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Lactic Acid Inhibits Lipopolysaccharide-Induced Mast Cell Function by Limiting Glycolysis and ATP Availability

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Sepsis has a well-studied inflammatory phase, with a less-understood secondary immunosuppressive phase. Elevated blood lactate and slow lactate clearance are associated with mortality; however, regulatory roles are unknown. We hypothesized that lactic acid (LA) contributes to the late phase and is not solely a consequence of bacterial infection. No studies have examined LA effects in sepsis models in vivo or a mechanism by which it suppresses LPS-induced activation in vitro. Because mast cells can be activated systemically and contribute to sepsis, we examined LA effects on the mast cell response to LPS. LA significantly suppressed LPS-induced cytokine production and NF-κB transcriptional activity in mouse bone marrow–derived mast cells and cytokine production in peritoneal mast cells. Suppression was MCT-1 dependent and reproducible with sodium lactate or formic acid. Further, LA significantly suppressed cytokine induction following LPS-induced endotoxemia in mice. Because glycolysis is linked to inflammation and LA is a byproduct of this process, we examined changes in glucose metabolism. LA treatment reduced glucose uptake and lactate export during LPS stimulation. LA 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 LA effects. Our work suggests that enhancing glycolysis and ATP production could improve immune function, counteracting LA suppressive effects in the immunosuppressive phase of sepsis. ***The Journal of Immunology*****, 2019, 203: 000–000.**

Sepsis is a common and deadly condition with life-threatening organ dysfunction and a dysregulated host response to infection. Sepsis can progress to septic shock, which is defined as a severe form of sepsis with circulatory, cellular, and metabolic dysfunction and has a higher risk of mortality (1). Although exact estimates of sepsis-induced death vary because of the occurrence of comorbidities such as cancer and aging-induced immunosuppression, conservative estimates suggest a fatality rate of at least 30%, which contributes to the death of >150,000 Americans annually (2). Alarmingly, there are no clinically available 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 proinflammatory cytokines leading to hypotension, vascular leak, tissue hypoxia, and organ failure (3). Although the inflammatory response is beneficial to fight the infection, prolonged or exaggerated inflammation can damage host tissue and lead to death. There have been >100 clinical trials aimed to dampen the immune response following sepsis, which had no effect or worsened patient outcomes and mortality (4, 5). Importantly, recent studies suggest that fluid resuscitation and antibiotics allow most patients to survive the initial hyperinflammatory phase, at which time they enter an immunosuppressed state marked by impaired immune cell activation (4, 6, 7). Patient death in the immunosuppressive phase is often attributed to failure to clear the initial infection or acquisition of a secondary infection. Additionally, >100 distinct biochemical mediators are intricately involved in sepsis pathogenesis (5), but little is known about their role and the optimal response for survival and bacterial clearance. To devise more effective interventions for sepsis, a better understanding of the cellular mechanisms behind immunosuppression is required.

Lactate and 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) (8–11). Whereas normal blood lactate is ∼0.5–2.5 mM (12), blood lactate concentrations ≥4 mM and impaired clearance have been independently associated with increased sepsis mortality (13–16). Because of these associations, lactate clearance has been added as a treatment guideline for sepsis to improve diagnosis and initial treatment for patients (1). This has reduced mortality in at least one study (17). Although these data suggest lactate and mortality are related, it is not known whether elevated lactate is a cause or consequence of the infection.

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; DC, dendritic cell; DCA, dichloroacetate; 2-DG, 2-deoxyglucose; KO, knockout; LTA, lipoteichoic acid; MCT, monocarboxylic transporter; OX, sodium oxamate; OX PHOS, oxidative phosphorylation; PMC, peritoneal mast cell; poly(I:C), polyinosinic-polycytidylic acid; qPCR, quantitative PCR; SCF, stem cell factor; $SCVO_2$, central venous oxygen saturation; WT, wild-type.

In the tumor microenvironment, evidence suggests that lactic acid suppresses immune cell activation and promotes regulatory subsets (e.g., M2 macrophage lineage and myeloid-derived suppressor cells) (18, 19). In the context of bacterial infection, lactic acid has been typically shown to suppress LPS-induced activation of monocytes, macrophages, and dendritic cells (DCs) (20–23). Our laboratory recently found that lactic acid suppresses IL-33–induced mast cell activation in vitro and in vivo (24); however, no studies have examined lactic acid effects on mast cells in the context of bacterial activation. Although mast cells are most often considered for their role in allergic disease, they contribute importantly to bacterial defense and septic inflammation. Specifically, local mast cell activation improves neutrophil recruitment and sepsis outcomes by the release of chemoattractants (25–31). Mast cell mMCP-4 enhances survival after cecal ligation and puncture–induced sepsis by degrading TNF and limiting the detrimental effects of TNF on the host (32). Furthermore, mast cells have been shown to contribute to the control of Gram-positive (TLR2-mediated) sepsis (33). Given the clinical correlations of lactate with sepsis outcomes and the role for mast cells in sepsis, understanding how lactic acid alters mast cell function is an important question.

The aim of this study was to understand lactic acid effects on LPS-mediated mast cell function and determine its mechanism of action. Our data suggest that lactic acid significantly suppresses LPS-induced mast cell responses by inhibiting glycolysis, effects that are reversed by increasing ATP availability. These results indicate that glycolytic suppression and ATP production are necessary and sufficient for lactic acid effects and suggest a means of circumventing immunosuppression associated with sepsis.

Materials and Methods

Reagents

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

Mice

C57BL/6J and NF-κB-luc transgenic mice, which have a luciferase transgene under the control of NF-κB binding sites, were purchased from The Jackson Laboratory (Bar Harbor, ME). Colonies were maintained in a pathogen-free facility with a standard chow diet. Bone marrow was extracted from mice at a minimum of 10 wk old. Sepsis studies were conducted between ages 8 and 10 wk with approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Mast cell culture

Mouse bone marrow was differentiated in IL-3–containing supernatant from WEHI-3 cells and stem cell factor (SCF)–containing supernatant from BHK-MKL cells, as described, to yield 90–99% FcεRI$^+$ and cKit$^+$ bone marrow–derived mast cells (BMMC) at 21 d (24, 34). 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 d to yield ∼85% FcεRI$^+$ and cKit$^+$ peritoneal mast cells (PMC). For most experiments, BMMC or PMC were plated at 2×10^6 cells/ml with IL-3 and SCF (20 ng/ml). Media or 25 mM lactic acid was added 1:1 for a final concentration of 1×10^6 cells/ml, 10 ng/ml IL-3 + SCF, and 12.5 mM lactic acid for all experiments, unless otherwise noted. LPS was added at 1 μg/ml for 16 h for ELISA and glucose/lactate analysis or for 2 h for quantitative PCR (qPCR) and luciferase analyses.

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; 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.

Real-time qPCR

TRIzol was purchased from Life Sciences (Grand Island, NY) and used to extract total RNA. 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 qPCR was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) 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 and 95°C. Results were normalized to GAPDH and the H_2O control by using the Relative Livak Method (ΔΔ cycle threshold).

Endotoxemia model

Age-matched groups of mice (∼8–10 wk old) were injected i.p. with ketoprofen (1 mg/kg) for pain relief 30 min prior to lactic acid (80 mg/kg) or PBS. After 20 h, a lethal dose of LPS (25 mg/kg) was injected i.p. to elicit septic shock. Core body temperature over 4 h was measured using a rectal microprobe (Physitemp Instruments). Additionally, observational score was assessed for posture, grooming, stool, locomotion, respiration, and eye discharge, with 0 as normal and 4 as the worst score for each measure. Mice were sacrificed prior to end point if they reached a score of 3 on three or more categories or if they reached a score of 4 on two or more categories. After 4 h of temperature and observational measures, mice were sacrificed. Blood was collected using cardiac puncture, and plasma cytokines were measured using ELISA.

Western blot

Cell lysates were collected using Protease arrest (G-Biosciences, Maryland Heights, MO) in cell lysis buffer (Cell Signaling Technology, Danvers, MA). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific); 4–20% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) were loaded with 30 μg protein, electrophoresed, and transferred to nitrocellulose (Pall Corporation, Ann Arbor, MI), and membranes were blocked for 60 min in Blocker casein in TBS (from Thermo Fisher Scientific). Blots were incubated with primary Abs overnight in block buffer + Tween 20 (1:1000) with rabbit anti-IκB and rabbit anti-actin (1:1000; Abs all purchased from Cell Signaling). Blots were washed six times for 5 min each in TBS–Tween-20, followed by incubation with secondary Ab (1:10,000) for 60 min at room temperature (Cell Signaling). Size estimates for proteins were obtained using m.w. standards from Bio-Rad. 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 actin calculated relative to unactivated samples, using Image Studio 4.0 (LiCor).

Luciferase

BMMC were differentiated from NF-κB-luc transgenic mouse 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; Thermo Fisher Scientific) and normalized to the unactivated control.

Flow cytometry

To determine viability, BMMC were incubated for 1 min with propidium iodide (10 μg/ml) prior to flow cytometry analysis with a BD FACSCelesta (Franklin Lakes, NJ). The gating strategy used was doublet exclusion

(forward scatter area [FSC-A] × FSC height [FSC-H]) and size and granularity (FSC × side scatter [SSC]). Percent positive and negative were recorded.

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).

Statistical analyses

For the glucose uptake, lactate export, and IκB data, a *t* test was used. For the rest of the data, a one-way ANOVA was used to detect differences between groups. Post hoc testing using Tukey 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 ± SEM with statistical significance as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

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

We have previously shown suppressive effects of lactic acid on mast cell activation by IL-33 (24). Therefore, we hypothesized that these effects could extend to LPS-induced mast cell function and determined the kinetics of this response. BMMC were pretreated with or without lactic acid (12.5 mM) for various times prior to LPS activation (1 μg/ml). Pretreatment from 0 to 48 h significantly suppressed LPS-induced IL-6 and TNF (Fig. 1A). In sepsis, lactate increases following infection because of tissue hypoperfusion, impaired pyruvate dehydrogenase activity, elevated catecholamine secretion, and increased immune cell glycolysis (8, 35–37), factors that may emerge after infection has begun. Therefore, we also treated BMMC with lactic acid after LPS activation. Lactic acid significantly suppressed IL-6 and TNF when given up to 4 h post-LPS activation for all cytokines measured (Fig. 1B). To determine optimal lactic acid concentrations for suppression, BMMC were treated with or without lactic acid for 24 h prior to LPS. IL-6 was significantly reduced with ≥6 mM lactic acid, whereas TNF was significantly decreased with ≥12.5 mM treatment (Fig. 1C). Importantly, there was no significant change in cell viability at these concentrations (Supplemental Fig. 1A).

For the remainder of the experiments, lactic acid treatment for 24 h at 12.5 mM was used for optimal suppression, unless otherwise stated. Additional cytokines and chemokines were examined under these conditions. IL-13, MCP-1, and MIP-1α concentrations were significantly reduced by lactic acid treatment (Fig. 1D). Furthermore, lactic acid significantly suppressed IL-6 mRNA expression (Fig. 1E), suggesting the effects were at or before the transcriptional level, not solely at the level of translation or secretion.

To ensure our results were not related to BMMC differentiation in vitro, PMC were used as a comparison population. PMC were extracted by peritoneal lavage and expanded in culture prior to treatment with lactic acid (12.5 mM) for 24 h prior to LPS activation. Lactic acid significantly suppressed LPS-induced IL-6, TNF, and MCP-1 in PMC, similar to the results observed with BMMC (Fig. 1F). These results suggest that lactic acid consistently suppresses LPS-mediated mast cell function in vitro and ex vivo.

Lactic acid similarly suppresses TLR-2, -3, and -4 effects

Sepsis can occur in response to a multitude of bacterial, viral, or fungal infections (38). Whereas LPS activation of TLR-4 is often used to mimic bacterial activation in vitro, 13 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 (39). Therefore, we extended this study to determine the effects of lactic acid on other common TLR signaling pathways. BMMC were treated with lactic acid prior to stimulation with 1 μg/ml LPS (TLR4 ligand), 5 μg/ml LTA (a TLR2 ligand), or 100 μg/ml poly(I:C) (a TLR3 ligand). Lactic acid significantly suppressed cytokine production by all three ligands (Fig. 2), 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. Because results were similar, we used LPS as our model stimulus for the remainder of the study.

Lactic acid suppresses cytokine production in a model of LPS-induced endotoxemia

Our results are consistent with lactic acid effects on other immune cell lineages (20–23). Therefore, we expanded our questions to examine the effects of lactic acid on LPS-induced cytokine production in vivo. Because there is a correlative relationship between septic mortality and blood lactate in patients, we modulated lactic acid levels in a model of sepsis to provide causal data. An i.p. injection of lactic acid (80 mg/kg) or PBS was given 20 h prior to an i.p. injection of LPS (25 mg/kg) or PBS (Fig. 3A). Lactic acid significantly suppressed LPS-induced plasma IL-6, TNF, and MCP-1 compared with the PBS control (Fig. 3B). It is important to note that LPS and lactic acid act on many other immune cell lineages in this model, supporting the interpretation that lactic acid–mediated suppression is a consistent immune response. We also noted that lactic acid did not affect body temperature or observational score (Fig. 3C, 3D), perhaps because of LPS effects on the vasculature. Overall, these data show that lactic acid can suppress LPS-induced cytokine production in vivo.

LPS effects involve the MCT-1 transporter, lactate, and H^+ ions

MCTs are a group of proton-linked transporters that shuttle single carboxylate molecules, including lactate, across the plasma membrane (40, 41). We previously found that MCT-1 is required for lactic acid to suppress IL-33–mediated mast cell activation (24) and therefore examined the role of MCT-1 in the LPS response. BMMC were cultured with or without AR-C155858 (MCT-1/2 inhibitor) or DMSO for 1 h prior to lactic acid treatment and LPS activation. Although lactic acid significantly suppressed IL-6 and TNF in the DMSO control, it had no effect in the presence of AR-C155858 (Fig. 4A). There was no effect on cell viability (Supplemental Fig. 1B). Mast cells express MCT-1 but not MCT-2 (24), therefore, MCT-1 transport appears to be required for the suppressive effects of lactic acid on mast cells.

MCTs transport both lactate and H^+ ions (40, 41). We previously published that lactic acid, but not lactate, suppresses IL-33–induced BMMC cytokine production (24). To determine if acidification is required for lactic acid effects on LPS activation, BMMC were cultured with or without 12.5 mM lactic acid (pK_a 3.86), sodium lactate, or formic acid (pK_a 3.75) for 24 h prior to LPS activation. Lactic acid and formic acid significantly suppressed IL-6 and TNF production (Fig. 4B). There was no significant effect with sodium lactate at 12.5 mM. Additionally, there was no effect on cell viability with formic acid or sodium lactate (Supplemental Fig. 1A). Although these data support our previous findings that indicate a pH-dependent mechanism (24), other studies have shown suppression by lactate with effects often observed at higher doses (23, 42, 43). Therefore, a dose response was conducted with sodium lactate for 24 h prior to LPS activation. At doses ≥20 mM, sodium lactate significantly suppressed cytokine production with no effects on viability

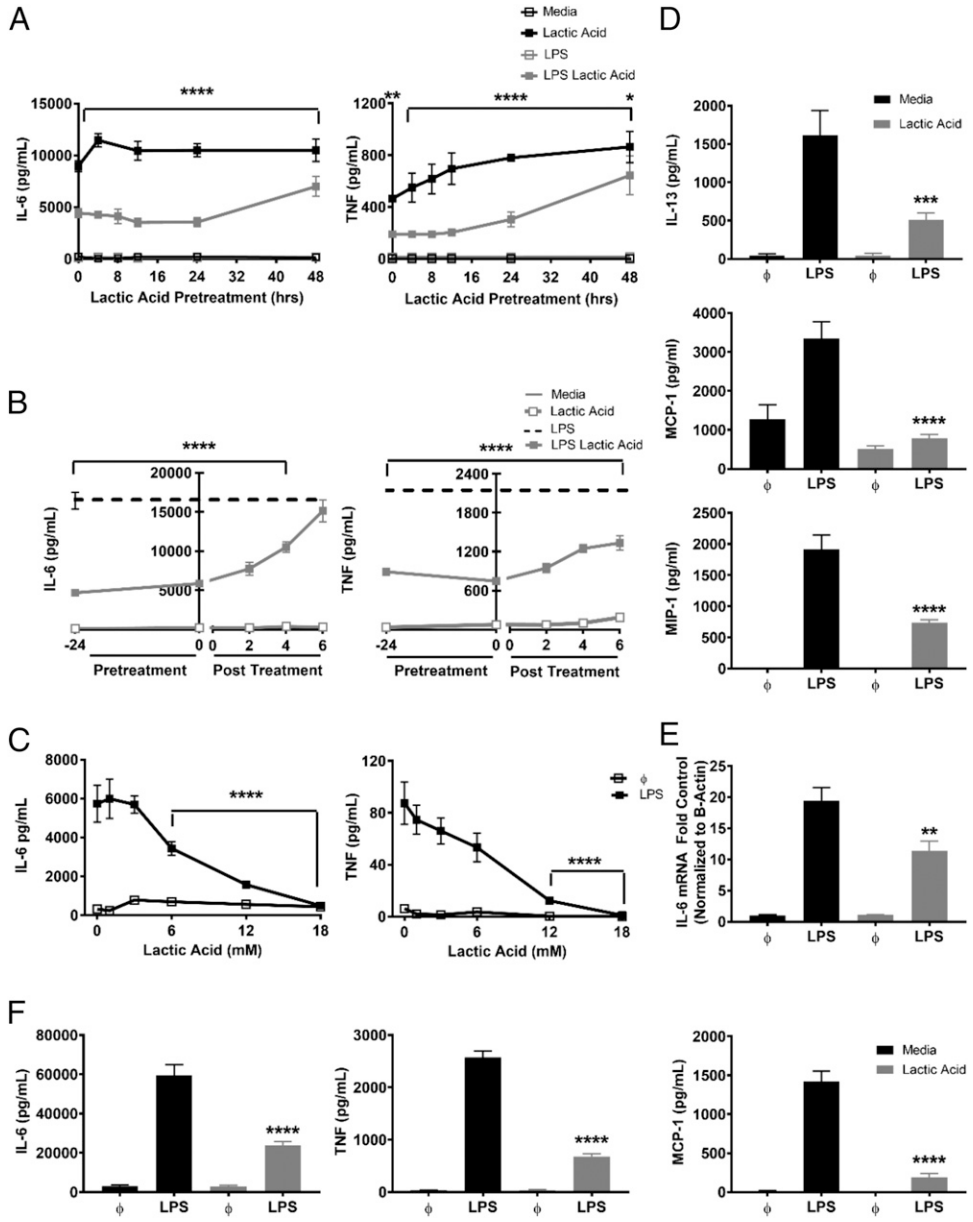

FIGURE 1. Lactic acid suppresses cytokine secretion and transcription in LPS-activated BMMC. (**A**) BMMC were pretreated with or without 12.5 mM lactic acid for the indicated time points and activated with or without LPS (1 μg/ml) for 16 h. (**B**) BMMC were treated with or without 12.5 mM lactic acid prior to, during, or following activation with or without LPS (1 μg/ml) for 16 h. (**C**) BMMC were treated for 24 h with or without the indicated doses of lactic acid and activated with or without LPS (1 μg/ml) for 16 h. (**D**) BMMC were treated for 24 h with or without 12.5 mM lactic acid and activated with or without LPS (1 μg/ml) for 16 h. ELISA analysis was used to determine supernatant cytokines. (**E**) BMMC were treated for 24 h with or without 12.5 mM lactic acid and activated with or without LPS (micrograms per milliliter) for 2 h. Real-time qPCR was used to determine IL-6 and actin mRNA expression. (**F**) PMC were treated for 24 h with or without 12.5 mM lactic acid and activated with or without LPS (1 μg/ml) for 16 h. ELISA analysis was used to determine supernatant cytokine concentrations. Data are means ± SEM of three populations, representative of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(Fig. 4C, Supplemental Fig. 1A), indicating suppressive effects independent of pH. These data suggest that both acidity and the lactate molecule have similar effects on cytokine production.

Once lactate and H^+ ions are released from the cell and into the bloodstream, buffering systems such as bicarbonate help to prevent acidosis (44, 45). Additionally, metabolic alkalosis sometimes occurs in patients with hyperlactemia because of saline resuscitation comorbidities (46, 47). Because our cultures are buffered by HEPES and FBS buffering systems, we monitored pH after lactic acid addition and examined kinetics of the buffering capacity. Upon addition to BMMC cultures, pH decreased to 6.7 (Fig. 4D). Within 2 h, the pH returned to 7.4 and was not different from our media control. These results suggest that lactic acid is buffered within our assays, similar to effects observed in blood systemically. Finally, because the pH returned to basal values within hours of addition, we investigated whether lactic acid effects persisted after plating treated cells in new media. BMMC were cultured with or without lactic acid for 24 h, washed, then activated in fresh media and compared with cells cultured with or without 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 secretion once lactic acid was washed out of the media (Fig. 4E). Together, these data suggest that both acidity and lactate impact mast cell responses

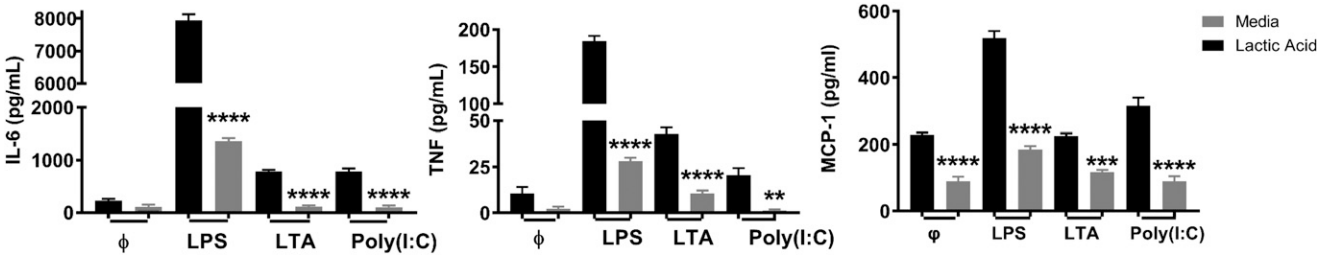

FIGURE 2. Lactic acid suppresses TLR-2, -3, and -4 effects on BMMC. BMMC were treated with or without 12.5 mM lactic acid for 24 h and activated with or without LPS (1 μg/ml), LTA (25 μg/ml), or poly(I:C) (100 μg/ml) for 16 h. Data are means ± SEM of three populations, representative of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

to LPS and that lactic acid effects are transient, requiring its presence at the time of activation.

Lactic acid suppresses NF-κB function and miR-155 expression

TLR4 signaling via MyD88 induces phosphorylation of IRAK, TRAF, TAK1, and MAP kinases and NF-κB (39). Previous publications have observed reduced p-NF-κB and nuclear translocation in monocytes with lactate and lactic acid treatment (22, 43). To determine the effects on NF-κB activity in mast cells, we first examined canonical NF-κB signaling by measuring IκB degradation. BMMC were treated with or without lactic acid for 24 h prior to LPS activation for 30 min. Although LPS activation decreased IκB expression relative to unactivated controls, this change was attenuated with lactic acid treatment (Fig. 5A). Because these results suggested that lactic acid reduces NF-κB activation, we further examined BMMC from NF-κB-luc transgenic mice, which have a luciferase transgene under the control of NF-κB binding sites. BMMC were treated with or without lactic acid for 24 h prior to LPS activation for 2 h. Lactic acid significantly suppressed LPS-induced NF-κB transcriptional activity as determined by luciferase expression (Fig. 5B).

miR-155 is an NF-κB–induced micro-RNA that enhances inflammation (48). We previously found that lactic acid inhibited IL-33–induced miR-155 expression, an effect that was required for lactic acid–mediated suppression (24). To determine if LPS-mediated miR-155 expression was similarly inhibited, BMMC were cultured with or without lactic acid for 24 h prior to activation. Lactic acid did not affect baseline miR-155 levels but antagonized LPS-induced miR-155-5p and miR-155-3p expression (Fig. 5C). To determine if changes in miR-155 expression are required for lactic acid effects, we compared wild-type (WT) and miR-155 knockout (KO) BMMC. As anticipated by the proinflammatory role of miR-155, LPS-stimulated KO cells had lower LPS-induced cytokine production than WT cells. Lactic acid similarly inhibited LPS-induced IL-6 (54% versus 60%) and TNF suppression (48% versus 35%) in WT compared with the miR-155 KO mast cells (Fig. 5D). Together, these data suggest that lactic acid suppresses NF-κB activation and downstream miR-155 induction, but whereas suppressing miR-155 (a proinflammatory

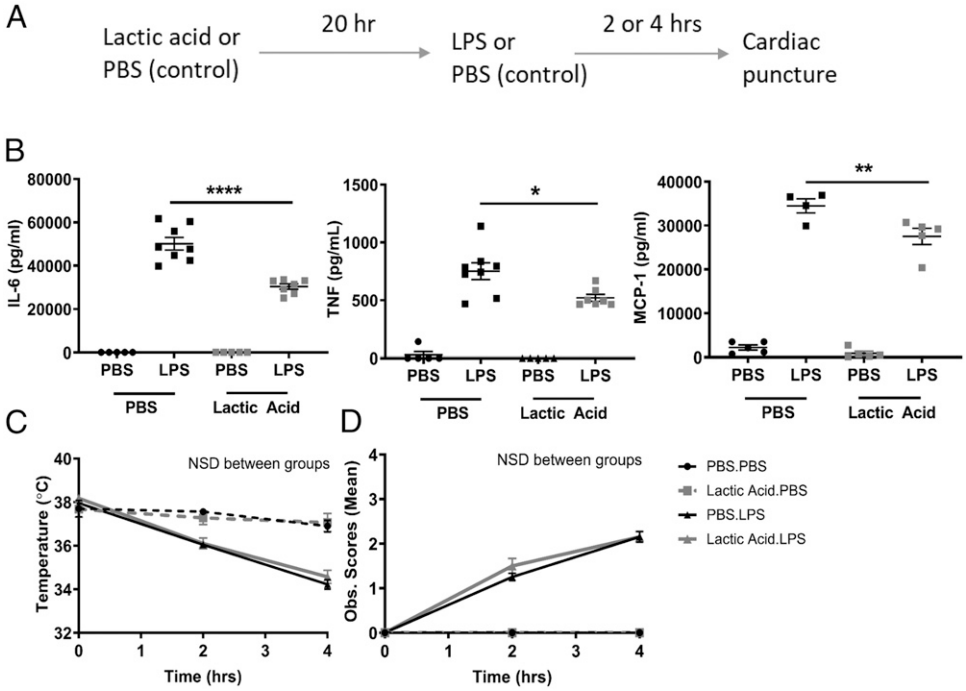

FIGURE 3. Lactic acid suppresses cytokine production in a model of LPS-induced septic shock. (**A**) Schematic. Lactic acid (80 mg/kg) or PBS was injected i.p. 20 h prior to LPS (25 mg/kg) or PBS. Mice were sacrificed at 2 or 4 h. (**B**) Plasma IL-6 was measured at 4 h, and TNF and MCP-1 were measured at 2 h by ELISA. (**C**) Rectal temperature and (**D**) Observational scores were measured over 4 h. Data are means of five mice per control group and eight mice per experimental group, representative of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. NSD, no significant difference.

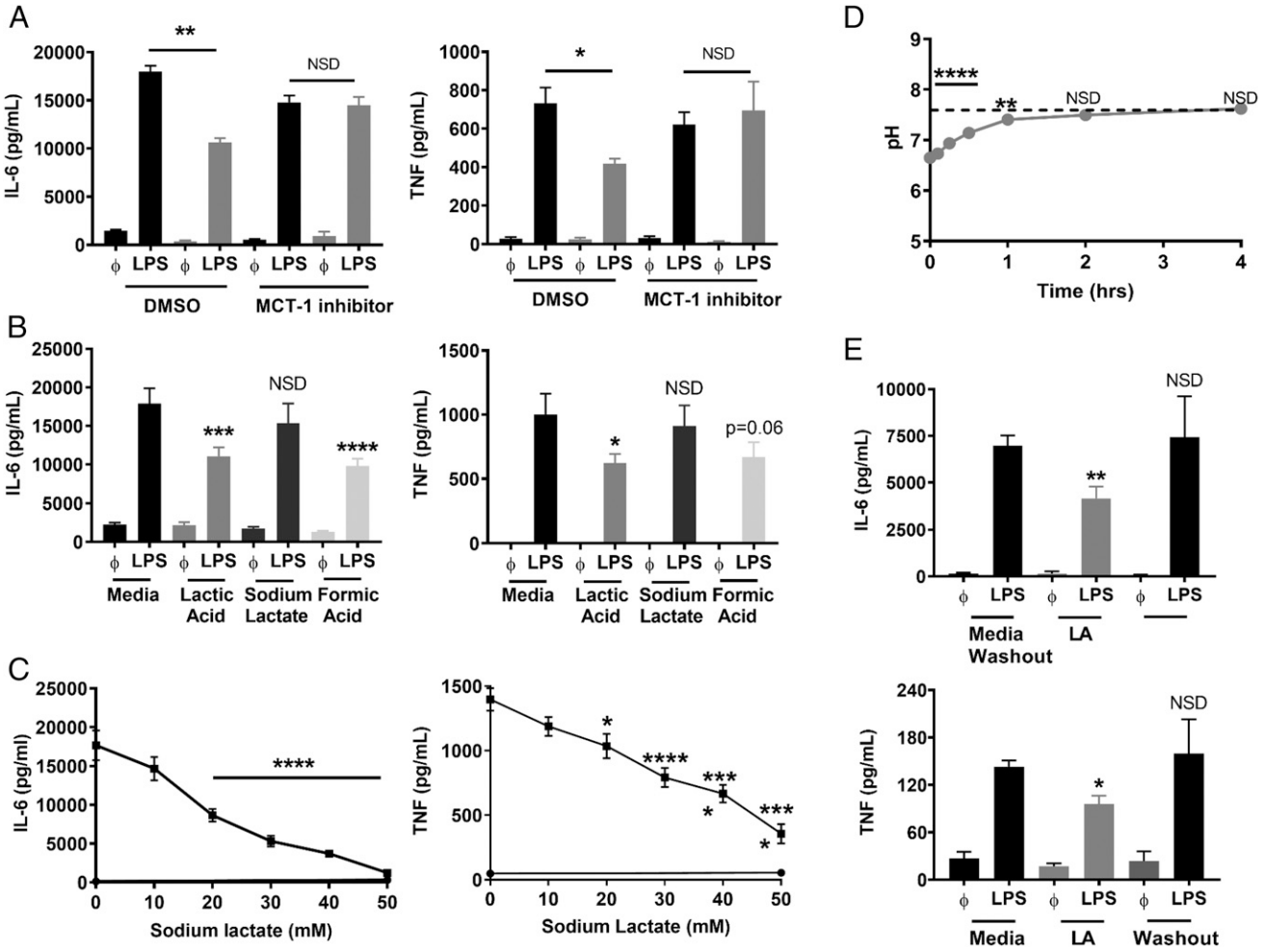

FIGURE 4. Lactic acid effects are dependent on MCT-1, lactate, and H^+ ions. (**A**) BMMC were treated with or without AR-C155858 (100 nM) or DMSO (vehicle) for 1 h, with or without lactic acid (12.5) mM for 16 h, and activated with or without LPS (1 μg/ml) for 16 h. (**B**) BMMC were treated with or without 12.5 mM lactic acid, sodium lactate, or formic acid for 24 h, then activated with or without LPS (1 μg/ml) for 16 h. (**C**) BMMC were treated with various doses of sodium lactate for 24 h and activated with LPS (1 μg/ml) for 16 h. (**D**) Lactic acid was added to cell culture media with BMMC plated at 1×10^6 cells/ml. pH was measured over 4 h. Dashed line indicates starting pH. (**E**) BMMC were treated with or without 12.5 mM lactic acid (LA) for 24 h, then replated in new media with or without 12.5 mM lactic acid and activated with or without LPS (1 μg/ml) for 16 h. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of three populations, representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. NSD, no significant difference.

mediator) inhibits LPS effects, miR-155 reduction is not the sole mechanism explaining suppression.

Lactic acid effects on glucose metabolism suppress cytokine production in BMMC

Lactate and associated H^+ ions are the byproduct of glycolysis, an important cellular pathway for the production of energy and intermediates for additional chemical reactions. Glycolysis produces ATP from glucose, ending with the conversion of pyruvate to lactate to recycle NADH and continue rapid ATP production during cellular activation. Importantly, other studies have shown that lactic acid and lactate suppress intracellular ATP, glucose uptake, and extracellular acidification in monocytes and $CD4^+$ T cells (21, 23, 49). Therefore, we examined how lactic acid modulates glucose metabolism in our system. BMMC were cultured with or without 12.5 mM lactic acid for 24 h prior to LPS activation for 16 h. LPS increased glucose uptake and lactate export, effects that were significantly reduced by lactic acid (Fig. 6A), and suggest that lactic acid suppresses glycolysis in our assays. These results support data from other systems and indicate that lactic acid acts as a negative regulator of glycolysis during LPS-mediated mast cell activation.

In the past 10 y, ample research has shown that cellular metabolism is closely linked to immune cell phenotype and effector function (50). To examine if glycolysis has a functional role in LPS-induced cytokine production, 2-DG was used to block hexokinase activity, directly reducing glycolysis, and OX was used to block lactate dehydrogenase activity, slowing glycolysis by preventing the recycling of NADH needed for glycolytic enzyme activity. BMMC were cultured with or without 2-DG (1 mM) or OX (40 mM) for 1 h prior to activation with LPS for 16 h. 2-DG and OX significantly suppressed LPS-induced cytokine production (Fig. 6B). Importantly, there was no effect on cell viability (Supplemental Fig. 1C). Additionally, 1 h of treatment with 2-DG and OX reduced NF-κB transcriptional activity, as determined by luciferase expression in NF-κB-luc BMMC (Fig. 6C). Because oxidative phosphorylation (OX PHOS) is also used to produce energy, we examined if inhibiting OX PHOS reduced cytokine production. BMMC were treated with etomoxir (200 μM) or rotenone and antimycin A (1 mM each) to inhibit OX PHOS 1 h prior to activation with LPS. There was no significant change in cytokine production following OX PHOS inhibition (Supplemental Fig. 2). Together, these data show that inhibiting glycolysis mimics

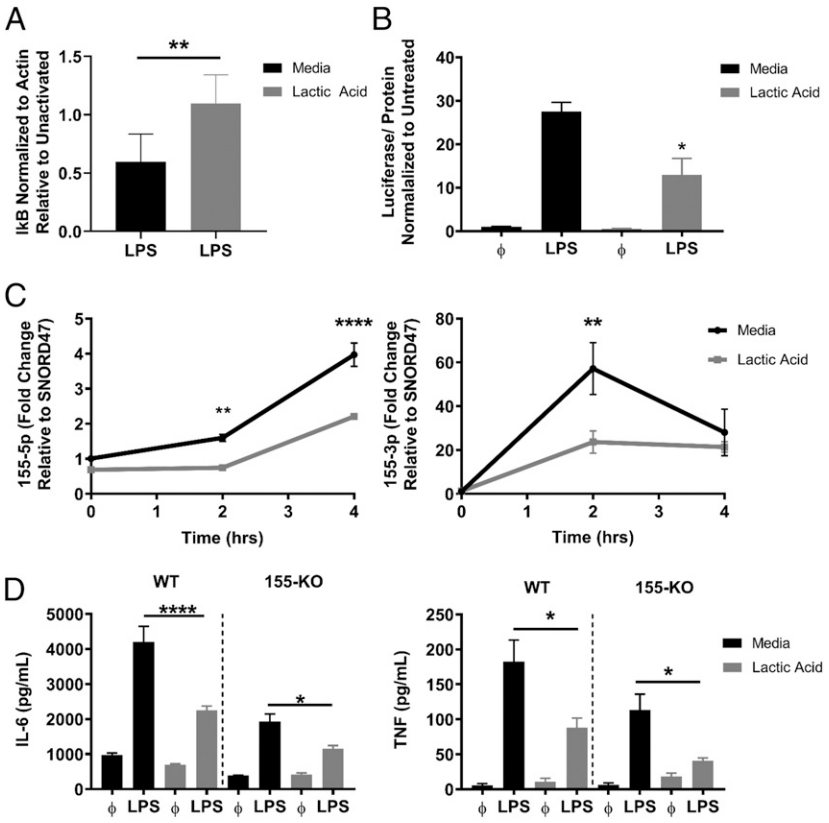

FIGURE 5. Lactic acid suppresses NF-κB function and miR-155 expression. (**A**) BMMC were treated with or without lactic acid (12.5 mM) for 24 h, then activated with or without LPS (1 μg/ml) for 30 min. IκB degradation was determined by LPS-activated expression normalized to β-actin and relative to unstimulated controls. (**B**) NF-κB-luc transgenic BMMC were treated with or without lactic acid (12.5 mM) for 24 h, then activated with or without LPS (1 μg/ml) for 2 h. Luciferase activity was measured with the Promega Luciferase Assay Substrate and Glomax Luminometer. (**C**) BMMC were cultured with or without lactic acid (12.5 mM) for 24 h, then activated with or without LPS (1 μg/ml) for 2–4 h. MicroRNAs were measured via qPCR and normalized relative to SNORD47. (**D**) C57BL/6 or miR-155 KO BMMC were treated with or without lactic acid (12.5 mM) for 24 h and activated with or without LPS (1 μg/ml) for 16 h. ELISA was used to measure cytokine concentrations in the cell supernatant. Data are means ± SEM of three populations, representative of two (A) or three (B–D) independent experiments. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

lactic acid effects and is sufficient to suppress both cytokine production and cell signaling.

ATP reverses lactic acid suppression

Glycolysis rapidly generates ATP, which is required for kinase activity and signaling, tRNA synthetase function, ion transport, and chromatin remodeling. These processes all play a role in cell signaling and cytokine production. In addition, using glycolysis allows immune cells to generate metabolites for many anabolic processes, such as cell growth, differentiation, and inflammatory function (50). Because LPS induced glycolysis and lactic acid suppressed glycolysis, we wanted to determine if reduced glycolytic ATP production was a critical mechanism of lactic acid–mediated suppression. BMMC were cultured with or without lactic acid and with or without ATP (10 μM) at the time of LPS activation. ATP reversed lactic acid effects on both cytokine secretion and NF-κB transcriptional activity (Fig. 7A, 7B). In a previous manuscript, we showed that micromolar quantities of labeled ATP diffuse into BMMC within 20 min (51), which can increase the ATP available in the cell for energy and bypass the need for ATP produced by glycolysis. These data, together with the experiments above, suggest that lactic acid suppresses LPS-induced glycolytic ATP production, effectively attenuating energy available for cell signaling and cytokine production (Fig. 8).

Discussion

Elevated blood lactate consistently correlates with increased mortality from sepsis (2, 13, 52–55); however, there is little understanding of a cause-and-effect relationship. Previous studies showed that lactic acid suppresses macrophages, DCs, and T cell activation in vitro (18, 20–24, 43), but there has been no clear mechanism of action and no in vivo studies to determine the direct effects of lactic acid in a sepsis model. Because mast cell function reduces sepsis-induced death in mouse models (25–33), we used mast cells as a model system to study lactic acid. We demonstrate that lactic acid suppresses LPS-mediated activation of BMMC and PMC. Additionally, these are the first data, to our knowledge, to demonstrate that lactic acid suppresses cytokine production in vivo, using a model of LPS-induced endotoxemia. Lactic acid has been shown to suppress glycolysis and intracellular ATP (21, 23, 49), and our data demonstrate glycolytic/ATP suppression is necessary and sufficient to inhibit LPS signaling and cytokine production (Fig. 8). These findings suggest that lactic acid, the end product of glycolysis, can act as a feedback regulator of mast cell activation to reduce the inflammatory response, specifically in sepsis. Additionally, these data support the hypothesis that targeting metabolic pathways may be an effective mechanism to modulate lactic acid effects in disease.

In BMMC and PMC, physiologically relevant lactic acid concentrations suppressed the LPS-induced production of several cytokines and chemokines. We observed significant effects between 6 and 12 mM, which are relevant to wound tissue lactate concentrations (5–20 mM; maximum 80 mM) (56, 57) and blood lactate concentrations (2–10 mM; maximum 20 mM) (15, 17, 54, 58). Importantly, many studies define high lactate as blood concentrations >4 mM (13, 14, 59). However, changes in tissue lactate concentration and pH can be more dynamic than blood. For example, one study showed that septic patients had no difference in blood pH between low- and high-lactate groups, but there was a significant difference in the gastric intramucosal pH (60). Additionally, another study demonstrated that muscle lactate concentrations were ∼1.5 mM higher than arterial lactate concentrations in septic shock patients (61). These data suggest that our results are particularly relevant to tissue-resident immune cells, such as mast cells, and immune cells recruited into the tissue.

Importantly, lactic acid suppressed cytokine production in a model of endotoxemia in vivo. We chose the LPS-induced model and the associated time points to directly extend our in vitro studies.

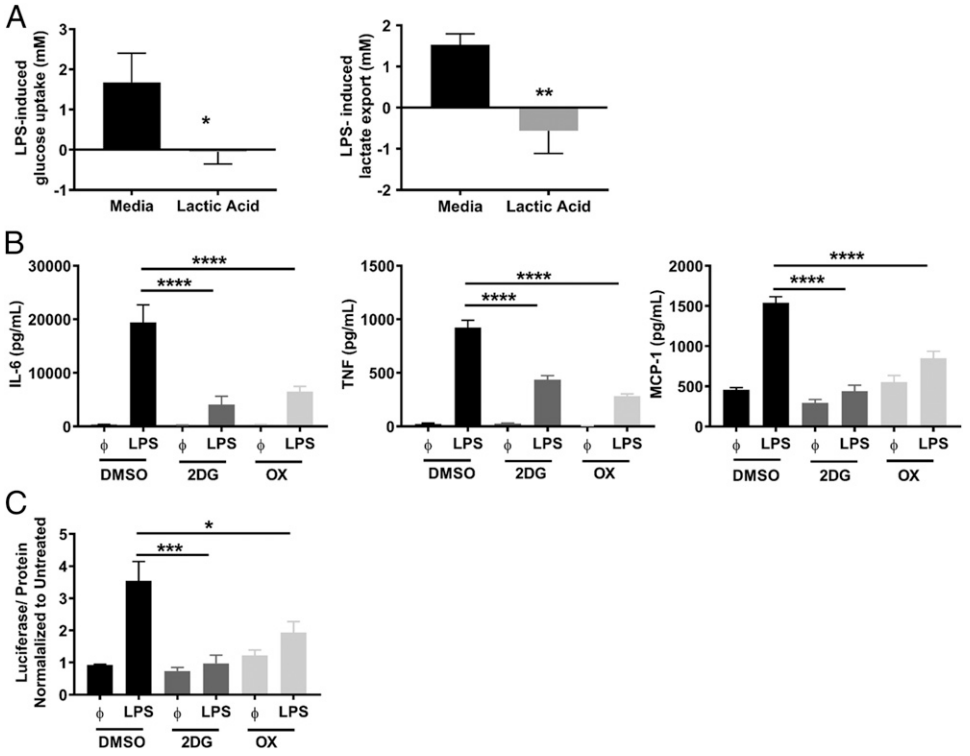

FIGURE 6. Lactic acid suppresses glycolysis, a necessary component of the LPS response. (**A**) BMMC were treated with or without 12.5 mM lactic acid for 24 h, then activated with or without LPS (1 μg/ml) for 16 h. Glucose and lactate were measured in the cell supernatant by colorimetric assay. Uptake and export were calculated from the difference between controls and activated samples. (**B**) BMMC were treated with or without 2-DG (1 mM) or OX (20 mM) for 1 h prior to LPS activation (1 μg/ml). After 16 h, cytokines were measured in supernatant by ELISA. (**C**) BMMC were treated with two DG (1 mM) or OX (20 mM) for 1 h prior to LPS activation (1 μg/ml). After 2 h, luciferase activity was measured from the NF-κB-luc transgenic BMMC on the Glomax Luminometer. Data are means ± SEM of three populations analyzed in triplicate, representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Lactic acid significantly reduced LPS-induced IL-6, MCP-1, and TNF, supporting the effects we observed in vitro. It is important to note that LPS-induced endoxemia only mimics the initial inflammatory stage of sepsis, which limits our ability to understand the role of lactic acid on the secondary immunosuppressive phase. Additionally, although we show that pretreatment with lactic acid reduces the production of inflammatory cytokines, our model does not directly show that lactic acid following the onset of sepsis has the same effect. Future experiments should modulate lactic acid after shock initiation and include study of the secondary immunosuppressive phase with reinfection models. 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. We hypothesize that after the onset of sepsis, lactate levels rise in the blood and could suppress inflammatory cytokine production, contributing to pathological immunosuppression.

Interestingly, there were no effects of lactic acid on hypothermia or observational score in the LPS endotoxemia model. We postulate that these clinical phenotypes are at least partly immune independent. LPS and lactic acid have been shown to have overlapping effects on endothelial cells, increasing NO production, vascular permeability, and vasodilation (62–70). In mice, low-dose infusions of HCl have been reported to drop blood pH to 7.13, increase endothelial NO, reduce blood pressure, and reduce temperature (66). Furthermore, these authors mentioned that lactic acid also reduced pH and blood pressure and increased endothelial NO. 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 (71), influencing locomotion, grooming, and overall observational scores. For ethical considerations and animal care, ketoprofen was injected prior to lactic acid. This limits our model, in that arachidonic acid metabolite production and pain responses are reduced. Future work should further examine the effects of lactic acid on different cell types and organ systems within a sepsis model to better study sepsis as a systemic disease.

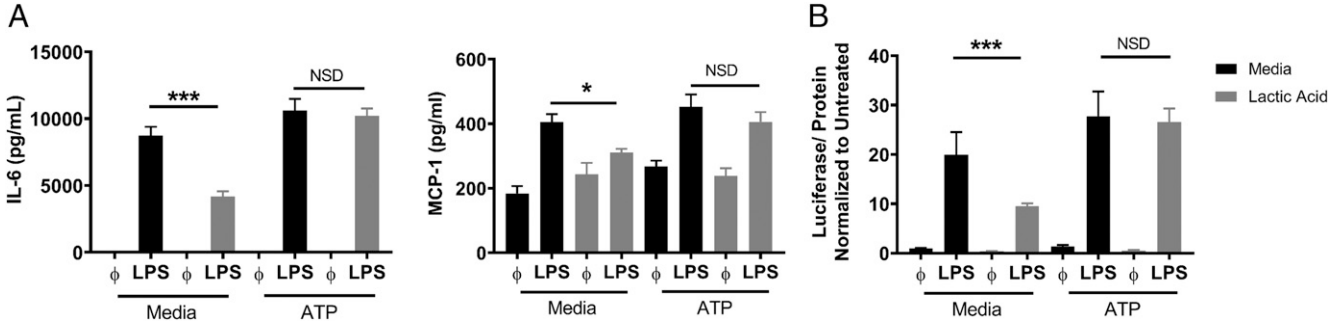

FIGURE 7. ATP reverses lactic acid effects. (**A**) BMMC were treated with or without 12.5 mM lactic acid, with or without ATP (10 μM), and with or without LPS (1 μg/ml) for 16 h, and ELISA analysis was used to determine supernatant cytokine concentrations. (**B**) BMMC were treated with or without 12.5 mM lactic acid, with or without ATP (10 μM), and with or without LPS (1 μg/ml). After 2 h, luciferase activity was measured from the NF-κB-luc transgenic BMMC on the Glomax Luminometer. Data are means ± SEM of three populations, representative of three independent experiments. $*p < 0.05$, $***p < 0.001$.

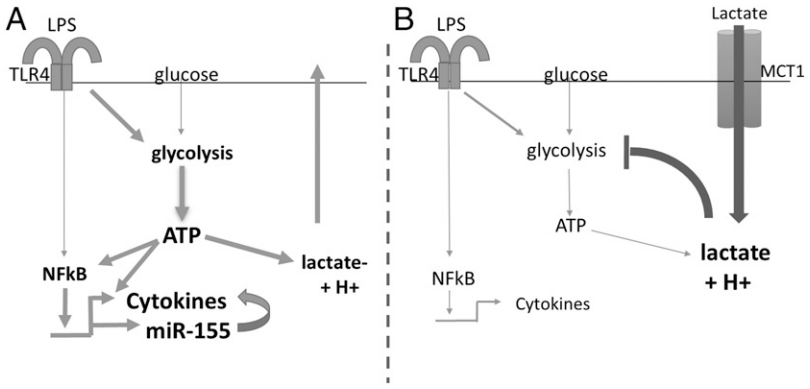

FIGURE 8. Schematic diagram. (**A**) LPS activates TLR4, signaling through NF-κB to induce cytokines and miR-155. LPS activation also increases glycolysis, providing ATP to augment NF-κB transcription and cytokine production. (**B**) Exogenous lactic acid imported through MCT-1 suppresses glycolysis, effectively suppressing NF-κB transcriptional activity.

Lactic acid effects on LPS-induced cytokine production were MCT-1 dependent, similar to our findings with lactic acid effects on IL-33–induced activation (24). MCT-1 is the primary transporter of lactic acid, known to cotransport lactate and H^+ (40), and in our experiments, there was no effect of lactic acid with MCT-1 inhibition, which suggests that lactate and/or H^+ must be transported into the cell for suppressive effects. We also observed that both acidification with formic acid and elevated concentrations of sodium lactate could mimic the suppressive effects of lactic acid. These results support many studies that have reported immunosuppressive effects of acidification (21, 72, 73), high concentrations of lactate (10–100 mM) (20–22), and slightly lower concentrations of lactic acid (5–20 mM) (23, 42, 43). These results also support our data, showing that lactic acid suppresses glycolysis because H^+ inhibits phosphofructokinase, the rate-limiting enzyme in the glycolytic pathway, and lactate can inhibit different isoforms of lactate dehydrogenase, an example of product inhibition that can further reduce glycolysis (74, 75). Our data have been corroborated in DCs, in which the inhibitory effects of 10 mM lactic acid were reverted after normalizing the pH (20). 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. We also observed a pH reduction from 7.4 to 6.7 upon lactic acid addition to media, rebounding within 2 h. This suggests that our in vitro media buffering systems at least partly mimic blood, in which deprotonated lactic acid and H^+ ions are buffered by the bicarbonate system.

When seeking a molecular mechanism, we noted that lactic acid reduced IκB degradation and the activity of NF-κB, a major transcription factor in LPS signaling that is important for cytokine expression (76–78). Previous studies reported reduced NF-κB phosphorylation and nuclear translocation in monocytes after lactate or lactic acid treatment (22, 43), data supported by our study. Our results suggest that lactic acid inhibits NF-κB activation and transcriptional activity, which may contribute to reduced cytokine production. We also examined miR-155, which functions to suppress the negative regulators SHIP1 and SOCS1, with an overall proinflammatory role in immune cell signaling (34, 79). We have previously published that lactic acid suppresses the IL-33–induced, NF-κB–dependent induction of miR-155 (24). In this study, lactic acid similarly suppressed LPS-mediated miR-155-5p and miR-155-3p expression. Although miR-155 KO BMMC were hyporesponsive to LPS, lactic acid had similar effects on LPS-stimulated cytokine secretion in these cultures. miR-155 suppression may be one anti-inflammatory effect of lactic acid; however, miR-155 suppression could not fully explain lactic acid effects, supporting additional mechanisms.

Our data suggest a primary mechanism of action is glycolytic inhibition, which is necessary and sufficient for lactic acid to suppress LPS-induced cytokine production. In BMMC, we observed a significant reduction in LPS-mediated glucose uptake and lactate export with lactic acid treatment. This is consistent with results in human monocytes and mouse macrophages stimulated with LPS (21, 23) as well as the known ability of H^+ ions to inhibit phosphofructokinase activity (80, 81), which reduces glycolysis and lactate production (82). Elevated lactate also suppresses pyruvate-to-lactate conversion by lactate dehydrogenase, an equilibrium enzyme. This effectively reduces NADH recycling required for glycolysis (83, 84). Interestingly, another study reported that blocking endogenous lactate export by MCT-4 in macrophages following LPS activation suppresses cytokine production and glycolysis (11). 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 2-DG and OX significantly suppressed cytokine production and NF-κB transcription, similar to lactic acid. Although 2-DG has been shown to suppress LPS-induced cytokine production (21, 23, 79), this is the first report, to our knowledge, to show an effect on NF-κB–mediated transcription. Collectively, our studies suggest that suppressing glycolysis is sufficient to limit inflammatory cytokine responses. ATP, the main product of glycolysis, is required for many cellular processes, including enzyme activity involved in cell signaling and cytokine production. Increasing ATP availability reversed the effects of lactic acid, restoring both NF-κB function 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 following the initiation of sepsis may suppress immune cell glycolysis and function, potentially contributing to the immunosuppression observed in the secondary phase of sepsis. In immunosuppression, immune cells have reduced glucose metabolism, cytokine production, Ag presentation, and cytolytic function (6), mirroring the effects of lactate treatment (19–23, 49, 85). 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; however, 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 ($SCVO_2$) (86). Although 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 RBCs, and dobutamine. The interventions were administered because of 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 (87). DCA treatment worsened patient survival, leading researchers to conclude that modulating blood lactate does not improve survival in sepsis. However, although DCA reduces lactate secretion by increasing pyruvate conversion to acetyl co-A in the mitochondria, it also inhibits glycolysis, effectively augmenting lactic acid effects (88–90). Furthermore, despite lowering blood lactate levels, i.v. DCA administration has been shown to reduce tissue ATP (91), 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 (92, 93).

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 inhibits LPS-induced glycolysis, suppressing cytokine production and NF-κB function, partly by reducing ATP availability. Future studies should directly examine lactic acid effects in a model of immunosuppression and target enhancing glycolysis and ATP production to improve immune function and counteract lactic acid effects in sepsis.

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Disclosures

The authors have no financial conflicts of interest.

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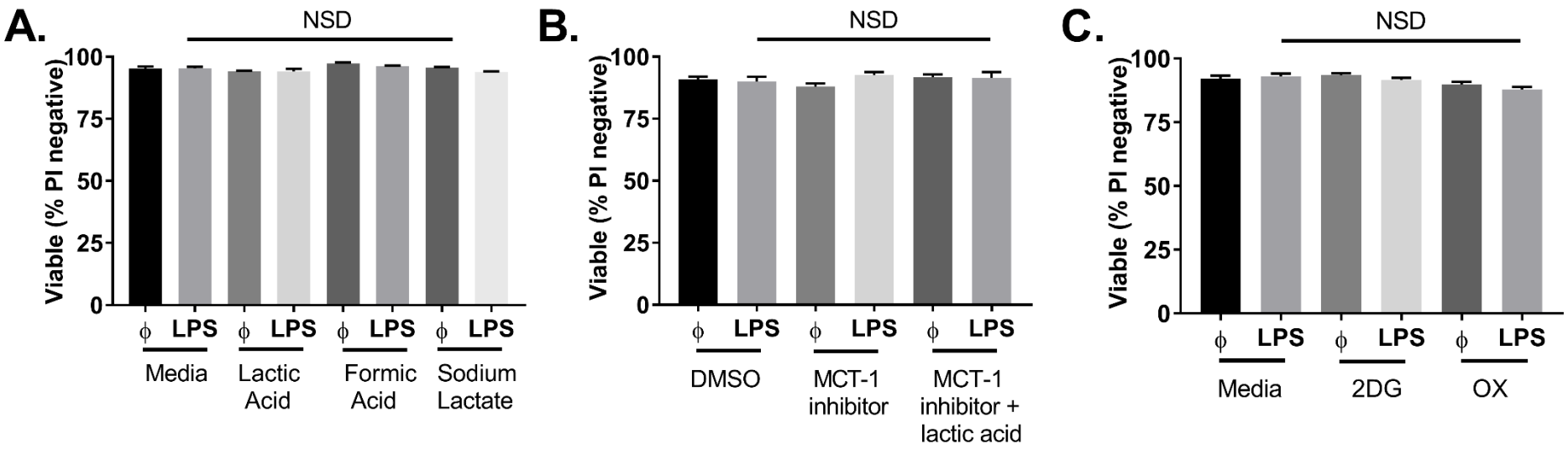
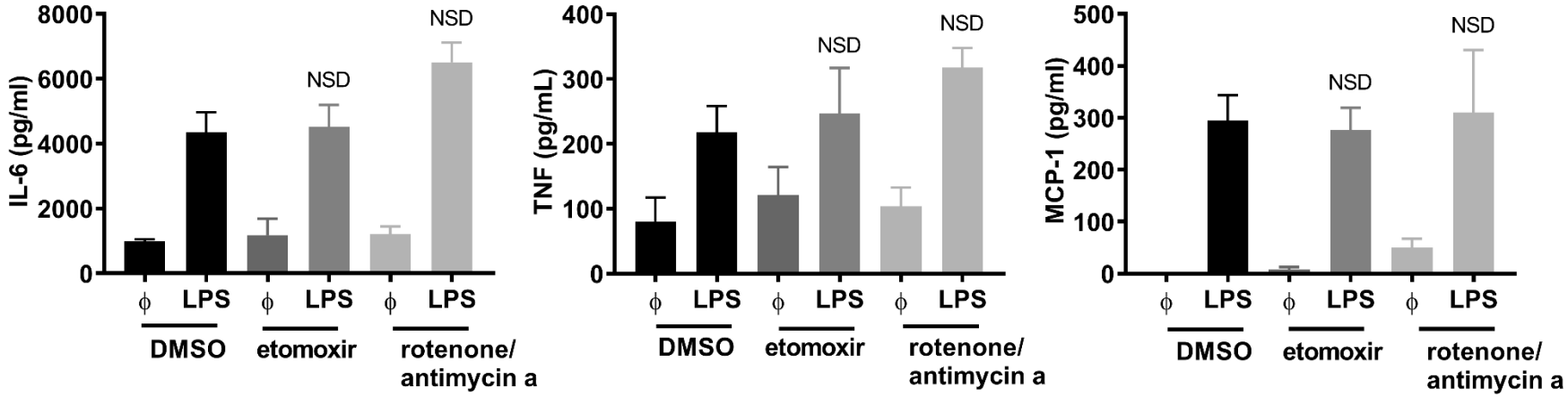


Supplemental Figure 1: Cell viability is not changed with treatments used. BMMC were treated A) for 24 hours with 12.5 mM lactic acid, formic acid, or sodium lactate, B) for 24 hours with DMSO or 100 nM AR-C155858, or C) for 1 hour 1 mM 2DG or 20 mM OX ± LPS (1 µg/mL) for 16 hours as in the corresponding experimental conditions. 10 µg/mL propidium iodide was then added for 1 min prior to flow cytometry analysis. Data are means ± SEM of 3 populations. NSD= No significant difference.



Supplemental Figure 2: OX PHOS inhibitors have no effect on cytokine production. BMMC were treated with DMSO (vehicle control), etomoxir (200 μM), or rotenone+antimycin A (1 μM) 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