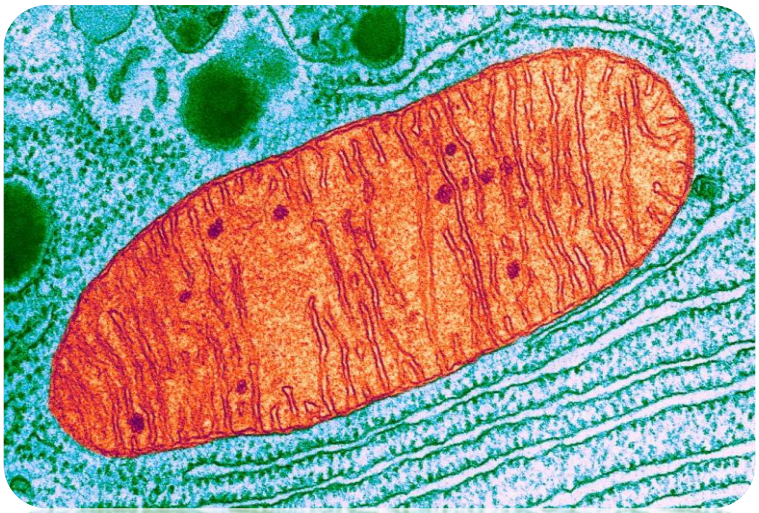
University of Groningen

The Effect of SK Channel Activation on Reverse Electron Transport in Inflammation

The effect of CyPPA treatment on ROS-RET generation in RAW macrophages



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Date: 1 August 2019

university of groningen

Preface

To fulfill my first research project of my study Medical Pharmaceutical Sciences at the University of Groningen, I have participated in a project from the department of Molecular Pharmacology of the University of Groningen. This project is titled “The Effect of SK Channel Activation on Reverse Electron Transport in Inflammation”.

It was my pleasure to work at this department, and I would like to thank my supervisors, A.M. Dolga, PhD, and I.E. Krabbendam, MSc. I would also like to thank the whole department for welcoming me and helping me with the project. A great thank you to all for understanding my health issues and adapting the project accordingly. I learned many new techniques, which will be very important for the future.

I wish all the students I met along the way the best of luck with their research projects, and I hope that the continuation of this project will lead to good results.

I hope you enjoy reading this report.

Rianne Kloosterman

Groningen, 1 August 2019

Abstract

BACKGROUND: An imbalance in reactive oxygen species (ROS) levels has been associated with cardiovascular and neurodegenerative diseases. ROS are produced in the electron transport chain (ETC). Succinate is a substrate for CII of the ETC, and a pro-inflammatory metabolite that is known to accumulate during macrophage activation. Accumulation of succinate can induce reverse electron transport (RET). Small conductance calcium-activated potassium (SK) channels may also be involved in ROS-RET. A recent study showed that activation of SK channels with cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (CyPPA) was able to slightly inhibit CI and CII of the ETC. The aim of this project is to study the role of SK channel activator CyPPA on succinate-induced ROS-RET in macrophages. It is expected that succinate treatment can induce the pro-inflammatory response in macrophages, and that SK channel activation by CyPPA will reduce the ROS generated during ROS-RET. **METHODS:** RAW 264.7 macrophages were either pre-treated and co-treated or only pre-treated with succinate, rotenone and/or CyPPA. The expression of pro-inflammatory cytokines will be assessed using RT-qPCR. Metabolic assays, such as seahorse and lactate assays, will be used to give an insight in the metabolic switch of activated macrophages. Macrophage activation will be studied using xCELLigence, and the ROS generation will be studied with different staining methods. **RESULTS:** The results showed that LPS was able to induce a morphological change, and induce the pro-inflammatory response in macrophages. The xCELLigence assays, metabolic assays, and the RT-qPCR results showed that succinate + CyPPA + LPS treatment could reduce the pro-inflammatory response. Succinate + LPS treatment did not further increase the pro-inflammatory response. **CONCLUSIONS:** LPS can activate the macrophages, inducing the pro-inflammatory response. This effect can be reduced by CyPPA co-treatment. Upon activation with CyPPA, macrophage activation is reduced, as well as the phagocyti activity.

List of abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
Acetyl-CoA	Acetyl-Coenzyme A
AMV RT	Avian Myeloblastosis Virus Reverse Transcriptase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BK channels	Large conductance calcium-activated potassium channels
BMDM	Bone Marrow-Derived Macrophages
CD14	Cluster Of Differentiation 14
cDNA	Complementary DNA
CI	Complex I or Cell Index
CII	Complex II
CoQ	Coenzyme Q
CyPPA	cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine
DMEM	Dulbecco's Modified Eagle Medium
DMM	Dimethylmalonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
ECAR	Extracellular Acidification Rate
ETC	Electron Transport Chain
FACS	Fluorescent-Associated Cell Sorting
FBS	Fetal Bovine Serum
FS	Forward Scatter
HIF-1α	Hypoxia-Inducable Factor 1α
IK channels	Intermediate conductance channels
IL-10	Interleukin 10
IL-1β	Interleukin 1β
K_{Ca} channels	Calcium-activated potassium channels
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharides
mRNA	messenger RNA
mtROS	Mitochondrial Reactive Oxygen Species

RET	Reverse Electron Transport
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT	Reverse Transcriptase, or room temperature
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SK channels	Small conductance calcium-activated potassium channels
TCA	Tricarboxylic Acid
TLR-4	Toll-like Receptor 4
TNFα	Tumor necrosis factor-α

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1.0 Introduction

Natural human antioxidant defenses are not always sufficient in maintaining a proper reactive oxygen species (ROS) balance [2]. Oxidative stress has been associated with aging and cell death, and ROS play a part in many age-associated diseases such as Alzheimer's disease, Parkinson's disease, and cardiovascular diseases [3-5]. ROS are also involved in tissue reperfusion, inducing oxidative damage and cell death [13].

ROS is a collective term for superoxide ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) [1]. ROS are molecules derived from oxygen, thought to mediate oxygen toxicity due to their high reactivity compared to oxygen [2]. They are byproducts of aerobic metabolism which can act as intracellular signaling molecules, with low levels of ROS being key to cell survival, and high levels of ROS being involved in inflammation and mediating in cell death.

Chronic or prolonged production of ROS is central in the progression of many inflammatory diseases [23]. Mitochondrial-derived ROS (mtROS) contribute to the production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα). mtROS are also involved in the regulation of the inflammasome, which activates inflammatory caspases and cytokines in macrophages.

It was recently proposed that mtROS also play a crucial role in several redox-dependent signaling processes as well as in aging [6]. Therefore, the mtROS formation and its role in both physiological and pathological processes is of great interest. mtROS are produced during oxidative phosphorylation by the electron transport chain (ETC). The ETC is a mitochondrial pathway consisting of five complexes (figure 1).

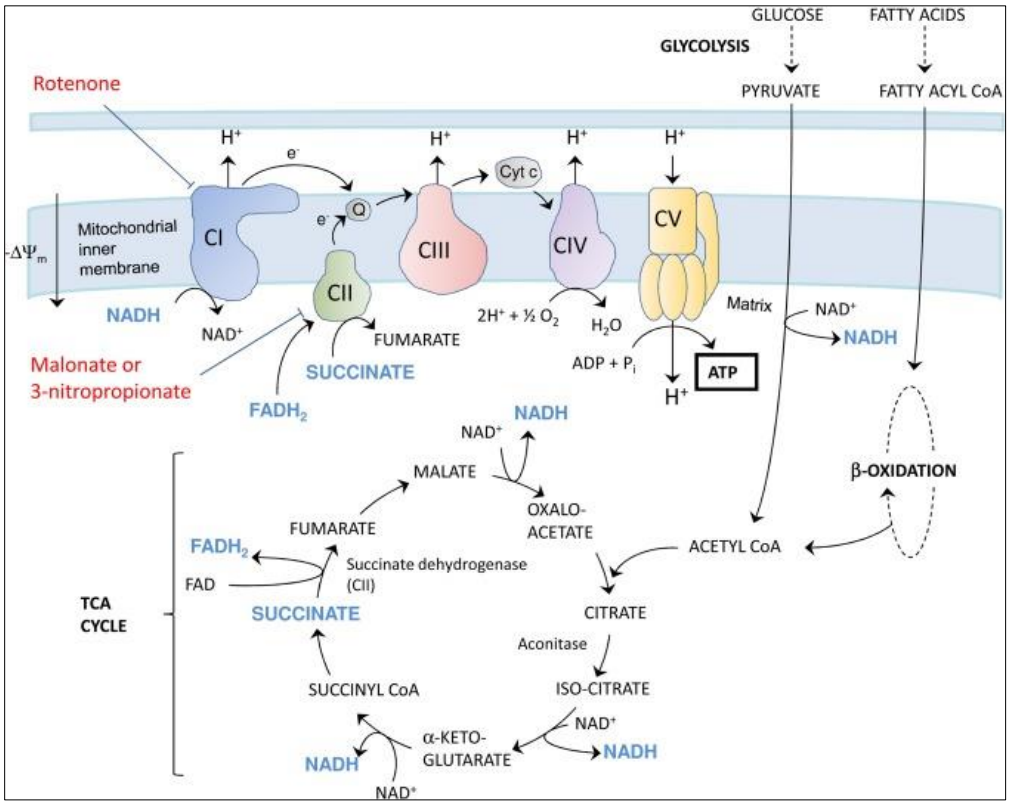

Figure 1: Overview of the ETC. In this figure, a schematic overview of the ETC is shown. The figure also shows the substrates for the complexes.

Succinate is a substrate for complex II of the ETC. It is a pro-inflammatory metabolite that is known to accumulate during macrophage activation [7]. Its accumulation leads to increased hypoxia-induced factor 1α (HIF-1α) activity, and increased interleukin 1β (IL-1β) expression [7].

Macrophages can be activated through different pathways, with the lipopolysaccharide/toll-like receptor-4 (LPS/TLR-4) pathway being the most common (figure 3). The TLRs are microbial-sensing proteins that are the first responders to danger signals and are therefore significant in combatting infectious and inflammatory diseases [8]. TLRs recognize pattern-associated molecular patterns (PAMPs), like LPS. LPS is an endotoxin present on the outer membrane of Gram-negative bacteria.

It has been shown that LPS induces metabolic repurposing from ATP synthesis to glycolysis, and blocking succinate-induced ROS-RET generation inhibited IL-1β expression [22]. Their results, shown in figure 2, showed that treatment of bone marrow-derived macrophages (BMDM) with diethylsuccinate significantly increased the IL-1β expression. These results may prove that succinate-induced ROS-RET is capable of significantly increasing the IL-1β expression in the presence of LPS.

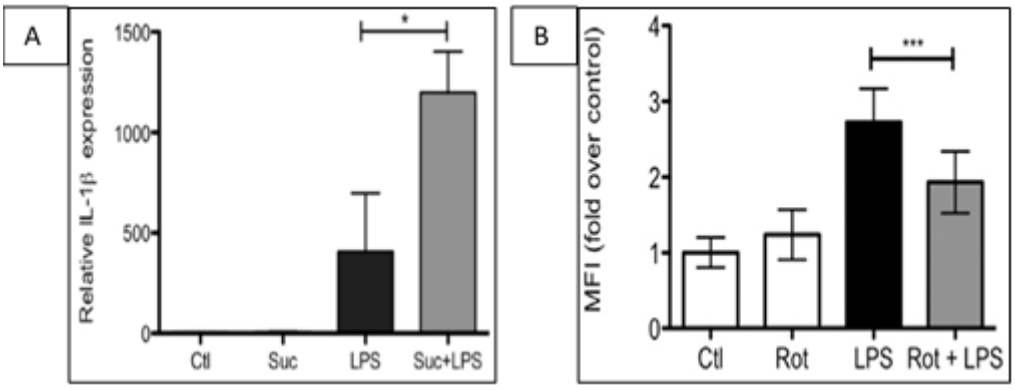

Figure 2: Results from Mills et al. (2016). (A) BMDM were pre-treated for 3 hours with 5 mM before being stimulated with LPS (100 ng/mL) for 48 hours. (B) BMDM were pre-treated for 3 hours with 0.5 µM rotenone before being stimulated with LPS (1 µg/mL) for 24 hours.

After LPS binding and recognition, NF-κB pathway is activated [10]. Activation of the NF-κB pathway leads to the formation of pro-inflammatory cytokines, such as IL-1β [11].

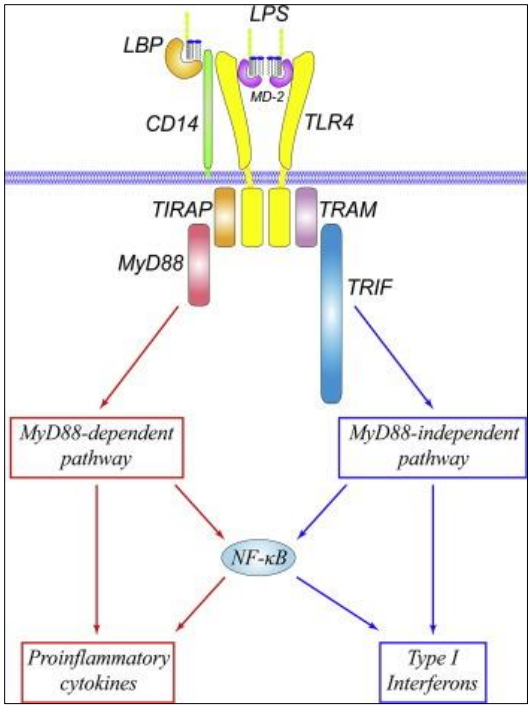

Figure 3: LPS binding to TLR-4. This figure shows LPS binding to TLR-4 along with the proteins involved in recognition and signal transduction.

During macrophage activation metabolic repurposing is key in regulating pro-inflammatory responses. Upon activation, macrophage metabolism switches from oxidative phosphorylation to glycolysis. An upregulation in glycolysis leads to higher ATP levels, necessary for maintaining the mitochondrial membrane potential and prevent apoptotic cell death [12].

However, in the switch from oxidative phosphorylation to glycolysis, the TCA cycle is disrupted in two places: after citrate and after succinate. This leads to increased synthesis of acetyl-coenzyme A (acetyl-CoA), and succinate accumulation. The increase in the succinate concentrations can induce reverse electron transport (RET) (figure 4). Therefore, succinate might contribute to inflammation caused by ROS produced via RET (ROS-RET), and also the metabolic shift in activated macrophages.

As a result of the metabolic repurposing in activated macrophages, there is an increase in ROS-RET, leading to oxidative stress and an increased expression of pro-inflammatory cytokines.

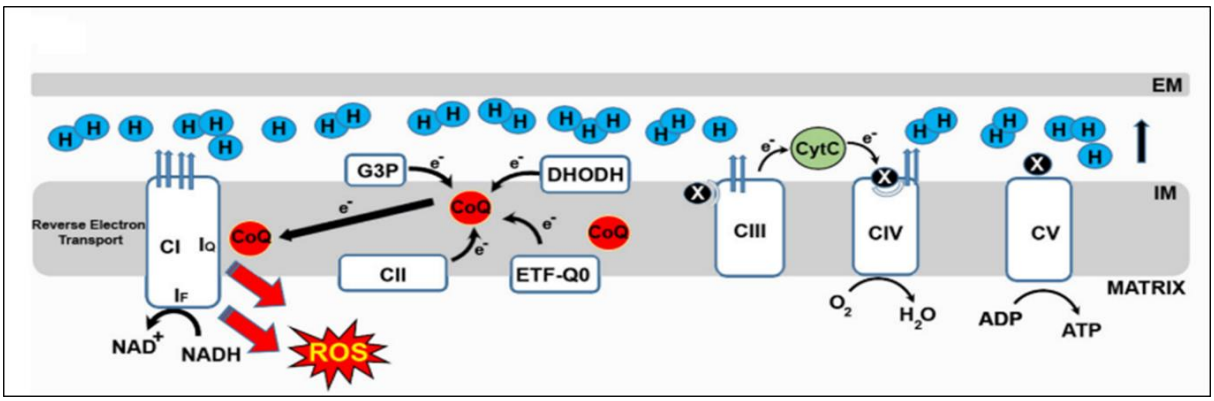

Figure 4: Reverse Electron Transport. In this figure a schematic overview of RET is shown. The arrows indicate the flow of electrons.

When complex I (CI)-linked substrates are used, such as glutamate and pyruvate in combination with malonate, the electrons move in forward direction [13]. When blocking the I_Q-site site with rotenone during RET, NADH accumulates and over-reduces the I_F-site. Electrons leak to oxygen, leading to superoxide formation. When complex II (CII)-linked substrates, such as succinate, are used, a portion of the electrons flow through CI in reverse direction. The ROS generated in these conditions can be reduced by rotenone.

SK channels may play a role in protection against oxidative stress by blocking complex I. In microglia SK channels protect against LPS [14]. SK channels belong to a large family of K^+ channels that were first identified in nervous tissues [19]. Calcium-activated K^+ (K_{Ca}) channels are expressed in a wide variety of tissues, including neuronal and cardiac cells, where they are involved in membrane potential regulation. K_{Ca} channels are classified based on their Ca^{2+} conductance, into large conductance (BK) and small conductance (SK) channels and intermediate conductance (IK) channels. BK channel activation is mediated by voltage changes, whereas SK channel activation depends on the binding of Ca^{2+} to calmodulin (figure 4) [19]. Activation of any type of K_{Ca} channels with pharmacological agents in pathological conditions is associated with protective effects, such as oxidative stress defense and DNA repair [20]. mtBK channel activation was shown to reduce ROS-RET [24].

In a recent study, it was shown that pharmacological activation of mitochondrial SK channels with cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (CyPPA) was able to slightly inhibit CI [17]. Another study showed that CyPPA significantly reduced LPS-stimulated activation in microglia [21]. This study also showed that CyPPA attenuated the LPS-induced elevation of the intracellular calcium concentration ($[Ca2+]_i$).

Ca2+ signaling, especially fluctuations in $[Ca2+]_i$ will affect intracellular structures such as the mitochondria.

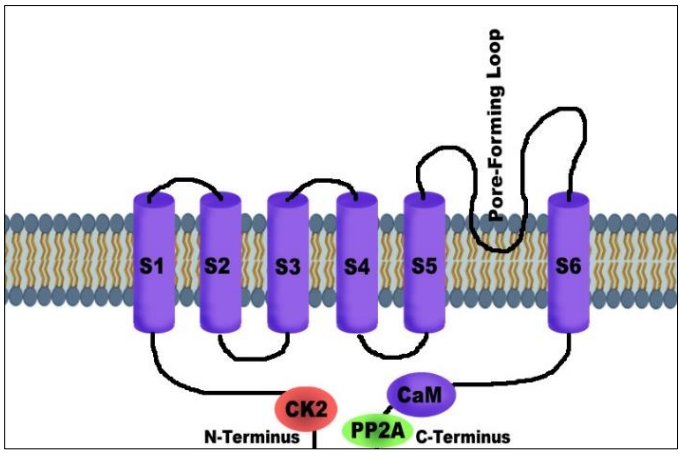

Figure 5: SK channel. In this figure a schematic overview of an SK channel is shown.

In summary, recent research has shown that SK channel activation in microglia reduces LPS-induced inflammation. Still unknown is whether SK channel activation is able to inhibit CI and thereby inhibit ROS-RET.

The aim of this research is to study the role of SK channel activator CyPPA on succinate-induced ROS-RET in macrophages. It is expected that succinate and LPS treatment induces the pro-inflammatory response in macrophages, therefore leading to an increase in the expression of pro-inflammatory cytokines and an increase in ROS generation.

It is hypothesized that SK channel activation by CyPPA will slightly inhibit CI, thereby reducing the succinate-induced ROS-RET generation by CI. The LPS-induced inflammation is expected to be reduced by SK channels, thereby reducing ROS-RET. This might be a potential mechanism by which SK channels activation reduce inflammation.

2.0 Materials and methods

RAW 264.7 macrophages were cultured and treated with succinate, rotenone, malonate, and CyPPA. Following the treatment, the macrophages were stimulated with LPS. The expression of pro- and anti-inflammatory cytokines was analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). mtROS generation was analyzed by different staining methods, including MitoSOX and DCF. Metabolic assays, such as seahorse and lactate assays, were used to give an insight into the effect of the compounds on metabolism. The protein expression was analyzed using Western Blot.

2.1 Cell Culturing

RAW 264.7 macrophages were cultured at 37°C with 5% CO_2, until further analysis.

The RAW 264.7 macrophages were cultured in T75 flasks in an incubator set at 37°C with 5% CO_2. The cells were split every three days with a ratio of 1:3. Before the weekend, the cells were split 1:5. The medium used is Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Thermo Fisher Scientific, Massachusetts, USA with 4.5g/L glucose, GlutaMAX-I, 1mM sodium pyruvate, 2mM L-Glutamin, 10µg/mL Gentamycin, and 10% Fetal Bovine Serum (FBS).

The cells were treated with diethylsuccinate (Sigma-Aldrich, Missouri, USA, Cas No. 123-25-1), rotenone, CyPPA (provided by Prof. dr. F.J. Dekker), dimethyl malonate (DMM) (Sigma-Aldrich, Missouri, USA, Cas No. 108-59-8), and LPS.

Prior to diethylsuccinate, dimethylsuccinate (Sigma-Aldrich, Missouri, USA, Cas No. 106-65-0) was used to treat the cells. However, a study by Mills et al. (2016) showed that diethylsuccinate can induce ROS-RET. Therefore, diethylsuccinate was used for further experiments.

2.2 Brightfield microscopy

Macrophage activation and viability after treatment with different compounds was assessed using microscopy.

RAW macrophages were seeded in 24 well plates and treated with different compounds, using four wells per condition. After the treatment brightfield images of the cells were taken with a light microscope connected to NIS Element Software (Nikon, Tokyo, Japan). The magnification was set to 100X.

2.3 xCELLigence real-time impendence measurement

To assess the viability and activation of the macrophages after treatment with different compounds, an xCELLigence assay was performed.

Macrophage activation was monitored in real-time with cell impedance measurements, using the xCELLigence system (Roche Diagnostics, Penzberg, Germany). The macrophages were seeded in the xCELLigence plate with 20,000 cells per well. Cell impedance was normalized to the time of treatment (normalized cell index), which is defined as the starting point (t = 0h) of the experiment.

2.7 Lactate assay

To analyze the lactate produced by macrophages, a lactate assay was performed.

RAW macrophages were cultured as previously described (chapter 2.1). The cells were seeded in 96 well plates with 20,000 cells per well, followed by treatments 24 hours later. Medium was collected and diluted with 10, 20, or 30X demi water prior to the analysis. A calibration curve of eight lactate standards ranging from 0 – 1.2 mM was prepared for quantification purposes. Subsequently, lactate was measured in a 96-well plate using 20 µl medium sample or lactate standard mixed with 225 µl reaction mixture (0.44 M Glycine/0.38 M Hydrazine [pH 9.0], 2.8 mM NAD) and 5 units L-lactic dehydrogenase followed by absorbance determination at 340 nM using Synergy™ H4. All chemicals were purchased from Merck Millipore. Background absorbance of the blank control (0.0 mM lactate standard) was subtracted from all sample readings and medium samples were corrected for dilution. Medium lactate concentrations were determined based on linear regression of the standard curve.

2.8 Seahorse extracellular flux analysis

To analyze the shift in metabolism towards glycolysis in treated macrophages, a Seahorse extracellular flux assay was performed.

RAW macrophages were cultured as previously described (chapter 2.1). The cells were seeded in a 96-well plate meant for Seahorse at a density of 20,000 cells per well. The corner wells were left empty as background controls. The cells were pre-treated with or without compounds, followed by an LPS treatment in the analyzer. In preparation for the analysis, the cartridges were hydrated with sterile UP and placed in an incubator overnight (37°C, without CO_2) together with the Seahorse calibration buffer. On the day of the assay, the UP was replaced with the calibration buffer and placed at 37°C. The assay buffer was prepared fresh on the day of the measurement and contained base-medium (1x DMEM-powder (Sigma D5030), 143 mM NaCl, 3mg/L Phenolred, pH 7,35), 1 mM sodium pyruvate, 2 mM L-Alanyl-L-Glutamine, and 0.4 mg/mL BSA, pH 7,35. The assay buffer was also placed at 37°C without CO_2. After warming the medium, the pH was set to 7.35 with 1 mM NaOH. 1 hour prior to starting the analysis, the medium in the plate was replaced by assay buffer and the plate was incubated at 37°C (without CO_2). The Seahorse XF Biosystem was used to assess the extracellular acidification rate (ECAR). Three baseline measurements (3x min mix, 0min delay, 3min measure = 3/0/3) were recorded followed by injection in port A with glucose, with or without LPS(0,1ug/ml), CyPPA (25µM) or the combination. Measurements were recorded for 24 h (3/15/3). Immediately after analysis, medium was removed from the plate, 50µl lysis buffer was added to each well, and the plate was incubated at -20ºC for 1 hour. After incubation, 50µl bicinchoninic acid (BCA) was added to each well, incubated for 1 hour at 37 ºC and absorbance was measured using a Synergy H1 Multi-mode reader (Biotek, Winooski, VT, USA) at 550nm, to quantify protein content. These data were then used to normalize the data obtained in the Seahorse analysis.

2.4 Flow cytometry

To analyze the ROS production, flow cytometric assays were done.

The macrophages were seeded in a 24-well plate with a density of 50,000 or 70,000 cells per well. The macrophages were (pre)treated with different compounds and stimulated with LPS for 24 hours.

2.4.1 Measurement of mitochondrial superoxide production

To analyze the amount of ROS, especially superoxide, was generated by the macrophages after dimethyl succinate and LPS treatment, a MitoSOX staining was done.

Formation of mitochondrial superoxides was determined with the MitoSOX dye (Fisher Scientific, Landsmeer, Netherlands, Catalog No. M36008). Cells were incubated with 2.5 µM MitoSOX dye for 30 min at 37°C and then harvested. Fluorescence was excited at 488 nm and detected at 690/50 nm using CytoFLEX (Beckman Coulter, Woerden, the Netherlands). Data was recorded from $1.5x10^4$ cells in triplicate per condition.

2.4.2 Measurement of intracellular ROS production

To analyze the intracellular ROS generation after treatment, a DCF staining was done.

Generation of intracellular ROS was determined with the DCF dye (Fisher Scientific, Landsmeer, Netherlands, Catalog No. C6827). Cells were incubated with serum-free medium containing 4 µM DCF dye for 30 min at 37°C and then harvested. The fluorescent signal was excited at 492-495 nm and detected at 517-527 nm using CytoFLEX (Beckman Coulter, Woerden, the Netherlands). Data were recorded from 1.5x104 cells in triplicate per condition.

2.5 RT-qPCR

To analyze the expression of pro- and anti-inflammatory cytokines, and SK2 and SK3 channel expression, RT-qPCR was performed.

RAW macrophages were seeded in a 6-wells plate with 300,000; 325,000; or 350,000 cells per well.

2.5.1 RNA isolation

In preparation for the RT-qPCR, RNA was isolated from the treated cells.

The surfaces were cleaned with RNaseZap (Sigma-Aldrich, Missouri, United States) prior to the RNA isolation. The medium was removed from the wells and washed with cold 1X PBS. After washing, RNA was isolated using TRIZOL reagent (TRI Reagent Solution, Applied Biosystems, the Netherlands) according to manufacturer's protocol. The RNA concentration was measured using NanoDrop.

2.5.2 cDNA synthesis

The isolated RNA was used for cDNA synthesis to allow for future qPCR.

The cDNA was synthesized from 500-1000 ng RNA using the Reverse Transcriptase System (Promega, Madison, WI, USA, Catalog No. A3500), and the following protocol: 10 min 25°C, 45 min 42°C, 5 min 99°C.

2.5.3 qPCR

To analyze the mRNA expression of pro- and anti-inflammatory cytokines, and SK2 and SK3 channel expression, a RT-qPCR was performed.

The PCR mastermix was added to the samples. Each mastermix contained forward primer, reverse primer (see table 1 for sequences), and SYBR® Green Mater (ROX) (Roche, Basel, Switzerland, Cat no. 28137500). The samples and mastermix were pipetted into 48-well PCR plates. The plate was sealed and centrifuged for 1 minute at 1000 rpm. The plate was immediately analyzed using the Illumina Eco Real-Time PCR System (Illumina, California, USA) using the following protocol: 10 min 95°C; 30 sec 95°C, 30 sec 54°C, 30 sec 72°C (45 cycles); 5 min 72°C; 15 sec 95°C; 15 sec 55 °C; 15 sec 95°C, or stored at -20°C.

Table 1: Primer sequences and manufacturers.

Name of primer	Sequence (5'-3')
18S Forward	AAACGGCTACCACATCCAAG
18S Reverse	CCTCCAATGGATCCTCGTTA
RPL13A Forward	AGAAGCAGATCTTGAGGTTACGG
RPL13A Reverse	GTTCACACCAGGAGTCCGTT
IL-1β Forward	TGCCACCTTTTGACAGTGATG
IL-1β Reverse	ATGTGCTGCTGCGAGATTTG
SK2 Forward	GAATGACCAAGCAAATACCCTAGT
SK2 Reverse	GTGACGATCCTTTTCTCAAAGTCT
SK3 Forward	GAAAAGAGAAAGCGACTGAGTGAC
SK3 Reverse	CATGGAATCCTTTGAGTACAAACC
IL-10 Forward	TAAGGCTGGCCACACTTGAG
IL-10 Reverse	GTTTTCAGGGATGAAGCGGC

2.5.4 Primer testing

In order to test the designed primers for SK2 and SK3, a RT-qPCR was executed and the product was run on an agarose gel.

RT-qPCR was executed as previously described. The primers for SK2 and SK3 were tested on control samples. After analysis of the primer efficiency (>1.8), the PCR products were analyzed on an agarose gel. Agarose was dissolved in 1X TBE and heated in the microwave. The solution was cooled down until it could be handled. SYBR Safe DNA Gel Stain (Invitrogen, California, USA, Lot no. 1821848) was added to the mixture, and it was poured into the mold. The combs were placed into the gel, and the gel was left to solidify. The PCR samples were diluted 5:1 with 6X DNA loading dye (Thermo Scientific, Massachusetts, USA, Lot no. 00389807) and were pipetted into the gel along with a GeneRuler 100 bp Plus DNA ladder (Thermo Scientific, Massachusetts, USA, Lot no. 00348102). The gel was analyzed in a Syngene GBox Chemi 16 Bio Imaging System (Syngene, Bangalore, India).

2.6 Western Blot

To analyze the inflammatory protein expression in macrophages, a Western Blot was executed.

Raw macrophages were cultured as previously described (chapter 2.1). The cells were seeded in 6 well plates with a density of 350.000, 325.000, or 300.000 cells per well. The cells were treated with different compounds, and after the treatment 100 µL lysis buffer was added to each well. The lysis buffer contained 1.06 mg β-glycerophosphate, 1 µL Apoprotein (1 mg/mL), 1 µL Leupeptin (1 mg/mL), 1 µL Leupeptin (1 mg/mL), 5 µL Na_3VO_4, and 1 µL NaF (200 mM). The plate was stored at -80°C until further analysis.

2.6.1 BCA assay

To determine the total amount of protein in each sample, a BCA assay was performed.

The plate was thawed on ice and scraped with a silicon scraper. The solutions were transferred to cups and centrifuged at 20.000xg for 20 minutes (4°C). The supernatant was transferred to new cups. The samples were diluted 5X with UP and 10 µL samples was pipetted in duplo into a 96 well plate. Standards for the BCA assay were thawed and 10 µL were pipetted in duplo in the 96 well plate. Next, a BCA reagent (Thermo Fisher Scientific, Massachusetts, USA, Catalog No. 23225) was prepared by mixing compound A and B at a 1:50 ratio. 200 µL reagent was added to each well and the plate was incubated on a shaker for 30 minutes (120 rpm, 37°C). After the incubation, the absorption was measured at 595 nm.

Each samples was diluted with UP to reach a concentration of 20 µg protein in each sample. The final volume, including the loading buffer, was 140 µL. The cups were placed a 95°C for 5 minutes and stored at -20°C after.

2.6.2 Electrophoresis and Blotting

In order to separate the proteins electrophoresis was done. Afterwards, the proteins were tranferred to membranes via blotting.

For the gel electrophoresis (and blotting), a Bio-Rad Electrophoresis System was used (Bio-Rad, California, USA). A 10% running gel and 5% stacking gel were prepared (1.5 mm thick). After polymerisation, 20 to 25 µL sample was loaded and the electrophoresis was started at 100V, until the samples alligned in the stacking gel. Afterwards, the voltage was set to 130V. ELFO (30.26 g/L Tris, 187.6 g/L glycin, 10 g/L SDS) was used as running buffer.

The blotting system was prepared and filled with transferbuffer (20% methanol, 70% UP, 10% 10X transferbuffer) and the cassette was prepared. The system was assembled and the blotting was set to 100V for 2 hours. After the blotting, the membrane was cut and placed in 1X Roti®-Block solution (Carl Roth, Mannheim, Germany, Art. No. A151) for 45 min. The primary antibody (HIF-1α, NSJ BioReagents, Cat No. R31666) was added (1:3000, diluted in TBST) and incubated for 2 hours. Afterwards, the membrane was washed with TBST and the second antibody (GAPDH) was added and incubated for 2 hours. The membrane was washed prior to placing it on a plastic sheet and adding ECL. The membrane was analyzed in a Syngene GBox Chemi 16 Bio Imaging System (Syngene, Bangalore, India).

2.7 Phagocytosis assay

In order to determine the effect of different compounds on the phagocytic activity of the cells, a phagocytosis assay was performed.

RAW macrophages were cultured as previously described (chapter 2.1). The cells were seeded in a 96 well plate with a density of 20.000 cells per well. The cells were pre-treated with different compounds, followed by LPS treatment with pHrodo Red S aureus BioParticles Conjugate (1 µg/0.1ml) (Thermo Scientific, Massachusetts, USA, Cat no. A10010). The plate was placed in an IncuCyte S3 Live-Cell Analysis System (Essen BioScience, Michingan, USA) in a cell incubator (37°C, 5% CO_2). Images were taken every hour.

2.8 Statistics

To test the significance and reliability of the results, multiple statistical tests were executed in GraphPad Prism.

To analyze the significance of the FACS, lactate, and RT-qPCR results, ordinary one-way analysis of variance (ANOVA) tests and Tukey's multiple comparisons tests were executed in GraphPad Prism 6. A one-way ANOVA allows for the analysis of the means of two or more samples. Tukey's multiple comparison test is one of several tests that can be used to determine which means amongst a set of means differ from the rest.

3.0 Results

3.1 Macrophages changed their morphology when treated with LPS

Since macrophages are known to alter their shape upon activation, the effect of succinate, CyPPA, and LPS on macrophage morphology was studied using microscopy. Cells were pre-treated with succinate (5 mM) and/or CyPPA (25 µM) for 3 hours, followed by either no other treatment, 24h LPS (0.1 µg/mL) treatment, or 24h LPS and CyPPA co-treatment.

As shown in figure 6, cells treated with LPS became activated and changed their morphology. Untreated controls and cells treated with succinate alone, did not change their morphology. Co-treatment of succinate with activated the cells, which was also the case with CyPPA and LPS treated cells. When treated with succinate, CyPPA and LPS the morphology of the cells appeared similar to LPS treated cells. Succinate and LPS treated cells also look similar to the LPS treated cells. These results suggest that LPS is able to activate the macrophages, which does not seem to be reduced when treated with succinate, CyPPA, and LPS.

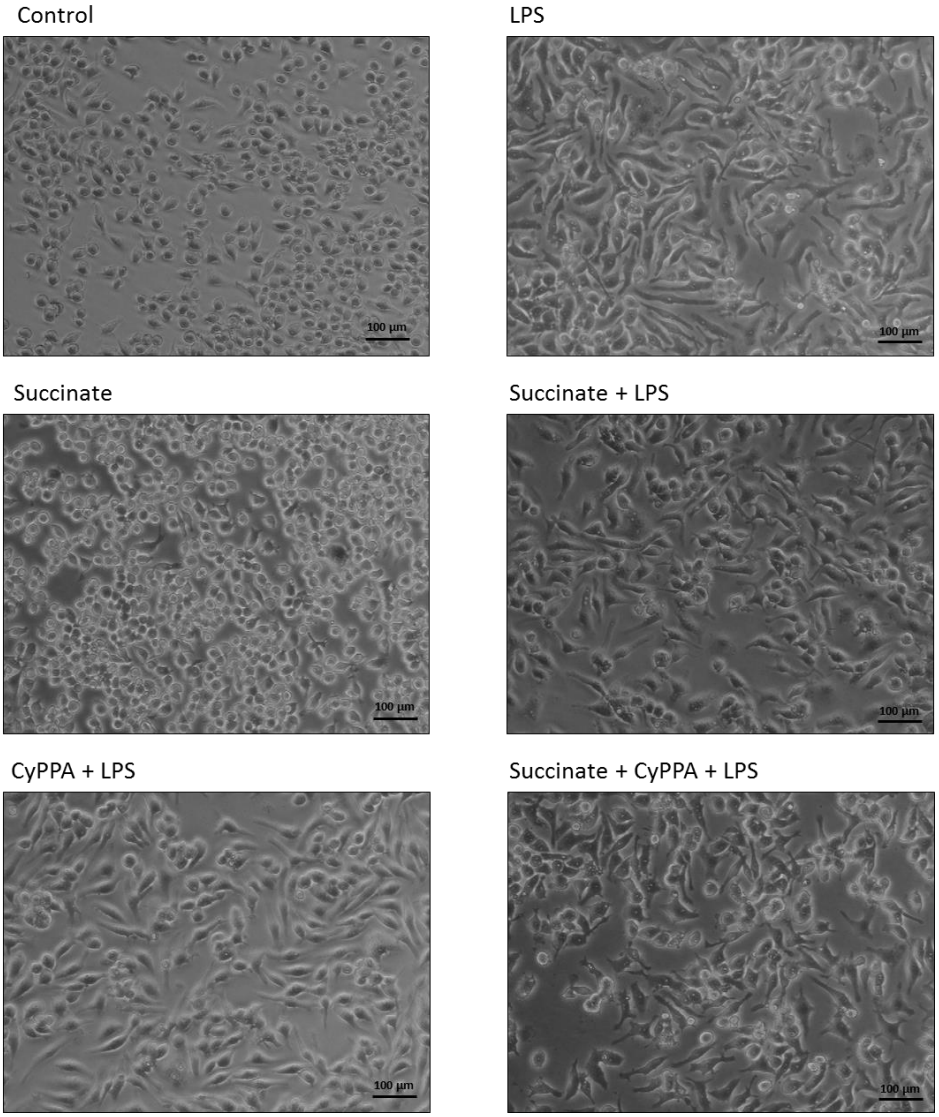

Figure 6: Macrophage viability and activation after treatment. The cells were pre-treated for 3 hours with succinate (5 mM) or CyPPA (25 µM), followed by 24 hour LPS (0.1 µg/mL) treatment, or LPS and CyPPA co-treatment.

3.2 CyPPA treatment reduced macrophage activation initiated by LPS

To assess the effect of pre-treatment with diethylsuccinate and/or CyPPA followed by LPS treatment or LPS and CyPPA co-treatment on macrophage activation, an xCELLigence real-time impedance measurement was performed. As shown in the figure below, LPS (0.1 µg/ml) treatment alone increased the cell index compared to untreated controls. Cells pre-treated 3h with succinate (5 mM) and CyPPA (25 µM), followed by co-treatment with CyPPA and LPS showed a lower the cell index compared to LPS alone. Not shown in this figure are the cells treated with succinate alone, succinate and LPS, and CyPPA and LPS.

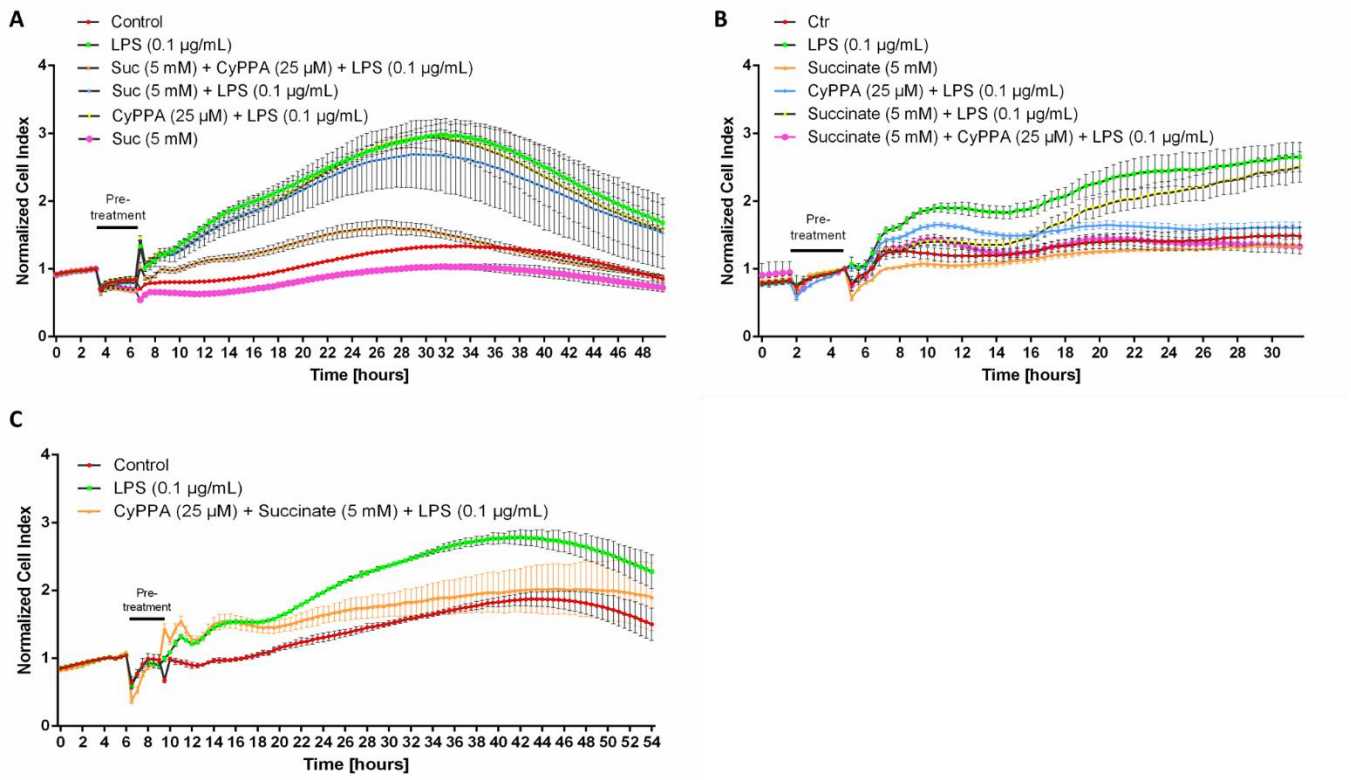

Figure 7: Real-time impedance measurements of cells treated with succinate, CyPPA, and LPS treated cells. This figure shows the normalized cell index (y-axis) of untreated, LPS (0.1 µg/mL) treated, and succinate (5 mM), CyPPA (25 µM), and LPS treated cells. The x-axis shows the time in hours. The 3 hour pre-treatment period is indicated in the figure. n=4 wells per condition.

Macrophages treated with LPS caused an increase in the cell index, as shown in fig. 7A-C, compared to control. Succinate and LPS treatment also increased the cell index in all graphs. CyPPA reduced the increase in cell index caused by succinate and LPS treatment (figure 7A-C).

LPS treatment alone increased the cell index compared to control. Succinate and LPS treatment, and CyPPA and LPS treatment increased the cell index comparable to LPS treatment alone. Macrophages treated with succinate, CyPPA, and LPS showed a reduction in the cell index, to a level similar to the control. Succinate treatment alone also shows a cell index similar to that of the control. Overall, the real-time impedance measurements suggest that LPS was able to increase the cell index, as well as succinate + LPS treatment. This increase was reduced with CyPPA, succinate, and LPS treatment. This may suggest that succinate + LPS can activate the macrophages, and succinate, LPS and CyPPA treatment can reduce this effect.

3.3 LPS treatment induced an increase in glycolysis which is diminished by CyPPA treatment

Upon activation, macrophages undergo a metabolic shift from oxidative phosphorylation to glycolysis [22], which can be analyzed using metabolic assays such as a Seahorse extracellular flux analysis. This method allows for measurement of the ECAR, which is an indication for glycolysis.

The cells were pre-treated with succinate (5 mM), CyPPA (25 µM), DMM (10 mM), and/or rotenone (0.5 µM), followed by 24h LPS (0.1 µg/mL) treatment, or 24h LPS and CyPPA co-treatment.

As shown in the figure below (figure 7) there was an immediate increase in ECAR at the start of the experiment. Another peak came after arond 700 minutes (12 hours after the start of the experiment). In this second peak, LPS increased the ECAR compared to the control. Cells treated with CyPPA and LPS showed an ECAR similar to the control. Both 5 mM succinate and 10 mM succinate caused an increase in ECAR, and succinate + LPS treatment resulted in even higher ECAR. When treated in combination with CyPPA, this increase is reduced to rates similar to the control.

The increase in ECAR may indicate increased glycolysis, as lactate is the primary source of free H^+ in the medium of cultured cells. This may imply that LPS increases glycolysis rates, whereas the presence of CyPPA decreased the LPS (+ succinate) induced glycolysis.

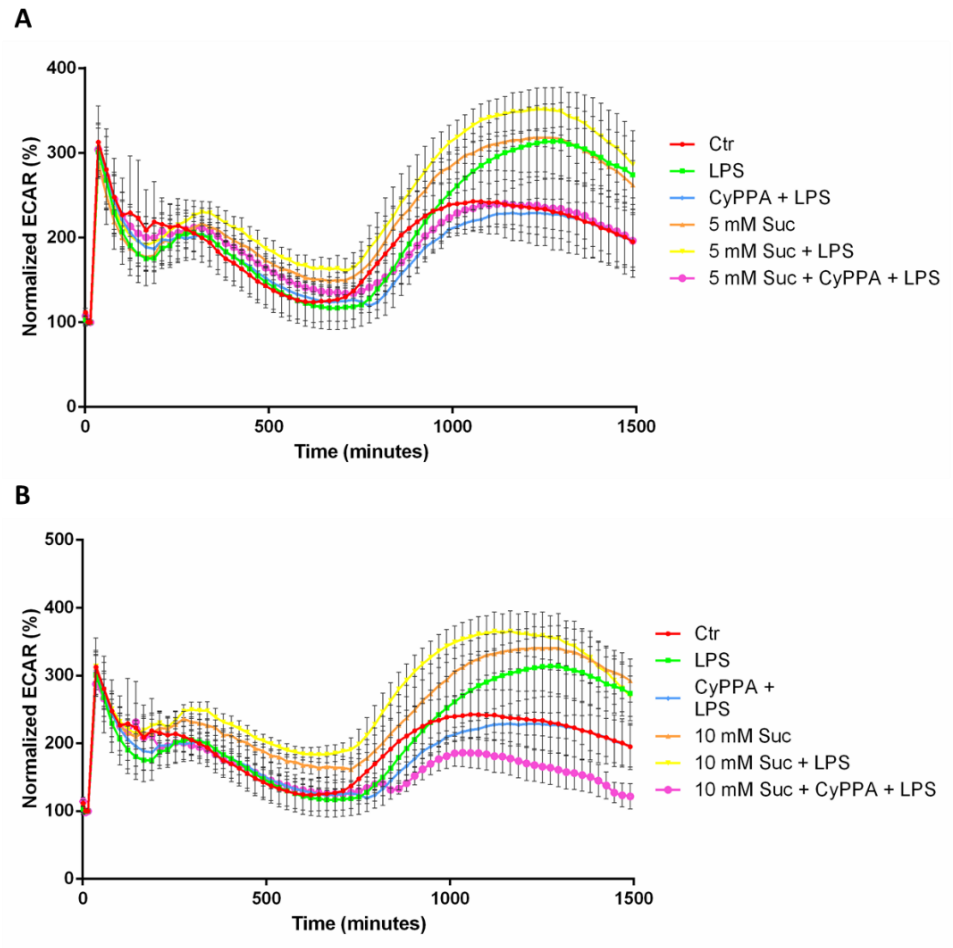

Figure 8: Seahorse assay. The y-axis shows the normalized ECAR value (normalized to protein), the x-axis shows the time in minutes. A: Seahorse assay with 5 mM succinate, CyPPA, and LPS. B: Seahorse with 10 mM succinate, CyPPA, and LPS. N=8 wells per condition (except control, N=14 wells per condition).

3.4 CyPPA may reduce the lactate production in LPS treated macrophages

Another method to study the glycolysis rate is a lactate assay, where the lactate concentrations in the medium can be measured.

The cells were pre-treated with succinate (5 mM), CyPPA (25 µM), rotenone (0.5 µM), or DMM (10 mM), followed by either no treatment, 24h LPS (0.1 µg/mL) treatment, or 24h LPS and CyPPA co-treatment. Rotenone is a CI inhibitor, and treatment with rotenone is expected to decrease macrophage activation and thereby the glycolysis rate. Treatment with DMM, a CII inhibitor, is also expected to decrease the glycolysis rate.

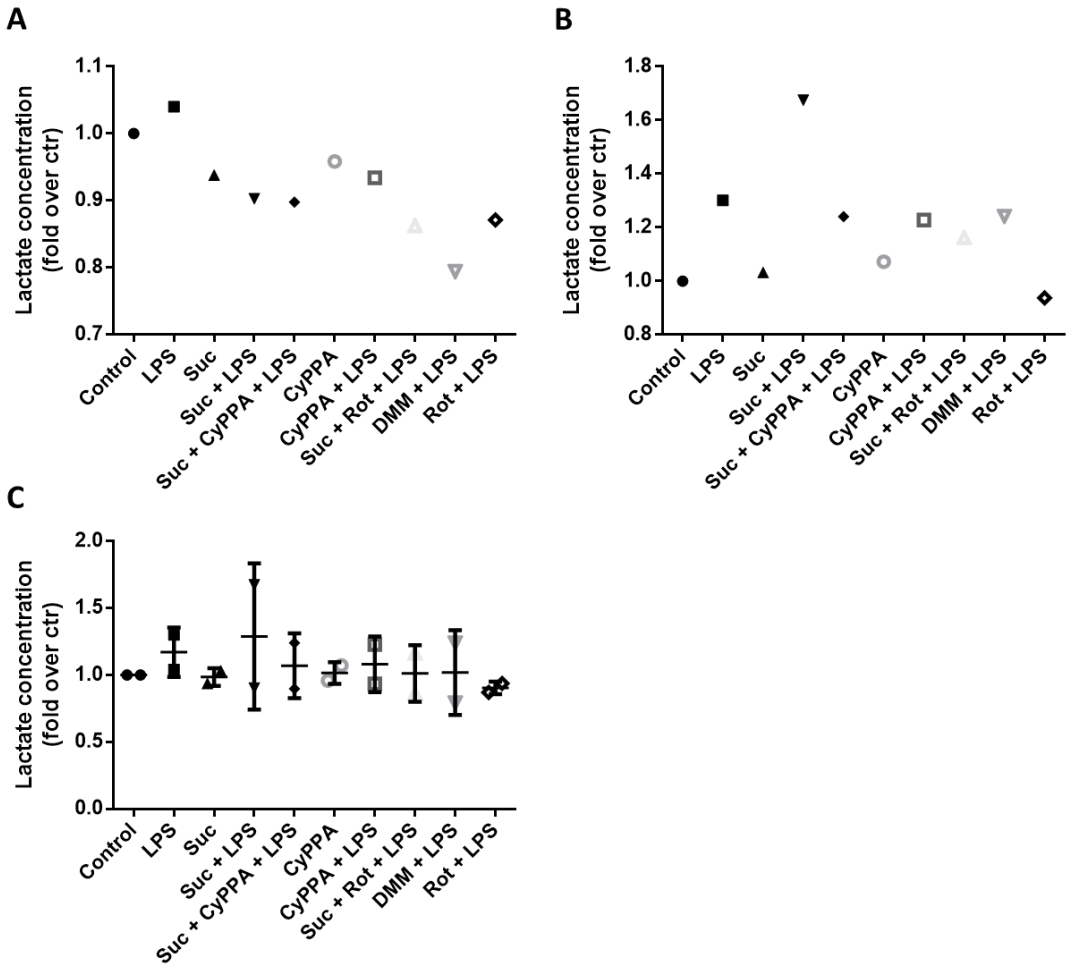

Figure 9: Lactate assay. The y-axis shows the lactate concentration in mM. A & B: Succinate, rotenone, DMM, CyPPA and LPS treated cells. N=4 wells per condition. C: Combined results. No significance was found.

Macrophages treated with LPS showed an increase in lactate production compared to control (figure 9A&B). Treatment of succinate with LPS further increased the lactate production in one experiment (figure 9B). Treatment with CyPPA, rotenone, and malonate resulted in similar lactate concentrations to the control. However, when looking at the combined results (figure 9C), there was almost no difference between the lactate concentrations of the treated and the untreated cells. The individual results may suggest that there is a difference in lactate production between treated and untreated cells. The increase in lactate production after LPS treatment, and succinate + LPS treatment, possibly indicating a shift to glycolysis in LPS treated cells, can be reduced in the presence of CyPPA.

3.5 LPS treatment significantly increased the ROS generation

With an increase in glycolysis rates, comes an increase in succinate and therefore ROS-RET, which can be measured in different ways. A MitoSOX staining was done to analyze the levels of mitochondrial ROS generated by the macrophages after LPS treatment.

The cells were treated with either 0.1, 0.5, or 1.0 µg/mL LPS for 24 hours. Compared to the control, 0.1 µg/mL LPS significantly increased the MitoSOX fluorencent signal (figure 10). However, higher concentrations of LPS reduced the signal compared to control. The results suggest that 0.1 µg/mL LPS treatment is sufficient in increasing the fluorescence signal, and therefore the superoxide generation. Higher concentrations of LPS might have a different impact on the cells, such as cell death.

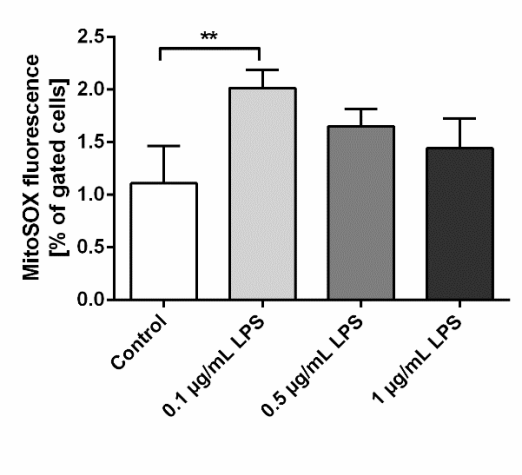

*Figure 10: MitoSOX staining on LPS treated macrophages. The y-axis shows the MitoSOX fluorescent signal. Significance is indicated with ** (p<0.01). The cells were either untreated, or treated with increasing LPS concentrations (0.1, 0.5, 1 µg/mL).*

To further analyze the ROS generated by treated macrophages, the intracellular ROS generation was analyzed using a DCF staining (figure 11).

The cells were untreated, or pre-treated with succinate (5 mM), CyPPA (25 µM), and/or rotenone (0.5 µM) for 3 hours, followed by either 24h LPS (0.1 µg/mL) treatment, or 24h LPS and CyPPA co-treatment.

The intracellular ROS generation, indicated with the fluorescent signal from DCF, significantly increased after LPS treatment compared to control (figure 11). Unexpectedly, no increase in ROS generation was observed when the cells were treated with LPS in the presence of succinate. Also, no decrease in ROS generation was observed in cells treated with CyPPA and rotenone (and succinate) compared to succinate + LPS/

However, as shown in figure 11A, succinate + CyPPA + LPS treatment significantly reduced the fluorescent signal compared to LPS, but not to succinate + LPS.

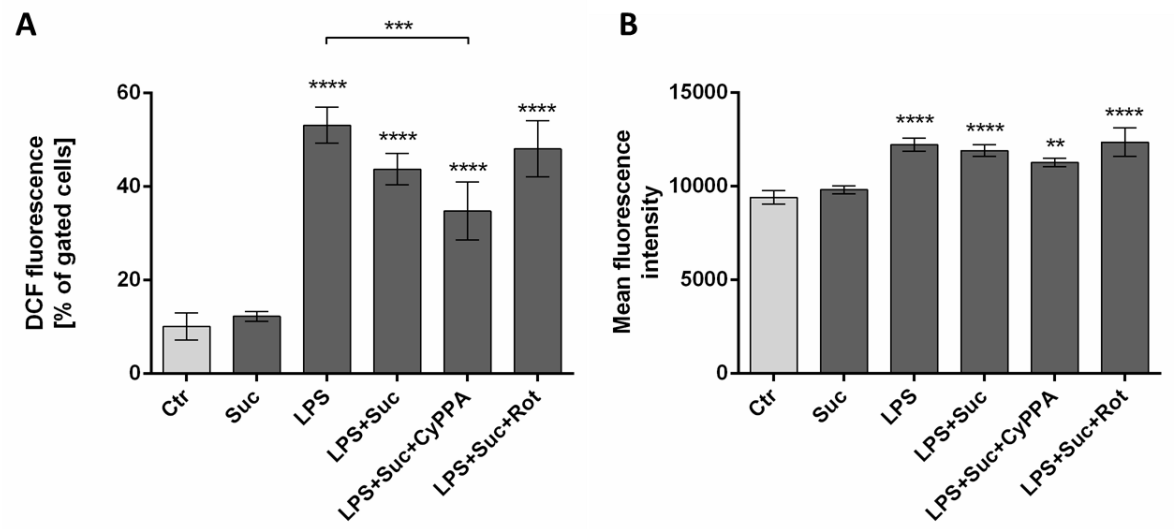

*Figure 11: DCF staining. Data are presented as mean ± SD. Significance is indicated with ** (p<0.01), *** (p<0.001), or **** (p<0.0001). A: Mean fluorescence intensity of untreated and treated cells. The cells were pre-treated with succinate (5 mM), CyPPA (25 µM), and/or 0.5 µM rotenone for 3 hours, followed by either 24h LPS (0.1 µg/mL) treatment or 24h LPS and CyPPA co-treatment. B: DCF fluorescence of treated and untreated cells. The cells were pre-treated with succinate (5 mM), CyPPA (25 µM), and/or 0.5 µM rotenone for 3 hours, followed by either 24h LPS (0.1 µg/mL) treatment or 24h LPS and CyPPA co-treatment.*

3.6 LPS-induced IL-1β expression may be decreased after CyPPA treatment

Upon macrophage activation, the IL-1β expression is increased. IL-1β is an important mediator of the inflammatory response [26].

Previous research suggested that LPS treatment can increase the IL-1β expression, which can be further increased with succinate + LPS treatment [7]. CyPPA was shown to reduce LPS-induced macrophage activation [21].

To test whether CyPPA decreases the LPS induced increase in IL-1β, RT-qPCR was done. The cells were pre-treated with succinate (5 mM), CyPPA (25 µM), rotenone (0.5 µM), or DMM (10 mM) for 3 hours, followed by either 24h or 48h LPS treatment, or 24h or 48h LPS and CyPPA co-treatment.

After 24 hour LPS treatment, the IL-1β is increased compared to the untreated cells (figure 12A-D).

As shown in figure 12A&B, the succinate + rotenone + LPS condition shows lower IL-1β expression compared to the succinate + LPS treated group. However, in other experiments (figure 12C&D) the rotenone treated group seems to further increase higher IL-1β expression. It was expected that rotenone (in combination with succinate and LPS) reduces the IL-1β expression compared to succinate + LPS, but this was not found in all experiments.

Succinate + CyPPA + LPS decreases the expression compared to LPS alone, but is similar to succinate + LPS. CyPPA + LPS shows lower expression levels compared to LPS alone. Succinate + DMM + LPS shows an increase in the expression of IL-1β compared to control (figure 12A).

The combined results show no significant differences between the treated and the untreated cells (figure 12E). There is a lot of variation within the LPS and succinate + rotenone + LPS treated groups. On the contrary, the succinate + CyPPA + LPS treated cells show very little variance. The mRNA expression level of this group is very close to the control. When looking at the individual experiments, this treatment always results in lower IL-1β expression levels compared to LPS treatment alone. Although the trends are similar in all individual experiments, the relative mRNA expression level highly varies between the experiments.

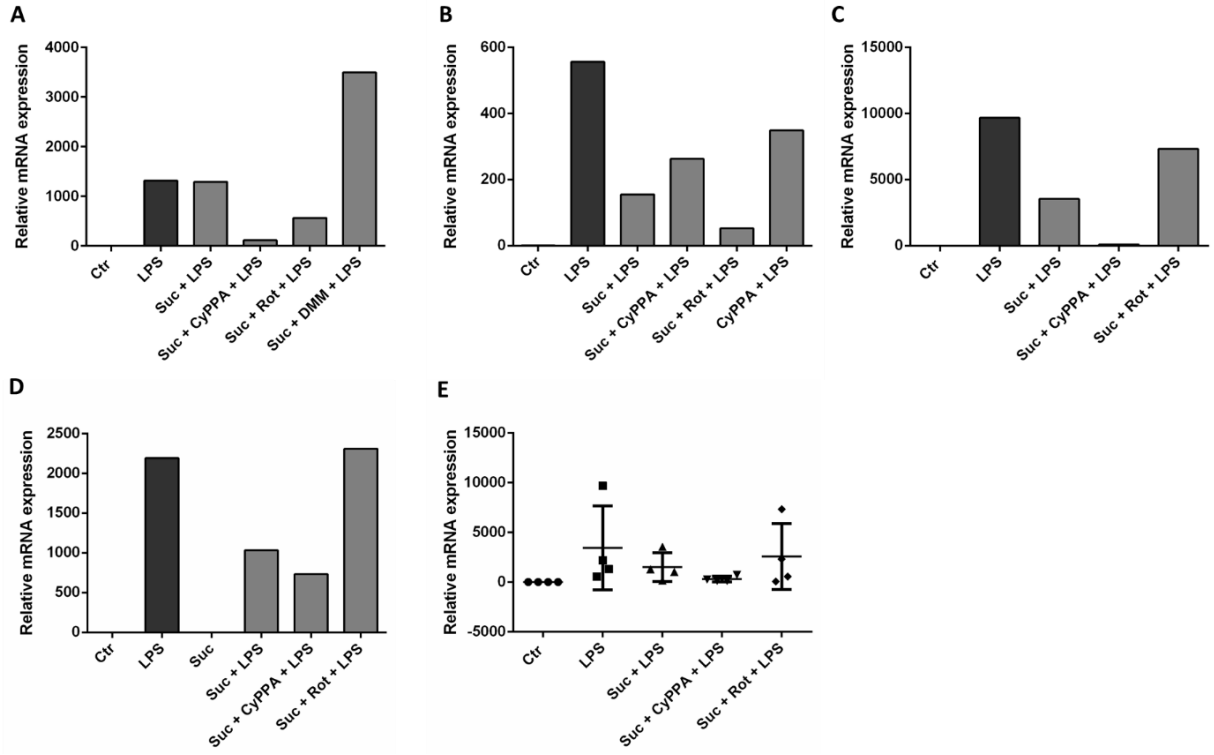

Figure 12: IL-1β expression of 5 mM succinate treated cells after 24 hour LPS. The y-axis shows the relative mRNA expression. A: Succinate (5 mM), CyPPA (25 µM), rotenone (0.5 µM) , DMM (10 mM), and LPS (0.1 µg/m:, 24h) treated cells. B,C,D: Succinate (5 mM) , CyPPA (25 µM), rotenone (0.5 µM), and LPS (0.1 µg/mL) treated cells. E: Combined results. No significance was found between the treated and untreated groups.

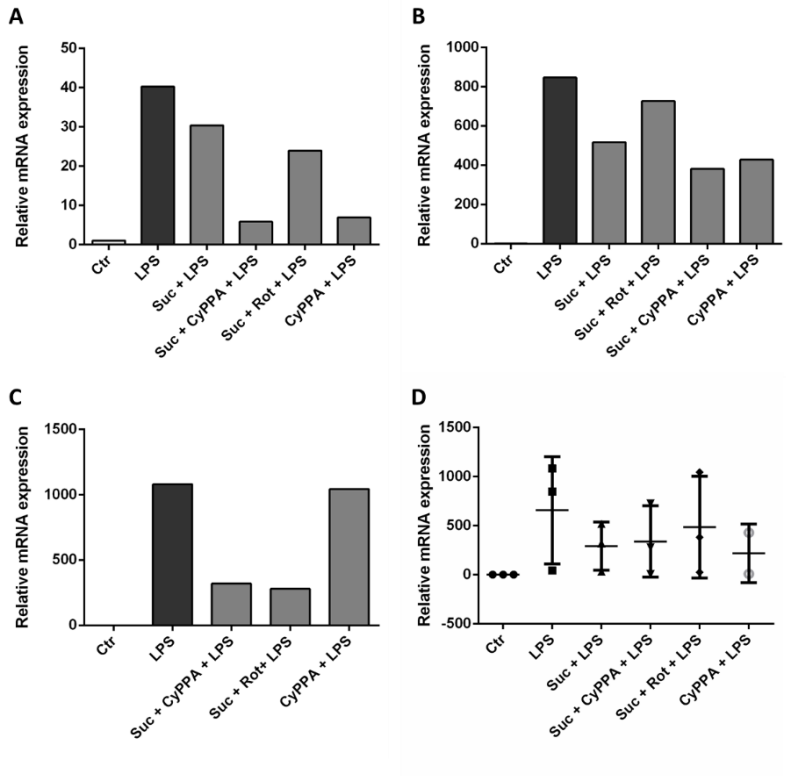

Figure 13: IL-1β expression of 5 mM succinate treated cells after 48 hour LPS. The y-axis shows the relative mRNA expression. A-C: Succinate (5 mM), CyPPA (25 µM), rotenone (0.5 µM), and LPS (0.1 µg/ml, 48h) treated cells. D: Combined results. No significance was found between the treated and untreated groups.

As shown in figure 13A-C, 48 hour LPS treatment increases the IL-1β expression compared to control. The succinate + LPS treatment group shows the same effect.

Compared to succinate + LPS, the succinate + CyPPA + LPS treatment was able to reduce the IL-1β expression (figure 13A&B). But, in the combined results (figure 13D), this effect is no longer visible. Succinate + rotenone + LPS was able to reduce the expression in one experiment (figure 13A), but not in another (figure 13B).

When the results are combined, there are no significant differences between the treated and untreated cells (figure 13D). Contrary to the results in figure 12E, there is variance in the mRNA expression levels of all treated groups. But, similar to the 24h LPS results, there is a trend in the individual results: succinate + CyPPA + LPS treatment reduces the IL-1β expression compared to succinate + LPS treatment.

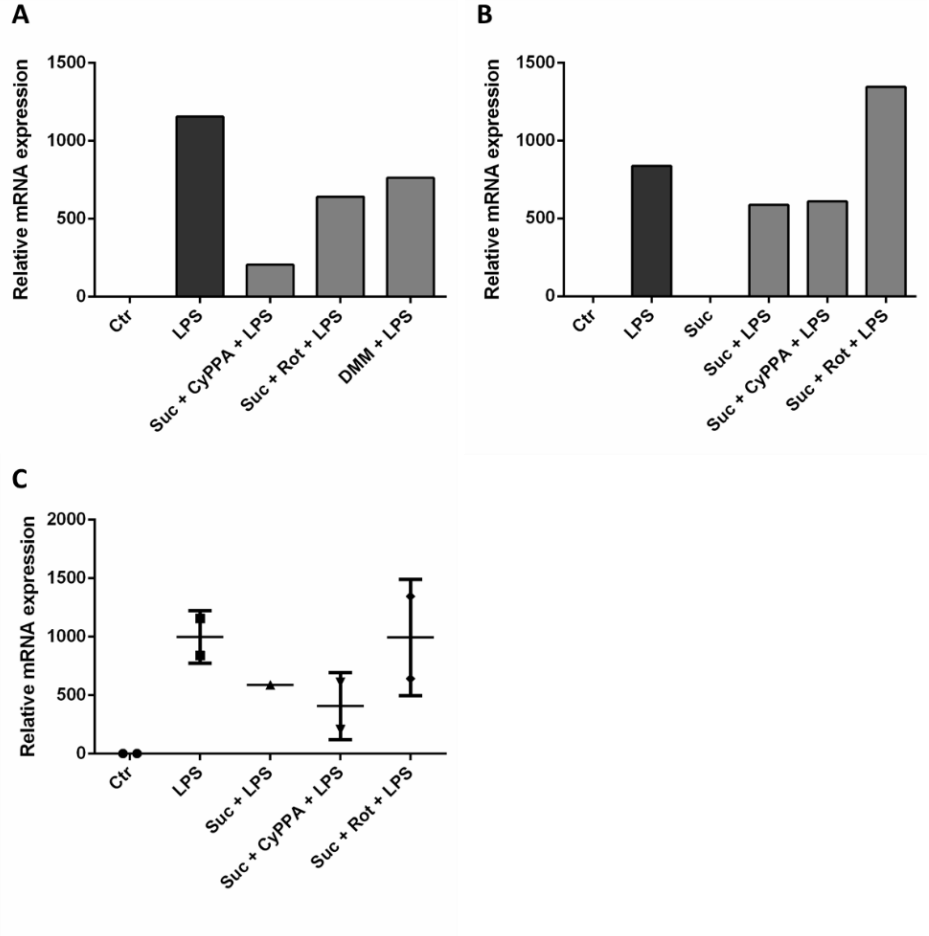

Figure 14: IL-1β expression in 10 mM succinate treated cells after 24 hours LPS. The x-axis shows the treatments, the y-axis shows the IL-1β expression. A & B: Relative mRNA expression in succinate (10 mM), CyPPA (25 µM), rotenone (0.5 µM), DMM (10 mM), and LPS (0.1 µg/mL) treated macrophages. C: Combined results.No significance was found between the treated and untreated groups.

24 hour LPS treatment increased the relative expression level of IL-1β compared to the control (figure 14A,B). Succinate + LPS treatment also increased the IL-1β expression (figure 14B). The mRNA value of succinate + LPS treatment in the graph shown in figure 15A was excluded. Succinate + rotenone + LPS treatment decreased the expression level compared to LPS in one experiment (figure 14A), but not in another experiment (figure 14B). Succinate + CyPPA + LPS decreases the IL-1β expression compared to LPS in both experiments, but was similar to succinate + LPS (figure 14B). Succinate + rotenone + LPS decreased the expression in one experiment (figure 14A), but not in the other (figure 14B). DMM + LPS treated cells also show a decrease in IL-1β expression.

In the combined results, no significant differences were found between the untreated and treated groups (figure 14C). However, it does look like LPS generally increases the IL-1β expression compared to control. Succinate + LPS treatment also appears to increase the relative expression compared to control, but the expression is not higher than LPS alone. Succinate + CyPPA + LPS treatment did not reduce the IL-1β expression compared to succinate + LPS. Succinate + rotenone + LPS treatment has a high variability between the experiments, and the mean relative IL-1β expression seems higher than succinate + LPS.

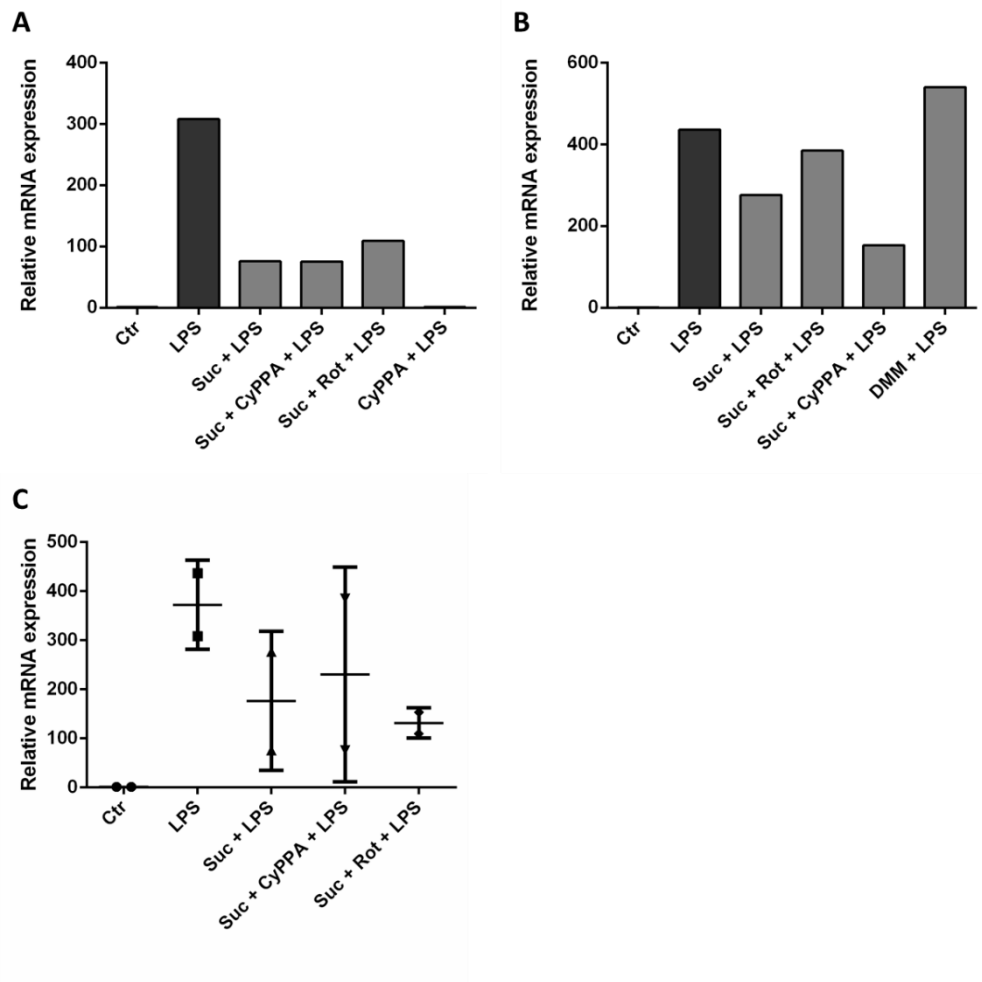

Figure 15: IL-1β expression in 10 mM succinate treated cells after 48 hours LPS. The x-axis shows the treatments, the y-axis shows the IL-1β expression. A & B: Relative mRNA expression in succinate (10 mM), CyPPA (25 µM), rotenone (0.5 µM), DMM (10 mM), and LPS (0.1 µg/mL) treated macrophages. C: Combined results. No significance was found between the treated and untreated groups.

As shown in the figure above, the IL-1β expression increases after 48h LPS treatment compared to the untreated cells (figure 15A,B). Succinate + LPS treatment also increases the expression level, but not as much as LPS alone. Succinate + CyPPA + LPS and succinate + rotenone + LPS treatment decreases the expression of IL-1β compared to LPS alone, but not succinate + LPS treatment. DMM + LPS treated increases the expression level even higher than LPS alone (figure 15B).

When looking at the combined results, no significant differences were found between the groups. However, it is apparent that LPS increases the IL-1β expression compared to the control. The succinate + rotenone + LPS treated groups seems to reduce the IL-1β expression compared to succinate + LPS.

3.7 SK2 and SK3 channels were present in RAW macrophages

To test the presence of SK2 and SK3 channels along with the efficiency of the designed SK2 and SK3 primers in RAW macrophages, a RT-qPCR was executed. In case of good primer efficiency, the PCR products were tested on an agarose gel.

SK2 and SK3 primers were designed, and tested on untreated (control) samples. The primer efficiency was analyzed, alongside the quality controls. If the primer efficiency was greater than 1.8 and the samples passed the quality controls, the PCR products were analyzed on an agarose gel.

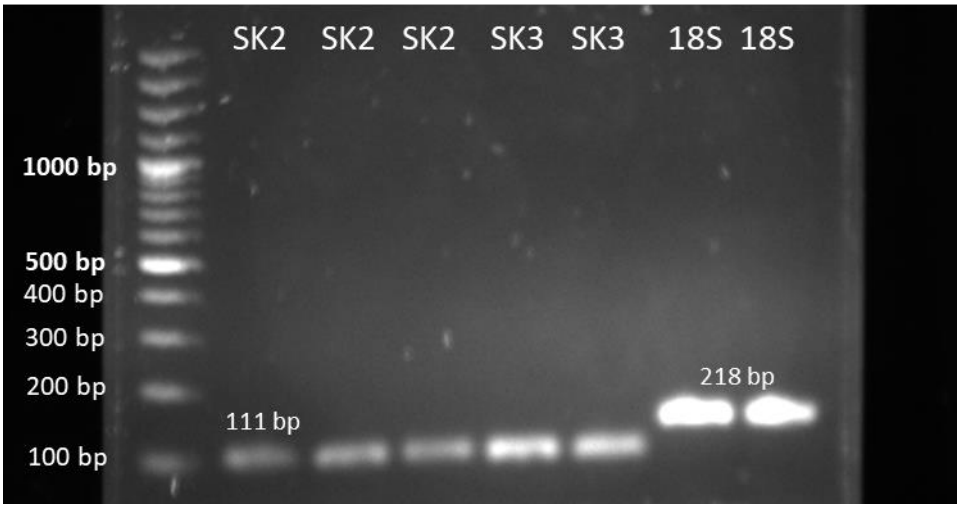

Figure 16: SK2 and SK3 primer testing. This figure shows the PCR product size of SK2, SK3, and 18S primers. The primers were tested on untreated cells. The ladder is in the left of the figure, indicated with the number of basepairs. The product size is also shown in the figure.

The expected product size of SK2 and SK3 is 111 bp, which can be seen in the figure above. The 18S primers, which were used as a control, have an expected product size of around 218 bp. In the gel there is a strong band for 18S, however the band seems slightly lower than 200 bp.

3.8 HIF-1α appeared to be degraded in untreated and treated macrophages

A recent study showed that succinate and LPS treatment induces not only IL-1β expression, but also boosts hypoxia inducible factor 1α (HIF-1α) protein levels [7]. HIF-1α is a regulator of the cellular and systemic response to hypoxia, by activating the transcription of genes involved in energy metabolism, angiogenesis, apoptosis and other genes [25].

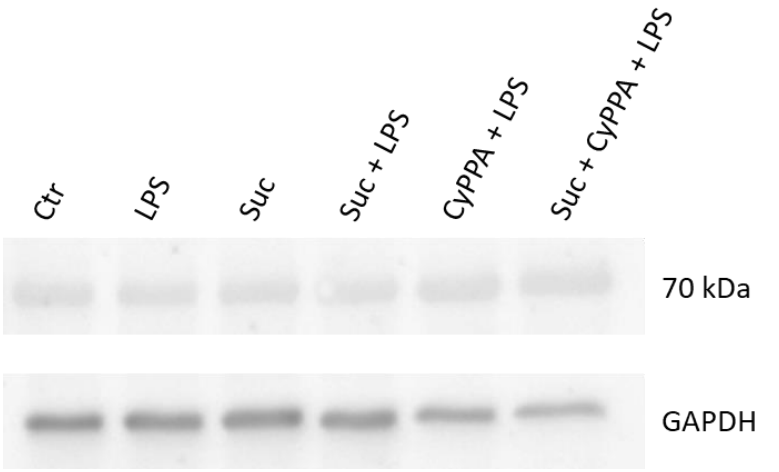

Figure 17: Western Blot for HIF-1α. Whole cell lysates were analyzed by western blotting for HIF-1α and GAPDH. The cells were pre-treated with succinate (5 mM) +/- CyPPA (25 µM), followed by 24 hour LPS treatment (+/- CyPPA) (0.1 µg/mL).

The HIF-1α protein is expected to have a size around 120 kDa. As shown in the result below, the blotting for HIF-1α resulted in a band at 70 kDa, independent of the treatments. This is likely due to degradation of the protein, as this may result in a band at 70 kDa [23].

3.9 CyPPA treatment reduced phagocytic activity in macrophages

As previously described, LPS can activate the macrophages and also lead to a significant increase in phagocytosis [27].

The cells were pre-treated for 3 hours with succinate (5 mM) ± CyPPA (25 µM), followed by 3 days of LPS (± CyPPA) treatment, including pHrodo Red S aureus BioParticles.

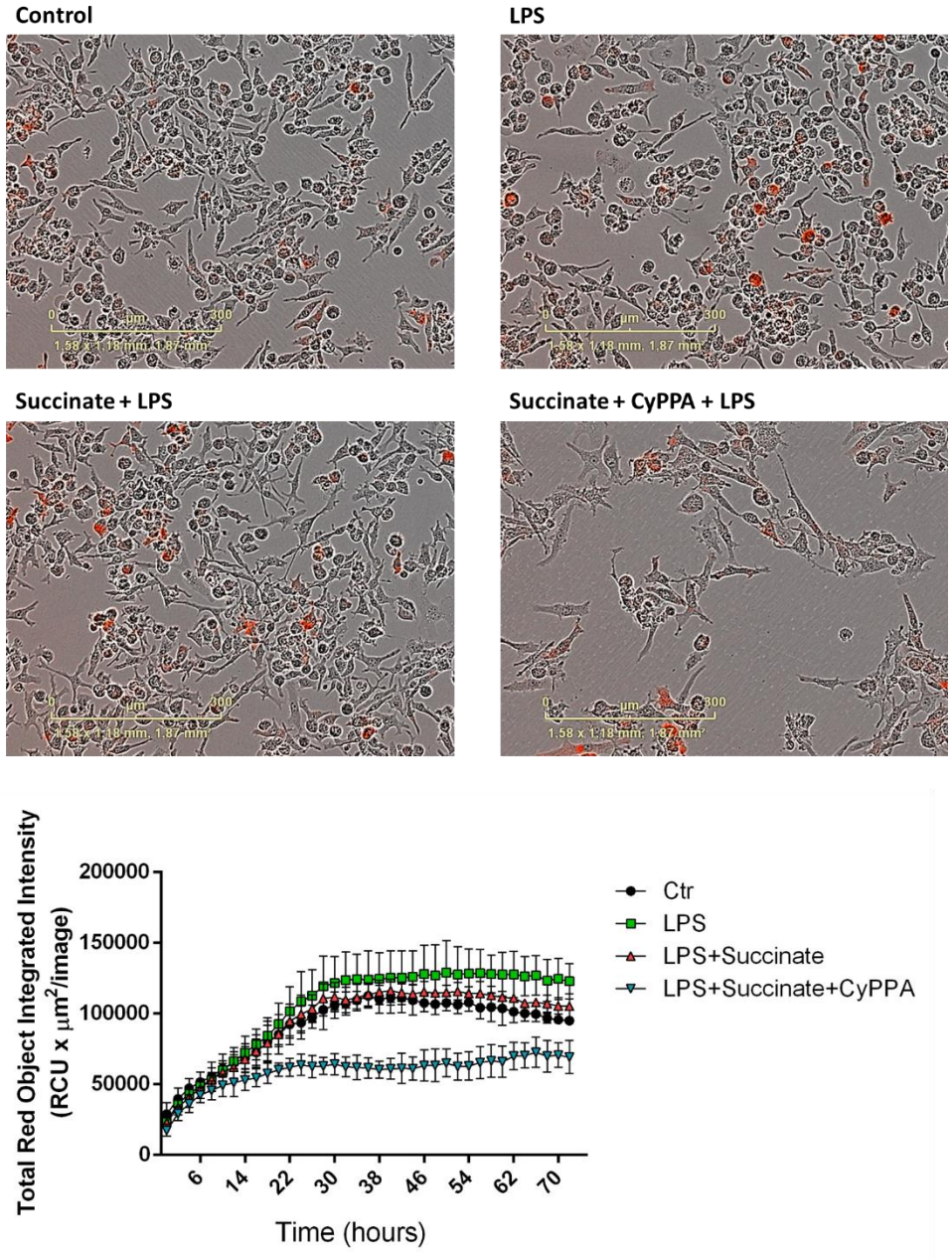

Figure 18: IncuCyte on phagocytosis. The top four panels show the final images (taken on day 3). The cells were pre-treated with succinate (5 mM) +/- CyPPA (25 µM), followed by 72 hour LPS treatment (+/- CyPPA) (0.1 µg/mL), in combination with 1 µg/0.1 mL pHrodo Red S aureus BioParticles Conjugate.

As shown in the top panels of figure 18, The LPS treated cells show more phagocytosis than the control. The succinate + LPS treated group does not seem to show more phagocytosis than LPS alone, but it is higher than the control. The succinate + CyPPA + LPS treatment group shows less cells, and also less phagocytosis. The findings from the pictures are confirmed by the graph. The data may imply that SK channel activation reduces phagocytosis, and possibly the pro-inflammatory response.

4.0 Conclusion and Discussion

The aim of this research was to study the role of SK channel activator CyPPA on succinate-induced ROS-RET in macrophages. It was expected that LPS treatment induces the pro-inflammatory response in macrophages, therefore leading to an increase in the expression of pro-inflammatory cytokines, a switch in metabolism, and an increase in ROS generation. It was expected that LPS treatment in combination with succinate is able to further increase the effect of LPS alone, possibly via RET. Also, it was expected that CyPPA treatment (in combination with succinate and LPS) will attenuate ROS-RET and also reduce the expression of pro-inflammatory response.

It was expected that LPS will activate macrophages, characterized by, among others, a change in morphology. As shown in figure 6, LPS treated macrophages showed a change in morphology, possibly indicating activation. Recent studies showed that succinate + LPS treatment would further activate the macrophages, an effect which can be attenuated with CyPPA [7]. In the previously mentioned figure, there did not seem to be more activation in the succinate + LPS treated cells, or less activation in the succinate + CyPPA + LPS treated cells. The figure did show that succinate alone has no effect on the morphology.

Despite the clear change in morphology after LPS treatment, it was difficult so see any small changes in morphology in the other treated groups. Microscopy is a great measure for clear changes in morphology, but not for subtle changes. Therefore, other analyses had to be done in order to determine macrophage activation.

An xCELLigence assay was done to further determine macrophage activation in both untreated and treated cells. The results, shown in figure 7, showed that LPS caused an increase in cell index, which could be reduced by succinate + CyPPA + LPS treatment. This increase in cell index is likely due to the morphological change, therefore the results may indicate that LPS activated the macrophages. However, contrary to the expectations, succinate + LPS treatment did not induce more activation. Although xCELLigence data may indicate a certain response, it does not prove it without the proper validation.

When macrophages become activated, there is also a switch in metabolism, from oxidative phosphorylation to glycolysis. The rate of glycolysis can be measured using many metabolic assays, including a lactate assay, and a Seahorse assay.

The results from the lactate assay further confirmed that LPS treatment induced macrophage activation, which could be reduced by succinate + CyPPA + LPS treatment (figure 9). However, succinate + LPS was expected to further increase macrophage activation, which it did not. Also, the increase by LPS and decrease by CyPPA were visible in the individual results, but not in the combined results. There was a lot of variation in the lactate concentrations between the individual results, which may have resulted in no visible trends in the combined results.

The results from the Seahorse assay showed similar trends as the lactate assay. LPS treatment caused an increase in ECAR, and therefore the glycolysis rate (figure 8). Contrary to the results of the lactate assay, succinate + LPS treatment further increased the ECAR, as was hypothesized. Succinate + CyPPA + LPS treatment reduced the ECAR to levels similar to the control. 10 mM succinate + CyPPA + LPS treatment even reduced the ECAR to levels below the control.

The results from the metabolic assays suggest that LPS is able to increase the glycolysis rate, with succinate + LPS treatment possibly increasing this rate even further, as could be seen with the Seahorse assay, but not with the lactate assay.

Besides the switch in metabolism, it was expected that the ROS generation in LPS treated macrophages would increase, with succinate + LPS treatment increasing this even more due to ROS-RET. Therefore, flow cytometry was done with MitoSOX or DCF. MitoSOX specifically indicates superoxide, whereas DCF detects cellular ROS.

The MitoSOX staining was done on untreated and LPS treated cells. Figure 10 showed that 0.1 µg/mL LPS treatment significantly increased the superoxide generation, whereas higher concentration of LPS only seemed to reduce this effect. The results may indicate that 0.1 µg/mL LPS is the optimal concentration for stimulating ROS generation in RAW macrophages. Higher LPS concentrations may lead to an increased superoxide generation, but free superoxide radicals are rapidly converted to H_2O_2 and O_2. This may explain why the higher LPS concentrations did not lead to an increase in MitoSOX fluorescence.

The DCF staining results showed that combinational treatment with CyPPA was able to reduce the ROS generation, possibly by blocking CI during succinate induced RET (figure 11). However, this reduction in ROS generation was not comparable to the level of the control.

Although the DCF staining gave a good result, one of the limitations of DCF stainings is that it does not directly react with H_2O_2 to form a fluorescent product [28]. It is therefore not a direct measure of H_2O_2. The probe preferred by most researchers when investigating ROS generation is Amplex (Ultra)Red [29]. It reacts with HRP-H_2O_2 to form a fluorescent compound. This compound is stable and able to detect low amounts of H_2O_2.

Next to ROS generation, activated macrophages increase the expression of pro-inflammatory cytokines, such as IL-1β [7]. The expression levels of IL-1β were analyzed using RT-qPCR. The results showed varying IL-1β expression after the treatments (figure 12-15). In general, LPS increased the expression, indicating that the macrophages have become activated. DMM and rotenone treatments showed different results. Succinate + DMM + LPS increased the expression, whereas DMM + LPS decreased the expression, possibly indicating that succinate is involved in macrophage activation. However, succinate + LPS treatment did not further increase the expression of IL-1β, contrary to the expectations. The rotenone treated cells showed a lot of variation, but in general succinate + rotenone + LPS treatment reduced the mRNA expression. In the 10 mM succinate treated group, there seemed to be more of a difference between the untreated and treated cells, but no significance was found. Concluding from the PCR data, LPS was able to increase the IL-1β expression, which was reduced with co-treatment with CyPPA.

The presence of SK2 and SK3 channels were also tested with RT-qPCR (figure 16). As the result showed, both SK2 and SK3 were present in the samples. In future research the effect of succinate, CyPPA, and LPS treatment on SK channels can be tested.

Next, a Western Blot for HIF-1α was done. HIF-1α protein levels were shown to be increased after LPS treatment [7]. It was expected that after succinate and LPS treatment the protein levels of HIF-1α would be even higher, possibly due to ROS-RET and the increase in the pro-inflammatory response. As shown in the results, there was a band at 70 kDa, which might indicate degraded HIF-1α. In the future, a different lysis buffer should be used to prevent degradation of HIF-1α.

Finally, a phagocytosis assay was done. The results, shown in figure 18, suggest that combinational treatment with CyPPA was able to reduce the phagocytic activity of the cells, but also seemed to reduce the cell viability as a reduction in cell number was visible. Nevertheless, the results may suggest that SK channel activation is able to reduce the phagocytic activity of the macrophages, thereby possibly reducing the pro-inflammatory response.

In summary, LPS treatment was able to activate the macrophages in 24 hours, inducing a change in morphology, and a metabolic shift to glycolysis. Also, the expression of IL-1β was increased after LPS treatment. Even though succinate + LPS treatment did not further increase the effect of LPS treatment alone, succinate + CyPPA + LPS treatment was able to reduce the effect that LPS treatment had on the cells. This last observation matched the hypothesis that SK channel activation will attenuate ROS-RET and will reduce the pro-inflammatory response. Only the Seahorse assay showed that succinate + LPS treatment was able to cause a higher increase in the glycolysis rate, compared to LPS treatment alone. This may imply that succinate + LPS treatment does increase macrophage activation, but this effect may only be visible in the metabolic shift in live cells. Although the lactate assay should give similar results, the Seahorse assay is a real-time assay with living cells, whereas the lactate assay only measures the lactate concentrations in the medium after 24 hours.

These findings may be important in the near future, as ROS-RET is involved in cell death occurring during reperfusion [13]. Finding means to reduce ROS-RET may allow for prevention of excessive cell death in disease, but also in case of a heart attack or stroke. Another major situation where reperfusion plays a role, is transplantation of organs. Being able to reduce oxidative cell damage and death by attenuating ROS-RET, will help maintain organ function and viability.

In the future, it is important to further research ROS-RET in macrophages by the analysis of more pro-inflammatory markers, but also anti-inflammatory markers such as IL-1, IL-6, IL-10, IL-11, and IL-13. Western Blot and ELISA will give insight in protein levels and the presence of the cytokines, respectively. An Amplex UltraRed assay can give more insight in the mitochondrial H_2O_2 production.

Also, it will be of importance to test pro- and anti-inflammatory markers in different cell lines, as RAW macrophages are murine cells. The next step would be to test the treatments in a human macrophage cell line.

5.0 References

Image on front page: Close-up of a mitochondrion. Photo by: Keith Porter/Science Source. Retrieved from: https://news.cals.wisc.edu/2016/12/27/the-mysteries-of-mitochondria-functions-behavior-and-implications-for-human-health/

Figure 1: Polyzos, A. A., & McMurray, C. T. (2017). The chicken or the egg: mitochondrial dysfunction as a cause or consequence of toxicity in Huntington's disease. Mechanisms of ageing and development, 161, 181-197.

Figure 2: Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. Cytokine, 42(2), 145-151.

Figure 3: Adapted from: Scialò, F., Fernández-Ayala, D. J., & Sanz, A. (2017). Role of mitochondrial reverse electron transport in ROS signaling: potential roles in health and disease. Frontiers in physiology, 8, 428.

Figure 4: SK channel, Wikipedia

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6.0 Appendices

6.1 Supplementary figures

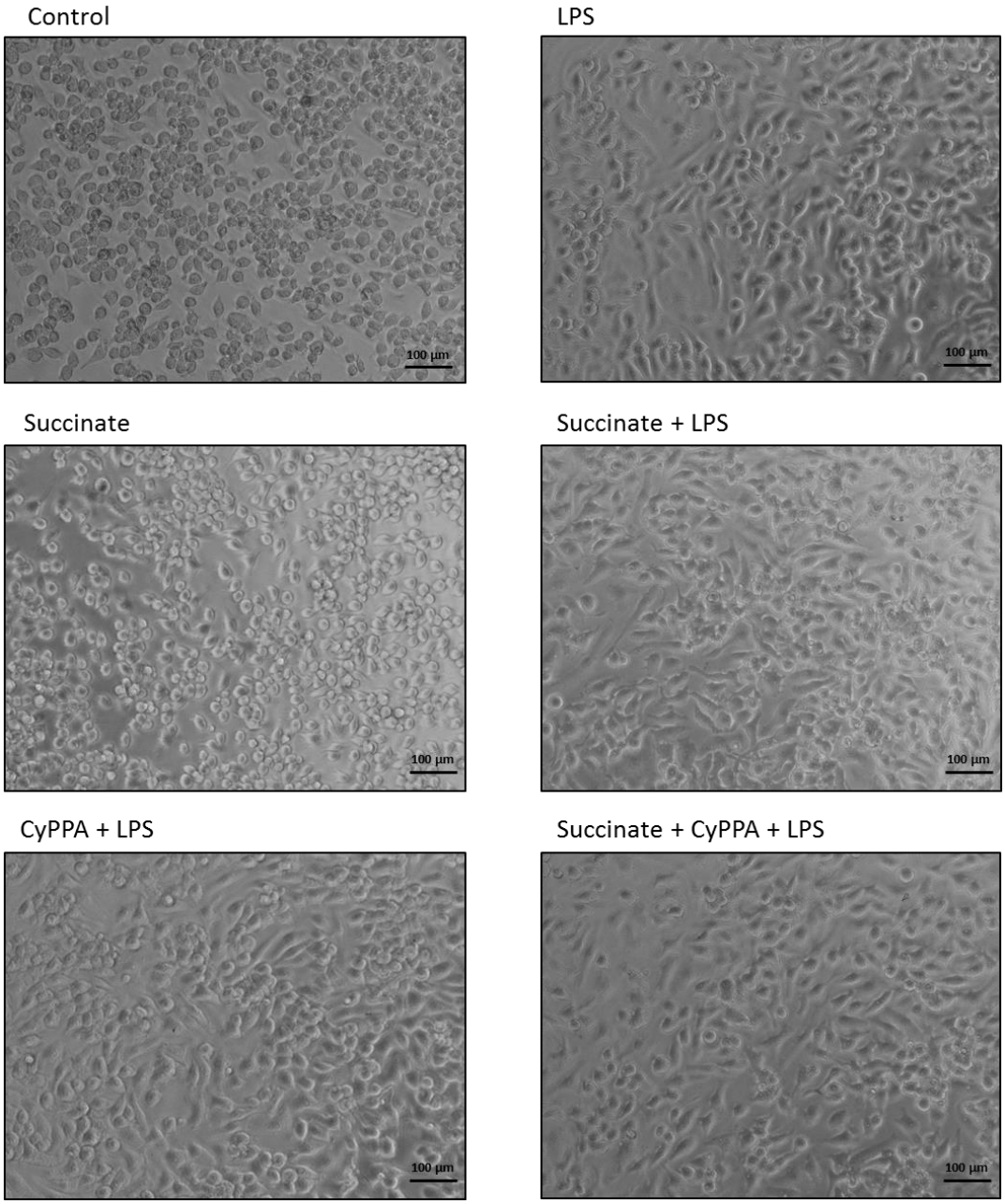

Figure S1: Macrophages treated with succinate, CyPPA, and LPS. The cells were pre-treated for 3 hours with 5 mM dimethylsuccinate and/or 25 µM CyPPA, followed by 24 hour 0.1 µg/mL LPS treatment. The pictures were taken at a 100X magnification. The scale bar is 100 µM .

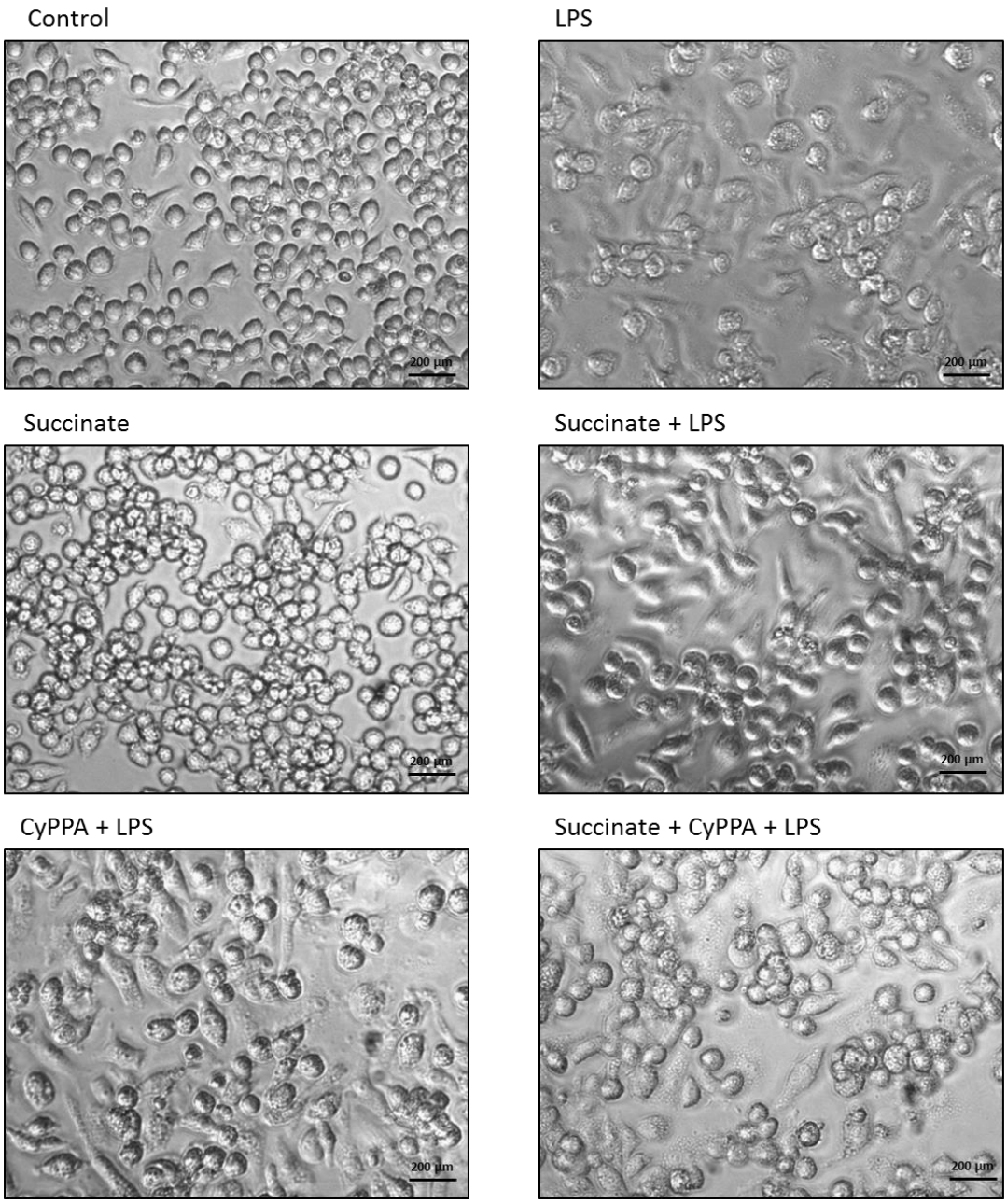

Figure S2: Macrophages treated with succinate, CyPPA, and LPS. The cells were pre-treated for 3 hours with 5 mM dimethylsuccinate and/or 25 µM CyPPA, followed by 48 hour 0.1 µg/mL LPS treatment. The pictures were taken at a 100X magnification.

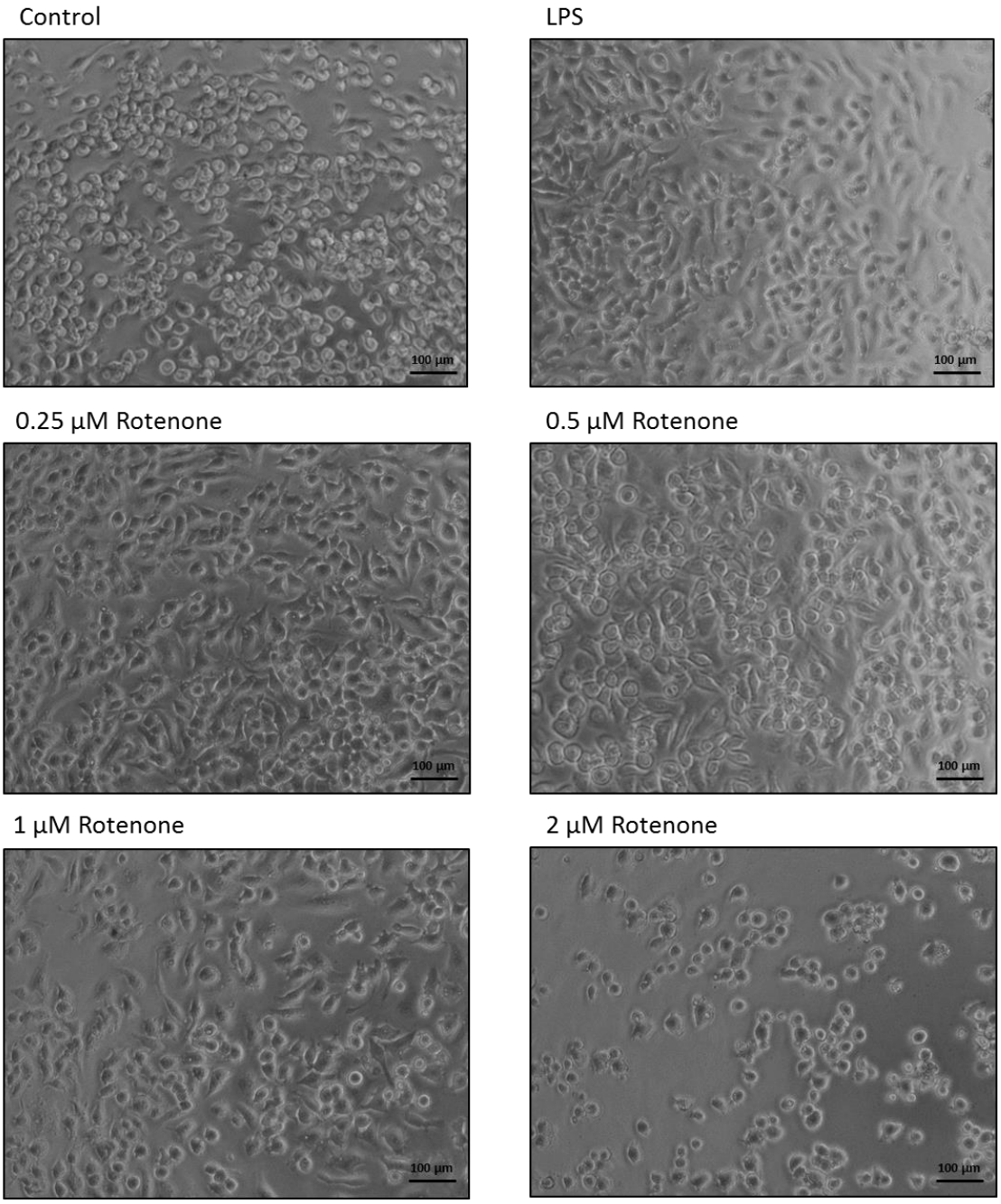

Figure S3: Macrophages treated with increasing rotenone concentrations. The cells were pre-treated for 3 hours with increasing rotenone concentrations (0.25 – 2 µM). Pictures were taken at a 100X magnification.

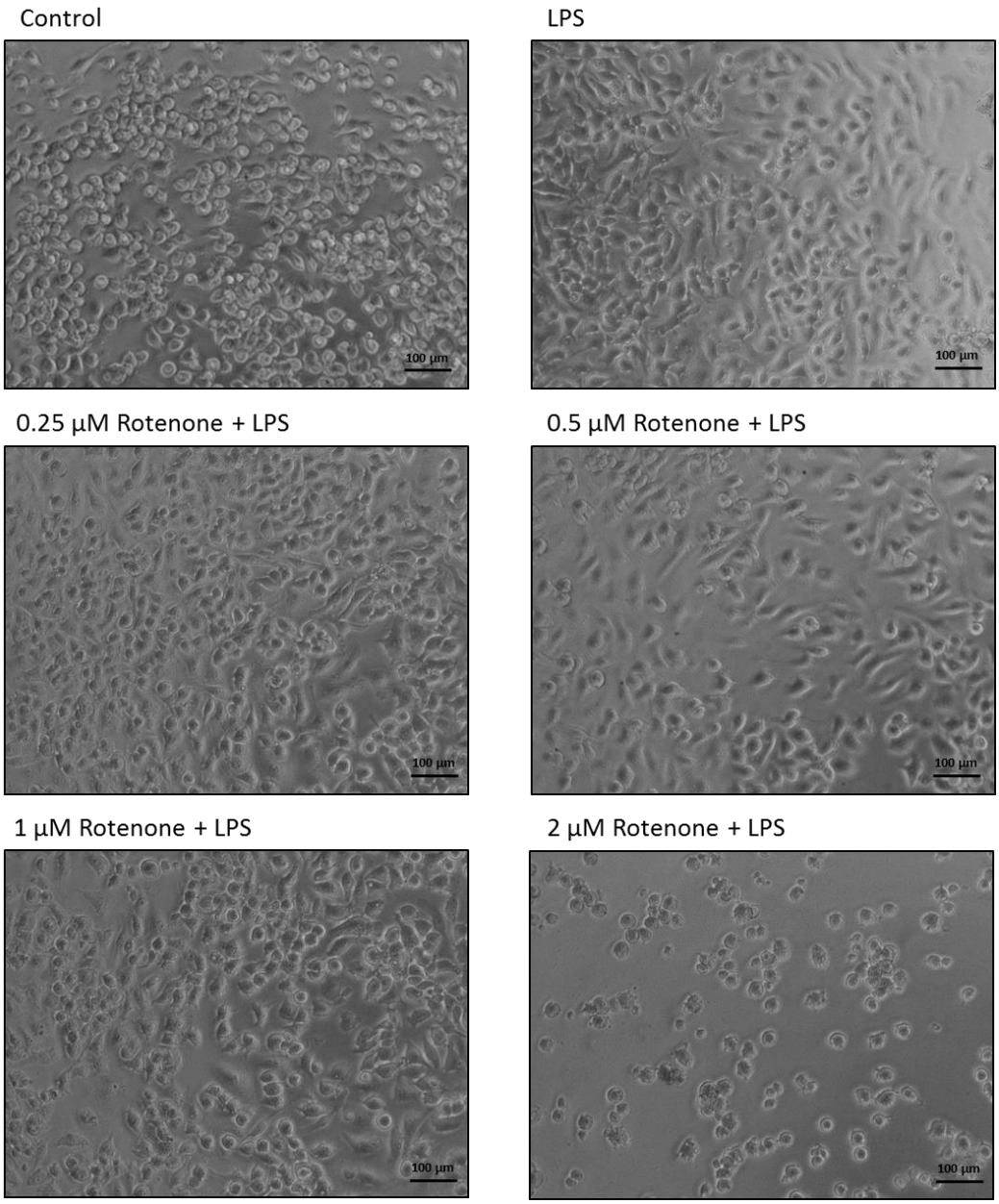

Figure S4: Macrophages treated with increasing rotenone concentrations and LPS. The cells were pre-treated for 3 hours with increasing rotenone concentrations (0.25 – 2 µM), followed by 24 hour 0.1 µg/mL LPS treatment. The pictures were taken at a 100X magnification.

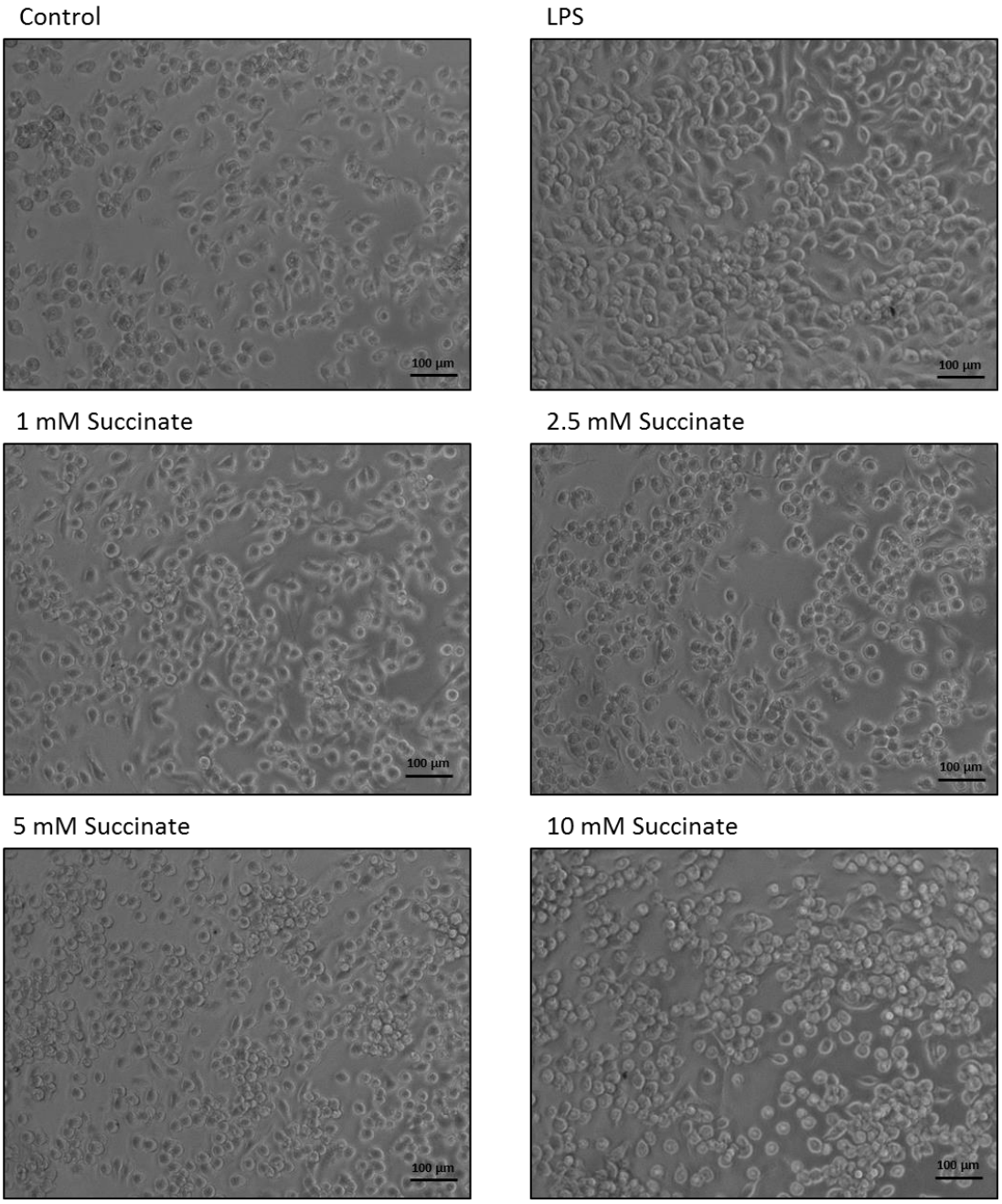

Figure S5: Macrophages treated with increasing succinate concentrations. The cells were pre-treated with increasing dimethylsuccinate concentrations (1 – 10 mM).

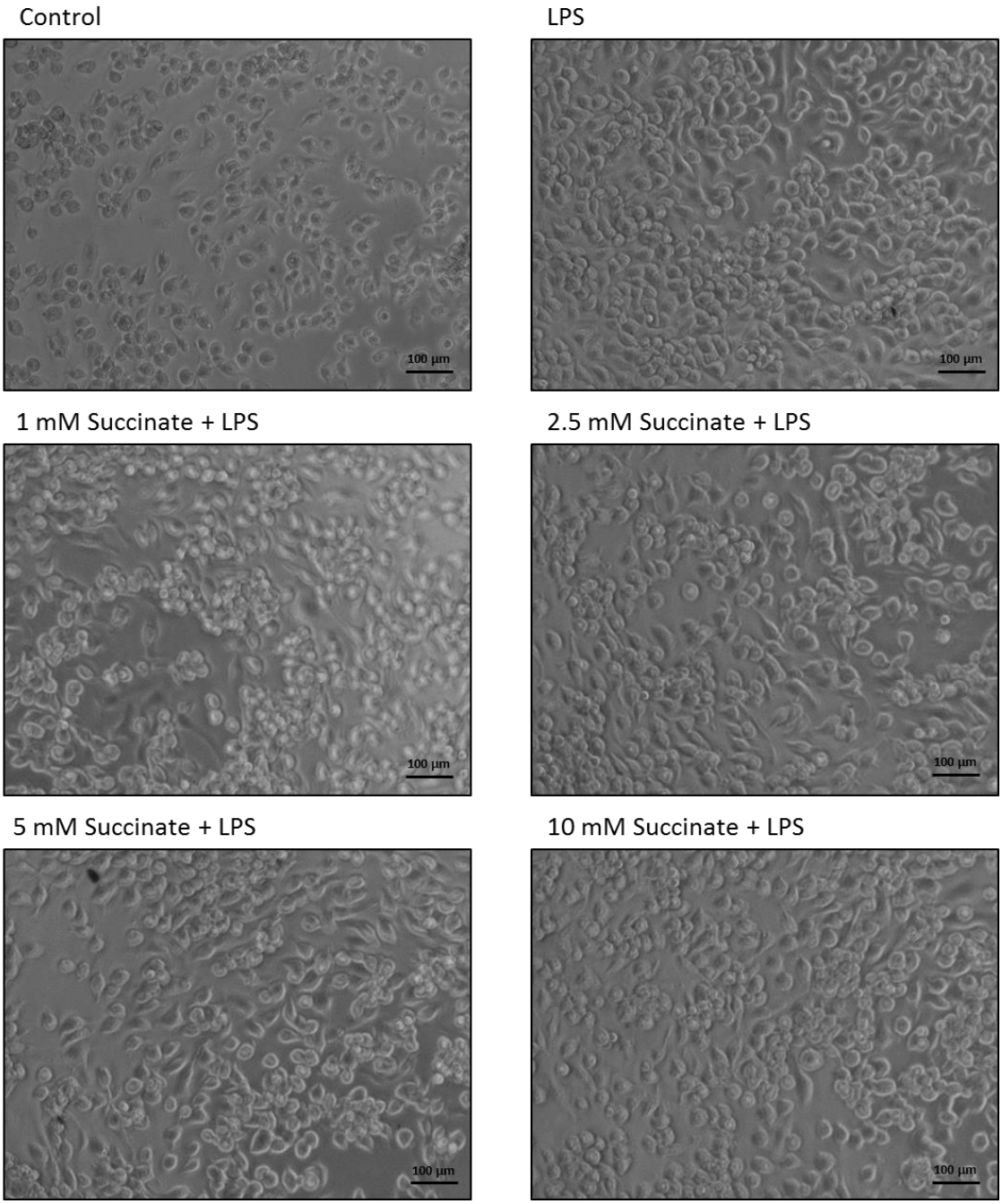

Figure S6: Macrophages treated with increasing succinate concentrations and LPS. The cells were pre-treated for 3 hours with increasing dimethylsuccinate concentrations (1 – 10 mM), followed by 24 hour 0.1 µg/mL LPS treatment. The pictures were taken at a 100X magnification.

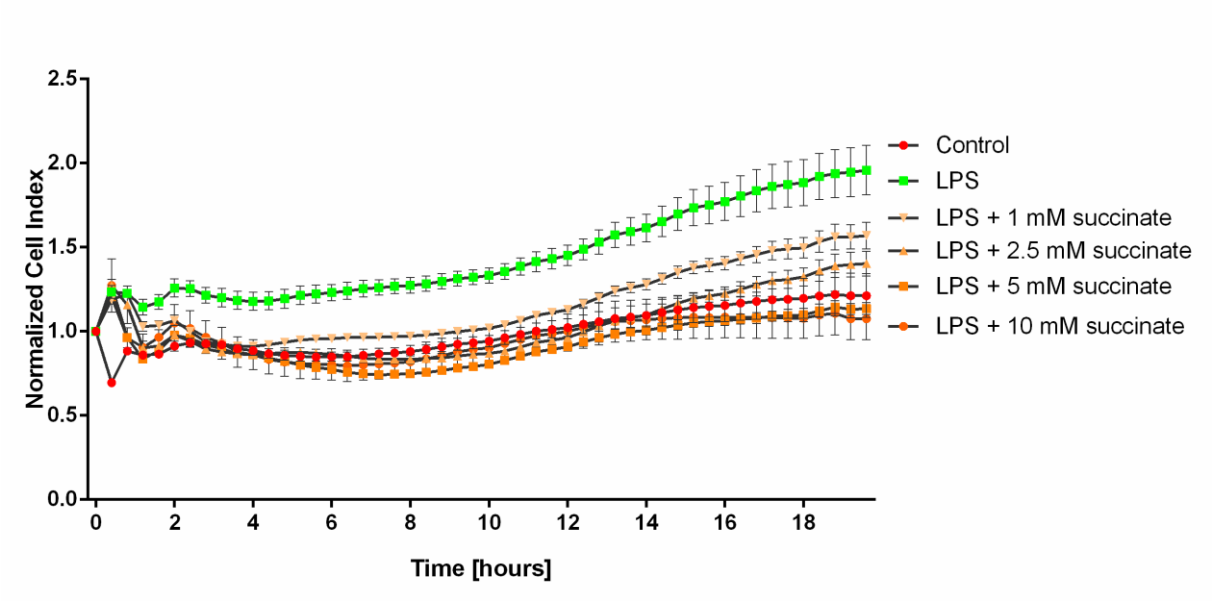

Figure S7: xCELLigence assay on succinate and LPS treated macrophages. The cells were treated with increasing dimethylsuccinate concentrations (1 mM – 10 mM) and 0.1 µg/mL LPS for 24 hours. The x-axis shows the time in hours, the y-axis shows the normalized cell index.

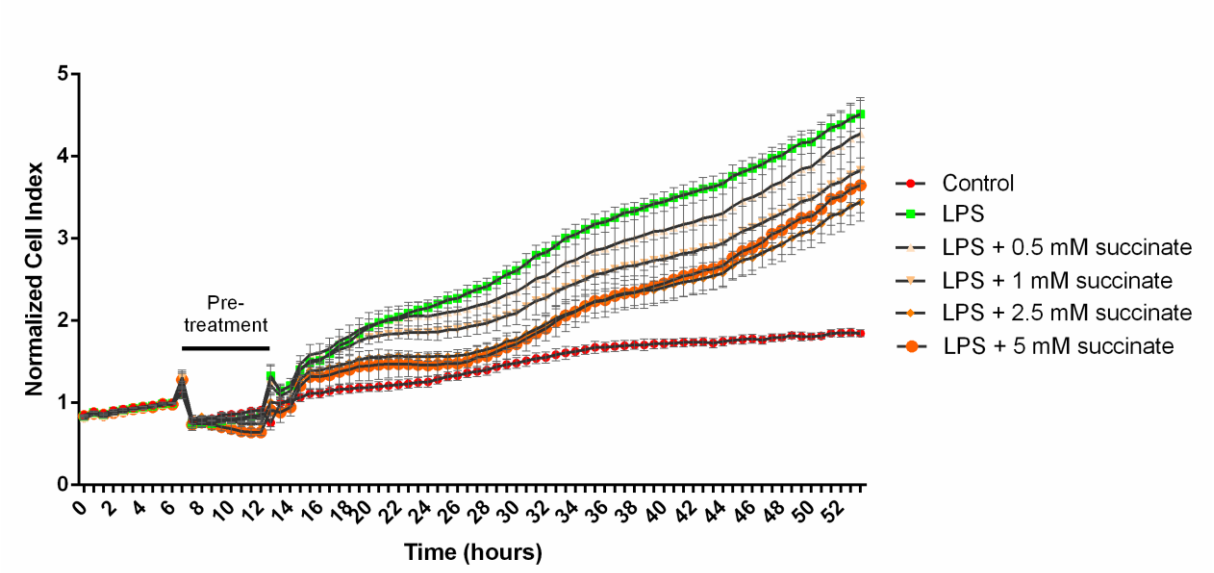

Figure S8: xCELLigence assay on succinate and LPS treated macrophages. The cells were pre-treated with increasing dimethylsuccinate concentrations (0.5 mM – 5 mM) for 3 hours, followed by 0.1 µg/mL LPS treatment. The x-axis shows the time in hours, the y-axis shows the normalized cell index.

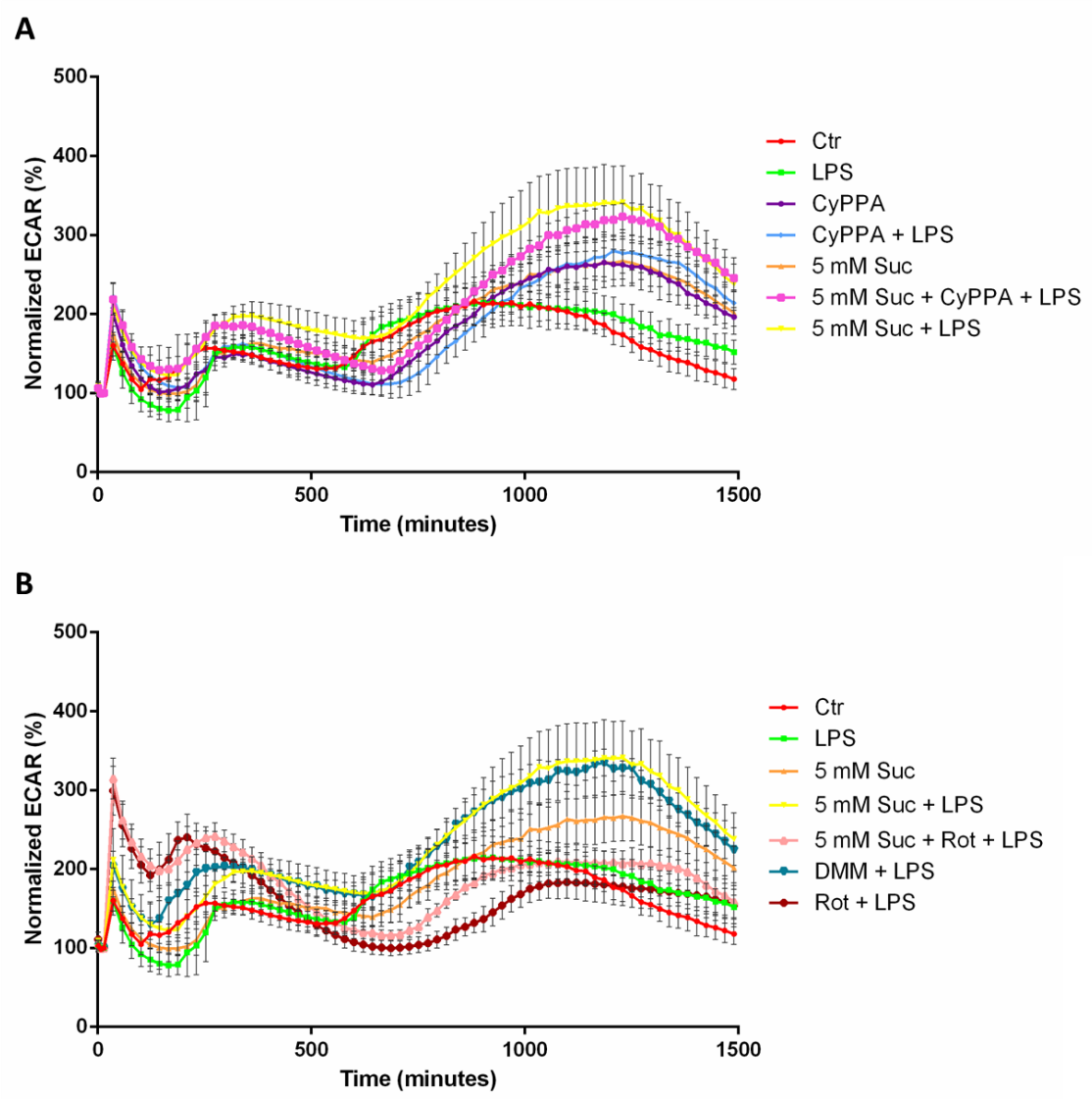

Figure S9: Seahorse assay on succinate, CyPPA, rotenone, DMM, and LPS treated cells. The x-axis shows the time in minutes, the y-axis shows the normalized ECAR (normalized to protein). A: The cells were pre-treated for 3 hours with succinate (5 mM) and/or CyPPA (25 µM), followed by 24h LPS (0.1 µg/mL) treatment, or 24h LPS and CyPPA co-treatment. B: The cells were pre-treated for 3 hours with succinate (5 mM), DMM (10 mM), or rotenone (0.5 µM), followed by 24h LPS (0.1 µg/mL) treatment. N=8 wells per condition, control: N=14 wells.

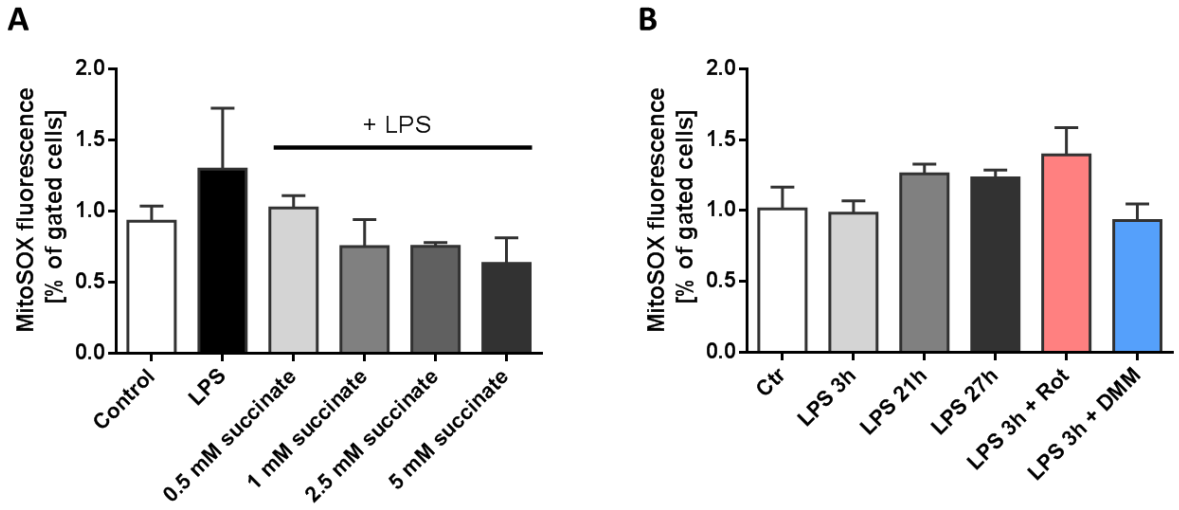

Figure S10: MitoSOX staining on succinate, rotenone, DMM, and LPS treated macrophages. The x-axis shows the treatment, the y-axis shows the MitoSOX fluorescence. A: The cells were pre-treated with increasing dimethylsuccinate concentrations (0.5 – 5 mM), followed by 24 hours 0.1 µg/mL LPS treatment. B: The cells were pre-treated with 0.5 µM rotenone or 10 mM DMM, followed by 3 hour LPS treatment. Other cells were treated with 3, 21, or 27 hours LPS treatment.

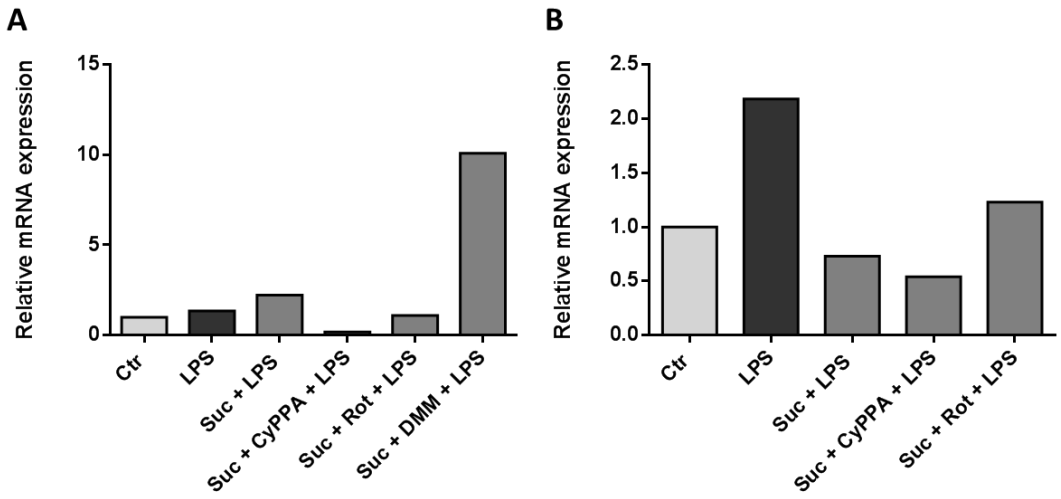

Figure S11: IL-10 expression in succinate, CyPPA, rotenone, DMM, and LPS treated cells. The x-axis shows the treatment, the y-axis shows the relative IL-10 expression. A: The cells were pre-treated with 5 mM diethylsuccinate for 3 hours, followed by 48 hour 0.1 µg/mL LPS treatment. Other cells were pre-treated with diethylsuccinate and 25 µM CyPPA, 0.5 µM rotenone, or 10 mM DMM, followed by 48 hour LPS treatment. The LPS control cells were treated for 48 hours with LPS. B: The cells were pre-treated with 5 mM diethylsuccinate for 3 hours, followed by 48 hour 0.1 µg/mL LPS treatment. Other cells were pre-treated with diethylsuccinate and 25 µM CyPPA, or 0.5 µM rotenone, followed by 48 hour LPS treatment. The LPS control cells were treated for 48 hours with LPS.

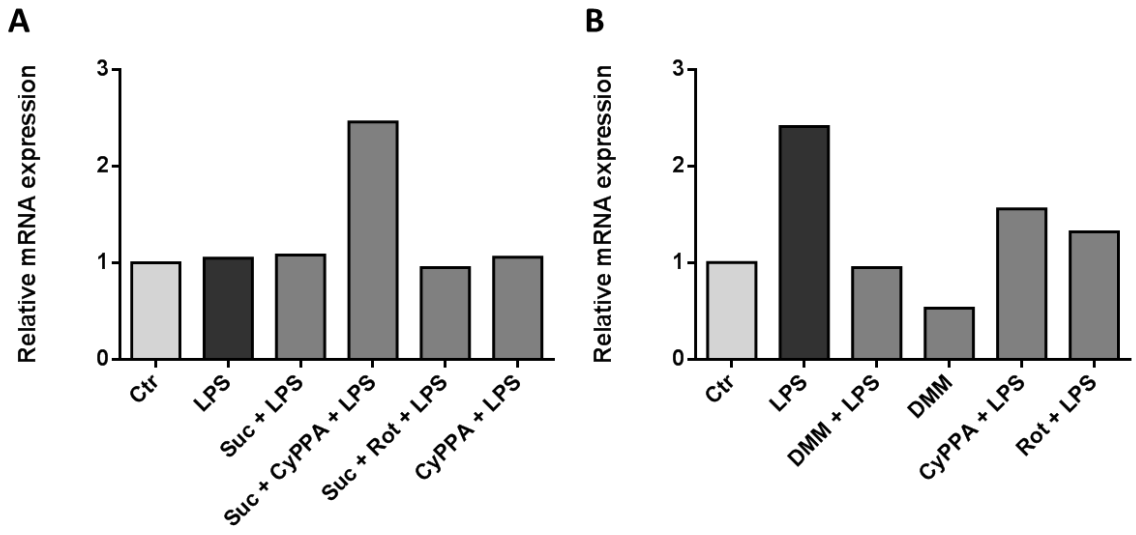

Figure S12: IL-10 expression in succinate, CyPPA, rotenone, DMM, and LPS treated cells. The x-axis shows the treatment, the y-axis shows the relative IL-10 experssion. A: The cells were pre-treated with 5 mM diethylsuccinate for 3 hours, followed by 24 hour 0.1 µg/mL LPS treatment. Other cells were pre-treated with diethylsuccinate and 25 µM CyPPA, 0.5 µM rotenone, or 10 mM DMM, followed by 24 hour LPS treatment. The LPS control cells were treated for 24 hours with LPS. The control cells only received fresh medium. B: The cells were pre-treated with 5 mM diethylsuccinate for 3 hours, followed by 24 hour 0.1 µg/mL LPS treatment. Other cells were pre-treated with diethylsuccinate and 25 µM CyPPA, or 0.5 µM rotenone, followed by 24 hour LPS treatment. The LPS control cells were treated for 24 hours with LPS. The control cells only received fresh medium.