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Dimethyl Malonate Protects the Lung in a Murine Model of Acute Respiratory Distress Syndrome

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Abstract

Introduction: Succinate is a pro-inflammatory citric acid cycle metabolite that accumulates in tissues during pathophysiological states. Oxidation of succinate after ischemia-reperfusion leads to reversal of the electron transport chain and generation of reactive oxygen species. Dimethyl malonate (DMM) is a competitive inhibitor of succinate dehydrogenase, which has been shown to reduce succinate accumulation. We hypothesized that DMM would protect against inflammation in a murine model of ARDS.

Methods: C57BL/6 mice were given ARDS via 67.7 ug of intra-tracheally administered lipopolysaccharide (LPS). DMM (50 mg/kg) was administered via tail vein injection 30 minutes after injury, then daily for 3 days. The animals were sacrificed on day 4 after bronchoalveolar lavage (BAL). BAL cell counts were performed to examine cellular influx. Supernatant protein was quantified via Bradford protein assay. Animals receiving DMM (n=8) were compared to those receiving sham injection (n=8). Cells were fixed and stained with FITC-labelled wheat germ agglutinin to quantify the endothelial glycocalyx (EGX).

Results: Total cell counts in BAL was less for animals receiving DMM (6.93×10^6 vs. 2.46×10^6, p=0.04). The DMM group had less BAL macrophages (168.6 vs. 85.1, p=0.04) and lymphocytes (527.7 vs. 248.3; p=0.04). DMM treated animals had less protein leak in BAL than

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sham treated (1.48 vs. 1.15 ug/ul, p=0.03). Treatment with DMM resulted in greater staining intensity of the EGX in the lung when compared to sham (12,016 vs. 15,186 Arbitrary Units, p=0.03). Untreated animals had a greater degree of weight loss than treated animals (3.7% vs. 1.1%, p=0.04). DMM prevented the upregulation of MCP-1 (1.66 vs. 0.92 RE, p=0.02) and ICAM-1 (1.40 vs. 1.01 RE, p=0.05).

Conclusions: DMM reduces lung inflammation and capillary leak in ARDS. This may be mediated by protection of the EGX and inhibition of MCP-1 and ICAM-1. DMM may be a novel therapeutic for ARDS.

Background

The importance of succinate as a pro-inflammatory metabolite of hypoxia and driver of reactive oxygen species formation in critically injured patients has recently come to light (1–5). Studies have shown that succinate is a better marker of shock than lactate in critically ill patients (1). Succinate levels correlate with mortality in patients with combat-related injuries (4). As a tricarboxylic acid cycle metabolite, succinate levels are known to increase in animal models of hemorrhage where there is hypoxia at the cellular level (6). In these periods of ischemia, succinate accumulation occurs due to reversal of succinate dehydrogenase, which is driven by fumarate overflow from purine nucleotide breakdown and partial reversal of the malate/aspartate shuttle (7). In ischemia-reperfusion models, during the reperfusion phase, there is increased availability of oxygen, and the excess succinate that accumulates in tissues during hypoxia becomes rapidly oxidized by succinate dehydrogenase, leading to a large mitochondrial membrane change and reversal of the electron transport change, which drives generation of reactive oxygen species (2, 7). Elevated succinate and reactive oxygen species have been linked to endothelial glycocalyx damage and coagulopathy (5).

Malonate esters have been used to inhibit succinate accumulation and decrease ischemia-reperfusion in several tissue beds such as the kidney, brain, liver, and heart (8–12). Dimethyl Malonate (DMM) is a cell permeable inhibitor of succinate dehydrogenase, a key enzyme of the citric acid cycle (8). In periods of ischemia, the citric acid cycle reverses, and succinate dehydrogenase converts fumurate to succinate. By inhibiting succinate dehydrogenase with malonate esters in periods of anaerobic metabolism, succinate accumulation can be decreased. Recent pre-clinical studies have demonstrated that in a swine model of ischemia-reperfusion, dimethyl malonate reduces succinate accumulation, resulting in decreased generation of reactive oxygen species and preserved endothelial glycocalyx.

The role of succinate accumulation in the lung and its role in development of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is not well defined. Slaughter et al. found that succinate accumulates in lung tissue during hemorrhagic shock, suggesting that succinate could be a potential inflammatory mediator leading to lung injury (13). Translational studies have shown that succinate accumulation may play a role in development of pulmonary hypertension (14, 15). Succinate can mediate an inflammatory response through the chemokine monocyte chemoattractant protein-1 (MCP-1) and the enzyme p300 (16–18). Hypoxia Inducible Factor-1α (HIF-1α) and Nuclear factor kappa-

light-chain-enhancer of activated B cells (NF-κB) also play an integral role in succinate mediated inflammation (17, 19). Interestingly, another study showed that succinate levels specifically in the epithelium of the lung had lung protective effects (20). In this study, we sought out to determine if DMM could be a novel therapeutic to treat ALI in a murine model of ARDS. We hypothesized that DMM would decrease lung succinate levels and protect against lung inflammation and damage to the pulmonary capillary endothelial glycocalyx.

Methods

Acute Lung Injury Model and DMM Treatment

The study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the the Institutional Animal Care and Use Committee (Protocol ID: 607). The study adhered to the ARRIVE guidelines as shown in Supplemental Figure 1. All surgery was performed under isoflurane anesthesia and protocols were designed to minimize suffering. All animals were monitored every 24 hours and each animal was examined for signs of distress or suffering such as hunched posture or respiratory distress. Any animals demonstrating suffering or distress were euthanized. The method of euthanasia was cervical dislocation and exsanguination under anesthesia.

Equal numbers of male and female, 6–8 week old C57BL/6 mice (Charles River Laboratories, Cambridge, MA) were given ALI via intra-tracheally administered lipopolysaccharide (LPS) as previously described (21). After obtaining appropriate depth of anesthesia using isoflurane, the animals received 67.7 ug of LPS administered intra-tracheally. Animals were randomized to control or the experimental group. Approximately 30 minutes after LPS administration, 50 mg/kg dimethylmalonate (DMM) was administered via tail vein injection for the experimental group (n=8), while sham animals (n=8) received an injection with phosphate-buffered saline (PBS). An additional dose of DMM or saline (for Sham) was administered every 24 hours for the next three days, until the conclusion of the experiment. All analyses were performed by a blinded reviewer.

Evaluation of Lung Injury

Euthanasia and bronchoalveolar lavage (BAL) were carried out on post-injury day 4. After obtaining appropriate levels of anesthesia with inhaled isoflurane, the trachea was cannulated using a 26 gauge needle and BAL was performed with three successive washes using 1 mL of phosphate buffered saline (PBS). Next, a small segment of the left lower lobe was removed flash frozen in optimal cutting temperature compound (O.C.T.) for glycocalyx measurement. Finally, 1 cc of 4% paraformaldehyde was injected to the lung for fixation.

The BAL fluid was then centrifuged at 500 x gravity for 5 minutes. Cells were obtained from the BAL after centrifuge and cell counts performed. Cells were then affixed to glass slides and stained with Wright's stain. To quantify protein in the BAL supernatant, a Bradford protein assay (Bio-Rad Laboratories) was performed. Protein was quantified by measuring absorbance at 595 nm on a BMG Labtech FLUOstar Optima plate reader. In addition, the BAL supernatant was used to measure pro-inflammatory cytokines

Interleukin-6 (IL-6), tumor necrosis factor-α (TNF- α), and interferon-γ (IFN-γ) using ELISA kits (Thermo Fisher Scientific).

Histopathological Evaluation

Immediately after sacrifice on post-injury day 4, lung tissue from the right lower lobe was fixed in 4% paraformaldehyde and cut into sections. The sections were stained with hematoxylin and eosin (H & E). Representative images were taken. We chose to sacrifice animals on post-injury day 4 as our prior studies have shown that this is when protein leak in the lung interstitium is most pronounced.

Glycocalyx Quantification

Flash-frozen lung tissue in O.C.T was sectioned in a cryostat at −20°C at a section thickness of 10 μm; the sections were affixed to room-temperature glass slides as described in (21). Fixation was performed by immersion in 4°C methanol for 10 min. Glycocalyx staining was performed with FITC-labelled wheat germ agglutin (1:1000) in PBS for 1 hour at room temperature with DAPI (4′,6-diamidino-2-phenylindole) and Alexa-Fluor 594 phalloidin to aid in identifying the vasculature. Sections were washed three times with PBS and coverslipped in Fluoro-Gel (Electron Microscopy Sciences). Glycocalyx was imaged on an Olympus BX51 fluorescence microscope. ImageJ software was used to quantify glycocalyx fluorescence intensity in the alveolar vessels from a minimum of 20 regions of interest from 3 mice per condition.

Quantification of Succinate Levels

Plasma succinate assay was performed using a colorimetric commercially available kit as per manufacturer instructions (LSBio) using venous plasma.

Immunofluorescence techniques were used to measure succinyl-lysine lysine levels as an indicator of succinate levels at the pulmonary endothelium (22). After sacrifice on post-injury day 4, lung tissue was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight. Paraformaldehyde-fixed lung segments were flash-frozen in Optimal Cutting Temperature (O.C.T.) compound (Sakura) and sectioned on a cryostat at −20°C. Tissue was then blocked in 1% bovine serum albumin (BSA) in PBS for one hour. Tissue was then incubated overnight in primary antibody for succinyl-lysine (PTM Biolabs PTM-401) diluted 1:100 in 1% BSA in PBS. Cells were then washed with PBS 3x. Cells were incubated with secondary antibody donkey anti-rabbit Alexa Fluor 488 (1:500, Invitrogen, A32790TR) diluted in 1% BSA in PBS along with 0.1 ug/ml of 4,6 diamidino-2phylindole (DAPI) (Sigma) and Alexa-Fluor 594 phalloidin for one hour, followed by three washes in PBS. Cells were then cover slipped with Fluoro Gel mounting medium and imaged on an Olympus BX51 fluorescence microscope. Fluorescence intensity was quantified using ImageJ.

HUVEC Culture

Human umbilical vein endothelial cells were purchased from Promocell. Cells were initially grown in 2% gelatin-coated 10-cm plastic dishes using Endothelial Growth Base Media (R&D Systems) supplemented with Endothelial Cell Growth Supplement (R&D Systems)

and penicillin/streptomycin in a cell culture incubator at 37°C with 5% CO_2 atmosphere as previously described (23). Cells were passaged by digestion in 0.25% trypsin in Hanks' Balanced Salt Solution (HBSS) after reaching 80% confluence. Cells were used for experiments between passages 1–3. All HUVEC experiments were repeated using 3 biological replicates. For succinate addition experiments, HUVECs were treated with the cell-permeable succinate analong dimethylsuccinate (DMS) at 50 μM for 24 hours. For lipopolysaccharide (LPS) addition experiments, cells were treated with 1 μg/ml for 24 hours. Some cells were treated with HIF-1α inhibitor GN44 (10 μM), NF-κB inhibitor SC514 (10 μM), or p300 inhibitor A485 (10 μM). These inhibitors were added concurrently with the LPS treatment.

SDS-Polyacrylamide Gel Electrophoresis Western Blots

HUVECs were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 M EDTA, 1% Triton X-100, and Halt™ protease inhibitor cocktail) as previously described(24). Proteins were quantified using Bio-Rad protein assay (Bio-Rad Laboratories), and 20–50 μg of protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–12% gradient acrylamide gel run at 100 V. Proteins were then transferred to 0.45 μm PVDF membrane at 30 V for 2 hours. Membranes were blocked in Tris Buffered Saline (TBS: 137 mM NaCl, 20 mM Tris Base), 0.1% Tween 20, and 5% bovine serum albumin (blocking solution) for 1 h, followed by overnight incubation with primary antibody diluted in TBS, 0.1% Tween 20, and 3% BSA, and 1 h incubation with horseradish peroxidase-conjugated secondary antibody diluted at 1:5,000. The primary antibody used for intercellular adhesion molecule-1 (ICAM-1) was Invitrogen MA5407 and the primary antibody for MCP-1 was Novus Biologicals NBP2-2215. Immunoreactive protein was detected using ECL (GE Healthcare) imaged on a Bio-Rad ChemiDoc™ MP Imaging System.

Statistical Analysis and Power Analysis

After checking for normal distribution, image staining intensity and western blot densitometry levels are presented as means ± standard error. A p-value of less than 0.05 was considered significant for all tests. For comparisons of more than 2 groups, one-way ANOVA was first performed with Tukey's multiple comparison post hoc test. All figures show means with error bars representing standard error.

Based on previous data showing a difference of approximately 25% and standard deviation of 16% of the mean (21), a power analysis and sample size determination were performed. With a significance level (alpha) of 0.05 and power (1−β) value of 0.8 was calculated; a sample number of 8 animals per group was required to achieve the 0.8 power value.

Results

Plasma and Endothelial Succinate Levels

Plasma succinate levels were not different in the DMM treated animals (118.3 vs. 155.3 mmol/L, p=0.64). High succinate levels lead to an increase in succinyl-lysine modification of proteins (5), allowing a cell-type specific determination of succinate elevations in tissue sections. To assess potential succinate elevations in the pulmonary endothelium in our

ARDS mouse model, succinyl-lysine stains were carried out and examined in lung tissue. Representative images of uninjured animals, LPS injured with sham treatment, and LPS injured with DMM treatment are shown in Figure 1A–C. As shown in Figure 1D, sham treated animals had higher levels of succinyl-lysine than uninjured (12,700 vs. 18,986 AU, p=0.004). Animals treated with DMM had lower succinyl-lysine levels in the endothelium than sham (18,986 vs. 13,818 AU, p=0.03) and similar levels as non-injured animals (13,818 vs. 12,700 AU, p=0.86).

Inflammatory Mediators

Measures of inflammatory cell influx into the bronchoalveolar lavage is shown in Supplemental Figure 2. Total cell count (6.93 vs. 2.46 × 10^6 total cells, p=0.04), macrophage count (168.6 vs. 83.5 macrophages, p=0.04) and lymphocyte count (527.7 vs. 279.5 lymphocytes, p=0.049) were all lower in the DMM treated. There were no neutrophils located in the BAL at sacrifice. Representative images of lung tissue on H & E staining are shown in Supplemental Figure 3.

To examine if alterations in pro-inflammatory protein expression in endothelial cells may mediate the effects of succinate elevation, additional experiments were performed in HUVECs to measure expression of MCP-1 and intercellular adhesion molecule 1 (ICAM-1). ICAM-1 is an important mediator of macrophage and lymphocyte influx in lung inflammation (25, 26). To examine if succinate alone will increase MCP-1 or ICAM-1, we exposed HUVECs to dimethyl succinate (DMS), a cell permeable succinate analog (Figure 2A–C). DMS alone increases both MCP-1 and ICAM-1 expression when compared to uninjured HUVECs. LPS has been previously been shown to elevate succinate in HUVECs (5). We measured MCP-1 and ICAM-1 in HUVECs treated with LPS and found that HUVECs with LPS injury only had higher MCP-1 and ICAM-1 expression than uninjured, while those treated with DMM had no difference when compared to uninjured animals (Figure 3A–C). In the presence of the LPS plus HIF-1α inhibitor GN44, relative expression of MCP-1 and ICAM-1 was greater than that of uninjured HUVECs. In the presence of the LPS plus NF-κB inhibitor SC514, relative expression of MCP-1 and ICAM-1 after LPS injury was higher than that of uninjured HUVECs (Figure 3A–C).

To examine if DMM may exert its anti-inflammatory effects through p300, we carried out experiments in HUVECs with a p300 inhibitor A485 (Figure 4A–C). Addition of A485 resulted in decreased expression of MCP-1 and ICAM-1 as compared to HUVECs injured with LPS, but expression of both MCP-1 and ICAM-1 was still higher that uninjured control (Figure 4A–C).

An examination of inflammatory cytokines in BAL (Figure 5) demonstrated that DMM treated animals had lower levels of interleukin-6 (427.5 vs. 93.6 pg/mL, p=0.001). Interferon-γ and tumor necrosis factor-α levels were not significantly different.

Pulmonary Capillary Leak

A comparison of BAL protein leak showed that treatment with DMM in LPS lung injured mice resulted in decreased protein leak (Supplemental Figure 4). When examining the pulmonary capillary endothelial glycocalyx, LPS injury with sham injection resulted in

decreased staining intensity of the endothelial glycocalyx, while treatment with DMM resulted in higher staining intensity than Sham, with no difference from uninjured animals (Figure 6A–D).

Weight Loss

A comparison of weight loss in mice with lung injury is shown in Supplemental Figure 5. Animals that received DMM had less weight loss from baseline than those that received sham injections.

Discussion

Prior pre-clinical research has shown that DMM and other malonate esters have therapeutic potential to protect against ischemia-reperfusion injury (8–12). In both small and large animal models of hemorrhagic shock and resuscitation, our group showed that DMM can protect against damage to the pulmonary endothelial glycocalyx (2, 5). This study is the first to examine the therapeutic use of a malonate ester in an acute lung injury model.

Malonate esters reduce succinate production and accumulation in animal models of ischemia-reperfusion injury (2, 27). This study did not observe a difference in plasma succinate levels with DMM treatment that has been seen in other injury models (2). This may be related to using a direct lung injury model, rather than an ischemia-reperfusion model, as seen with myocardial infarction or hemorrhagic shock injury models (2, 8). However, the present study is the first to show that succinate is produced in the pulmonary vasculature endothelium in a model of acute lung injury and that DMM reduces succinate accumulation in this model (Figure 1). Our study and others indicate that succinate production leads to increased inflammation (19). We observed decreased inflammatory cell influx into the lungs, including macrophages and lymphocytes. In addition, we found decreased IL-6 in lung BAL. Other preclinical studies have corroborated this link between succinate and inflammatory cell function (28). In a mouse model of endotoxin sepsis, decreasing succinate with DMM results in decreased inflammatory macrophages (29). Inhibiting succinate production also results in decreased macrophage activation in a mouse model of myocardial infarction (30). In addition, succinate has been linked to lymphocyte infiltration in other animal models (31, 32).

We found decreased expression of pro-inflammatory cytokine MCP-1 with DMM treatment. MCP-1 plays a key role in mediating inflammation when succinate is produced.(17) Our study corroborates that DMM has an anti-inflammatory effect by decreasing succinate and MCP-1 production. Other studies have shown that the inflammation caused by succinate in vivo is mediated by HIF-1α and NF- κB. However, we found that the anti-inflammatory effect of DMM could not be explained by HIF-1α and NF- κB. Lung injury experiments with HIF-1α and NF- κB inhibitors did not mimic DMM's effects, suggesting that other mechanisms are responsible for the observed anti-inflammatory effect (17, 19, 33, 34).

The cell adhesion molecule ICAM-1 plays an integral role in inflammatory cell influx into the lungs after injury (35, 36). Our data indicates that succinate causes an increase in ICAM-1 expression. Furthermore, treatment with DMM's resulted in decreased expression

of ICAM-1. Similar to MCP-1, this could not be explained through HIF-1α and NF- κB. Further studies are needed to better elucidate how ICAM-1 is regulated through succinate production.

Prior studies have shown that succinate accumulation can also lead to succinylation of histones (37). Histone modifications help regulate chromatin structure, DNA unwrapping, and protein binding, playing an important role in gene expression and transcription. The p300 protein is a histone acetyltransferase that is known to succinate histones leading to an increased inflammatory cascade (16). We found that p300 is, at least partially, responsible for the endothelial inflammatory reaction to LPS. The p300 protein partially decreases both MCP-1 and ICAM-1 expression when compared to LPS injury alone, although not down to levels seen in uninjured HUVECs. This suggests that DMM at least partially inhibits the histone succinylation that contributes to the inflammatory cascade. Histone succinylation can also occur non-enzymatically, potentially explaining why p300 inhibition had only a partial effect (5). Future work will be needed to investigate this possibility.

This study agrees with our prior work that has shown that succinate is injurious to the endothelial glycocalyx (2, 5). In a rat hemorrhagic shock and resuscitation model, succinate accumulation led to damage to the endothelial glycocalyx. Treatment of animals with DMM preserved the endothelial glycocalyx. The damage to the glycocalyx was thought to be mediated through matrix metalloproteinase-24 and −25 (5). In a translational model, using swine that underwent hemorrhagic shock, followed by resuscitation, DMM was again found to protect the endothelial glycocalyx in the pulmonary vasculature (5). In the present study, direct acute lung injury with LPS led to succinate in the pulmonary vascular endothelium. This also resulted in damage to the endothelial glycocalyx. Furthermore, treatment with DMM resulted in decreased succinate in the pulmonary vascular endothelium and prevented damage to the endothelial glycocalyx. This resulted in improved pulmonary vasculature barrier function as animals treated with DMM had decreased protein leak into the lung interstitium. Interestingly prior studies have shown that epithelial succinate in acute lung injury has a protective role. Intratracheal dimethylsuccinate administration has been shown to reduce inflammation, improve alveolar barrier function, and decreased histopathological injury in a mechanical ventilation induced lung injury model. Further studies are needed to define how succinate in the endothelium and epithelium can have opposing effects in acute lung injury and ARDS.

Our study supports others that suggest malonate esters such as DMM may have therapeutic potential in treating severely injured trauma patients. Trauma patients that develop ARDS have often suffered ischemia-reperfusion injury in the setting of hemorrhagic shock and resuscitation (38). In addition, multiple system organ failure is a risk factor for developing ARDS in trauma patients (39). Malonate esters can protect multiple organ systems, including the lung, in ischemia-reperfusion injury (8–12). Furthermore, succinate appears to be a mediator of injury in trauma patients (1). Collectively, this body of data suggests that malonate esters may be a potential therapeutic in the organ failure that occurs after traumatic hemorrhage.

This study is not without limitations. First, we used the LPS model of acute lung injury as it is commonly used and easily reproducible. Further studies are needed to test DMM in in infectious and trauma models of ARDS to determine if this may change findings. A more comprehensive analysis of inflammatory cytokines, as well as other laboratory parameters such as coagulation studies, blood cell counts, and platelet function are needed. Repeating experiments with genetically altered knockout mice may also shed new light on the role of succinate in lung injury. Physiological studies are also needed to determine if DMM preserves pulmonary mechanics and compliance after lung injury. Furthermore, large animal models of ARDS are needed for translation of DMM from bench to bedside. In addition, there are potential toxicities that could be associated with malonate esters. A comprehensive metabolomics analysis using isotope-labeled tracers in vivo to better define reduced de novo fluxes into succinate would describe potential byproducts of inhibiting succinate dehydrogenase with malonate esters. Finally, some mechanistic studies were performed in vitro using HUVECs. More in vivo mechanistic studies are needed to better define the deleterious effects of succinate in ARDS.

In conclusion, DMM may have a therapeutic benefit in preventing and treating acute lung injury and ARDS. DMM reduces succinate in the endothelium of the pulmonary vasculature and results in decreased inflammation in the lungs. This is at least partially mediated by inhibiting succinylation of histones by p300. In addition, DMM prevents damage to the pulmonary endothelial glycocalyx, which results in decreased protein leak into the lung parenchyma. Additional studies are needed to determine how malonate esters can be used in patients with ARDS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Conflicts of Interest and source of funding:

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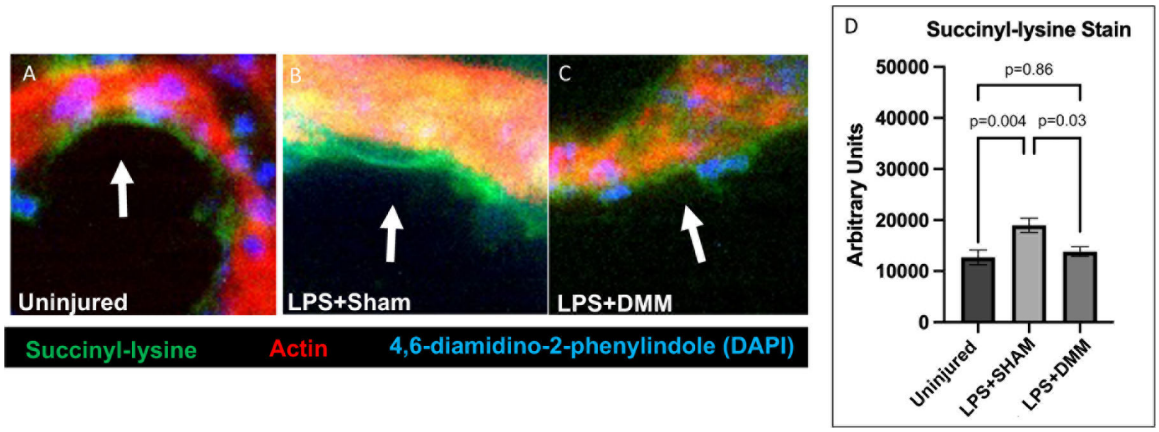

Figure 1 –.

Succinyl-lysine stains were performed in the pulmonary capillaries to examine succinate levels in the pulmonary epithelium. Representative images show tissue from A) uninjured animas, B) LPS injured animals treated with sham injections, and C) LPS injured animals treated with dimethyl malonate. A comparison (D) shows sham treated animals had more succinate in the pulmonary endothelium than control. Succinate levels in DMM treated were no different than uninjured and were less than sham treated.

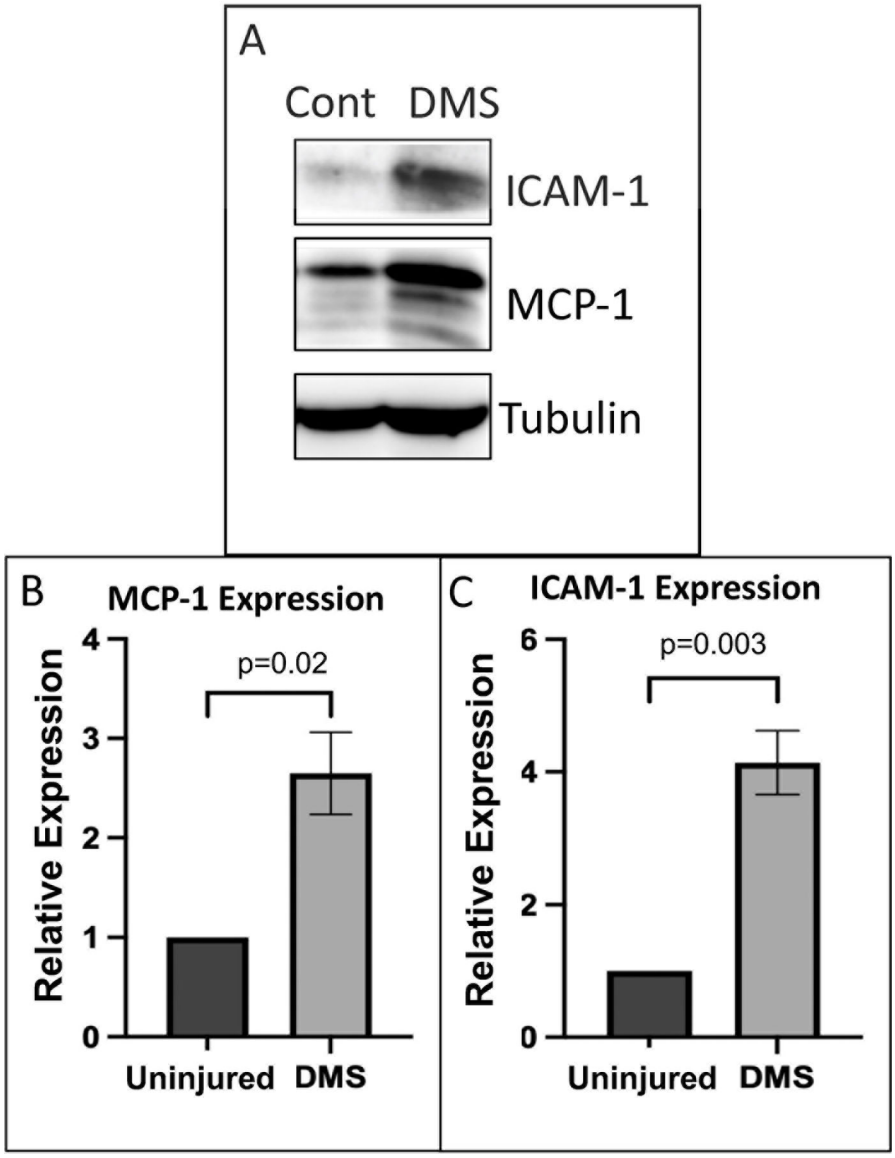

Figure 2 –.

Human umbilical vein endothelial cells (HUVECs) were exposed to dimethyl succinate (DMS) and compared to uninjured cells. Western Blot (A) shows increased B) MCP-1 and C) ICAM-1 expression in HUVECs exposed to DMS when compared to uninjured cells.

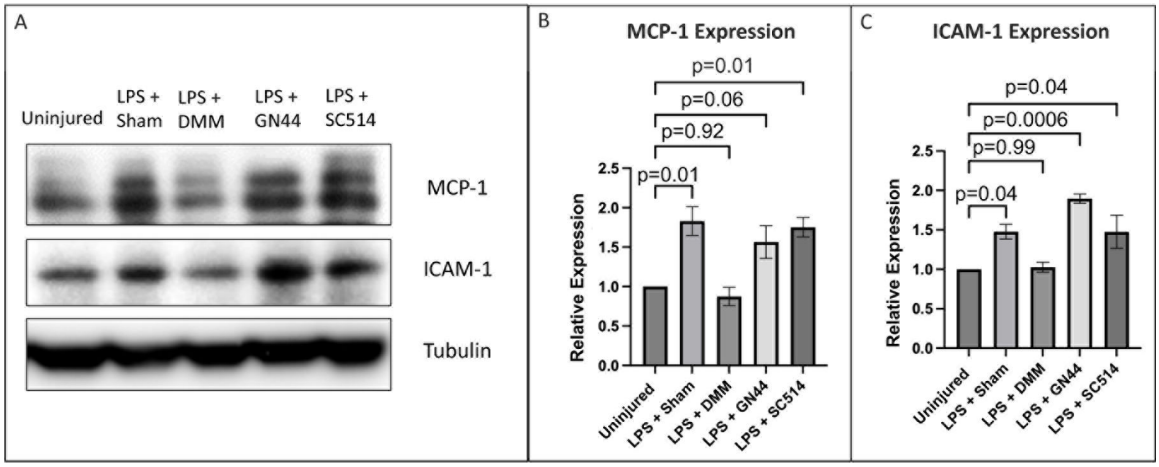

Figure 3 -

Human umbilical vein endothelial cells (HUVECs) were exposed to DMM, a HIF-1α inhibitor (GN44), and an NF- κB inhibitor (SC514). Western Blot analysis (A) shows that DMM resulted in decreased B) MCP-1 and C) ICAM-1. Addition of GN44 and SC514 did not decrease expression of MCP-1 and ICAM-1.

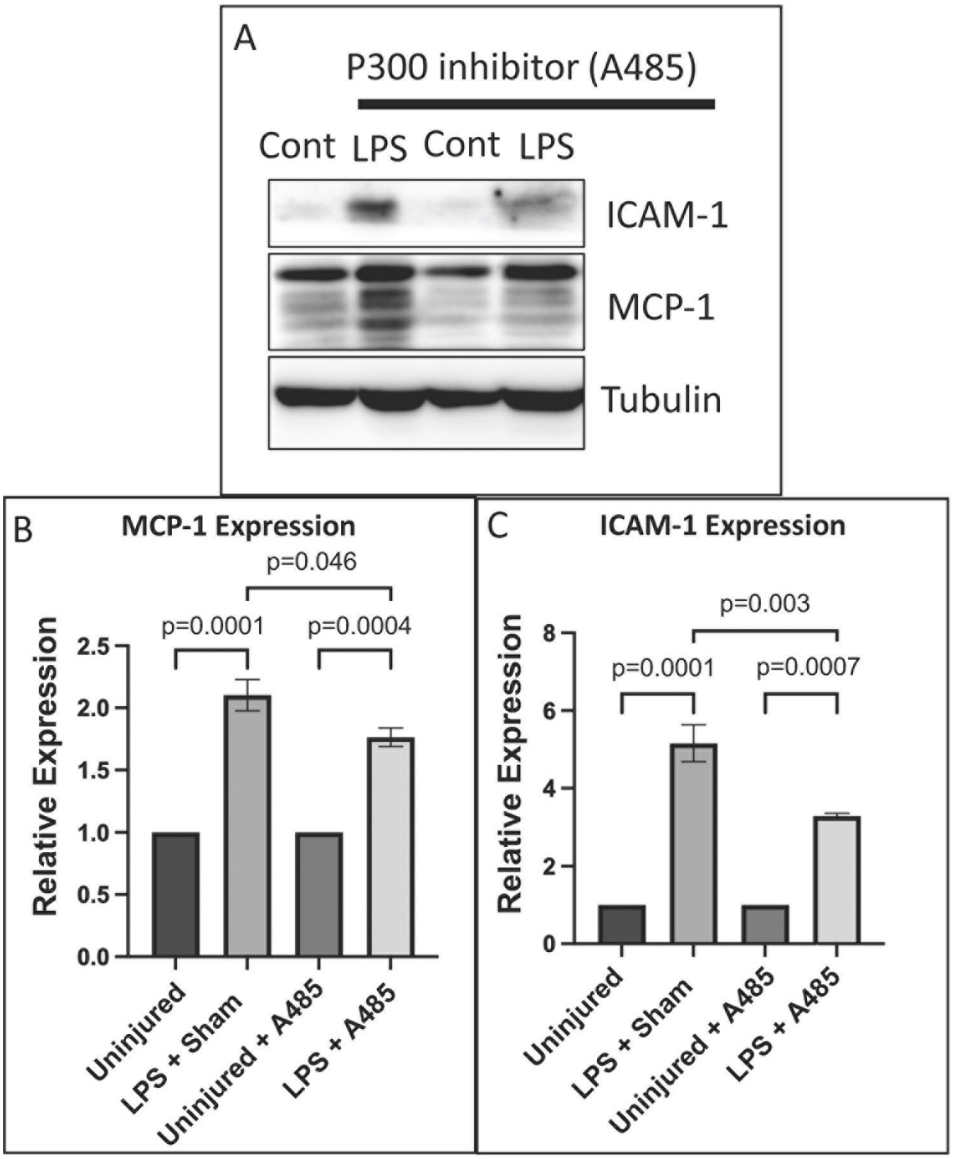

Figure 4 –.

Experiments in HUVECs were repeated with a P300 inhibitor (A485). Western Blot (A) shows that the presence of A485 results in decreased B) MCP-1 and C) ICAM-1 expression in the presence of A485 as compared to HUVECs that get LPS injury and sham treatment.

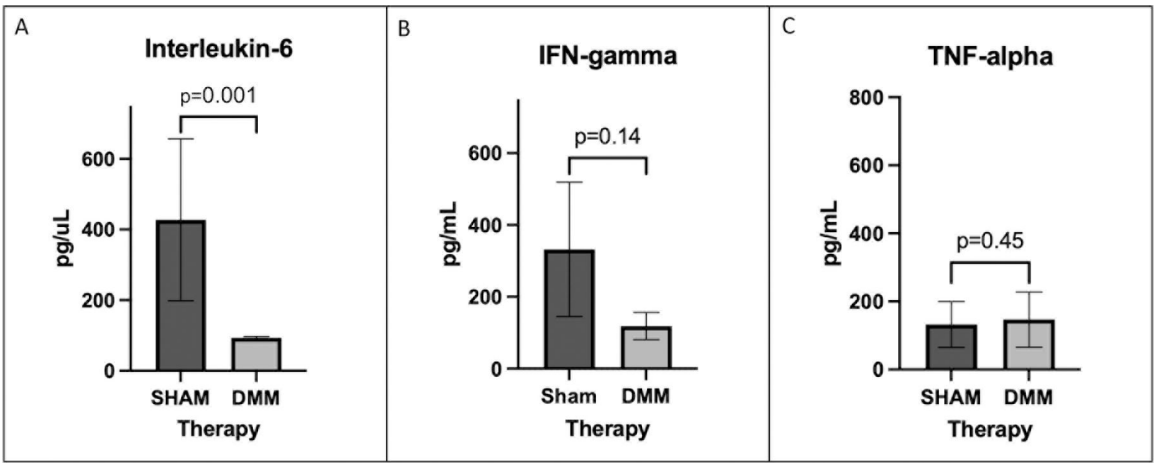

Figure 5 -

Treatment of LPS injured mice with DMM resulted in decrease A) interleukin-6 in bronchoalveolar lavage fluid when compared to sham animals. DMM did not change the amount of B) interferon- γ or C) tumor necrosis factor-α in bronchoalveolar lavage fluid.

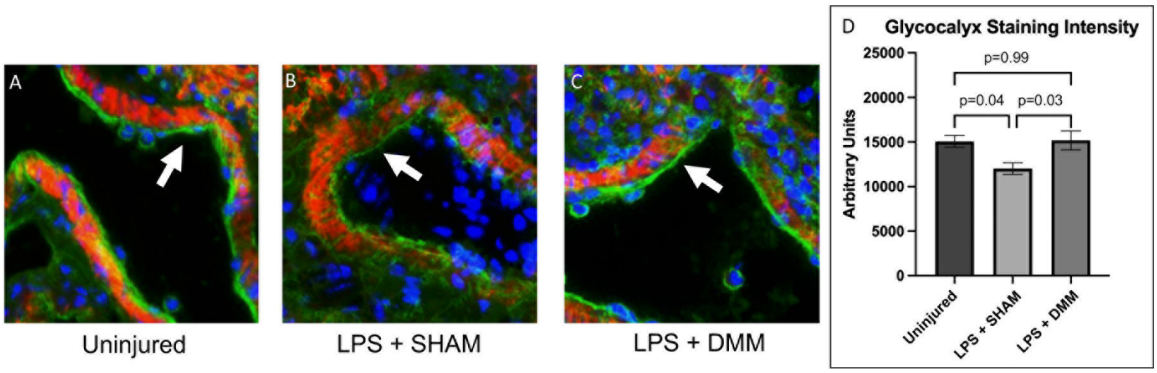

Figure 6 –.

Representative images of the pulmonary vasculature of the endothelial glycocalyx are shown in A) uninjured animals, B) animals receiving LPS injury with sham treatment, and C) animals receiving LPS injury and DMM treatment. Treatment with DMM resulted in D) endothelial glycocalyx staining intensity that was less than sham treated and no different from the control.