

2016

Metabolism of Herpes Simplex Virus 1 Infected Raw 264.7 Macrophages

Mary K. Jenkins
Wright State University

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Metabolism of Herpes Simplex Virus 1 infected RAW 264.7 macrophages

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By
Mary Kathryn Jenkins
B.S., Bowling Green State University, 2011

2016
Wright State University

WRIGHT STATE UNIVERSITY
GRADUATE STUDIES

August 22, 2016

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Mary K. Jenkins ENTITLED Metabolism of HSV-1 Infected RAW 264.7 Macrophages BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

Nancy J. Bigley, Ph.D.
Thesis Director

Committee on Final Examination

Barbara E. Hull, Ph.D.
Director of Microbiology and
Immunology Program,
College of Science and
Mathematics

Nancy J. Bigley, Ph.D.
Professor of Microbiology and Immunology

Debra A. Mayes, Ph.D.
Assistant Professor, Department of Neuroscience, Cell Biology and Physiology

Courtney Sulentic, Ph.D.
Associate Professor, Department of Pharmacology and Toxicology

Robert E.W. Fyffe, Ph.D.
Vice President for Research and Dean of the Graduate School

ABSTRACT

Jenkins, Mary Kathryn. M.S. Department of Microbiology and Immunology, Wright State University, 2016. Metabolism of Herpes Simplex Virus 1 infected RAW 264.7 macrophages.

Macrophages are immune cells that phagocytize pathogens and act as antigen presenting cells. Macrophages are critical in regulating the adaptive immune response and play a key role in neutralizing infection. Macrophages exhibit a variety of phenotypes which have different energy requirements. In the case of HSV-1 infection the virus has been shown to alter metabolic processes of its host. Utilizing a XF24 Extracellular Flux Analyzer the metabolic activity of M0, M1, and M2 (IL-4, IL-13, and IL-10) RAW 264.7 macrophages was quantified for both uninfected cells and in response to HSV-1 infection. The analysis showed uninfected M0 and M2 (IL-4, IL-13, IL-10) macrophages undergo glycolysis and mitochondrial respiration for energy metabolism while M1 macrophages only undergo glycolysis. HSV-1 showed the ability to alter metabolism in macrophages by inhibiting oxidative phosphorylation. This inhibition leads to increased rates of glycolysis with variability shown among the macrophage phenotypes. As HSV-1 is dependent upon its host cell for energy production the degree of response can be correlated to viral replication.

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List of Abbreviations

2-DG – 2-deoxy-glucose
ATP – adenosine triphosphate
ECAR – extracellular acidification rate
FCCP – carbonyl cyanide-4 (trifluoromthoxy) phenylhydrazone
LPS – lipopolysaccharide
IL – interleukin
MHC – major histocompatibility complex
NO – nitric oxide
OCR – oxygen consumption rate
PFK-1 – 6-phosphofructo-1-kinase
ROS – reactive oxygen species

Acknowledgements

I would like to thank Dr. Nancy Bigley for all the support and guidance she has provided through my graduate studies. I would like to thank Dr. Debra Mayes for her support, guidance, and the use of the Seahorse XF flux analyzer. Also, I would like to thank Dr. Courtney Sulentic for her support and guidance. I would also like to acknowledge my lab associates, who expanded my knowledge and provided guidance in my experimentation.

Dedication

I would like to dedicate my thesis research to my husband for his immeasurable support and love. Achieving my goals is made easier with him by my side. Also, to my mother who always expressed the significance of education and work hard.

Introduction

Herpes simplex virus 1 and humans evolved together creating a host environment for the double stranded DNA virus to thrive in (7). HSV-1 is endemic with 50-90% of the world's population carrying the lifelong latent virus, which can become serious if the virus disseminates (20). There is no cure and the only treatment for HSV-1 infection is administration of antivirals. Virus metabolism via cellular components relies on glycolysis and the pentose phosphate pathway (PPP). Glycolysis provides a quick source of energy while PPP helps in de novo nucleotide biosynthesis to create an abundance of viral DNA. HSV-1 forces cells to commit to glycolysis by catalyzing fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP (1). The virus depletes mitochondrial DNA to impair oxidative phosphorylation and inhibits the citric acid cycle, but when mitochondrial DNA depletion occurs the innate immune response is primed (24).

Macrophages compose an important part of the innate immune response due to their ability to phagocytize pathogens as well as being involved in homeostasis, tissue repair, and development (26). Macrophages are able to respond to changes within a host environment directed by chemokines, fatty acids, cytokines and toll-like receptors (19). Macrophages exist in two phenotypic states denoted as M1 and M2, which are examined in this study. M1 or classically activated macrophages are a pro-inflammatory response induced by LPS, the production of IFN- γ , or both. These macrophages are involved in type I inflammation and help with the clearance of pathogens. M1 macrophages also

produce nitric oxide via nitric oxide synthase in order to produce a cytotoxic atmosphere. M2 or alternatively activated macrophages are an anti-inflammatory response induced by IL-4, IL-13, or IL-10. M2 macrophages are involved in type II inflammation, allergies, tissue repair and remodeling, immunoregulation, and encapsulation of parasites (13).

Macrophages produce energy based on their phenotype (10). M1 macrophages typically undergo glycolysis and utilize the pentose phosphate pathway while M2 macrophages undergo oxidative phosphorylation and fatty acid oxidation (FAO). M1 macrophages need a fast source of energy in order to phagocytosis pathogens within hours to days. Glycolysis has the ability to provide the rapid energy requirements by M1 macrophages. M1 macrophages enhance the uptake of glucose and found different methods to use the ATP generated by mitochondria more efficiently. The PPP is necessary for the production of NO by providing NADPH. As NO is an inhibitor of cytochrome c oxidase subsequently oxidative phosphorylation is reduced.

Huang et al. showed M0 and M2 (IL-4) macrophages undergo mitochondrial respiration, but M2 macrophages depend on fatty acid oxidation (FAO) as well (10). Mitochondrial respiration and FAO provide energy over a longer period of time which is beneficial for M2 macrophages due to their functions of type II inflammation, tissue repair and remodeling, and immunoregulation. M0 and M2 macrophages have the ability to proliferate as well as retain functional plasticity.

Utilizing new technology of a XF24 Extracellular Flux Analyzer the oxygen consumption and extracellular pH of RAW 264.7 macrophages were examined here

under different phenotypes and conditions. Specifically, glycolysis and mitochondrial respiration were examined in inactivated (M0), M1, and M2 induced by IL-4, IL-13, or IL-10 phenotypes to capture baseline metabolic rates for the polarized and non-polarized RAW 264.7 macrophages. After completing the baseline groups, M0, M1, and M2 (IL-4) macrophages were challenged with HSV-1 and the oxygen consumption and extracellular pH examined to study metabolic changes. Oxygen consumption shows the how much cells are utilizing oxygen to generate ATP which is an indicator of mitochondrial respiration. Mitochondrial respiration must have an available source of oxygen while glycolysis does not. Extracellular pH is a measurement of free protons in the media due to pyruvate being converted to lactic acid, independent of oxygen.

The XF24 Extracellular Flux analyzer measures fluctuations in the media due to metabolic output of the macrophages. The XF24 Extracellular Flux analyzer utilizes reagents to block glycolytic or mitochondrial respiration metabolic processes in order to analyze step of macrophage metabolism in live cells. Three reagents are provided within the glycolysis/mito stress test kit to help discover glycolytic or mitochondrial respiration variations. The glycolysis stress test reagents are glucose (to promote conversion of glucose to pyruvate), oligomycin (to inhibit ATP synthase), and 2-deoxyglucose (to inhibit glycolysis). Glycolysis is indirectly measured by extracellular acidification rate (ECAR), the rate of conversion of pyruvate to lactic acid, which is the outcome of pyruvate under anaerobic conditions. The mito stress test reagents are oligomycin (to inhibit respiration from ATP synthase), FCCP (to uncouple oxygen consumption from

ATP production), and rotenone/antimycin A (inhibits complex I and complex III, respectively). Mitochondrial respiration is measured by oxygen consumption rate (OCR), which is the rate of oxygen consumption by the macrophages.

Hypotheses:

1. M2 (IL-4, IL-13, IL-10) macrophages utilize oxidative respiration while M1 macrophages do not and M1 macrophages perform glycolysis.
2. HSV-1 drives macrophages to perform glycolysis.

Literature Review

Macrophage Function and Polarization

Macrophages are important immune cells derived from myeloid precursors in bone marrow, spleen, and fetal liver. Macrophages are differentiated from monocytes, which can develop into dendritic cells in a host immune stimulus-dependent fashion. Macrophages are located throughout an organism in all tissues and are considered the first line of host defense due to their ability to phagocytose pathogens (viral, bacterial, or parasitic) as well as dying and dead cells. Macrophage response goes beyond immunity; cells are also involved in homeostasis, tissue repair, and development (26). With the ability to recruit other phagocytic cells, such as neutrophils from the blood, activated macrophages also signal the adaptive immune system via chemokines and cytokines. While the importance of macrophages certainly is not completely understood the ability of a macrophage to respond to changes within the host environment is crucial for the survival of the organism.

Macrophages serve as scavenger cells and are induced by phagocytizing pathogens to present foreign antigens to naïve T cells. Macrophage responses can be directed by chemokines, fatty acids, cytokines and toll-like receptors (19). With a variety of receptors that recognize microbial surface components, macrophages are able to polarize into pro-inflammatory and anti-inflammatory phenotypes. Macrophages can shift between types within the same population, and once the phenotype is no longer needed cells can revert back to their original state (19). Macrophages are considered to be

inactivated or resting (M0) until a stimulus activates the cells via one of two routes: classically activated (pro-inflammatory, M1), and alternatively activated (anti-inflammatory, M2) (Figure 1). Cytokines (IFN- γ , IL-4, IL-13, or IL-10) or foreign materials (LPS) can act in a paracrine or autocrine way to polarize macrophages and are highly potent at picomolar concentrations (5). Macrophage activation can occur from a wide variety of signals, the state of macrophage activation could be considered a continuum. M1 and M2 macrophages both respond to the JAK-STAT pathway (13).

Classically activated M1 macrophages are a pro-inflammatory response to LPS or the production of IFN- γ or tumor necrosis factor. M1 macrophages are involved in type I inflammation and provide host protection against intracellular bacteria or viruses (13). IFN- γ and LPS can polarize a macrophage into M1 independently or together having different gene expression profiles (13). IFN- γ is produced by natural killer and natural killer T cells to activate macrophages via MHCII, which directly inhibits viral replication. IFN- γ is critical for innate and adaptive immunity against foreign pathogens. Once the macrophage is activated, the adaptive immune response may be required to help in clearance of pathogens. The macrophage will polarize a naïve T helper cell into a CD4 T helper 1 cell or a CD8 cytotoxic T lymphocyte, which in turn will start to produce IFN- γ in a positive feedback loop (11). LPS is found on the outer membrane of gram-negative bacteria and when a macrophage comes into contact with the outer membrane, toll-like receptor two and four on the surface of macrophages is signaled to begin an immune response. M1 phenotype, through the stimulation of IFN- γ , produces nitric oxide (NO) or

reactive oxygen species (ROS) via nitric oxide synthase (iNOS) to create a cytotoxic environment helping to rid the organism of pathogens. Due to the cytotoxicity of NO or ROS, after prolonged exposure the host organism may need tissue repair and restoration by the M2 phenotype.

M2 macrophages are considered to be alternatively activated and anti-inflammatory. They are involved in type II inflammation, allergies, tissue repair and remodeling, immunoregulation, and encapsulation of parasites (13). The M2 phenotype can be induced by IL-4, IL-13, or IL-10. IL-4 and IL-13 induce polarization of naïve helper T cells to T helper 2 cells in a positive feedback loop but the gene expression profiles are different (11). IL-4 and IL-13 are known to increase endocytosis of receptor-mediated processes, down regulate phagocytosis, and inhibit the production of NO or ROS (12). IL-13 induces IgE secretion from B-cells (11). IL-10 is produced by monocytes and T helper 2 cells to enhance B-cell survival, proliferation, and antibody production but inhibit T helper 1 cells and MHCII antigens. Whether polarization occurs or not each pathway requires different energy demands and can have different metabolic patterns (18).

Herpes Simplex Virus-1

HSV-1 is considered to be an ancient virus, which evolved to replicate and exist within human hosts (7). HSV-1 is an alphaherpesvirus with a double-stranded DNA genome of 152kb which encodes 84 polypeptides. The host cells begin to copy and shed

live virus 8 to 12 hours after entering the host (5). The virus contains an icosahedral nucleocapsid, enveloped with ten different glycoproteins in the membrane. The glycoproteins, gB or gC, binds to heparan sulfate (found on the membrane of host cells), while gD binds to the entry receptor and invades cells (mainly neurons) (7). The viral/cellular components and precise method of attachment and fusion are still unclear. Early host control of HSV-1 is essential to host defense, and macrophage activation is induced early in response to HSV-1 (7). The advancement of infection depends on little to no exposure to extracellular substances. Infecting adjacent cells directly through cellular junctions helps to increase the spread of infection to guarantee escape from the adaptive immune response.

HSV-1 is endemic with 50-90% (higher among developing countries) of the world's population carrying the virus (20). Neonates still have a 30% mortality rate if the infection becomes disseminated (7). Reviewed in *Macrophages and Cytokines in the Early Defense against Herpes Simplex Virus*, once contracted HSV-1 becomes a lifelong latent infection in neurons (7). This causes a persistent viral infection which can lie dormant for years. The virus is transmitted oral to oral through saliva or contact with an open sore but can also be transmitted oral to genital. HSV-1 is generally asymptomatic, but can cause painful blisters or open sores as well as tingling, itching, and burning. A small fraction of infected individuals can develop central nervous system encephalitis when HSV-1 localizes to the frontal and temporal lobes of the brain. There is no cure for

herpes but it can be treated with antiviral Acyclovir. Acyclovir reduces localized immune response within the ganglion to decrease severity and frequency of symptoms.

Metabolism

Metabolism is the process of producing energy by way of physical and chemical reactions. Individual cells need to metabolize resources to produce energy in order to function. Metabolism is linked and each cell type may use one process or many processes to complete the task (Figure 2). Cells produce energy through multiple processes, including: glycolysis, pentose phosphate pathway, the citric acid cycle, mitochondrial respiration (oxidative phosphorylation), and fatty acid oxidation (9).

Glycolysis is the process of breaking down ATP and glucose to create ATP and pyruvate. Glycolysis is not an effective way to maintain energy over time but can create energy in the form of ATP very quickly and is often utilized even when oxygen isn't limiting (17). Once pyruvate is synthesized it is either broken down into lactate or ethanol (in microorganisms) under anaerobic conditions or broken down into acetyl-CoA for fuel for the citric acid cycle under aerobic conditions. Both glycolysis and the citric acid cycle generate NADH and FADH₂ and in order to produce ATP from these molecules they are oxidized via mitochondrial respiration. Mitochondrial respiration is an efficient way to generate energy for long term use. A proton gradient forms to drive ATP synthesis (complex V of the electron transport chain).

Another way for cells to generate energy and pyruvate (indirectly) for the citric acid cycle is the pentose phosphate pathway (PPP). The PPP provides NADPH as well as ribose-5-phosphate essential for nucleic acid synthesis (9). NADPH is used in reductive biosynthesis reactions, such as fatty acid synthesis. Once fatty acids are created they will need to be broken down eventually for energy. Fatty acid oxidation (FAO) breaks down fatty acids into acetyl-CoA, which is used to generate energy via the citric acid cycle. FAO is used by cells for a long term energy source (9).

Metabolism of Macrophages

Macrophages can create energy utilizing glycolysis, PPP, mitochondrial respiration, and fatty acid oxidation, which metabolic process is used depends on the activation state of the macrophage (10). Glycolysis and mitochondrial respiration were examined in the current studies using RAW 264.7 cells which are considered to have higher metabolic fluxes than peritoneal macrophages (18). Macrophages require energy for activation. Early in the polarization process (approximately one hour after the addition of cytokines) metabolism can adapt (15). M1 macrophages typically utilize a higher amount of glycolysis and PPP to produce while M2 macrophages undergo oxidative phosphorylation and FAO. 90% of macrophages perform glycolysis regardless of phenotype (4).

The function of M1 macrophages (especially phagocytosis of pathogens) requires rapid energy, hours compared to days. Anaerobic or aerobic glycolysis has the ability to

produce energy quickly for short term use (18). Overexpression of glucose transporter 1 (GLUT-1) occurs with enhanced uptake of glucose allowing for faster transport of glucose across the plasma membrane. Along with GLUT-1, hexokinase 2 is highly expressed. Hexokinase 2 and adenine nucleotide translocator (ANT) helps M1 macrophages to use ATP generated by the mitochondria more efficiently (4). Pentose phosphate pathway is also essential for M1 to generate NADPH to create reactive oxygen species (ROS) for host defense (16). This reduces mitochondrial respiration due to NO acting as an inhibitor of cytochrome c oxidase in the oxidative phosphorylation process.

M2 macrophages are involved in tissue repair, wound healing, and parasite clearance, which require a source of energy for a longer period of time (19). M2 macrophages have a higher level of mitochondrial oxygen consumption when compared to resting or M1 macrophages but depends on fatty acid oxidation (FAO) (10). Even though M2 macrophages rely on mitochondrial oxygen consumption and FAO, they still retain a modest dependence on glycolysis similar to basal macrophages. Resting macrophages depend on glycolysis, but convert 95% of glucose to lactate (24). The reliance on mitochondrial oxygen consumption and FAO can be correlated to the ability of M2 macrophages to proliferate (under certain conditions), while M1 macrophages cannot proliferate (16). Proliferating macrophages require different energy needs (24).

Herpes Simplex Virus 1 and Metabolism

Viruses have to reprogram the metabolism of an infected cell due to not having the mechanisms for channeling energy (6). Generally viruses will decrease mitochondrial oxygen consumption in cells and compensate by increasing glycolysis. HSV-1 is no exception and localizes in the mitochondria to rapidly deplete mitochondrial DNA, which impairs oxidative phosphorylation and can inhibit the citric acid cycle (25).

Mitochondrial DNA stress is a necessary part of engaging the antiviral response from the host and prime the innate immune response, which is a drawback for the virus. In order to effectively counteract the positive host response HSV-1 requires a quick source of energy.

For HSV-1 to alter a cell's predisposition towards to glycolysis, the virus forces 6-phosphofructo-1-kinase (a key rate limiting step in glycolysis due to the utilization of ATP) (PFK-1) to catalyze the conversion of fructose 6-phosphate (an intermediate in glycolysis) and ATP to fructose 1,6-bisphosphate and ADP (1). HSV-1 increases translation and transcription of PFK-1, glucose uptake, lactate efflux, drives ATP syntheses and diverges glucose carbons for PPP. PPP is essential for HSV-1 to amass DNA quickly via de novo nucleotide biosynthesis. Eventually HSV-1 will decrease the uptake of glucose and lactate to infect adjacent cells (22). When an infected cell has replicated so much viral protein that it is about to go through apoptosis, then the artificially upregulated glucose and lactate will be brought back to normal levels.

Macrophages are primary defense against pathogens and are the first to interact with HSV-1 virus. Understanding basic metabolism of macrophages will help in furthering how they will respond when an infection arises. Learning the basic metabolism of a macrophage allows for further understanding of metabolism when an infection occurs and how the metabolism is altered for the benefit of the virus. An HSV-1 infection occurs and the rate of glycolysis is increased while mitochondrial respiration is inhibited. The macrophages metabolism is transformed to suit the needs of the virus. Using new technology to measure glycolysis and mitochondrial respiration will give a more refined view of the metabolic processes.

Materials and Methods

Cell Line

Purchase from American Type Culture Collection (ATCC), RAW 264.7 macrophage cell line is created from a tumor induced by Abelson murine leukemia virus derived from an adult male BALB/c mouse. RAW 264.7 cells are grown in Biolife 25 cm² vented cap cell culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) with 10% donor bovine serum (DBS) and 1% penicillin/streptomycin. Cells are incubated in a water-jacketed incubator at 37°C and 5% CO₂. To split cells a cell scraper is used to remove cells from the bottom of the flasks and poured into a 15mL centrifuge tube. Cells are typically cultured in a 1:10 ratio and split two to three times weekly. All materials (culture flasks, growth medium, and DBS) were purchased from Fisher Scientific.

Polarization Treatment

RAW 264.7 cells are grown 24 hours (~70-80% confluency) prior to adding cytokines and LPS to the media cells are grown in to achieve a desired phenotype. To achieve M1 phenotype cells are treated with IFN- γ (20 ng/mL) and LPS (100 ng/mL) for 24 hours. The M2 phenotype can be achieved by adding IL-4 (20 ng/mL), IL-13 (20 ng/mL), or IL-10 (20 ng/mL) for 24 hours. Cytokines are purchased from Peprotech and LPS is purchased from Chondrex.

Virus Treatment

Herpes Simplex Virus one strain 17+ was obtained from the University of Cincinnati Children's Hospital. A plaque assay was performed to find the viral titer in order to find the correct multiplicity of infection (MOI). If the titer was higher than the MOI, the virus needs to be serially diluted to achieve the correct MOI. The MOI of 0.1 was utilized, so for every 100,000 cells, 10,000 cells should be infected. The virus titer utilized was 1.75×10^7 therefore 0.4 μL was added to 200 μL to infect 60,000 cells.

Laminin Treatment

The laminin (1 milligram, at 1.96 milligram per milliliter) was thawed on ice and diluted with 1mL of 1xphosphate-buffered saline (PBS) ($\text{Ca}^{++}/\text{Mg}^{++}$). The laminin was aliquoted into sterile plastic tubes at 1.96 μL per tube. When needed the aliquoted laminin should be thawed and added to 10 mL of sterile, serum-free media. Once the stock solution was created, it was added to the culture surface and incubated at room temperature for 1 hour. After the hour the stock solution was vacuumed out and the culture surface was washed one time with 1xPBS. The 1xPBS was removed by vacuum.

Cell Viability

In 6-well plates, RAW 264.7 cells were grown to ~70-80% confluency. The cells were either not polarized (control) or polarized (see Polarization Treatment). After 4, 8, 12, and 24 hours cells were removed from the bottom of the wells and collected. The cells were centrifuged; the supernatant was aspirated, and then resuspended in 1mL of

media. Trypan Blue was added and both live (clear) and dead (blue) cells were counted using a hemocytometer to determine viability.

Metabolism Assays

Mito Stress Test (measuring OCR) and Glycolysis Stress Test (measuring ECAR) is a three day process. On day one XF24 cell culture microplates (24 wells) were treated with 200 μ L of Laminin (1.96 mg/mL) in each well for 1 hour, and then the media was vacuumed out of each well. The wells are washed once with 1xPBS and then the 1xPBS was vacuumed out (see Laminin Treatment). After the wells are washed, 200 μ L of RAW 264.7 cells, seeded at a density of 6×10^5 were added to 20 wells leaving 4 wells (A1, B4, C3, D6) filled with growth media (for background control for the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, MA, USA)) and incubated for 24 hours at 37°C and 5% CO₂.

On day two the cells were treated with the appropriate cytokine and LPS for 24 hours at 37°C and 5% CO₂ (see polarization treatment). When utilizing HSV-1, the virus was added at the same time as the polarization treatment to both polarized and non-polarized cells for 24 hours at 37°C and 5% CO₂ (see Virus Treatment). Of the 20 wells used to seed cells, 10 wells were used for one non-polarization or polarization treatment. In addition to treating the cells, the XF24 extracellular sensor cartridge was hydrated with 1 mL of Seahorse XF Calibrant. After adding the calibrant to the plate, the cartridge lid was replaced. Confirmation the cartridge was fully submerged occurred before and the

XF24 extracellular sensor cartridge was wrapped in plastic wrap. The cartridge was incubated in a non-CO₂ incubator at 37°C for 24 hours.

On day three XF base medium was prepared. When running a Mito Stress Test, 1mM sodium pyruvate (2mL), 2mM glutamine (2mL), and 10mM D-glucose (0.45g) were added to the XF base medium per 96mL (creates 100mL). When running a Glycolysis Stress Test, 2mM glutamine (2mL) was added to the XF base medium per 98mL (creates 100mL). After the media had been prepared the pH and temperature were adjusted utilizing a magnetic stirrer with hot plate. The media was adjusted to a pH of 7.4 with 0.1 NaOH at 37°C before being filtered with a disposable 250mL vacuum filter system.

After the prepared media was filtered, the wells were washed. 150 µL of the 200 µL of the current growth media (DMEM+DBS+ penicillin/streptomycin) were removed from all 24 wells in the microplate. Once removed, the wells were carefully washed twice with 1mL of prepared media; taking caution not to remove any cells. 475 mL of prepared media was added to each well for a total of 525 mL. After the wells were washed and the prepared media was added, the microplate was placed in a non-CO₂ incubator at 37°C for 1 hour.

During the 1 hour, reagents from the stress test kits were created to measure mitochondrial function or glycolysis. The reagents were created for constant volume by re-suspending them in the prepared media (Prepared in accordance to the Mito Stress Test Kit and Glycolysis Stress Test Kit User Guides provided by Seahorse Bioscience):

Mitochondrial Respiration: 1.0 μ m Oligomycin (Port A), 1.0 μ m FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) (Port B), 5 μ m Rotenone/Antimycin A (Port C)

Glycolysis: 10mM Glucose (Port A), 1.0 μ m Oligomycin (Port B), 50mM 2-Deoxyglucose (Port C)

Once the compounds (3 for each stress test kit) were created they were loaded into the ports on the hydrated sensor cartridge. Utilizing a multi-channel pipette, 75 μ L of each compound was injected into the correct ports. After the compounds were loaded into the correct port for each column of the sensor cartridge; the sensor cartridge was inserted into the XF24 Extracellular Flux Analyzer, which runs a calibration test for 20 minutes. After an hour of incubation, the test plate was inserted into the XF24 Extracellular Flux Analyzer and the stress test ran for 2 hours. Each well was measured three times before a reagent was injected into the well. Real-time analysis of OCR and ECAR rates were performed with the XF24 Extracellular Flux Analyzer.

Statistical Significance

Statistical analysis was performed in Microsoft Excel utilizing one way ANOVA and t-test: Two-Sample for Means. This analysis calculates a P-value to show the significance of $P < .05$, $P < .01$, and $P < .001$. All experiments were performed at least 3 times.

Results

Inactivated and activated macrophages metabolize energy via glycolysis

Measuring glycolysis by ECAR demonstrates all macrophage phenotypes produce energy under glycolytic conditions (Figure 3). When exposing RAW 264.7 macrophages to glucose they respond by utilizing and consuming the glucose to create ATP, NADH, water, and protons. The influx of protons creates an increase of ECAR among all macrophage phenotypes. After the injection of oligomycin the glycolytic capacity increases for M0 and M2 phenotypes to meet the metabolic stress of the cell, but a decrease occurs under M1 phenotype conditions. With an injection of 2-DG all macrophage phenotypes drastically reduces ECAR showing the ECAR produced during the experiment is due to only glycolysis. A higher non-glycolytic acidification rate has been shown in IL-4 and IL-13 induced macrophages that the remaining ECAR is not traceable to glucose metabolism. With glycolytic reserve showing an increase in glycolysis due to shut down of any ATP production via mitochondrial respiration; M0 and M2 phenotypes are similar with IL-13 induced macrophage having the highest rate, but glycolytic reserve for M1 phenotype is negative.

Inactivated and M2 macrophages metabolize energy via oxidative phosphorylation

Measuring oxidative phosphorylation by OCR demonstrates only M0 and M2 macrophages produce energy under mitochondrial stress conditions (Figure 4). Basal

respiration is highest among IL-4 and IL-13 induced macrophages, while M0 and IL-10 induced macrophages have a similar basal respiration, and M1 having the lowest basal respiration. Exposing RAW 264.7 macrophages to oligomycin decreases OCR among all phenotypes (slight decrease in M1 phenotype) showing proton leak (basal respiration that is not coupled to ATP production). ATP production by the mitochondria is highest among IL-4 and IL-13 induced phenotypes and lowest in the M1 phenotype. After collapse of the proton gradient via FCCP, OCR dramatically increases among M0 and M2 macrophages and continues to utilize oxygen at maximum OCR. M1 phenotype conditions slightly decrease. With an injection of rotenone/Antimycin A M0 and M2 phenotypes drastically reduce OCR with higher non-mitochondrial respiration rates among M0 and M2 phenotypes. Spare respiratory capacity is the theoretical maximum. M2 phenotypes are similar with IL-13 having the highest spare respiratory capacity. M0 and IL-10 induced macrophages have lower spare respiratory capacity. The coupling efficiency shows all phenotypes $\geq 80\%$ of basal respiration used to drive ATP synthesis, but only $< 20\%$ of basal respiration is used for M1 phenotype. The average spare respiratory capacity (as fold-increase) shows how closely mitochondrial respiration is working at maximum. IL-4 induced phenotype has the highest spare respiratory capacity (as fold-increase) compared to M0, IL-13, and IL-10 induced macrophages with similar rates working close to max, but M1 has the lowest rate.

HSV-1 diminishes glycolysis and shuts down oxidative phosphorylation

When RAW 264.7 macrophages are infected with HSV-1 the effects on glycolysis are diminished, but still apparent (Figure 3). Infected RAW 264.7 ECAR increases with an injection of glucose. There is an increase in glycolytic capacity among M0 and IL-4 induced macrophages, but a slight decrease in M1 phenotype. 2-DG drastically reduces ECAR in all phenotypes with a higher non-glycolytic acidification rate in the IL-4 induced phenotype and M1 having half the non-glycolytic acidification rate than M0 macrophages. Glycolytic reserve is highest in IL-4 induced macrophages.

Under mitochondrial stress conditions infected macrophages basal respiration is recorded for all phenotype, but higher for IL-4 induced macrophages than M0 and M1 (Figure 4). Once oligomycin is injected all phenotypes OCR decrease. ATP production is highest in IL-4 induced phenotype. After the injection of FCCP, OCR decreases for all phenotypes. The last injection of rotenone/Antimycin A show a decrease in the IL-4 induced phenotype, but M0 and M1 phenotypes seem to level off (no increase or decrease). Non-mitochondria-derived OCR shows no difference between phenotypes. The spare respiratory capacity is negative among all phenotypes with the highest spare respiratory capacity in the M1 phenotype. Coupling efficiency shows >60% of basal respiration is used to drive ATP synthesis. Comparing ECAR to OCR in uninfected macrophages shows higher OCR and ECAR in IL-4 and IL-13 induced phenotypes, while M0 and IL-10 induced phenotype have similar rates between each other (Figure 5). M1 macrophages have higher ECAR than M0 and IL-10 induced macrophages, but lower

OCR. When comparing infected to uninfected, infected macrophages have a lower OCR than their counterparts, and a slightly lower ECAR in M1 and IL-4 induced macrophages, but higher ECAR in M0.

Measurements of Glycolysis comparing non-virus to virus

Glycolysis is a metabolic process of converting glucose to pyruvate and measured by ECAR. The average glycolysis is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 6). When comparing the statistical significance of all five conditions; M1 is significantly different M0 ($p < .001$), IL-4 ($p = .003$), IL-13 ($p = .004$), and IL-10 ($p < .001$) induced macrophages. M0 is significant when compared to IL-4 ($p = .02$), but not IL-13 or IL-10 induced macrophages. IL-10 induced macrophages are significant when compared to IL-4 ($p < .001$) and IL-13 ($p = .002$) induced macrophages. Comparing non-virus to HSV-1 treatment, IL-4 induced phenotype shows a slight decrease, but M0 and M1 are diminished. All macrophage phenotypes utilize glycolysis but infected macrophages at a diminished capacity.

Glycolytic Capacity is the maximum ECAR macrophages reach. The average glycolytic capacity is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 7). When comparing the statistical significance of all five conditions; M1 is significantly different M0 ($p < .001$), IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p < .001$) induced macrophages. M0 is significant when compared to IL-4 ($p = .048$) induced macrophages, but not IL-13 or IL-10 induced macrophages. IL-10

induced phenotype is significant when compared to IL-4 ($p=.012$), but not IL-13 induced macrophages. Comparing non-virus to HSV-1 treatment, IL-4 induced phenotype shows a slight decrease, but M0 and M1 are diminished. Maximum ECAR is highest in M0 and M2 uninfected phenotypes, but diminished in infected phenotypes.

Glycolytic reserve is the ability of macrophages to respond to energetic demands. The average glycolytic reserve is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 8). When comparing the statistical significance of all five conditions; M1 is significantly different than M0 ($p<.001$), IL-4 ($p<.001$), IL-13 ($p<.001$), and IL-10 ($p<.001$) induced phenotypes. M0 is significant when compared to IL-13 ($p=.001$) and IL-10 ($p=.022$) induced macrophages, but not IL-4 induced macrophages. IL-4 and IL-13 induced phenotypes are significant ($p<.001$) when compared to each other. IL-10 induced macrophages are significant when compared to IL-4 ($p<.001$), but not IL-13 induced macrophages. Comparing non-virus to HSV-1 treatment, IL-4 induced macrophages shows a slight decrease, but M0 and M1 are diminished. M0 and M2 macrophages in both uninfected and infected respond to cellular energy demands but infected macrophages at a diminished rate.

Non-glucose-derived ECAR is all ECAR that is not attributed to glycolysis. The average non-glucose-derived ECAR is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 9). When comparing the statistical significance of all five conditions; M1 is significantly different than M0 ($p<.001$), IL-4 ($p<.001$), IL-13 ($p<.001$), and IL-10 ($p<.001$) induced macrophages. M0 is significant

when compared to IL-4 ($p < .015$) and IL-10 ($p < .04$) induced macrophages, but not IL-13 induced macrophages. IL-10 induced phenotype is not significant when compared to IL-4 and IL-13 induced macrophages. Comparing non-virus to HSV-1 treatment, IL-4 induced macrophages and M1 shows a slight decrease, but M0 is diminished. All phenotypes have ECAR that is not attributed to glycolysis.

Glycolytic Reserve Capacity (as fold-increase) is how closely glycolysis is working at maximum. The average glycolytic reserve capacity (as fold-increase) is significantly different than M2 (IL-4, IL-13, or IL-10) macrophages than M1 macrophages (Figure 10). When comparing the statistical significance of all five conditions; M1 is significant different than all other phenotypes ($p < .001$). M0 is not significant when compared to IL-4, IL-13, or IL-10 induced macrophages. IL-10 induced phenotype is significant when compared to IL-4 ($p = .003$), but not IL-13 induced macrophages. Comparing non-virus to HSV-1 treatment, M1 shows a slight increase, but M0 and IL-4 induced macrophages are slightly decreased. All phenotypes are working close to maximum glycolysis.

Measurements of Mitochondrial Respiration comparing non-virus to virus

Basal respiration is the baseline respiration used to meet ATP demand and drive proton leak. The average basal respiration is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 11). When comparing the statistical significance of all five conditions; M1 is significant different than M0 ($p < .001$),

IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p < .001$) induced macrophages. M0 is significant when compared to IL-4 ($p < .001$) and IL-13 ($p = .005$) induced phenotypes, but not IL-10 induced macrophage. IL-10 induced phenotype is significant when compared to IL-4 ($p = .005$) and IL-13 ($p = .014$) induced macrophages. Comparing non-virus to HSV-1 treatment, M1 shows no significant difference, but M0 and IL-4 induced macrophages are greatly reduced. Basal respiration is the highest among M0 and M2 uninfected macrophages but severely reduced in M1 uninfected and all infected phenotypes.

The proton leak is the portion of basal respiration being leaked across the mitochondrial inner membrane and not coupled to ATP production. The average proton leak is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 12). When comparing the statistical significance of all five conditions; M1 is significantly different than M0 ($p < .001$), IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p < .001$) induced macrophages. M0 is significant when compared to IL-4 ($p = .002$), IL-13 ($p = .002$), and IL-10 ($p = .004$) induced phenotypes. IL-10 induced phenotype is significant when compared to IL-4 ($p < .001$) and IL-13 ($p < .001$) induced phenotypes. Comparing non-virus to HSV-1 treatment, M1 shows a slight decrease, but M0 and IL-4 induced phenotype are decreased. All phenotypes have some proton leak with the highest among M0 and M2 uninfected macrophages.

The ATP production is the portion of basal respiration used to produce ATP. The average ATP production is significantly different among M2 (IL-4 and IL-13)

macrophages than M1 or M0 macrophages (Figure 13). When comparing the statistical significance of all five conditions; M1 is significantly different than M0 ($p < .001$), IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p < .001$) induced phenotypes. M0 is significant when compared to IL-4 ($p < .001$) and IL-13 ($p = .008$) induced macrophages, but not IL-10 induced macrophages. IL-10 induced phenotype is significant when compared to IL-4 ($p = .026$) induced macrophages. Comparing non-virus to HSV-1 treatment, M1 shows a slight increase, but M0 and IL-4 induced phenotype are decreased. ATP production is the highest among M0 and M2 uninfected macrophages but severely reduced in M1 uninfected macrophages and all infected phenotypes.

The maximal respiration is the maximum rate of oxygen consumption. The average maximal respiration is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 14). When comparing the statistical significance of all five conditions; M1 is significantly different than M0 ($p < .001$), IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p < .001$) induced macrophages. M0 is significant when compared to IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p = .019$) induced phenotypes. IL-10 induced phenotype is significant when compared to IL-4 ($p < .001$) and IL-13 ($p < .001$) induced phenotypes. Comparing non-virus to HSV-1 treatment, M1 shows a slight decrease, but M0 and IL-4 induced phenotype are decreased. Maximal respiration is the highest among M0 and M2 uninfected macrophages but severely reduced in M1 uninfected macrophages and all infected phenotypes.

Spare respiratory capacity is the ability of the macrophage to respond to energy demands. The average spare respiratory capacity is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 15). When comparing the statistical significance of all five conditions; M1 is significantly different than IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p = .03$) induced phenotypes. M0 is significant when compared to IL-4 ($p = .002$) and IL-13 ($p = .002$), but not to IL-10 induced phenotypes. IL-10 is significant when compared to IL-4 ($p = .013$) and IL-13 ($p = .013$) induced phenotypes. Comparing non-virus to HSV-1 treatment, M1 shows a slight decrease, but M0 and IL-4 induced macrophages are decreased. M0 and m2 uninfected macrophages are able to respond to cellular demands.

Non-mitochondrial respiration is all oxygen consumption not attributed to mitochondrial respiration. The average non-mitochondrial respiration is significantly different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 16). When comparing the statistical significance of all five conditions; M1 is significantly different than M0 ($p < .001$), IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p = .001$) induced macrophages. IL-10 induced phenotype is significant when compared to IL-4 ($p = .002$) induced phenotype. Comparing non-virus to HSV-1 treatment, M1 shows a slight decrease, but M0 and IL-4 induced macrophages are decreased. All phenotypes have OCR not attributed to mitochondrial respiration.

The coupling efficiency shows the portion of basal respiration that drives ATP synthesis rather than the proton leak. The average coupling efficiency is significantly

different among M2 (IL-4 and IL-13) macrophages than M1 or M0 macrophages (Figure 17). When comparing the statistical significance of all five conditions; M1 is significantly different than M0 ($p < .001$), IL-4 ($p < .001$), IL-13 ($p < .001$), and IL-10 ($p < .001$) induced macrophages. M0 is significant when compared to IL-10 ($p = .002$) induced phenotype but not to IL-4 or IL-13 induced phenotypes. IL-10 induced phenotype is significant when compared to IL-4 ($p = .004$) and IL-13 ($p < .001$) induced phenotypes. Comparing non-virus to HSV-1 treatment, M1 shows a massive increase, but M0 and IL-4 induced macrophages are decreased. The coupling efficiency of all phenotypes but M1 uninfected is $>60\%$, which drives ATP synthesis.

The spare respiratory capacity (as fold-increase) is how close the macrophage is working at maximum capacity during basal respiration. The average spare respiratory capacity (as fold-increase) is significantly different among M2 (IL-13) macrophages than M1 or M0 macrophages (Figure 18). When comparing the statistical significance of all five conditions; M1 is significantly different than IL-4 ($p < .001$) and IL-13 ($p < .001$) induced phenotype. M0 is significant when compared to IL-13 ($p < .001$) induced phenotype but not to IL-4 or IL-10 induced phenotypes. IL-10 induced phenotype is significant when compared to IL-13 ($p < .02$) induced phenotype, but not IL-4 induced phenotype. Comparing non-virus to HSV-1 treatment, M1 shows a slight increase, but M0 and IL-4 induced macrophages are decreased. IL-13 induced phenotype is working close to max but the other phenotypes are not.

Discussion

In our study we discovered inactivated and M2 (IL-4, IL-13, IL-10) macrophages metabolize energy via glycolysis and oxidative respiration, while M1 macrophages metabolize energy by glycolysis. An analysis of the literature did not show that M2 cells utilize glycolysis. In this study we conclusively demonstrated that M2 cells do undergo glycolysis and at a higher rate than M1 cells. Interestingly M1 and M2 cells responded to HSV-1 in a similar fashion with both phenotypes showing a relative decrease in glycolytic activity. HSV-1 has an anti-apoptotic effect in RAW 264.7 macrophages (14). Due to the anti-apoptotic effect of HSV-1, macrophages continue to metabolize via cellular machinery. Although glycolysis has been diminished when the virus is added, macrophages still utilize glucose for the production of energy. The use of glycolysis by M0, M1 and M2 (IL-4, IL-13, IL-10) macrophages with and without the addition of HSV-1 is prospectively due to a need for a faster generation of ATP necessary for formation of intermediates required by other metabolic processes (oxidative phosphorylation, PPP, etc.).

The XF24 Extracellular Flux Analyzer adds glucose to saturate the macrophages and promotes the conversion of glucose to pyruvate measured against extracellular acidification rate. After the addition of glucose M0 macrophages convert glucose faster than M1 or M2 (IL-4, IL-13, IL-10) macrophages. M1 macrophages convert glucose at a slower rate than M0 and M2 phenotypes. HSV-1 addition shows IL-4 converts glucose at a higher rate than M0 or M1 (lowest rate). As M0 macrophages are not active when HSV-1

infection occurs their lower energy requirements could explain the decreased acidification rate relative to IL-4. The faster glucose is converted the faster pyruvate can be used in other sources of metabolism. This could mean M0 and M2 phenotypes need to convert pyruvate at a faster rate because the macrophages also utilize other sources of energy, such as oxidative phosphorylation, which require pyruvate as an intermediate.

When oligomycin is added oxidative phosphorylation shuts down leading to an increase in glycolysis due to the Macrophage no longer receiving energy from ATP synthase. Macrophage's glycolytic capacity is higher in the M0 phenotype, but again M2 phenotypes are not far behind. M1 has a lower glycolytic capacity than M0 and M2 phenotypes. With the addition of HSV-1 the glycolytic capacity is higher in IL-4, than M0 or M1 (lowest rate). The maximum conservation of glucose for M0 and M2 could mean their cellular processes require more energy to continue function, whereas M1 macrophages do not perform the same cellular processes.

An interesting finding in the M1 phenotype in non-virus and virus is after the addition of oligomycin, which blocks mitochondrial ATP synthesis, there is a decrease in glycolytic capacity versus the increase in inactivated and activated M2 phenotype response. More research will need to be executed to conclude the exact cause of the decrease. Potential explanations for this observation include some dependence on ATP synthesis for energy, the higher increase in hexokinase, or an unknown interaction between oligomycin and macrophages/cytokines/products of activation.

Glycolytic reserve helps to understand the macrophage's response to the demand for energy as well as determining if the macrophage is working at the glycolytic theoretical maximum. M2 (IL-4, IL-10) have higher glycolytic reserve than M0, IL-13, and M1. With the addition of HSV-1 IL-4 has a higher glycolytic reserve than M0 or M1 (lowest rate). M2 (IL-4, IL-10) responds to energy demands faster than M0, IL-13, and M1 due to the involvement in endocytosis, antibody production along with inhibiting M1 responses (T helper 1 cells, production of NO, etc.), which could explain the need for a quicker response. M1 macrophages with and without the addition of virus have a negative glycolytic reserve, which could be attributed to the inability of the cell to respond at a faster rate or to the decrease after the addition of oligomycin. When comparing theoretical max (glycolytic reserve) to max (glycolytic capacity) none of the phenotypes with or without the addition of virus are working at theoretical max meaning there is a possibility for more glycolytic potential within the macrophages.

After the addition of 2-DG (an inhibitor of hexokinase) the ability to discover all other contributions to the ECAR is possible. When comparing phenotypes M0 has the highest ECAR contributions from other sources followed by M2 phenotypes with M1 showing the lowest ECAR. When virus is added IL-4 has the highest ECAR contributions than M0 or M1 (lowest). A potential source of ECAR can be contributed to the production of CO₂ during substrate oxidation where CO₂ become H₂CO₃ to dissociate to HCO₃⁻+H⁺. M0 and M2 phenotypes utilize oxidative phosphorylation and the electron transport chain whereas M1 macrophages do not, leading to an increase in other sources

of ECAR. This would indicate that when glycolysis is shut down there are other potential sources of metabolizing energy.

When measuring mitochondrial respiration with the XF24 Extracellular Flux Analyzer we have found that uninfected M0 and M2 (IL-4, IL-13, IL-10) phenotypes have significantly more potential for mitochondrial respiration than M1 or infected macrophages (M0, M1, IL-4). Potential explanations for mitochondrial respiration being utilized by M0 and M2 macrophages are M0 and M2 having the ability to proliferate, the functional plasticity of M0 and M2 macrophages, and M2 macrophages need to have a longer source of energy. When considering the differences between M0, M1, and M2 phenotypes the ability of M0 and M2 macrophages to switch between phenotypes and to proliferate has a significant impact on mitochondrial respiration. M1 macrophages typically do not revert back to M0 or even to go as far as becoming an M2 macrophage and do not proliferate. M1 macrophages will typically signal apoptosis after phagocytizing pathogens or the creation of a cytotoxic environment due to NO being produced. M2 macrophages need energy stores for a longer period to execute their functions because their functions take longer than hours to days versus clearance of pathogens, which needs a quicker response. The variable energetic demands of the different phenotypes therefore correspond to variation in metabolic response to HSV-1 infection as it decreases the macrophages ability to perform mitochondrial oxidation. As the HSV-1 infection depletes mitochondrial DNA the resulting change in cellular metabolism trends toward glycolysis and increased available energy compounds. This

response could allow for HSV-1 to generate energy quickly in order to facilitate the creation of large quantities of viral DNA via de novo nucleotide biosynthesis.

Baseline conditions are measured to determine macrophage ATP demand via oxygen consumption as well as mitochondrial proton leak contributions. When comparing phenotypes, M2 (IL-4, IL-13) consumes oxygen at a higher rate than M0, M2 (IL-10), and M1. With the addition of HSV-1 IL-4 consumes oxygen at a higher rate than M0 or M1 (lowest). Energy demands are higher among M0 and M2 (IL-4, IL-13, IL-10) macrophages due to the ability to proliferate or their plasticity. HSV-1 basal respiration is lower than non-virus due to mitochondrial DNA being depleted by the virus.

Once oligomycin is added successfully inhibiting ATP synthase ATP production and the proton leak can be measured. The oxygen consumption coupled to ATP production of the basal respiration is higher in M2 phenotypes than M0 or M1 (lowest). With the addition of virus IL-4 is higher than M0 or M1 (lower) but is significantly lower than non-virus ATP production. Proton leak shows the basal respiration not coupled to ATP production and can show mitochondrial damage. When looking at the proton leak M2 (IL-4 and IL-13) is higher than M0, M2 (IL-10), and M1 (lowest). With the addition of virus IL-4 is higher than M0 or M1 (lower), but is significantly lower than non-virus proton leak. Comparing ATP production to proton leak ATP production contributes the majority of basal respiration with or without the addition of virus. The majority of energy macrophages require is due to ATP detonating oxidative phosphorylation is an important metabolic process for M0 and M2 phenotypes.

FCCP is added to mimic a demand for energy and can show the maximal respiration macrophages are able to achieve. After the collapse of the proton gradient via FCCP, M2 (IL-13, IL-4) macrophages have a higher rate of oxygen consumption than M0, IL-10, or M1 (lowest). With the addition of virus IL-4 is higher than M0 or M1 (lower), but is significantly lower than non-virus maximal respiration. Knowing the maximal respiration can tell how often or how long the macrophages are working at max oxidative phosphorylation. M0 and M2 phenotypes are able to meet a metabolic challenge by oxidizing substrates at a higher rate for their cellular processes.

Spare respiratory capacity shows the ability of the macrophage to respond to a demand of energy as well as the theoretical max. M2 phenotypes have a higher response than M0 or M1 (lowest). With the addition of virus M1 is higher than M0 or IL-4 (lower), but is significantly lower than non-virus maximal respiration. M2 macrophages are involved in tissue repair and remodeling meaning that they require higher energy demands than M0 macrophages. Comparing theoretical max to the maximal respiration M0 and M2 phenotypes are working very close to their maximum energy output.

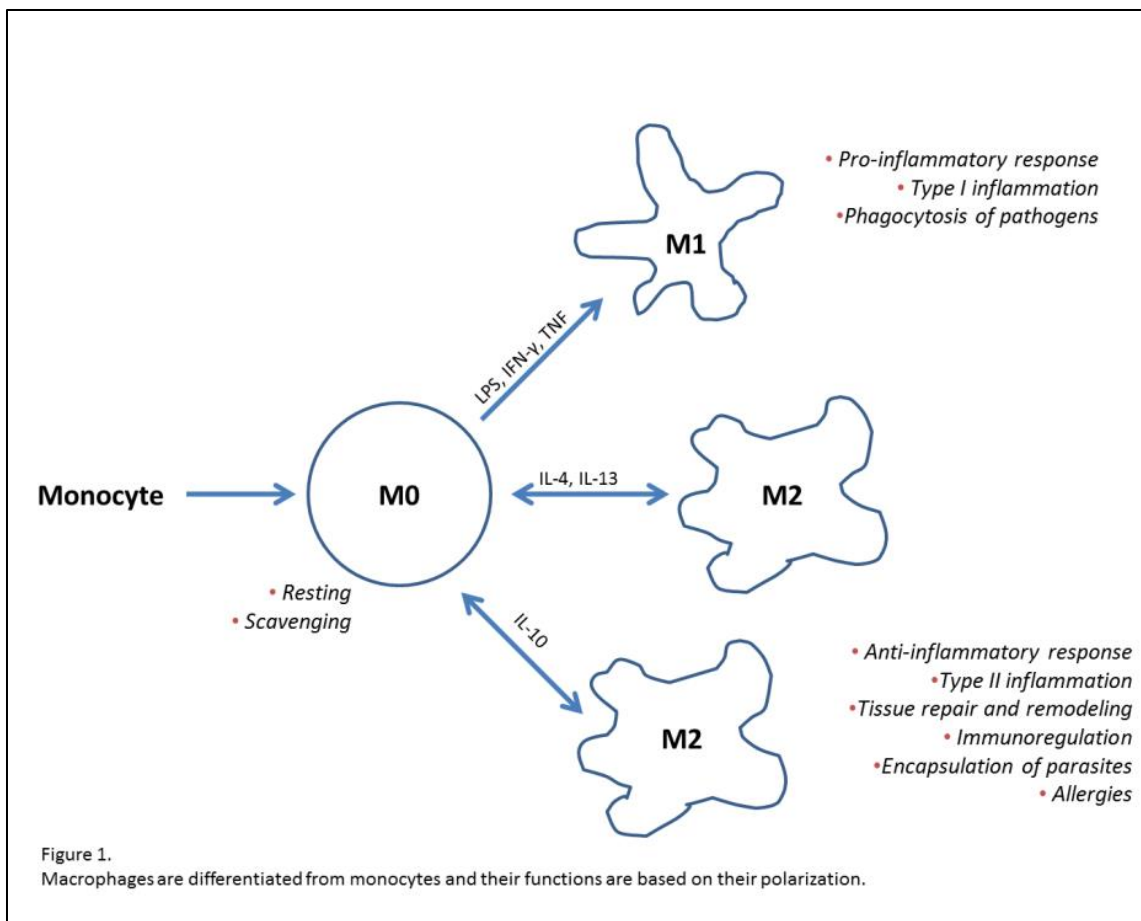
When rotenone and antimycin A are added oxidative phosphorylation is shut off and the remaining oxygen consumption rate can be contributed to other sources. M2 (IL-4, IL-13) are slightly higher than M0 and IL-10, but M1 is the lowest. With the addition of virus IL-4 is slightly higher, while M0 and M1 are very similar. A possible source for the oxygen consumption is glycolysis via NADH. NADH is oxidized to NAD⁺ and the H⁺ consumes oxygen to become water (H₂O).

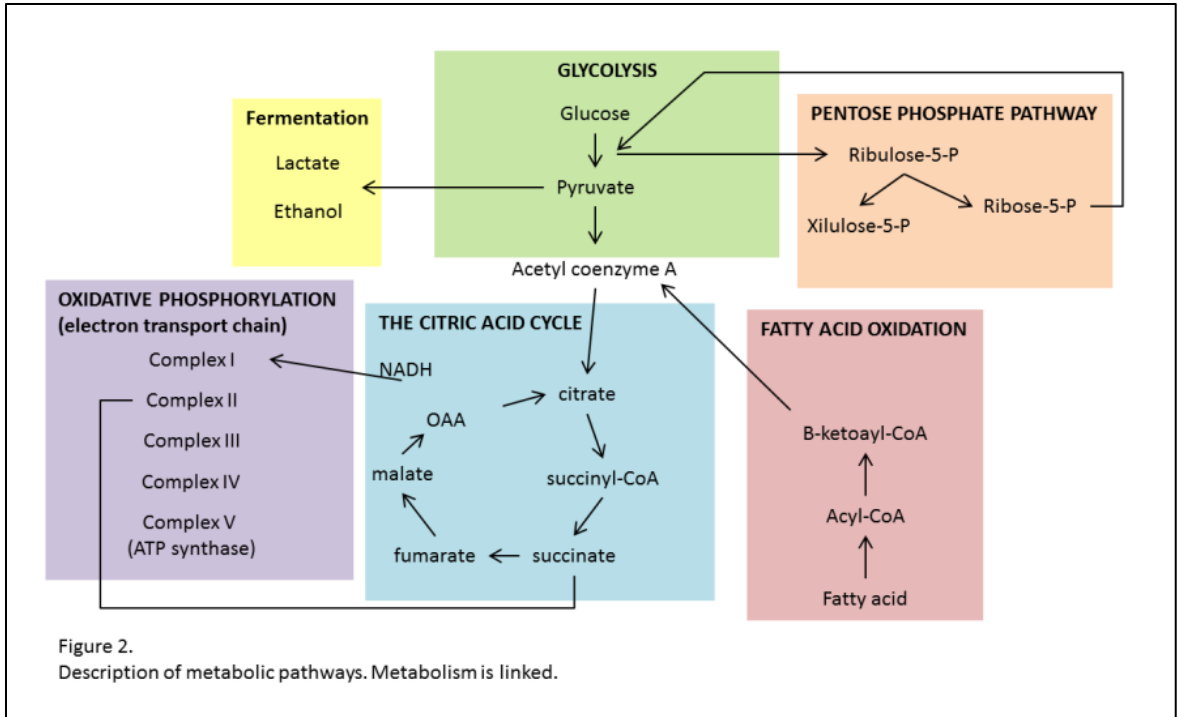
We found M2 (IL-4, IL-13, IL-10) macrophages utilize oxidative respiration while M1 macrophages do not but glycolysis is performed by all macrophages not just M1 macrophages. HSV-1 drives macrophages to perform glycolysis and shuts down oxidative phosphorylation.

Metabolic changes can occur within macrophages based on their phenotype. Using the XF24 Extracellular Flux analyzer we found M0 macrophages along with all three M2 induced phenotypes (IL-4, IL-13, and IL-10) do utilize oxidative respiration but discovered the utilization glycolysis by these macrophage phenotypes to generate energy. M1 macrophages perform glycolysis and inhibit oxidative phosphorylation. Viruses do not have the ability to metabolize energy and requires cellular machinery to generate a source of energy. HSV-1 does replicate and infect macrophages while causing metabolic changes within the macrophages. The macrophages metabolism is altered to perform glycolysis, while mitochondrial respiration is inhibited in all three infected phenotypes. Since the virus utilizes the cellular machinery, a way to prevent or slow down infection by blocking or shutting down glycolysis in the cell would be beneficial in combating HSV-1. The infectivity of the macrophages will be decreased and prevent further infection among macrophages as well as in neurons.

In future studies it would be beneficial to explore HSV-1 virus treatment, M1 possibilities for the decrease in glycolysis, and M2 macrophages utilization of glycolysis. With the addition of HSV-1 we discovered inactivated and activated macrophages perform glycolysis at a diminished capacity but do not undergo oxidative

phosphorylation. The HSV-1 was added 24 hours prior to running the experiment which could make time intervals a reason for diminished glycolysis. While previous research has shown that the uptake of glucose eventually decreases and the reasons for this decrease are not completely understood. I propose running glycolysis at four, eight, twelve, and 48 hours along with 24 hours again to see if there is a difference among time treatments. Another future study is to explore the decrease in glycolysis for M1 macrophages and why a decrease occurs when observing the maximum in glycolytic activity versus an increase to max. Exploring M2 macrophages utilization of glycolysis can be beneficial to discover the reasons behind utilizing glucose for energy.





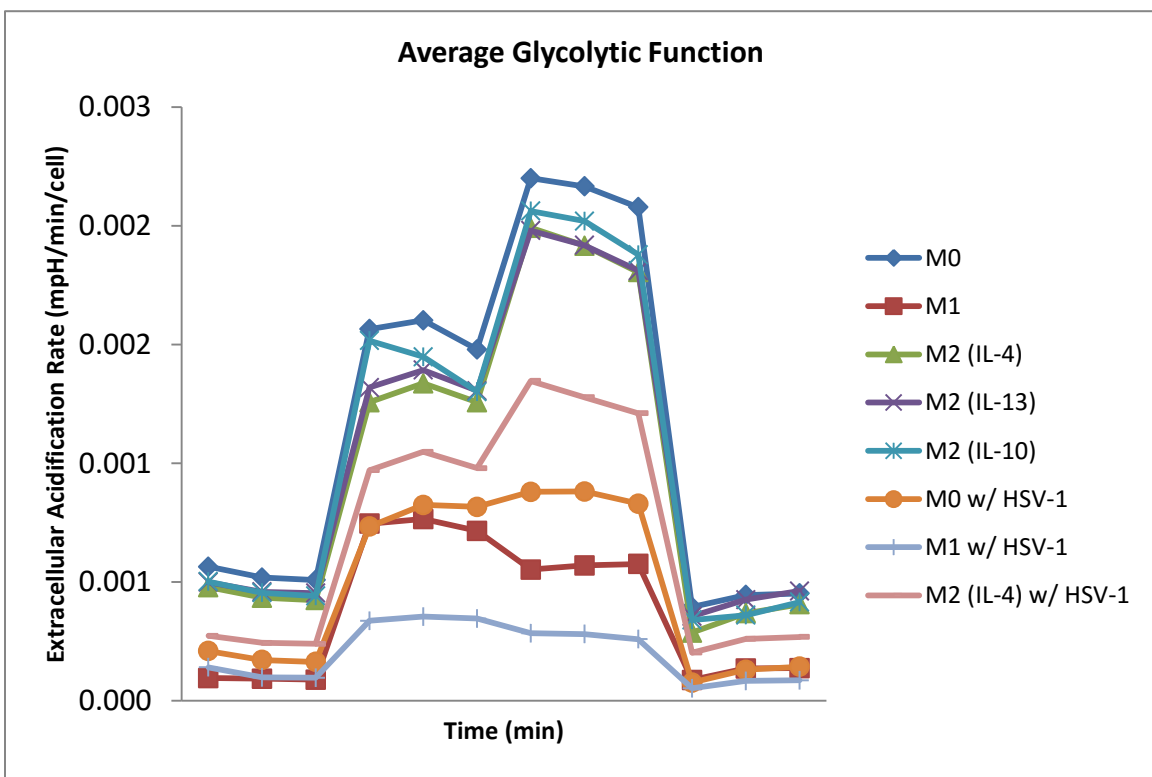


Figure 3. Glycolytic Function of uninfected and infected RAW 264.7 macrophages. RAW 264.7 macrophages undergo glycolysis. Infecting macrophages diminishes glycolysis response.

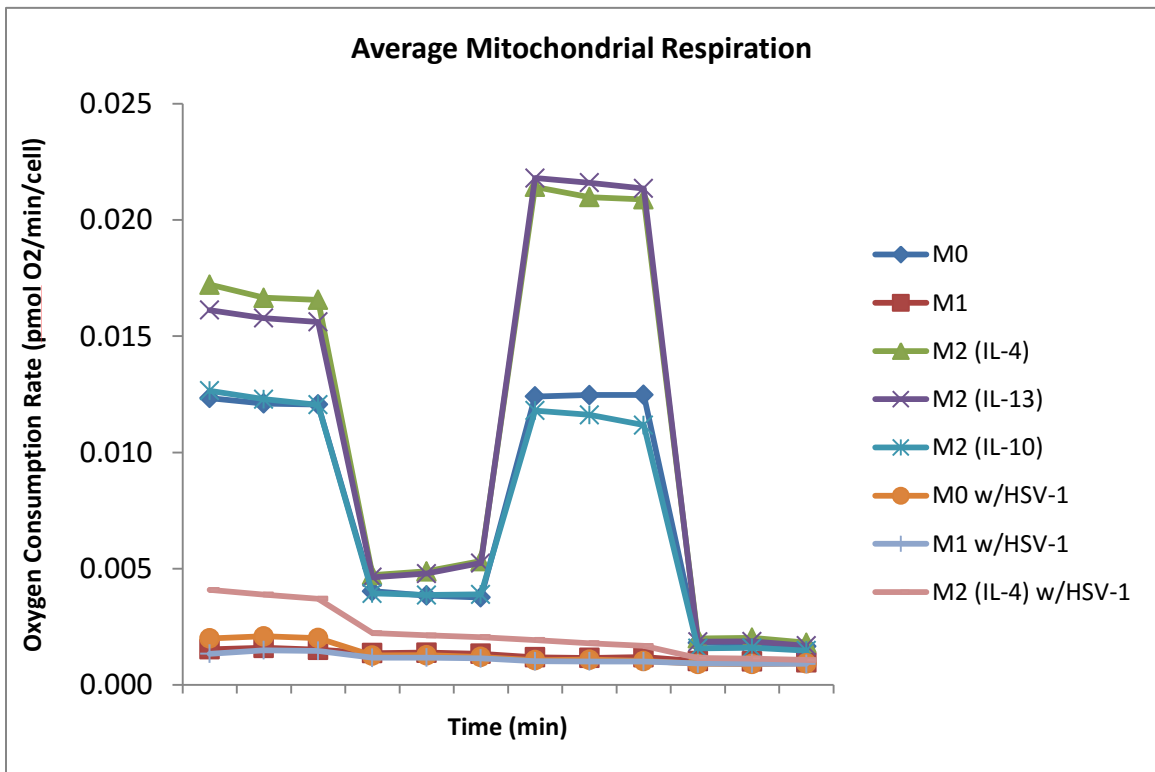


Figure 4. Mitochondrial Respiration of uninfected and infected RAW 264.7 macrophages.

Basal and M2 RAW 264.7 macrophages undergo mitochondrial respiration, but M1 RAW 264.7 does not. Addition of HSV-1 shuts down mitochondrial respiration.

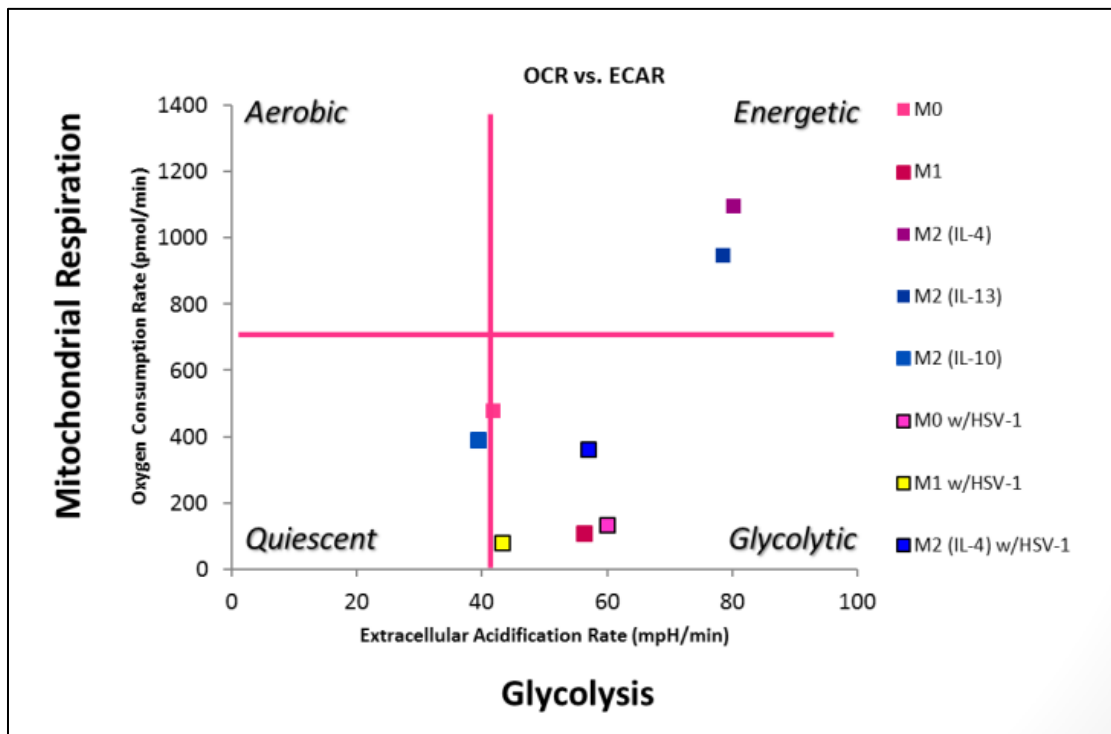


Figure 5.

OCR versus ECAR in uninfected and infected RAW 264.7 macrophages.

Oxygen Consumption Rate (measurement of oxidative phosphorylation) versus Extracellular Acidification Rate (measurement of glycolysis). M2 IL-4 and IL-13 induced macrophages (uninfected) are considered to be energetic utilizing both glycolysis and mitochondrial respiration. M0 and M1 (uninfected) along with all three infected phenotypes are more glycolytic.

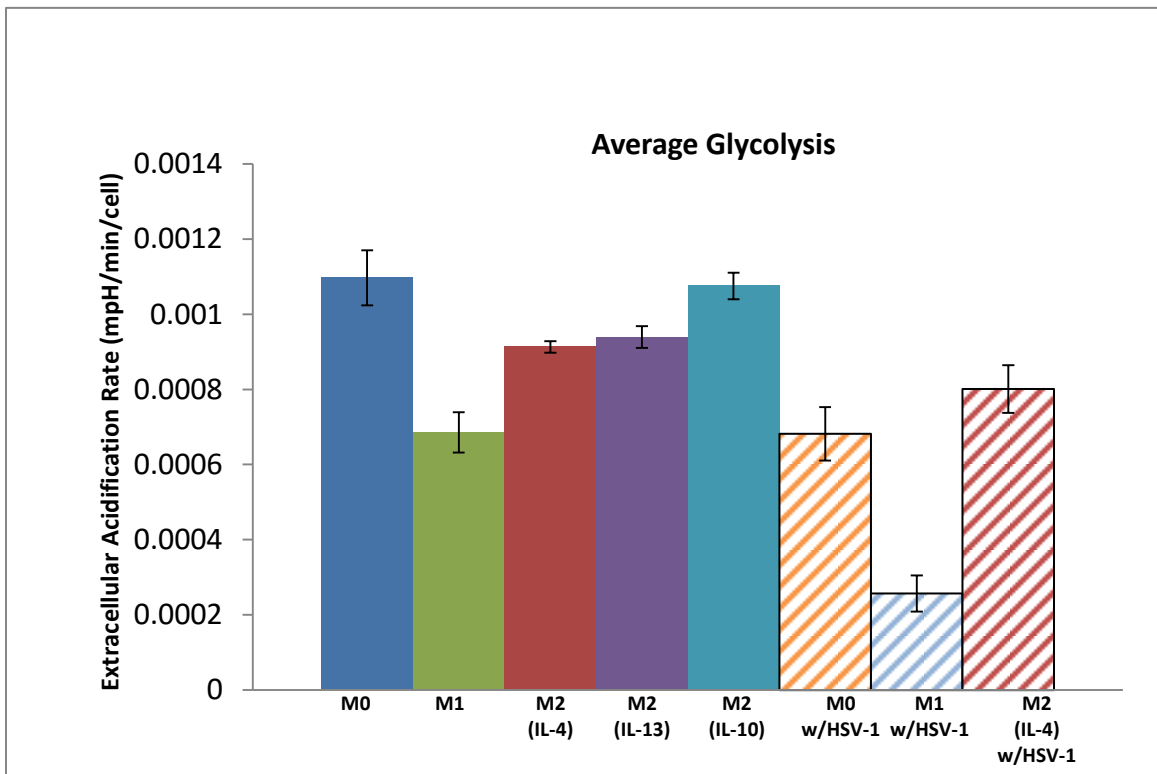


Figure 6.
Glycolysis of uninfected and infected RAW 264.7 macrophages.

Conversion of glucose to pyruvate.

Error bars represent mean values \pm SEMs from 3 independent replicates.

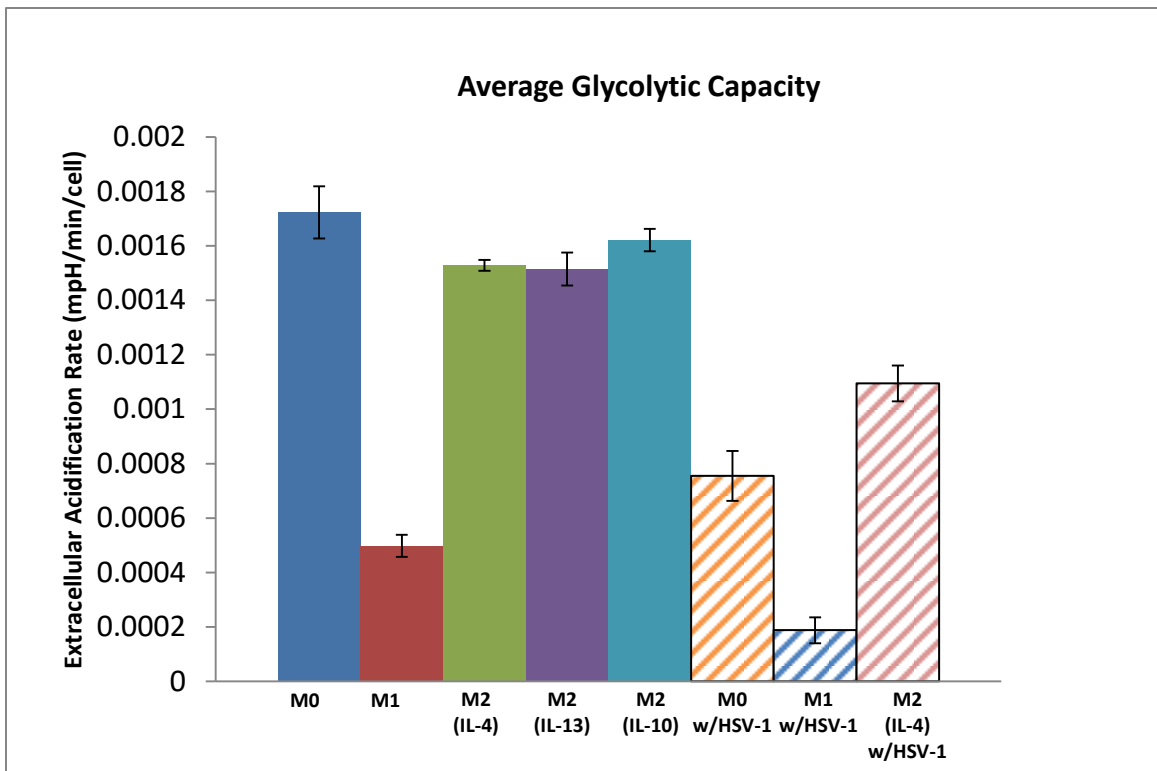


Figure 7. Glycolytic Capacity of uninfected and infected RAW 264.7 macrophages, a measurement of glycolytic function.

Maximum glycolysis the cell is able to reach after oxidative respiration is successfully shut down by oligomycin. Error bars represent mean values \pm SEMs from 3 independent replicates.

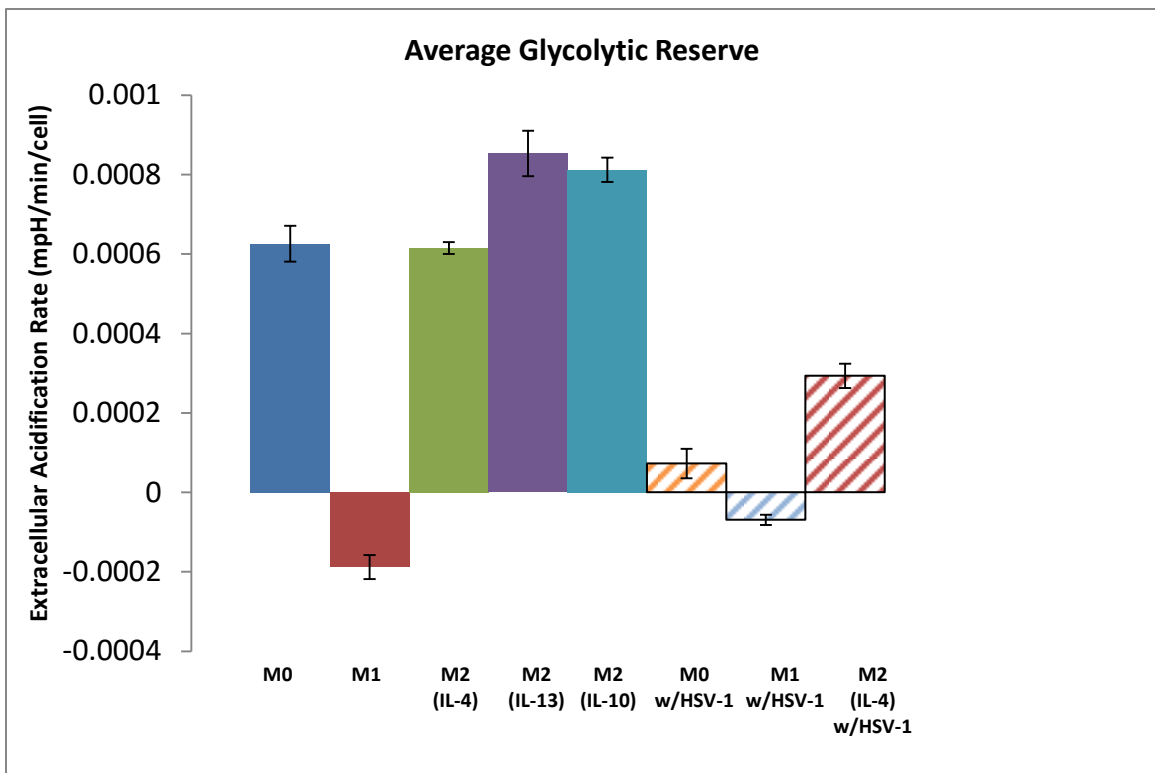


Figure 8. Glycolytic Reserve of uninfected and infected RAW 264.7 macrophages, a measurement of glycolytic function. Increase in glycolysis needed to meet energy demands without mitochondrial ATP production. Error bars represent mean values \pm SEMs from 3 independent replicates.

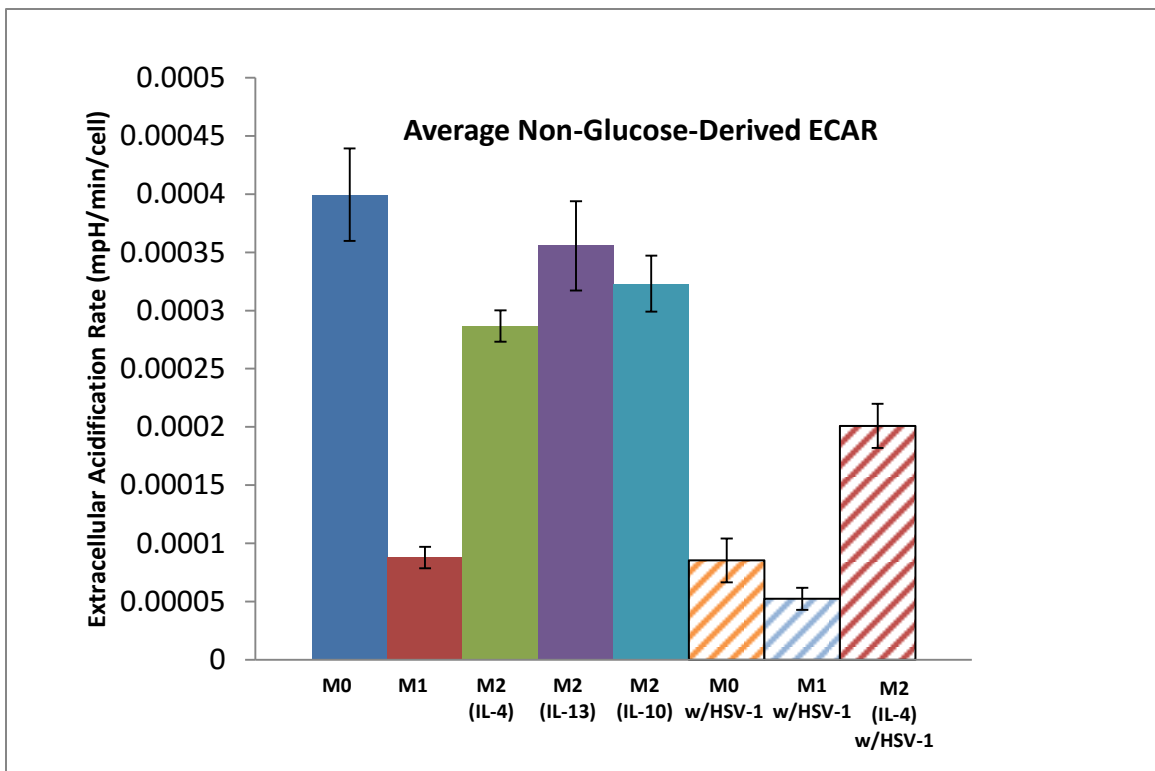


Figure 9. Non-glucose derived ECAR of uninfected and infected RAW 264.7 macrophages, a measurement of glycolytic function.

ECAR not contributed by glycolysis. Error bars represent mean values \pm SEMs from 3 independent replicates.

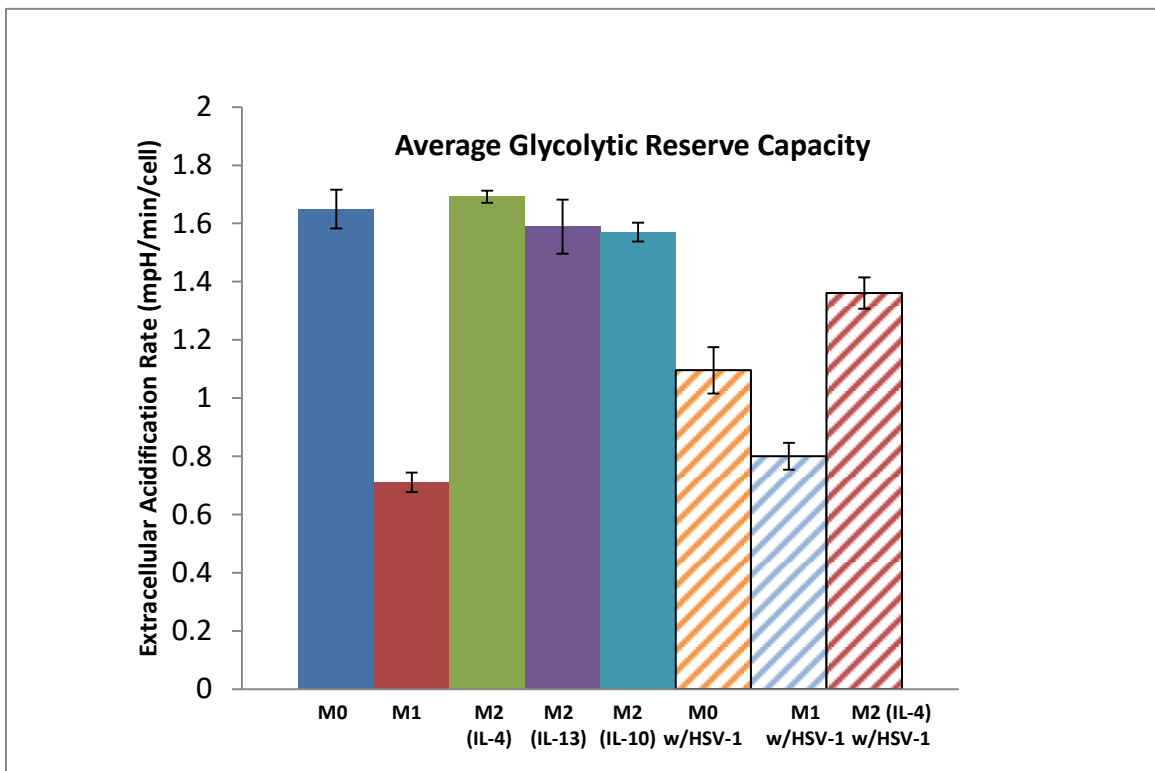


Figure 10. Glycolytic Reserve Capacity (as fold-increase) of uninfected and infected RAW 264.7 macrophages, a measurement of glycolytic function.

How closely glycolysis is working at maximum. Error bars represent mean values \pm SEMs from 3 independent replicates.

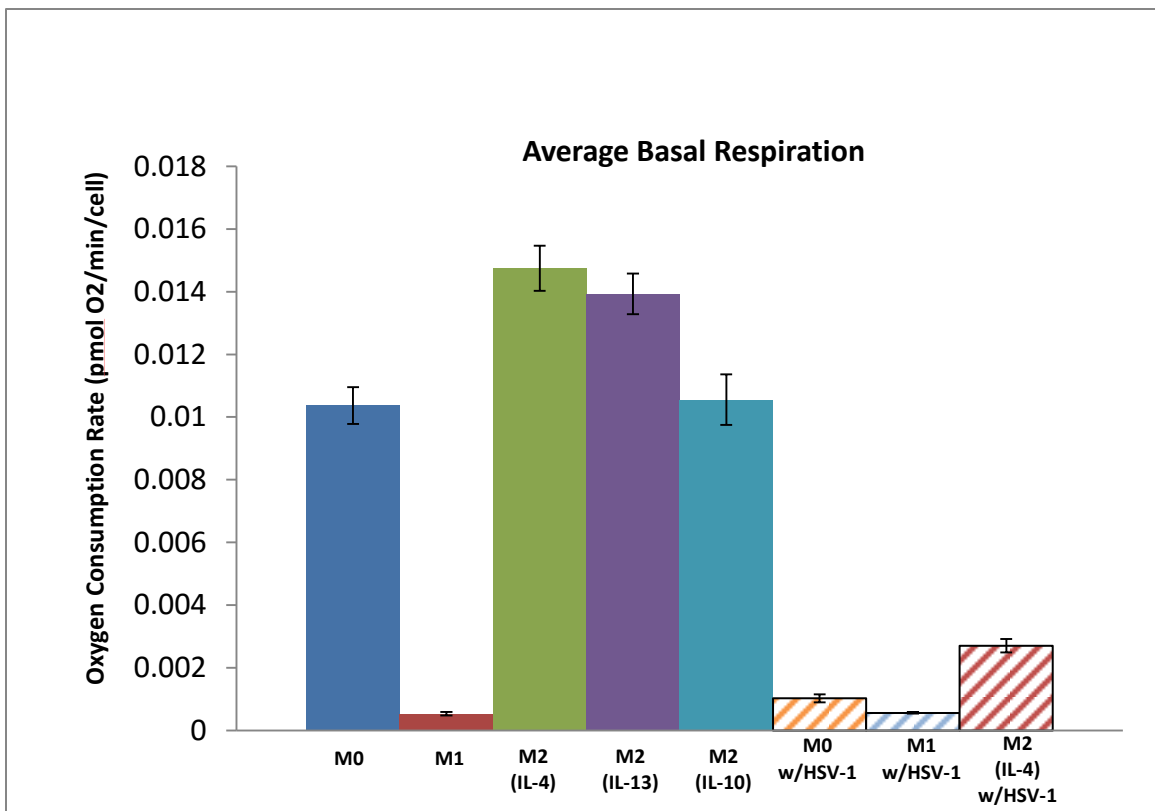


Figure 11. Basal Respiration of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

Energy requirements under baseline conditions. The respiration used to meet the cell's ATP demand and drive mitochondrial proton leak. Error bars represent mean values \pm SEMs from 3 independent replicates.

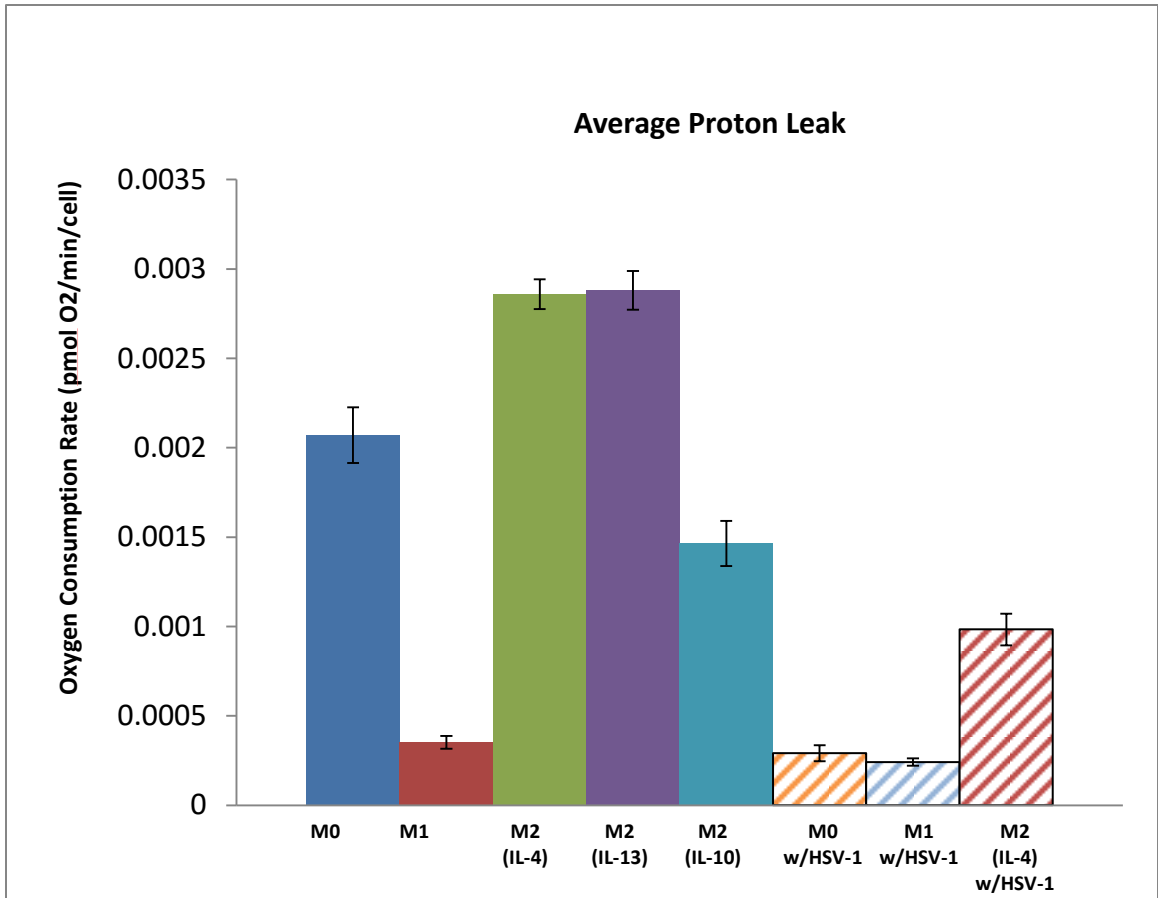


Figure 12.

Proton Leak of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

Basal Respiration not coupled to ATP production due to proton leak across mitochondrial membrane. Error bars represent mean values \pm SEMs from 3 independent replicates.

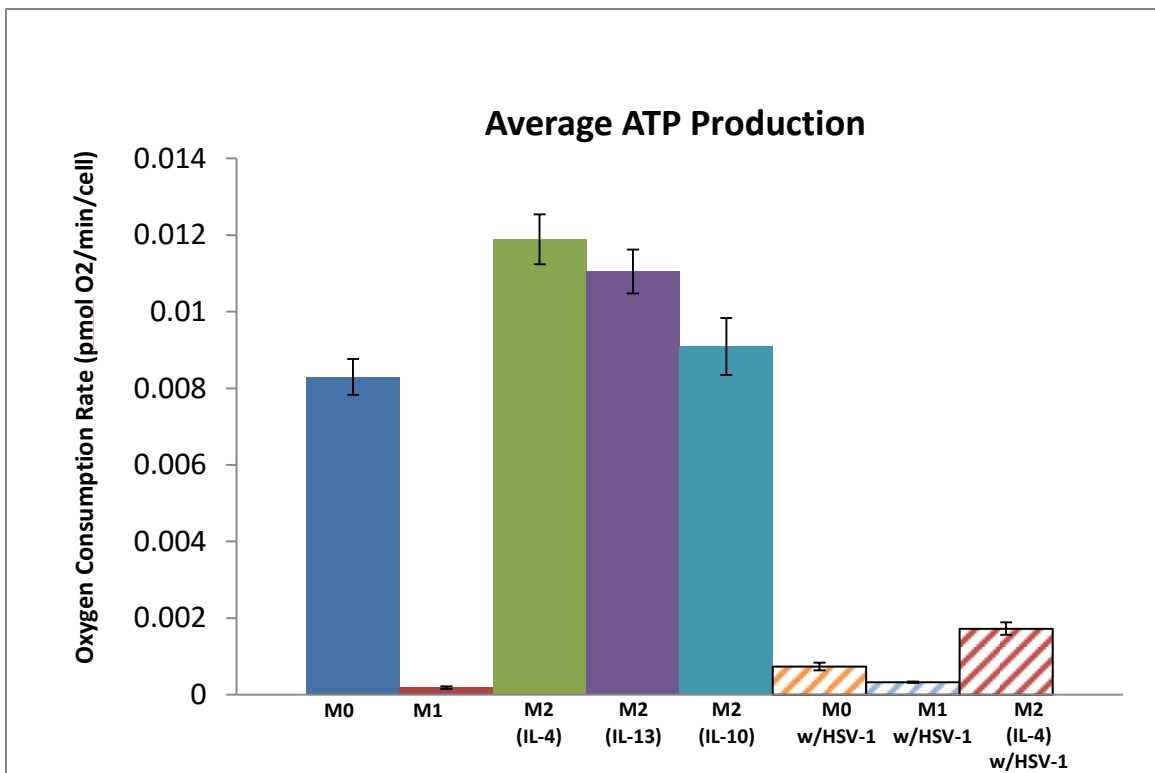


Figure 13.

ATP Production of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

Respiration used to drive ATP production and meet energetic needs under basal conditions. Error bars represent mean values \pm SEMs from 3 independent replicates.

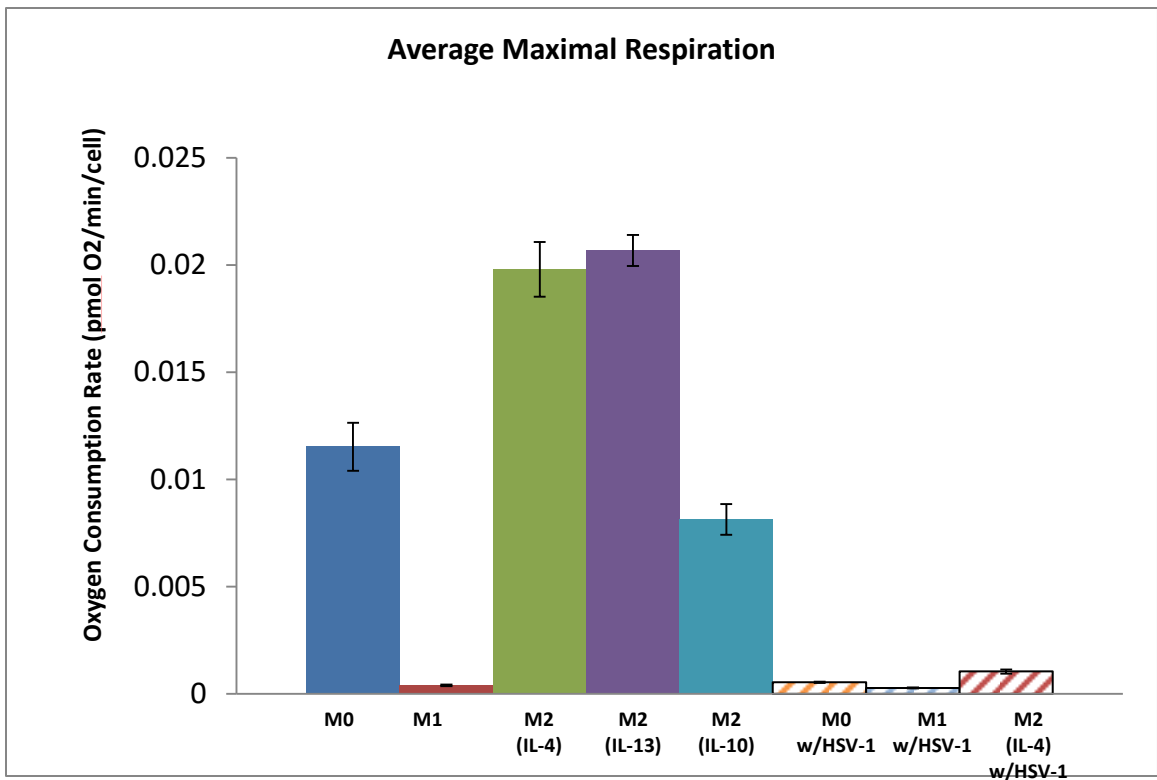


Figure 14.

Maximal Respiration of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

Maximum oxygen consumption, macrophages rapidly oxidize substrates to meet the metabolic change. Error bars represent mean values \pm SEMs from 3 independent replicates.

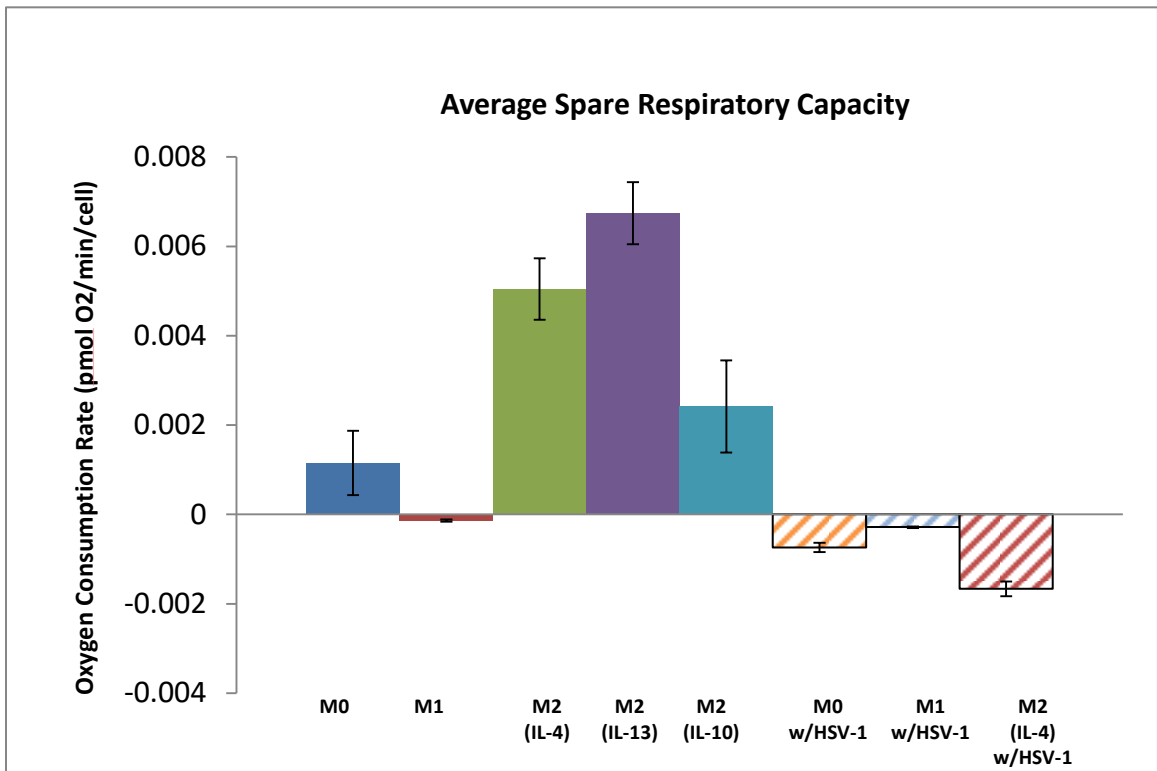


Figure 15.

Spare Respiratory Capacity of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

How closely the macrophage is running at it' theoretical maximum and the capability to respond to energy demands. Error bars represent mean values \pm SEMs from 3 independent replicates.

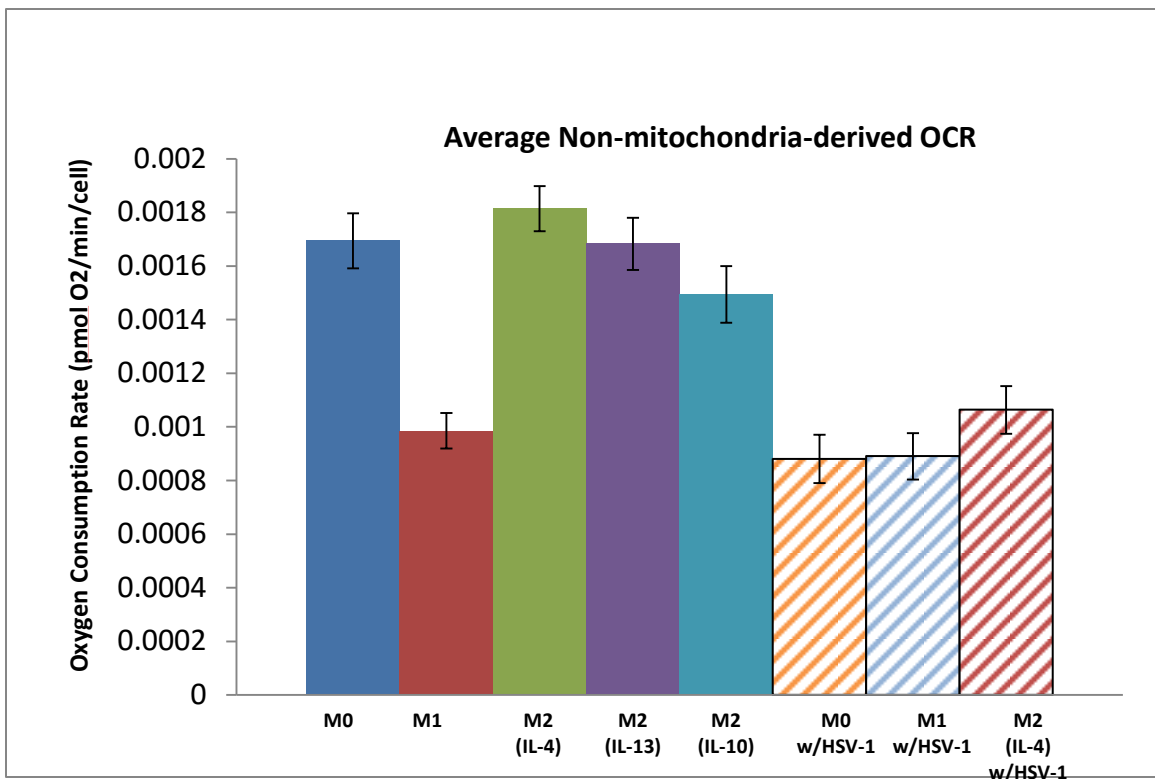


Figure 16.

Non-mitochondria derived OCR of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

Oxygen consumption not contributed to mitochondrial respiration. Error bars represent mean values \pm SEMs from 3 independent replicates.

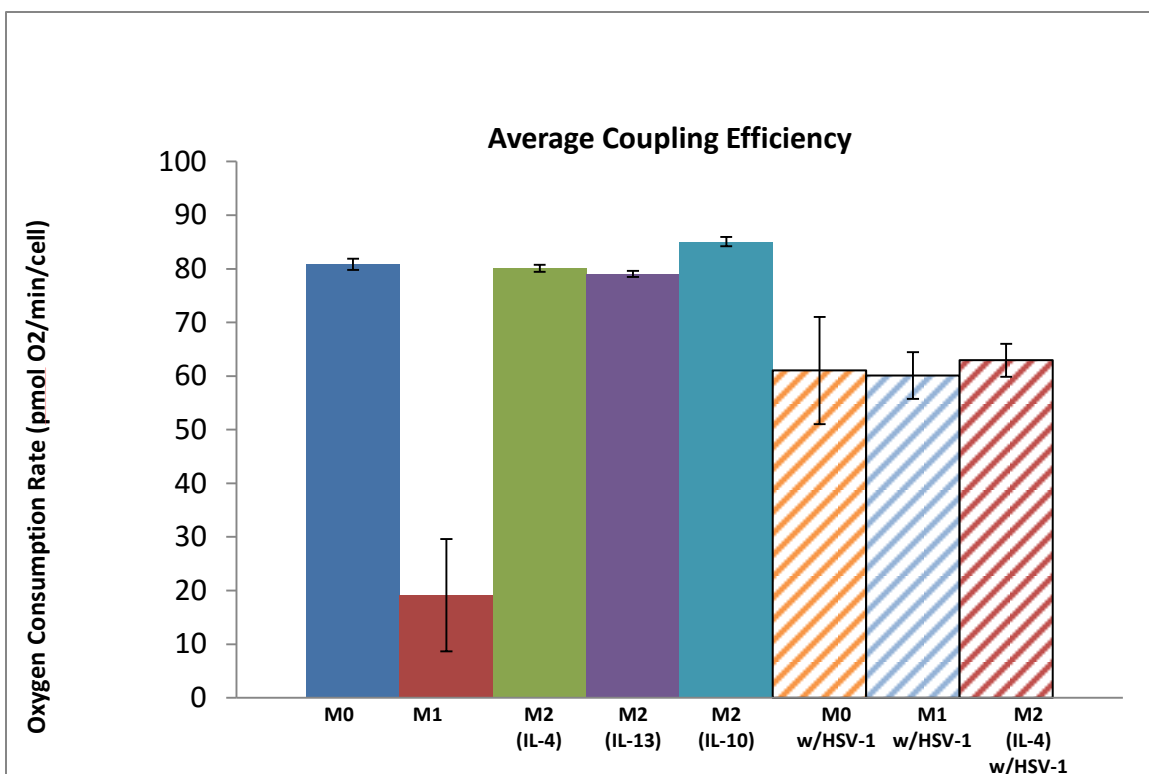


Figure 17. Coupling efficiency of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

The fraction of basal respiration used to drive ATP synthesis versus the proton leak. Error bars represent mean values \pm SEMs from 3 independent replicates.

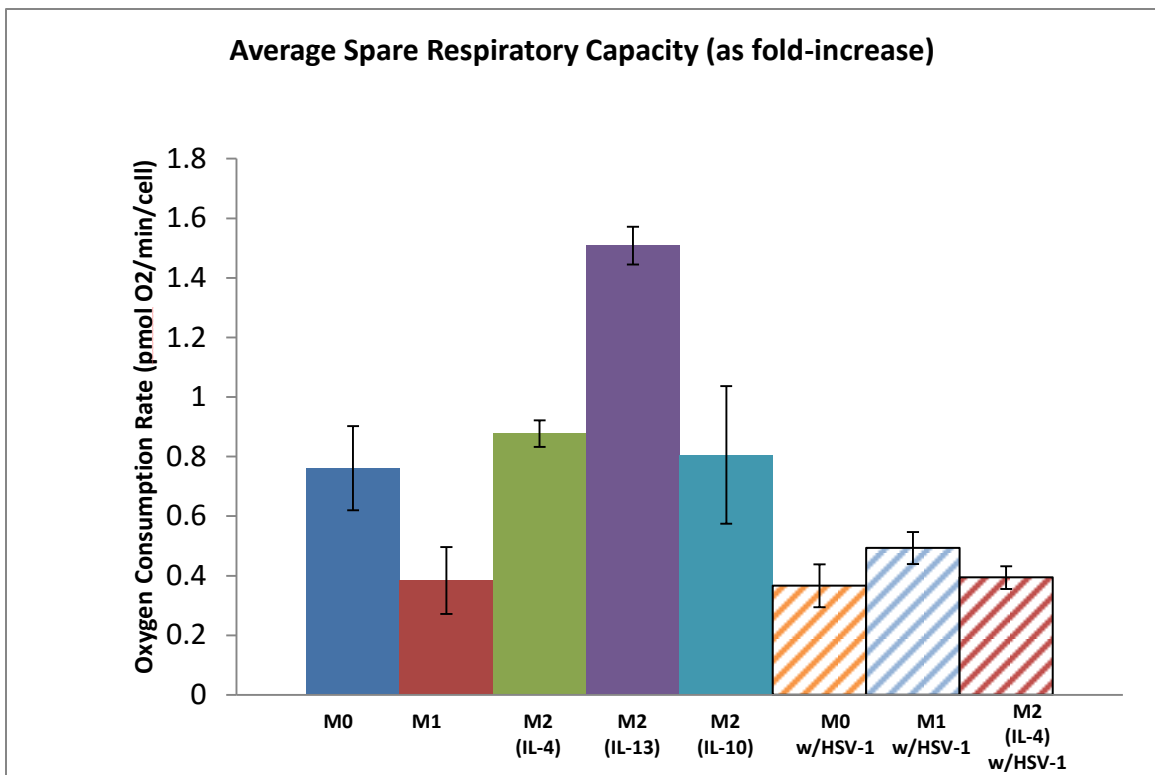


Figure 18.

Spare Respiratory Capacity (as fold-increase) of uninfected and infected RAW 264.7 macrophages, a measurement of mitochondrial respiration.

How closely mitochondrial respiration is working at maximum. Error bars represent mean values \pm SEMs from 3 independent replicates.

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