HEME-INDUCED ACUTE LUNG INJURY BY INFLAMMASOME ACTIVATION IN VASCULAR ENDOTHELIUM

by

Bethany J. Flage

BA, Malone University, 2014

Submitted to the Graduate Faculty of

Infectious Diseases and Microbiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2018

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This thesis was presented

by

Bethany J. Flage

It was defended on

April 17, 2018

and approved by

Moses Bility, PhD, Assistant Professor, Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Samit Ghosh, PhD, Research Assistant Professor, Medicine
School of Medicine, University of Pittsburgh

Thesis Director: Robbie Mailliard, PhD, Assistant Professor
Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Robbie Mailliard, PhD

HEME-INDUCED ACUTE LUNG INJURY BY INFLAMMASOME ACTIVATION IN VASCULAR ENDOTHELIUM

Bethany J. Flage, MS

University of Pittsburgh, 2018

ABSTRACT

Acute lung injury (ALI) is a form of acute respiratory failure, having an estimated annual incidence of 200,000 with a 40% mortality rate in the United States alone. Patients suffering from hemolytic diseases such as sepsis, malaria, and sickle cell disease are placed at a greater risk of developing ALI because of the excess amount of heme release that occurs during the pathogenesis of these diseases. Free heme is known to be a danger associated molecular pattern (DAMP) molecule and previous reports from our lab have identified particularly toxic effects apply to the lung endothelial barrier, ultimately contributing to ALI. However, the cellular mechanism of how heme is able to cause endothelial damage remains largely unknown. Recent studies have revealed that heme is able to induce the activation of nod-like receptor 3 (NLRP3) inflammasome in monocytes, which is followed by the maturation and release of inflammatory molecules Interleukin-1β (IL-1β) and Interleukin-18 (IL-18). Therefore, in this study we hypothesized that heme induces NLRP3 in pulmonary microvascular endothelial cells, resulting in destructive inflammatory activity that leads to the development of ALI. To test this hypothesis, we challenged human primary microvascular endothelial cells (HMVEC-Ls) with heme and assessed cell death, barrier integrity, cytokine release, and NLRP3 activation. Heme challenge of HMVEC-Ls

produced a 75% decrease in barrier resistance as compared to vehicle, measured via electrical cell-substrate impedance sensing (ECIS). This barrier instability was concomitant with a significant 3-fold increase in IL-18 ($p=0.046$) release and an increase in NLRP3 protein, measured through extracellular supernatant and western blot respectively. Importantly, inhibition of NLRP3 resulted in a 35% increase in barrier integrity, which corresponded to a 20% decrease in lung wet-to-dry weight ratio in mice that were injected with heme. These results demonstrate that heme mediated endothelial damage involves an inflammatory response associated with NLRP3 activation, which is a potential target for therapeutic intervention. Future studies are warranted to confirm heme-mediated inflammasome formation and activation. Identifying specific molecular and cellular mechanisms by which heme damages the vascular endothelium in the pathogenesis of ALI is important for decreasing morbidity among patients and improving the overall health of the public.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

I would like to give the utmost thanks to my advisor and thesis director, Dr. Robbie Mailliard, for his constant guidance and encouragement throughout this project. I would also like to thank my committee members, Dr. Samit Ghosh for his devotion and support to the project, and Dr. Moses Bility, for his insightful feedback and advisement. Special thanks to Dr. Solomon Ofori-Acquah for his incredible support and cooperation throughout my completion of this MS program, and to Dr. Aisha Walker, Dr. Rimi Hazra, Dr. Maureen Mburu, Diane Lenhart, and Danielle Crosby for their willingness to provide feedback every step of the way and overall enthusiasm in assistance.

1.0 INTRODUCTION

1.1 EPIDEMIOLOGY

Acute lung injury (ALI) is a form of respiratory failure with an estimated annual incidence of 200,000 and a devastating mortality rate of 40% in the United States alone. Several conditions resulting from disease or physical trauma may predispose certain patients to develop ALI, while factors such as age and race have been implicated in affecting overall mortality. For example, it is established that younger patients are more resilient, and therefore survive ALI at a much higher rate than their older counterparts. In addition, racial inequalities are shown to result in higher 60-day mortality rates among African-Americans and Hispanics as compared to Caucasians (1-4). Predisposing conditions or clinical factors for the development of ALI include sepsis, trauma, blood transfusions, smoke or gas inhalation, sickle cell disease (SCD), pneumonia, and pancreatitis (5-9).

Over the past two decades there has been an apparent downward trend in mortality associated with ALI (10, 11). However, this trend should be received with caution as a precise clinical definition for ALI was only recently reached in 1994. Prior to this, the diagnosis criteria varied greatly, making the epidemiologic assessment of ALI incidence

extremely challenging. According to the American-European Consensus Conference Committee, the official definition of ALI occurs in a patient that meets the flowing criteria:

- arterial oxygen partial pressure to fractional inspired oxygen ratio (PaO_2/FiO_2) $\leq$ 300
- chest radiograph of acute onset bilateral pulmonary infiltration
- absence of left atrial hypertension (12).

The definition has become widely used because of its simplicity and effectiveness in quantifying the overall severity of lung injury (4, 13), however the objective approach that is required in order to quantify chest radiographs has been called in to question, potentially indicating the need for a revision (14, 15).

1.2 PATHOGENESIS

In general, the pathogenesis of ALI is caused by the acute agitation of the alveolar-capillary membrane. The alveolar-capillary membrane, as depicted in figure 1, involves the microvascular endothelial cells, which line the blood vessels of the lung, and the alveolar epithelial cells which line the alveolar sacs of the lung. The alveolar epithelial cells are further divided into two types: type I and type II. The vast majority of the alveolar surface area is composed of fragile type I cells, leaving only about 10 percent of total area for the more resistant type II cells. Type II cells are also responsible for surfactant production, ion transportation, and epithelial cell repair. Under normal physiological circumstances, shown on the left of figure 1, endothelial cells form a tight barrier, disallowing the circulating cells from passing through into the interstitial

space, while healthy alveoli are free of excess fluid and contain only inactivated circulating alveolar macrophages, surfactant, and inspired air.

During ALI, as shown on the right of figure 1, the barriers made by the cells of the microvascular endothelium and the alveolar epithelium are disrupted. Initial damage to the endothelial barrier leads to an increase in pro-inflammatory molecules as a direct result of endothelial cell damage. Barrier instability and an uptick of adhesion molecules along the endothelial cells increases barrier permeability, allowing circulating and recruited neutrophils to enter the interstitial space. Activated neutrophils further produce pro-inflammatory cytokines and pro-apoptotic mediators, extrapolating the recruitment of additional neutrophils through chemotaxis. As capillary permeability continues to increase, protein rich fluid floods the interstitial space. This pressure and influx of pro-inflammatory and pro-apoptotic mediators causes barrier instability between type I and type II alveolar epithelial cells, allowing excess fluid into airspaces of the alveoli, clinically recognized as pulmonary edema (16). Interruption of the normal fluidic balance between the epithelial barrier prevents type II epithelial cells from pumping out edema flooding, broadening the existing trauma. Continued damage to type II cells causes dysfunction of surfactant production, staging the alveoli for a surfactant imbalance which may result in long-term surfactant dysregulation. Additionally, damage to the type II cells leads to unsystematic repair of the epithelial barrier, potentially promoting the formation of fibrotic tissue (17).

Resolution of ALI is dependent on the restoration of gas exchange, requiring correction in the fluidic imbalance between the alveolar epithelial barrier. Though neutrophil depletion has been shown to be a protective strategy against ALI, the

condition’s development in the absence of neutrophils indicates alternative pathways to dangerous inflammation (18-20). Pharmacological treatments include the use of β-2 agonists, which increase alveolar fluid transport, and the use of bone marrow-derived mesenchymal stem cells (MSCs), which aim to improve endothelial and epithelial barrier integrity.

ALI can progress rapidly leading to pulmonary vascular destruction, pulmonary fibrosis, and multiple organ failure due to low oxygen levels, the culmination of which ultimately concludes in death. Due to the severe damage that occurs in just one episode of ALI, those that survive often experience long-term physical, cognitive, and mental adverse effects (21).

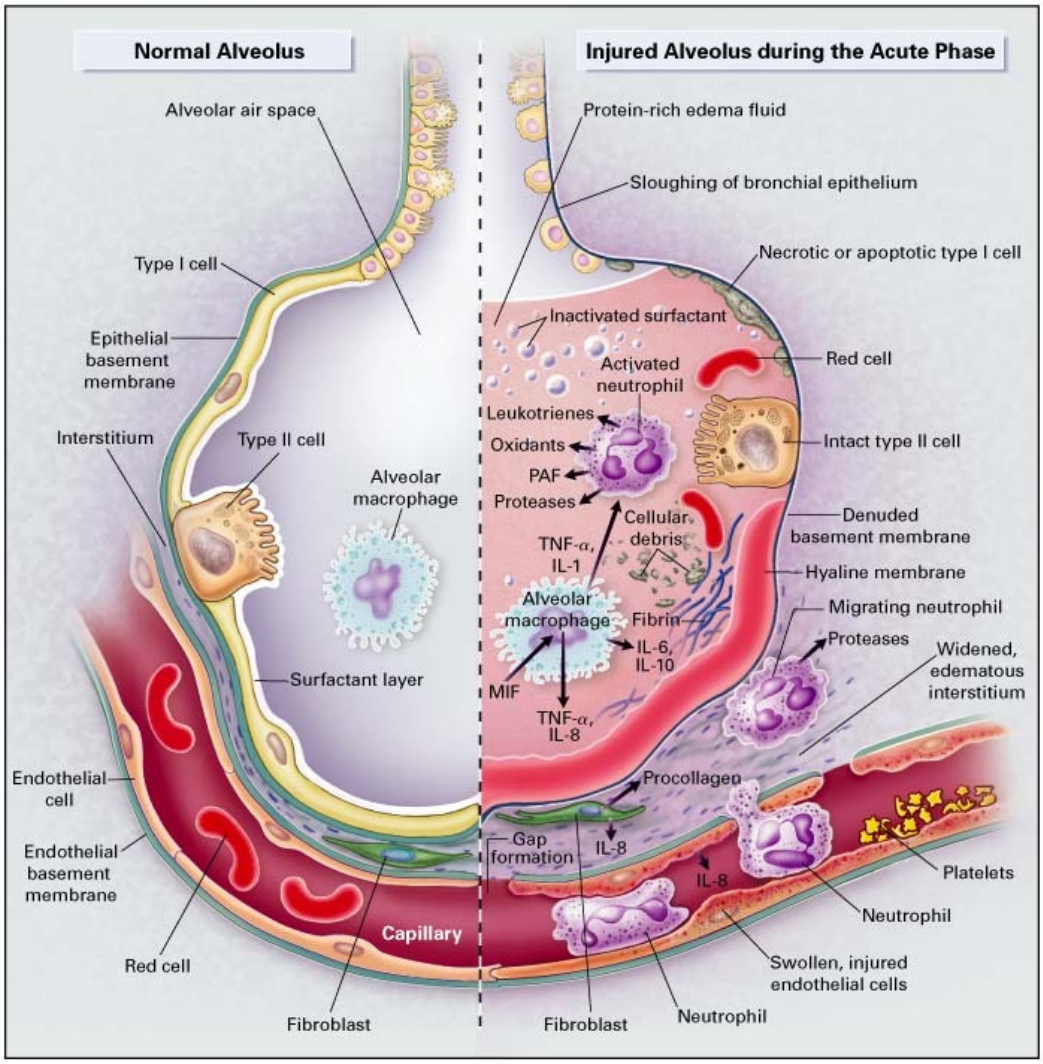

Figure 1. Normal alveolus (left) compared to alveolus during acute lung injury. When the lung endothelial barrier is disrupted, neutrophils are recruited and migrate into the interstitial space. Pro-inflammatory molecules cause further disruption of the alveolar epithelial barrier, allowing a flood of edema fluid into the alveolar space. Continued damage results in cell death among both epithelial and endothelial cells. Figure reproduced with permission from The New England Journal of Medicine (22), Copyright Massachusetts Medical Society.

The pathophysiology of ALI development may differ between stimuli and has been shown to be an important predictor of outcome. For example, infection-onset ALI has a higher mortality rate than trauma-induced ALI (23). It is also known, as previously mentioned, that certain ailments may predispose patients to ALI development. The

majority of cases develop in the presence of an infection, the most common of which being primary pneumonia (4). Sepsis is the second most common cause, and contrary to pneumonia may result from a non-pulmonary associated source. Other non-pulmonary associated sources include malaria and SCD, and though these diseases differ greatly, they are all associated with a high degree of intravascular hemolysis. Moreover, studies of sepsis have actually found that the higher the degree of intravascular hemolysis, the poorer the clinical outcome (24-26). Together these indicate a viable link between a large degree of intravascular hemolysis and the development of ALI.

1.3 HEMOLYSIS, HEME, AND ALI

The large degree of intravascular hemolysis that occurs in SCD, malaria, and sepsis may be a primary stimulus for ALI in patients. As demonstrated in figure 2, hemolysis is the rupture of red blood cells (RBCs) which allows for the release of hemoglobin directly into the bloodstream. Ferrous (Fe^{2+}) hemoglobin, when free from the RBC, is easily oxidized to ferric (Fe^{3+}) hemoglobin. Upon the addition of a single oxygen molecule, four heme molecules are released from hemoglobin. Heme is the oxygen binding portion of hemoglobin and holds essential functions in aerobic organisms including chemical catalysis and electron transfer. However, upon release from the hemoglobin molecule, heme becomes “free” and is toxic to the body via the formation of reactive oxygen species (ROS) and ability to trigger strong inflammatory responses. Free heme is a known danger-associated molecular pattern (DAMP), which

is an endogenous molecule capable of exacerbating the immune response. During cases of severe hemolysis, such as the aforementioned malaria, sickle cell disease, and sepsis, large amounts of hemoproteins are released into the circulation, thus leading to the release of large amounts of toxic free heme. The accumulation of this endogenous toxic molecule poses a problem specifically to the cells lining the blood vessels, including the vascular endothelium of the lung capillary-alveolar membrane.

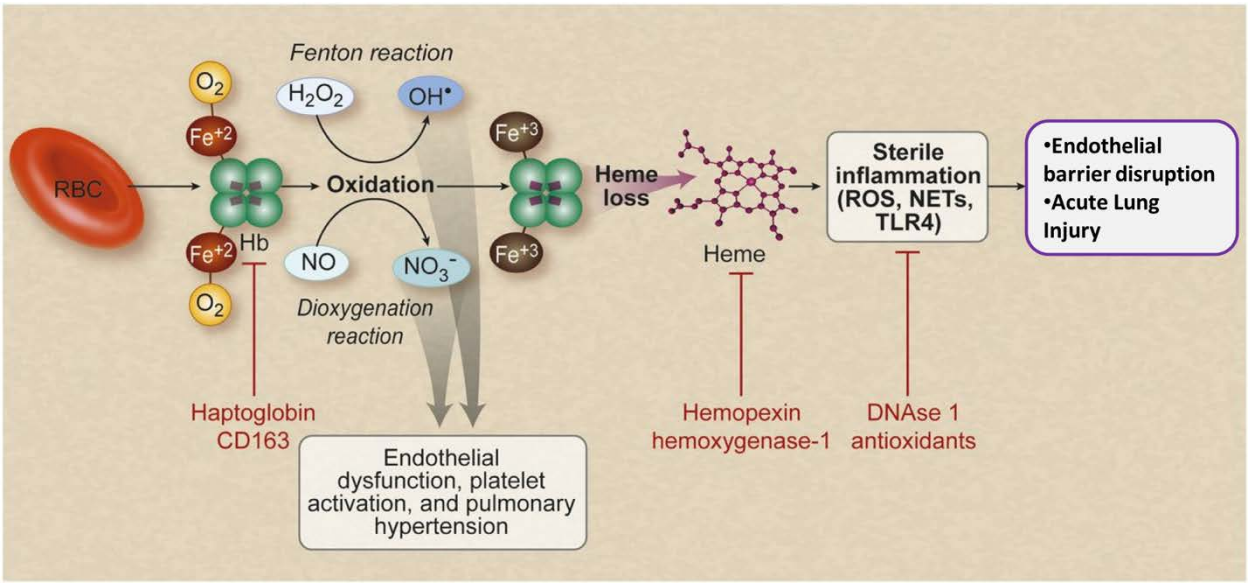

Figure 2. Hemolysis results in the release of free heme, designated as an erythroid DAMP molecule.
Excess circulating heme can result in vascular injury through sterile inflammation, which may contribute to the pathogenesis of hemolytic diseases. Original figure adapted with permission from Blood (27).

1.3.1 Mechanisms of Heme induced lung injury

The connection between hemolysis and induced lung injury has been exhibited in numerous experimental animal models, including those of sepsis and SCD (26, 28 R 2010). It is known that endothelial cells of the alveolar-capillary membrane are receptive

to heme through the pattern recognition receptor (PRR) toll-like receptor 4 (TLR4) (29). Endothelial cells activated by heme promote the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin, aiding in the migration of neutrophils across the endothelial barrier (29-32). Furthermore, heme induces interleukin-8 (IL-8), an important chemoattractant of neutrophils and recognized bio-marker of ALI.

Importantly, it has been established that rescue from ALI is possible through the use of heme-scavengers, namely hemopexin (Hx) and haptoglobin (Hp). These endogenous molecules exist to scavenge heme under normal physiologic circumstances. Hx in particular, binds heme with high affinity and transports it to the liver for degradation. However, these scavengers become exhausted in individuals suffering from severe hemolytic crisis, resulting in an amass of toxic heme throughout the circulation. Experimentally, it has been shown that acute exacerbation of heme followed by therapeutic treatment with Hx alleviates respiratory failure in a murine model of ALI (33). In murine models of sepsis, prophylactic treatment with Hx prevented mortality (26), while clinically, high Hp levels are associated with lower mortality rates in adults suffering from sepsis (25).

Together these studies strongly suggest that heme and defective heme-scavenging mechanisms is a significant contributor to the pathophysiology of ALI. Unfortunately targeting and replacing the Hx scavenger may have limited therapeutic value in patients, for though it is successful in resolving ALI, significant liver damage occurs (34). Therefore, additional studies to elucidate other mechanistic pathways, such

as intracellular inflammatory signaling pathways of the endothelial cells, are needed to identify new potential targeted-therapies to prevent or treat heme-induced ALI.

1.4 TLR4 AND INFLAMMASOME ACTIVATION

TLR4 activation is also known to induce the assembly of inflammasomes, which are multiprotein complexes of the innate immune response functioning to activate pro-inflammatory molecules that lead to an inflammatory cascade. The exact composition of the inflammasome complex depends on the stimulus, but generally consists of a caspase protein, the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (35), and either sensor protein family NOD-like or AIM-like receptors, as displayed in figure 3. After recognition of a specific stimulus by the sensor protein, the adaptor protein, ASC, recruits the caspase protein (for simplicity, caspase-1) which is then cleaved into its active subunits p10 and p20. Once activated, caspase-1 can cleave immature inflammatory cytokines, pro-interleukin-18 (pro-IL-18) and pro-interleukin-1β (pro-IL-1β) to their active form interleukin-18 (IL-18) and interleukin-1β (IL-1β), which is illustrated by figure 4. Upon release on the systemic level, IL-1β causes fever and leukocyte migration, while IL-18 induces interferon-γ (IFNγ) and has been shown to have natural killer cell priming activity (36).

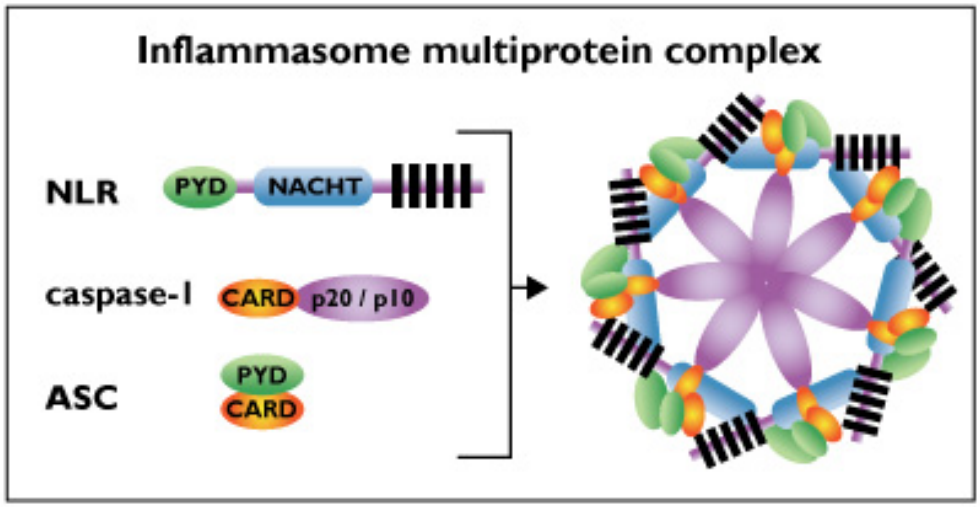

Figure 3. General inflammasome composition.
Original picture reproduced from InvivoGen. "Inflammasomes-Review" (2012). www.invivogen.com

1.4.1 The NLRP3 Inflammasome

Of the known inflammasome complexes, the nod-like receptor protein-3 (NLRP3) is the most widely characterized and is known to be activated by a variety of stimuli of both endogenous and pathogenic nature. In human disease, NLRP3 has been found to be the cause of cryopyrin-associated periodic syndromes (CAPS) which are a group of recognized genetic autoinflammatory diseases (37, 38). In addition to CAPS, NLRP3 also has implications in gout, type-II diabetes, obesity-induced insulin resistance, cancer, asbestosis, and Alzheimer's disease (39-50).

The exact course of NLRP3 activation can be a debatable subject, as there seem to be a variety of mechanistic pathways that each involve multi-step signal activation. The two major recognized activation pathways are broken down into the canonical pathway and the non-canonical pathway. For the canonical pathway, depicted in figure 4, two step-wise signals are required for activation. First, TLR

stimulation, which seems to function in order to initiate the transcription of immature cytokines via cleavage by caspase-1. While these cell damage signals are still under investigation, popular signals seem to include potassium efflux (51) and ROS generation (52).

The non-canonical pathway, as depicted in figure 5, is considered dependent on m-caspase-11 (mouse-caspase-11; human-caspase-4 and capsase-5) (53, 54), whereby once activated, it promotes the activation of NLRP3 and indirectly enhances pro-IL-18 and pro-IL-1β (35, 55-61). Pryoptosis, a highly inflammatory form of programmed cell death follows NLRP3 initiation of the pro-inflammatory cascade in both pathways.

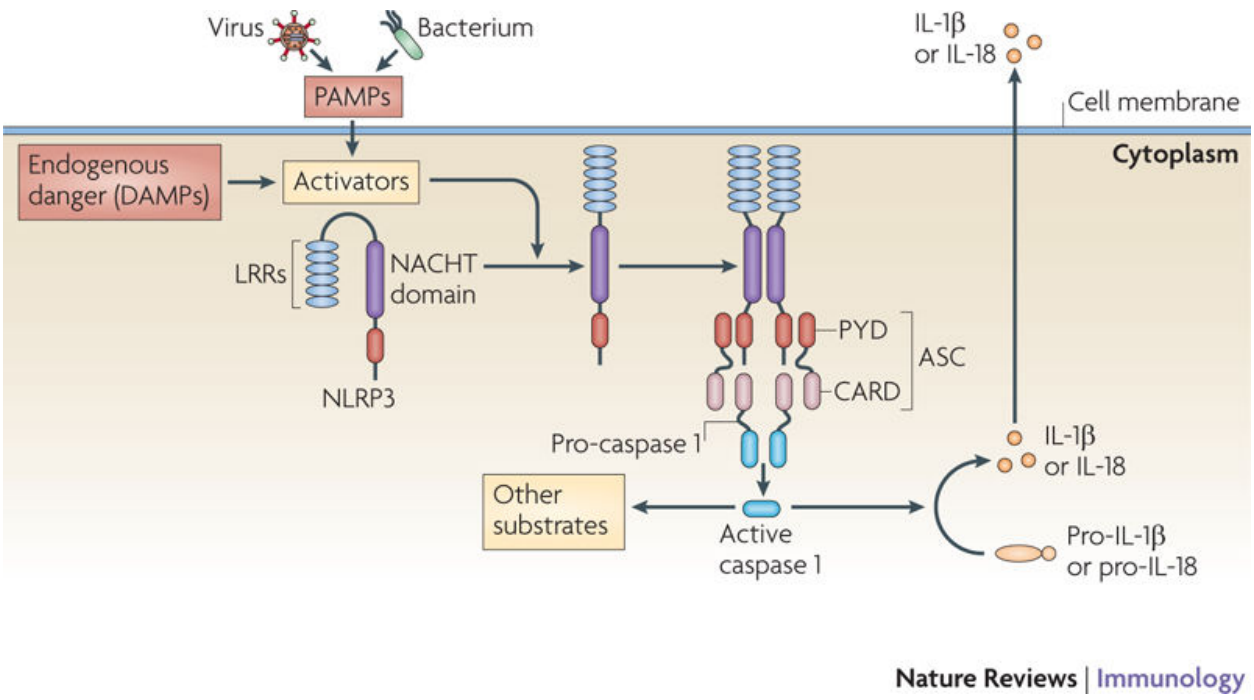

Figure 4. Stimulation caused by PAMP or DAMP molecules causes oligomerization of NLRP3 inflammasome.
When activated, inflammasome cleave caspase-1 for activation which leads to the cleavage of pro-IL-18 and pro-IL-1β into mature forms prepared for release from the cell. Reproduced with permission from Nature Reviews Immunology (62).

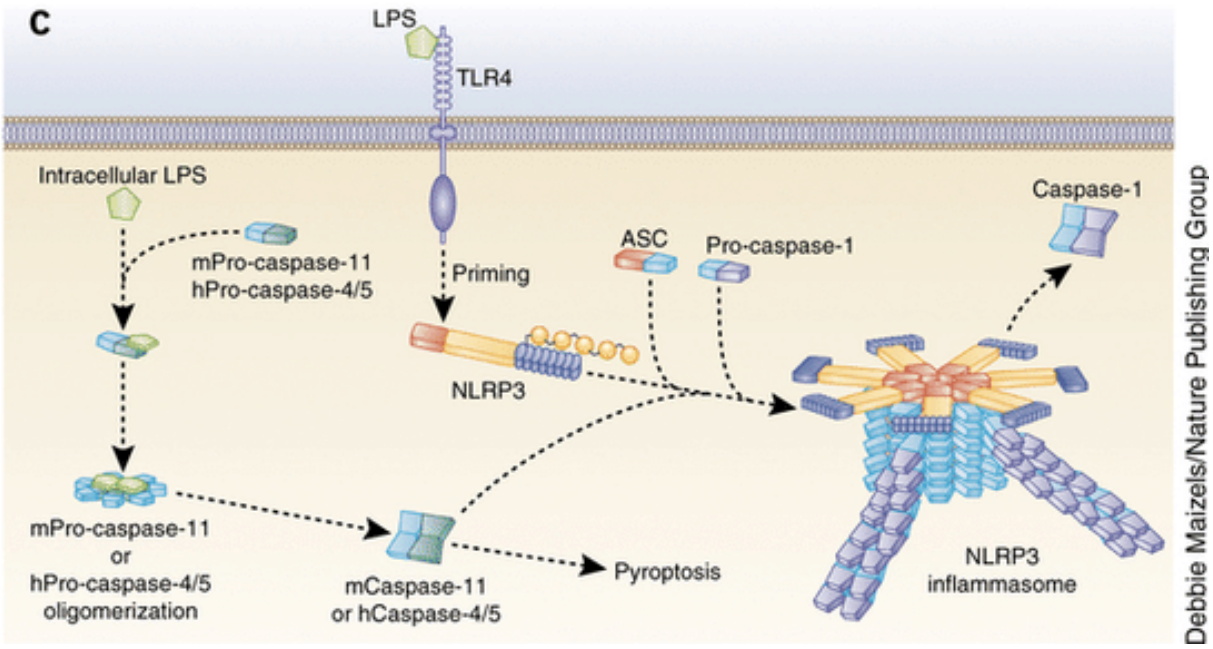

Figure 5. Non-Canonical NLRP3 Activation.
Intracellular stimulation of caspase-11 indirectly increases pro-IL-18 and pro-IL-1β and causes oligomerization of NLRP3 inflammasome. Reproduced with permission from Nature Medicine (63).

What separates these two pathways is the stimulus. The non-canonical pathway is known to be induced by several gram-negative bacteria, but not gram-positive bacteria, which indicates a specific role for TLR4. Studies employing cytosolic LPS have been shown to activate the non-canonical pathway (57, 64), indicating an independence from TLR4. However, both canonical and non-canonical pathway signaling are increasingly being found to have an additional dependence on caspase-8 for priming and activation of TLR-associated stimuli (65-67). Furthermore, studies have indicated

that different stimuli result in a differential balance of IL-1β and IL-18 release (68, 69) potentially concluding in a different immune response.

1.4.2 Heme as an Activator of NLRP3

Several studies have shown that inflammasomes play a critical role in sepsis and mechanical ventilation induced ALI development (70, 71). Furthermore, inflammasome activation, specifically NLRP3, via heme stimulus has been reported, but only in combination with lipopolysaccharide (LPS) priming in macrophages (72). While we have previously shown that acute exacerbation of circulatory heme, in the absence of LPS, triggers endothelial barrier disruption and ALI in vivo (33), the exact cellular mechanism behind this damage remains largely unknown. The association between heme and ALI combined with the fact that heme functions as a DAMP molecule able to signal through TLR4, lends the question of possible inflammasome activation via heme. However, inflammasome activation as a result of heme stimulus in the absence of LPS has not been studied in the context of ALI development.

2.0 STATEMENT OF THE PROJECT

In this project, we seek to investigate the pathobiological role of free heme in activating the NLRP3 inflammasome within vascular endothelial cells. Inflammasome activation within vascular endothelial cells of the alveolar-capillary membrane would contribute to the inflammatory response recognized during ALI as well as the dysfunction of the endothelial barrier that causes ALI. We will test the overall hypothesis that **extracellular heme induced acute lung injury is mediated by the activation of the endothelial NLRP3 inflammasome.**

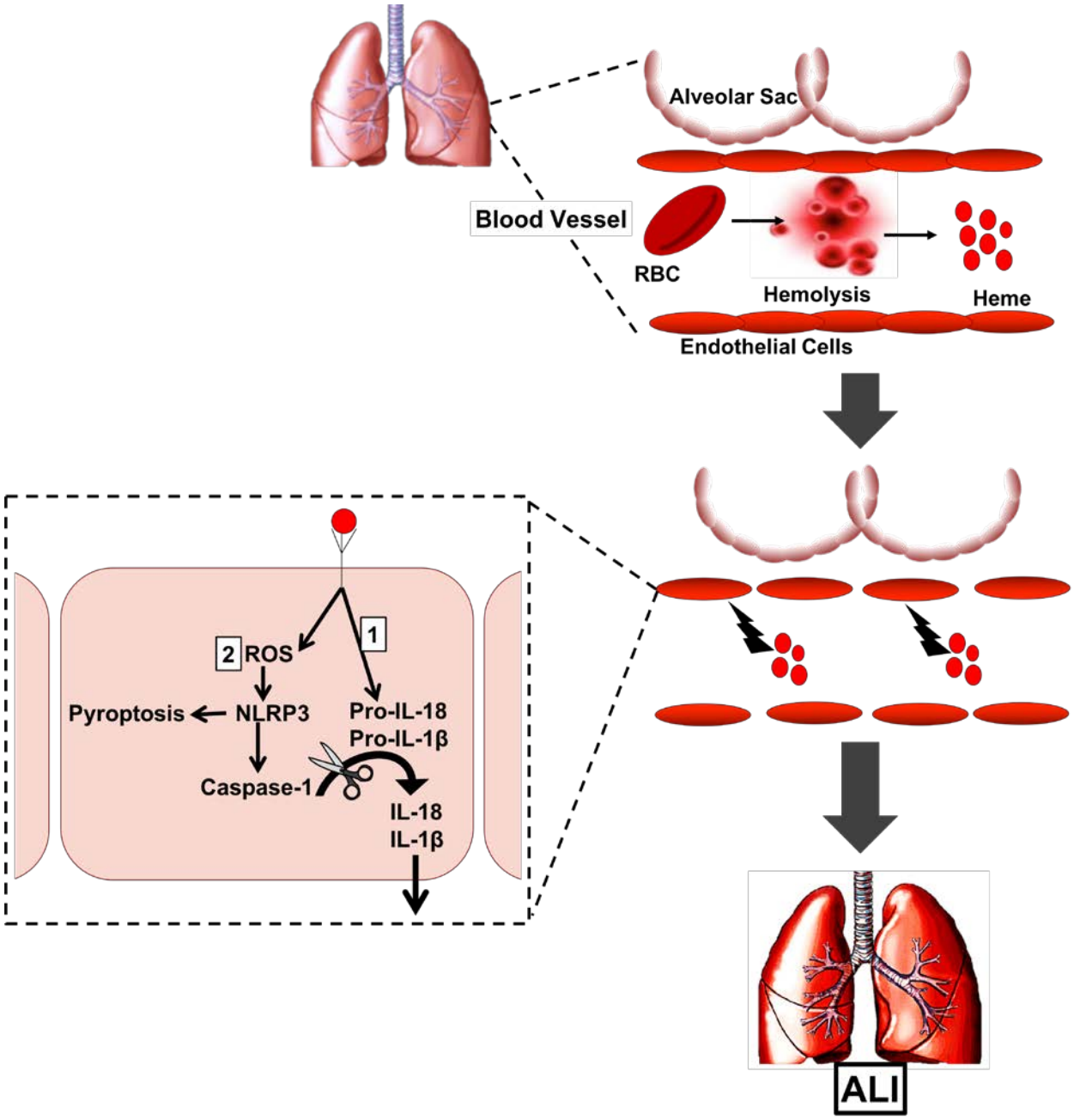

Figure 6. Graphical Abstract.

3.0 SPECIFIC AIMS

Two Specific Aims are formulated to test this hypothesis:

3.1 [AIM 1] DETERMINE THE ABILITY OF HEME TO INDUCE NLRP3 IN PULMONARY ENDOTHELIAL CELLS.

We will confirm that heme alone will induce NLRP3 in endothelial cells by measuring caspase-1 activity through the expression of IL-1β and IL-18 release in human primary microvascular endothelial cells (HMVEC-Ls). We will also test whether activation of NLRP3 is associated with endothelial barrier disruption. The following methods will be utilized to test the stated aim:

1. Assess impact of heme treatment on cell death and barrier stability using cultured HMVEC-Ls. Cell death will be quantified through lactate dehydrogenase assay, while specific barrier function will be assessed through electric-cell substrate impedance sensing technology (ECIS).
2. Evaluate potential presence of NLRP3 inflammasome machinery by measuring the output of IL-18 and IL-1β through ELISA.
3. Detect presence of NLRP3 inflammasome protein through western blot analysis.

3.2 [AIM 2] DETERMINE THE IMPORTANCE OF NLRP3 DURING HEME INDUCED ACUTE LUNG INJURY.

We will investigate whether NLRP3 activation is a critical component during the progression of heme induced acute lung injury. We will examine the effects of inhibition of NLRP3 in vivo, utilizing our model of heme induced ALI in transgenic sickle mice. The following methods will be used to fulfill the stated aim:

1. Establish the efficacy of NLRP3 inhibition in improving barrier dysfunction and cell death during heme treatment in HMVEC-Ls. Previously mentioned measurements of cell death and barrier stability will be utilized to measure potential rescue of cells during heme treatment.
2. Verify the inhibition of NLRP3 through expression of IL-18 and IL-1β via ELISA.
3. Determine efficacy of NLRP3 inhibition during in vivo heme-induced ALI.

At the end of this project, we will able to confirm whether NLRP3 is a significant factor in heme induced acute lung injury.

4.0 MATERIALS AND METHODS

4.1 REAGENT PREPARATION

4.1.1 Heme

Hemin (Frontier Scientific, Logan, UT) was dissolved in 0.25M sodium hydroxide (Fisher Chemical, Pittsburgh, PA) and pH was adjusted to 7.5 with 12M hydrochloric acid (Fisher Scientific). The solution was adjusted to the appropriate concentration in cell culture media or 1xPBS (Hyclone, GE Healthcare Life Sciences, Chicago, IL) for in vitro or in vivo experiments respectively. When necessary, hemin was dissolved in DMSO (Sigma, St. Louis, MO) and again brought to the appropriate concentration using cell culture media or saline (Thermo Scientific). For the vehicle analog, 0.25M sodium hydroxide was adjusted to pH 7.5 with 12M hydrochloric acid and the appropriate dilution was prepared using cell culture media.

4.1.2 MCC950

MCC950 (InvivoGen-10mg, San Diego, CA) was dissolved in DMSO and diluted to the appropriate concentration in cell culture media or saline. To avoid DMSO toxicity in mouse experiments, total volume of MCC950 was doubled using saline. For all

experiments with MCC950, heme was dissolved in DMSO as previously described to serve as the most appropriate vehicle.

4.2 CELL CULTURE

Human microvascular lung endothelial cells (HMVEC-L, Lonza, Basel, Switzerland) were cultured according to manufacturer's protocols in Endothelial Cell Growth Medium (EBM-2, Lonza) with added supplement pack (EGM-2MV SingleQuots, Lonza) containing 10% fetal bovine serum. Cells were plated at 125,000 cells/well on six-well plates, 20,000 cells/well on ECIS arrays, and 8,000 cells/well on 96-well plates and allowed to grow to confluence. On day of confluence, typically 48 hours after plating, cells were treated with the appropriate stimulant or vehicle. All experiments were performed using serum-free media.

4.2.1 Cell Lyses and Sample Collection

Samples were collected at pre-designated times, 3-12 hours after treatment. For supernatant sample collection, cell media was aspirated from cells and immediately frozen at -80°C until further analysis. For cell lysate sample collection, Pierce™ IP Lysis Buffer (ThermoFisher Scientific) containing Halt™ Protease Inhibitor (ThermoFisher Scientific) was placed directly on cells and incubated at 4°C for 10-15 minutes on a rocking plate. Cells lysates were then scraped from the plate and frozen at -80° C until further analysis.

4.3 LACTATE DEHYDROGENASE (LDH) ASSAY

HMVEC-Ls were cultured as previously described on 96-well plates. On day of confluence, typically 48 hours after plating, cells were stimulated with heme or heme+MCC950 with appropriate vehicle controls. At pre-designated times, entire supernatant volumes were transferred to a “collection” 96-well plate. Collection plate was placed in -30°C freezer in between different points of collection. After the collection of all samples, collection plate was brought to room temperature for quantification of LDH using QuantiChrom™ LDH Cytotoxicity Assay Kit (BioAssay Systems, San Francisco, CA) according to manufacturer’s protocols.

4.4 ELECTRIC CELL-SUBSTRATE IMPEDANCE SENSING (ECIS)

The Electric Cell-Substrate Impedance Sensing (ECIS) system (Applied BioPhysics, Troy, NY) uses impedance-based technology to detect cell activity and movement in response different stimulants. HMVEC-Ls were cultured as previously described and plated at 20,000 cells/well without serum on 8W10E+ PET arrays, which are arrays specially fitted with electrodes. On day of confluence, typically 48 hours after plating, arrays were placed on the ECIS apparatus. Within the ECIS incubator, a small current (I) applied to the arrays causes the cells to act as insulators, resulting in a measurable potential energy (V) across the cell monolayer. Together, the impedance (Z) is quantified using Ohm’s law, where Z=V/I. Under confluent conditions with no stimulant, the impedance remains constant. When a stimulant (for example, heme) is

added, or the cells are disturbed, their insulating activity decreases, corresponding to a decrease in impedance and decrease in resistance. For all experiments, baseline measurements were taken for at least two hours prior to the addition of stimulant to ensure consistent baseline quantities.

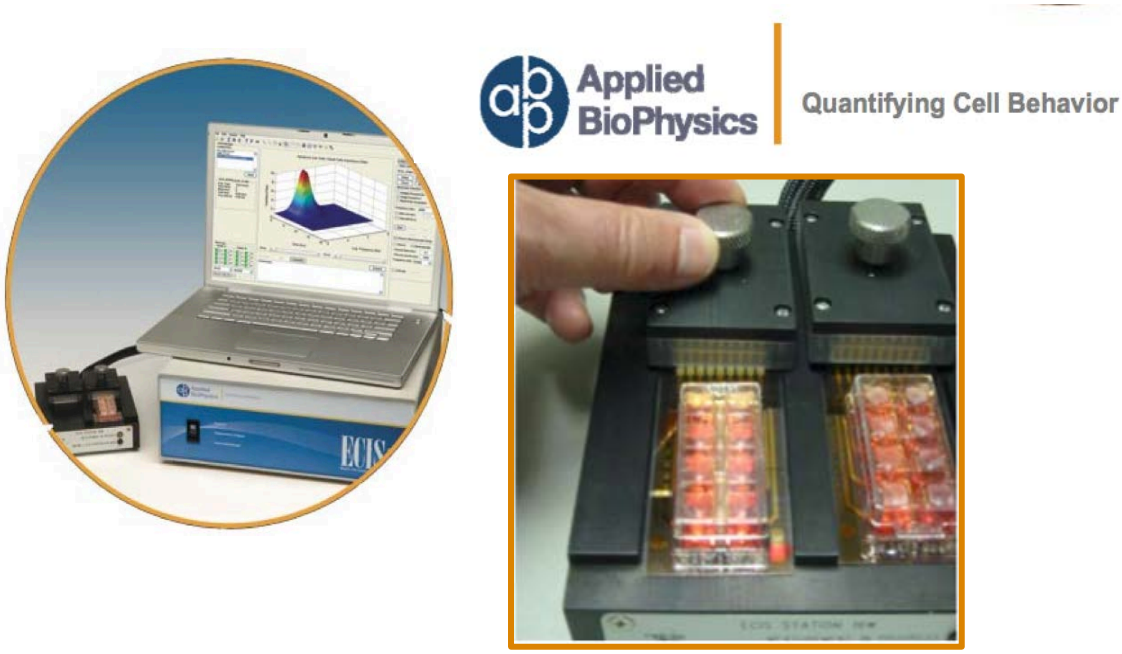

Figure 7. Electric Cell Substrate Impedance Sensing Apparatus.
Main photo depicts culture arrays on electric platform.

4.5 IL-18 AND IL-1β (ELISA)

IL-18 and IL-1β ELISA kits were purchased from R&D Systems (Minneapolis, MN). Concentrated cell supernatant was used for detection of either IL-18 or IL-1β expression via ELISA to determine mature cytokine release from treated cells. Protein

concentration (Pierce™ BCA Protein Assay Kit; ThermoFisher Scientific) was determined for normalization of ELISA results for all values reported as pg/mg protein.

4.5.1 Protein Concentration

Cell supernatant collected from treated HMVEC-Ls was concentrated using Amicon Ultra-2 mL Centrifugal Filters (Millipore Sigma, Burlington, MA). Briefly, previously collected supernatant was placed in filter units and spun 2272 x g for 90 minutes at 4° C. Filter units were then reversed and re-spun at 2200 x g for 10 minutes at 4° C, allowing for the concentrate to collect at the bottom of the collection tube. Filters were rinsed with 20 μl cold 1xPBS and spun for an additional 15 minutes at 2200 x g to ensure proper rinse of filters.

4.6 WESTERN BLOT ANALYSIS

Cell lysates of HMVEC-Ls treated with heme were concentrated using Amicon Ultra-0.5mL Centrifugal Filter units (Millipore Sigma). Protein concentration was determined using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Samples were placed in 4x laemeli sample buffer (BioRad, Hercules, CA) and diluted in nuclease-free water to equilibrate all protein levels prior to heating at 95°C for five minutes. Equal amounts of protein were then separated on pre-cast 7.5% TGX gel (BioRad). Protein was transferred to nitrocellulose membrane (BioRad) and blocked for two hours in 5% milk in TBST (BioRad). Blots were then probed for NLRP3 (Cell

Signaling, Danvers, MA, 1:5000) with the secondary antibody anti-rabbit (Vector, Burlingame, CA, 1:5000). Alpha-tubulin (Abcam, Cambridge, UK, 1:10000) was used for the loading control, with the secondary antibody anti-mouse (Vector, 1:10000).

4.7 HEME INDUCED ALI IN MICE

Transgenic Townes' sickle mice (73) expressing exclusively human sickle hemoglobin (SS) and hemizygous (AS) age-matched controls were used for animal studies. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (IACUC).

4.7.1 Physiological Measurement of Pulmonary Hypoxemia

Mice, 10-12 weeks of age were anaesthetized using isoflurane and shaved around the collar using Nair™ Hair Remover Cool Gel (Church & Dwight CO, Inc. Ewing, NJ) 48 hours prior to experimental procedure. On day of procedure, mice were again anaesthetized using isoflurane (Henry Schein, Melville, NY) and attached to a mouse-adapted pulse-oximeter (Mouse-Ox Plus, Starr Life Sciences, Oakmont, PA). Mice were then monitored to obtain baseline measurements and subsequently injected with freshly prepared solutions of Saline+MCC950 (i.p.), to which Saline+DMSO (i.p.) served as the vehicle, and heme (70 μmoles/kg, i.v.). Mice were monitored for up to two hours or until oxygen saturation levels dropped below 85% for a continuous period of five minutes. Mice were then sacrificed using a CO_2 chamber.

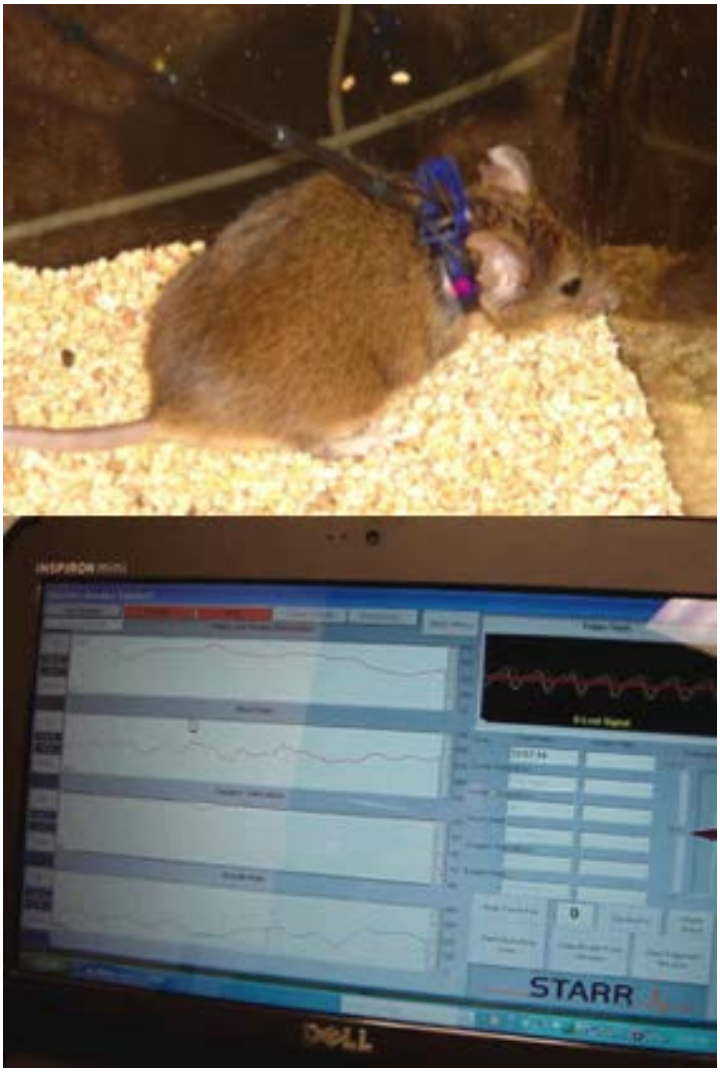

Figure 8. Mouse-Ox Plus Pulse-Oximeter.
Image of awake mouse attached to Mouse-Ox Plus apparatus and corresponding output of respiratory measurements**.**

4.7.2 Pulmonary Edema

Gravimetric analysis of the large left lobe of the mouse lung was used as a measure of pulmonary edema. Immediately after death, the left lung lobe was harvested and weighed using an isometric transducer (Harvard Apparatus, Holliston, MA). Lungs

were then dried in an oven containing desiccant crystals at 80°C for 48 hours. Dry weight was then determined in the same manor and wet/dry ratios were calculated.

5.0 RESULTS

5.1 [AIM 1] DETERMINE THE ABILITY OF HEME TO INDUCE NLRP3 IN PULMONARY ENDOTHELIAL CELLS.

5.1.1 Heme is cytotoxic and causes barrier disruption among human microvascular endothelial cells

Human microvascular endothelial cells (HMVEC-Ls) were cultured to form a confluent monolayer. Using release of intracellular lactate dehydrogenase (LDH) as a measure of cell death, cells challenged with heme (10 μM) showed a 75% increase in cytotoxicity at three hours post treatment as compared to vehicle controls (figure 9A). This cytotoxicity decreased at six hours, indicating slight recovery, but increased again up to 100% at 12 hours post treatment. This indicates that heme is toxic to endothelial cells and capable of inducing a large degree of endothelial cell death.

Loss of vascular barrier integrity is a characteristic feature of ALI. Therefore, we tested whether heme induced cell death is associated with the disruption of cell-to-cell contacts within a monolayer of HMVEC-Ls. Using ECIS technology, heme treatment was found to cause significant barrier disruption among a confluent monolayer of HMVEC-Ls (figure 9B-D). Figure 9B is a representative graph displaying the typically

observed drop in normalized resistance as a result of heme stimulation. Thrombin, known to cause endothelial barrier disruption, is used as a positive control (74-76), and shows a slight recovery of barrier integrity over time, while heme challenge failed to show any recovery (figure 9C,D).

Taken together, these data demonstrate endothelial barrier disruption as a result of cell death due to heme stimulation. As a DAMP molecule, heme may trigger multiple intracellular antecedent events including inflammasome activation that eventually leads to cell death.

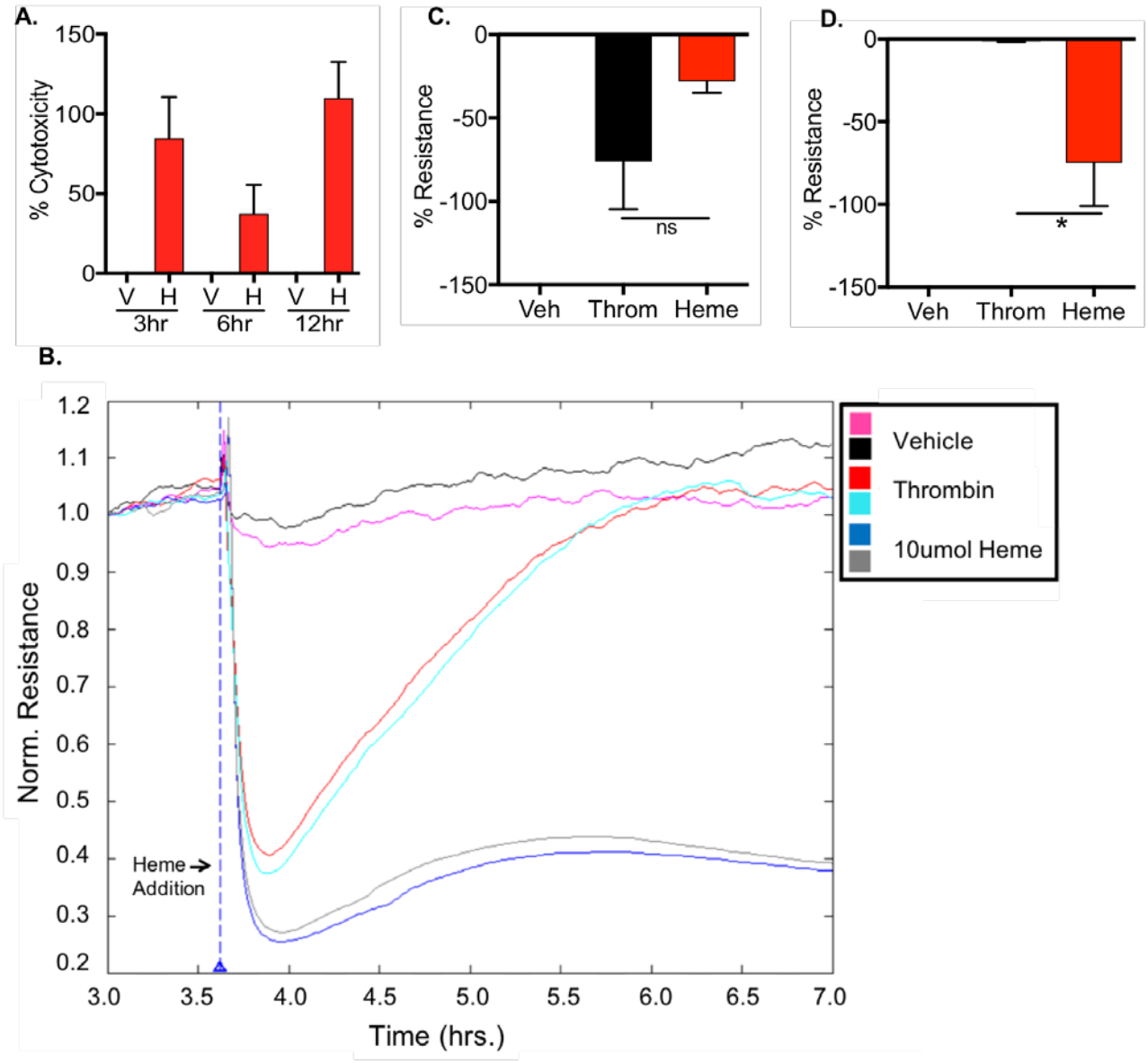

Figure 9. Heme causes cytotoxicity and endothelial barrier disruption in HMVEC-Ls.

(A) Percent cytotoxicity quantified via LDH release as compared to vehicle at 3, 6, and 12 hours post 10 μM heme challenge. Data represented as a mean±SEM of three separate experiments (n=3). **(B)** ECIS output of HMVEC-Ls treated with 10 μM heme. Output displays up to three hours post heme challenge. Apparent recovery of thrombin treated cells is evident via gradual rise in normalized resistance (red and light blue lines). **(C, D)** Corresponding percent resistance drops in heme and thrombin challenged wells at 30 minutes and 3-hours (p=0.048) post heme and thrombin addition. Data represents results of two separate experiments. All data normalized to vehicle.

5.1.2 Heme induces IL-1β and IL-18 secretion from HMVEC-Ls

Activation of NLRP3 inflammasomes results in the induction of caspase-1, which in turn cleaves pro-inflammatory cytokines Pro-IL-18 and Pro-IL-1β. They are then released from the cell in their mature form of IL-18 and IL-1β. Therefore, in order to determine the possible induction of NLRP3, supernatant from HMVEC-Ls treated with heme (10 μM) was collected and subsequently analyzed for the presence of IL-18 and IL-1β. We consistently observed a significant increase in IL-18 at the three-hour ($p=0.046$, $n=4$) and 12-hour ($p=0.0024$) time points post treatment (figure 10A). These results align with the time points of highest cytotoxicity observed in figure 9A. Though increases in IL-1β (pg/mL) were not found to be significant (figure 10B) we did observe up to a 25% increase in IL-1β ($n=4$, figure 10D) compared to vehicle when displayed in this manner.

The increased expression of both IL-18 and IL-1β indicates the induction of cytokine processing machinery as a result of heme challenge. Because activation of NLRP3 occurs upstream of the cellular events required for IL-18 and IL-1β release, we have evidence for finding NLRP3 expression in heme treated endothelial cells.

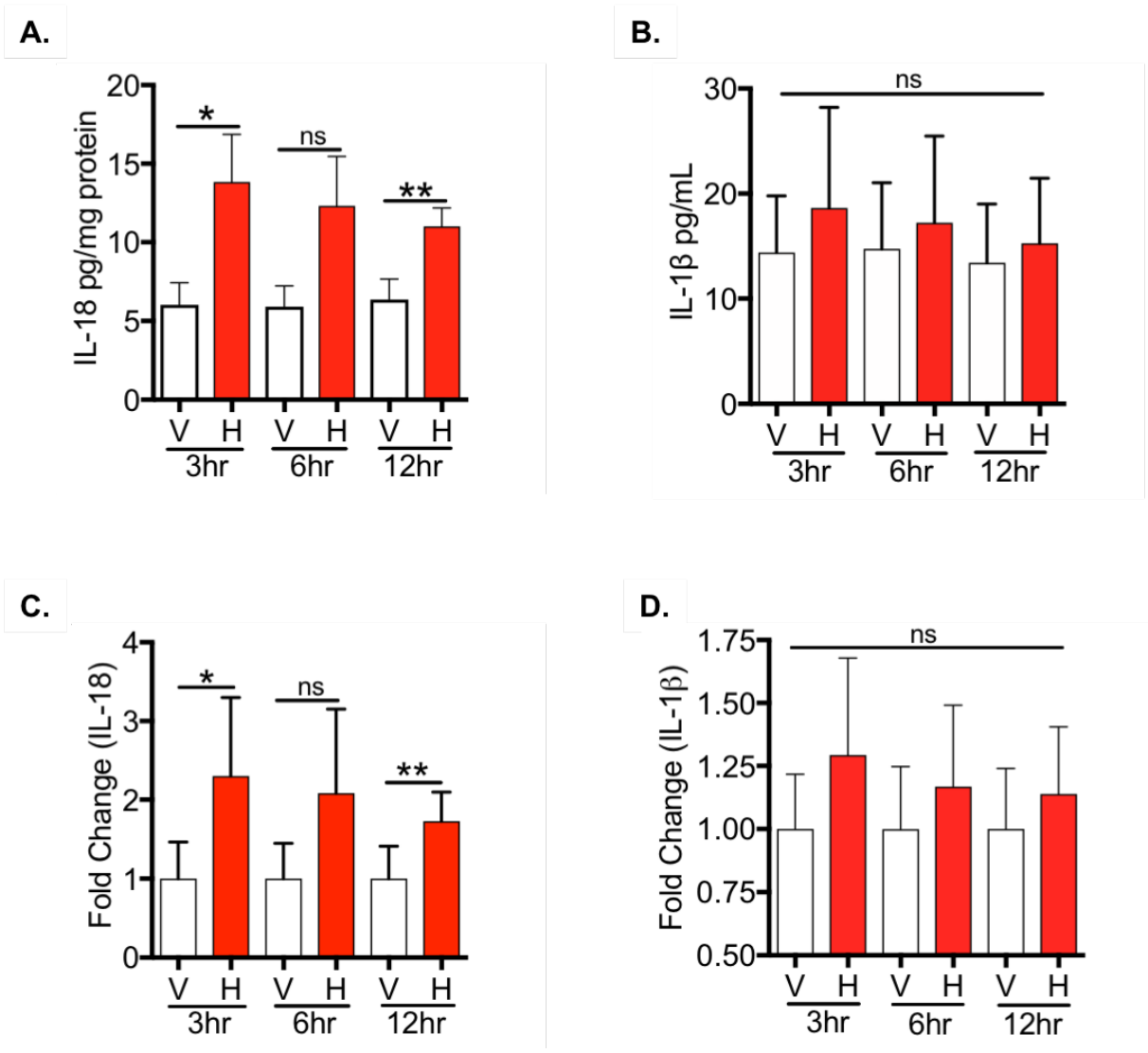

Figure 10. IL-18 and IL-1β expression after heme treatment.
(A) IL-18 release expressed as pg/mg protein significantly increases at three (p=0.046) and 12 hours (p=0.0024) post heme challenge. Data represents four separate experiments (n=4). **(B)** IL-1β expressed as total pg/mL, results represent four separate experiments (n=4). **(C, D)** Fold change in IL-18 (*p=0.046, **p=0.0024) and IL-1β expression as compared to vehicle at each respective time point.

Differential NLRP3 expression is apparent in HMVEC-Ls treated with heme

Western blot analysis reveals a slight difference in NLRP3 expression, most notably at three hours post treatment with 10 μM heme (figure 11). Alpha-tubulin used as the house keeping protein reveals unequal protein loading, however, because the

loading appears consistent within the indicated time points (excluding 12 hour), it is apparent that an increase in NLRP3 protein expression occurred after 3 hours of heme challenge. This is preliminary data and requires confirmation. Nonetheless, this data further supports the significant increase in extracellular IL-18 (Figure 10) and indicates that heme stimulates NLRP3 activity within endothelial cells.

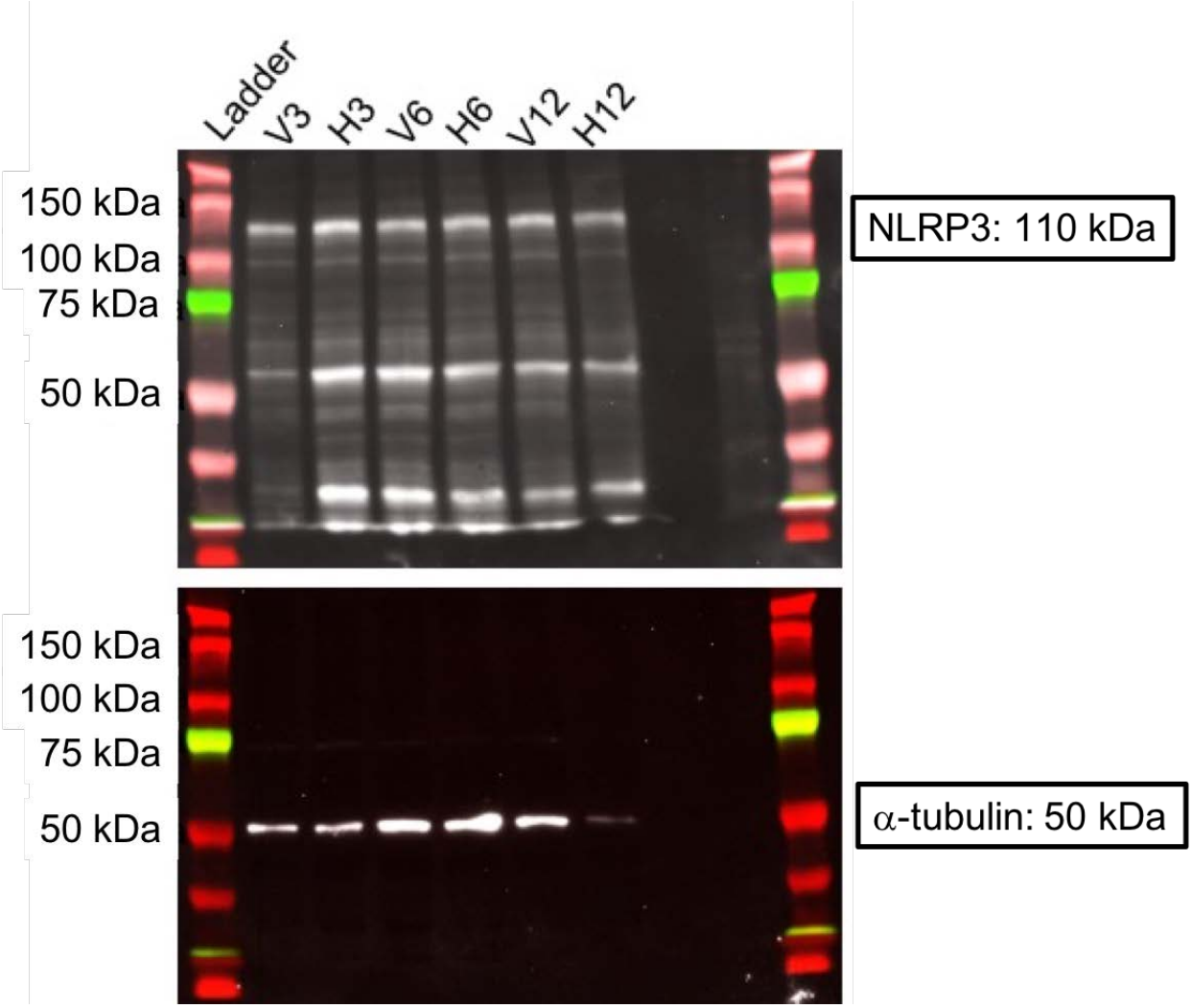

Figure 11. Western Blot of NLRP3.
Top panel NLRP3, bottom panel house-keeping alpha-tubulin. Total protein was quantified using BCA protein assay. Whole cell lysates of HMVEC-Ls, with 15 µg protein loaded in each lane.

5.2 [AIM 2] DETERMINE THE IMPORTANCE OF NLRP3 DURING HEME INDUCED ACUTE LUNG INJURY.

5.2.1 Inhibition of NLRP3 with MCC950 does not attenuate cell death in heme-treated HMVEC-Ls

MCC950 is a selective inhibitor of NLRP3, known to prevent its activation without interfering with the activation of other inflammasomes or TLR4 signaling (77). As a consequence of MCC950 treatment, studies have reported a decrease in IL-1β production (77, 78). In order to determine whether NLRP3 inhibition would alleviate some of the cytotoxic effects of heme, we again measured the amount of LDH in cultured HMVEC-Ls that were simultaneously treated with heme (10 μM) and MCC950 (50-100 μM) (figure 12). The combination of two experiments indicated a significant increase in cytotoxicity as a result of both 50μM ($p=0.0008$) and 100μM ($p=0.0422$) MCC950 treatment at three-hours of treatment, when compared to vehicle. However, visual analysis of the cells at this time point did not correlate to the supposed 100% death that the LDH analysis suggests. Furthermore, the six, and 12-hour time points indicate no significant change in cytotoxicity.

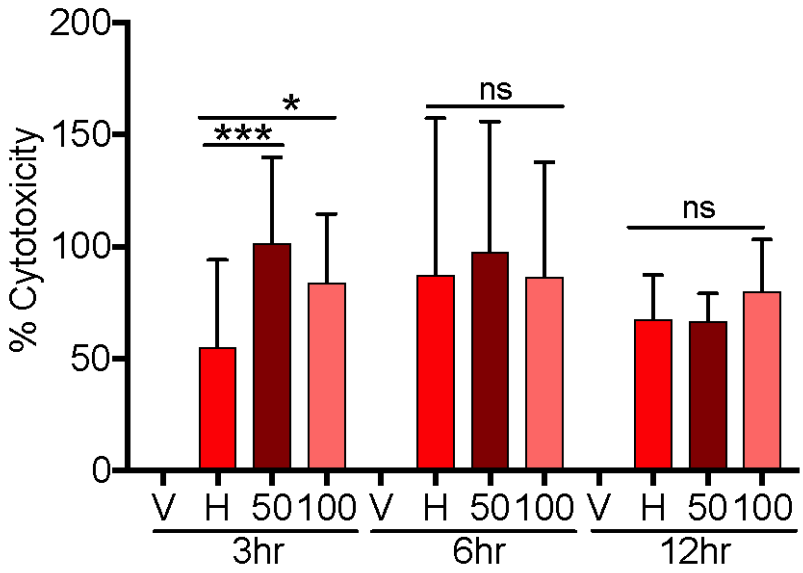

Figure 12. Cytotoxicity with MCC950.
Percent cytotoxicity quantified via LDH release in HMVEC-Ls treated with 10 μM heme. Significant increase with 50μM (p=0.0008) 100μM (p=0.0422) MCC950 observed at three hours of treatment. Data result of two separate experiments.

5.2.2 MCC950 attenuates barrier disruption in HMVEC-Ls treated with heme

Though we did not observe an overall rescue of cells based on the LDH cytotoxicity assay, we did observe attenuation of the endothelial barrier disruption 30 minutes and three hours after 10 μM heme treatment (figure 13A,B). Figure 13C is a representative graph displaying the slight rescue of barrier integrity with 50 μM MCC950, as a gradual increase in normalized resistance is observed between 3-4.5 hours. This rescue continues throughout three hours, following the trend of the vehicle treated cells. This indicates that there is some potential salvaging quality to NLRP3 inhibition via MCC950.

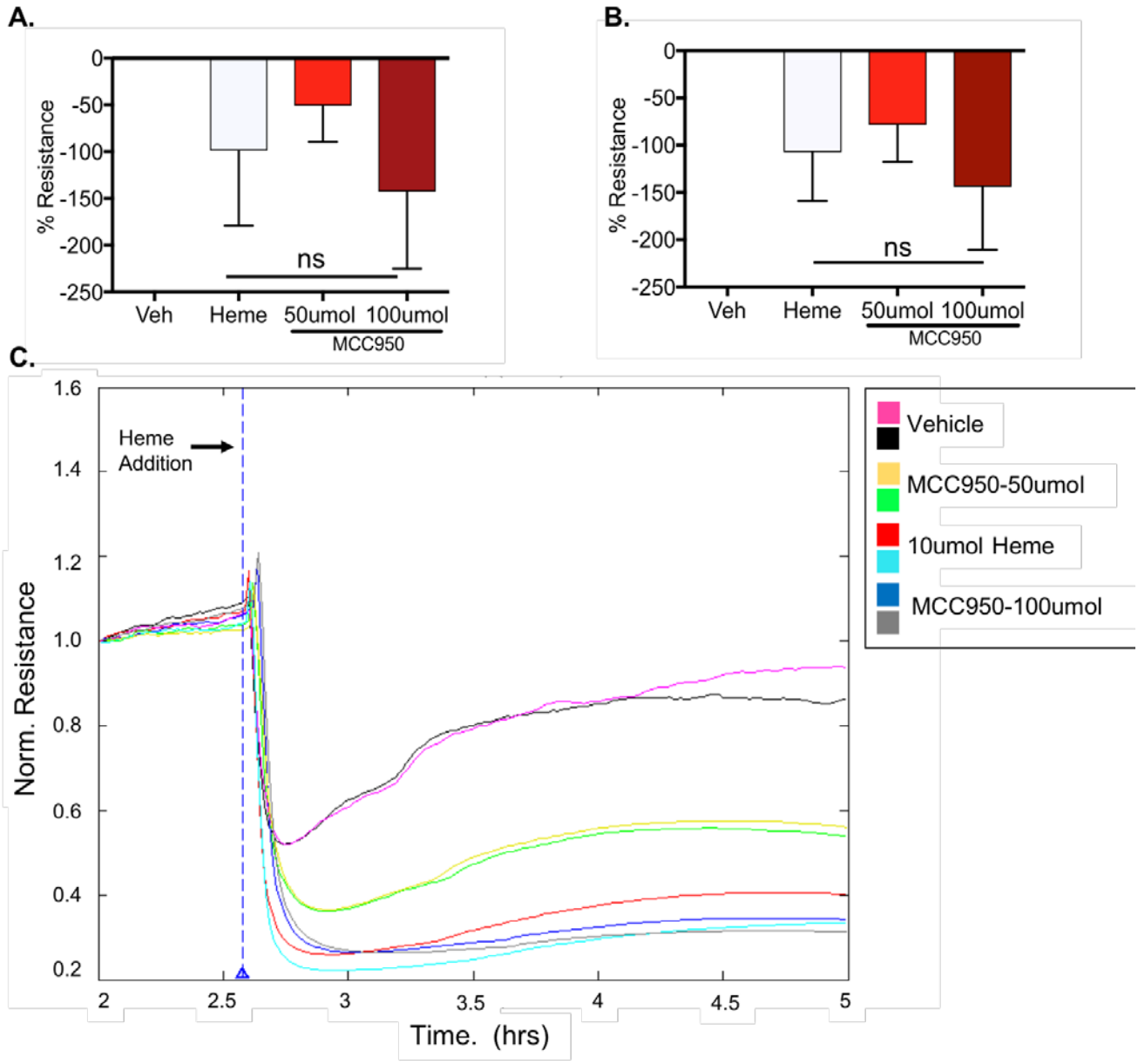

Figure 13. Barrier integrity is increased with NLRP3 inhibition via MCC950. All data represents three separate experiments, and all treatment conditions are normalized to vehicle. **(A)** Percent normalized resistance 30 minutes after heme, heme+MCC950, or vehicle addition. **(B)** Percent normalized resistance 3-hours after heme, heme+MCC950, or vehicle addition. **(C)** Representative graph of normalized resistance up to two hours post heme addition. Gradual recovery of MCC950 treated cells is observed by the slight rise in normalized resistance (green and yellow lines).

5.2.3 Different doses of MCC950 has varying effects on IL-18 and IL-1β release from HMVEC-Ls treated with heme

In order to determine whether the doses and treatment regimen of MCC950 were able to inhibit the processing of IL-18 and IL-1β, supernatant from HMVEC-Ls that were simultaneously treated with both heme and MCC950 was collected and analyzed for the two analytes using ELISA. When compared to vehicle, we observed a significant increase in IL-18 ($p=0.008$) with sole heme treatment, as well as a significant increase with 10 μM ($p=0.0191$) and 100 μM ($p=0.0072$) MCC950+heme treatment (figure 14A,C). However, no significant change in IL-18 release was noted between the heme +50 μM MCC950 dose and vehicle (figure 14A,C). Furthermore, an apparent but not significant decrease in IL-18 is noted between heme and heme+50 μM MCC950 (figure 14A,C). This indicates that the 50 μM dose of MCC950 has potential of inhibiting IL-18 processing in HMVEC-Ls treated with heme (50 μM). We also observed a non-significant decrease in IL-1β with the 50 μM MCC950 dose (figure 14B,D). Further dose optimization is needed in order to indicate whether MCC950 is able to completely reduce cytokine levels to baseline or vehicle expression. If dose optimization does not result in significantly reduced cytokine levels, we can conclude that other mechanistic pathways aside from NLRP3 are responsible for IL-18 and IL-1β release.

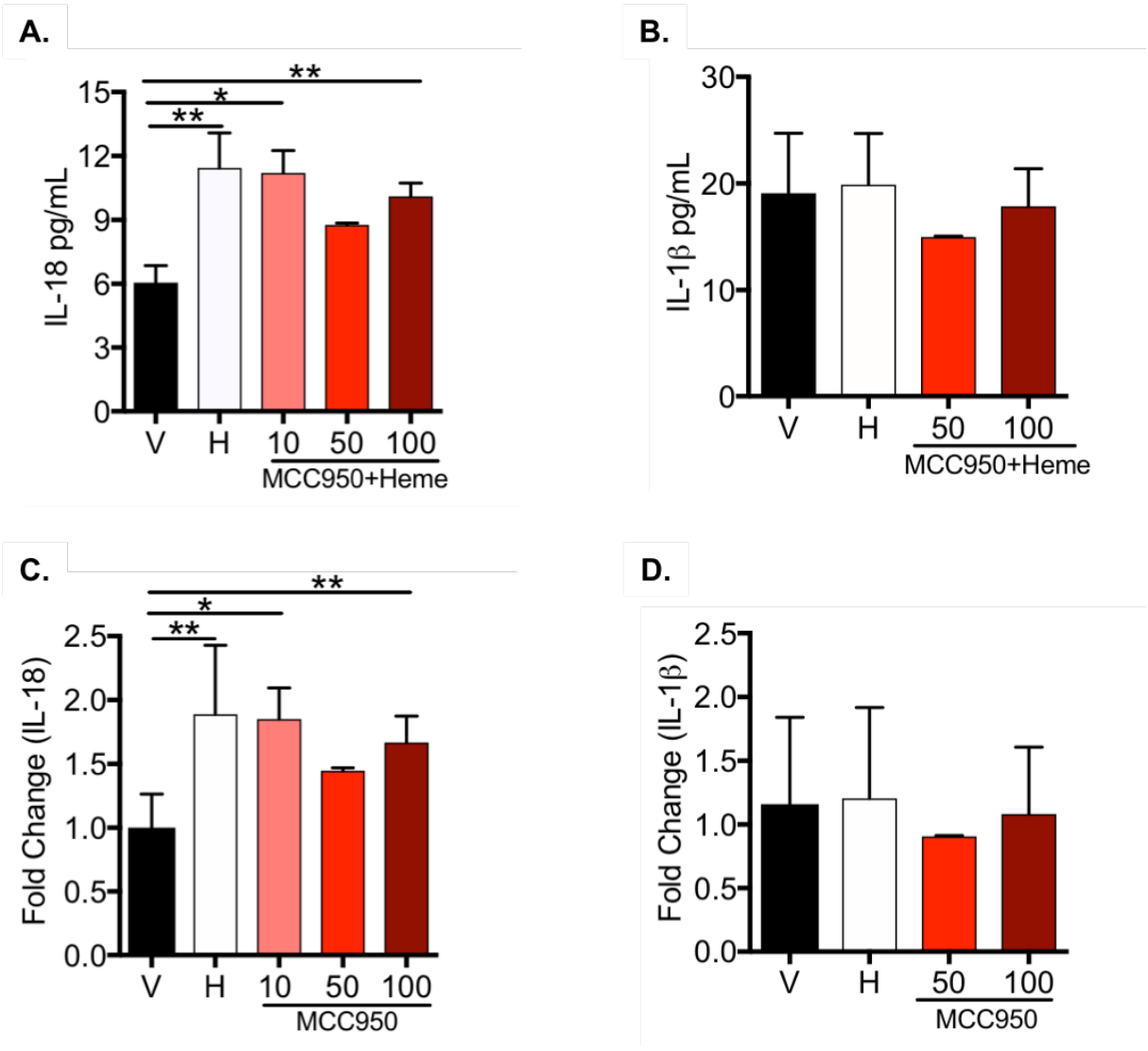

Figure 14. IL-18 and IL-1β release after heme+MCC950 addition.
(A) Release of IL-18 expressed in total pg/mL after 6-hours of 10 μM heme+MCC950 treatment. Significant increase in IL-18 with heme (p=0.008), heme+10μM MCC950 (0.0191), and heme+100μM MCC950 (p=0.0072). All data represents two separate experiments. **(B)** Release of IL-1β in cell supernatant expressed in total pg/mL after 6-hours of 10 μM heme+MCC950 treatment. All data represents two separate experiments. **(C-D)** Fold change of IL-18 and IL-1β expression compare to vehicle. Significant increase in IL-18 with heme (p=0.008), heme+10μM MCC950 (p=0.0191), and heme+100μM MCC950 (p=0.0072).

5.2.4 Prophylactic treatment with MCC950 is unable to prevent mortality in a murine model of ALI

Because of the previously observed preventative qualities of MCC950 in barrier disruption and, though weak, inhibition of pro-inflammatory cytokine release, we questioned whether there was potential for condition attenuation during our murine model of ALI. As described in the Methods, mice (10-12wk) were attached to a mouse-adapted pulse-oximeter and injected with MCC950+Saline (i.p., 25μg/g) (77) or DMSO+Saline (i.p.) (figure 15A). One vehicle mouse expired prior to the injection of heme via tail vein, while the remaining were monitored for one hour before infusing 70 μmoles/kg heme (i.v.) to induce ALI (figure 15A,B). All mice experienced a sudden and severe drop in oxygen saturation (figure 15D), a characteristic of acute lung injury, and expired prior to an hour and 30 minutes after heme injection (figure 15B).

Though NLRP3 inhibition had no effect on mortality, it did appear to have an effect on the lung wet-to-dry weight ratio, as MCC950 treated mice displayed a 20% decrease (figure 15C), indicating vehicle mice suffered more severe pulmonary edema than MCC950 treated mice. Ultimately, this implies a decrease in overall vascular leakage, which does corroborate previous findings of increased barrier integrity from the ECIS. However, great caution is taken in interpreting these results, as only two vehicle mice survived long enough to gate the measurement. Repeated cohorts are needed to confirm results.

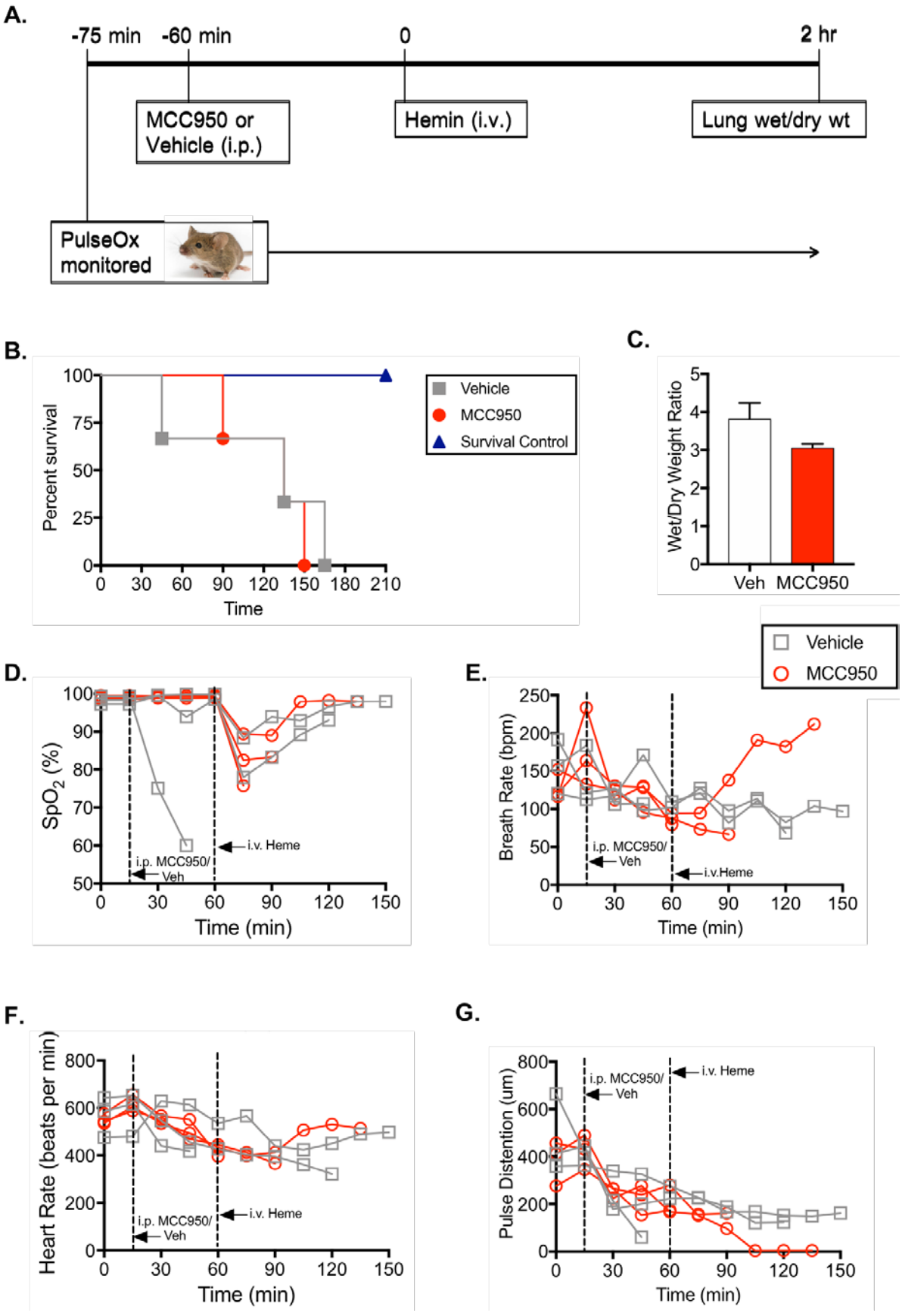
A.
-75 min
-60 min
0
2 hr
MCC950 or Vehicle (i.p.)
Hemin (i.v.)
Lung wet/dry wt
PulseOx monitored
B.
Percent survival
Time
Vehicle
MCC950
Survival Control
C.
Wet/Dry Weight Ratio
Veh
MCC950
D.
SpO2 (%)
Time (min)
i.p. MCC950/ Veh
i.v. Heme
E.
Breath Rate (bpm)
Time (min)
i.p. MCC950/ Veh
i.v.Heme
Vehicle
MCC950
F.
Heart Rate (beats per min)
Time (min)
i.p. MCC950/ Veh
i.v. Heme
G.
Pulse Distention (um)
Time (min)
i.p. MCC950/ Veh
i.v. Heme

Figure 15. MCC950 prophylaxis in a murine model of ALI.
(A) Experimental design. **(B)** Survival curve including survival control (AS mouse) which did not succumb prior to two hours. n=7 total, MCC950 n=3, Vehicle n=3, AS control n=1. **(C)** Wet-to-dry weight ratio of MCC950 treated mice compared to vehicle treated mice. MCC950 n=3, Vehicle n=2 **(D-G)** Real time physiological measurement of **(D)** percent oxygen saturation, **(E)** breath rate, **(F)** heart rate, and **(G)** pulse distention during baseline, MCC950 prophylaxis, and after heme infusion. MCC950 n=3, Vehicle n=3

6.0 DISCUSSION

The onset of ALI stems primarily from the disruption of the lung endothelial barrier. Heme, well-known as a DAMP molecule, is increased in patients suffering from several forms of hemolytic crisis, all of whom are placed at an increased risk of developing ALI (4, 23). Portions of this study exhibit the damaging effects that heme can cause on lung endothelial cells, supporting the theory of heme-induced ALI. However, the exact cellular mechanism underlying how heme stimulates endothelial cell damage that may propagate barrier disintegration is largely unknown. Previous reports of heme-induced inflammasome activation within monocytes initiated the speculation as to whether the inflammatory effects caused by heme are occurring through the activation of inflammasomes (72, 79). The NLRP3 inflammasome specifically is known to be activated by a variety of stimuli, including those of endogenous nature, making it a prime candidate for primary investigation (72, 79-81). Therefore, the goal of this project was to determine whether heme is able to induce NLRP3 inflammasome activation within endothelial cells. If true, this would likely be a major component in the onset of ALI that could be targeted through therapeutic agents.

In order to determine the potential activation of NLRP3, it was foremost necessary to quantitate the damage heme causes to lung endothelial cells. Using HMVEC-Ls, cell death was found to be consistently increased across all time points

compared to vehicle in three separate experiments, exhibiting 75% cell death after three hours of treatment. This death corresponded to a 75% decrease in barrier resistance measured through ECIS, physiologically translating to severe cell-to-cell breakage throughout the endothelial cell barrier ultimately resulting in vascular leakage.

This observed cell death and endothelial barrier disruption reveals that heme is able to trigger a large degree of cell stress responses. In order to determine whether this stress was related to inflammasome activation, subsequent analysis for the presence of IL-18 and IL-1β was performed. The maturation of both cytokines is known to be largely reliant on the activation of caspase-1, which must first be cleaved to its active form by the NLRP3 inflammasome upstream in the sequence of cellular events (See figure 4,5). Therefore, the analysis of supernatant taken from HMVEC-Ls treated with heme was used as a primary step in determining whether there was a possibility of NLRP3 activation. Supernatant taken directly from the HMVEC-Ls showed an elevation of both IL-18 and IL-1β, confirming an increase of extracellular release for both cytokines compared to vehicle treated cells.

Though the rise in both cytokine levels is a compelling factor in support of the overall hypothesis, alternative cytokine maturation pathways are possible. Therefore, it was further necessary to detect actual protein levels of NLRP3 within the endothelial cells. Through western blot analysis, an apparent increase in NLRP3 expression in HMVEC-Ls can be observed at three hours post treatment. This increase in expression aligns with the increased release of IL-18 and IL-1β, indicating a prominent role of NLRP3 in the maturation and release of these cytokines.

After determining a conceivable link between the amplified release of IL-18 and IL-1β and the activation of NLRP3, it was vital to proceed with experiments using an NLRP3 inhibitor, MCC950. The specific inhibition of NLRP3 via MCC950 has been shown in multiple publications and was desirable for use in these experiments particularly because of its previous use in in-vivo experimentation (77, 82, 83). The purpose of experiments utilizing MCC950 proved to be two-fold. First, it helped clarify the relationship between cytokine release and NLRP3. Second, it aided in establishing whether inhibition of NLRP3 resulted in an improvement of the negative heme-induced conditions. Though initial cytotoxicity experiments with MCC950 were not overall promising, I believe much of the observed cytotoxicity to be culprit of the solvent rather than a direct result of MCC950 treatment. Subsequent experiments investigating the use of DMSO as a heme solvent as compared to sodium hydroxide revealed an increase in overall cytotoxicity among the prior group (data not shown). Therefore, upon continuation of experiments using MCC950, heme was also dissolved in DMSO to better equilibrate DMSO concentrations between treatment conditions. With this correction, ECIS experiments continuously showed an improvement in barrier dysfunction with 50 μM MCC950 use.

The increased barrier integrity displayed through the ECIS experiments seemed promising and poised the question of whether NLRP3 inhibition could be a potential therapeutic target in an in-vivo model of ALI. The large concentration of DMSO was a considerable concern for animal experiments, however, because manufacturer's protocols called for the dilution of MCC950 only in DMSO, experiments proceeded accordingly. In an attempt to circumvent the possible toxicity of DMSO within the

animals, the total volume of injected amount was doubled with sterile saline. Furthermore, in order to determine whether DMSO toxicity was a legitimate problem within the experimental design, one mouse was injected with only saline. For all animal experiments we used transgenic homozygous sickle (SS) mice or hemizygous sickle (AS) mice. Our sickle mice at baseline levels experience an increased degree of intravascular hemolysis. During adulthood, their Hx levels have substantially decreased, rendering them particularly sensitive to ALI development (84). The ALI model in use requires a 70 μmol/kg body weight injection of heme for full ALI symptoms. The use of these mice more accurately mimics human disease particularly because of their lifetime in existence with the complications of SCD. By the time of adulthood, the mice typically develop noticeable multi-organ damage (33), making them a suitable substitute for what occurs clinically in patients.

As suspected, all mice injected with DMSO+Saline and MCC950+Saline showed signs of slight distress after i.p. drug administration, while the single mouse injected solely with saline showed minimal signs of pain. This indicates that DMSO is responsible for any observed toxicity, and the increase in total volume injected and MCC950 were not. Unfortunately, one vehicle mouse did succumb prior to heme injection, however I would like to emphasize that due to the nature and sensitivity of these mice, sudden mortality due to stressors such as drug injection and location movement are not completely uncommon. Other than this mouse, all other mice retained consistent oxygen saturation levels, indicating no mice were in respiratory distress prior to heme injection. While some decrease in pulse distention does seem to occur after MCC950 pretreatment, both breath rates and heart rates maintained

expected levels. After heme treatment, all mice experienced a rapid drop in oxygen saturation, revealing severe and sudden respiratory distress indicative of ALI. These problems continued until death as some mice made slight oxygen saturation recovery, but none returned to baseline levels. This is a noted characteristic of our ALI model.

While MCC950 treatment was unable to have any effect on survival, a 20% decrease in the wet-to-dry lung weight ratio was observed. The wet-to-dry ratio is a measurement of pulmonary edema whereby an increase in the ratio specifies a greater severity in vascular leakage. Theoretically if in-line with my hypothesis, the observed decrease indicates that MCC950 was able to inhibit NLRP3 activation which foremost resulted in a decrease in pro-inflammatory cytokine activity and lessened endothelial barrier breakage. Overall, this would lead to a decrease in total cell death and inflammation. These results are particularly promising because they corroborate with the previous finding that MCC950 treatment did result in some increase in endothelial barrier integrity after heme treatment.

6.1 IMPLICATIONS

This project demonstrates that sole heme treatment causes devastating effects to the lung endothelial barrier, which likely contributes to the development of ALI. Evidence of NLRP3 activation in response to heme was found via the increase in IL-18 and IL-1β and furthermore through the apparent increase in NLRP3 protein levels. Though inhibition of NLRP3 via MCC950 failed to reduce cytokine expression to baseline levels and displayed no decrease in overall cytotoxicity, this could be culprit of

the lack of a standardized treatment protocol. The non-significant decrease in expression of both cytokines, the increase in barrier integrity, and the reduced wet-to-dry lung weight ratio as a result of MCC950 treatment is promising in that it indicates inhibition of NLRP3 has some kind of positive effect in battling heme induced damage of the endothelial barrier. When applied on a physiologic level, this translates to a decrease in the severity of vascular permeability during an episode of ALI.

When applying these findings on a broad level, it is important to again consider the mechanism of NLRP3 activation. As previously discussed, NLRP3 activation can occur as a result to a broad range of stimuli, and furthermore through a variety of pathways. Initially, this is one of the reasons NLRP3 seemed like a reasonable target for study-its response to many stimuli including those of endogenous nature, such as heme. Because NLRP3 can react to such an extensive group of stimulants, it has additionally been speculated as to whether or not NLRP3 actually recognizes specific activating motifs of differing stimuli, or whether the inflammasome responds to general cell homeostasis disturbances (85). While this goes along with the “cell damage” second signal needed for canonical activation, it does not fully explain the initial TLR-associated signal.

Considering heme is known to signal through TLR4, but also considering the possible transporter influx of heme allows for the postulation of how the non-canonical and canonical pathway may work together for overall activation (86). Though the pathways are considered independent, m-caspase-11 (human caspase-4 -5) is able to enhance the processing capability of caspase-1 in the presence of certain stimuli (ex. Cholera toxin, E. coli) (55, 87). Therefore, in considering diseases such as sepsis, the

prospect of an enhanced or even subdued signal resulting from the combination of multiple stimuli is distinctly possible. For example, a study by Li, H. et al (88) showed that the induction of heme-oxygenase-1 (HO-1) actually attenuated NLRP3 activation induced by LPS in gingival epithelial cells. What is most interesting is that the authors induced HO-1 by using heme (20 μM/L). Though this higher dose instigates question as to whether this decrease in expression was due to an increase in cell death, it could very well be an indicator of how variable the response to heme can be between different cell types, and also of the importance of heme balance within the cell. Furthermore, recent data from our own lab have indicated that heme also activates NLRP3 inflammasomes in human renal proximal tubular epithelial cells (HK-2) treated with heme, which also results in IL-18 and IL-1β release. Together these additional studies support the finding of NLRP3 activation within lung endothelial cells, and further indicate the need to tease out specific mechanisms of activation as well as the differential outcomes of activation.

The concept of heme-induced NLRP3 activation in endothelial cells is to our knowledge novel. Furthermore, the concept of NLRP3 induction by heme without the priming of LPS has not been shown. Though these results need validation, there is at the very least additional evidence for NLRP3 induction within endothelial cells in response to heme treatment.

6.2 AREAS OF DISCUSSION

Of note for discussion are the previously mentioned loading concerns in figure 11, the possible discrepancy in band identification in figure 11, the use of DMSO throughout the study, and the loss of a vehicle mouse prior to heme challenge which resulted in an n=2 in figure 15C. In considering figure 11, regardless of protein normalization between samples, there are apparent loading differences throughout the blot. However, loading between samples of the same time point seems to be consistent, excluding the 12 hour which was not considered for analysis. Additional concerns within the same figure include the identification of the correct band and the presence of multiple bands through the entire blot. The specified size of NLRP3 is 110kDa. As seen on the blot, two bands appear between the 100kDa and 150kDa markers. While the top band is more prominent, it seems to consistently run closer to 125kDa, while the bottom band runs consistently at 100kDa. A clear positive control is needed to amend this discrepancy, and ideally, a clear and evenly loaded repeat blot is also needed for full confirmation of the experiment.

The use of DMSO throughout the cell and animal experiments did appear to have an effect on the overall cytotoxicity of the cells and mortality of the animals. Attempts to correct this were made and appeared to correct differences within the in-vitro results, but it is difficult to fully interpret in-vivo challenges. Therefore, the use of a different solvent is crucial for full confirmation of these experiments. Also, as previously mentioned, the results of the wet-to-dry weight ratio should be interpreted with caution, as one vehicle did succumb prior to heme stimulation. Unfortunately, because of time and availability of reagent constraint, these experiments could not be repeated.

One point to additionally consider is whether NLRP3 activation is directly in response to heme or in response to the death of surrounding cells. For example, adenosine triphosphate (ATP) is known to be released from cells in result to sterile injury and has also been characterized as an NLRP3 activator (89-91). Therefore, it is possible that heme is causing cell death, which results in the release of ATP (or other NLRP3 activators), which in turn activates NLRP3 within neighboring cells. This would result in an overall increase in all of the previously identified factors. Although it could be argued that heme is still ultimately causing the cascade, it technically would not be the only mediator of NLRP3 activation. This discrepancy could be solved through intracellular staining, or somehow interfering with ATP release from dead cells.

7.0 FUTURE DIRECTIONS

Based on the performed experiments, and knowing TLR4 is a receptor for heme, it is easy to assume that heme acts through TLR4 to induce the canonical pathway of NLRP3 activation in lung endothelial cells. For verification of this speculation, it is foremost necessary confirm induction of NLRP3 protein and perform further protein analysis for caspase-1 and caspase-11 (human caspase-4 -5). This could help differentiate the canonical versus non-canonical pathway, further identifying the mechanistic routes of heme induced inflammation and also shedding light on both NLRP3 pathways. Furthermore, characterizing the role of TLR4 through the use of TLR4 inhibitors such as TAK-242 will help in distinguishing between pathways (92). In this study, we did not address the effect NLRP3 may have on junctional dysfunction between endothelial cells. Future studies aimed at identifying whether junctional disruption or cell death is primarily responsible for a decrease in barrier integrity would aid in developing targeted therapeutics.

It is important to dissect out the specific intermediate intracellular steps within the NLRP3 pathway that is responsible for the inflammatory outcome of heme induced damage. This can be achieved by performing gain-of-function and loss-of-function experiments for the various previously-identified intermediates that include TLR4, NLRP3 and Caspase-1. In vitro experiments using siRNAs to knock-down specific gene

expression will confirm gene involvement. This set of experiments can be additionally corroborated with gene over-expression (using cDNA or viral transfection) to potentiate the role of TLR4, NLRP3 or Caspase-1 in endothelial cell response to extracellular heme. Similar in vivo experiments evaluating the effect of heme induced lung injury using specific gene knockout mice (for example, $NLRP3^{-/-}$, $Caspase\text{-}1^{-/-}$ mice) will highlight the precise gene that can be targeted therapeutically to protect from ALI.

Though we have shown that heme causes extensive damage to lung endothelium, the exact contribution of heme to the induction of ALI is likely co-dependent on the stimulus that initiated the increase in free heme (bacterial sepsis, genetics, etc.). When considering the addition of any infectious agent or other DAMP on top of the already prominent hemolysis, there is a possibility for greater amplification of inflammatory response resulting in a different mechanistic route of response. IL-18 and IL-1β release appeared to be only partially lessened with NLRP3 inhibition, thus calling for the assumption that there are other cellular mechanisms responsible for their maturation and release. Therefore, an interesting area for future study will be the combinatorial effects of heme and other stimuli on NLRP3 expression. Experiments should focus on how the combination of different stimuli affects NLRP3 expression, and also how the quantified release of IL-18 and IL-1β may differ between stimuli. If identified, this differential cytokine expression would have major implications in how the body ultimately is responding to stimuli.

8.0 PUBLIC HEALTH SIGNIFICANCE

As a condition that is the potential result of multiple diseases, ALI research is of great public health concern, both on a national and global level. This study was primarily focused on the ability of heme to effectively induce NLRP3 inflammasome formation within endothelial cells, with the ultimate goal of defining a mechanism for heme-induced acute lung injury (ALI). Research in to the cellular mechanisms of ALI are not only applicable to the stimuli (which could be of infectious, genetic, or injury-onset nature) which cause ALI, but also potentially to those that cause the pathogenesis of acute respiratory distress syndrome (93). Additionally, this work sheds light on other aspects of endothelial barrier dysfunction induced by heme, which open up doors to the heme-induced damage of other cell types. As diseases associated with vasculature disruption and dysfunction become more common, any insight into how these cells react to stimuli is of benefit. Furthermore, the potential therapeutic aspect of NLRP3 inhibition in rescue of pulmonary edema as a result of ALI has clinical significance for the ultimate goal of improving patient outcome.

BIBLIOGRAPHY

1. **Flori HR, Glidden DV, Rutherford GW, Matthay MA.** 2005. Pediatric acute lung injury: prospective evaluation of risk factors associated with mortality. Am J Respir Crit Care Med **171:**995-1001.
2. **Johnston CJ, Rubenfeld GD, Hudson LD.** 2003. Effect of age on the development of ARDS in trauma patients. Chest **124:**653-659.
3. **Erickson SE, Shlipak MG, Martin GS, Wheeler AP, Ancukiewicz M, Matthay MA, Eisner MD, National Institutes of Health National Heart L, Blood Institute Acute Respiratory Distress Syndrome N.** 2009. Racial and ethnic disparities in mortality from acute lung injury. Crit Care Med **37:**1-6.
4. **Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD.** 2005. Incidence and outcomes of acute lung injury. N Engl J Med **353:**1685-1693.
5. **Doyle RL, Szaflarski N, Modin GW, Wiener-Kronish JP, Matthay MA.** 1995. Identification of patients with acute lung injury. Predictors of mortality. Am J Respir Crit Care Med **152:**1818-1824.
6. **Sloane PJ, Gee MH, Gottlieb JE, Albertine KH, Peters SP, Burns JR, Machiedo G, Fish JE.** 1992. A multicenter registry of patients with acute respiratory distress syndrome. Physiology and outcome. Am Rev Respir Dis **146:**419-426.
7. **Pepe PE, Potkin RT, Reus DH, Hudson LD, Carrico CJ.** 1982. Clinical predictors of the adult respiratory distress syndrome. Am J Surg **144:**124-130.
8. **Fowler AA, Hamman RF, Good JT, Benson KN, Baird M, Eberle DJ, Petty TL, Hyers TM.** 1983. Adult respiratory distress syndrome: risk with common predispositions. Ann Intern Med **98:**593-597.
9. **Hudson LD, Milberg JA, Anardi D, Maunder RJ.** 1995. Clinical risks for development of the acute respiratory distress syndrome. Am J Respir Crit Care Med **151:**293-301.
10. **Villar J, Slutsky AS.** 1989. The incidence of the adult respiratory distress syndrome. Am Rev Respir Dis **140:**814-816.
11. **Milberg JA, Davis DR, Steinberg KP, Hudson LD.** 1995. Improved survival of patients with acute respiratory distress syndrome (ARDS): 1983-1993. JAMA **273:**306-309.
12. **Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L, Lamy M, LeGall JR, Morris A, Spragg R.** 1994. Report of the American-European

Consensus conference on acute respiratory distress syndrome: definitions, mechanisms, relevant outcomes, and clinical trial coordination. Consensus Committee. J Crit Care **9:**72-81.

13. **Johnson ER, Matthay MA.** 2010. Acute lung injury: epidemiology, pathogenesis, and treatment. J Aerosol Med Pulm Drug Deliv **23:**243-252.
14. **Rubenfeld GD, Caldwell E, Granton J, Hudson LD, Matthay MA.** 1999. Interobserver variability in applying a radiographic definition for ARDS. Chest **116:**1347-1353.
15. **Meade MO, Cook RJ, Guyatt GH, Groll R, Kachura JR, Bedard M, Cook DJ, Slutsky AS, Stewart TE.** 2000. Interobserver variation in interpreting chest radiographs for the diagnosis of acute respiratory distress syndrome. Am J Respir Crit Care Med **161:**85-90.
16. **Pugin J, Verghese G, Widmer MC, Matthay MA.** 1999. The alveolar space is the site of intense inflammatory and profibrotic reactions in the early phase of acute respiratory distress syndrome. Crit Care Med **27:**304-312.
17. **Zapol WM, Trelstad RL, Coffey JW, Tsai I, Salvador RA.** 1979. Pulmonary fibrosis in severe acute respiratory failure. Am Rev Respir Dis **119:**547-554.
18. **Shasby DM, Vanbenthuysen KM, Tate RM, Shasby SS, McMurtry I, Repine JE.** 1982. Granulocytes mediate acute edematous lung injury in rabbits and in isolated rabbit lungs perfused with phorbol myristate acetate: role of oxygen radicals. Am Rev Respir Dis **125:**443-447.
19. **Shasby DM, Fox RB, Harada RN, Repine JE.** 1982. Reduction of the edema of acute hyperoxic lung injury by granulocyte depletion. J Appl Physiol Respir Environ Exerc Physiol **52:**1237-1244.
20. **Abraham E.** 2000. What role does neutrophil apoptosis play in acute respiratory distress syndrome? Crit Care Med **28:**253-254.
21. **Hough CL, Herridge MS.** 2012. Long-term outcome after acute lung injury. Curr Opin Crit Care **18:**8-15.
22. **Ware LB, Matthay MA.** 2000. The acute respiratory distress syndrome. N Engl J Med **342:**1334-1349.
23. **Eisner MD, Thompson T, Hudson LD, Luce JM, Hayden D, Schoenfeld D, Matthay MA, Acute Respiratory Distress Syndrome N.** 2001. Efficacy of low tidal volume ventilation in patients with different clinical risk factors for acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med **164:**231-236.
24. **Janz DR, Ware LB.** 2014. Plasma biomarkers in acute respiratory distress syndrome: a work in progress*. Crit Care Med **42:**755-756.
25. **Janz DR, Bastarache JA, Sills G, Wickersham N, May AK, Bernard GR, Ware LB.** 2013. Association between haptoglobin, hemopexin and mortality in adults with sepsis. Crit Care **17:**R272.
26. **Larsen R, Gozzelino R, Jeney V, Tokaji L, Bozza FA, Japiassu AM, Bonaparte D, Cavalcante MM, Chora A, Ferreira A, Marguti I, Cardoso S, Sepulveda N, Smith A, Soares MP.** 2010. A central role for free heme in the pathogenesis of severe sepsis. Sci Transl Med **2:**51ra71.
27. **Gladwin MT, Ofori-Acquah SF.** 2014. Erythroid DAMPs drive inflammation in SCD. Blood **123:**3689-3690.

28. **Adisa OA, Hu Y, Ghosh S, Aryee D, Osunkwo I, Ofori-Acquah SF.** 2013. Association between plasma free haem and incidence of vaso-occlusive episodes and acute chest syndrome in children with sickle cell disease. Br J Haematol **162:**702-705.
29. **Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, Smith A, Nath KA, Hebbel RP, Vercellotti GM.** 2014. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood **123:**377-390.
30. **Wagener FA, Feldman E, de Witte T, Abraham NG.** 1997. Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. Proc Soc Exp Biol Med **216:**456-463.
31. **Graca-Souza AV, Arruda MA, de Freitas MS, Barja-Fidalgo C, Oliveira PL.** 2002. Neutrophil activation by heme: implications for inflammatory processes. Blood **99:**4160-4165.
32. **Porto BN, Alves LS, Fernandez PL, Dutra TP, Figueiredo RT, Graca-Souza AV, Bozza MT.** 2007. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. J Biol Chem **282:**24430-24436.
33. **Ghosh S, Adisa OA, Chappa P, Tan F, Jackson KA, Archer DR, Ofori-Acquah SF.** 2013. Extracellular hemin crisis triggers acute chest syndrome in sickle mice. J Clin Invest **123:**4809-4820.
34. **Spiller F, Costa C, Souto FO, Vinchi F, Mestriner FL, Laure HJ, Alves-Filho JC, Freitas A, Rosa JC, Ferreira SH, Altruda F, Hirsch E, Greene LJ, Tolosano E, Cunha FQ.** 2011. Inhibition of neutrophil migration by hemopexin leads to increased mortality due to sepsis in mice. Am J Respir Crit Care Med **183:**922-931.
35. **Sander LE, Davis MJ, Boekschoten MV, Amsen D, Dascher CC, Ryffel B, Swanson JA, Muller M, Blander JM.** 2011. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature **474:**385-389.
36. **Chaix J, Tessmer MS, Hoebe K, Fuseri N, Ryffel B, Dalod M, Alexopoulou L, Beutler B, Brossay L, Vivier E, Walzer T.** 2008. Cutting edge: Priming of NK cells by IL-18. J Immunol **181:**1627-1631.
37. **Neven B, Callebaut I, Prieur AM, Feldmann J, Bodemer C, Lepore L, Derfalvi B, Benjaponpitak S, Vesely R, Sauvain MJ, Oertle S, Allen R, Morgan G, Borkhardt A, Hill C, Gardner-Medwin J, Fischer A, de Saint Basile G.** 2004. Molecular basis of the spectral expression of CIAS1 mutations associated with phagocytic cell-mediated autoinflammatory disorders CINCA/NOMID, MWS, and FCU. Blood **103:**2809-2815.
38. **Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD.** 2001. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet **29:**301-305.
39. **Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J.** 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature **440:**237-241.
40. **Donath MY, Shoelson SE.** 2011. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol **11:**98-107.

41. **Schroder K, Zhou R, Tschopp J.** 2010. The NLRP3 inflammasome: a sensor for metabolic danger? Science **327:**296-300.
42. **Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, Ravussin E, Stephens JM, Dixit VD.** 2011. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat Med **17:**179-188.
43. **Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, Brickey WJ, Ting JP.** 2011. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nat Immunol **12:**408-415.
44. **Stienstra R, Joosten LA, Koenen T, van Tits B, van Diepen JA, van den Berg SA, Rensen PC, Voshol PJ, Fantuzzi G, Hijmans A, Kersten S, Muller M, van den Berg WB, van Rooijen N, Wabitsch M, Kullberg BJ, van der Meer JW, Kanneganti T, Tack CJ, Netea MG.** 2010. The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity. Cell Metab **12:**593-605.
45. **Lee HM, Kim JJ, Kim HJ, Shong M, Ku BJ, Jo EK.** 2013. Upregulated NLRP3 inflammasome activation in patients with type 2 diabetes. Diabetes **62:**194-204.
46. **Guo B, Fu S, Zhang J, Liu B, Li Z.** 2016. Targeting inflammasome/IL-1 pathways for cancer immunotherapy. Sci Rep **6:**36107.
47. **Lin C, Zhang J.** 2017. Inflammasomes in Inflammation-Induced Cancer. Front Immunol **8:**271.
48. **Villani AC, Lemire M, Fortin G, Louis E, Silverberg MS, Collette C, Baba N, Libioulle C, Belaiche J, Bitton A, Gaudet D, Cohen A, Langelier D, Fortin PR, Wither JE, Sarfati M, Rutgeerts P, Rioux JD, Vermeire S, Hudson TJ, Franchimont D.** 2009. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. Nat Genet **41:**71-76.
49. **Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J.** 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science **320:**674-677.
50. **Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, Fitzgerald KA, Latz E, Moore KJ, Golenbock DT.** 2008. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol **9:**857-865.
51. **Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J.** 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ **14:**1583-1589.
52. **Zhou R, Yazdi AS, Menu P, Tschopp J.** 2011. A role for mitochondria in NLRP3 inflammasome activation. Nature **469:**221-225.
53. **Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, Hu L, Shao F.** 2014. Inflammatory caspases are innate immune receptors for intracellular LPS. Nature **514:**187-192.
54. **Kajiwara Y, Schiff T, Voloudakis G, Gama Sosa MA, Elder G, Bozdagi O, Buxbaum JD.** 2014. A critical role for human caspase-4 in endotoxin sensitivity. J Immunol **193:**335-343.
55. **Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M, Dixit**

VM. 2011. Non-canonical inflammasome activation targets caspase-11. Nature **479:**117-121.

56. **Pellegrini C, Antonioli L, Lopez-Castejon G, Blandizzi C, Fornai M.** 2017. Canonical and Non-Canonical Activation of NLRP3 Inflammasome at the Crossroad between Immune Tolerance and Intestinal Inflammation. Front Immunol **8:**36.
57. **Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, Miyake K, Zhang J, Lee WP, Muszynski A, Forsberg LS, Carlson RW, Dixit VM.** 2013. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science **341:**1246-1249.
58. **Gurung P, Malireddi RK, Anand PK, Demon D, Vande Walle L, Liu Z, Vogel P, Lamkanfi M, Kanneganti TD.** 2012. Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4 (TLR4) protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens. J Biol Chem **287:**34474-34483.
59. **Schauvliege R, Vanrobaeys J, Schotte P, Beyaert R.** 2002. Caspase-11 gene expression in response to lipopolysaccharide and interferon-gamma requires nuclear factor-kappa B and signal transducer and activator of transcription (STAT) 1. J Biol Chem **277:**41624-41630.
60. **Rathinam VA, Vanaja SK, Waggoner L, Sokolovska A, Becker C, Stuart LM, Leong JM, Fitzgerald KA.** 2012. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. Cell **150:**606-619.
61. **Broz P, Ruby T, Belhocine K, Bouley DM, Kayagaki N, Dixit VM, Monack DM.** 2012. Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. Nature **490:**288-291.
62. **Tschopp J, Schroder K.** 2010. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? Nat Rev Immunol **10:**210-215.
63. **Guo H, Callaway JB, Ting JP.** 2015. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med **21:**677-687.
64. **Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA.** 2013. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science **341:**1250-1253.
65. **Ganesan S, Rathinam VAK, Bossaller L, Army K, Kaiser WJ, Mocarski ES, Dillon CP, Green DR, Mayadas TN, Levitz SM, Hise AG, Silverman N, Fitzgerald KA.** 2014. Caspase-8 modulates dectin-1 and complement receptor 3-driven IL-1beta production in response to beta-glucans and the fungal pathogen, Candida albicans. J Immunol **193:**2519-2530.
66. **Gurung P, Kanneganti TD.** 2015. Novel roles for caspase-8 in IL-1beta and inflammasome regulation. Am J Pathol **185:**17-25.
67. **Allam R, Lawlor KE, Yu EC, Mildenhall AL, Moujalled DM, Lewis RS, Ke F, Mason KD, White MJ, Stacey KJ, Strasser A, O'Reilly LA, Alexander W, Kile BT, Vaux DL, Vince JE.** 2014. Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. EMBO Rep **15:**982-990.

68. **Schmidt RL, Lenz LL.** 2012. Distinct licensing of IL-18 and IL-1beta secretion in response to NLRP3 inflammasome activation. PLoS One **7:**e45186.
69. **Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J.** 2010. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat Immunol **11:**136-140.
70. **Grailer JJ, Canning BA, Kalbitz M, Haggadone MD, Dhond RM, Andjelkovic AV, Zetoune FS, Ward PA.** 2014. Critical role for the NLRP3 inflammasome during acute lung injury. J Immunol **192:**5974-5983.
71. **Kuipers MT, Aslami H, Janczy JR, van der Sluijs KF, Vlaar AP, Wolthuis EK, Choi G, Roelofs JJ, Flavell RA, Sutterwala FS, Bresser P, Leemans JC, van der Poll T, Schultz MJ, Wieland CW.** 2012. Ventilator-induced lung injury is mediated by the NLRP3 inflammasome. Anesthesiology **116:**1104-1115.
72. **Silveira AA, Cunningham C, Corr E, Ferreira WA, Costa FF, Almeida CB, Conran N, Dunne A.** 2016. Heme Induces NLRP3 Inflammasome Formation in Primary Human Macrophages and May Propagate Hemolytic Inflammatory Processes By Inducing S100A8 Expression. Blood **128**.
73. **Ryan TM, Ciavatta DJ, Townes TM.** 1997. Knockout-transgenic mouse model of sickle cell disease. Science **278:**873-876.
74. **Rabiet MJ, Plantier JL, Rival Y, Genoux Y, Lampugnani MG, Dejana E.** 1996. Thrombin-induced increase in endothelial permeability is associated with changes in cell-to-cell junction organization. Arterioscler Thromb Vasc Biol **16:**488-496.
75. **Garcia JG, Siflinger-Birnboim A, Bizios R, Del Vecchio PJ, Fenton JW, 2nd, Malik AB.** 1986. Thrombin-induced increase in albumin permeability across the endothelium. J Cell Physiol **128:**96-104.
76. **Grand RJ, Turnell AS, Grabham PW.** 1996. Cellular consequences of thrombin-receptor activation. Biochem J **313 (Pt 2):**353-368.
77. **Coll RC, Robertson AA, Chae JJ, Higgins SC, Munoz-Planillo R, Inserra MC, Vetter I, Dungan LS, Monks BG, Stutz A, Croker DE, Butler MS, Haneklaus M, Sutton CE, Nunez G, Latz E, Kastner DL, Mills KH, Masters SL, Schroder K, Cooper MA, O'Neill LA.** 2015. A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. Nat Med **21:**248-255.
78. **LaRock CN, Todd J, LaRock DL, Olson J, O'Donoghue AJ, Robertson AAB, Cooper MA, Hoffman HM, Nizet V.** 2016. IL-1beta is an innate immune sensor of microbial proteolysis. Sci Immunol **1:**eaah3539.
79. **Dutra FF, Alves LS, Rodrigues D, Fernandez PL, de Oliveira RB, Golenbock DT, Zamboni DS, Bozza MT.** 2014. Hemolysis-induced lethality involves inflammasome activation by heme. Proc Natl Acad Sci U S A **111:**E4110-4118.
80. **Martinon F, Tschopp J.** 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. Cell **117:**561-574.
81. **Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N, Nunez G.** 2006. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. J Biol Chem **281:**36560-36568.

82. **Ismael S, Nasoohi S, Ishrat T.** 2018. MCC950, the selective NLRP3 inflammasome inhibitor protects mice against traumatic brain injury. J Neurotrauma doi:10.1089/neu.2017.5344.
83. **Dempsey C, Rubio Araiz A, Bryson KJ, Finucane O, Larkin C, Mills EL, Robertson AAB, Cooper MA, O'Neill LAJ, Lynch MA.** 2017. Inhibiting the NLRP3 inflammasome with MCC950 promotes non-phlogistic clearance of amyloid-beta and cognitive function in APP/PS1 mice. Brain Behav Immun **61:**306-316.
84. **Muller-Eberhard U, Javid J, Liem HH, Hanstein A, Hanna M.** 1968. Plasma concentrations of hemopexin, haptoglobin and heme in patients with various hemolytic diseases. Blood **32:**811-815.
85. **He Y, Hara H, Nunez G.** 2016. Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends Biochem Sci **41:**1012-1021.
86. **Khan AA, Quigley JG.** 2011. Control of intracellular heme levels: heme transporters and heme oxygenases. Biochim Biophys Acta **1813:**668-682.
87. **Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, Cuellar T, Haley B, Roose-Girma M, Phung QT, Liu PS, Lill JR, Li H, Wu J, Kummerfeld S, Zhang J, Lee WP, Snipas SJ, Salvesen GS, Morris LX, Fitzgerald L, Zhang Y, Bertram EM, Goodnow CC, Dixit VM.** 2015. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature **526:**666-671.
88. **Li H, Zhou X, Zhang J.** 2014. Induction of heme oxygenase-1 attenuates lipopolysaccharide-induced inflammasome activation in human gingival epithelial cells. Int J Mol Med **34:**1039-1044.
89. **Amores-Iniesta J, Barbera-Cremades M, Martinez CM, Pons JA, Revilla-Nuin B, Martinez-Alarcon L, Di Virgilio F, Parrilla P, Baroja-Mazo A, Pelegrin P.** 2017. Extracellular ATP Activates the NLRP3 Inflammasome and Is an Early Danger Signal of Skin Allograft Rejection. Cell Rep **21:**3414-3426.
90. **Zha QB, Wei HX, Li CG, Liang YD, Xu LH, Bai WJ, Pan H, He XH, Ouyang DY.** 2016. ATP-Induced Inflammasome Activation and Pyroptosis Is Regulated by AMP-Activated Protein Kinase in Macrophages. Front Immunol **7:**597.
91. **Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM.** 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature **440:**228-232.
92. **Matsunaga N, Tsuchimori N, Matsumoto T, Ii M.** 2011. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. Mol Pharmacol **79:**34-41.
93. **Vuckovic S, Vandyke K, Rickards DA, McCauley Winter P, Brown SHJ, Mitchell TW, Liu J, Lu J, Askenase PW, Yuriev E, Capuano B, Ramsland PA, Hill GR, Zannettino ACW, Hutchinson AT.** 2017. The cationic small molecule GW4869 is cytotoxic to high phosphatidylserine-expressing myeloma cells. Br J Haematol **177:**423-440.