Complex I inhibition decreases inflammasome-activated IL-1 β secretion in iBMDMs.

iBMDMs were seeded at 2.5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of rotenone (0.5 nM–1 μ M) for 30 min before stimulation with LPS (100 ng/mL) for three hours, followed (A) media or (B) ATP (5 mM) for one hour. ELISA was used for analysis of supernatants for IL-1 β . Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

A



B

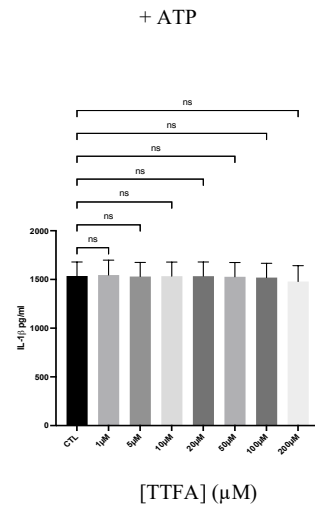
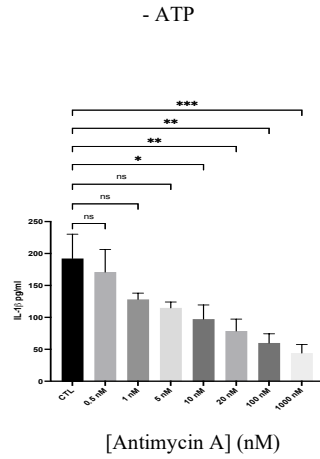


Figure 5.9. Complex II inhibition does not affect inflammasome-activated IL-1 β secretion in iBMDMs.

iBMDMs were seeded at 2.5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of TTF (1 μ M-200 μ M) for 30 min before stimulation with LPS (100ng/mL) for three hours, followed (A) media or (B) ATP (5mM) for one hour. ELISA was used for analysis of supernatants for IL-1 β . Data are presented as mean $\pm$ SEM, n=3. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

A



B

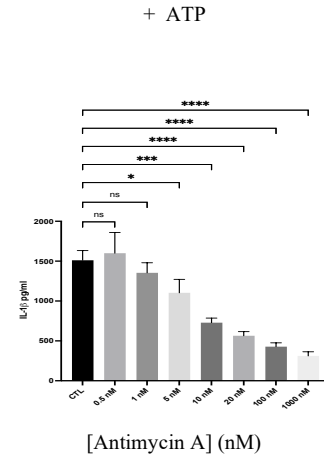


Figure 5.10. Complex III inhibition decreases inflammasome-activated IL-1 β secretion in iBMDMs.

iBMDMs were seeded at 2.5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of antimycin A (0.5 nM–1 μ M) for 30 min before stimulation with LPS (100 ng/mL) for three hours, followed (A) media or (B) ATP (5 mM) for one hour. ELISA was used for analysis of supernatants for IL-1 β . Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.2.11 Complex I inhibition decreases TNF- α secretion in LPS-activated BMDMs.

Rotenone dose-dependently (0-1000 nM) inhibited LPS- induced TNF- α secretion in BMDMs. **Figure 5.11** showed that in BMDMs treated with different concentrations of rotenone for 30 minutes, then stimulated with 100 ng/ml LPS for 3 h, followed by 5 mM ATP for one 1h, TNF- α secretion was decreased up to $\sim 50\%$.

5.2.12 Complex II inhibitor decreases TNF- α secretion in LPS-activated BMDMs.

Figure 5.12 shows that TNF- α secretion from BMDMs was significantly decrease by $\sim 15\%$ at the higher concentrations of complex II inhibitor, TTFA (0 - 200 μ M), when cells were LPS-activated for 3 h and treated with 5 mM ATP for 1h.

5.2.13 Complex III inhibition decreases TNF- α secretion in LPS-activated BMDMs.

Antimycin A (0-1000 nM) inhibits TNF- α secretion in LPS-activated BMDMs. **Figure 5.13** shows that in BMDMs treated with different concentrations of antimycin A for 30 minutes then stimulated with 100 ng/ml LPS for 4 h and 5 mM ATP for 1 h, TNF- α secretion was decreased by $\sim 25\%$ at the highest antimycin A concentrations.

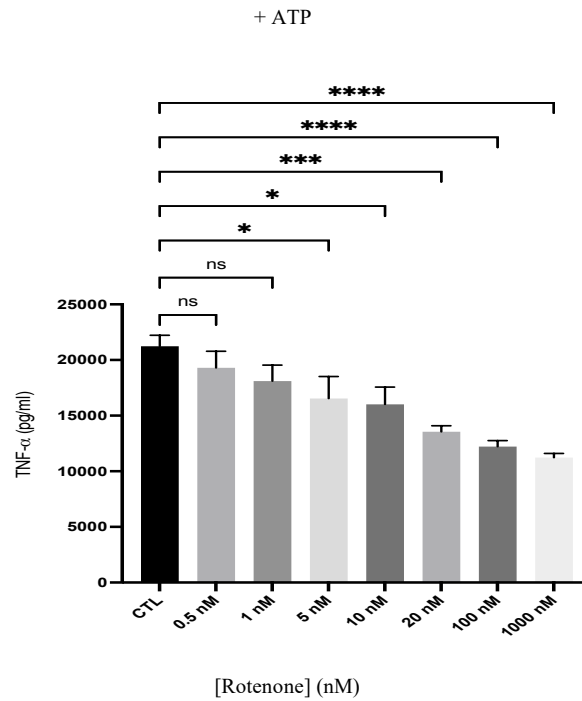


Figure 5.11. Complex I inhibition decreases TNF- α secretion in LPS-activated BMDMs.

BMDMs were seeded at 5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of rotenone (0.5 nM–1 μ M) for 30 min before stimulation with LPS (100 ng/mL) for 3 h, followed by ATP (5 mM) for one hour. ELISA was used for analysis of supernatants for TNF- α . Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

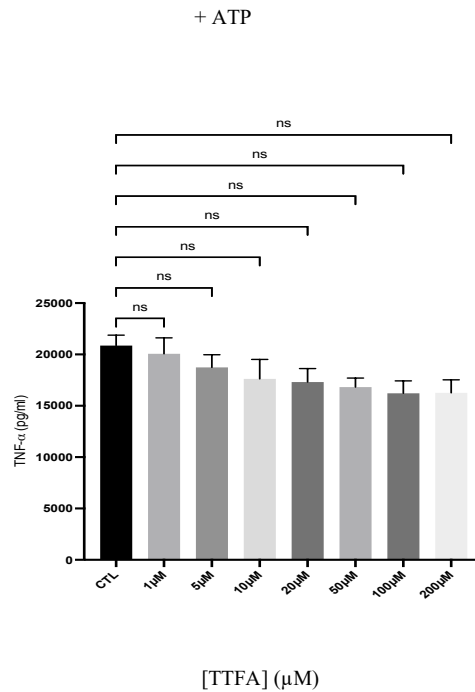


Figure 5.12. Complex II inhibition decreases TNF- α secretion in LPS-activated BMDMs.

BMDMs were seeded at 5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of TTFA (1 μ M-200 μ M) for 30 min before stimulation with LPS (100 ng/mL) for 3 h, followed by ATP (5 mM) for 1 h. ELISA was used for analysis of supernatants for TNF- α . Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

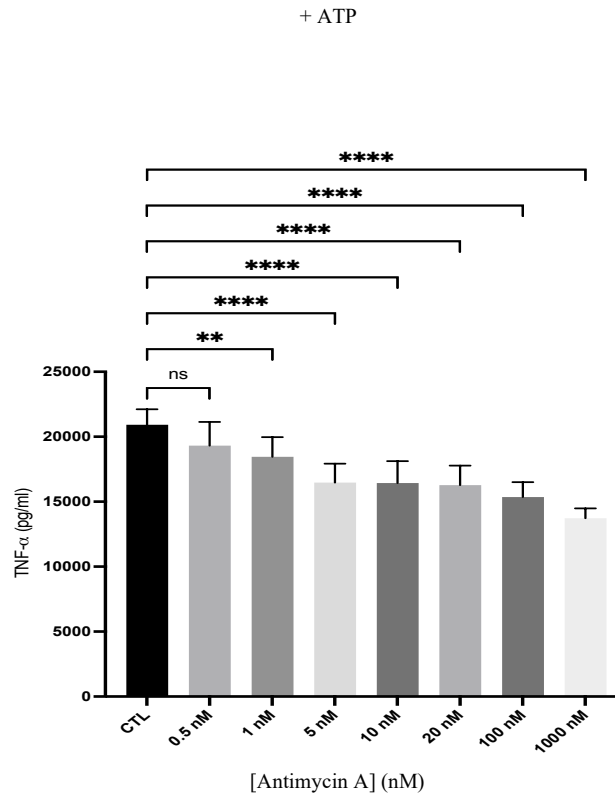


Figure 5.13. Complex III inhibition decreases TNF- α secretion in LPS-activated BMDMs.

BMDMs were seeded at 5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of antimycin A (0.5 nM–1 μ M) for 30 min before stimulation with LPS (100 ng/mL) for 3 h, followed by ATP (5 mM) for 1 h. ELISA was used for analysis of supernatants for TNF- α . Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.2.14 Complex I affects the inflammasome activated IL-1 β secretion in BMDMs

Rotenone (0-1000 nM) inhibits LPS- induced IL-1 β secretion in BMDMs. **Figure 5.14 (A)** shows that in BMDM treated with different concentration of rotenone for 30 min, then stimulated with 100 ng/ml LPS for 4 h, IL-1 β secretion was decreased to ~ 25 % over the rotenone concentration range of 0 - 1000 nM. **Figure 5.14 (B)** shows a 5-fold increase in IL-1 β secretion following inflammasome activation (LPS+ATP). In BMDMs treated with different concentrations of rotenone for 30 min, then stimulated with 100 ng/ml LPS for 3 h, followed by 5 mM ATP (inflammasome activation conditions) for 1 h, IL-1 β secretion was decreased to ~ 90 % over the rotenone concentration range of 0 - 1000 nM.

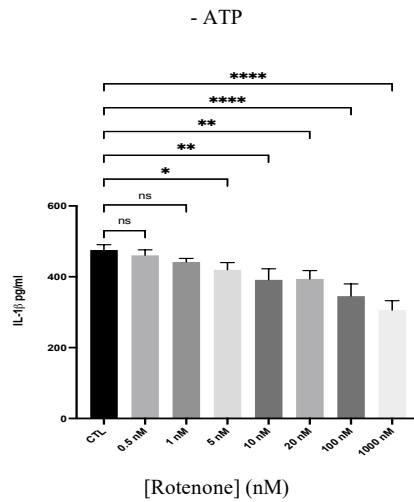
5.2.15 Complex II has no effect on IL-1 β secretion in BMDMs.

TTFA did not affect the IL-1 β secretion by LPS-activated BMDMs in the absence or presence of inflammasome activation conditions (**Figure 5.9 (A)** and **(B)**).

5.2.16 Complex III affects the inflammasome activated IL-1 β secretion in BMDMs

Antimycin A (0-1000 nM) inhibits IL-1 β secretion by LPS-activated iBMDMs. **Figure 5.16 (A)** shows that in BMDM treated with different concentration of antimycin A for 30 min, then stimulated with 100 ng/ml LPS for 4 h, IL-1 β secretion was decreased by ~ 30 % over the antimycin A concentration range of 0-1000 nM. **Figure 5.16 (B)** shows a 5-fold increase in IL1 β secretion following inflammasome activation. In BMDMs treated with different concentration of antimycin A for 30 min, then stimulated with 100 ng/ml LPS for 3 h, followed by 5 mM ATP (inflammasome activation conditions) for 1 h, IL-1 β secretion was decreased ~ 85 % over the antimycin A concentration range of 0-1000 nM.

A



B

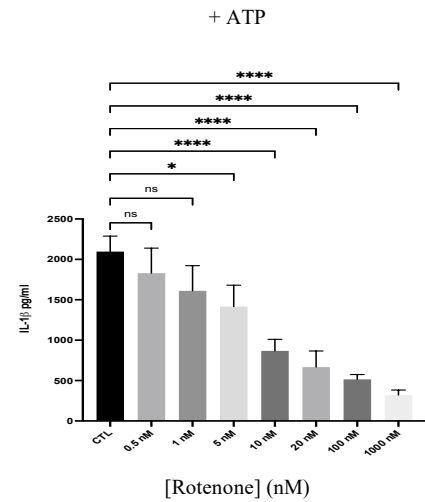
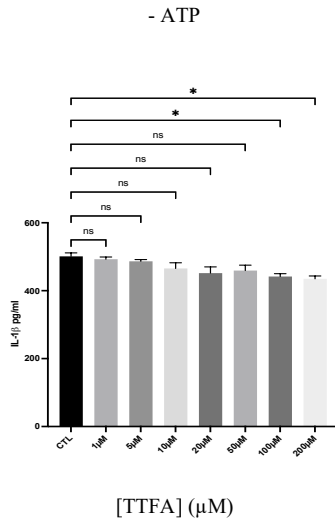


Figure 5.14. Complex I inhibition decreases inflammasome-activated IL-1 β secretion in BMDMs.

BMDMs were seeded at 5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of rotenone (0.5 nM–1 μ M) for 30 min before stimulation with LPS (100 ng/mL) for three hours, followed (A) media or (B) ATP (5 mM) for one hour. ELISA was used for analysis of supernatants for IL-1 β . Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

A



B

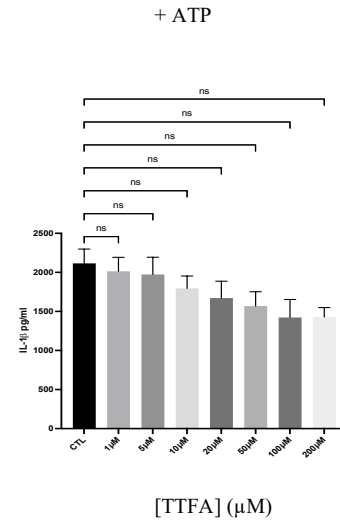


Figure 5.15. Complex II inhibition does not affect inflammasome-activated IL-1 β secretion in BMDMs.

BMDMs were seeded at 5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of TTFA (1 μ M-200 μ M) for 30 min before stimulation with LPS (100ng/mL) for three hours, followed (A) media or (B) ATP (5mM) for 1 h. ELISA was used for analysis of supernatants for IL-1 β . Data are presented as mean $\pm$ SEM, n=3. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

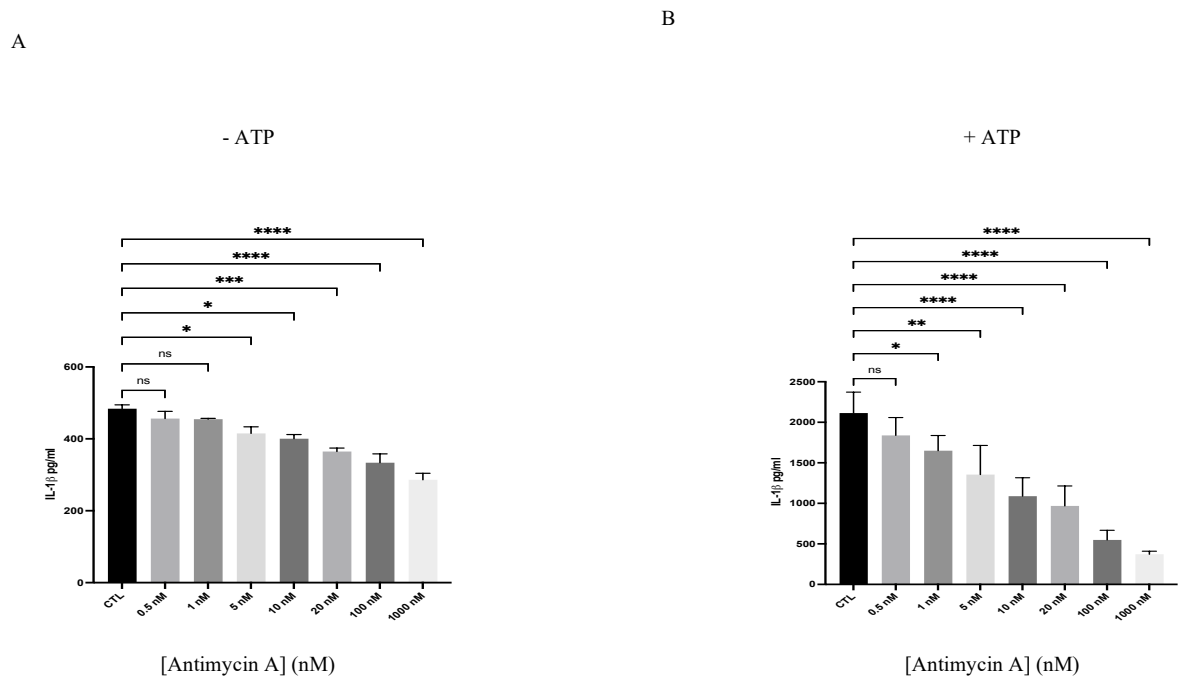


Figure 5.16. Complex III inhibition decreases inflammasome-activated IL-1 β secretion in BMDMs.

BMDMs were seeded at 5×10^5 cells/mL in DMEM with 200 μ L per well in a 96-well plate and incubated at 37°C overnight. Cells were treated with the indicated concentration of antimycin A (0.5 nM–1 μ M) for 30 min before stimulation with LPS (100 ng/mL) for three hours, followed (A) media or (B) ATP (5 mM) for one hour. ELISA was used for analysis of supernatants for IL-1 β . Data are presented as mean $\pm$ SEM, $n=3$. P values were calculated using one-way ANOVA. * for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$ and **** for $p \leq 0.0001$.

5.3 Discussion

Mitochondria are commonly known as a potential source of reactive oxygen species (ROS), however, mitochondria also possess a strong capacity for ROS consumption. Controlling the level of ROS production can prevent DNA damage for all cell types, such as cancer cells, parasites and memory T cells. Studies dating back to the 1970s established that LPS attenuates macrophage respiration by inhibiting complexes II and III and slowing state III respiration (what study is this reference it).

The ROS produced are generally neutralized by several antioxidant compounds, including proteins. Large amounts of ROS can also be produced by inflammatory processes, ionizing radiation, and many chemotherapeutic drugs. When the production of ROS exceeds the capacity of antioxidant proteins, it can cause what is known as "oxidative stress". In biological terms, oxidative stress can be broadly defined as the imbalance between the production of oxidants and the antioxidant capacity of cells to prevent oxidative damage. Macrophage colony-stimulating factor (MCSF) is a secretory cytokine that can induce the differentiation of hematopoietic cells into macrophages. This cytokine promotes the survival of BMDM *in vitro*. Furthermore, it has been reported that MCSF induces ROS production in human monocytes, and ROS activates the MAPK/ERK pathway to induce monocyte proliferation (Hole et al., 2010).

Previous studies have shown that, in addition to the respiratory burst (NADPH Oxidase), LPS increases mitochondrial ROS production as an important response for bacterial killing in macrophages, but the mechanism still remains to be elucidated. A decrease in NO is coupled to an increase in mitochondrial respiratory function and glycolytic levels

comparable to that seen in untreated macrophages (Mills and O'Neill, 2016, Moreno-Sánchez et al., 2013).

In this study, we tested the effect of rotenone, TTFA and antimycin A, which are complex I, complex II and III inhibitors, respectively, on ROS production in iBMDMs and BMDMs. A surprising result from these experiments was that the ROS production increased in BMDMs but not in iBMDMs when activated with 100 ng/ml LPS for 4 hours and 24 hours. This is could be because iBMDMs lack functions or characteristics of normal cells. The effect of rotenone (**Figure 5.2** (A) and (B)) on ROS production was slightly less in BMDMs than iBMDMs. TTFA did not affect the ROS production in iBMDMs or BMDMs (**Figure 5.3** (A) and (B)). The effect of antimycin A (**Figure 5.4** (A) and (B)) on ROS production was slightly less in BMDMs than iBMDMs. It was only when the complex I or complex III was fully inhibited that ROS production increased. The nature of ROS production in mitochondria is complicated and varies from cell type to cell type. The rotenone binding site in complex I is a well characterised site for leakage of electrons. Antimycin A inhibits complex III by disrupting the electron flow from cytochrome b to cytochrome c and this disruption makes it possible for electrons leak onto molecular oxygen to form superoxide. A study by (Gattermann et al., 2004) highlighted the fact that the effect of rotenone and antimycin A were similar on primary human keratinocytes and 143B cells, a human osteosarcoma cell line.

The ETC comprises two electron carriers and four respiratory complexes (CI–IV) and these complexes (except CII) can accumulate as super-complexes. Super-complex formation is thought to improve coupling and lower ROS production. However, it is diminished when macrophages encounter bacterial products or living bacteria (Van den Bossche et al., 2017).

There are several possible sources of ROS signals emitted from mitochondria in response to succinate oxidation and elevated membrane potential, including complex I and complex III. One hypothesis is that the ROS signal is induced by reverse electron transport (RET) at complex I of the ETC. Complex I can induce large amounts of ROS by fine-tuning RET when the CoQ pool is significantly reduced, and the mitochondrial membrane potential is high, which together provide the thermodynamic dynamics to push electrons along in the opposite direction to the ROS production site in the complex I (Chandel et al., 2000, Chance and Hollunger, 1961b). The role of RET in metabolic signalling has been implicated in several recent studies, such as ischemia/reperfusion injury, mitochondrial ROS production during ageing in *Drosophila*, status detection in *Drosophila* and lack of oxygen in the carotid body (Chouchani et al., 2014, Scialò et al., 2016, Fernández-Agüera et al., 2015).

Some consequences of mitochondrial ROS production have been identified in M1 macrophages. ROS has direct bactericidal properties. LPS signalling induces tumour necrosis factor receptor-associated factor 6 (TRAF6)-associated factor 6. TRAF6-mediated recruitment of evolutionarily conserved signalling in toll-collecting (ECSIT) pathways in the mitochondrial outer membrane has been established . The possibility that this directly interacts with the ETC complex I promoting subsequent ROS assembly and production is unknown. Bactericidal ROS enters phagocytosis containing intracellular bacteria and promotes infection clearance (West et al., 2011a). ROS can affect the production of inflammatory cytokines, including IL-6 and TNF- α . ROS prevents the dephosphorylation of mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), extracellular signal-regulating kinase (ERK), and p38 MAPK phosphorylation in response to LPS, and may contribute to increased cytokine production (Bulua et al., 2011).

Antimycin A, in particular, prevents electron transport at the Q_i site, which is required for semiquinone radical reduction to quinol. As shown in experiments with antimycin A-treated and DHE-loaded neutrophils, inhibiting particular Q cycle-related activities causes semiquinone radical build up and O₂[•] production within the mitochondria (Chen et al., 2003).

The limitation of the DHE fluorescence flow cytometer is that the technique detected intracellular ROS production. Also, DHE fluorescence flow cytometry cannot separate 2-hydroxyethidium (2-OH-E⁺) from ethidium, so it is difficult to find out whether the oxidative fluorescence is from O₂^{•-} or other species of ROS (Fan and Li, 2014).

Most of the literature on macrophage immunometabolism has focused on the use of LPS, a Gram-negative bacterial component, to induce a pro-inflammatory response. LPS which is an outer membrane component of Gram negative bacteria, is a potent activator of monocytes and macrophages. The major cell surface receptor for LPS on macrophages is CD14 and it induces the generation of cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-1, and IL-6. Activation of macrophages by LPS has generated a great amount of information on the metabolic pathways and ROS-related reprogramming engaged during inflammation (O'Neill and Pearce, 2016, Meng and Lowell, 1997, Hirschfeld et al., 2001)

The rotenone and antimycin A treated cells showed a lot of variation. In the present study, it was demonstrated that rotenone and antimycin A significantly decreased inflammatory pro-inflammatory cytokines such as TNF- α and IL-1 β , but this effect was not seen with TTFA. Kelly, Murphy and O'Neill suggested that rotenone mimics the effect of metformin

on LPS-induced cytokines. Metformin (dimethylbiguanide) was discovered in the 1920. It is widely used for non-insulin-dependent diabetes treatment.

Metformin is also an inhibitor for complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial electron transport chain (Owen et al., 2000, El-Mir et al., 2000). Here the effects of metformin on cytokine production in LPS-stimulated BMDMs was examined and it was found that metformin decreased LPS-induced production of the proinflammatory cytokine pro-IL-1 β at both the mRNA and protein levels. IL-10 production was also boosted, in agreement with previous findings in human monocyte-derived macrophages (Kelly et al., 2015, Lee et al., 2013).

In contrast to some studies that report that rotenone-induced oxidative stress and the increased release of pro-inflammatory mediators through a p38-mediated cascade are involved in the development of Parkinson's disease (Borrajó et al., 2014, Gao et al., 2013), the experiments in this thesis showed that rotenone inhibited the release of TNF- α and IL-1 β induced by LPS in both iBMDMs and BMDMs.

Complex I inhibition partially decreases TNF- α secretion in iBMDMs and BMDMs (**Figures 5.5 and 5.11**). Similarly, complex III inhibition partially decreases TNF- α secretion in iBMDMs and BMDMs (**Figures 5.7 and 5.13**). However, complex II inhibition only slightly decreased TNF- α secretion in iBMDMs and BMDMs at very high concentrations of 200 μ M (**Figures 5.6 and 5.12**). On the other hand, complex I inhibition substantially decreased inflammasome-activated IL-1 β secretion in iBMDMs and BMDMs (**Figures 5.8 and 5.14**). Complex III inhibition also substantially decreased inflammasome-activated IL-1 β secretion in iBMDMs and BMDMs stimulated by LPS and ATP while only

partially decreasing it in iBMDMs and BMDMs stimulated by LPS only (**Figures 5.10 and 5.16**). **Figures 5.9** and **5.15** showed that Complex II inhibition does not affect inflammasome-activated IL-1 β secretion in iBMDMs and BMDMs.

Many reports have suggested that the cellular energy sensor AMP-activated protein kinase (AMPK) can serve as an anti-inflammatory molecule is a marker of M2 macrophages. Also, it is known to be an indirect target of metformin (Zhou et al., 2001, Sag et al., 2008, Meng et al., 2015).

The role of AMPK in the effect of metformin on LPS-induced cytokines was examined by using pharmacological and genetic manipulation of this enzyme. LPS decreased AMPK activity. AMPK is able to switch macrophages from a pro- to an anti-inflammatory functional state by differential regulation of transcription factors, inhibition of NF- κ B (pro-inflammatory) and activation of Akt and cAMP response element binding (CREB) (anti-inflammatory), which subsequently decrease pro-inflammatory genes but increase anti-inflammatory ones, for example, IL-10. Sag et al. previously showed LPS-induced inhibition of AMPK in the context of macrophage polarization (Sag et al., 2008). Metformin helped reduce AMPK activity during LPS-activation. Metformin can restore AMPK activity in mice with experimental autoimmune encephalomyelitis as well as in a mouse model of diet-induced obesity (Nath et al., 2009, Woo et al., 2014).

CHAPTER 6

GENERAL DISCUSSION

6.1 General Discussion

The mitochondrial electron transport chain (ETC) is an essential component of bioelectricity generation, biosynthesis, and redox control. Several small molecules have been described as inhibitors of ETC with varying consequences for mitochondrial biogenesis, cell viability and proliferation. There are many studies that have sought to determine the mechanisms of mitochondrial ROS production. Inhibitors that act at various points in the electron transport chain (ETC) are widely used to localize and quantify mitochondrial ROS production. The redox centres of complexes I and III are involved as major sites of mitochondrial ROS production, and some studies suggest that complex II can also produce ROS (Sugioka et al., 1988, St-Pierre et al., 2002).

The very first observation of increased glycolysis and decreased OXPHOS in macrophages happened in 1969 and since then insights into the signalling pathways were garnered important molecules that govern this switch during both macrophage and DC activation (Ryan and O'Neill, 2017). Hard presented that activated murine peritoneal macrophages had lower levels of oxygen consumption than resting macrophages as well as higher levels of glycolysis. Studies by Newsholme and colleagues in the 1980s provided more evidence supporting this idea, as they were able to demonstrate that enzymes involved in glucose metabolism have higher enzymatic activities in macrophages, resulting in high rates of glucose and glutamine consumption (Galván-Peña and O'Neill, 2014b).

Recent studies have noticed that infectious stimuli alter the metabolic phenotype of macrophages or dendritic cells, and that the metabolic switch from mitochondrial oxidative phosphorylation (OXPHOS) to glycolysis is essential for innate immunity (Krawczyk et al., 2010, Pantel et al., 2014, Tannahill et al., 2013).

By inhibiting cytochrome c oxidase, nitric oxide (NO) may have a normal role in controlling mitochondrial O₂ consumption. The fact that the apparent K_m for O₂ in whole cells is higher than in isolated mitochondria could be explained by the fact that NO inhibits cytochrome oxidase. Although NO inhibition of cytochrome oxidase is easily reversible, in pathophysiological disease conditions, it can have significant consequences for the cell states (Sharpe and Cooper, 1998, Brown, 1995, Brown and Cooper, 1994).

LPS affects the Krebs cycle, leading to a situation where it is inhibited at two points: after citrate formation and after succinate formation (Jha et al., 2015). Many studies have shown that LPS increases mitochondrial ROS production which is an important responsibility for killing bacteria in macrophages (West et al., 2011a).

Itaconate is known to regulate succinate levels, inflammatory cytokine production, mitochondrial respiration, and electron transport chain directionality. Experiments in Chapter 3 showed that high levels of itaconic acid inhibited some mitochondrial ETC activities. In iBMDMs and BMDMs, itaconate was found to inhibit complex I, II, and II/III specific activities. Succinate build-up is known to enhance ROS generation in mitochondria and the cell when the respiratory chain complex II is inhibited. However, itaconic acid has capacity to prevent the reverse electron transfer from ubiquinol to complex I. The inhibitor of complex II malonate has previously been found to greatly reduce the rate of H₂O₂ generation by mitochondria (Zhang et al., 2020, Belosludtsev et al., 2020, Williams and O'Neill, 2018a).

Itaconate inhibits complex II activity in iBMDMs and BMDMs in a time-dependent and competitive manner. This partly agrees with the O'Neill group who showed, in 2016, that itaconate's main immunomodulatory effect was determined to be SDH inhibition. While itaconate has been known as a competitive inhibitor of SDH for decades due to its structural resemblance to succinate, its physiological relevance as an endogenous inhibitor of SDH has yet to be investigated. SDH inhibition limits the oxidation of succinate to fumarate, preventing the production of reactive oxygen species in the mitochondrial complex (mtROS) (Coelho, 2022, Bezawork-Geleta et al., 2017).

Different stimuli cause macrophage polarization. For example, TLR agonists (such as LPS) or IFN- γ mediate classical activation, and IL4 and IL13 mediate alternative activation of macrophages. The activated macrophages are often referred to as M1 and M2 macrophages, respectively. As part of these different phenotypes, there are certain changes in the molecules involved in metabolism. M1 macrophages upregulate and promote hypoxia-inducible factor (HIF) 1 α , glucose transporter channel 1 (GLUT1), and ubiquitous phosphofructokinase 2 (PFK2), a positive regulator of glycolysis increase (Stein et al., 1992, Rodríguez-Prados et al., 2010, Mantovani et al., 2004).

The mitochondrial respiration chain and OXPHOS component cytochrome oxidase (Complex IV) are well-known for adding two pairs of electrons to water to decrease molecular oxygen (mitochondrial consumed oxygen). NADH and FADH₂ reducing equivalents that obtained electrons through substrate oxidation, notably during the Krebs cycle, are the source of these electrons. After that, the electrons move through the electron carriers of the mitochondrial electron transport chain (ETC), creating a proton gradient or proton motive force (p) by pumping out protons from the mitochondrial matrix to the

intermembrane space. Then, like a hydroelectric pump, this force pumps protons back into the matrix by a nanomachine called ATP synthase (complex v), which makes energy (ATP from ADP and Pi).

In the study by (Garaude et al., 2016), *E. coli*–stimulated BMDMs had much lower oxygen consumption on a glucose substrate than that of resting BMDMs, while they exerted a high glycolytic activity that increased their extracellular acidification rate (ECAR) and lactate production at 18 h after infection relative to that of resting BMDMs. Our study demonstrated that the OCR was decreased rapidly and the ECAR increased rapidly when the iBMDMs were treated with LPS.

In fact, these studies show that when macrophages are activated by LPS, metabolic reprogramming from oxidative phosphorylation to glycolysis occurs. Our current research looks at the underlying mechanisms of these events and considers the possibility that changes in ETC activity may underlie the switch to glycolysis.

Therefore, a metabolic flux control analysis was carried out in which the control of complex I activity on oxygen respiration rates in iBMDMs and primary BMDMs was studied. The results of this study suggest that small perturbations in complex I activity (using low concentrations of rotenone) in untreated iBMDMs decrease the OCR relative to the control. In addition, performing a similar complex I activity titration in untreated iBMDMs and primary BMDMs indicates that primary BMDMs have lower reserves of complex I activity. The reasons for this may be due to the fact that, iBMDMs are immortalised cell lines and have different bioenergetic properties compared to primary BMDM cultures. These studies

highlight on how cellular metabolism of BMDMs is affected by LPS treatment and further experiments in this area need to be fully explored.

We investigated the effects of rotenone and antimycin A, both complex I and III inhibitors, on ROS generation in iBMDMs and BMDMs in this study. When stimulated with 100 ng/ml LPS for 4 hours and 24 hours, ROS production increased in BMDMs but not in iBMDMs, which was a surprising finding. Rotenone had a slightly lower effect on ROS generation in BMDMs than in iBMDMs. Antimycin A had a slightly lower effect on ROS generation in BMDMs than in iBMDMs. ROS generation increased only when complex I or complex III were fully inhibited. The nature of ROS generation in mitochondria is complex and differs between cell types. The rotenone binding region in complex I has been widely studied for electron leakage. Antimycin A inhibits complex III by disrupting the electron transport from cytochrome b to cytochrome c, which prevents CoQ from passing its electrons to cytochrome b.

M1 macrophages additionally increase expression of the mitochondrial citrate carrier, which is important for NO, ROS and prostaglandin production. In conjunction with IL-6, IL-12 and TNF- α expression this enhances their antimicrobial and inflammatory phenotype, respectively. However, M2 macrophages induce oxidative phosphorylation of mitochondria via upregulation of the peroxisome proliferator-activated receptor γ -coactivator 1 β (PGC1 β) and components of the electron transport chain (Mills et al., 2000, O'Neill, 2015, Jha et al., 2015, Infantino et al., 2011, Shao et al., 2010, St-Pierre et al., 2003).

6.2 Conclusion

The use of LPS to activate macrophages has generated a substantial amount of information about metabolic pathways and reprogramming programs during inflammation.

Recently, numerous studies have demonstrated that inhibition of mitochondrial respiratory complex I in LPS-stimulated neutrophils leads to increased mitochondrial ROS generation, increased intracellular H_2O_2 concentration, and decreased production of mitochondrial cytokines, such as $\text{TNF-}\alpha$.

Most notably, we found that the inhibition of complex I, II and II/III during the challenge of iBMDMs and BMDMs with LPS decreased the secretion of $\text{IL-1}\beta$ and $\text{TNF-}\alpha$. Thus, establishing a functional association between pattern recognition receptors, ETC organization and subsequent inflammatory immune responses could offer significant advantages for vaccine design and delivery. Valuable new targets for pharmacological intervention in both the infectious phase and in inflammatory metabolic disorders.

6.3 Future work:

- Further investigations into the effects of itaconate on ETC complexes (I-IV) in iBMDMs and BMDMs activated with LPS are required.
- Further investigations into the effects of IL-10 on ETC complexes (I-IV) in iBMDMs and BMDMs activated with LPS are required.
- Further investigations of the spread of control of the respiratory chain components, complex I, and complex II/III over oxygen consumption in LPS-activated BMDMs $\pm$ IL-10 are warranted, by determining their flux control coefficients and inhibition thresholds.

CHAPTER 7

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