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Glycolytic ATP production is required for innate mast cell activation and is limited by lactic acid, which effectively reduces LPS-induced cytokine production in mast cells and in vivo

Heather Caslin
Virginia Commonwealth University

Glycolytic ATP production is required for innate mast cell activation and is limited by lactic acid, which effectively reduces LPS-induced cytokine production in mast cells and in vivo

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Integrative Life Sciences at Virginia Commonwealth University

By
Heather Caslin
M.S. Virginia Commonwealth University

PhD Mentor: John J.Ryan, Professor, Department of Biology

Virginia Commonwealth University
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Abstract

GLYCOLYTIC ATP PRODUCTION IS REQUIRED FOR INNATE MAST CELL ACTIVATION AND IS LIMITED BY LACTIC ACID, WHICH EFFECTIVELY REDUCES LPS-INDUCED CYTOKINE PRODUCTION IN MAST CELLS AND IN VIVO

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The metabolic pathways required for adenosine triphosphate (ATP) production within the cell are well understood, however recent publications suggest that metabolic pathways are closely linked to immune cell activation and inflammatory diseases. There has been little examination of the metabolic pathways that modulate mast cell activation and the feedback regulator lactic acid. Here we examine metabolic pathways and regulation within mast cells in the context of lipopolysaccharide (LPS) and interleukin (IL-33) activation, for which there has been little to no reported studies. First, we examine the effects of lactic acid, previously considered only a by-product of glycolysis and now understood to act as a negative feedback regulator of inflammation in the context of LPS activation and sepsis. Lactic acid is elevated in septic patients and associated with mortality, potentially due to suppressive effects on LPS signaling and

contribution to late phase immunosuppression. By attenuating glycolysis and reducing ATP availability for signaling and cytokine transcription, lactic acid impairs the function of immune cells to fight the initial or subsequent infections. We support this with *in vitro* and *in vivo* data. Additionally, our lab has published that lactic acid can suppress IL-33 activation, potentially by metabolic modulation as with LPS activation; however there has been no study of the metabolic requirements for IL-33 activation. We report here that glycolysis is required for ATP and reactive oxygen species (ROS) production to augment signaling and cytokine production downstream of the IL-33 receptor. Together, these studies examine the contribution of metabolism to mast cell activation and may provide potential targets for treatments of diseases that involve LPS- or IL-33-dependent mast cell activation.

Chapter 1: Introduction

1.1 The Immune System

The immune system is an elaborate system of cells, tissues, and organs that function to protect us from foreign pathogens, including bacteria, viruses, fungi, and parasites, by discriminating pathogens from "self", commensal bacteria, and harmless elements of the environment. In addition, the immune system plays an important role in maintaining tissue and nutrient homeostasis in many other organs, such as the liver, gut, adipose tissue, and skin[1–6]. While responses against dangerous foreign pathogens are ordinarily protective, inappropriate immune activation against allergens or one's own body can promote allergic disease and autoimmunity, such as allergic rhinitis, asthma, food allergy, arthritis, multiple sclerosis, lupus, and type I diabetes. Additionally, inflammation is associated with diseases such as obesity, type II diabetes, and depression, and while the cause and effect is not entirely elucidated, inflammation and pathogenesis of these diseases may augment each other[7–12].

There are two main arms of the immune system: innate and adaptive immunity. The innate immune system provides a rapid, but non-specific, defense. This response occurs primarily at the site of infection or injury, with tissue resident cells initiating antigen presentation, cytokine production, phagocytosis, and cell recruitment. These cells respond to pathogen "patterns" - proteins and lipids commonly found on the surface of pathogens. The adaptive immune system requires time to develop a specific response to the pathogen of interest and develops memory for secondary exposures. This response occurs mainly in the lymph nodes, where T and B cells are trained to respond to a

specific protein, referred to as an antigen, on one particular pathogen and produce antibodies against that antigen. Memory cells are produced following this response, and upon subsequent exposures, the adaptive response is much faster and more effective.

Many immune cells participate in these responses. Innate lymphoid cells (ILCs), macrophages, and mast cells are tissue resident innate immune cells that respond to non-specific pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). These cells release cytokine and chemokine signals to initiate the immune response and recruit cells to the area where they are needed. Neutrophils and monocytes are recruited from the blood into the tissue by cytokine and chemokine signals and phagocytize pathogens. Macrophages play an additional role by phagocytosis of dead neutrophils after an immune response has completed, and initiating wound healing to repair damaged tissue. Dendritic cells are the primary tissue resident cells to play a role in antigen presentation to T cells. Upon seeing antigen, dendritic cells travel to the nearest lymph node and present that antigen to a T cell. T cells then find B cells and induce B cells to produce of antigen-specific antibodies. Together, these cells tailor the immune system to fight the specific pathogen of interest.

1.2 Mast Cells

The focus of the work in our laboratory is on the mast cell. Discovered in 1878 by Paul Erlich, mast cells were found to produce heparin and histamine as the main drivers of anaphylaxis in 1937 and 1950, respectively[13]. Today, mast cells are known to secrete many additional mediators, including an array of cytokines. Mast cells are found in all tissues except blood, and are numerous at sites of contact with the outside environment,

including the skin, respiratory tract, and gastrointestinal tract. Localization in different tissues can elicit distinct mast cell protease and receptor expression[14–16]. Moreover, mast cell phenotype is highly "tunable" based on short term modulation by inflammatory stimuli, growth factors, cytokines, and metabolites [15,17–19]. Mast cells are typically recognized for their role in Th2 immunity, including their detrimental role in allergic disease and their protective role against parasites and venoms[20–23]. However, mast cells also play a role in defense against bacterial and viral pathogens[24–26] and have been implicated in many diseases[27], including atherosclerosis[28], multiple sclerosis[29,30], rheumatoid arthritis[31], and tumor progression[32,33].

The best-studied mechanism of mast cell activation is IgE. This was discovered by two groups in the late 1960's by the Ishizakas, Bennich and Johansson[34–36]. As described by Johansson, the Ishizakas found an antiserum that could precipitate a serum fraction, resulting in a decrease in PK activity and provisionally called it γE-globulin while Bennich and Johansson discovered a patient's serum with an atypical myeloma protein and was also shown to blocked the PK test[37]. This immunoglobulin was provisionally called IgND. In 1967, when both groups shared reagents, the antiserum to γE-globulin reacted with isolated IgND, and that purified IgND could block the reaction of anti-γE in a biological test system for reaginic activity. It seemed that the γE and IgND shared antigenic determinants and biological activity. The World Health Organization Immunoglobulin Reference Centre in Lausanne officially named this molecule IgE in 1968.

For mast cell activation, antigens crosslink (XL) monomeric IgE bound to the alpha subunit of the high affinity FcεRI receptor, resulting in subunit aggregation which

induces phosphorylation and signaling through Fyn, Lyn, Syk, LAT, PI3K, PLCγ, and MAPKs[38]. In response to IgE XL, these signals induce calcium flux, degranulation, and cytokine production[21]. Mast cell granules contain histamine, lipid mediators, proteases, and cytokines, which are released upon activation to elicit the symptoms of allergic disease, including itching, swelling, warmth, and redness, and pain[39,40]. Additionally, the *de novo* synthesis of arachidonic acid metabolites, cytokines, and chemokines follows, which initiate a greater immune response, including recruitment of neutrophils and eosinophils, B cell class switching, and the activation of T cells[41–43].

Mast cells can also recognize PAMPs and DAMPs. PAMPs are non-specific antigens from bacterial, viral, and fungal pathogens that activate Toll-like receptors (TLRs) and NOD-like receptors (NLRs)[24,25]. A well-studied PAMP is LPS from gram negative bacterial cell walls, which binds to TLR4. DAMPs include proteins released following cell damage, necrosis, or activation, such as high-mobility group box 1 (HMGB1), IL-33, and ATP. LPS and IL-33 have both been implicated in initiating mast cell activation and augmenting activation by IgE, as well as allergic disease and inflammation *in vivo*[44–49]. While the importance and role of LPS and IL-33 may differ in each mast cell-related diseases, their downstream signaling is very similar[50]. LPS binding to TLR4 or IL-33 binding to ST2 both induce signaling through myeloid differentiation primary response 88 (MyD88), tumor necrosis factor (TNF) receptor associated factors (TRAFs), interleukin-1 receptor-associated kinase (IRAK), transforming growth factor beta-activated kinase (TAK1), mitogen-activated protein kinases (MAPKs; p38, JNK, and ERK). These activate transcription factors, including nuclear factor kappa B (NFκB), activator protein-1 (AP-1), and cAMP response element-

binding protein (CREB). Both LPS and IL-33 induce cytokine production from mast cells[51–54], eliciting similar downstream effects. Importantly, mast cell-dependent neutrophil recruitment and TNF, IL-6, and leukotriene production has been shown *in vivo* using IL-33- and bacterial-induced peritonitis. These studies showed that IL-33- and LPS-induced mast cell activation is critical for the immune response[55–62].

Current therapeutics for mast cell-related diseases like allergy and asthma are incompletely effective and have many side effects. Traditional antihistamines and leukotriene modifiers block the effects of one type of mast cell mediator, however a plethora of signaling molecules are released and independently contribute to symptoms. While steroids are generally effective, they impair protective immune responses to infection, and patients can develop steroid resistance and serious side effects. Specifically, adverse effects and reduced efficacy is evident during viral-induced exacerbations, ibn asthmatics who smoke, and in patients with more severe forms of the disease[63,64]. Additionally, the mast cell stabilizer disodium cromoglycate is approved for some mast cell proliferative and activation diseases[65], but the mechanism of action is poorly understood[66], the time to effect is slower than drugs like antihistamines, and there is evidence that mast cell stabilizers are less efficacious than inhaled corticosteroids for asthma[67,68]. Progess is being made. The newer generation of antihistamines have better binding affinity for histamine receptors, reduced adverse side effects, and often have mast cell stabilizing and anti-inflammatory properties in addition to their effects on histamine, which may provide better relief for patients[67]. Additionally, the humanized anti-IgE antibody, omalizumab, is a common and preferred treatment for chronic urticaria[69] and shows efficacy for the treatment of some asthma[70], however like many drugs, there

appear to be responders and non-responders[71]. More research is needed to develop effective treatment strategies for all patients with allergic disease.

Our group studies the molecular mechanisms of mast cell activation and inherent controls in order to determine specific targets for therapeutic development. We are particularly interested in new uses for drugs that are FDA-approved or in development for other diseases, repurposing them to modulate mast cell activation and better understand how modulating receptor signaling changes function. Recent publications in the past 10 years from our lab have focused on the contribution of kinases (Lyn, Fyn), transcription factor functions (Stat5A/ B), and the effects of cytokines that are elevated within inflammatory diseases like transforming growth factor (TGF)-β and IL-10 on mast cell function[54,72–75]. Additionally we have published the suppressive effects of fluvastatin, dexamethasone, and Didox on mast cell function[53,76–78]. Recently we have become interested in the suppressive effects of lactic acid on mast cell activation by IL-33 and IgE[52]. Lactate and the associated H^+ ions are a product of glycolytic energy production that is elevated in many inflammatory environments, such as cancer, wounds, colitis, sepsis, and metabolic syndrome (obesity and type II diabetes)[79–84]. While we showed that the suppressive effects of lactic acid involved monocarboxylic transporter (MCT)-1, micro RNA (miR)-155, and hypoxia inducible factor (HIF)-1α[52], our first publication did not examine the full mechanism of action or the effects on mast cell metabolism, which have potential implications for mast cell function.

1.3 Mast Cell Metabolism

Cellular metabolism is the production of energy (ATP) from sugars, fats, and proteins by glycolysis, the pentose phosphate pathway, the Kreb's cycle, β-oxidation, and the electron transport chain. While scientists generally understand the importance of energy for basic cell function and human life, immunologists have recently begun to appreciate the contribution of different energy systems to support immune cell activation and effector functions. Immune cells require energy for homeostasis, maintenance, and proliferation, but also for differentiation and activation, phenotypic changes, effector functions, and overall inflammation[85]. Researchers generally agree that glycolysis is the primary energy production pathway used by inflammatory cells, because activation of T-helper (Th)1, Th17, macrophage (M)1, and dendritic cells (DCs) induces glycolysis, while oxidative phosphorylation (OX PHOS) in the electron transport chain (ETC) is the primary energy production pathway used by regulatory cells such as T-regulatory (Treg), M2 macrophages, and monocyte derived suppressor cells (MDSC)[86–89].

Utilizing glycolysis may appear counterproductive, since it is inefficient, producing only 2 ATP per glucose molecule compared with 32 per glucose in OX PHOS. Because ATP provides phosphates and bond energy for kinase activity, receptor signaling, transcription, actin polymerization, vesicle trafficking and fusion, and macromolecule biosynthesis, greater ATP levels are needed for inflammatory functions. However, the benefits of utilizing glycolysis during activation and proliferation are multi-fold and mimic the Warbug effect in tumors[90,91]. Glycolysis rapidly increases ATP availability, operates under low oxygen tension, and provides intermediates for the pentose phosphate pathway and Kreb's cycle - intermediates needed for anabolic pathways synthesizing nucleotides, amino acids, and lipids[86,92]. This has been most

extensively studied in T cells, which undergo dynamic and complex metabolic reprogramming in response to activation, cytokine stimulation, and other changes in their microenvironment[88,89,93].

However, there is little understanding of the metabolic requirements for mast cell activation. Evidence suggests that rat mast cells release lactate upon activation with compound 48/80 and polymyxin B[94]. Additionally, glycolytic blockade by 2-deoxyglucose (2-DG), iodoacetate, fluoride and oxamate suppressed glucose-sustained, compound 48/80 and IgE XL-induced histamine release in rat mast cells[95], suggesting that glycolysis is used. Additionally, studies in LAD2 and HMC1 mast cells suggest that high glucose media can increase reactive oxygen species, TNF expression, p-ERK, p-JNK, and p-p38 following IgE-mediated activation[96]. Furthermore, Kitahara et al. reported that long-term (7 days), but not shorter term (3 h, 1 day, or 3 days) culture of bone marrow-derived mouse mast cells (BMMC) with high-glucose (33 mM) medium enhanced the IgE XL-mediated degranulation and LTC_4 production[97], suggesting that glucose metabolism increases inflammatory functions in mast cells. Interestingly, these results were not due to changes in FcεRI expression, intracellular ATP levels, or calcium signaling, but high glucose significantly increased $cPLA_2$ phophorylation and COX-1 expression. In a more recent publication, IgE-XL increased p-ERK, mitochondrial p-STAT3 (ser727), and OX PHOS activity[98]. Furthermore, protein inhibitor of activated STAT3 (PIAS3) was found to inhibit OXPHOS and mast cell degranulation, suggesting these proteins are important for mitochondrial ATP production and degranulation. And lastly, mouse mast cell activation in response to IgE XL was recently characterized using the Seahorse analyzer[55]. Glycolysis quickly increased, while OX PHOS was elevated ~2

hrs after stimulation. Interestingly, blocking glycolysis with dichloroacetate, increasing the conversion of pyruvate into acetyl CoA at the expense of lactate production, or blocking OX PHOS with rotenone, suppressed cytokine production and degranulation. There was no effect of etomoxir, which inhibits fatty acid oxidation, suggesting that cytokine production and degranulation are dependent upon glucose metabolism.

While these recent studies have begun to unravel mast cell metabolism in the context of IgE activation, there have been relatively few studies regarding cellular metabolism in mast cells and little to no studies examining other means of activation such as IL-33 and LPS. Additionally, there have been no studies to examine IL-33-induced changes in metabolism in any cell type, and none regarding the effects of lactic acid on LPS-mediated mast cell activation. To address these gaps in the literature, Chapter 2 will examine effects of lactic acid on LPS-activation in mast cells, the mechanisms of action, and the relevance *in vivo*. Chapter 3 will follow up with a review of lactic acid effects in immune cells and the implications for health and disease, including the data from Chapter 2. And lastly, Chapter 4 will examine metabolic requirements of IL-33 activation. Together, this work contributes to our fundamental understanding of mast cell function in health and disease and will hopefully provide targets for future therapeutics.

Chapter 2: Lactic acid inhibits LPS-induced mast cell function by limiting glycolysis and ATP availability.

Authors: Heather L. Caslin, Daniel Abebayehu, Amina Abdul Qayum, L., Patrick Paez, Alexis Hoeferlin, Charles E. Chalfant, John J. Ryan

2.1 Abstract

Sepsis, an exaggerated and life threatening response to infection, has a well-understood initial cytokine storm, with a less understood secondary immunosuppressive phase. Elevated blood lactate and slow lactate clearance are associated with mortality, however, any regulatory role is currently unknown. We hypothesized that lactic acid contributes to the late phase of sepsis and is not solely a consequence of bacterial infection. Thus far, no studies have examined the effect of lactic acid on sepsis models *in vivo* or a mechanism by which they suppress LPS activation *in vitro*. Mast cells can be activated systemically and contribute to sepsis, and therefore, our purpose was to examine the effects of lactic acid in mast cells and in a model of septic shock. Lactic acid significantly suppressed LPS-induced cytokine production and NFκB transcription in bone marrow derived and peritoneal mast cells. Suppressive effects were dependent upon MCT-1 transport and observed with both sodium lactate and formic acid. Further, lactic acid significantly suppressed cytokine induction following LPS-induced septic shock *in vivo*. As a feedback regulator of glucose metabolism, lactic acid treatment reduced glucose uptake, lactate export, and hexokinase-2 expression following LPS activation. Lactic acid effects were mimicked by glycolytic inhibitors and reversed by increasing ATP availability. These results indicate that glycolytic suppression and ATP production

are necessary and sufficient for lactic acid effects. Future studies should enhance glycolysis and ATP production to improve immune function and counteract the suppressive effects of lactic acid in the immunosuppressive phase of sepsis.

2.2 Introduction

Sepsis is a common and deadly condition with life-threatening organ dysfunction and a dysregulated host response to infection. This can progress to septic shock, a subset of sepsis with circulatory and cellular/metabolic dysfunction associated with higher risk of mortality[100]. As the single most expensive condition treated in US hospitals, sepsis accounts for 5% of all hospital costs (>$20B)[101]. These costs are less concerning than the high death rate of septic patients, which ranges from 30-50%. While exact estimates of sepsis-induced death vary due to the occurrence of co-morbidities such as cancer and aging-induced immunosuppression, conservative estimates are that >150,000 Americans die of sepsis annually[102]. Alarmingly, there are no targeted molecular treatments for sepsis, as we still do not understand the underlying disease progression and mechanisms.

Sepsis is traditionally characterized by high levels of pro-inflammatory cytokines leading to hypotension, vascular leak, tissue hypoxia, and organ failure[103]. The initial inflammatory response activates a secondary cascade of cytokines, lipid mediators, reactive oxygen species, and coagulation factors. Reactive oxygen species increase vasodilation and slow local blood flow. Additionally, pro-inflammatory cytokines and chemokines recruit neutrophils and macrophages to the site of infection and initiate fever. This secondary mediator cascade is an important contributor to the vascular collapse and organ failure.

While the inflammatory response is beneficial to fight the infection, prolonged or considerable inflammation can damage host tissue and lead to death. There have been more than 100 clinical trials aimed to dampen the immune response following sepsis, which had no effect on or worsened patient outcomes and mortality[104,105]. Importantly, recent studies suggest that fluid resuscitation and antibiotics allow most patients to survive the initial hyperinflammatory phase, at which time they enter a immunosuppressed phase marked by impaired immune cell activation[104,106,107]. It is important to note that these phases have been distinctly studied as the systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS), however the current consensus is that both may occur in a typical sepsis case[108].

Patient death in the immunosuppressive phase is often attributed to failure to clear the initial infection or acquisition of a secondary infection. In order to devise more effective interventions for sepsis, a better understanding of the cellular mechanisms behind immunosuppression is required. More than 100 distinct biochemical species are intricately involved in sepsis pathogenesis, since blockade or administration of these can alter mortality risk[105]. But less is known about the regulation of these mediators during sepsis and the optimal response for survival and bacterial clearance. This understanding may provide us with better treatments for septic patients.

Lactate and the associated H^+ ions are produced following glycolytic energy production by hypoxic tissues or in tissues requiring a rapid supply of ATP (i.e. during cellular activation)[109–112]. Normal blood lactate is approximately 0.5-2.5 mM[113], while increased blood lactate (≥4 mM) and impaired clearance have been independently

associated with increased mortality in sepsis patients[114–117]. From these data, lactate clearance has been added as a treatment guideline for sepsis[100], and at least one hospital has reported reduced patient mortality after lactate levels >4mM were added to a laboratory critical call list, in which the results are quickly reported to the physician and time to treatment is reduced[118]. While these data suggest lactate and mortality are related, it is not known whether elevated lactate is a cause or consequence of the infection.

Our lab has recently published that lactic acid suppresses IL-33-induced mast cell activation both *in vitro* and *in vivo*[52], suggesting that lactic acid may play a feedback role in suppressing the immune response following the initiation of sepsis. In the tumor microenvironment, evidence suggests that lactic acid suppresses immune cell activation and promotes regulatory cell subsets (M2 macrophages, MDSCs)[119,120]. In the context of bacterial infection, lactic acid has been typically shown to suppress LPS-induced activation of monocytes, macrophages, and dendritic cells[121,121–124], however a select few publications report enhanced LPS activation with lactic acid[125–127]. Furthermore, no studies have examined the effects on lactic acid on mast cells in the context of bacterial activation and there is little understanding of the mechanism of action.

Mast cells, similar to macrophages, are tissue resident innate immune cells that contribute to bacterial defense and septic inflammation. Specifically, local mast cell activation improves neutrophil recruitment and sepsis outcomes by the release of IL-6, MCP-1, and leukotrienes[55,58–60,128]. Mast cell mMCP-4 enhances survival after cecal ligation and puncture (CLP)-induced sepsis by degrading TNF and limiting the detrimental effects of TNF on the host[57]. Furthermore, mast cells have been shown to contribute to control of gram positive (TLR2-mediated) sepsis[129], with enhanced myeloid

recruitment, CD8+ Tcell recruitment, Th1 immnty, and production of TNF, IL-6, and IL-1β, further expanding their role *in vivo*. Understanding the effects of lactic acid on mast cells in the context of bacterial infection and altering lactic acid concentrations *in vivo* would improve our understanding of the role of lactate in sepsis and may provide a potential drug target. Therefore, the purpose of this study was to better understand the role of lactic acid in the context of sepsis, with an emphasis on the effects of lactic acid in mast cells and more broadly *in vivo*.

2.3 Methods

2.3.1 Reagents

Recombinant mouse IL-33 was purchased from Shenandoah Biotechnology (Warwick, PA). L-lactic acid was ordered from MP Biosciences (Santa Ana, CA). Lipopolysaccharide (LPS) from Escherichia coli (E.Coli) 055:B5 (catalog L4524) for *in vitro* studies, LPS from E.coli 0111:B4 (catalog L3024) for *in vivo* studies, polyinosinic-polycytodylic acid (Poly (I:C; catalog P1530), L-sodium lactate, and formic acid were purchased from Sigma (St Louis, MO). Lipoteichoic acid (LTA) was purchased from AbD Serotec (BioRad). The MCT-1/2 inhibitor AR-C155858 was purchased from Tocris Bioscience, part of Bio-techne (Minneapolis, MN). Sodium oxamate and 2-deoxyglucose (2DG) were purchased from Alfa Aesar (Tewksbury, MA). Etomoxir and rotenone were purchased from Cayman Chemical (Ann Arbor, MI) and antimycin A was purchased from Chem Cruz via Santa Cruz Biotechnology (Dallas, TX). ATP disodium salt was purchased from Tocris via Biotechne Corporation (Minneapolis, MN).

2.3.2 Mice

Mouse C57BL/6J and NFκB-luc breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME) and colonies were maintained in a pathogen free facility with a standard chow diet. Bone marrow was extracted from mice at a minimum of 10 weeks old and sepsis studies were conducted between 8-10 weeks under protocol approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

2.3.3 Mast cell culture

Mouse bone marrow was differentiated in IL-3-containing supernatant from WEHI-3 cells and SCF-containing supernatant from BHK-MKL cells as described to yield 90-99% FcεRI$^+$ and cKit$^+$ bone marrow derived mast cells (BMMC) at 21 days[52,75]. Additionally, mast cells from mouse peritoneal lavage were expanded in complete RPMI 1640 medium containing 10% FBS and IL-3+SCF (10 ng/mL) for 7-10 days to yield ~85% FcεRI$^+$ and cKit$^+$ peritoneal mast cells (PMCs). These cells matured *in vivo* and were expanded *ex vivo*. They were used to support BMMC data to ensure results were not an effect of *in vitro* differentiation. Following differentiation and expansion, BMMC and PMC were plated at 2x10^6/mL with IL-3 and SCF (20 ng/mL). Media or 25 mM lactic acid was added 1:1 for a final concentration of 1x10^6 cells/mL, 10 ng/mL IL-3 and SCF, and 12.5 mM lactic acid for all experiments unless otherwise noted. LPS was added at 1 μg/mL for 16 hours for ELISA and glucose/ lactate analysis, 2 hours for qPCR analysis, or 2 hours for luciferase analysis.

2.3.4 ELISA

ELISA analysis was used to measure cytokine concentrations in the cell culture supernatant. IL-6, TNF, and MCP-1 (CCL2) murine ELISA kits were purchased from Biolegend and IL-13 and MIP-1α (CCL3) murine ELISA kits were purchased from Peprotech (Rocky Hill, NJ). Assays were performed in duplicate or triplicate according the manufacturers' protocols.

2.3.5 RT-qPCR

TRIzol (Life Sciences, Grand Island, NY) was used to extract total RNA and nucleic acid purity was measured using a Nanodrop 1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized with the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) following the manufacturer's protocol. To determine IL-6 and GAPDH mRNA expression, real time quantitative PCR (RT-qPCR) was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and PerfeCTa SYBR Green SuperMix (Quanta Biosciences). Primers for IL-6 (forward: 5'-TCCAGTTGCCTTCTTGGGAC-3', reverse: 5'-GTGTAATTAAGCTCCGACTTG-3') and GAPDH (GAPDH forward, 5'-GATGACATCAAGAAGGTGGTG-3', reverse, 5'-GCTGTAGCCAAATTCGTTGTC-3') were purchased from Eurofins MWG Operon (Huntsville, AL). Amplification conditions were as follows: 95°C (2 min) followed by 40 cycles of 95°C (15 s), 55°C (30 s), and 60°C (1 min). A melting curve analysis was performed between 50°C and 95°C. Results were normalized to GAPDH and the H_20 control by using the Relative Livak Method (ΔΔCt).

2.3.6 Septic Shock Model

Age-matched groups of mice (~8-10 weeks old) were injected intraperitoneal (IP) with lactic acid (80 mg/kg) or PBS 20 hours prior to sepsis. A lethal dose of LPS (25 mg/kg) was injected IP to elicit septic shock. See Figure 3-8A for a schematic diagram. The core body temperature of each mouse was measured using a rectal microprobe for 4 hours (Physitemp Instruments). Additionally, observational score was assessed for posture, grooming, stool, locomotion, respiration, and eye discarge, with 0 as normal and 4 as the worst score for each measure. Mice were sacrificed prior to endpoint if they reached a 3 on 3 or more categories or if they reached a 4 on two or more categories. After 4 hours of temperature and observational score measures, mice were sacrificed. Plasma cytokines were measured from cardiac puncture.

2.3.7 Luciferase

BMMC were differentiated from NFkB-luc transgenic bone marrow as above. Following lactic acid treatment and LPS activation, cells were lysed and luciferase activity was measured with the Promega Luciferase Assay Substrate and Glomax 20/20 Luminometer (Promega, Madison, WI). Luciferase expression is reported relative to protein concentration (Pierce BCA Protein Assay Kit, Thermofisher, Waltham, MA) and normalized to the unactivated control.

2.3.8 Flow Cytometry

Cells were collected in 1.6% paraformaldehyde and permeablized with methanol for p-ERK analysis. Cells were stained with anti-CD16/32 (clone 2.4G2, BD Pharmingen via BD Biosciences, San Jose, CA) and APC-anti-H/M pERK1/2 (clone MILAN8R, eBioscience, via Thermofischer, Waltham, MA) or the isotype control (APC mouse IgG1; eBioscience) at 2 μg/mL for 30 minutes at 4°C, and analyzed by flow cytometry with the FACsCelesta (BD Biosciences). The gating strategy used was doublet exclusion (FSC-A x FSC-H) and size and granularity (FSC x SSC). MFI was recorded for all samples.

2.3.9 Cellular Metabolism

To determine glucose uptake and lactate export, supernatant concentrations were measured using the Glucose Assay Kit 1 and L-Lactate Assay Kit 1 from Eton Bioscience (San Diego, CA). Glucose uptake was calculated as [glucose in unactivated supernatant] – [glucose in activated supernatant]. Lactate export was calculated as [lactate in activated supernatant] – [lactate in unactivated supernatant].

2.3.10 Western Blot

Cell lysates were collected using Protease arrest (GBiosciences, Maryland Heights, MO) and lysis buffer (Cell Signaling Technology, Danvers, MA). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermofisher, Waltham, MA). 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio–Rad, Hercules, CA) were loaded with 30 mg protein, proteins were transferred to nitrocellulose (Pall Corporation, Ann Arbor, MI), and membranes were blocked for 60 minutes in

Blocker casein in TBS (from Thermofisher, Waltham, MA). Blots were incubated with primary antibodies overnight in block buffer + Tween20 (1:1000) ± rabbit anti-HK2 (1:750), rabbit anti-PKM1/2 (1:750), rabbit anti-GAPDH (1:1000). Blots were washed six times for 5 minutes each in TBST, followed by incubation with secondary antibody (1:10,000) for 60 minutes at room temperature. All antibodies were purchased from Cell Signaling (Danvers, MA). Size estimates for proteins were obtained using molecular weight standards from Bio–Rad (Hercules, CA). Blots were visualized and quantified using a LiCor Odyssey CLx Infrared imaging system (Lincoln, NE). After background subtraction, fluorescence intensity for the protein of interest was normalized to the signal intensity for the relevant loading control, using Image Studio 4.0 (LiCor).

2.3.11 Statistical analyses

For all data, a one-way analysis of variance (ANOVA) was used to detect differences between groups. Post hoc testing using Tukey's multiple comparisons was used to determine which conditions were significantly different from the media/vehicle control. GraphPad Prism software was used for all statistical analyses. Data are expressed as mean ± standard error of mean (SEM) with statistical significance: $*p < .05$, $**p < .01$, and $***p < .001$.

2.4 Results

2.4.1 Lactic acid suppresses cytokine transcription and secretion in LPS-activated BMMC.

Numerous publications suggest that lactic acid suppresses LPS activation in macrophages, dendritic cells, and T cells[121–124], and we have previously shown suppressive effects of lactic acid on mast cell activation by IL-33[52]. Therefore, we hypothesized that lactic acid suppression extends to LPS-induced mast cell activation and determined the kinetics of this response. Bone marrow derived mast cells (BMMC) were pretreated ± lactic acid (12.5 mM) for various times prior to LPS activation (1 μg/mL). Pre-treatment from 0-24 hours significantly suppressed LPS-induced IL-6, TNF, and MCP-1 (Figure 2-1). IL-6 and TNF production were also significantly reduced after 48-hour treatment, however MCP-1 was no longer different. In sepsis, lactate increases due to tissue hypoperfusion, impaired pyruvate dehydrogenase activity, elevated catecholamine secretion, and increased immune cell glycolysis during the systemic response[109,130–132]. Therefore, we also treated BMMC with lactic acid pretreatment, simultaneously with LPS-activation, or following LPS-activation. At these timepoints, lactic acid significantly suppressed cytokine production similar to pretreatment between 2-6 hours post-activation (Figure 2-2).

To determine the optimal dose, BMMC were treated ± lactic acid for 24 hours prior to LPS activation. IL-6 was significantly reduced with ≥6 mM treatment, while TNF and MCP-1 were significantly decreased with ≥12.5mM treatment (Figure 2-3). Importantly, there was no significant change in cell viability at these doses (Figure 2-4).

For the remainder of the experiments, lactic acid treatment for 24 hours at 12.5 mM was used for optimal suppression unless otherwise stated. Additional cytokines and chemokines were examined under these conditions. IL-13 and MIP-1α concentrations were significantly reduced with lactic acid treatment (Figure 2-5). Furthermore, lactic acid significantly suppressed IL-6 mRNA expression (Figure 2-6), suggesting the effects were at the transcriptional level or above, not solely at the level of translation or secretion. Together, these results support reported effects of lactic acid on immune cells and suggest that lactic acid modulates mast cells in the context of bacterial infection.

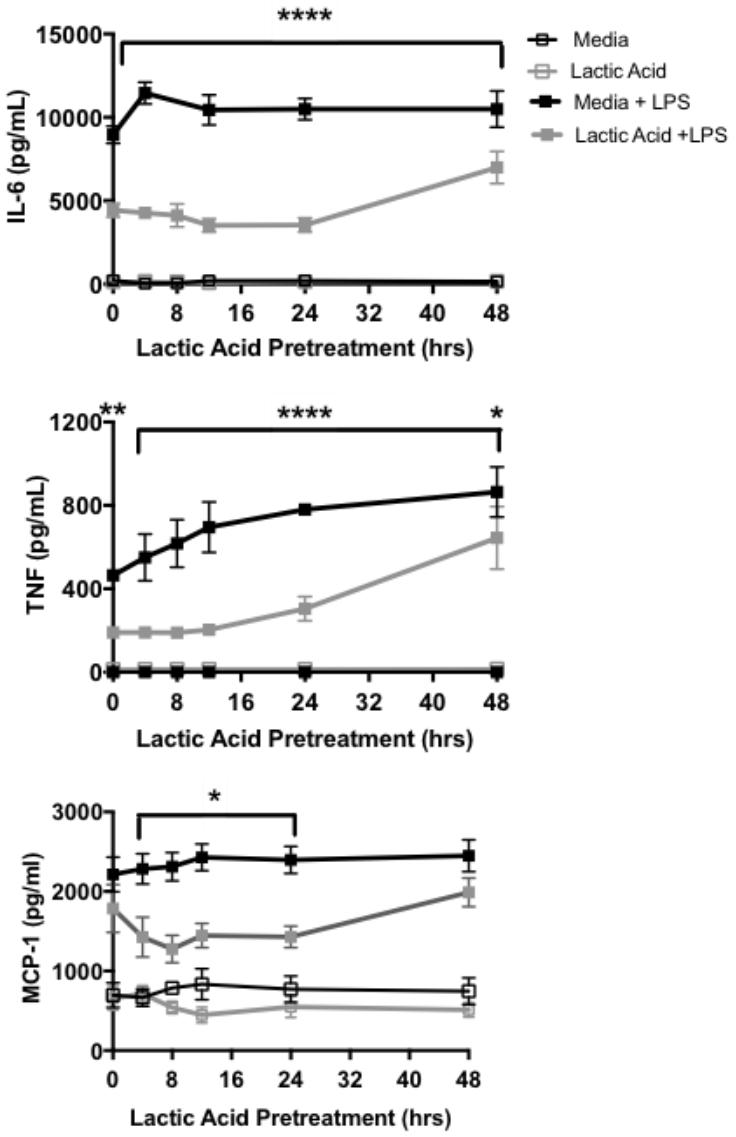

Figure 2-1. Lactic acid pre-treatment suppresses cytokine production following LPS activation. BMMC were pre-treated ± 12.5 mM lactic acid for the indicated time points and activated ± LPS (1 μg/mL) for 16 hours. ELISA analysis was used to determine supernatant concentrations of IL-6 and TNF. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$, ****$p < .0001$

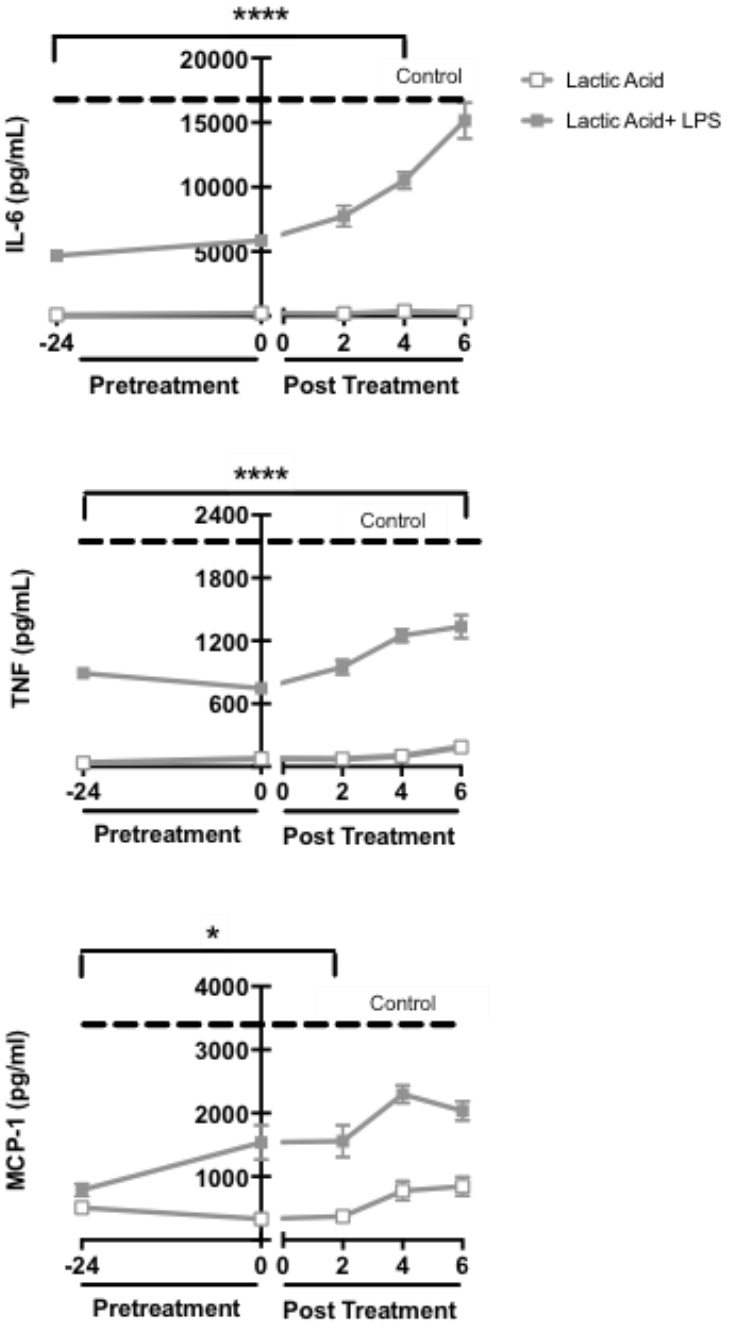

Figure 2-2. Lactic acid post-treatment suppresses cytokine production following LPS activation. BMMC were pre-treated, treated simultaneously, or post-treated ± 12.5 mM lactic acid prior to, during, or following activation ± LPS (1 μg/mL) for 16 hours. ELISA analysis was used to determine supernatant concentrations of IL-6 and TNF. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *p < .05, ****p < .0001

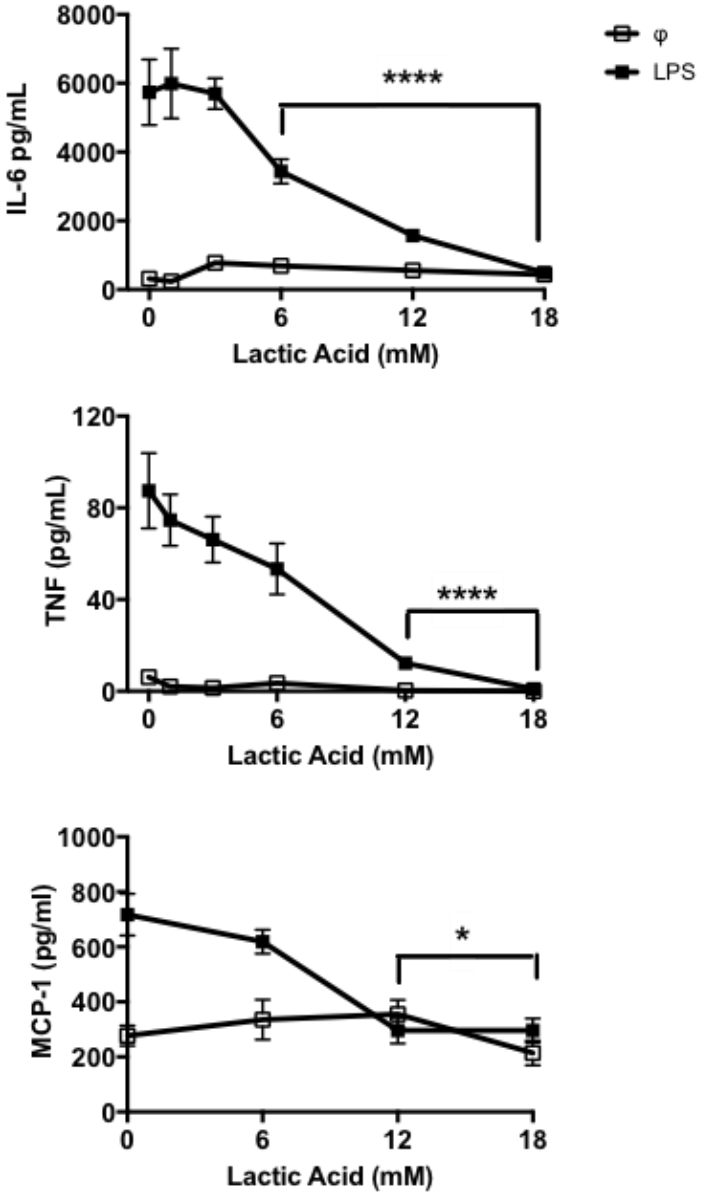

Figure 2-3. Lactic acid suppression is dose dependent. BMMC were treated for 24 hours ± the indicated doses of lactic acid and activated ± LPS (1 μg/mL) for 16 hours. ELISA analysis was used to determine supernatant concentrations of IL-6 and TNF. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *p < .05, ****p < .0001

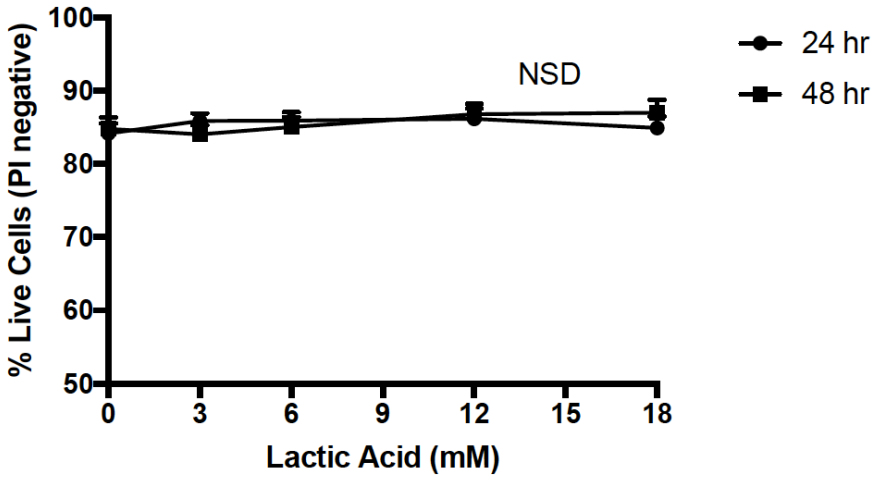

Figure 2-4. Lactic acid does not affect cell viability over 48 hours. BMMC were treated ± the indicated doses of lactic acid over 24 or 48 hours. PI exclusion was used to determine viability by flow cytometry. Data are means ± SEM of 3 populations, representative of 2 independent experiments. NSD= no significant difference.

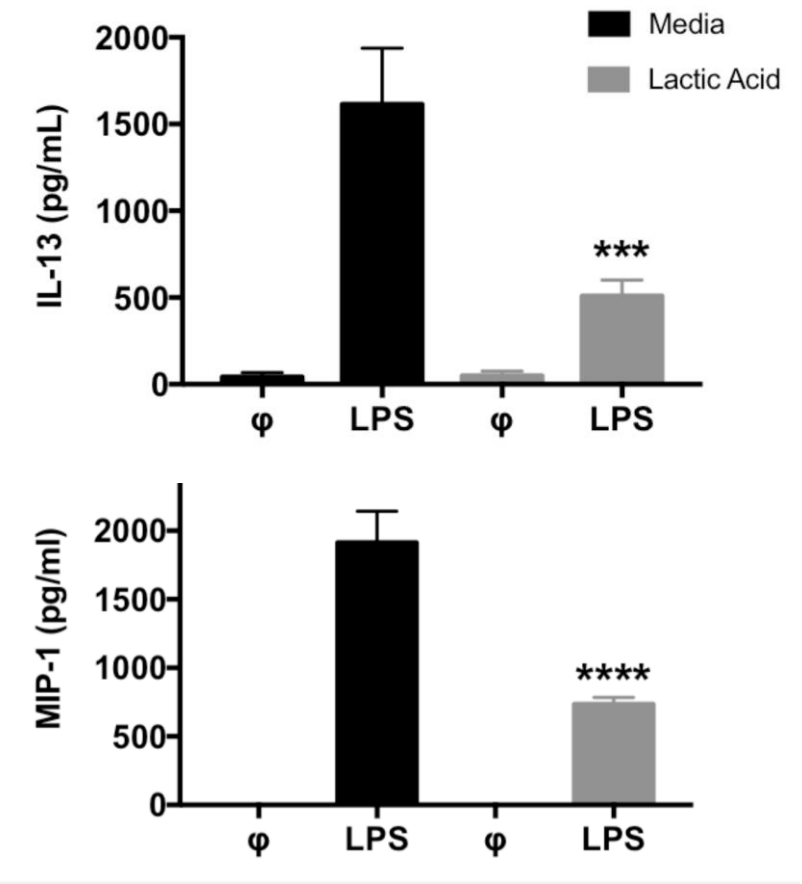

Figure 2-5. Lactic acid suppresses additional cytokines and chemokines following LPS activation. BMMC were treated for 24 hours ± 12.5 mM lactic acid and activated ± LPS (1 μg/mL) for 16 hours. ELISA analysis was used to determine supernatant concentrations of IL-13, MCP-1, and MIP-1α. Data are means ± SEM of 3 populations, representative of 3 independent experiments. ***$p < .001$, ****$p < .0001$

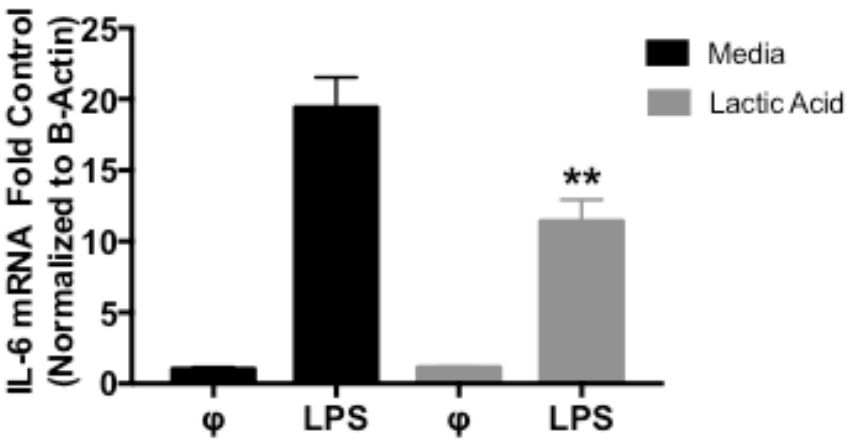

Figure 2-6. Lactic acid suppresses transcription of IL-6 mRNA following LPS activation. BMMC were treated for 24 hours ± 12.5 mM lactic acid and activated ± LPS (μg/mL) for 2 hours. qPCR was used to determine IL-6 and expression. Data are means ± SEM of 3 populations, representative of 3 independent experiments. ** $p < .01$

2.4.2 Lactic acid suppresses LPS-induced cytokine production in peritoneal mast cells and in a sepsis model.

While the BMMC data above concur with literature reports in other cell types, we wanted to ensure that our results were not an artifact of culture conditions because we differentiate and expand BMMC *in vitro*. Peritoneal mast cells (PMCs), differentiated *in vivo*, were extracted by peritoneal lavage and expanded in culture for 7-10 days with IL-3 and SCF. Lactic acid significantly suppressed IL-6, TNF, and MCP-1 in PMCs, similar to the results observed with BMMC (Figure 2-7). These results suggest that lactic acid effects in mast cells are not due to in vitro differentiation or culture techniques.

Moreover, our results in mast cells support the results observed with other immune cell lineages and lactic acid has not been administered *in vivo*. Therefore we expanded our questions to examine the effects of lactic acid on cytokine production *in vivo* in a model of sepsis in order to provide causal data to support the correlative relationship between septic mortality and blood lactate. An intraperitoneal (IP) injection of lactic acid 20 hours prior to an IP injection of LPS significantly suppressed plasma IL-6, TNF, and MCP-1 compared to the PBS control (Figure 2-8 A and B). We should note that lactic acid did not affect body temperature or observational score (Figure 2-8 C and D), which we attribute to non-immune effects and speculate about in the discussion. Overall, these data suggest that lactic acid suppresses LPS-induced cytokine production in mast cells in culture and more broadly *in vivo*.

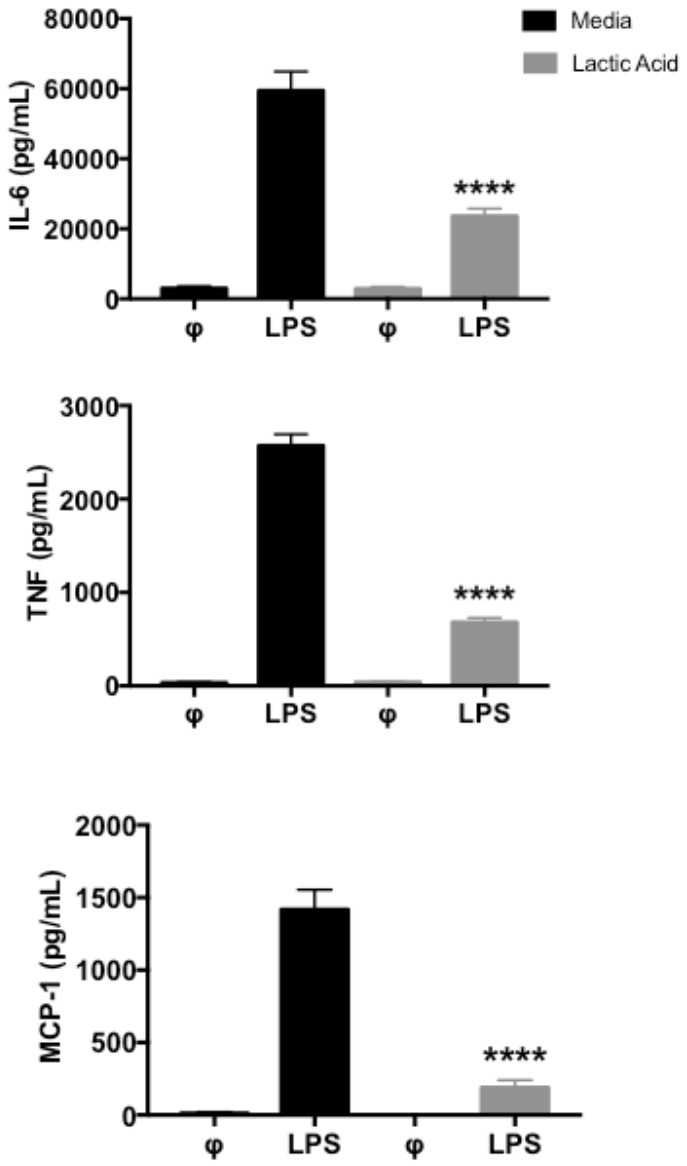

Figure 2-7. Lactic acid suppresses cytokine production in peritoneal mast cells. A) PMC were treated for 24 hours ± 12.5 mM lactic acid and activated ± LPS (1 μg/mL) for 16 hours. ELISA analysis was used to determine supernatant cytokine concentrations. Data are means ± SEM of 3 populations, representative of 3 independent experiments. ****$p < .0001$

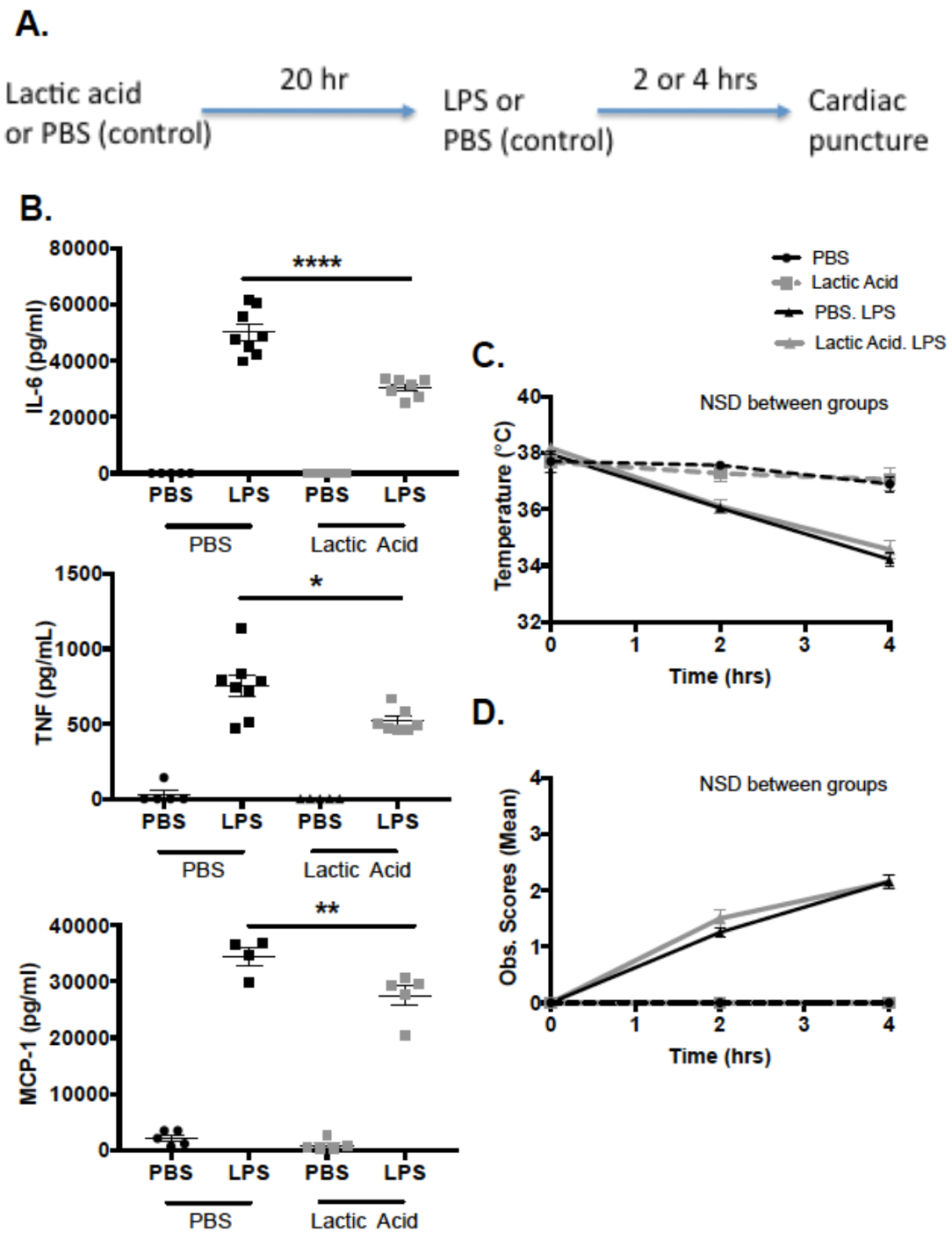

Figure 2-8. Lactic acid suppresses cytokine production following LPS-induced sepsis in vivo. A) Schematic. Lactic acid (80 mg/kg) or PBS was injected IP 20 hours prior to LPS (25 mg/kg) or PBS. Mice were sacrificed at 2 or 4 hour. B) Plasma IL-6 was measured at 4 hours, TNF and MCP-1 were measured at 2 hours by ELISA. C) Rectal temperature and D) Observational scores were measured over 4 hours. Data are means of 5 mice/control group and 8 mice/experimental group, representative of 2 independent experiments. NSD= no significant difference. *$p < .05$, **$p < .01$, ****$p < .0001$

2.4.3 Lactic acid suppression extends to TLR2 and TLR3 agonists

Sepsis can occur in response to a multitude of bacterial, viral, and even fungal infections[133]. While LPS activation of toll-like receptor-4 (TLR4) is often used to mimic bacterial activation in vitro, thirteen TLRs have been classified with different bacterial and viral ligands. Extracellular signals from bacterial cell walls, like LPS, and intracellular signals from bacterial and viral replication, like dsRNA, have similar signaling pathways in mast cells[51]. Many publications have reported lactic acid effects on LPS activation, and therefore we extended this study to determine the effects of lactic acid on activation of other common TLR signaling pathways. BMMC were treated with lactic acid prior to activation with 1 μg/mL LPS (TLR4 ligand), 5 μg/mL lipoteichoic acid (LTA, TLR2 ligand), and 100 μg/mL polyinosinic:polycytidylic acid (PolyI:C, TLR3 ligand). Lactic acid significantly suppressed cytokine production by all three ligands (Figure 2-9), suggesting the effects are not specific to TLR4, and that lactic acid may play a broader role in the context of bacterial and viral infections.

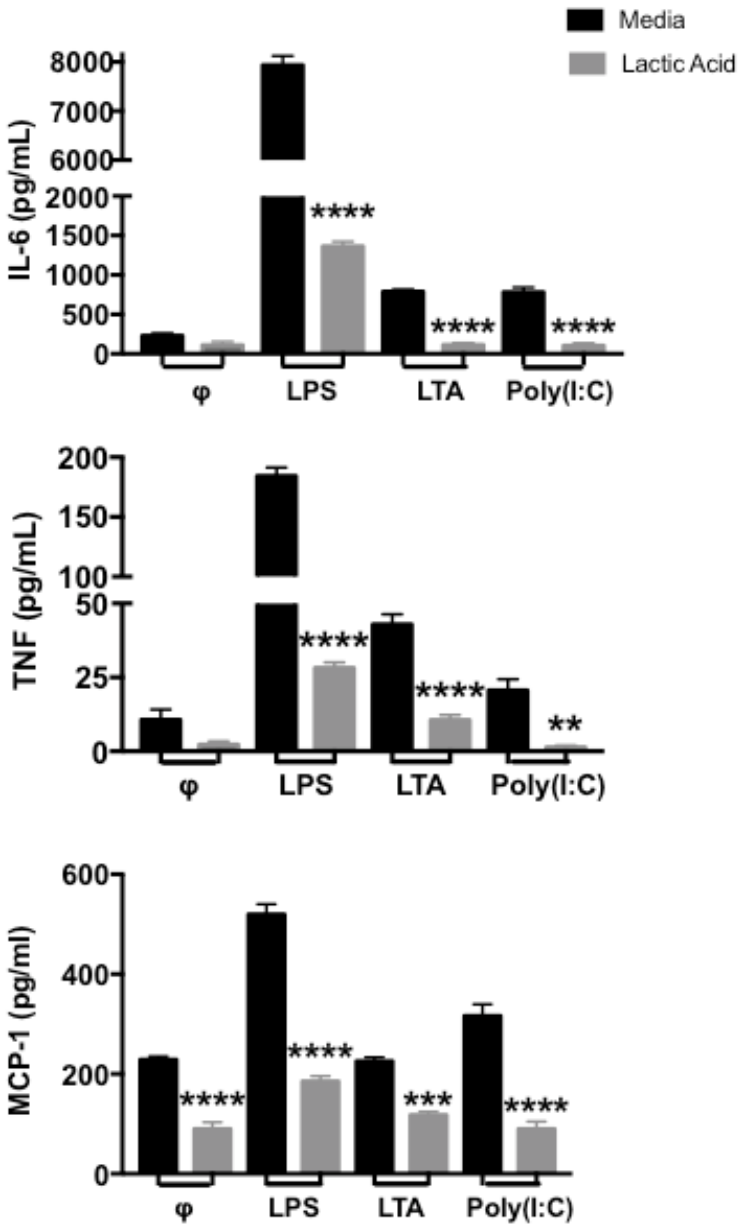

Figure 2-9. Lactic acid suppression extends to TLR2 and TLR3 agonists. BMMC were treated ± 12.5 mM lactic acid for 24 hours and activated ± LPS (1 μg/mL), LTA (25 μg/mL), or Poly (I:C) (100 μg/mL) for 16 hours. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of 3 populations, representative of 3 independent experiments. $^{**}p < .01$, $^{****}p < .0001$

2.4.4 Suppression is dependent upon MCT-1 transporter expression.

Monocarboxylic transporters are primarily responsible for lactic acid transport[134,135], acting as proton-linked transporters of lactate and H^+ ions. We previously published that MCT-1 expression is required for lactic acid to suppress of IL-33-mediated mast cell activation[52], and therefore examined the role of MCT-1 in the LPS system. BMMC were cultured ± AR-C155858 (MCT-1/2 inhibitor) or DMSO prior to lactic acid treatment and LPS activation. While lactic acid significantly suppressed IL-6 and TNF in the DMSO control, there was no effect of lactic acid when cultured with AR-C155858 (Figure 2-10). We have previously reported that mast cells express MCT-1 but not MCT-2[52], therefore, MCT-1 transport appears to be required for the suppressive effects of lactic acid in mast cells activated by LPS.

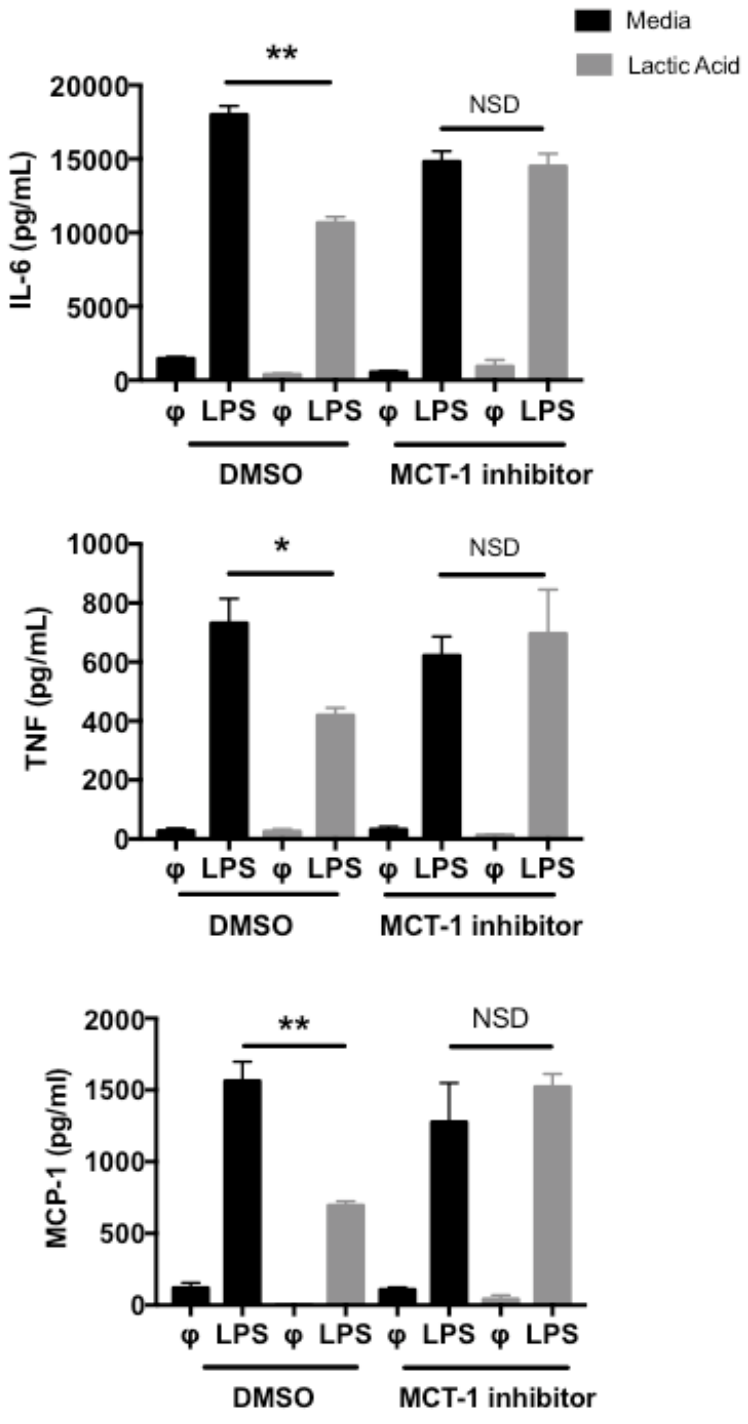

Figure 2-10. Lactic acid suppression is MCT-1 dependent. BMMC were treated ± AR-C155858 (100 nM) for 1 hour ± lactic acid (12.5) mM for 16 hours and activated ± LPS (1 μg/mL) for 16 hours. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$, **$p < .01$

2.4.5 The lactate molecule and H^+ ions both contribute to suppression

At physiological pH, lactic acid is deprotonated, existing as lactate and H^+ ions, which suggests that the effects of lactic acid on cell function can occur due to lactate, H^+ ions, or both. We previously published that lactic acid but not lactate suppress cytokine production in BMMC following IL-33 activation[52], however others have shown a role for lactate alone[124,136–138]. To determine if acidification is required for lactic acid effects on LPS activation, BMMC were cultured ± 12.5 mM lactic acid (pKa 3.86), sodium lactate, or formic acid (pKa 3.75) for 24 hours prior to LPS activation. Lactic acid significantly suppressed IL-6, TNF, and MCP-1 production, while formic acid had significant effects on IL-6 and TNF, with a trend towards significance with MCP-1 (Figure 2-11). There was no significant effect with sodium lactate at 12.5 mM (Figure 2-11). Additionally, there was no effect on cell viability with formic acid or sodium lactate (Data not shown). While these data support our previous findings that indicate a pH effect[52], many other studies have published suppression by lactate alone with effects often observed at higher doses[124,136,137]. Therefore, a dose response was conducted with sodium lactate for 24 hours prior to LPS activation. At doses ≥20 mM, sodium lactate significantly suppressed cytokine production with no effects on viability (Figure 2-12 and data not shown), suggesting suppressive effects of the lactate molecule independent of pH.

Once lactate and H^+ ions are released from the cell and into the bloodstream, acid-base buffering systems such as the bicarbonate buffering system help to prevent acidosis[139,140]. Additionally, hypochloremic metabolic alkalosis sometimes outweighs acidosis in patients with hyperlactemia due to saline resuscitation practices and underlying health conditions like chronic obstructive pulmonary disease [141,142]. Because

our media is highly buffered by HEPES and FBS buffering systems, we monitored pH after addition of lactic acid to our culture and examined kinetics of the buffering capacity. Upon addition to culture media, pH decreased to 6.7. Within 2 hours, the pH returned to 7.4 and was not different than our media control (Figure 2-13). These results suggest that lactic acid is buffered within our assays similar to effects observed in blood systemically. Because the pH returned to basal values within hours of addition, we determined if lactic acid effects persisted after plating treated cells in new media. BMMC were cultured ± lactic acid for 24 hours, washed, then activated in fresh media and compared to cells cultured ±lactic acid throughout the duration of the experiment. Continuous culture in lactic acid suppressed cytokine production, but there was no suppression of IL-6 and TNF once lactic acid was washed out of the media (Figure 2-14). Together, these data suggest that both acidity and lactate impact mast cell function following LPS activation and that lactic acid effects are transient, requiring the presence of an acid and/or lactate at the time of activation.

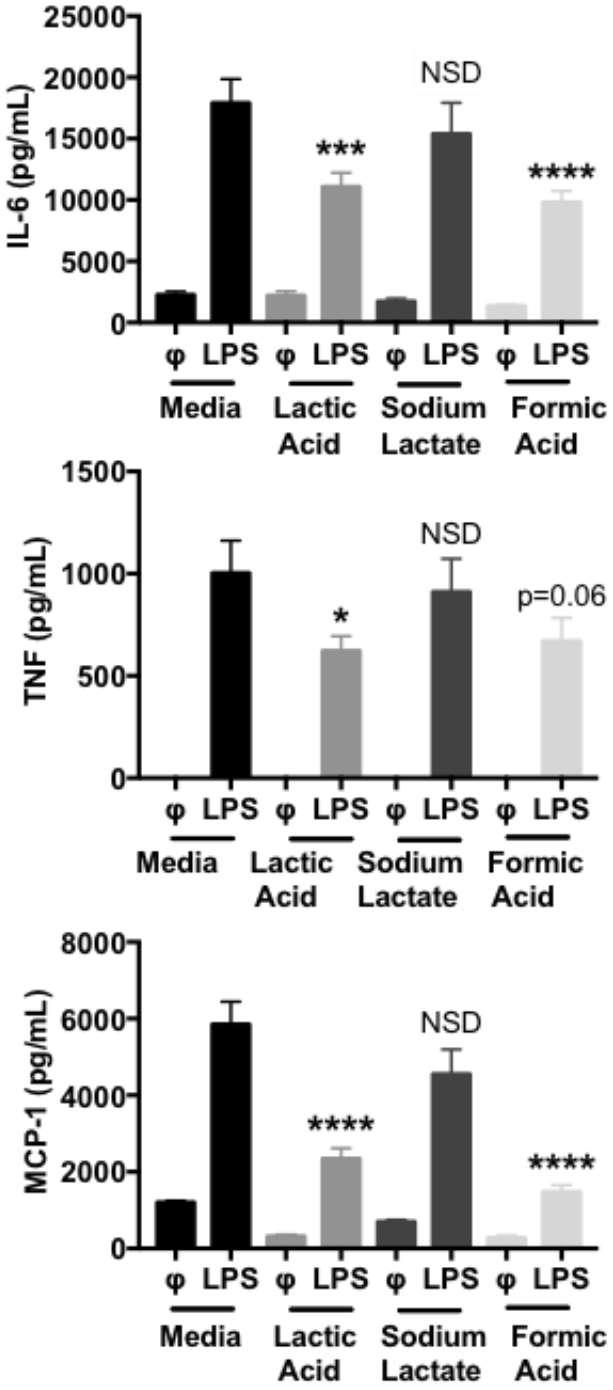

Figure 2-11. Formic acid mimics lactic acid at the same concentration. BMMC were treated ± 12.5 mM lactic acid, sodium lactate, or formic acid for 24 hours and activated ± LPS (1 μg/mL) for 16 hours. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$, ***$p < .001$, ****$p < .0001$, NSD= no significant difference

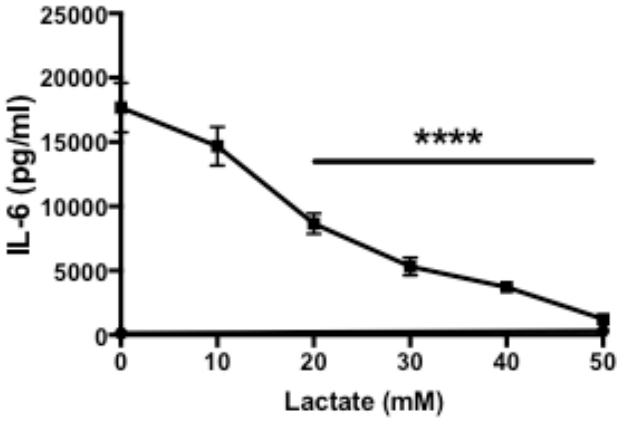

Figure 2-12. Lactate effects are dose dependent, albeit at much higher doses. BMMC were treated with varying doses of lactate for 24 hours and activated with LPS (1 μg/mL) for 16 hours. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of 3 populations, representative of 3 independent experiments. ****$p < .0001$

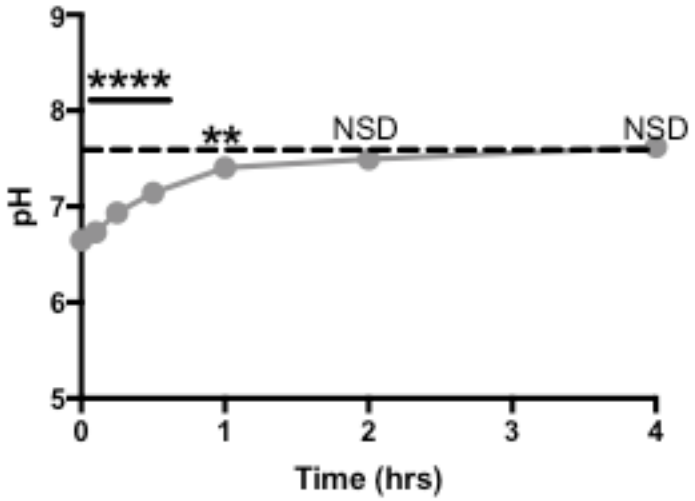

Figure 2-13. pH is buffered following lactic acid addition. Lactic acid was added to cell culture media with BMMC plated at 1x10^6/mL. pH was measured over 4 hours. Data are means ± SEM of 3 populations, representative of 3 independent experiments. $^{**}p < .01$, $^{****}p < .0001$

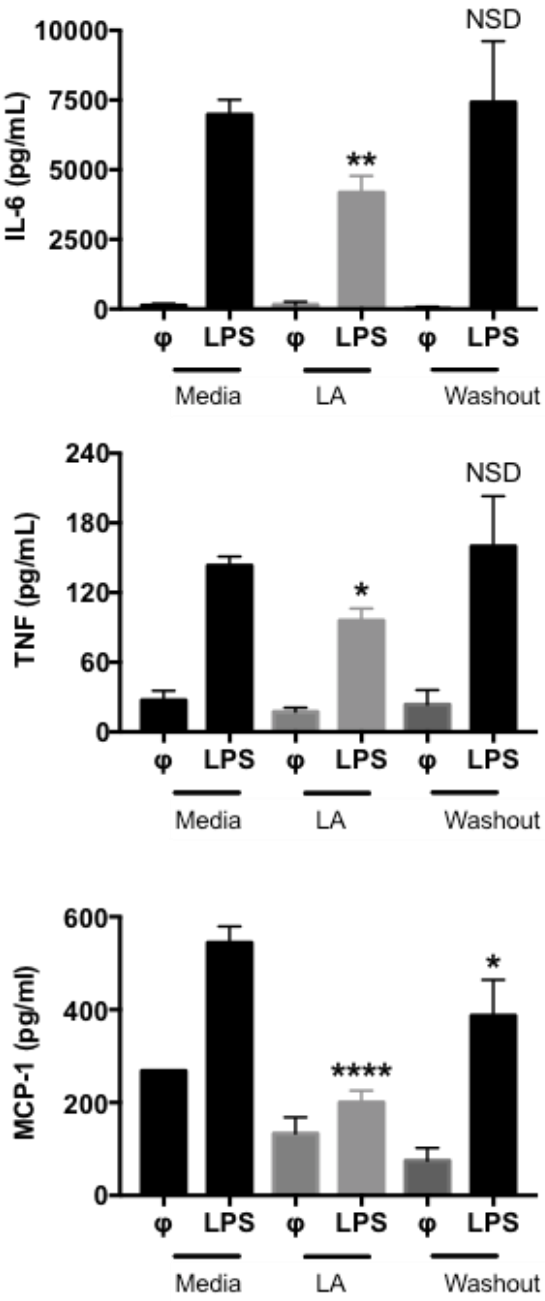

Figure 2-14. Lactic acid must be present during activation for suppression. BMMC were treated ± 12.5 mM lactic acid (LA) for 24 hours, then re-plated in new media ± 12.5 mM lactic acid and activated ± LPS (1 μg/mL) for 16 hours. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$, **$p < .01$, ****$p < .0001$

2.4.6 Lactic acid suppresses NFκB transcription downstream of TLR4

TLR4 signaling via MyD88 induces protein phosphorylation of IRAK, TRAF, TAK1, MAP kinases, and NFκB[51]. Previous publications have observed reduced p-NFκB and nuclear translocation in monocytes with lactate and lactic acid treatment [123,137]. To determine the effects on NFκB activity in our system, we differentiated BMMC from NFκB-luc transgenic mice, which have a luciferase transgene under the control of NFκB binding sites. BMMC were treated ± lactic acid for 24 hours prior to LPS activation for 2 hours. Lactic acid significantly suppressed LPS-induced NFκB transcriptional activity as determined by luciferase expression (Figure 2-15).

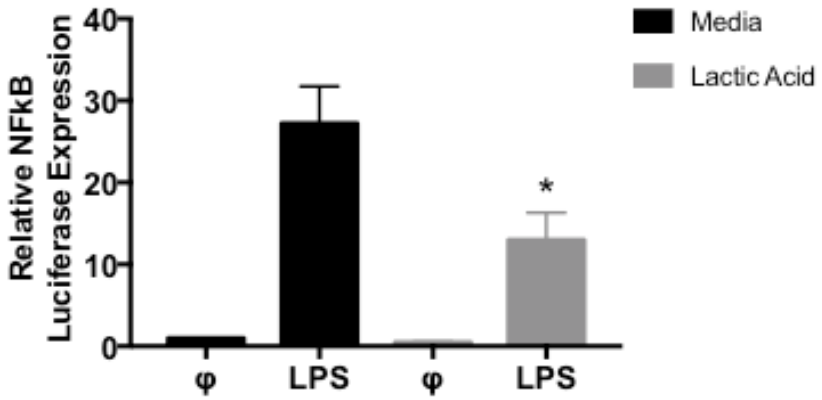

Figure 2-15. LA suppresses NFκB transcription. NFκB-luc transgenic BMMC were treated ± lactic acid (12.5 mM) for 24 hours and activated ± LPS (1 μg/mL) for 2 hours. Luciferase activity was measured with the Promega Luciferase Assay Substrate and Glomax Luminometer. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$

2.4.7 mir-155 suppression contributes to but does not fully explain lactic acid effects.

MiR-155 is an inflammatory micro-RNA that has been shown to enhance signaling. It is suppressed by lactic acid, contributing to effects downstream of IL-33 and IgE in BMMC[52,75]. To examine the role of miR-155 in our system, BMMC were cultured ± lactic acid for 24 hours prior to activation. Lysates were collected at 0, 2, and 4 hours for miR analysis by qPCR. Lactic acid did not affect baseline miR-155 levels, but LPS-induced suppressed miR-155-5p and miR-155-3p expression (Figure 2-16 A). To determine if miR-155 suppression is required for lactic acid effects, we compared wild type (WT) and miR-155 KO BMMC. As anticipated by the known pro-inflammatory role of miR-155, LPS-stimulated KO cells had lower LPS-induced cytokine production compared than WT cells. However, lactic acid further reduced LPS-induced cytokines in miR-155 KO mast cells (Figure 2-16 B). These data suggest that lactic acid inhibitory effects include suppressing miR-155, but that lactic acid has additional effects beyond suppressing miR-155.

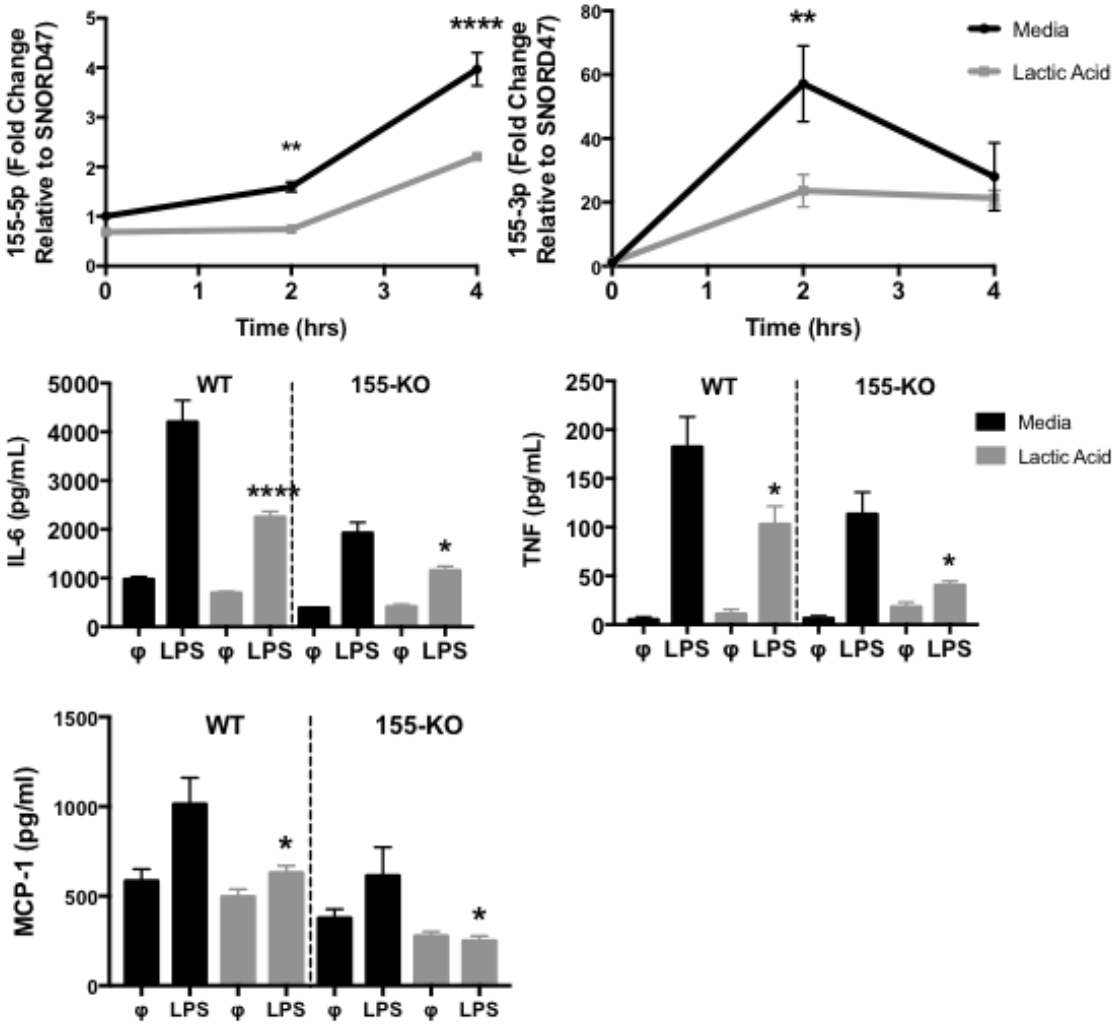

Figure 2-16. mir-155 suppression contributes to lactic acid effects. A) BMMC were cultured ± lactic acid (12.5 mM) for 24 hours and activated ± LPS (1 μg/mL) for 8 hours. miRs were measured via qPCR at various times relative to SNORD47. B) C57Bl/6 or mir-155 KO BMMC were treated ± lactic acid (12.5 mM) for 24 hours and activated ± LPS (1 μg/mL) for 16 hours. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$, **$p < .01$, ****$p < .0001$

2.4.8 Lactic acid suppresses glycolysis

Lactate and associated H^+ ions are the byproduct of glycolysis, with pyruvate conversion to lactate serving to recycle NADH and continue rapid ATP production during cellular activation. Lactic acid and lactate have been published to suppress intracellular ATP, glucose uptake, and extracellular acidification in monocytes and CD4 T cells[122,124,138]. Additionally, LPS is known to enhance the rate of glycolysis and the expression of glycolytic enzymes like hexokinase (HK) in other cell types[110–112]. Therefore, we examined how lactic acid modulates glucose metabolism in mast cells.

BMMC were cultured ± 12.5 mM lactic acid for 24 hours prior to LPS activation for 16 hours. LPS increased glucose uptake and lactate export, effects that were significantly suppressed by lactic acid (Figure 2-17), and suggesting that lactic acid suppresses glycolysis in our system. To determine if lactic acid affects glycolytic enzyme induction, BMMC were cultured ± 12.5 mM lactic acid for 24 hours prior to LPS activation for 8 hours, and lysates were used for western blot analysis. Lactic acid suppressed LPS-mediated HK2 and PKM2 induction (Figure 2-18). These results support data from other systems, and suggest that lactic acid acts as a negative feedback regulator of glycolysis during LPS-mediated mast cell activation.

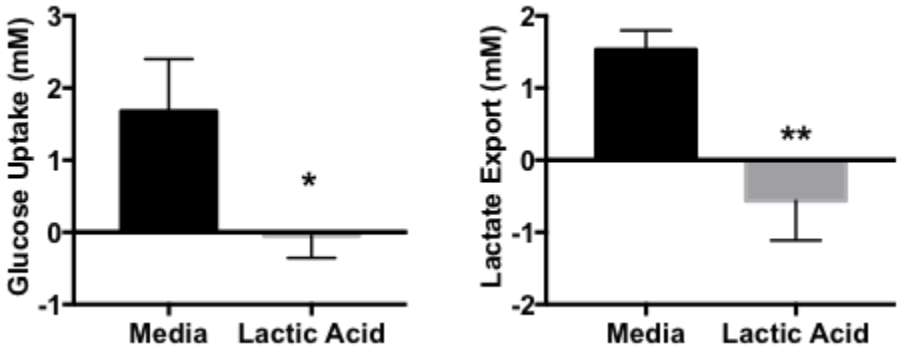

Figure 2-17. Lactic acid suppresses glycolysis. BMMC were treated ± 12.5 mM lactic acid for 24 hours and activated ± LPS (1 μg/mL) for 16 hours. Glucose and lactate were measured in the cell supernatant by colorimetric assay. Uptake and export were calculated from the difference between controls and activated samples. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$, **$p < .01$

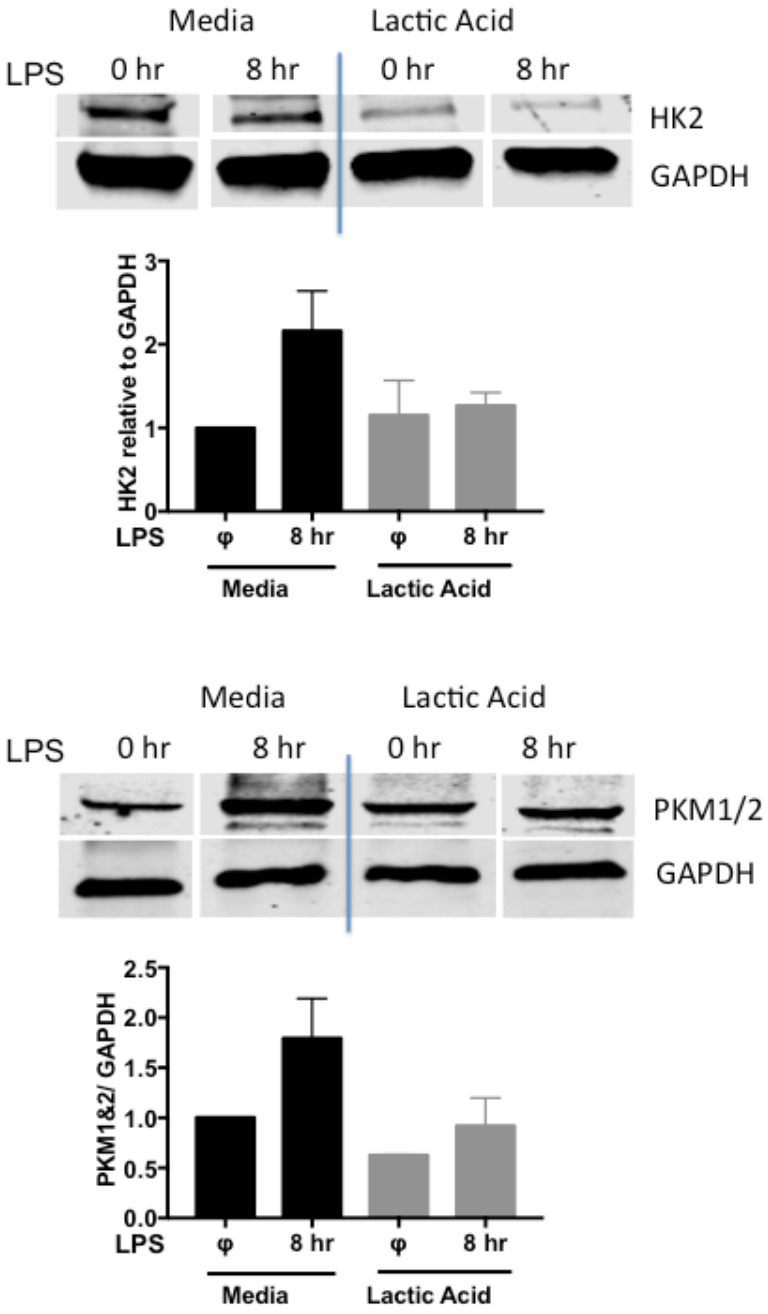

Figure 2-18. Lactic acid suppresses HK2 and PKM2. BMMC were treated ± 12.5 mM lactic acid for 24 hours and activated ± LPS (1 μg/mL) for 8 hours. Lysates were analyzed by western blot for hexokinase and pyruvate kinase. Data are means ± SEM of 2 populations. **p* < .05, ***p* < .01

2.4.9 Glycolytic inhibitors mimic lactic acid

In the past 10 years, ample research has been published suggesting that immune cell metabolism is closely linked to phenotype and effector function[85]. To examine mast cell reliance on glycolysis for cytokine production in response to LPS, 2-deoxyglucose (2-DG) was used to block hexokinase activity, directly reducing glycolysis, and sodium oxamate (OX) was used to block lactate dehydrogenase activity, slowing glycolysis by preventing the recycling of NADH needed for glycolytic enzyme activity (Figure 2-19). BMMC were cultured ± 2-DG (1 mM) or OX (40 mM) for 1 hour prior to activation with LPS for 16 hours. 2DG and OX significantly suppressed IL-6 and TNF following LPS activation (Figure 2-20). Additionally, 1 hour treatment with 2-DG and OX significantly suppressed NFκB transcriptional activity following LPS activation (Figure 2-21). Together, these data suggest that inhibiting glycolysis mimics lactic acid effects and is sufficient to suppress both cytokine production and cell signaling.

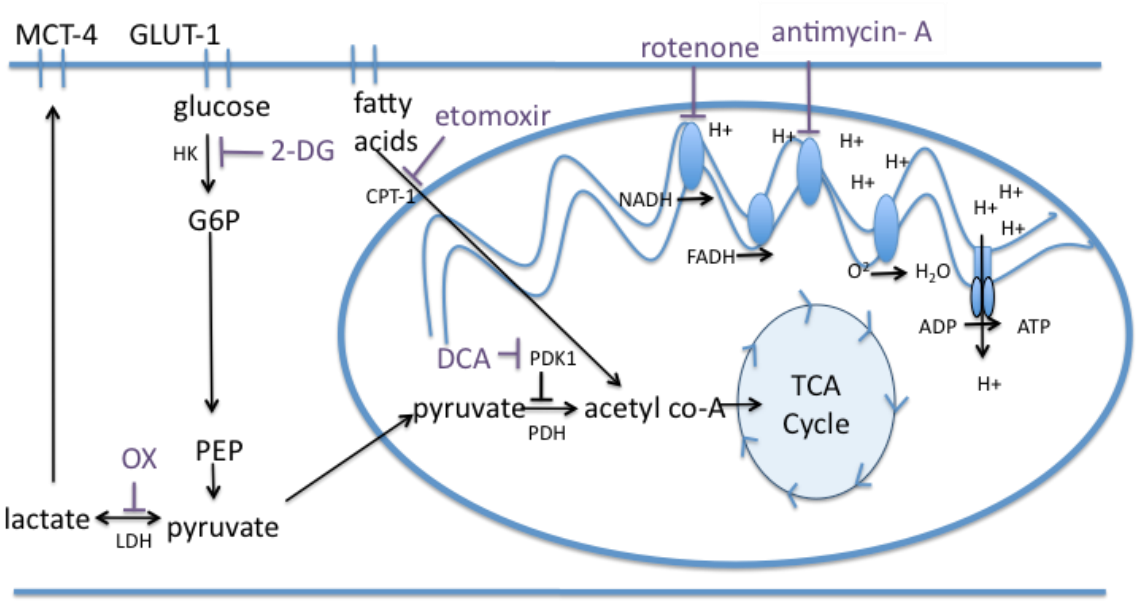

Figure 2-19. Metabolic Inhibitor Map: 2-deoxyglucose (2-DG), sodium oxamate or oxamic acid (OX), and dicholoracetate (DCA) inhibit glycolysis. Etomoxir, rotenone, and anti-mycin A inhibit OX PHOS.

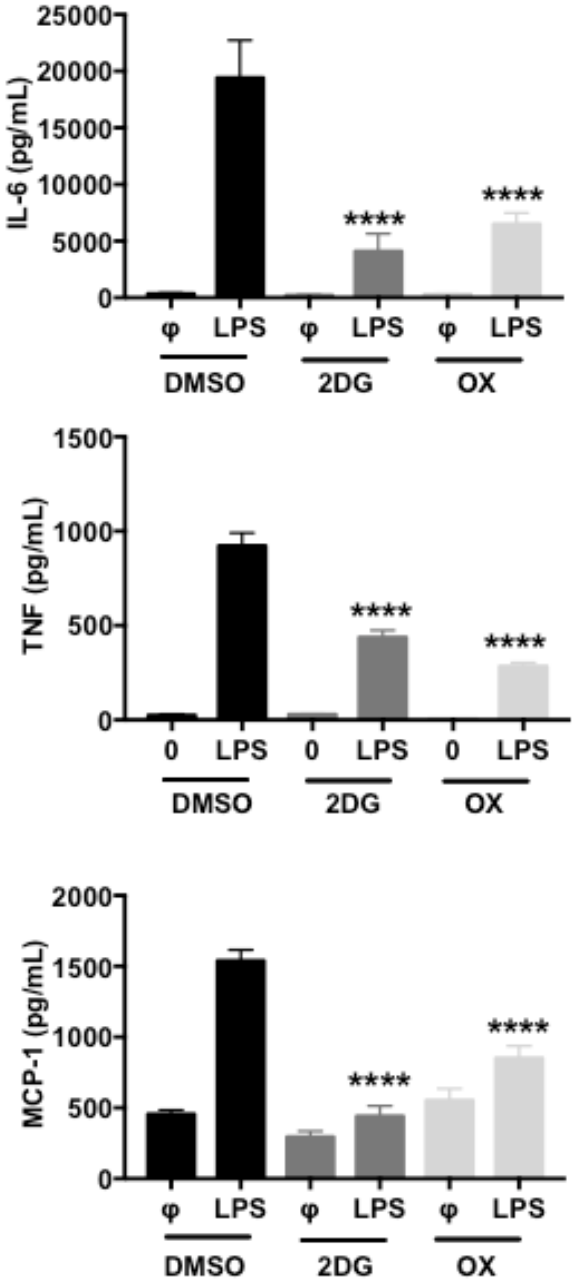

Figure 2-20. Glycolytic inhibitors suppress cytokine production. BMMC were treated with 2DG (1mM) or OX (20 mM) for 1 hour prior to LPS activation (1 μg/mL). After 16 hours, cytokines were measured in supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. ****$p < .0001$

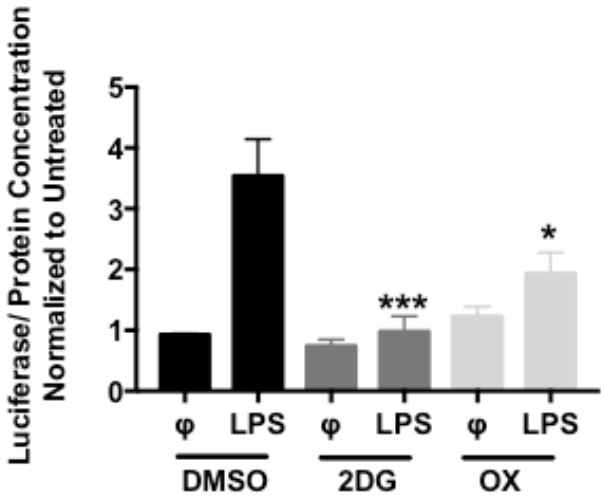

Figure 2-21. Glycolytic inhibitors suppress NFκB transcriptional activity. BMMC were treated with 2DG (1mM) or OX (20 mM) for 1 hour prior to LPS activation (1 μg/mL). After 2 hours, luciferase activity was measured from the NFkB-luc transgenic BMMC on the Glomax Luminometer. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. *$p < .05$, ***$p < .001$,

2.4.10 OX PHOS inhibition does not affect cytokine production.

We next examined if inhibiting OX PHOS had any effect on cytokine production. BMMC were treated with etomoxir (200 μM) or rotenone and antimycin A (1 mM each) to inhibit OX PHOS 1 hour prior to activation with LPS. There was no significant change in cytokine production following OX PHOS inhibition (Figure 2-22). These data suggest that OX PHOS is not required for LPS-induced cytokine production.

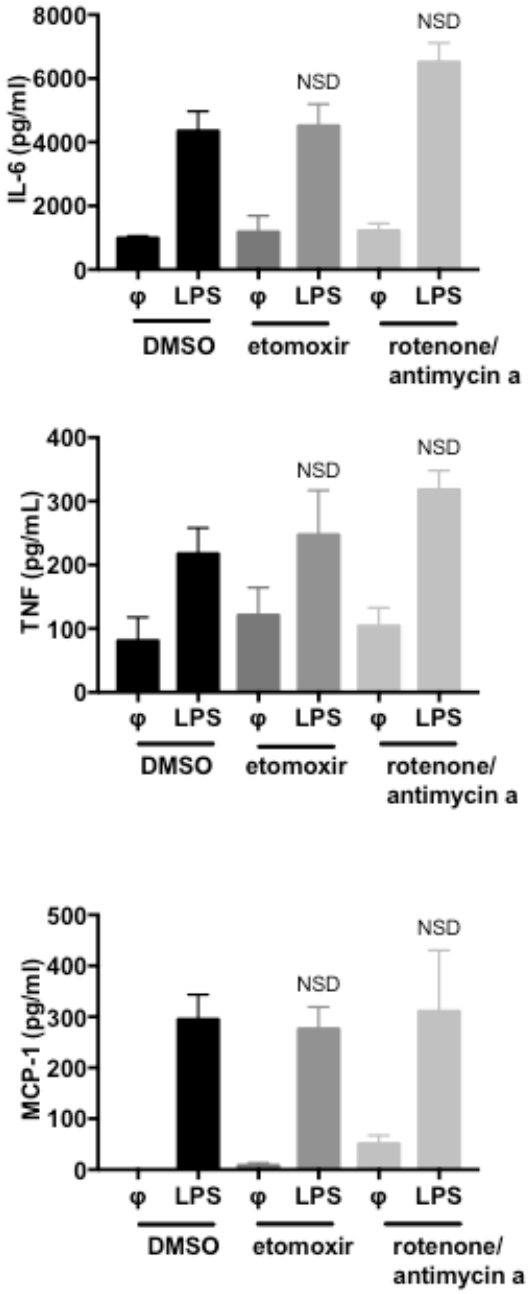

Figure 2-22. OX PHOS inhibition does not affect cytokine production. BMMC were treated with DMSO (vehicle control), etomoxir (200 uM), or rotenone+antimycin A (1 uM) for 1 hour prior to LPS activation (1 μg/mL) for 16 hours. Cytokines were measured in supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. NSD= no significant difference

2.4.11 ATP reverses LA suppression.

Glycolysis is a rapid pathway by which cells produce ATP, the energy currency of the cell required by many enzymes and kinases for optimal function. We hypothesized that ATP requirements increase LPS-induced glycolysis, which is suppressed by lactic acid. To examine if ATP can reverse lactic acid effects, BMMC were cultured ± lactic acid and ± ATP (10 μM) at the time of LPS activation. BMMC treated with lactic acid and ATP were not different than the media control, whereas BMMC treated with vehicle and lactic acid had significantly reduced cytokine levels (Figure 2-23). Additionally, ATP reversed lactic acid effects on NFκB transcriptional activity, with no significant difference between lactic acid and media control conditions (Figure 2-24). These data suggest that suppressing ATP availability is necessary for lactic acid suppression. These data, together with data from Figures 2-18 to 2-22 above, support the theory that lactic acid suppresses glycolytic ATP production following LPS activation, which effectively attenuates TLR4 signaling and cytokine production.

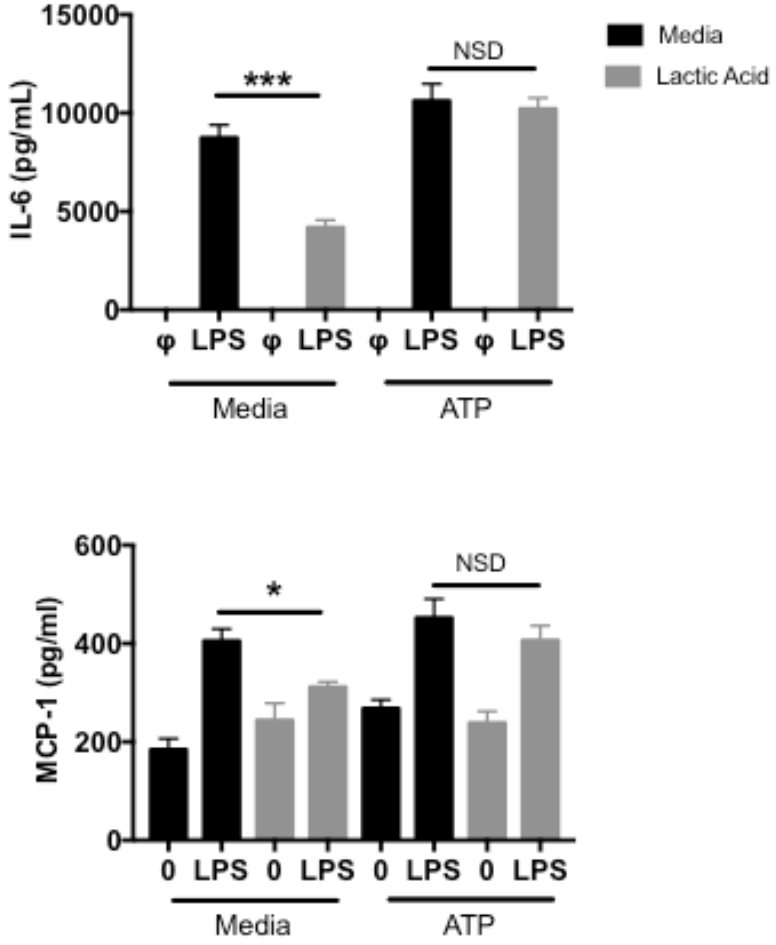

Figure 2-23. ATP reverses cytokine suppression by lactic acid. BMMC were treated ± 12.5 mM lactic acid, ± ATP (10 uM) and ± LPS (1 μg/mL). After 16 hours, ELISA analysis was used to determine supernatant cytokine concentrations. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *p < .05, ***p < .001

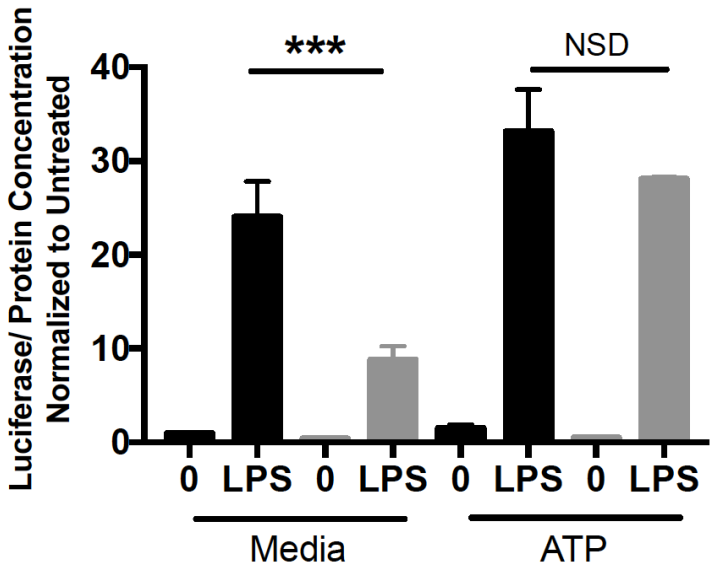

Figure 2-24. ATP reverses NFκB transcriptional suppression by lactic acid. BMMC were treated ± 12.5 mM lactic acid, ± ATP (10 uM) and ± LPS (1 μg/mL). After 2 hours, luciferase activity was measured from the NFκB-luc transgenic BMMC on the Glomax Luminometer. Data are means ± SEM of 3 populations, representative of 3 independent experiments. ****p* < .001

3.5 Discussion

Elevated blood lactate has been consistently shown to correlate with increased mortality in sepsis patients[102,114,143–146], however there is little understanding of a cause and effect relationship. This is the first study to examine the effects of lactic acid on mast cell activation in the context of sepsis. Previous studies suggest that lactic acid suppresses macrophages, DCs, and T cell activation in vitro[52,119,121–124,137], however there is no clear mechanism of action and no experimental studies to determine the direct effects of lactic acid within a sepsis model. In this study, we demonstrate that lactic acid suppresses LPS-mediated activation of bone marrow derived- and peritoneal mast cells. Additionally, we are the first to demonstrate that lactic acid suppresses cytokine production *in vivo*, in a model of LPS-induced septic shock. While lactic acid has been previously shown to suppress glycolysis and intracellular ATP[122,124,138], these are the first data demonstrating that lactic acid suppression of glycolysis is necessary and sufficient to inhibit cell signaling and cytokine production (Figure 2-25). This research suggests that lactic acid, the end product of glycolysis, can act as a feedback regulator of mast cell activation to modify and affect the immune response, specifically in sepsis. Additionally, targeting metabolic pathways may be an effective mechanism to modulate lactic acid responses in disease.

In BMMC and peritoneal mast cells, lactic acid concentrations that are physiologically relevant to disease states suppressed the LPS-induced production of many cytokine and chemokine mediators. We observed significant effects between 6-12 mM, which are relevant to wound tissue lactate concentrations often between 5-20mM, (maximum 80 mM)[84,147] and blood lactate concentrations in sepsis of 2-10mM

(maximum 20 mM)[116,118,145,148]. Lactate can also be measured in mg/dL (convert mg/dL to mmol/L by dividing by 9 or mmol/L to mg/dl by multiplying by 0.111). Importantly, many studies define high lactate as concentrations greater than 4 mM in the blood[83,114,115]. Changes in tissue lactate and pH can be much more dramatic than in the blood. For example one study showed that septic patients had no significant difference in blood pH between low and high lactate groups, while there was a significant different in the gastric intramucosal pH[149]. Additionally, another study demonstrated that muscle lactate concentrations were ~1.5 mM higher than arterial lactate concentrations in septic shock patients[150]. These data suggest that our results seem to be particularly relevant to tissue resident immune cells, such as mast cells, and immune cells recruited into the tissue.

Additionally, lactic acid suppression does not require substantial time to impact mast cell function, as suppression was evident with simultaneous or even delayed addition of lactic acid after LPS. These kinetics may be particularly relevant to sepsis. Lactate elevation occurs following bacterial infection and the subsequent immune response, due to tissue hypoperfusion, impaired pyruvate dehydrogenase activity, elevated catecholamine secretion, and increased immune cell glycolysis during the systemic response[109,130–132]. Thus immune cells are activated before and during lactate accumulation. But clinical data suggest that lactate clearance even after the systemic response is occurring has significant benefits. One study showed that 10% lactate clearance improved mortality by up to 40%[115], measuring clearance at 6 hours following hospital admission and diagnosis. Another study found that septic non-survivors had lactate levels of ~6 mM at 24 hours following hospital admission[116]. Further, others have reported that septic patients with the lowest lactate levels 24 hours post-admission had

the highest survival rate, compared with patients with the same initial lactate concentration[149,151], suggesting that lactate levels over time do influence patient mortality. Together, these results suggest that systemic lactate (and likely tissue lactate) may be elevated for many hours while immune cells are recruited to the site of infection. Both immediate and long term effects may be important for understanding of lactic acid actions.

Importantly, lactic acid suppressed cytokine production in a model of septic shock *in vivo*. We chose the LPS-induced model to directly extend our *in vitro* studies. Lactic acid significantly suppressed LPS-induced IL-6, MCP-1, and TNF, which directly supports the effects we observed *in vitro*. Future experiments should modulate lactic acid after the initiation of septic shock and broaden the sepsis model to the polymicrobial fecal slurry model or cecal ligation and puncture. *In vitro,* we observed that lactic acid suppressed activation by TLR-2, 3, and 4 agonists, suggesting that lactic acid may antagonize immune responses more broadly *in vivo*. Interestingly, there were no effects of lactic acid on hypothermia or observational score. We believe these effects may be immune independent. LPS has many direct effects on endothelial cells, including vasodilation, vascular bed relaxation, reduced sympathetic vascular tone, increased vascular leakiness, barrier function disruption, actin depolymerization, increased apoptosis, and increases nitric oxide production[152–156]. Lactic acid has also been shown to increase Akt phosphorylation[157], which is associated with increased NO production in endothelial cells[158], and to increase VEGF[159], which enhances vascular permeability[160]. In mice, low dose infusions of HCl to drop the blood pH to 7.13 increased endothelial NO, reduced BP, and reduced temperature, in the animals[156]. Furthermore, the authors noted

in the discussion that lactic acid reduced pH and BP with a small increase in endothelial NO, however these data were not shown. Thus, we hypothesize that lactic acid contributes to vasodilation and vascular leak in sepsis, while reducing inflammatory cytokine production from immune cells. Furthermore, lactate and/or the associated changes in pH may contribute to pain within incisions or wounds[161], influencing locomotion, grooming, and overall observational scores. Future work should further examine the effects of lactic acid on different cell types and organ systems within a sepsis model to better determine the effects on sepsis as a systemic disease.

Lactic acid suppression of LPS-induced cytokine production was MCT-1 dependent, similar to our findings with lactic acid effects on IL-33-induced activation[52]. MCT-1 is the primary transporter of lactic acid, known to co-transport lactate and H^{+}[134]. In our experiments, there was no effect of lactic acid with MCT-1 inhibition, suggesting that lactate and/or H^{+} must be transported into the cell for suppressive effects. Interestingly, we also observed reduced LPS effects after acidification with formic acid or high concentrations of sodium lactate. These results are supported by many studies which have found immunosuppressive effects of acidification[122,162,163], higher concentrations of lactate(10-100 mM)[121–123], and slightly lower concentrations of lactic acid (5-20 mM)[124,136,137]. Our data have been corroborated in DCs, in which the inhibitory effects of 10mM lactic acid were reverted after normalizing the pH[121]. Together, these results suggest that without a change in pH, a much higher concentration of lactate is required for transport into the cell by MCT-1. Importantly, we observed a pH reduction from 7.4 to 6.7 upon lactic acid addition to media, with a rebound within 2 hours. This suggests that our buffer systems in culture mimic the human body, as lactic acid

deprotonates to lactate and H^+ ions that are buffered by the bicarbonate system in the blood.

When seeking a molecular mechanism, we noted that lactic acid reduces NFκB activity. NFκB is a major transcription factor in LPS signaling, important for cytokine transcription[164–166]. Previous studies report reduced p-NFκB and nuclear translocation in monocytes with lactate or lactic acid treatment [123,137], which were supported in our system. Our data suggest that lactic acid inhibited NFκB transcriptional activity, as observed by a luciferase reporter system, which could reduce cytokine production. We have previously published that lactic acid suppresses the NFκB-dependent induction of miR-155 by IL-33[52]. miR-155 functions to suppress the negative regulators Shp-1 and SOCS1, with an overall pro-inflammatory role in immune cell signaling[75,167]. Lactic acid similarly suppressed LPS-mediated miR-155-5p and miR-155-3p expression. But differences in the two studies were noted. Genetic deletion of miR-155 demonstrated its critical role in cytokine production. miR-155 KO BMMC had weaker responses to LPS, as we found with IL-33. Lactic acid did not further suppress cytokine secretion from miR-155 KO BMMC stimulated with IL-33. However, LPS-induced cytokines were further reduced by lactic acid when studying miR-155 KO mast cells. Thus these data show that miR-155 suppression is an anti-inflammatory effect of lactic acid, but that its suppression alone does not account for all of the lactic acid effects.

Our data suggest that inhibiting glycolysis is necessary and sufficient for lactic acid to suppress LPS-induced cytokine production. In BMMC, we observed a significant reduction in LPS-induced glucose uptake and lactic export with lactic acid treatment. This is consistent with the known ability of H^+ ions to inhibit phosphofructokinase

activity[168,169], effectively reducing glycolysis and lactate production[170]. Elevated lactate also increases the production of pyruvate by lactate dehydrogenase, an equilibrium enzyme, effectively reducing NADH recycling required for glycolysis[171,172]. Our data support previous publications showing that 20 mM lactic acid, 68 mM lactate, and acidification to a pH of 6.6 suppress glucose uptake, lactate export, and intracellular ATP in human monocytes and mouse macrophages stimulated with LPS[122,124]. Additionally, this has been shown in CD4 T cells, in which 10 mM sodium lactate suppressed ECAR at rest and following CXCL10 activation[138]. Lactic acid also suppressed HK2 and PKM2 expression in mast cells following LPS activation. These data support two studies showing reduced HK1 expression in CD4 T cells cultured with sodium lactate[138,169]. Interestingly, another study reported that blocking endogenous lactate export by MCT4 in macrophages following LPS activation suppresses cytokine production, glycolysis, and HK2 expression[112]. Together, these results suggest that elevated lactic acid, whether endogenous or exogenous, suppresses cytokine production and immune cell glycolysis needed for inflammatory responses.

Notably, inhibiting glycolysis mimicked lactic acid effects on cytokine production. Treatment with the glycolytic inhibitors 2DG and OX significantly suppressed cytokine production and NFκB transcription, similar to lactic acid. While the suppressive effects of 2DG on cytokine production have been reported following LPS activation in other immune cells[122,124] and following IgE-induced mast cell activation[99], this is the first report in immune cells to directly show an effect on NFκB transcription. We additionally found that 2DG and OX suppress cytokine production and NFκB transcription with mast cells following IL-33 activation (data not shown). Collectively,

our studies suggest that suppressing glycolysis is sufficient to limit inflammatory cytokine responses. This effect could be due to reduced ATP production. In fact, we found that increasing ATP availability reversed the effects of lactic acid, restoring both NFκB function and cytokine production. ATP is important for kinase activity, tRNA synthetase function, ion transport, and chromatin remodeling, which play a role in cell signaling and cytokine production. Thus by acting on glycolysis, lactic acid can blunt LPS-induced ATP production that is broadly required for the inflammatory response.

Together these data support the theory that high lactate levels early in sepsis may suppress immune cell glycolysis and function, contributing to the immunosuppression observed in the secondary phase of sepsis. In immunosuppression, immune cells have reduced glucose metabolism, cytokine production, antigen presentation, and cytolytic function[106], similar to effects observed with lactate treatment[120–124,138,173]. We hypothesize that reducing lactate accumulation, improving lactate clearance, or reversing lactic acid effects may improve immune function in the secondary phase of sepsis. However, no studies have directly examined the effects of these interventions on immune cell function. Some studies conflict with this hypothesis, reporting no significant improvement in patient outcomes after reducing blood lactate. We believe that the study designs may limit the interpretation of these results. In one study, a randomized clinical trial was conducted to determine if targeting lactate clearance provided lower patient mortality than targeting central venous oxygen saturation ($SCVO_2$)[174]. While management to normalize blood lactate clearance did not improve survival compared with interventions normalizing $SCVO_2$, both patient groups received the same interventions, including isotonic crystalloid, vasopressors, packed red blood cells, and dobutamine. The

interventions were administered due to different lactate or $SCVO_2$ indicators, however the protocol was nearly identical and the lactate and $SCVO_2$ levels were not significantly different between the groups at either time point measured. These results suggest that protocols to normalize lactate are similarly effective to protocols to normalize $SCVO_2$, however there was no control group to adequately compare the overall effectiveness of these interventions. Another study administered dichloroacetate (DCA) to reduce the production of blood lactate in sepsis patients[175]. DCA treatment worsened patient survival, leading researchers to conclude that modulating blood lactate does not improve survival in sepsis. However, while DCA reduces lactate secretion by increasing pyruvate conversion to acetyl co-A in the mitochondria (see Figure 3-19), it also inhibits glycolysis, effectively augmenting lactic acid effects[176–178]. Furthermore, despite lowering blood lactate levels, intravenous DCA administration has been shown to reduce tissue ATP[179], mimicking lactic acid effects. Future studies should examine the effects of early lactate clearance on immune cell metabolism and function in septic patients during the immunosuppressive phase of the disease. Additionally, future studies should aim to increase glycolysis to reverse lactic acid suppression, potentially by targeting known glycolytic regulators such as mTORC, Akt2, Myc, or HIF-1 signaling[180,181].

In summary, lactic acid suppresses LPS-induced mast cell activation and LPS-induced cytokine production *in vivo*. These effects are dependent upon MCT-1 transport into the cell, and both lactate and H^+ ions independently reduce activation. Moreover, lactic acid suppresses LPS-induced glycolysis, suppressing cytokine production and NFκB, partly by reducing ATP availability. Future studies should target enhancing

glycolysis and ATP production to improve immune function and counteract lactic acid effects in sepsis.

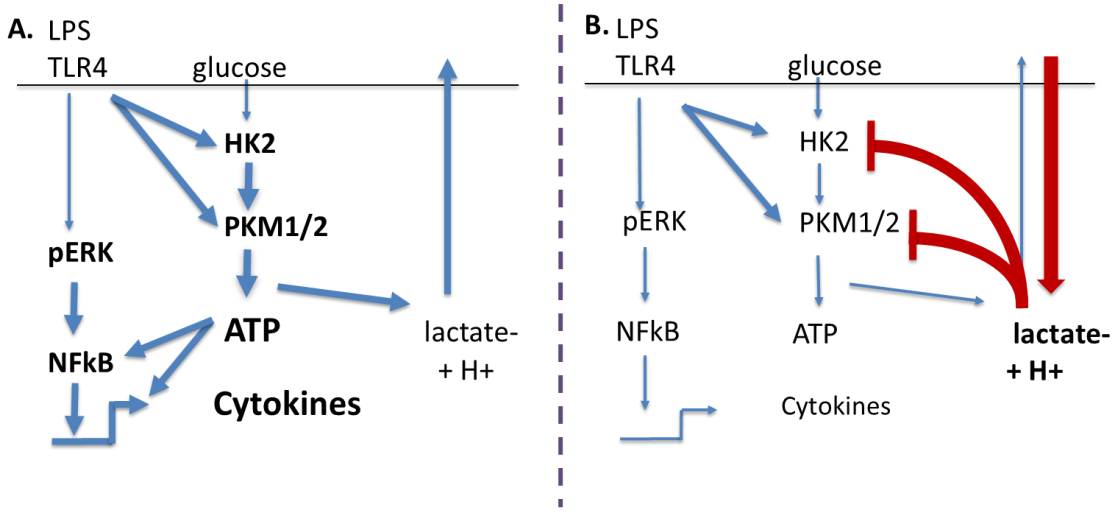

Figure 2-25: Schematic Diagram. A) LPS activates TLR4, signaling through NFκB to induce cytokine production. LPS activation also increases glycolysis to provide ATP to augment NFκB transcription and cytokine production. B) Exogenous lactic acid suppresses glycolysis, effectively suppressing NFκB transcription and cytokine production.

3.6 Acknowledgements

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Chapter 3: No longer solely a metabolic by-product, lactic acid shapes fate and function of immune cells in health and disease

Authors: Heather L. Caslin and John J. Ryan

3.1 Abstract

Lactic acid is still introduced in many biochemistry and general biology textbooks as a metabolic by-product within fast glycolysis or oxygen-independent glycolysis. The role of lactic acid as a metabolite, feedback regulator, and signaling molecule has been recently appreciated in the context of cancer and other organ systems. However, there is no comprehensive review of the role for lactate and lactic acid in immune cells. Here we summarize the known immunosuppressive effects of lactic acid and lactate on immune cells, the mechanisms of action, and implications in disease.

3.2 History and Biochemistry

Lactic acid was first discovered in sour milk in 1780 by the Swedish chemist Karl Wilhelm Scheele[182]. In 1843, the German physician–chemist Johann Joseph Scherer produced evidence for lactic acid in human blood under pathological conditions[182]. Carl Folwarczny subsequently demonstrated the presence of lactic acid in the blood of a living patient in 1858[182]. At the same time, Louis Pasteur recognized that some types of yeast fermented sugar to lactate under anaerobic conditions[183], a phenomenon known as the Pasteur Effect[184,185]. Later, a parallel phenomenon was noted in skeletal muscles with the conversion of glucose to lactate in anaerobic frog muscles[186]. Stimulation rapidly increased the lactate concentrations, which were reduced back to resting concentrations in

an oxygen rich environment. Importantly, it was later shown that lactate production can occur in muscle despite adequate oxygenation[187].

In the early 1940's, Meyerhof demonstrated that glycogen is a precursor for lactate in isolated muscles and the full glycolytic pathway was determined[188]. One glucose molecule, transported from the bloodstream or broken down from stored glycogen, is converted to two ATP, two NADH, and two pyruvate molecules. There are two different metabolic fates for pyruvate when energy is required[184]. It can be converted to acetyl-coA in the mitochondria for further catabolism in the Kreb's cycle and electron transport chain, as part of oxidative phosphorylation (OX PHOS). Alternatively, pyruvate can be converted to lactate, the end product of glycolysis[184]. Lactate dehydrogenase (LDH) bi-directionally converts pyruvate to lactate and has higher enzyme activity than the enzymes that oxidize pyruvate[189,190], suggesting that some lactate is always being produced from pyruvate, even when pyruvate primarily enters the mitochondria for OX PHOS. Importantly, with the conversion of pyruvate to lactate, NADH is also recycled to NAD^+, which can be recycled for continued glycolysis. While there is evidence of a shuttle for lactate to enter the mitochondria and contribute to mitochondrial ATP production[184], a majority is quickly shuttled out of the cell to ensure that glycolysis continues.

There has been recent debate as to whether or not lactic acid is produced from glycolysis or if lactate and H^+ ions are produced separately during circumstances in which glycolysis occurs[191–195]. It is difficult to examine the exact chemical reactions because lactic acid has a pKa of 3.86 and immediately and almost completely dissociates into lactate and H^+ions. H^+ ions are produced by many enzymes that use ATP hydrolysis

within and outside of the glycolytic pathway, and ATP utilization (hydrolysis) is one mechanism that increases glycolysis. Studies using magnetic resonance spectroscopy suggest that lactic acid is produced in muscle tissue[196,197] without any changes in ATP hydrolysis[198]. However, others contend that glycolytic intermediates are always in the carboxylic ion form, not the carboxylic acid form[195], and that the H^+ ions are produced solely from ATP hydrolysis, by phosphofructokinase and GAPDH in glycolysis, and by energy consuming pathways which fuel the need for glycolysis[195]. Interestingly, pyruvate kinase and lactate dehydrogenase consume a H^+ ion to maintain homeostasis within the system[191], however, there is still a net release of H^+ ions, evident by a drop in pH as glycolysis continues with the equal production of lactate and H^+ ions[199].

While the source of H^+ ions is interesting from a biochemical perspective, both lactate and H^+ ions are produced during circumstances that increase glycolysis. From a physiological perspective, we believe the functions of both products, lactate and H^+ ions, are important for cellular function and feedback in response to glycolysis, and therefore we have tailored this review and our own studies to the combined cellular effects. We should note that in living systems, lactate and H^+ (measured via pH) are often measured separately because they are dissociated, and therefore we will be specific when referring to these measures in cited studies. Additionally, H^+ ions are buffered by the bicarbonate system in the blood[139,140] and acidosis can occur without a concomitant rise in lactate levels[142]. Therefore, we will also be specific when referring to hyperlactemia or acidosis in cited studies. Finally, lactic acid has been used both *in vivo* and *in vitro* to measure direct effects of both the lactate ion and the change in pH, and we will specifically use the term lactic acid when referring to these experiments.

3.3 Lactic acid effects on immune cells

Many publications have examined lactic acid and/or lactate effects on immune cells. Interestingly, while most researchers have found significant effects with lactic acid, only some have observed differences with lactate treatment, suggesting a role for changes in pH. Additionally, while many of the reported effects are inhibitory, there are some contradictory reports of enhanced activation of the same cell type at similar concentrations. We will examine some of these results below.

The effects of lactic acid and lactate on LPS activation of monocytes and macrophages, both human and mouse, have been the most often published. Added simultaneously to activation, 5-10 mM lactic acid has been reported to suppress LPS-induced TNF-α, while 20 mM was required to significantly suppress IL-6 in human monocytes[122]. These results were pH-dependent, as the same concentrations of sodium lactate had no effect, but a pH of 6.6 or the addition of HCl with sodium lactate (pH: 6.6 or 7.1) did suppressed TNF-α. Additionally, adding NaOH to lactic acid reversed the effects. Another study showed that the LPS-induced upregulation of CCL2, CCL7, PTX3, EGR2, GEM, TNF-α, and IL-8 were delayed in the presence of lactic acid in human monocytes, while IL-23 was enhanced[200], suggesting a specific effect and not a general inhibitory effect of lactic acid.

Similar to lactic acid, lactate concentrations between 10-100 mM have been shown to inhibit LPS-induced IL-1β and CD-40 from bone marrow-derived macrophages (BMDM), while 50-100 mM was required to inhibit IL-6 and IL-12 mRNA and protein[124]. Similar trends were observed within mouse peritoneal macrophages. Lactate treatment (15 mM) 15 minutes prior to LPS stimulation suppressed pro-IL-1β and pro-IL-

18 expression in human peritoneal macrophages and PBMCs, with no effect on IL-10[137], and 20 mM lactate inhibited LPS-induced TNF-α and IL-6 release as well as the migration of human monocytes[136].

Acidity alone may impact macrophage function. A pH <7 has been shown to suppress TNF-α production from rabbit alveolar macrophages, while a pH <6 reduced viability[162]. Exposure to a pH of 1.75 for 1 minute has been shown to suppress the production of TNF-α, MCP-1, MIP-2, and IFN-β from rat alveolar macrophages following LPS or LTA stimulation for 24 hours[163] Furthermore, a low pH and high lactate concentration, as observed in peritoneal dialysis fluid, has been shown to suppress LPS- induced TNF-α and IL-1β in human PBMCs and macrophages[201]. Together, these publications suggest that lactic acid, lactate, and acidity all impact macrophage and monocyte responses to LPS, and suggest that these generally inhibitory effects are dependent on concentration, pH, and overall inflammatory function.

Similar to monocytes and macrophages, lactic acid suppresses proliferation and function in dendritic cells, T cells, and NK cells. In dendritic cells, lactic acid (10 mM or endogenous production in culture) reduces dendritic cell maturation, as measured by CD1a, and suppressed dendritic cell IL-12, IL-6, TNF-α, and IL-23 production following LPS activation[202,203]. There was no significant change in IL-10, indicating some specificity. Lactic acid also suppressed T cell proliferation following dendritic cell co-culture and stimulation, suggesting a larger impact on the immune system than solely modulation of cytokines[202]. In T cells, 15-20 mM lactic acid decreased CTL proliferation, IFNγ and IL-2 production, CD107a/b and perforin expression, and cytolytic activity[173,204]. Results in these studies were dependent reduced pH, with no effect when lactic acid was

buffered, no effect of sodium lactate, and no effect when lactic acid was washed out prior to activation[204]. However, both sodium lactate and lactic acid have been shown to inhibit CD4+ and CD8+ T cell motility, while lactic acid, but not sodium lactate, reduced CD8 cytolytic function[138]. Furthermore, lactate and acidity inhibited cytolytic function of both human and mouse NK cells, and lactate decreased the expression of perforin, granzyme, and NKp46 in NK cells[120], similar to the reported effects in T cells. Together, these results suggest that lactic acid consistently inhibits pro-inflammatory T cell functions, with differential effects of lactate alone.

Contributing to the greater effect of immunosuppression, lactate augments anti-inflammatory responses. Lactate increases the differentiation of myeloid derived suppressor cells (MDSC) following GM-SCF and IL-6 treatment[120]. Additionally, lactic acid promotes M2 polarization, increasing Arg-1, Fizz1, Mgl1, Mgl2, and VEGF[119]. Furthermore, 30 mM lactate increased IL-2 mRNA and protein when added 0-8 hr after stimulation of CD4 but not CD8+ T cells[205]. IL-2 promotes T-regulatory cell (Treg) development contributing to immunosuppression and innate control of the immune system[206]. Lactate secretion by umbilical cord derived mesenchymal stromal cells induced M2 polarization and promoted Th2 polarization by monocyte derived dendritic cells[207]. These data further suggest a context-specific effect of lactic acid and suggest a systemic suppressive effect on Th1-like immune activation.

Our lab has examined the effects of lactic acid on mast cells. Lactic acid suppressed cytokine and chemokine production in BMMC activated with IL-33 and LPS in bone marrow derived and peritoneal mast cells[52] (and Chapter 2- data not published). Interestingly, while lactic acid suppressed IL-6, TNF-α, IL-13, MCP-1, and MIP-1α,

lactic acid increased the production of the endothelial growth factor VEGF. These data support the above publications showing a selective suppression of inflammatory mediators and functions, yet enhanced Th2, regulatory, and wound healing phenotypes.

In contrast to the majority of studies showing suppressive effects, several publications show enhanced inflammatory function induced by lactic acid and/or lactate. Sodium lactate has been reported to increase IL-17 production from CD4+ cells[138]. Related to this, lactic acid added simultaneous to LPS or peptidoglycan (PGN) increased IL-23 transcription in monocytes, macrophages, and tumor infiltrating immune cells, which subsequently enhanced IL-17 production from TLR-stimulated splenocytes[126]. IL-17 and IL-23 are pro-inflammatory cytokines that contribute to autoimmune diseases[208,209]. In two studies other studies, sodium lactate was shown to enhance the secretion of IL-6, MMP1, and IL-1β from U397 and human monocyte-derived macrophages when pretreated for 24 hours and activated by LPS[125,127]. While the explanation for some divergence in lactate/lactic acid effects is unclear, a better understanding of the mechanism of action may show us experimental variables that impact the role of lactic acid on the system of interest.

3.4 Mechanism of Lactic Acid Effects

There has been little direct examination of how lactic acid impacts immune cell function. An initial issue is how lactate or lactic acid from the microenvironment enters cells. Data from our lab and others suggest that lactic acid utilizes MCT-1-mediated import, and not the lactic acid receptor GPR81, for functional effects[52,124,138] (and Chapter 2- data not published). Broadly blocking the MCTs with α-cyano-4-hydroxycinnamate,

or specifically blocking MCT-1 and -2 with AR-C155858 reversed lactic acid effects on T cells and mast cells [52,138] (and Chapter 2- data not published). Importantly, MCT-2 is not expressed on mast cells[52] and has not been shown to be expressed on any other immune cell. The predominant role of MCT transporters, like MCT-1, unidirectional proton-linked transport of L-lactate across the plasma membrane[134], and hence optimal transport of lactate or lactic acid should occur in an acidic environment. However, it is also possible that at higher concentrations, lactate can move across the gradient without a 1-to-1 exchange with H^+ ions. MCT1 can also exchange one monocarboxylate for another without net movement of protons[134]. In this regard, we noted in mast cells both lactic acid and sodium lactate suppressed LPS-induced cytokine production. However, this required a 2-fold greater concentration of lactate (Chapter 2- data not published). Additionally, Gottfried et al. showed that the inhibitory effect of 10 mM lactic acid was reversed by adjusting the pH to 7.4, but at concentrations above 10 mM, adjusting the pH only partially reversed lactic acid effects[202]. One interpretation is that MCT-1 offers more efficient transport in acidic conditions, while greater concentrations access a proton-independent transport mechanism.

Regarding effects on signal transduction, lactic acid has been shown to suppress several inflammatory pathways. It delays Akt phosphorylation and IκB-alpha degradation, suppressing NFκB nuclear accumulation following LPS activation in human monocytes[200]. These data were corroborated by a similar study showing reduced p-NFκB in LPS-activated human peritoneal macrophages and PBMCs[137]. Additionally, lactic acid reduced p-JNK, p-cJun, and p-p38 without MEK1 and ERK effects in TCR-stimulated CTL[204]. Our lab has observed that lactic acid inhibits p-ERK, JNK, TAK1, p-p65, p-p38,

downstream of IL-33[52] and reduced LPS-induced NFκB transcription (Chapter 2- data not published).

How lactic acid suppresses these cell-signaling cascades is not clear. We noted decreased miR-155 expression after lactic acid treatment, and reduced LPS-mediated induction of this inflammatory microRNA[52](and Chapter 2- data not published). miR-155 is known to suppress expression of inhibitory proteins such as Ship-1 and SOCS1, effectively increasing signaling strength[210,211]. Overexpressing miR-155-5p prevented lactic acid-mediated suppression of IL-33-induced cytokine production[52], and miR-155 deficiency reduced LPS-mediated cytokine production in mast cells. But surprisingly, lactic acid still suppressed LPS-induced cytokine production in miR-155 KO mast cells (Chapter 2- data not published). These results show that lactic acid can inhibit miR-155 expression, possibly due to reduced NFκB activity, but this does not fully explain lactic acid effects.

We and others have observed a role for HIF-1α in lactic acid effects[52,119]. While HIF-1α is known to induce VEGF and angiogenesis, consistent with lactic acid effects, HIF-1α is typically increases inflammatory gene transcription and glycolysis, activities we note are suppressed by lactic acid and further discuss below. Further research should examine HIF-1α translation, stability, and transcriptional activity on angiogenic, inflammatory, and metabolic genes following lactic acid treatment.

Our current mechanism of interest is the role of lactic acid as a feedback regulator for cellular metabolism and ATP production. This is consistent with the known ability of H^+ ions to inhibit phosphofructokinase activity[168,169], reducing glycolysis and lactate production[170]. Additionally, elevated lactate increases pyruvate production by lactate

dehydrogenase, which reduces NADH recycling needed for glycolysis[171,172]. However there is little understanding of this feedback loop in immune cells. Previous work has shown that in human monocytes, lactic acid reduced glucose uptake and intracellular ATP, with no effect on amino acid uptake following LPS activation[122]. Another study in T cells showed that 68 mM lactate could decrease extracellular acidification rate (ECAR), a surrogate measure of glycolysis, with and without LPS activation[124]. Furthermore, an acidic pH of 6.7 reduced glucose-stimulated ECAR while increasing both basal and maximal oxygen consumption rate (OCR), a surrogate measure of OX PHOS, in T cells[212]. While these studies do not directly examine how lactate and lactic acid affect oxidative phosphorylation, our data suggest that glycolysis is more important for mast cell function in response to LPS and that lactic acid effects on glycolysis are critical for inflammatory functions (Chapter 2- data not published).

Our data show that lactic acid suppresses LPS-induced glucose uptake, lactate export, and protein expression of the glycolytic enzymes hexokinase and pyruvate kinase (Chapter 2-data not published). Similarly, others have found reduced basal and CXCL10-induced hexokinase expression in CD4 T cells treated with lactate[16,28]. The glycolytic inhibitors 2-deoxyglucose and sodium oxamate also suppressed cytokine production and NFκB transcription, mimicking lactic acid effects. Moreover, increasing ATP availability reversed lactic acid suppressive effects in mast cells. Together, these data suggest that suppressing ATP production by glycolysis is necessary and sufficient for lactic acid effects. Furthermore, these data may explain why lactic acid augments MDSC development and M2 differentiation, as these cell types rely on OX PHOS more than glycolysis.

3.5 Lactic acid in inflammatory environments and implications in disease

3.5.1 Tumors

In the 1920's, it was initially shown that tumor cells consumed substantial glucose and secreted lactate even in the presence of adequate oxygen[213,214]. This is now known as the Warburg Effect[215] and is a hallmark characteristic of cancer[216]. In the tumor microenvironment, lactate levels can reach as high as 40mM[81], while extracellular pH can be as low as 6-6.5[217], in comparison to normal cell environments with a lactate level ~1 mM and pH of 7.5. Additionally, high lactate levels are associated with increased metastasis and decreased survival [81,218], suggesting that lactate may be used as a prognostic parameter in the clinic.

Although glycolytic ATP production is less efficient than OX PHOS, increasing glycolysis allows the cell to continue ATP production while using metabolic intermediates from glycolysis, the pentose phosphate pathway, and the Krebs cycle to provide building blocks for proliferation[215]. The effects are also selective for tumor growth. Lactic acid generally suppressing immune cell function and reprograms immune cell metabolism[219], aiding tumor escape and immune evasion[220]. Lactic acid from the tumor microenvironment has been shown to suppress T cell activation and induce an M2 phenotype, characteristic of tumor-associated macrophages[119]. Lactate and lactic acid can increase HIF-1α and VEGF production from mast cells[52], macrophages[119], and endothelial cells[159], contributing to angiogenesis and endothelial cell migration. Additionally, cancer cells have a reduced uptake of alkaline chemotherapeutics in a low pH environment[221]. Together, these data suggest that the Warburg Effect and lactate

production by tumors provides inherent benefits to the tumor cell and modulates the microenvironment to promote immune evasion and tumor growth.

There have been a few approaches *in vivo* to directly target lactic acid effects on tumor growth and immune function. Many studies have added bicarbonate to drinking water to neutralize tumor acidity without causing systemic alkalosis or effecting sodium load in mice[212,221]. In melanoma models models using T cell adoptive transfer, bicarbonate treatment reduced tumor size, increased in tumor-associated CD8+ cells, and increased survival[212]. Furthermore, combining bicarbonate therapy with anti-CTLA-4 or anti-PD1 improved antitumor responses in multiple models in the same study. In another study, bicarbonate treatment improved doxorubicin treatment, reducing the size and growth rates of MCF-7 human breast cancer xenografts[221]. Furthermore, systemic treatment of B16 melanoma-bearing mice with proton pump inhibitors, which increase tumor pH, significantly increased survival and T cell function in active or adoptive immunotherapy models[222]. Importantly, proton pump inhibitors did not affect T cells in healthy tissues, likely because the drugs are acid-activated. Together, these studies suggest that modulating pH in the tumor microenvironment may reduce tumor evasion and increase immune cell function.

3.5.2 Wounds

Lactate levels are elevated in the local wound environment, typically reported between around 20mM, with a range between 5-80 mM[84,147]. This is due to poor tissue perfusion, poor oxygenation, or atypical bacterial colonization and immune activation[147]. Lactate and lactic acid concentrations between 10-20 mM promote fibroblast

proliferation, myofibroblast differentiation via TGF-β production, and collagen deposition[223–225]. Additionally, lactate concentrations between 12.5- 25mM induce revascularization and angiogenesis via VEGF production[52,119,226–228] and enhance M2 polarization[119], contributing to wound closure and healing[228]. However, others have shown that lactate concentrations above 20 mM can reduce fibroblast and endothelial cell viability and reduce wound closure[147]. Further understanding of the mechanisms by which lactic acid signals to and within immune cells may help us to develop direct targets to improve wound healing.

3.5.3 Sepsis

Blood lactate concentrations in sepsis are often between 2-10, however concentrations have been reported as high as 20 mM[118,145,148]. Many studies show that increased blood lactate (≥4 mM) and impaired clearance are independently associated with increased mortality in septic patients[114–117]. Because of this, lactate clearance is a treatment guideline for sepsis[100]. At least one hospital has reported reduced patient mortality after lactate levels >4mM were added to a laboratory critical call list, in which the results are quickly reported to the physician and treatment time is reduced[118]. While these data show that lactate and sepsis severity are related, it is not known whether elevated lactate is a cause or consequence of the infection.

We and others report that lactic acid and lactate suppress LPS-induced cell metabolism and immune cell function in culture [122,124,200,202] (and Chapter 2- data not published). We have also shown that intraperitoneal lactic acid injections can suppress LPS-induced cytokine production *in vivo* (Chapter 2- data not published). Additionally,

another study has reported that pulmonary acidic aspiration impaired clearance of *S. pneumoniae* and *E. coli* (via intratrachael administration)[163], suggesting reduced immune function. These data support the theory that high lactate levels early in sepsis may suppress immune cell glycolysis and function, contributing to immunosuppression observed in the secondary phase of sepsis. In this secondary immunosuppressive phase, immune cells have reduced glucose metabolism, cytokine production, antigen presentation, and cytolytic function[106], similar to effects observed with lactate treatment[120–124,138,173]. We hypothesize that reducing lactate buildup, improving lactate clearance, or reversing lactic acid effects may improve immune function in the secondary phase of sepsis, however no studies have directly examined the effects of these interventions on immune cell function. Future studies should directly examine the effects of early lactate clearance on *ex vivo* immune cell metabolism and function in septic patients during the immunosuppressive phase of the disease.

3.5.4 Other Inflammatory Diseases

Systemic elevations of lactate have been been reported in obesity, asthma, allergic disease, and colitis. Patients with obesity, with or without glucose intolerance have higher plasma lactate concentrations than controls[79]. Additionally, asthmatic patients had elevated plasma lactate, which correlated with reduced FEV1[82]. While the serum lactate levels in these patients is elevated by 1 mM or less, patients with stable asthma, rhinitis, and eczema have lower lung pH as measured by exhaled breath condensate (EBC) than controls. Additionally, acute asthmatics had the lowest EBC pH levels compared to each of the other groups[229]. Additionally, EBC-pH has been associated with EBC-lactate in

patients with lung injury[230]. These data suggest that local lactate concentrations may be much greater than systemic measures indicate, and suggest future studies examine the tissue concentrations of lactate in the lung of asthmatics and the adipose tissue of obese individuals. Furthermore, in patients with ulcerative colitis, those with moderate and severe colitis have low fecal pH and high fecal lactate, produced by the inflamed colonic muscosal cells[80,231].

The functional role of high lactate in these diseases remains unclear. Direct lactate administration and treatment with probiotic *lactobacilli* strains have been shown to reduce symptoms and inflammation in mouse models of colitis and IL-33-induced inflammation. Probiotic treatments have also been beneficial in patients with allergic disease and colitis[52,232–235]. The high lactate levels observed within these inflammatory diseases, coupled with beneficial effects of lactate administration, support the theory that lactate is an inherent feedback regulator of inflammation, however it is unknown if this feedback is dysregulated in disease. Positive results in clinical trials using probiotics is reason for optimism, since lactic acid administration has little potential as a therapy. Future studies should specifically examine the efficacy and safety of specific probiotic strains in animal models and in randomized, double-blind placebo-controlled clinical trials for different inflammatory diseases. The kinetics of treatment and delivery methods will also need to be examined. Probiotic administration may work better prior to the onset of disease or flare to reduce the initial inflammatory response, however it the efficacy is unclear once the disease has peaked and lactate levels are already high.

3.6 Conclusions

Immunologists are just now beginning to understand the important roles lactic acid appears to have as a metabolite, feedback regulator, and signaling molecule. While lactate and H^+ ions primarily suppress inflammatory mediators and functions, there are also effects that enhance Th2 immunity, regulatory functions, and wound healing. Lactate and H^+ ions suppress glycolytic ATP production, contributing to reduced cell signaling, transcription, cytokine production, and migration. Lactate levels are high in the tumor microenvironment as well as wounds, sepsis, colitis, asthma, and obesity, suggesting a plausible target for the treatment of these diseases. Future studies should directly modulate lactate levels and target lactate effects for therapeutic development. Proton pump inhibitors and bicarbonate treatment may limit immunosuppression in cancer and the later stage of sepsis. Probiotics may reduce inflammation in autoimmunity and allergic disease. And finally, a better understanding of lactic acid effects and optimal cell metabolism may also allow us to target known glycolytic regulators such as mTORC, Akt(2), myc, or HIF-1α signaling[180,181]. There is still much to learn, but one thing is clear: lactic acid is much more than a metabolic by-product.

Chapter 4: Inhibiting glycolysis and ATP production attenuates IL-33-mediated cytokine production in mast cells and IL-33-induced peritonitis

Authors: Heather L. Caslin, Marcela T. Taruselli, Brian O. Barnstein, John J. Ryan

4.1 Abstract

Cellular metabolism and energy sensing pathways are closely linked to inflammation and immune cell function, however there is little understanding of metabolism directly linked to mast cells or IL-33. Mast cells are a major effector cell in allergy and asthma. IL-33 is an alarmin linked to both mast cell activation and allergic disease. The purpose of this study was to determine the metabolic requirements for IL-33-mediated mast cell activation in hope of finding targets for controlling IL-33-mediated inflammation. Our data suggest that IL-33-increases glycolysis, glycolytic protein expression, and oxidative phosphorylation (OX PHOS). Inhibiting OX PHOS had no effect on cytokine production, while inhibiting glycolysis by 2-deoxyglucose and oxamate suppressed inflammatory cytokine production *in vitro* and *in vivo*. ATP reversed this suppression. Glycolytic blockade also suppressed IL-33 signaling *in vitro* (p-ERK and NFκB transcription) and IL-33 induced neutrophil recruitment *in vivo*. To test a clinically relevant way to modulate these pathways, we examined the effects of the FDA approved drug metformin on IL-33 activation. Metformin activates AMPK, which suppresses glycolysis in immune cells. We found that metformin suppressed cytokine production *in vitro* and *in vivo*, effects that were reversed by ATP, mimicking the actions of the glycolytic inhibitors we tested. Together, these data suggest that glycolytic ATP

production is important for IL-33-induced mast cell activation, and targeting this pathway may be useful in allergic disease.

4.2 Introduction

Cellular metabolism and energy sensing pathways control the breakdown of carbohydrates, fatty acids, and proteins when energy is required. While we know that cells require energy for homeostasis, maintenance, and proliferation, it is now understood that metabolism is closely linked to immune cell differentiation and activation, impacting phenotype, effector functions, and overall inflammation[85]. Researchers have consistently found that glycolysis is the primarily energy production pathway used by inflammatory cells, such as T-helper (Th)1, Th17, macrophage (M)1, and dendritic cells (DCs) during acute activation, while oxidative phosphorylation (OX PHOS) in the electron transport chain (ETC) is the primary energy production pathway used by regulatory cells such as T-regulatory (Treg), M2, and monocyte derived suppressor cells (MDSC)[86–89]. While glycolysis is inefficient, with only 2 ATP produced per glucose compared with 32 per glucose in OX PHOS, the benefits of utilizing glycolysis during activation and proliferation are multi-fold. Glycolysis rapidly increases ATP availability, operates under low oxygen tension, and provides pentose phosphate pathway- and Kreb's cycle-intermediates to anabolic pathways which synthesize nucleotides, amino acids, and lipids[86,92]. This has been most extensively studied in T cells, which undergo dynamic and complex metabolic reprogramming in response to activation, cytokine stimulation, and other changes in their microenvironment[88,89,93]. There is limited information on how metabolism is modulated in mast cells.

Mast cells are tissue resident myeloid cells that reside in both mucosal and connective tissue. These cells are typically recognized for their effector function in Th2 immunity: specifically their detrimental role in allergic disease and protective role against parasites and venoms[20–23]. While much is known about mast cell activation, little data concerning the role of glucose metabolism in mast cell responses has been published. Early studies from the 1990's suggest that adequate glucose and ATP are required for full mast cell function[96,97,236], while lactate was released upon activation in response to compound 48/80 and polymyxin B in rat mast cells[94]. Additionally, earlier studies by Chakravarty suggest that glycolytic blockade via by 2-deoxyglucose (2-DG), iodoacetate, fluoride and oxamate (OX) suppressed compound 48/80 and antigen-induced histamine release in rat mast cells[95,237]. Recently, an extraceullular flux analyzer (Seahorse device) was used to show that IgE XL rapidly increases glycolysis while OX PHOS increases ~2 hours after stimulation[99]. The same study showed that suppressing glycolysis with dichloroacetate (DCA) and inhibiting complex I of the electron transport chain (ETC) with rotenone suppressed cytokine production and degranulation, while etomoxir, inhibiting fatty acid oxidation, had no effect. Additionally, OX PHOS activity has been shown to increase following IgE XL in mast cells via p-ERK and mitochondrial Stat3[98]. These results suggest that IgE activation requires glycolysis and ETC activity, however the metabolic requirements for other important mast cell activators have not been examined.

IL-33 is cytokine mediator that is also considered to be an alarmin, released by endothelial, epithelial, and fibroblast cells upon cell damage, as well as by mast cells following activation[238–240]. IL-33 activates most immune cell types, including mast cells,

Th2, and innate-like lymphoid cell- (ILC)2. It augments IgE-induced inflammation[46,241] and is elevated in asthma and atopic dermatitis[242–245]. IL-33 administration promotes disease phenotypes in animal studies, while anti-IL-33 or anti-ST2 antibody treatments can alleviate disease pathologies[246,247], making it an important activator to understand in the realm of allergic disease. We have recently shown that lactic acid, a byproduct of glycolysis, can suppress IL-33-induced mast cell activation[52]. This prompted interest in how metabolism may contribute to IL-33 function, which has not been studied in immune cells.

Our purpose was to determine the metabolic requirements for IL-33 activation in mast cells and examine potential targets for controlling IL-33-mediated inflammation. Our data suggest that IL-33-induced cytokine production requires glycolysis for ATP production and that blockade suppresses inflammatory cytokine production *in vitro* and *in vivo*. To test proof of principle and suggest a clinically relevant way to modulate these pathways in humans, we report the effects of the FDA approved drug metformin on IL-33 activation. AMPK induction by metoformin, which suppresses glycolysis in immune cells, inhibited cytokine production *in vitro* and *in vivo*. These effects were reversed by ATP, mimicking the actions of glycolytic inhibitors.

4.3 Methods

4.3.1 Animals

Mouse C57BL/6J and NFκB-luc breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME) and colonies were maintained in a pathogen free facility. Bone marrow from male and female mice was extracted from mice at a minimum of 10

weeks old and IL-33-induced peritonitis studies were conducted around 12 weeks of age under protocol approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

4.3.2 Mast cell culture

Mouse bone marrow cells were differentiated in WEHI-3 cell supernatant containing IL-3 and BHK-MKL cell supernatant containing SCF as described to yield 90-99% FcεRI^{+} and cKit^{+} bone marrow derived mast cells (BMMC) at 21 days[52,75]. Following differentiation and expansion, BMMC were plated at 1x10^{6}/mL with IL-3 and SCF for all experiments (10 ng/mL). Cells were treated as described and activated ± IL-33 at 50 ng/ mL unless otherwise stated. Recombinant mouse IL-3, SCF, and IL-33 for in vitro experiments were purchased from Shenandoah Biotechnology (Warwick, PA). Sodium oxamate and 2-deoxyglucose (2DG) were purchased from Alfa Aesar (Tewksbury, MA). Etomoxir and rotenone were purchased from Cayman Chemical (Ann Arbor, MI) and antimycin A was purchased from Chem Cruz via Santa Cruz Biotechnology (Dallas, TX). ATP disodium salt was purchased from Tocris via Biotechne Corporation (Minneapolis, MN). Metformin was purchased from MP Biosciences (Santa Ana, CA), A769662 was purchased from Med Chem Express (Monmouth Junction, NJ), and SRT1720 was purchased from Cayman Chemical (Ann Arbor, MI).

4.3.3 Cellular Metabolism

To measure the extracellular acidification rate (ECAR), proton production rate (PPR), and oxygen consumption rate (OCR) as surrogates for glycolysis and oxidative phosphorylation, a Seahorse XFp analyzer (Aligent, Santa Clara, CA) was used. Cells were plated in duplicate at 200,000/ well on 4.6 μg/mL Cell Tak in miminal DMEM, 10 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% FBS. The protocol was as follows: initialization, 3 cycles baseline, inject IL-3/ SCF (10 ng/mL), 3 cycles, inject IL-33 (100 ng/mL), 5 cycles. For each condition, an average was taken across all wells.

To determine glucose uptake and lactate export, cell supernatants were analyzed for glucose and lactate concentrations 16 hours after activation using the Glucose Assay Kit 1 and L-Lactate Assay Kit 1 from Eton Bioscience (San Diego, CA). Glucose uptake was calculated as [glucose in unactivated cell supernatant] – [glucose in activated cell supernatant]. Lactate export was calculated as [lactate in activated cell supernatant] – [lactate in unactivated cell supernatant].

4.3.4 Western Blot

To determine protein concentration and protein phosphorylation within the cell, cell lysates were collected using Protease arrest (GBiosciences, Maryland Heights, MO) in cell lysis buffer (Cell Signaling Technology, Danvers, MA). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermofisher, Waltham, MA). 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio–Rad, Hercules, CA) were loaded with 30 μg protein, proteins were transferred to nitrocellulose (Pall Corporation, Ann Arbor, MI), and membranes were blocked for 60 minutes in Blocker casein in TBS (from Thermofisher, Waltham, MA). Blots were incubated with primary antibodies

overnight in block buffer + Tween20 (1:1000) ± rabbit anti-p-AMPK (1:750), rabbit anti-AMPK (1:1000), rabbit anti-HK2 (1:750), rabbit anti-PKM1/2 (1:750), rabbit anti-GAPDH (1:1000, antibodies all purchased from Cell Signaling, Danvers, MA). Blots were washed six times for 5 minutes each in TBST, followed by incubation secondary Ab (1:10,000) for 60 minutes at room temperature (Cell Signaling, Danvers, MA). Size estimates for proteins were obtained using molecular weight standards from Bio–Rad (Hercules, CA). Blots were visualized and quantified using a LiCor Odyssey CLx Infrared imaging system (Lincoln, NE). After background subtraction, fluorescence intensity for the protein of interest was normalized to the signal intensity for the relevant loading control and unactivated samples, using Image Studio 4.0 (LiCor).

4.3.5 ELISA

ELISA analysis was used to measure cytokine concentrations from the cell culture supernatant 16 hours after activation and from the plasma 4 hours after IL-33 induced peritonitis (further explained below). IL-6, TNF, and MCP-1 (CCL2) murine ELISA kits were purchased from Biolegend and MIP-1α (CCL3) murine ELISA kits were purchased from Peprotech (Rocky Hill, NJ). Assays were performed in duplicate (plasma) or triplicate (cell supernatant) according the manufacturers' protocols.

4.3.6 Flow cytometry

For cell signaling studies, cells were treated ± 2DG or OX for 1 hour and activated for 15 minutes. Cells were collected with 1.6% paraformaldehyde fixation and permeablized with methanol for p-ERK analysis. Cells were stained with anti-CD16/32

(clone 2.4G2, BD Pharmingen via BD Biosciences, San Jose, CA) and APC-anti-H/M pERK1/2 (clone MILAN8R, eBioscience, via Thermofischer, Waltham, MA) or the isotype control (APC mouse IgG1; eBioscience) at 2 μg/mL for 30 minutes at 4°C, and analyzed by flow cytometry with the FACsCelesta (BD Biosciences). The gating strategy used was doublet exclusion (FSC-A x FSC-H) and size and granularity (FSC x SSC). MFI was recorded for all samples.

For oxidative stress measures, cells were treated ± 2DG or OX for 1 hour then activated with IL-33 for 2 hours. Cells were then washed and re-suspended in Hank's buffered saline solution (HBSS) + 2',7' Diochlorofluorescin Diacetate (DCFH-DA , 5 μM, Millipore, Burlington, MA) ± 2DG or OX ± IL-33 for 30 minutes at 37°C. Cells were analyzed in the FITC channel by flow cytometry. The gating strategy used was doublet exclusion (FSC-A x FSC-H) and gating on size and granularity (FSC x SSC). MFI was recorded for all samples.

Following IL-33 induced peritonitis (described below), peritoneal lavage cells were collected, red blood cells were lysed, and rinsed pellets were stained with anti-CD16/32 (clone 2.4G2, BD Pharmingen), PE rat anti- mouse Ly6G (clone1A8, BD Pharmingen), APC-anti mouse CD45 (clone 30-F11, Biolegend) or the isotype controls PE rat IgG2a (BD Pharmingen) and APC rat IgG2b (Biolegend) all at 2μg/mL for 30 minutes at 4°C, and analyzed by flow cytometry with the FACsCelesta (BD Biosciences). The gating strategy used was doublet exclusion (FSC-A x FSC-H), size and granularity (FSC x SSC), lymphocytes (CD45+), and neutrophils (Ly6G++). Percent positive was reported from total leukocyte (CD45+) events.

4.3.7 Luciferase

BMMC were differentiated from NFκB-luc transgenic bone marrow as above. Following treatment ± 2DG or OX for 1 hour and IL-33 activation for 2 hours, cells were lysed and luciferase activity was measured with the Promega Luciferase Assay Substrate and Glomax 20/20 Luminometer (Promega, Madison, WI). Luciferase expression is reported relative to protein concentration (Pierce BCA Protein Assay Kit, Thermofisher, Waltham, MA) and normalized to the un-activated control.

4.3.8 *In vivo* studies

Mouse recombinant IL-33 for *in vivo* experiments was purchased from Biolegend (San Diego, CA). Age- and sex- matched groups of mice (~12 weeks old) were injected intraperitoneal (IP) with 1: 2-DG (1 g/kg, ~100 uL), sodium oxamate (15 mg/kg, ~100 μL) or PBS (100 uL) or 2: Metformin (100 mg/kg, ~100 μL) or PBS (100 μL) for 1 hour prior to IL-33. IL-33 was injected IP at 1 ug/mouse (100 μL) to elicit peritonitis and mice were sacrificed at 4 hours. Plasma cytokines were measured from cardiac puncture with ELISA and neutrophil recruitment was determined from peritoneal lavage cells run on flow cytometry as reported in 2.7.

4.3.9 Statistical analyses

Glucose uptake, lactate export, enzyme expression, and AMPK phosphorylation (Figure 4-2, 4-3, 4-8, and 4-12; comparison of two groups) were analyzed by t-test. The remainder of the data (3+ groups) were analyzed by a one-way analysis of variance (ANOVA) to detect overall differences between groups. With F-statistic significance,

Tukey's multiple comparisons were used as post hoc tests to determine which conditions were significantly different from the control. GraphPad Prism software was used for all statistical analyses. Data are expressed as mean ± standard error of mean (SEM) with statistical significance: *p< .05, **p< .01, ***p< .001, ****p< .0001, NSD= no significant difference.

4.4 Results

4.4.1 IL-33 activation induces glycolysis

To determine the effects of IL-33 on mast cell metabolism, we analyzed extracellular acidification rate (ECAR) and proton production rate (PPR) as indicators of H^+ production and a surrogate for glycolytic rate. Oxygen consumption rate (OCR) was used as an indicator of mitochondrial OX PHOS. Bone marrow derived mast cells (BMMC) were measured at baseline, following IL-3/SCF addition, and following IL-33 activation (Figure 4-1). ECAR, OCR, and OX PHOS were all significantly elevated following growth factor addition and IL-33 activation. We confirmed the induction of glycolysis by IL-33 with measures of glucose uptake and lactate export[122,248]. Following IL-33 activation for 16 hours, glucose uptake and lactate export were calculated using concentrations in the cell supernatants from the IL-33 activated and control groups. IL-33 activation significantly increased glucose uptake and lactate export (Figure 4-2), supporting enhanced glycolysis as measured by ECAR and PPR.

Lipopolysaccharide (LPS) is an innate activation signal that shares downstream signaling cascades with IL-33[50]. LPS has been shown to increase immune cell glycolysis, similar to IL-33 effects we observed in Figure 4-1 and 4-2. LPS effects occur in 2 stages:

a rapidly increased glycolytic enzyme activity and inhibition of OX PHOS, and a prolonged increase in enzyme expression[86,112,248]. To determine if IL-33 has prolonged effects, BMMC were activated for 8 hours ± IL-33 and protein expression was determined by Western blotting of cell lysates. IL-33 significantly enhanced hexokinase (HK)2 and pyruvate kinase (PK)M2 expression (Figure 4-3).

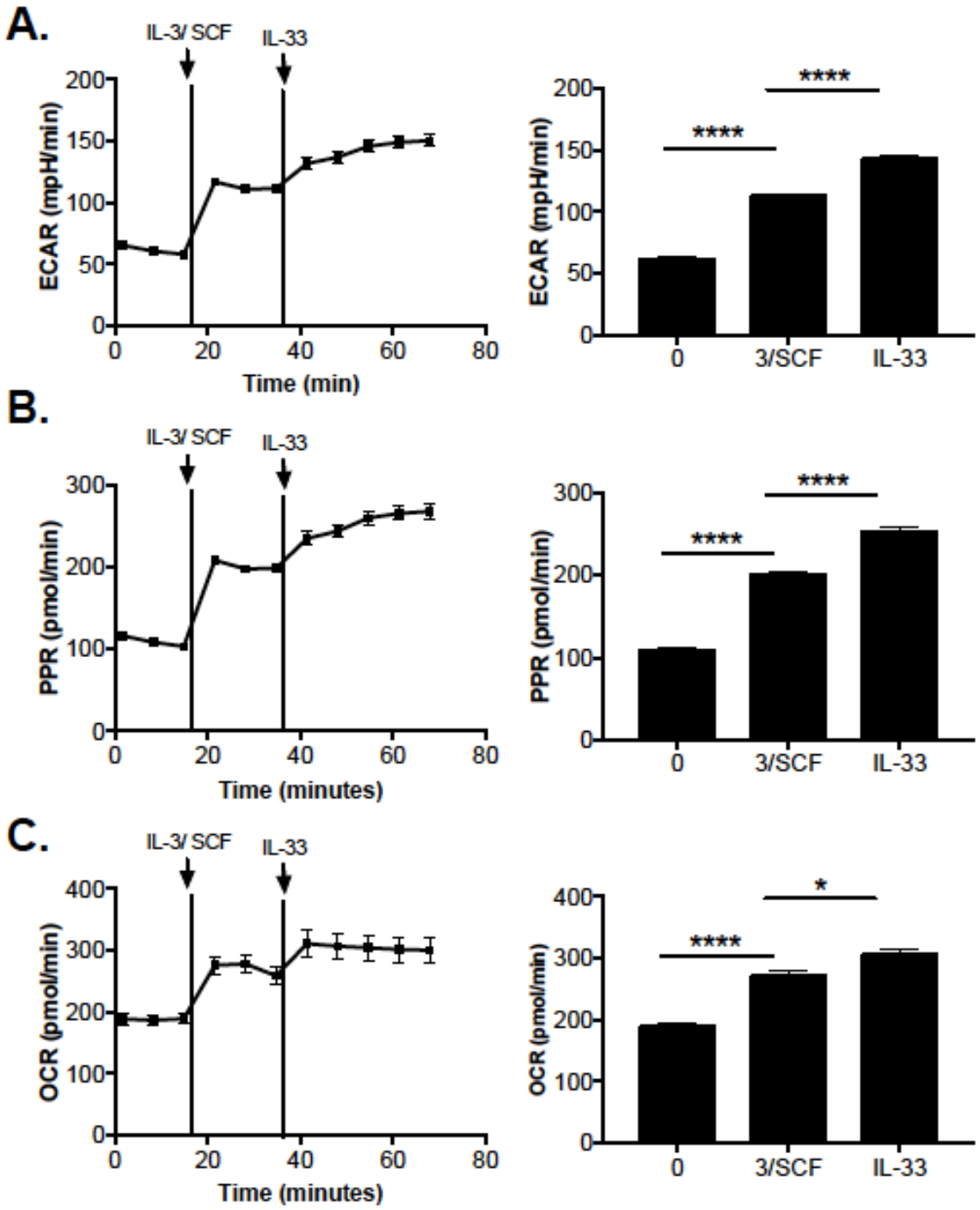


Figure 4-1. IL-33 activation induces glycolysis and OX PHOS. Seahorse metabolic analysis was used to measure basal metabolism in BMMC, followed by IL-3/SCF (10 ng/mL) and IL-33 (100 ng/mL). A) ECAR B) PPR and C) OCR measurements averaged over time are shown. Data are representative of 3 independent populations measured in duplicate, representative of three independent experiments *$p < .05$, ****$p < .0001$

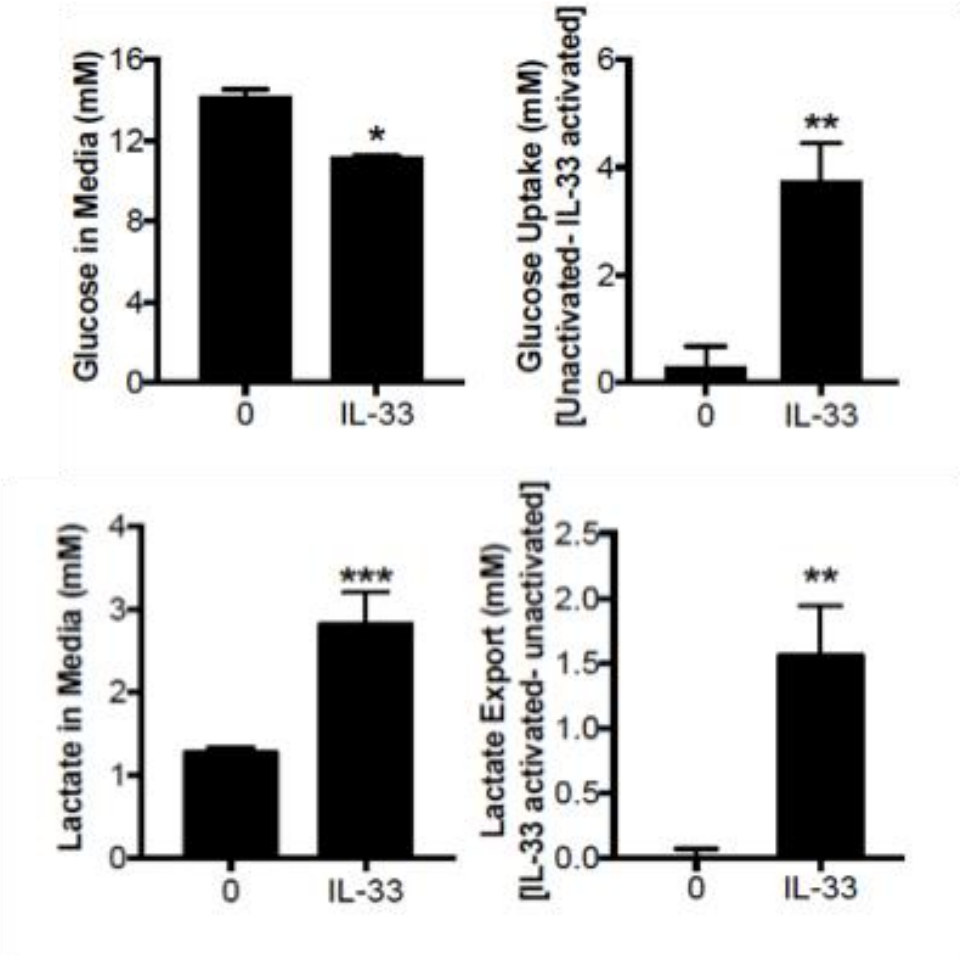


Figure 4-2. IL-33 activation induces glucose uptake and lactate export. BMMC were activated for 16 hours ± IL-33 (50 ng/mL). Glucose and lactate were measured in the cell supernatant by colorimetric assay. Uptake and export were determined by the difference between activated and unactivated groups. Data are means ± SEM of 3 populations, representative of 3 independent experiments. *$p < .05$, **$p < .01$, ***$p < .001$

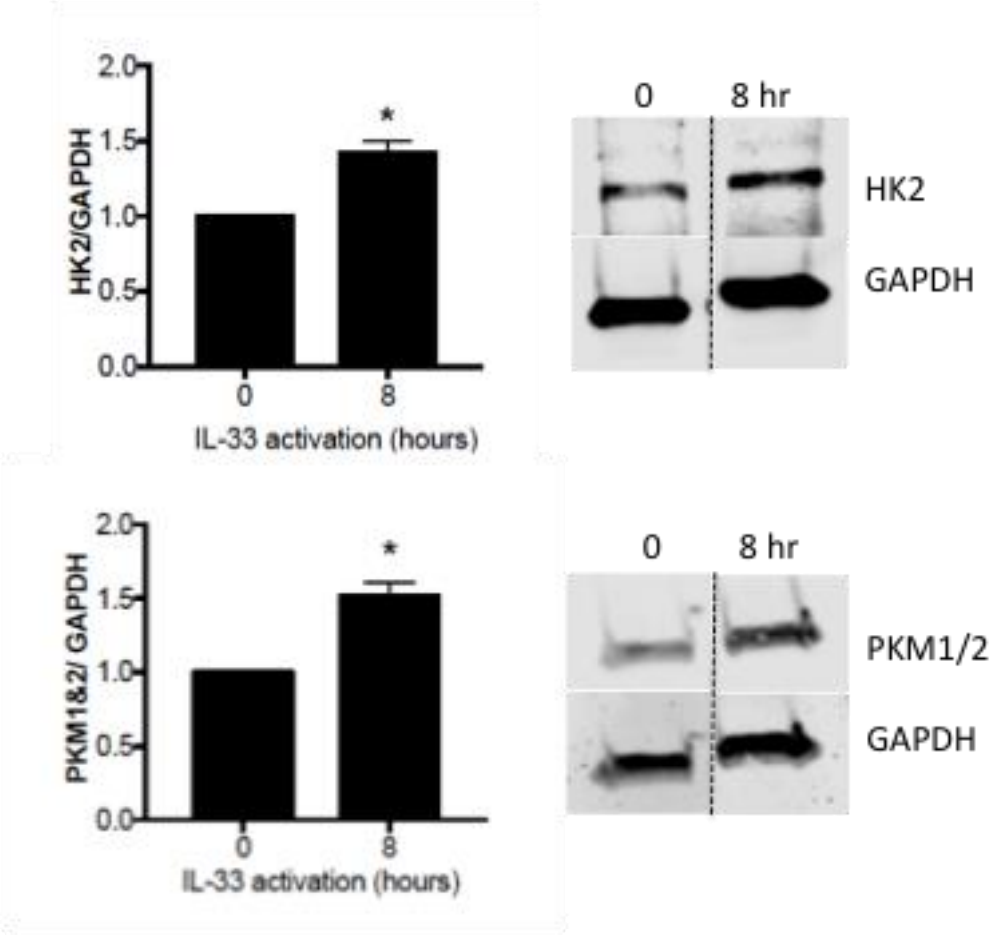


Figure 4-3. IL-33 activation increases hexokinase and pyruvate kinase expression. BMMC were activated with IL-33 (100 ng/mL) for 8 hours. Lysates were analyzed by western blot for hexokinase and pyruvate kinase. Data are representative of 3 independent populations *$p < .05$, **$p < .01$, ***$p < .001$

4.4.2 Glycolytic inhibition suppresses cytokine production following IL-33 activation

To determine the importance of glycolysis for IL-33-mediated mast cell function, we employed chemical antagonists. BMMC were treated with the glycolytic inhibitors 2DG (1 mM) or OX (20 mM) for 1 hour prior to IL-33 activation for 16 hours. There was no detectable change in cell viability at these doses over the duration of the experiment (Data not shown). 2DG and OX significantly suppressed IL-33-induced IL-6, TNF, MCP-1, and MIP-1α (Figure 4-4). Importantly, BMMC treated for 1 hour with etomoxir (Eto, 200 μM), inhibiting fatty acid oxidation (FAO), and rotenone+antimycin A (1 μM), inhibiting complex I and II of the ETC, had no effect on IL-6, TNF, and MCP-1 production (Figure 4-5). Interestingly, these inhibitors did suppress MIP-1α production, suggesting differential transcriptional or post-transcriptional regulation compared with the other cytokines measured (Figure 4-5). Additionally, the MIP-1α data provide evidence that doses were sufficient to induce cellular changes. Rotentone and antimycin A were used at the highest dose at which they did not increase cell death over 24 hours, similar to concentrations published to suppress IgE XL in mast cells[99]. Together with data from Figures 4-1 and 4-2, these data suggest that the increase in glycolysis observed following IL-33 activation is functionally important for cytokine production in mast cells.

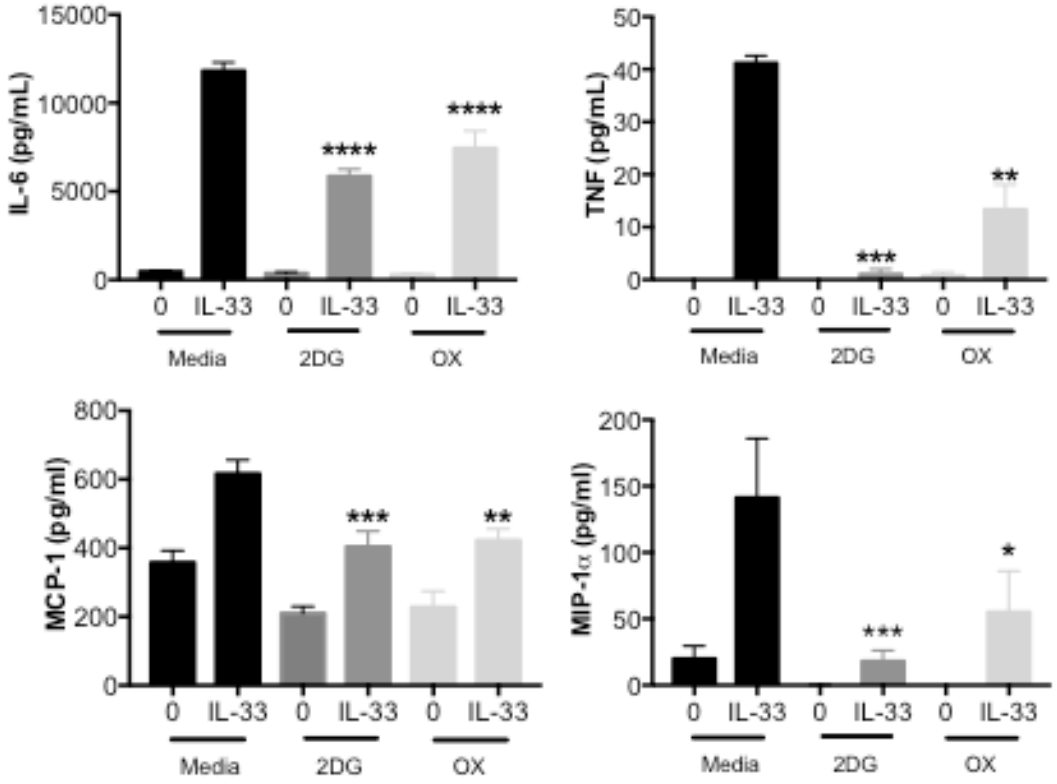


Figure 4-4. Glycolytic inhibition suppresses cytokine production following IL-33 activation. BMMC were treated with 2DG (1mM) or OX (20 mM) for 1 hour prior to IL-33 (50 ng/mL) activation for 16 hours. Cytokines were measured in supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$

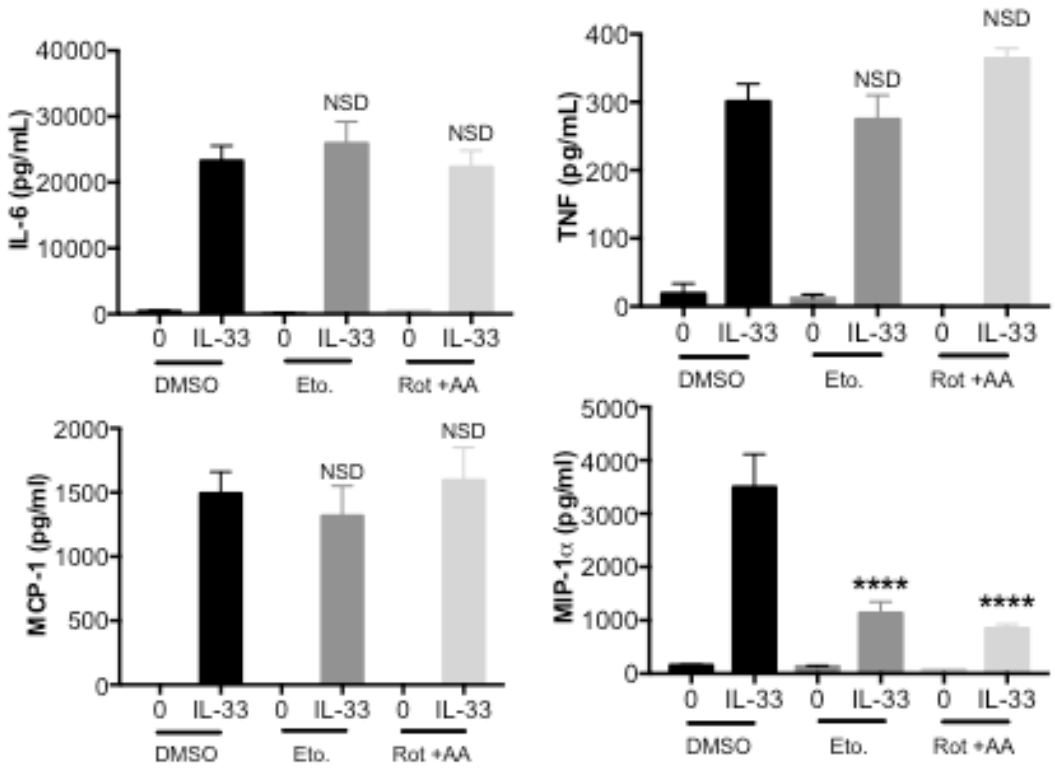


Figure 4-5. OX PHOS inhibition does not effect cytokine production following IL-33 activation. BMMC were treated with DMSO (vehicle control), etomoxir (Eto, 200 μM), or rotenone+antimycin A (Rot+AA, 1 μM) for 1 hour prior to IL-33 (50 ng/mL) activation for 16 hours. Cytokines were measured in supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$, NSD= no significant difference

4.4.3 Glycolytic inhibition suppresses p-ERK and NFκB transcription

While these data suggest that glycolytic inhibition affects cytokine release following IL-33, it is unclear if early receptor-signaling events are similarly affected. Therefore, p-ERK and NFκB transcriptional activity were measured. BMMC were treated with the glycolytic inhibitors 2DG and OX for 1 hour prior to IL-33 activation for 15 minutes. Phosphorylation events were determined by flow cytometry. Treatment with 2DG and OX significantly suppressed p-ERK following IL-33 activation (Figure 4-6). Additionally NFκB-luc BMMC bearing a luciferase gene driven by two copies of the NF-κB regulatory element were also treated with 2DG and OX for 1 hour prior to IL-33 activation for 2 hours. Luciferase expression was measured as a surrogate for NFκB-induced transcription. Similar to p-ERK, 2DG and OX significantly suppressed luciferase expression (Figure 4-7). Together, these data suggest that inhibition of glycolysis suppesses IL-33-induced cell signaling cascades.

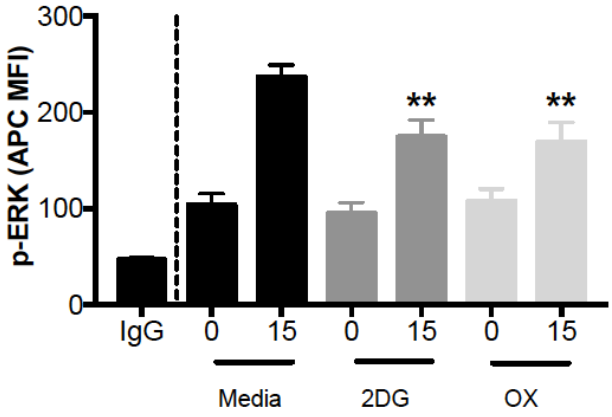


Figure 4-6. Glycolytic inhibition suppresses p-ERK. BMMC were treated with 2DG (1mM) or OX (20 mM) for 1 hour and activated for 0 or 15 min with IL-33 (100 ng/mL). p-ERK was analyzed by phospho-flow. Data are means ± SEM of 6 populations run in duplicate, representative of 3 independent experiments. ***$p < .001$, ****$p < .0001$

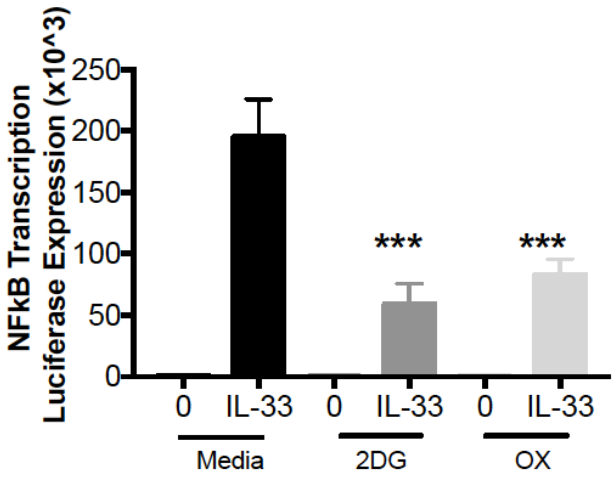


Figure 4-7. Glycolytic inhibition suppresses NFκB transcription. NFκB-luc transgenic BMMC were treated with 2DG (1mM) or OX (20 mM) for 1 hour and activated for 2 hours with IL-33 (100 ng/mL). Luciferase activity was measured with the Promega Luciferase Assay Substrate and Glomax Luminometer and normalized to the unactivated media control. Data are means ± SEM of 3 populations run in duplicate, representative of 3 independent experiments. ***$p < .001$

4.4.4 Exogenous ATP restores cytokine production following glycolytic inhibition

ATP is required for kinase activity, tRNA synthetase activity, ion transport, and chromatin remodeling, all of which play a role in cell signaling and cytokine production. Glycolysis can be favored to provide a rapid source of ATP and/or to provide pentose phosophate pathway- and Kreb's cycle- intermediates for nucleotide, amino acid, and lipid synthesis[86]. To determine if ATP alone was sufficient to restore cytokine production following glycolytic blockade, BMMC were treated for 1 hour with 2DG and OX prior to activation with IL-33 ± ATP (10 μM) for 16 hours. While 2DG and OX treatment significantly suppressed cytokine production similar to Figure 4-2, these inhibitors had no effect when ATP was added (Figure 4-8). Together, these data suggest that IL-33-induced glycolysis yields ATP that is critical for inflammatory function.

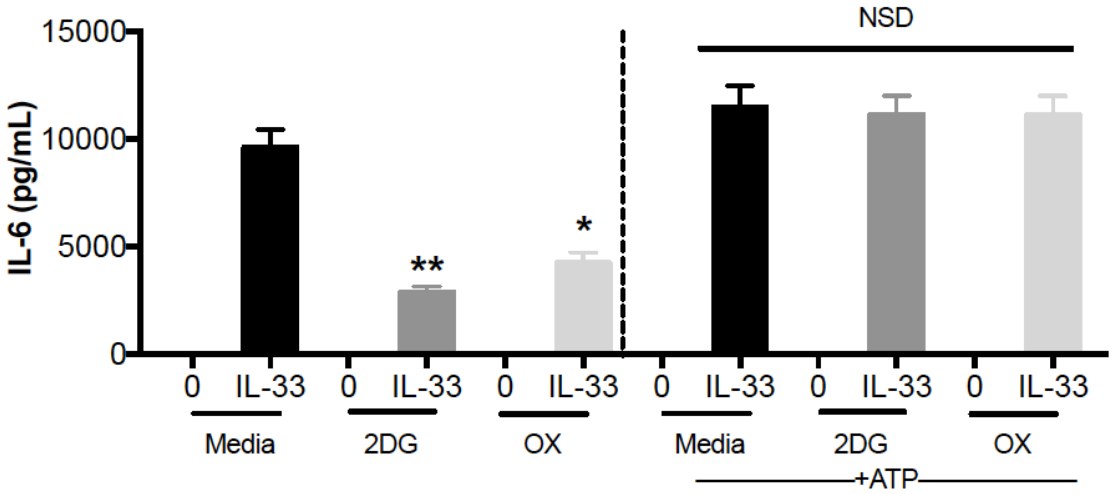


Figure 4-8. Increased ATP availability restores cytokine production following glycolytic inhibition. BMMC treated with 2DG (1mM) or OX (20 mM) for 1 hour and activated for 16 hours with IL-33 (50 ng/mL) and/or ATP (10 μM). IL-6 was measured in cell supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. *$p < .05$, **$p < .01$, NSD= no significant difference

4.4.5 Glycolytic inhibition suppresses ROS production

Increasing glycolysis is well known to increase the conversion of glucose to ATP and lactate, however glycolysis also effectively increases ROS production by the pentose phosphate pathway[86,249]. Additionally, reduced ETC-mediated ATP production increases ROS production in the mitochondria[250,251].We have previously published that IL-33 induced-cytokine production is suppressed using the antioxidant n-acetylcysteine[77], however ROS production following IL-33 activation has not been investigated. Therefore, we examined the effect of glycolytic inhibition on ROS production. BMMC were treated with 2DG or OX for 1 hour and activated with IL-33 for 2 hours. IL-33 significantly increased DCFH-DA fluorescence, a measure of ROS production. 2DG and OX suppressed DCFH-DA fluorescence, suggesting that IL-33-induced glycolysis is required for ROS production (Figure 4-9).

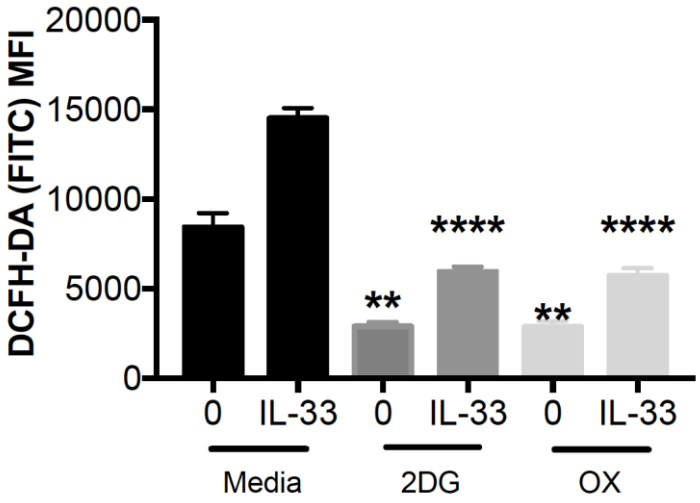


Figure 4-9. Glycolytic inhibition suppresses ROS production. BMMC were treated with 2DG (1mM) or OX (20 mM) for 1 hour and activated for 2 hour with IL-33 (50 ng/mL). Oxidative stress was analyzed by DCFH-DA fluorescence by flow cytometry. Data are means ± SEM of 3 populations run in duplicate, representative of 3 independent experiments. $^{**}p < .01$, $^{****}p < .0001$

4.4.6 Glycolytic inhibitors suppress IL-33 induced neutrophil recruitment and cytokine production *in vivo*

To test the importance of IL-33-induced glycolysis *in vivo*, we used a model of IL-33-induced peritonitis in which neutrophil recruitment was shown to be mast cell dependent[62]. As shown in the schematic (Figure 4-10 A), mice were injected intraperitoneally (IP) with 2DG (0.75 g/kg), OX (15 mg/kg), or PBS (control). After 1 hour, mice were injected IP with IL-33 (1 μg) or PBS. After 4 hours, mice were sacrificed for peritoneal lavage and cardiac puncture. 2DG and OX significantly suppressed neutrophil (Ly6G++) recruitment in the peritoneum compared with the PBS control group (Figure 4-10 B). Similarly, 2DG and OX significantly suppressed plasma IL-6 and MIP-1α (Figure 4-10 C). These data suggest the suppressive effects of glycolytic inhibition extend to IL-33 activation in vivo.

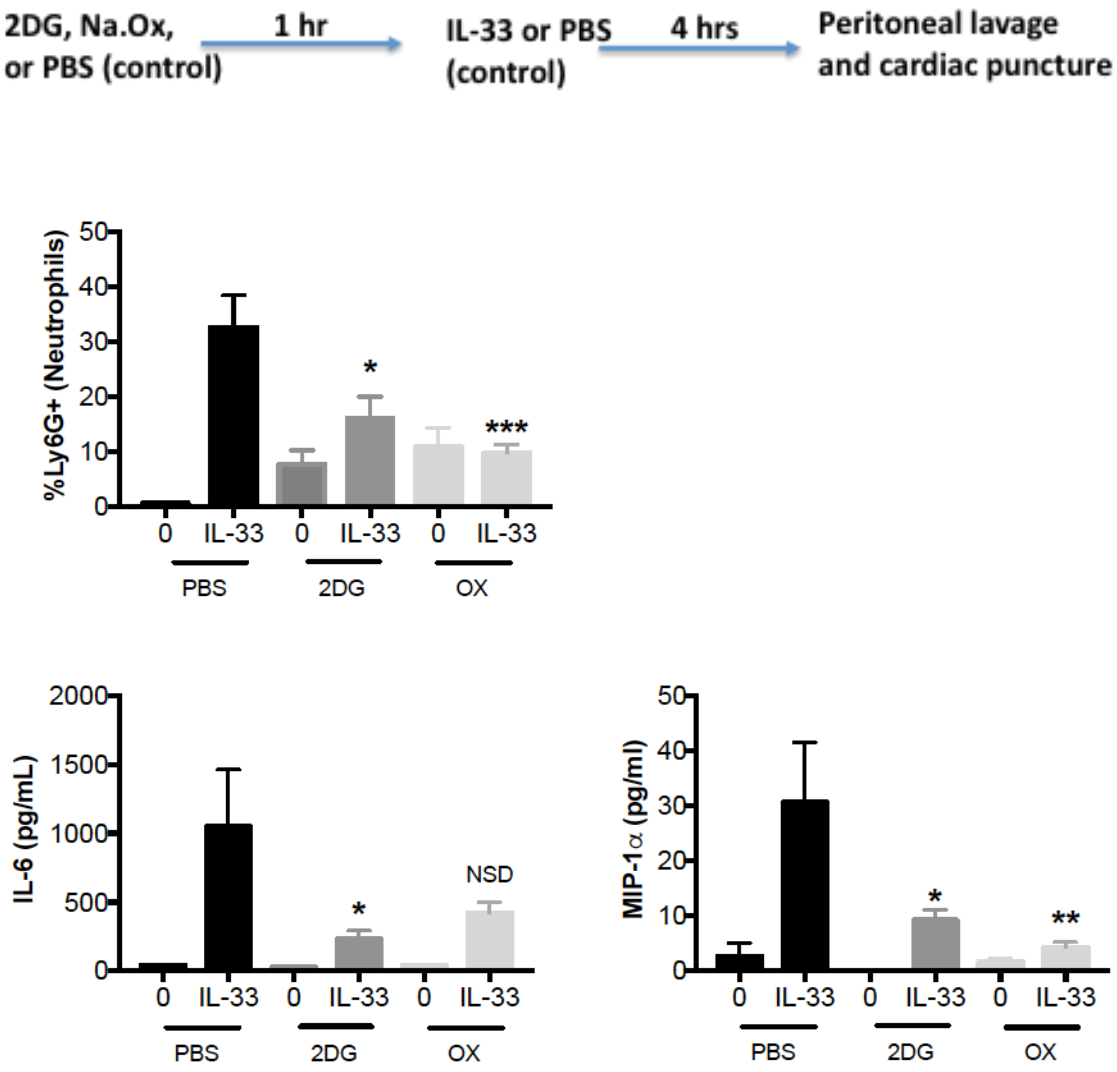


Figure 4-10. Glycolytic inhibitors suppress IL-33 induced neutrophil recruitment and cytokine production in vivo. A) Schematic diagram: C57BL6 males and females were injected intraperitoneal (IP) with 2DG (0.75 g/kg), OX (15 mg/kg), or PBS. After 1 hour, mice were injected IP with IL-33 (1 μg) or PBS. All groups were sacrificed after 4 hours. B) Peritoneal lavage was used to collect cell infiltrates and analyze neutrophil (Ly6G+) recruitment via flow cytometry. C) Cardiac puncture was used to collect plasma for cytokine analysis via ELISA. Data are means ± SEM of 2 mice/control group and 5 mice/peritonitis group run in triplicate (flow cytometry) and duplicate (ELISA), representative of 2 independent experiments. *$p < .05$, **$p < .01$, ***$p < .001$, NSD= no significant difference

4.4.7 AMPK agonists suppress cytokine production following IL-33 activation

While 2DG has been used in a phase I trial for cancer treatment, the cMAX reported for the recommended phase II dose of 2DG was 450 μM (for 45 mg/kg)[252], well under the doses used in this study. Fatigue, dizziness, and dose dependent cardiac QTc prolongation were also observed in a few patients[252]. Similarly, OX is known to have poor cell membrane permeability and concentrations sufficient for LDH effects cannot be reached *in vivo*[253]. Therefore, we became interested in AMPK as another way to target glycolysis in IL-33-related diseases.

AMPK is a protein well known for its role in energy sensing, activated in response to fasting and exercise. AMPK switches the cell from anabolic pathways to catabolic pathways, utilizing all potential energy in the form of glucose and lipids by both glycolysis and OX PHOS in liver, kidney, and skeletal muscle[86]. Interestingly, AMPK increases OX PHOS while inhibiting glycolysis in immune cells. This fits with the role of AMPK, since glycolysis is considered anabolic, increasing mitochondrial intermediates use in generating protein and lipid signaling messengers needed for proliferation[86,110]. AMPK phosphorylation is reduced following LPS-activation[110,254], and we see that this pathway is similarly affected by IL-33 (Figure 4-11). Interesting, IL-33 activation also suppressed total AMPK expression. These data indciate that IL-33 activation limits AMPK function, suggesting that increasing AMPK activity might inhibit IL-33 effects.

Metformin is an FDA-approved AMPK activator, widely prescribed for the treatment of diabetes. BMMC were treated with metformin at physiological doses (10, 50, 100 μM) for 24 hours before activation with IL-33 for 16 hours[255,256]. For all doses, we observed significant suppression with metformin treatment (4-12). Similar

suppression was observed with 1 hour treatment, albeit at higher doses (data not shown). To ensure specificity, BMMC were treated with A799662 (a specific AMPK agonist, 100 μM) or SRT1720 (a specific agonist for SIRT1, a protein downstream of AMPK, 5 μM) for 1 hour prior to activation with IL-33 for 16 hours. Supporting our metformin data, both agonists significantly suppressed IL-6 and TNF production (4-13). These data suggest that AMPK activation can suppress IL-33-induced mast cell activation.

To confirm that the effects of metformin were due to reduced glycolysis and ATP availability, BMMC treated with metformin (100 μM) for 24 hours were activated with IL-33 ± ATP (10 μM) for 16 hours. ATP reversed the suppression by metformin (Figure 4-14). These results suggest that suppressing glycolysis and ATP production by increasing AMPK function is an effective way to limit cytokine production following IL-33 activation.

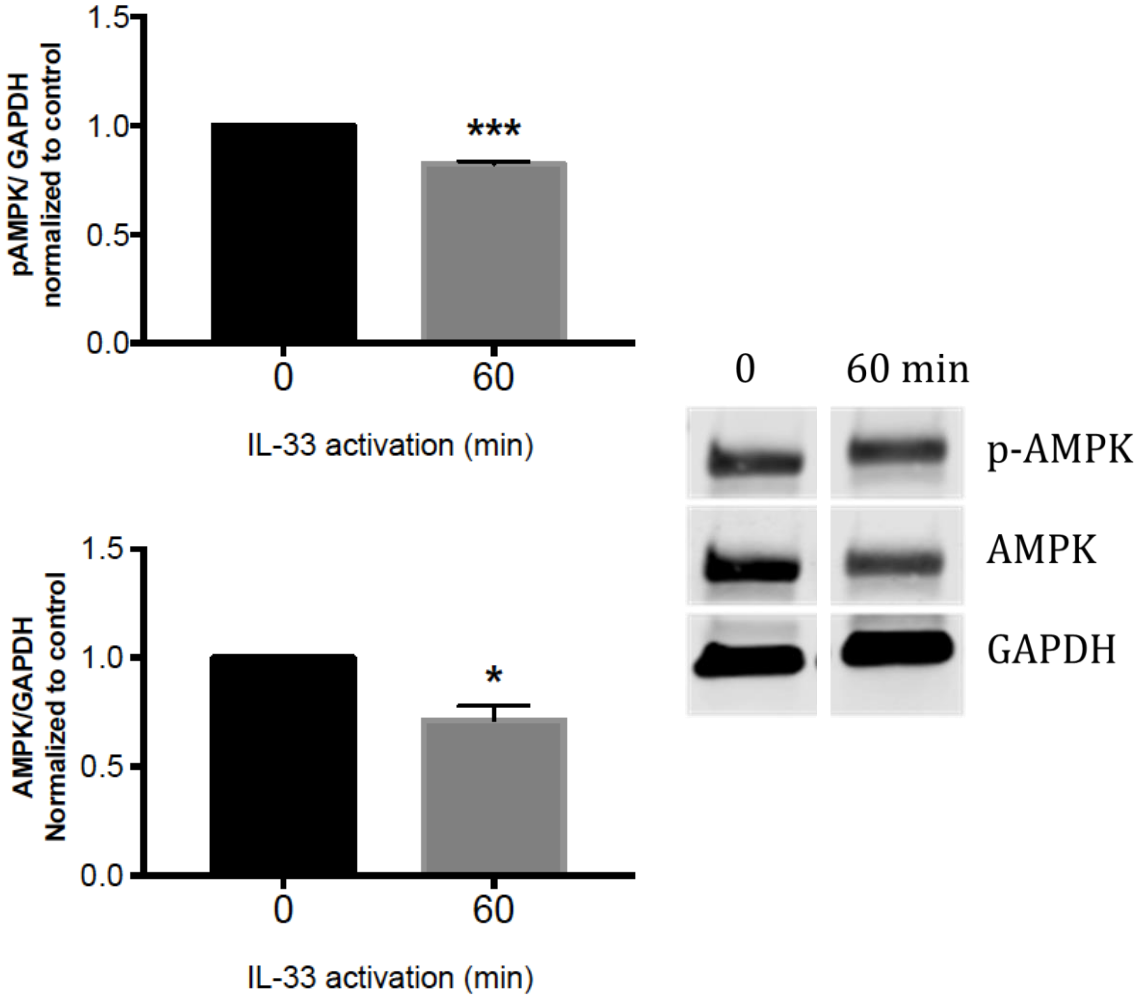


Figure 4-11: IL-33 activation reduces AMPK activation. BMMC were activated for 1 hour ± IL-33 (100 ng/mL). p-AMPK, AMPK, and GAPDH were determined by Western blot of cell lysates. Data are means ± SEM of three independent populations. Blot is representative of one population *$p < .05$, ***$p < .001$

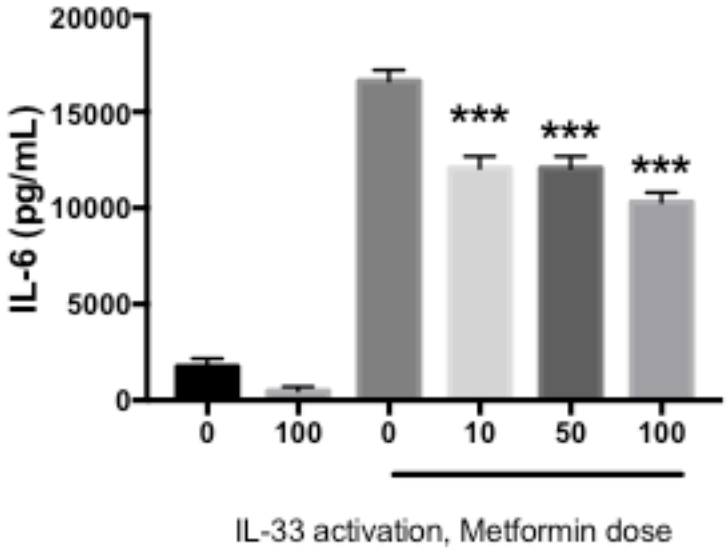


Figure 4-12. Low dose metformin suppresses cytokine production following IL-33 activation. BMMC were treated ± metformin (10, 50, 100 μM) for 24 hours and activated with IL-33 (50 ng/mL) for 16 hours. Cytokines were measured in supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$

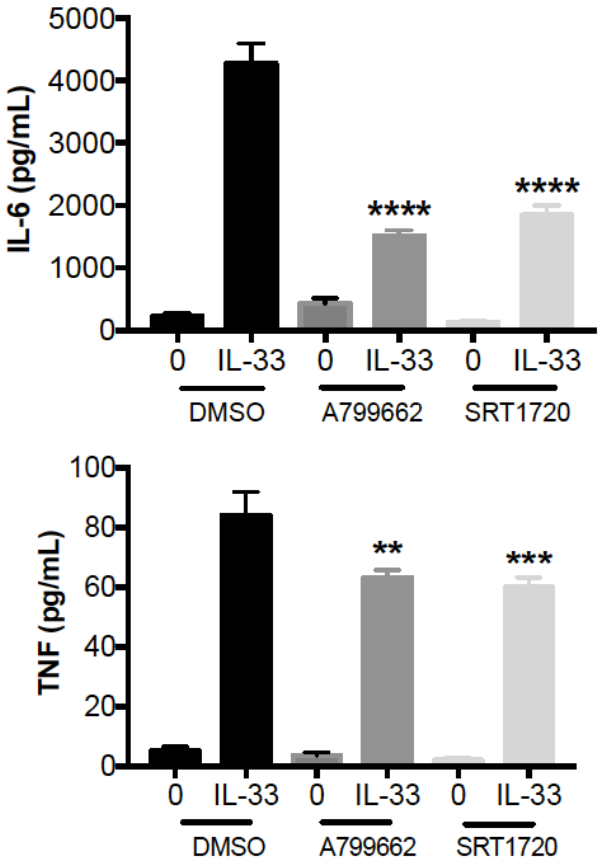


Figure 4-13. AMPK and SIRT1 agonists suppress cytokine production following IL-33 activation. BMMC were treated with DMSO (vehicle control), A799662 (AMPK agonist, 100 μM) or SRT1720 (SIRT1 agonist, 5 μM) for 1 hour and activated with IL-33 (50 ng/mL) for 16 hours. Cytokines were measured in supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$

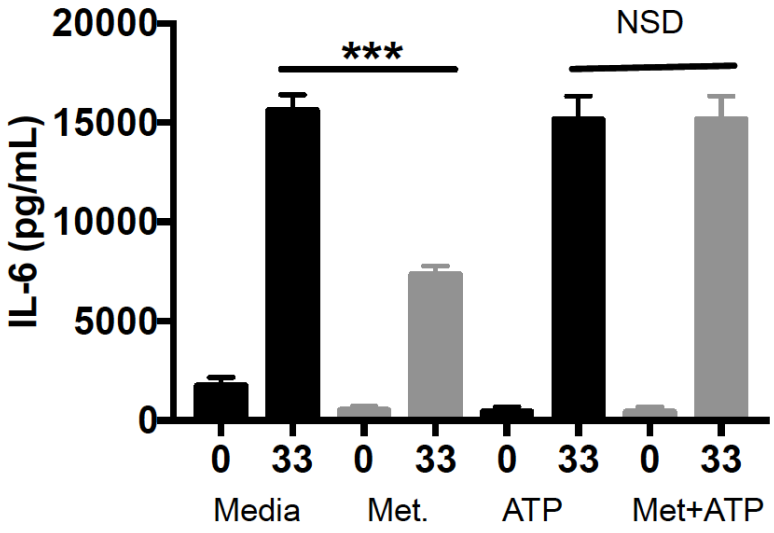
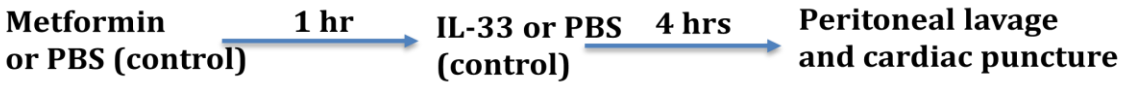


Figure 4-14. ATP reverses metformin suppression. BMMC were treated ± metformin (Met, 100 μM) for 1 hour and activated with IL-33 (50 ng/mL) ± ATP (10 μM) for 16 hours. Cytokines were measured in supernatant by ELISA. Data are means ± SEM of 3 populations run in triplicate, representative of 3 independent experiments. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$

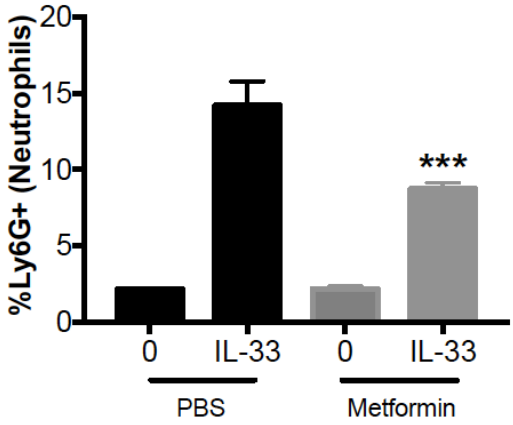
4.4.8 Metformin suppress IL-33 induced neutrophil recruitment and cytokine production in vivo

To establish proof of principle *in vivo*, metformin treatment was used with the IL-33-induced peritonitis model. As shown in the schematic (Figure 4-15 A), both mice received metformin (IP, 100mg/kg, ~100 μL) or PBS (control, 100 μL). After 1 hour, mice were injected IP with IL-33 (1 ug, 100 μL) or PBS (100 μL). After 4 hours, mice were sacrificed for peritoneal lavage and cardiac puncture. Metformin significantly suppressed neutrophil (Ly6G++) recruitment into the peritoneum compared with the PBS control group (Figure 4-15 B). Furthermore, metformin significantly suppressed plasma MCP-1 (Figure 4-15 C). These data support the theory that indirectly targeting glycolysis with an AMPK agonist can suppress IL-33-mediated inflammation *in vivo*.

A.



B.



C.

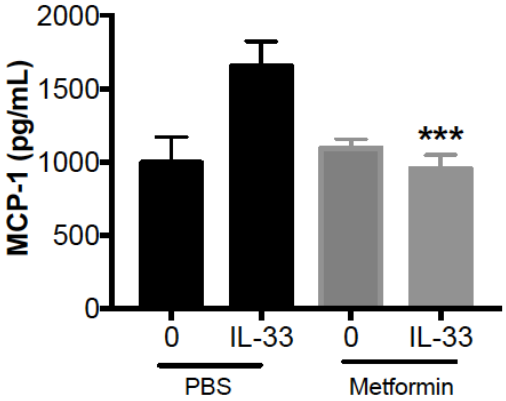


Figure 4-15. Metformin suppress IL-33 induced neutrophil recruitment and cytokine production in vivo. A) Schematic diagram: C57BL6 males and females were injected intraperitoneal (IP) with metformin (100 mg/kg) or PBS. After 1 hour, mice were injected IP with IL-33 (1 μg) or PBS. All groups were sacrificed after 4 hours. B) Peritoneal lavage was used to collect cell infiltrates and analyze neutrophil (Ly6G+) recruitment via flow cytometry. C) Cardiac puncture was used to collect plasma for cytokine analysis via ELISA. Data are means ± SEM of 3 mice/ control group and 8 mice/ peritonitis group run in triplicate (flow cytometry) or duplicate (ELISA), representative of 2 independent experiments. ***$p < .001$

4.5 Discussion

Immune cell metabolism is closely linked to phenotype and effector functions. While T cell and macrophage metabolism have been highly studied over the past decade, mast cell metabolism and IL-33-mediated activation have received little attention. This study is the first to report that IL-33 increases glycolysis, generating ATP that is necessay and sufficient for inflammatory cytokine production by mast cells (Figure 4-16). Targeting glycolytic ATP production by inhibiting glycolysis with 2-DG and OX, or by activating AMPK with metformin were sufficient to reduce IL-33-mediated cytokine production *in vitro* and *in vivo*. These data advance our understanding of IL-33 activation and suggest that regulators of glycolysis, like AMPK, may be potential targets for treating inflammatory diseases mediated by IL-33.

Previous to this report, only one paper using small-cell lung cancer (SCLC) cells reported metabolic effects of IL-33, showing an increase in glucose uptake and lactate export by IL-33[257]. We are the first to show similar effects in immune cells, showing an increase in glycolysis in BMMC with IL-33 activation. Additionally, we observed a slight increase in OX PHOS. We hypothesize that IL-33 signaling in other $ST2^+$ cells, including Th2 and ILC2 cells, would also require glycolysis for optimal function, however this remains to be determined. We observed early increases in glycolysis immediately with IL-33 activation using the Seahorse analyzer, which may be attributed to increased glycolytic enzyme activity. Additionally, metabolic enzyme expression was elevated at 8 hours and glucose uptake and lactate export remained different at 16 hours. We saw an increase in HK2 and PKM1/2 expression, while Wang et al. observed an increase in

Glut1 surface expression in SCLC cells following IL-33 activation[257], suggesting that IL-33 may regulate the expression of many different proteins important for glycolysis.

Our data suggest a functional role for enhanced glycolysis following IL-33 activation. In BMMC, the glycolytic inhibitors 2DG and OX suppressed ERK signaling, NFκB transcription, and cytokine production. This is supported in other systems, as 2DG and OX can block IgE XL-induced cytokine secretion in rat mast cells[95], cytokine mRNA and protein secretion, cytolytic activity, and cell cycle progression in CD8 T cells[258], and ERK phosphorylation in SCLC cells[259]. Furthermore, our data show that 2DG and OX suppressed both cytokine production and neutrophil recruitment in a model of IL-33-induced peritonitis *in vivo*. Together, these data suggest that glycolysis plays a critical role in IL-33-mediated inflammation.

ATP, the primary product of glycolysis, provides a phosphate group and energy for kinases and enzymes involved in signaling and transcription. Our data show that exogenous ATP can restore IL-33-induced cytokine production that in the presence of glycolytic inhibitors. In fact, increased ATP availability also limited the increase in glycolysis following IL-33 activation. These data support the hypothesis that BMMC require glycolytic ATP production for IL-33-induced activation and suggest that glycolytic blockade is sufficient to suppress IL-33 activation *in vivo*.

In addition to ATP, our data suggest that glycolysis contributes to ROS production following IL-33 activation. As the flux of glucose through glycolysis increases, glucose 6-phosphate is shuttled through the pentose phosphate pathway where NADPH oxidase increases ROS production[86,249]. Additionally, ROS production is increased in the ETC when glycolysis is the primary source of ATP production[250,251]. Our

data show that ROS production increases following IL-33 activation, which can be suppressed by glycolytic blockade. ROS production is typically considered pro-inflammatory, as oxidation can increase phosphorylation at tyrosine resides and transcription factor activity[260,261]. Furthermore, we previously published that the antioxidant n-acetylcysteine suppresses cytokine production following IL-33 activation[77]. Together, these data suggest that IL-33 increases glycolysis, ATP, and ROS production, which augment mast cell cytokine production and effector function.

While 2DG and OX have been used in humans, there is little potential for clinical use due to the dosage required and side effects. Our data suggest that targeting AMPK, an upstream mediator of cell metabolism, may be effective in IL-33-related diseases. Until now, there has been no evidence to link AMPK to IL-33 in immune cells, although systemic administration of an anti-ST2 antibody increased p-AMPK in the renal parenchyma of mice[262]. We show that p-AMPK is slightly but significantly reduced by IL-33 signaling, supporting data reported for other pro-inflammatory signals like LPS[110,254]. This inhibition was largely explained by reduced AMPK expression. Additionally, activating AMPK with metformin or with the specific agonist A799662 suppressed IL-33-induced cytokine production. Furthermore, activating SIRT1, a deaceytlase downstream of AMPK known to play a suppressive role in signal transduction and glycolysis[263–265], similarly suppressed cytokine production. This provides another possible clinical target for modulating IL-33-induced inflammatory responses and suggests that other Sirtuin family members should be studied as a target in IL-33-related disease. ATP was able to reverse metformin effects, suggesting that inhibiting glycolytic ATP production is the primary mechanism of action. With

metformin use *in vivo*, we also provide proof of principle for its use to suppress cytokine production as well as neutrophil recruitment in IL-33-related pathologies. These data support the idea that metformin provides anti-inflammatory effects beyond lowering blood glucose, and suggests that AMPK is a reasonable target for suppressing IL-33-mediated cytokine production and effector functions *in vivo*.

Interestingly, IL-33 increased both glycolysis and OX PHOS in mast cells, similar to IgE XL. However, blocking OX PHOS did not influence cytokine production, in contrast to the reported with IgE XL[99]. Larry Kane's group observed that rotenone (blocking complex I) suppressed IL-6 production and degranulation in IgE XL[99], yet in our studies with IL-33, rotenone and antimycin A (blocking both complex I and complex III) had no effect on IL-6, TNF, or MCP-1. The dose of rotenone (1 μM) was the same in each study. These studies suggest important differences regarding the use and control of energy pathways downstream of IgE and IL-33 signaling cascades.

Future work should examine the signaling mechanisms that are directly responsible for changes in metabolism and cytokine transcriptional changes. Transcription of both glycolytic enzymes and cytokines are linked to HIF-1α following LPS activation. TLR4 signaling can induce NADPH oxidase activity and ROS production, which stabilizes HIF-1a[266], and ASK1, p38, and ERK signaling can also contributes to HIF-1a accumulation[267–269], which suggests that similar pathways may play a role in IL-33 mediated changes. Furthermore, glycolysis induction is known to provide intermediates for lipid synthesis and histone acetylation[86,92], in addition to generating ATP and ROS. The importance of these intermediates should be examined in the context of IL-33 activation.

This work emphasizes the importance of glucose metabolism in mast cell function, supporting recent publications by Ehud Razin and Larry Kane's labs which examine IgE activation of mast cells[98,99]. IL-33 activation increases glycolysis to provide ATP and ROS for optimal receptor signaling, cytokine production, and effector functions. Direct glycolytic blockade and AMPK activation were sufficient to reduce cytokine production both *in vitro* and *in vivo*. Together, these data advance our understanding of IL-33 activation and suggest that AMPK and glycolysis may be potential targets for treating IL-33 mediated diseases.

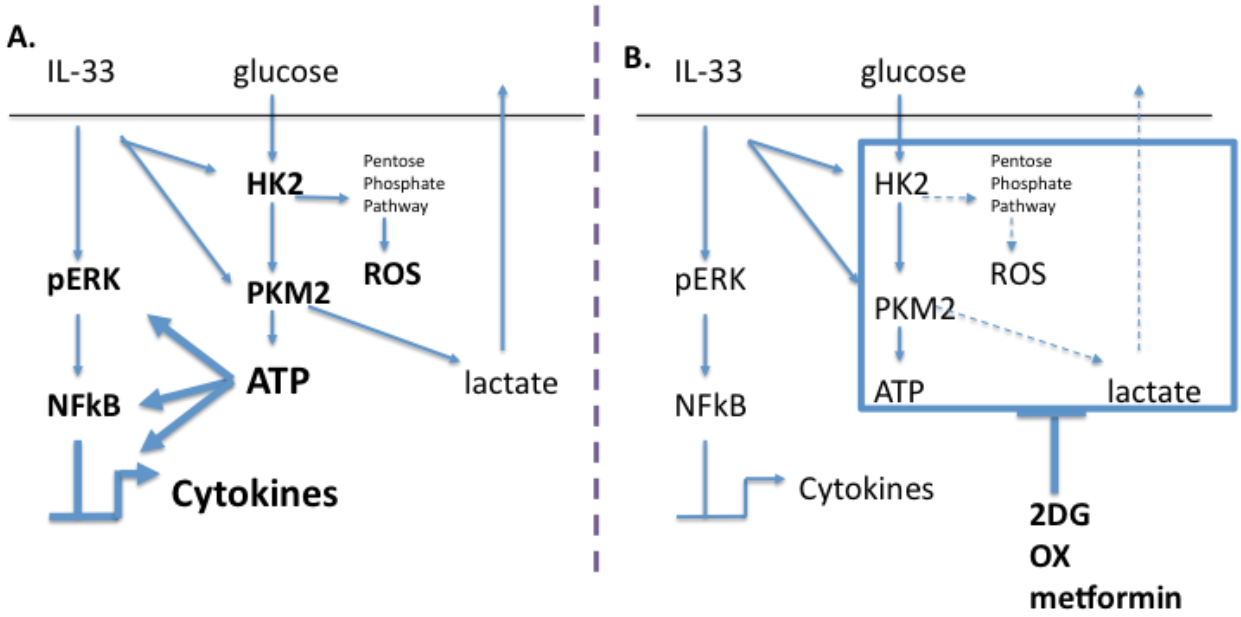


Figure 4-16. Schematic Diagram: A) IL-33 activation increases glycolysis to make ATP available for increased cell signaling and cytokine production. B) Glycolytic blockade by 2DG, OX, or metformin reduce ATP availability, effectively suppressing cell signaling and cytokine production in vitro and in vivo.

4.6 Acknowledgements

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Chapter 5: Conclusions and future directions

Aligning with the work that has been published in other immune cell types, it is clear that cellular metabolism plays an important role in mast cell function. Optimal cellular signaling and cytokine production following IL-33 and LPS activation of mast cells requires ATP production, which can be inhibited by glycolytic inhibitors or lactic acid. Future work should further examine the signaling mechanisms that are directly responsible for changes in metabolism and cytokine transcriptional changes downstream of each receptor. Potential mechanisms of interest include signaling proteins known to play a role in metabolic function and inflammation include mTOR, AMPK, Akt, HIF1, and myc[180,181]. Additionally, glycolysis is primarily utilized in other immune cell subsets during activation to provide metabolic intermediates for lipid synthesis and histone acetylation[86,92]. Blocking or increasing the expression of these intermediates may help to clarify the intermediates most important for mast cell effector functions. Moreover, glutamine, fatty acids, and proteins may enter the metabolic cascades, impacting function, roles which have not been clarified in mast cell survival, differentiation, and function. Finally, additional work may be completed in additional animal models of sepsis, colitis, wounds, and obesity to help us understand the role of lactic acid in both health and disease as well as potential targets for therapeutic treatments. Further work in these animal models may help us to better understanding how modulating metabolism at the organismal scale may impact mast cell function, function of all immune cells, and overall inflammation.

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Vita

Heather Caslin was born on March 9th, 1990 in Colorado Springs, CO and is a US citizen. In middle school, her family moved to Round Hill, VA. She graduated from Loudoun Valley High School in 2008. She received her Bachelor of Science in Health, Physical Education, and Exercise Science from Virginia Commonwealth University in 2012 with university honors. She then became interested in cell biology and research and completed her Master of Science in Health and Movement Science at VCU in 2014. Following, she enrolled in the Integrative Life Sciences program at VCU in 2014 to work and study in the laboratory of John Ryan.