PROHIBITIN: A NOVEL REGULATOR OF INFLAMMATORY CELL DYNAMICS

By

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Inflammation is a complex mechanism primarily driven by the immune system to eradicate pathogens/foreign substances and restore tissue homeostasis. Despite the beneficial effects that inflammation employs, signaling can often become dysregulated leading to uncontrolled systemic inflammation and irreversible host tissue damage. Therefore, regulating the cellular and physiological mechanisms of inflammation constitutes a viable avenue of research to mitigate inflammatory disease progression. Herein, we evaluated prohibitins (PHB1 and PHB2), pleiotropic homologous proteins with known anti-inflammatory and antioxidant capabilities, in the context of systemic inflammation as well as macrophage-specific inflammatory signaling. Using two *in vivo* models of systemic inflammation, we found that PHB1 levels were increased in serum, suggesting a potential signaling role for PHB. Moreover, recombinant PHB1 treatment mitigated systemic inflammation and tissue/organ injury and modulated the phenotype of circulating immune cells. When investigating the role of PHB specifically in monocytes/macrophages, we found that PHB not only increased populations of

pro-inflammatory monocytes *in vivo* but also regulated vital macrophage inflammatory signaling (as shown *in vitro*). We determined that PHB is a scaffold protein important for macrophage lipid raft formation and subsequent receptor trafficking. PHB modulation of macrophages influenced cell surface display of lipid-raft-dependent receptors and downstream inflammatory signaling cascades. To our knowledge, these are the first data to reveal PHB's pro-inflammatory effects in macrophages and its mechanistic operation of lipid-raft-dependent signal transduction in macrophages. In this report, we provide insight into the diverse yet complementary roles of PHB in regulating various aspects of immune-driven inflammatory processes.

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By

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TO

My Phenomenal Parents

Thank you for showing me the meaning of hard work and for teaching me the value of education.

My Wonderful Sister

Thank you for guiding me throughout my entire life. I could not have accomplished all of this without you. You paved the way through undergrad and graduate school, exposed me to job opportunities and developed my presentation and social skills, helped me make notecards for late-night study sessions, and provided coffee and home-cooked meals any time of the day. Thank you for pushing me forward when I have tried to turn around and for giving me a hand when I have missed a step from the path.

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LIST OF SYMBOLS OR ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
CFU	Colony forming units
CXCL1	Keratinocyte chemoattractant
CXCL2	Macrophage inflammatory protein-2
DAMP	Damage associated molecular pattern
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GP	Generalized polarization
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-10	Interleukin-10
i.p.	Intraperitoneal
KC	Keratinocyte chemoattractant
K.p.	*Klebsiella pneumoniae*
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MFI	Mean fluorescence intensity
MIP-2	Macrophage inflammatory protein-2
mtDNA	Mitochondrial DNA
NF-κB	Nuclear factor-kappa B
o.p.	Oropharyngeal
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PHB	Prohibitin

PHB1	Prohibitin-1
PHB2	Prohibitin-2
PMN	Neutrophils
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real time-polymerase chain reaction
ROS	Reactive oxygen species
RIPA	Radio immunoprecipitation assay buffer
rPHB1	Recombinant prohibitin 1
Scr	Scrambled control
SEM	Standard error of the mean
TLR	Toll like receptors
TLR4	Toll like receptor 4
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1

CHAPTER ONE: GENERAL INTRODUCTION

1.1 SEPSIS – AN INFLAMMATORY RESPONSE

Sepsis is a systemic inflammatory condition that remains a leading cause of mortality worldwide. Currently, treatment of septic patients costs more than $24 billion each year in the United States alone (1), indicating that this overt inflammatory response is a significant healthcare and economic burden (2, 3). The pathophysiology of sepsis is initiated by the innate immune system identifying invading pathogen associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs) and inducing downstream signal transduction to recruit additional immune cells, such neutrophils (PMNs) and monocytes, and promote eradication of the pathogen (4). Although the innate immune response is critical for host defense, it can also augment the production of pro-inflammatory cytokines and elicit increased accumulation of PMNs, leading to excessive oxidative stress and irreversible host tissue injury (5). Current treatment modalities aim to combat the progression of the inflammatory pathophysiology via emergency maintenance of vital signs and prevention of organ dysfunction/failure.

1.1.1 Systemic organ dysfunction and failure

The mortality rate in septic patients is exceedingly high (>35%), due in large part to the organ failure that accompanies the dysregulated inflammatory response to infection. Development of multiple organ failure is initiated by electro-mechanical dysfunction of the heart and/or heart failure. Cardiac dysfunction is caused by the acute systemic inflammation that disrupts a variety of mechanisms. Increased levels of the pro-inflammatory cytokines TNF-α and

IL-1β during sepsis have been shown to have a negative ionotropic effect on the heart by causing decreased cardiomyocyte contraction (6). Additionally, previous studies have demonstrated that TNF-α and IL-1β cause the sarcoplasm reticulum to leak Ca^{2+} leading to systolic and diastolic dysfunction and increased adenosine triphosphate (ATP) expenditure (7, 8). Inflammation during sepsis has also been shown to increase reactive oxygen species (ROS) levels which can trigger mitochondrial damage and dysfunction. This is important in the context of sepsis because sustained mitochondrial function is required to meet the high energy demand in cardiomyocytes. Ultimately, dysfunction of the heart and decreased cardiac output lead to hypoperfusion, or decreased delivery of oxygen to the organs (9).

Moreover, dilation of the vasculature occurs and results in a subsequent decrease in function of the endothelial barrier and leakage of fluids into the surrounding areas. With hypoperfusion, multiple organ dysfunction and failure occur in rapid sequelae, increasing patient morbidity and mortality. Therapeutic interventions include administration of antibiotics, crystalloid fluids and vasopressors for hypotension, and oxygen for tissue hypoxia (10). However, recent studies have reported that pro-inflammatory innate immune cells, such as macrophages, switch their metabolism from oxidative phosphorylation to glycolysis to quickly generate ATP (11). This increase in ATP supports further production of cytokines/chemokines and rapid cell proliferation, and a prolonged pro-inflammatory immune response can lead to both short- and long-term patient health effects. Hence, organ injury during severe sepsis is attributed to excessive PMN recruitment and accumulation in the tissues. Here PMNs act to combat invading pathogens by producing nitric oxide; however, inappropriate systemic overexpression of cytokines and chemokines can lead to host tissue damage. While physicians may utilize steroids to dampen the innate immune response, additional treatment methods are needed to

manage both the inflammation and mitochondrial damage that cause tissue/organ injury during sepsis.

1.1.2 Innate immune response

The pathophysiology of sepsis is driven by the innate immune system identifying the invading pathogen via pattern recognition receptors (PRR) and inducing a cascade of signals to react to the threat (4, 12). Toll-like receptor (TLR) is a type of PRR and is responsible for the subsequent signaling cascade to initiate inflammation and immune cell recruitment to the site of infection. Specifically, TLR4 is characterized as the PRR for Gram-negative pathogens as well as endotoxin LPS. The CD14/TLR4/MyD88 complex recognizes these PAMPs and activates intracellular signaling pathways. This signal transduction stimulates nuclear factor-kappa B (NF-κB) to translocate into the nucleus and promote the transcription of cytokines/chemokines and production of adhesion molecules (4). These cytokines and chemokines are released to recruit immune cells, such as PMNs and macrophages, which phagocytize pathogens and promote anti-inflammatory mediators to initiate the resolution phase, including wound healing/ tissue repair. Despite the beneficial effects that inflammation employs, many cellular and physiological mechanisms can become disrupted due to inappropriate systemic overexpression of cytokines, also known as cytokine storm (5). If unchecked, the hyper-inflammatory response can cause excessive host tissue damage leading to organ dysfunction (4, 12).

1.2 PROHIBITIN

1.2.1 Prohibitins are pleiotropic proteins

Prohibitins (PHB1 and PHB2) are mitochondrial proteins that assemble in hetero-oligomeric complexes within the mitochondrial inner membrane where they regulate

mitochondrial morphology and antioxidant mechanisms (13-17). The active form of PHB is a large ring-like protein complex comprised of interdependent homologs PHB1 and PHB2 (18). Studies have indicated that deletion of an individual PHB1 or PHB2 gene leads to a loss of both protein subunits, and degradation of one protein prevents assembly of the complex (19-22). PHBs also have critical roles in multiple biological processes including cell proliferation, apoptosis (23), oxidative stress (24), signal transduction (25, 26), and metabolism (27). Notably, overexpression of PHB in cardiomyocytes and in intestinal epithelial cells has been shown to be protective against oxidative stress (13, 16), whereas knockdown of PHB results in progression of inflammatory disease such as liver fibrosis and irritable bowel disease (14, 27, 28).These diverse functions are largely attributed to PHB's post-translational modifications and its ability to traffic to numerous cellular components (i.e. mitochondria, plasma membrane, Golgi, etc.).

1.2.2 Prohibitins are lipid raft proteins

PHBs are ubiquitously expressed proteins that belong to the SPFH-family (stomatin/prohibitin/flotillin/HflK/C), a group of proteins localized to lipid-rich cellular compartments, including plasma membrane lipid rafts (29). PHBs are highly expressed in immune cells (30) and proteomic analyses have identified PHBs in the lipid rafts of mast cells, macrophages, and lymphocytes (31-33). Recent studies have indicated PHB influences immune cell function by regulating CD86 phosphorylation of IκBα in B lymphocytes. PHB was identified as a scaffold protein, binding to CD86 and mediating signal transduction to ultimately activate NF-κB p50/p65 of B lymphocytes (33). Furthermore, one study has documented PHB's scaffolding function in mast cells to regulate degranulation and FcεRI signaling (34). These findings add to the growing evidence of PHB as a regulator of immune cell signaling.

CHAPTER TWO: IDENTIFYING THE ROLE OF CIRCULATING PROHIBITIN IN INFLAMMATORY SIGNALING DURING SEPSIS

2.1 ABSTRACT

Sepsis is a dysregulated inflammatory response to infection characterized by overt overexpression of pro-inflammatory cytokines and tissue damage resulting from excess immune cell infiltration and increased oxidative stress. Damaged mitochondria contribute to systemic inflammation by producing reactive oxygen species (ROS), cytochrome c, and mitochondrial DNA (mtDNA). In addition, recent immunometabolism studies have revealed pro-inflammatory macrophages switch their metabolic profile to glycolysis to quickly meet the high demand for ATP, and prolonged glycolytic metabolism can lead to mitochondrial dysfunction. Due to the augmented systemic inflammatory response and the mitochondrial dysfunction that accompany sepsis, novel therapeutic strategies are necessary to manage both the inflammation and mitochondrial damage. Prohibitins (PHB1 and PHB2) are mitochondrial proteins that assemble in hetero-oligomeric complexes within the mitochondrial inner membrane where they are at the nexus of metabolic and pro-survival decisions including inflammation. PHBs are important for maintaining mitochondrial structure and function and are known to have anti-inflammatory and antioxidant roles in chronic inflammatory diseases. However, the role of PHBs in the systemic inflammation that drives sepsis is currently unknown. Using an established *in vivo* model of sepsis, we found that mice intraperitoneally (i.p.) injected with lipopolysaccharide (LPS) had increased serum PHB1. This was confirmed with an additional model of sepsis, as *Klebsiella pneumoniae* (*K.p.*) infection also yielded increased serum PHB1. Treatment of LPS-induced septic mice with exogenous recombinant PHB1 (rPHB1) decreased serum levels of IL-6 and lactate dehydrogenase (LDH). Moreover, rPHB1 treatment mitigated LPS-induced expression of

pro-inflammatory cytokines (TNF-α and IL-6) and neutrophil-recruiting chemokines (MIP-2 and KC) in the kidney, lung, and liver tissues. To determine PHB1's effects on the circulating immune cell populations known to influence organ damage during sepsis, peripheral blood was analyzed by flow cytometry. Systemic rPHB1 treatment attenuated LPS-induced neutrophil (PMN) expression of the adhesion molecule CD11b. Interestingly, rPHB1 post LPS increased circulating inflammatory monocytes ($CD45^{+}CD115^{+}CD11b^{+}Ly6C^{hi}$). These data provide novel evidence of rPHB1's role in blunting inflammation-induced organ injury and modulating phenotypes of circulating immune cells. Herein, we reveal a potential novel role for PHB1 in regulating the immune-mediated inflammatory response during sepsis.

2.2 INTRODUCTION

Sepsis is a systemic inflammatory response to infection that remains a leading cause of mortality worldwide. Currently, treatment of septic patients costs more than $24 billion each year in the United States alone (1), indicating that this overt inflammatory response is a significant healthcare and economic burden (2, 3). The pathophysiology of sepsis is initiated by the innate immune system identifying invading pathogens and inducing downstream signal transduction to recruit additional immune cells, such PMNs and monocytes, and promote eradication of the pathogen. Although the innate immune response is critical for host defense, it can also augment the production of pro-inflammatory cytokines and elicit increased accumulation of PMNs, leading to excessive oxidative stress and irreversible host tissue injury (4, 5). Damaged mitochondria contribute to systemic inflammation (35, 36). Recent immunometabolism studies have reported that pro-inflammatory innate immune cells, such as macrophages, switch their metabolism from oxidative phosphorylation to glycolysis to quickly generate ATP (11). This increase in ATP supports further production of cytokines/chemokines and rapid cell proliferation. However, prolonged glycolysis in macrophages often becomes dysregulated leading to mitochondrial dysfunction and augmented oxidative stress and organ injury (37, 38). Therefore, targeting both the overt overexpression of pro-inflammatory cytokines and the mitochondrial dysfunction during sepsis may constitute a viable avenue of research to reduce the inflammation and oxidative damage which drive the disease progression.

Prohibitins (PHB1 and PHB2) are mitochondrial proteins that assemble in hetero-oligomeric complexes within the mitochondrial inner membrane where they regulate mitochondrial morphology and antioxidant mechanisms (13-17). PHBs have been implicated as potential therapeutic targets for inflammatory diseases due to their cytoprotective characteristics

(39). Notably, overexpression of PHB in cardiomyocytes and in intestinal epithelial cells has been shown to be protective against oxidative stress (13, 16), whereas knockdown of PHB results in progression of inflammatory disease such as liver fibrosis and irritable bowel disease (14, 27, 28). Despite PHB's beneficial anti-inflammatory and antioxidant effects, the role of PHB in the systemic inflammatory response to sepsis is currently unknown.

Herein, we present data suggesting PHB is a novel therapeutic target for regulating systemic inflammation during sepsis. We found that mice i.p. injected with LPS had increased serum PHB1which was further confirmed with *K.p.* infection. Injection of rPHB1 post LPS decreased serum pro-inflammatory cytokine IL-6 and mitigated serum LDH, a clinical marker for tissue/organ injury. Additionally, rPHB1 treatment mitigated LPS-induced expression of pro-inflammatory cytokines and neutrophil-recruiting chemokines in the kidney, lung, and liver tissues. We found that mice treated with rPHB1 had decreased CD11b cell surface display on circulating neutrophils but had increased percentage of inflammatory monocytes. These data provide novel evidence of PHB's role in modulating immune cells and blunting inflammation-induced organ injury. This study is the first to identify PHB1 as a potential modulator of immune-mediated inflammation and indicates a role for PHB as an extracellular signaling molecule. Furthermore, we introduce PHB as a novel therapeutic target for regulating the systemic inflammation present during sepsis.

2.3 MATERIALS AND METHODS

2.3.1 Reagents

Escherichia coli 0111:B4 Lipopolysaccharide was purchased from Sigma-Aldrich (St. Louis, MO); Recombinant PHB1 was purchased from OriGene (Rockville, MD); and Phosphate-buffered saline, Tween-20, and fetal bovine serum were purchased from Thermo Fisher Scientific (Waltham, MA).

2.3.2 Murine LPS exposure

C57Bl/6J male mice, 8-10 weeks old and weighing 18-22g, were purchased from Jackson Laboratories (Bar Harbor, ME). All experiments were conducted with approval from the Institutional Animal Care and Use Committee at East Carolina University. Mice were injected intraperitoneally (i.p.) with LPS (12mg/kg) or sterile saline control at baseline; then mice were injected i.p. with recombinant Prohibitin 1 (rPHB1) (12µg/kg) 2 hr, 8 hr, and 14 hr post first injection. Mice were euthanized at 7 hr or 16 hr post first injection and samples were collected.

2.3.3 Murine *Klebsiella pneumoniae* infection

C57Bl/6J male mice, 8-10 weeks old and weighing 18-22g, were purchased from Jackson Laboratories (Bar Harbor, ME). All experiments were conducted with approval from the Institutional Animal Care and Use Committee at East Carolina University. Mice were instilled oropharyngeally (o.p.) with 2,000 colony forming units (CFU) of *Klebsiella pneumoniae (K.p.)*. Mice were euthanized 6, 24, or 48 hr post treatment. Uninfected control mice were euthanized at 0 hr.

2.3.4 Bio-Plex assay

Serum cytokines were analyzed with Bio-Plex mouse cytokine panel (Bio-Rad; Hercules, CA) including TNF-αand IL-6. Samples were analyzed according to the manufacturer's recommended protocol and read using MAGPIX Instrument (Luminex; Austin, TX). Data were analyzed using Milliplex analyst software.

2.3.5 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) was measured in serum 16 hours post LPS injection using a commercially available kit (Abcam; Cambridge, MA) according to the manufacturer's instructions. Briefly, 50µL of serum diluted in LDH Assay Buffer was added to an optically clear 96-well plate. LDH Reaction Mix was added to each well and absorbance was measured at 450nm for 30-60 minutes in kinetic capture mode at 37°C and OD values were compared to standard curve.

2.3.6 RNA isolation, cDNA, and Polymerase chain reaction

RNA was isolated from various tissues via RNeasy kit (Qiagen; Hilden, Germany). RNA was transcribed into cDNA using a high Capacity cDNA Reverse Transcription kit from Applied Biosystems (Thermo Fisher Scientific; Waltham, MA) according to manufacturer's protocol. Real-time PCR was performed in duplicate for PHB1 (Mm01627033_g1), PHB2 (Mm00476104_m1), TNF-α (Mm00443258_m1), IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1), IL-10 (Mm00439614_m1), chemokine ligand-1 (Cxcl1) (Mm04207460_m1), and chemokine ligand-2 (Cxcl2) (Mm00436450_m1). All primer/probes were purchased from Applied Biosystems (Foster City, CA). Gene expression was normalized to

the house keeping gene 18S (Hs99999901_s1), and expression levels were normalized to saline-treated controls using the relative quantification method (Boulter 2016).

2.3.7 Flow cytometry analysis of blood monocytes, neutrophils, and CD11b

After mice were treated as described above, blood samples were processed for flow cytometry. Briefly, red blood cells were lysed, washed, and resuspended in FACS buffer. Cells were blocked using 5% normal mouse serum and 5% normal rat serum (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) and 1% FCR block in FACS buffer for 15 min. After blocking, samples were incubated with anti-mouse CD45 PB (cat# 103126), Ly6G FITC (cat# 127606), CD11b APC (cat# 101212), CD115 PE (cat# 135506), and Ly6C AmCyan/BV510 (cat# 128033) antibodies purchased from BioLegend (San Diego, CA). Flow cytometry was performed using an LSR II (BD Biosciences, Ontario, Canada) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

2.3.8 Enzyme-linked immunosorbent assay of PHB1

Blood was drawn via cardiac puncture of mice at 7 or 16 hours following i.p. injections of LPS-sepsis-model as described above. Also, blood was drawn via cardiac puncture of mice at 0, 6, 24, or 48 hours following o.p. infection of *K.p.* sepsis model as described above. Samples were centrifuged at 500x g for 5 minutes to separate the serum. Quantification of PHB1 in circulation was determined by an enzyme-linked immunosorbent assay (ELISA) approach developed by Dr. Ethan Anderson's laboratory. Briefly, standards and diluted serum (1:5) samples were added to an Immunolon-coated 96-well plate (Fisher Scientific; Hampton, NH). Samples were incubated overnight at 4°C, and the plate washed with PBS+0.05% Tween-20 and blocked for 2 hours with 10% fetal bovine serum (Sigma-Aldrich; St. Louis, MO) diluted in

PBS. Samples were then incubated with anti-PHB1 antibody (1:200 in PHB+0.05% BSA) (Abcam; Cambridge, MA) for 2 hours at 37°C. Samples were washed with PBS+0.05% Tween-20 and incubated with secondary antibody (1:1000 in PBS-Tween) (goat anti-rabbit HRP, Bio-Rad) for 2 hours at room temperature. Following this incubation, samples were washed as before and incubated with TMBZ for 20 minutes at RT. The reaction was quenched with 1M sulfuric acid, and the absorbance measured at 450nm. Total quantities of serum PHB1 in each group were determined using standard curve of rPHB1 (OriGene; Rockville, MD), and absolute amount was defined as ng/ml.

2.3.9 Statistical analysis

Analysis was performed using GraphPad Prism software (San Diego, CA). Data are represented as mean ± SEM. Two-tailed student's t test was applied for comparisons of two groups, and ANOVA for comparisons of >2 groups. For all tests, $p<0.05$ was considered significant.

2.4 RESULTS

2.4.1 Recombinant PHB1 treatment during sepsis decreases serum proinflammatory cytokines and lactate dehydrogenase.

Previous studies have reported that overexpression of PHB1 is cytoprotective, having antioxidant and anti-inflammatory effects in various models of disease (13-17). However, no studies have investigated the role of PHB1 in systemic models of inflammation. Based on a study indicating that incubation of pancreatic beta cells with rPHB1 exhibits cytoprotective effects similar to PHB1 overexpression (40), we hypothesized rPHB1 treatment has systemic anti-inflammatory and organ-protective effects in sepsis.

To test this hypothesis, C57Bl/6J male, 8-10-week-old mice were injected i.p. with LPS (12mg/kg), to initiate a previously-defined model of severe sepsis (41), or sterile saline control (Veh) at baseline. Then mice were injected i.p. with rPHB1 (12µg/kg) or Veh at each individual time point: 2, 8, and 14 hours post initial injection. Samples were collected at 7 hours (total of 2 injections) or 16 hours (total of 4 injections) (**Figure 2.1**). The purpose of these treatment parameters was to determine the effect of one or three successive injections of rPHB1 after LPS-induced systemic inflammation. Previous findings suggest rPHB1 to be a quickly-degrading protein (41); hence the decision to deliver multiple injections.

As expected, mice injected with LPS had significantly increased serum pro-inflammatory cytokine IL-6 production compared to Veh at the 16-hour time point (**Figure 2.2A**). Of note, rPHB1 treatments alone did not increase inflammatory cytokine TNF-α nor IL-6. Interestingly, mice treated with three rPHB1 doses had a complete mitigation of this LPS-induced IL-6 suggesting rPHB1 treatment decreases systemic pro-inflammatory cytokine production.

Organ injury and dysfunction are key contributors to increased mortality in patients with sepsis. To determine systemic tissue damage in our model of sepsis, serum was analyzed for LDH activity, an oxidoreductase and widely used clinical marker for cell/tissue toxicity. As expected, LPS challenge initiated a drastic increase in serum LDH at the 16-hour time point (**Figure 2.2B**). However, rPHB1 treatment blunted LDH release into the serum suggesting rPHB1 mitigated the detrimental effects of LPS. Taken together, these data provide evidence of rPHB1 blunting systemic inflammation during sepsis.

2.4.2 rPHB1 treatment during sepsis decreases organ pro-inflammatory cytokine expression.

In order to more closely determine the role of PHB1 in decreasing systemic inflammation, we next analyzed tissues for pro-inflammatory cytokine expression. RNA was isolated from (**Figure 2.3A**) kidney, (**Figure 2.3B**) lung, and (**Figure 2.3C**) liver tissue at the 16-hour time point and mRNA expression of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β was profiled by quantitative real time-PCR. Expression of TNF-α, IL-6, and IL-1β pro-inflammatory cytokines were increased in all organs of LPS-challenged mice. However, there was a uniform abrogation of LPS-induced IL-6 expression in kidney, lung, and liver tissues after three doses of rPHB1. Interestingly, increases in LPS-induced TNF-α were mitigated in the kidney tissue of rPHB1-treated mice (**Figure 2.3A**) but not in the lung or liver (**Figure 2.3B-C**). Lastly, LPS-induced increases of IL-1β were not affected by rPHB1 treatments. These data further characterize PHB's role in dampening systemic inflammation.

2.4.3 rPHB1 treatment during sepsis decreases organ chemokine expression.

Organ injury during severe sepsis is attributed to excessive neutrophil recruitment and accumulation in the tissues. Here neutrophils act to combat invading pathogens by producing nitric oxide; however, inappropriate systemic overexpression of cytokines and chemokines can lead to host tissue damage. To determine whether PHB1 affects chemokine expression in tissues, RNA was isolated from (**Figure 2.4A**) kidney, (**Figure 2.4B**) lung, and (**Figure 2.4C**) liver at the 16-hour time point and mRNA expression of neutrophil-recruiting chemokines MIP-2 and KC was profiled by quantitative real time-PCR. As anticipated, MIP-2 and KC expression was considerably increased in all tissues of LPS-challenged mice. However, rPHB1-treated mice had blunted MIP-2 and KC expression compared to LPS alone **(Figure 2.4A** and **Figure 2.4C**). Interestingly, rPHB1 treatment decreased MIP-2 (but not KC) expression in lung tissue (**Figure 2.4B**). These data suggest rPHB1 decreases expression of neutrophil-recruiting chemokines in kidney, lung, and liver which may provide an explanation for the decreased tissue injury in rPHB1-treated mice represented in **Figure 2.2B**.

2.4.4 rPHB1 treatment during sepsis alters anti-inflammatory cytokine IL-10 expression in tissue-specific manner.

Next, we identified whether PHB1 plays a role in regulating IL-10, an anti-inflammatory cytokine important in resolution of systemic inflammation (42). Kidney, lung, and liver were collected from 16-hour treated mice, RNA was isolated, and IL-10 was profiled by quantitative real time-PCR. As shown in **Figure 2.5A**, mice with LPS challenge had increased kidney IL-10 mRNA expression which was unchanged with rPHB1 treatments. However, LPS-challenge did not influence lung IL-10 expression, but mice injected with LPS and rPHB1 had an interesting increase in IL-10 (**Figure 2.5B**). Liver IL-10 expression increased with LPS challenge which was mitigated with rPHB1 treatments (**Figure 2.5C**). These varied results suggest rPHB1 has

diverse effects on regulation of anti-inflammatory cytokine IL-10 that may indicate a complex tissue-specific mechanism.

2.4.5` rPHB1 treatment during sepsis modulates circulating immune cell populations.

Given that immune cells are critical for the initiation of systemic inflammation, we next analyzed immune cell populations circulating in the blood of mice from the four different treatment groups. Mice were injected i.p. with LPS or Veh at baseline. Then mice were injected i.p. with rPHB1 or Veh at 2 hours post initial injection. Blood was collected at 7 hours (total of 2 injections) and samples were processed for flow cytometry. Blood inflammatory monocytes were defined as $CD45^{+}CD115^{+}CD11b^{+}Ly6C^{hi}$ and values are displayed as percentage of total circulating monocytes (defined as $CD45^{+}CD115^{+}CD11b^{+}$) (**Figure 2.6A**). Interestingly, percentage of inflammatory monocytes (of total circulating monocytes) did not differ in mice treated with rPHB1 alone or challenged with LPS. However, there was nearly a 20% increase in circulating inflammatory monocytes for mice treated with both LPS and one treatment of rPHB1. Although there were no statistically significant differences in the percentages of circulating blood neutrophils (defined as $[CD45^{+}Ly6G^{+}]$ of total circulating immune cells $[CD45^{+}]$) (**Figure 2.6B**), CD11b expression of neutrophils was significantly decreased in mice treated with rPHB1 alone or in conjunction with LPS. This suggests PHB plays a role in modulating systemic immune cells, potentially inhibiting their recruitment to target tissues.

2.4.6 Serum PHB1 is elevated during LPS model of sepsis.

Elevated serum levels of PHB have been used as a biomarker for patients with cancer due to PHB's effects on cell proliferation and survival (43). Additionally, cellular PHB levels have been shown to fluctuate in response to cellular stress (33), and emerging evidence suggests PHBs

have important roles in signal transduction pathways (44). However, no studies have investigated PHB protein production or expression during systemic inflammation. As shown in **Figure 2.7A**, mice treated with one injection of rPHB1 had increased serum PHB1 compared to Veh. Interestingly, serum PHB1 drastically increased in LPS-challenged mice and mice treated with one dose of rPHB1 post LPS. This suggests PHB1 may act as a circulating signal during early sepsis. At the 16-hour time point, serum PHB1 was not significantly elevated.

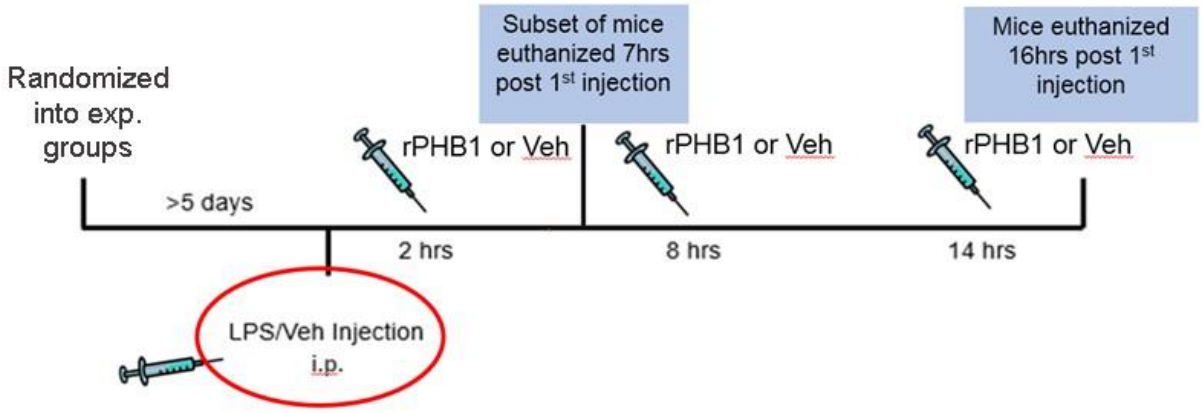
Next, RNA was isolated from kidney, lung, and liver tissue at the 16-hour time point, and mRNA expression of PHB1 was profiled by quantitative real time-PCR. As shown in **Figure 2.7B**, PHB1 mRNA expression decreased in kidney and liver tissues with LPS challenge and/or rPHB1 treatments suggesting a negative feedback regulation. Interestingly, lung PHB1mRNA expression was only upregulated with LPS challenge.

2.4.7 Serum PHB1 is elevated during *Klebsiella pneumoniae* model of sepsis.

In order to further confirm our results, the clinically-relevant *K.p.* was used an additional model of sepsis. C57Bl/6J male, 8-10-week-old mice were instilled oropharyngeally (o.p.) with 2,000 colony forming units (CFU) of *K.p*. Mice were euthanized 6, 24, or 48 hours post treatment (**Figure 2.8A**). To determine whether PHB1 is released into circulation upon the inflammatory response to *K.p.*, blood was collected from mice and serum was analyzed for PHB1. As shown in **Figure 2.8B**, serum PHB1 was elevated at 6 and 24 hours post *K.p.* infection. This provides further evidence that PHB1 is released into circulation during systemic inflammation and may be acting as a regulatory signal.

2.5 FIGURES

Figure 2.1: Experimental design – rPHB1 treatment in murine LPS model of sepsis. C57Bl/6J male, 8-10-week-old mice were injected intraperitoneally (i.p.) with lipopolysaccharide (LPS) (12mg/kg) or sterile saline control (Veh) at baseline; then mice were injected i.p. with recombinant Prohibitin 1 (rPHB1) (12µg/kg) or Veh at each individual time point: 2 hr, 8 hr, and 14 hr post initial injection. Mice were euthanized 7 hr (total of 2 injections) or 16 hr (total of 4 injections) post baseline and samples were collected. Experimental groups include: Vehicle, rPHB1, LPS, or LPS+rPHB1 treated mice.



Wild Type - C57Bl/6J

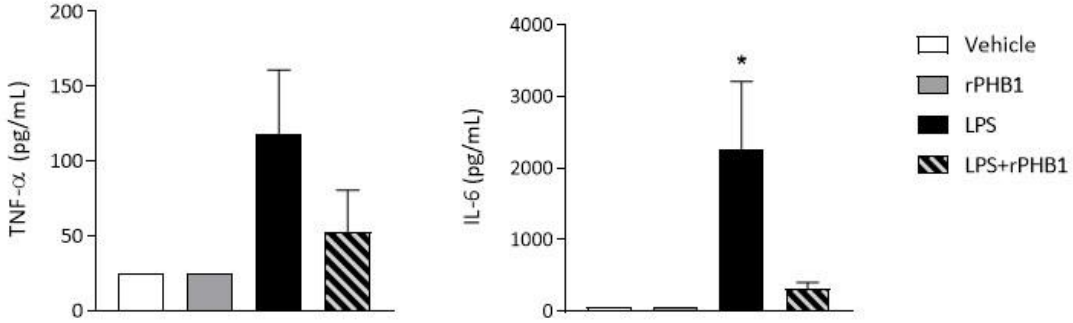
Exp. Groups

Group 1 - Vehicle
Group 2 - Vehicle + rPHB1
Group 3 - LPS + Vehicle
Group 4 - LPS + rPHB1

Figure 2.2: rPHB1 treatment during sepsis decreases serum proinflammatory cytokines and lactate dehydrogenase.

C57Bl/6J male mice were injected intraperitoneally (i.p.) with LPS (12mg/kg) or sterile saline control (Veh) at baseline; then mice were injected i.p. with rPHB1 (12μg/kg) or Veh at each individual time point: 2 hr, 8 hr, and 14 hr post initial injection. Blood was drawn via cardiac puncture at 16 hr and serum was harvested. **A)** Serum levels of pro-inflammatory cytokines TNF-α and IL-6 were measured by Multiplex ELISA. **B)** Serum lactate dehydrogenase (LDH) activity assay. All data are expressed as mean +/- SEM and derive from 2 independent experiments. n=6/group. *, $P<0.05$; ***, $P<0.001$.

A)


TNF-α (pg/mL)
200
150
100
50
0
IL-6 (pg/mL)
4000
3000
2000
1000
0
*
Vehicle
rPHB1
LPS
LPS+rPHB1

B)

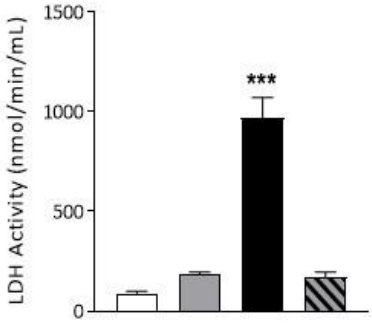

LDH Activity (nmol/min/mL)
1500
1000
500
0
***

Figure 2.3. rPHB1 treatment during sepsis decreases organ pro-inflammatory cytokine expression.

C57Bl/6J male mice were injected intraperitoneally (i.p.) with LPS (12mg/kg) or sterile saline control (Veh) at baseline; then mice were injected i.p. with rPHB1 (12μg/kg) or Veh at each individual time point: 2 hr, 8 hr, and 14 hr post initial injection. Mice were euthanized at 16 hr and RNA was isolated from (**A**) kidney, (**B**) lung, and (**C**) liver and expression of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β was profiled by quantitative real time-PCR normalized to 18S as arbitrary units (AUs). All data are expressed as mean +/- SEM and derive from 2 independent experiments. n=6/group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to vehicle. #, $P<0.05$; ##, $P<0.01$; ###, $P<0.001$ compared to LPS+rPHB1.

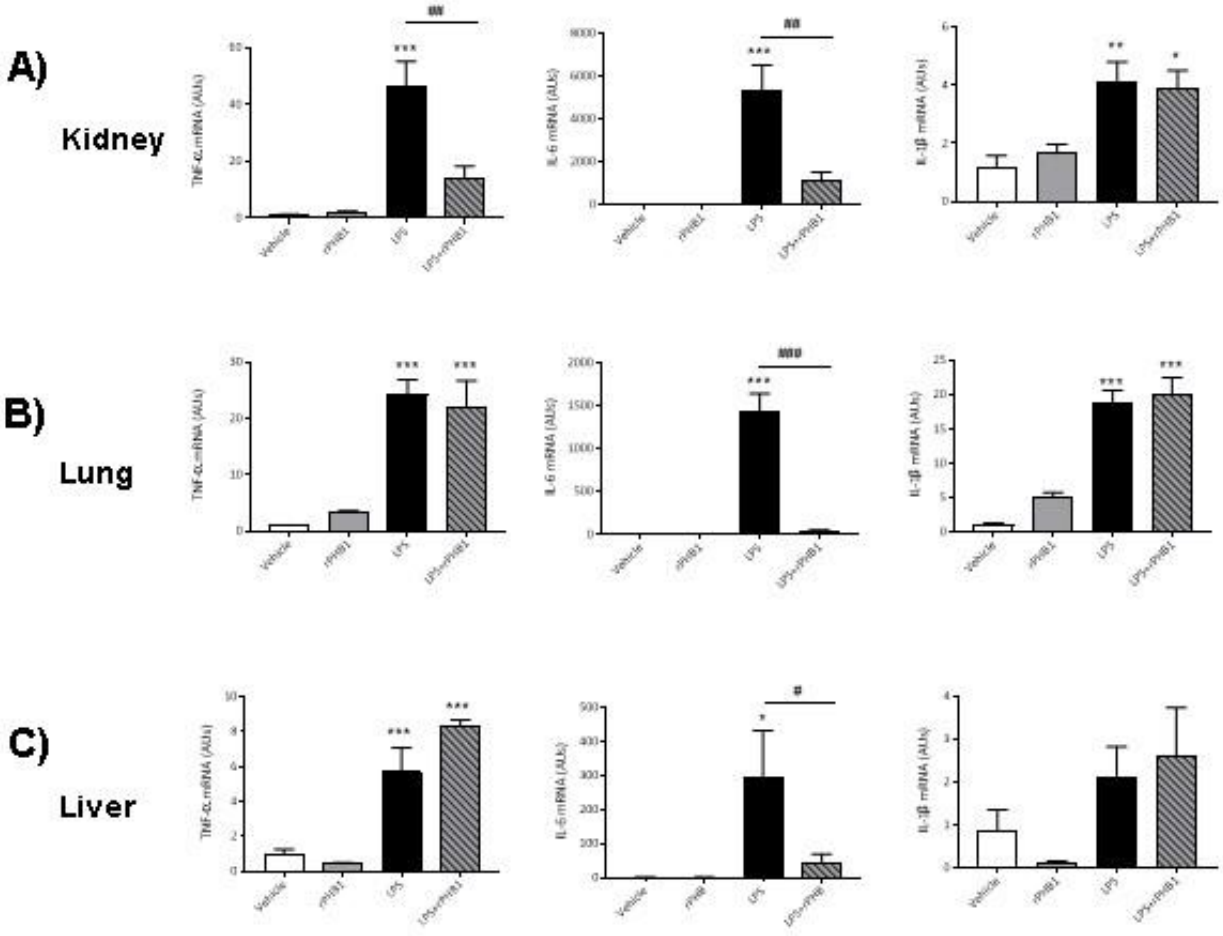

A)
Kidney
B)
Lung
C)
Liver
TNF-α mRNA (AUs)
IL-6 mRNA (AUs)
IL-1β mRNA (AUs)
Vehicle
rPHB1
LPS
LPS+rPHB1

Figure 2.4: rPHB1 treatment during sepsis decreases organ chemokine expression. C57Bl/6J male mice were injected intraperitoneally (i.p.) with LPS (12mg/kg) or sterile saline control (Veh) at baseline; then mice were injected i.p. with rPHB1 (12µg/kg) or Veh at each individual time point: 2 hr, 8 hr, and 14 hr post initial injection. Mice were euthanized at 16 hr and RNA was isolated from (**A**) kidney, (**B**) lung, and (**C**) liver and expression of neutrophil-recruiting chemokines MIP-2 and KC was profiled by quantitative real time-PCR normalized to 18S as arbitrary units (AUs). All data are expressed as mean +/- SEM and derive from 2 independent experiments. n=6/group. **, $P<0.01$; ***, $P<0.001$ compared to vehicle. #, $P<0.05$; ##, $P<0.01$; ###, $P<0.001$ compared to LPS+rPHB1.

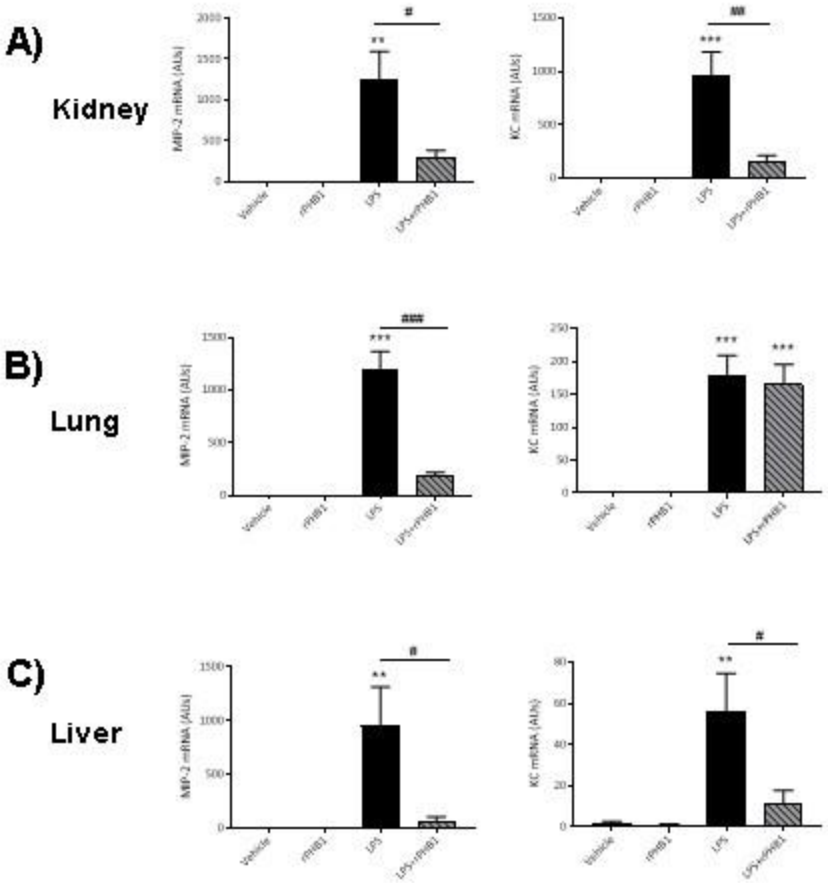

A)
Kidney
B)
Lung
C)
Liver
MIP-2 mRNA (AUs)
KC mRNA (AUs)
Vehicle
rFNB1
LPS
LPS+rFNB1

Figure 2.5: rPHB1 treatment during sepsis alters anti-inflammatory cytokine IL-10 expression in tissue-specific manner.

C57Bl/6J male mice were injected intraperitoneally (i.p.) with LPS (12mg/kg) or sterile saline control (Veh) at baseline; then mice were injected i.p. with rPHB1 (12µg/kg) or Veh at each individual time point: 2 hr, 8 hr, and 14 hr post initial injection. Mice were euthanized at 16 hr and RNA was isolated from kidney, lung, and liver tissues. **A)** Expression of anti-inflammatory cytokine IL-10 was profiled by quantitative real time-PCR normalized to 18S as arbitrary units (AUs). All data are expressed as mean +/- SEM and derive from 2 independent experiments. n=6/group. *, $P<0.05$; ***, $P<0.001$ compared to vehicle. #, $P<0.05$ compared to LPS+rPHB1.

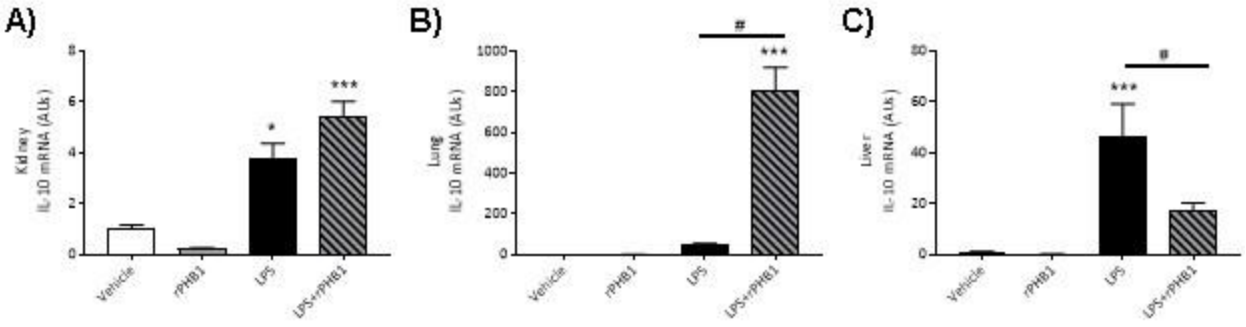

A)
Kidney
IL-10 mRNA (A.U.)
Vehicle
rPHB1
LPS
LPS+rPHB1
*
***
B)
Lung
IL-10 mRNA (A.U.)
#
***
C)
Liver
IL-10 mRNA (A.U.)
#
***

Figure 2.6: rPHB1 treatment during sepsis modulates circulating immune cell populations.

C57Bl/6J male mice were injected intraperitoneally (i.p.) with LPS (12mg/kg) or sterile saline control (Veh) at baseline; then mice were injected i.p. with rPHB1 (12µg/kg) or Veh at 2 hr post initial injection (for a total 2 injections). Blood was drawn via cardiac puncture at 7 hr and samples processed for flow cytometry. **A)** Blood inflammatory monocytes were defined as $CD45^{+}CD115^{+}CD11b^{+}Ly6C^{hi}$. Values are displayed as percentage of total circulating monocytes (defined as $CD45^{+}CD115^{+}CD11b^{+}$). **B)** Blood neutrophils were identified as $CD45^{+}Ly6G^{+}$. Values are displayed as percentage of total circulating immune cells ($CD45^{+}$). **C)** CD11b mean fluorescent intensity (MFI) on circulating neutrophils ($CD45^{+}Ly6G^{+}$). All data are expressed as mean +/- SEM and derive from 2 independent experiments. n=6/group. ***, $P<0.001$.

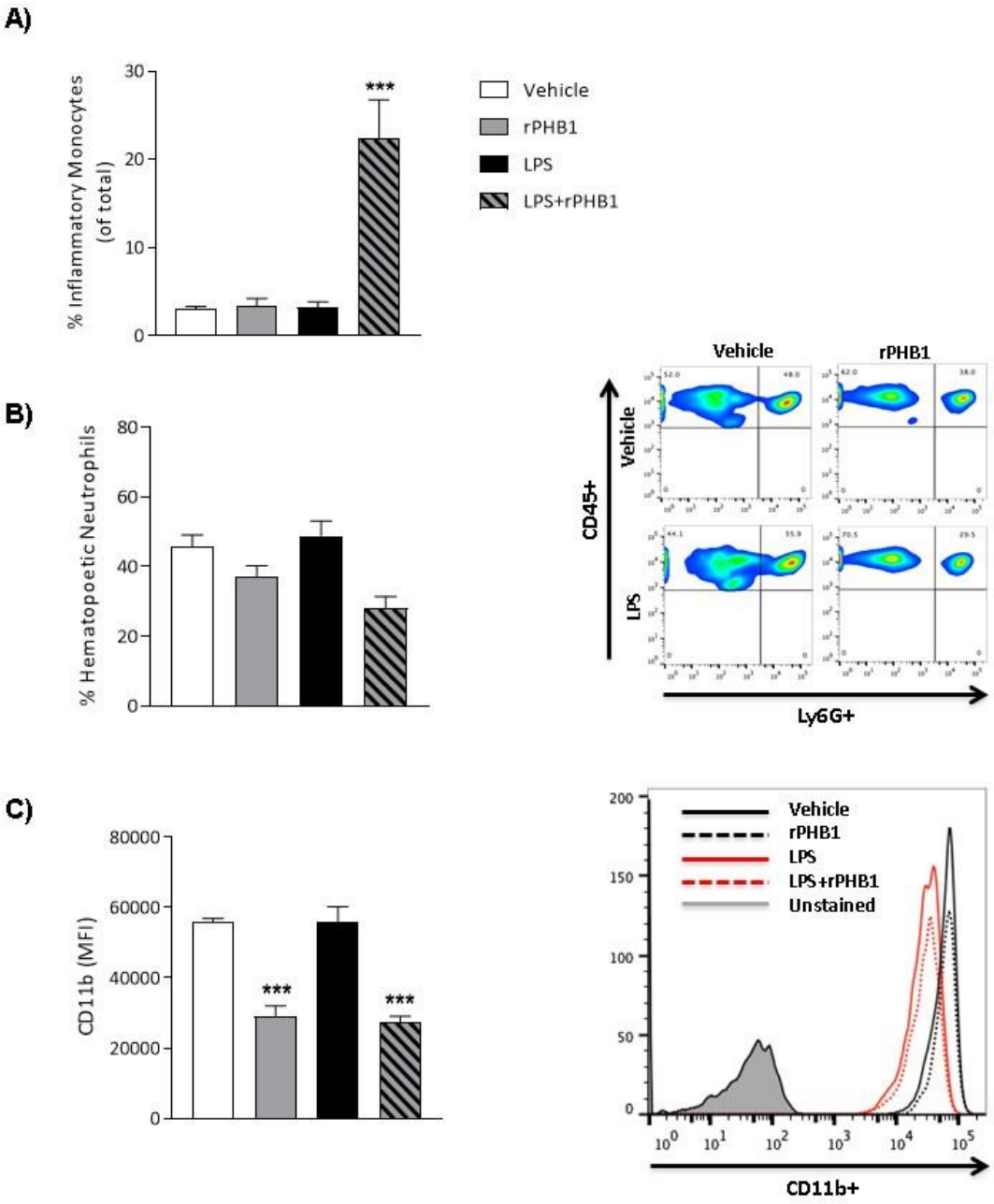

A)
% Inflammatory Monocytes (of total)
30
20
10
0
***
Vehicle
rPHB1
LPS
LPS+rPHB1
B)
% Hematopoetic Neutrophils
80
60
40
20
0
Vehicle
rPHB1
CD45+
Vehicle
LPS
Ly6G+
C)
CD11b (MFI)
80000
60000
40000
20000
0
***
***
Vehicle
rPHB1
LPS
LPS+rPHB1
Unstained
200
150
100
50
0
10^0 10^1 10^2 10^3 10^4 10^5
CD11b+

Figure 2.7: Serum PHB1 is elevated during LPS model of sepsis.

C57Bl/6J male mice were injected intraperitoneally (i.p.) with LPS (12mg/kg) or sterile saline control (Veh) at baseline; then mice were injected i.p. with rPHB1 (12µg/kg) or Veh at each individual time point: 2 hr, 8 hr, and 14 hr post initial injection. **A)** Blood was drawn via cardiac puncture at 16 hr and serum PHB1 protein levels were measured by ELISA. **B)** Kidney, lung, and liver were collected from mice euthanized at 16 hr. RNA isolated from tissues and mRNA expression of PHB1 was profiled by quantitative real time-PCR normalized to 18S as arbitrary units (AUs). All data are expressed as mean +/- SEM and derive from 2 independent experiments. n=6/group *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to vehicle.

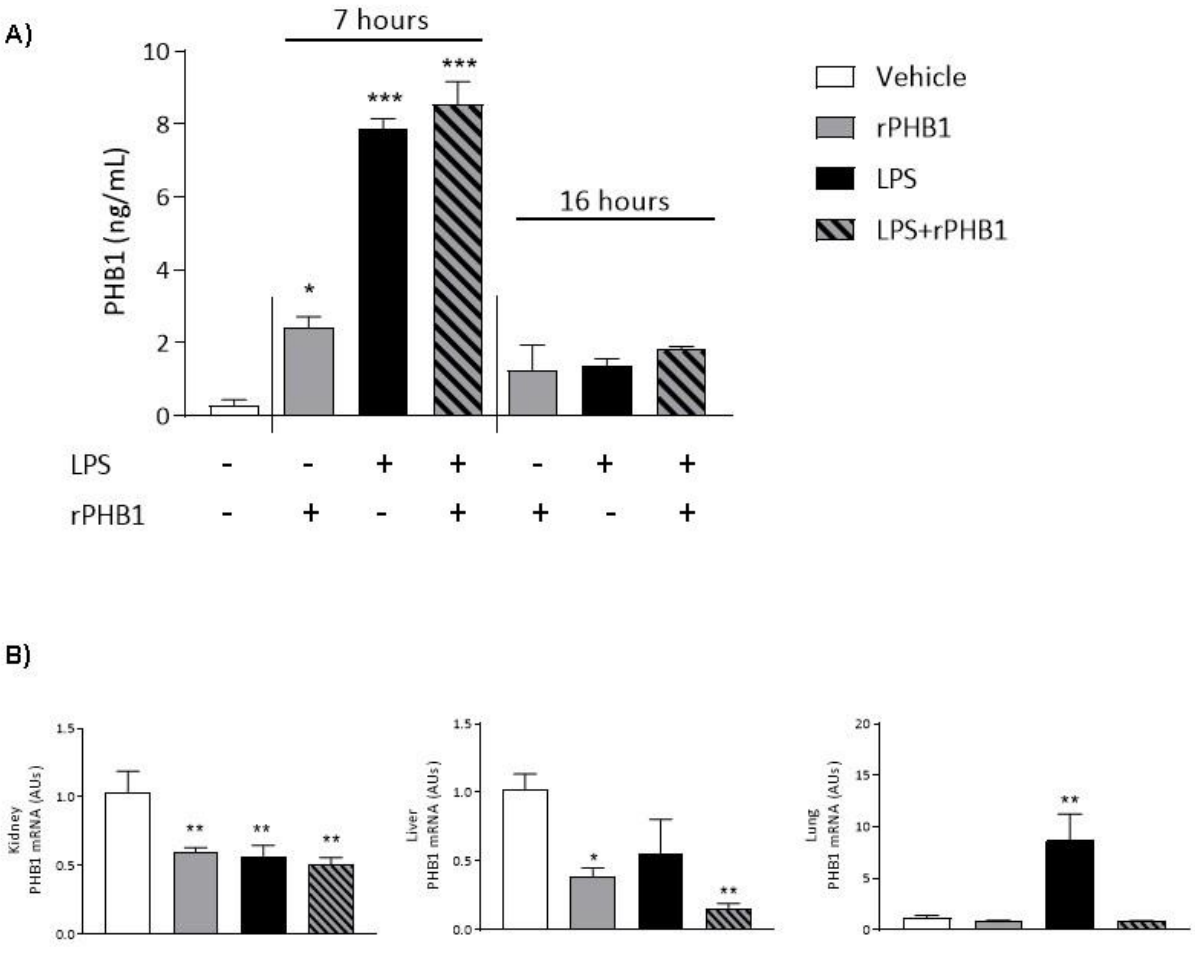
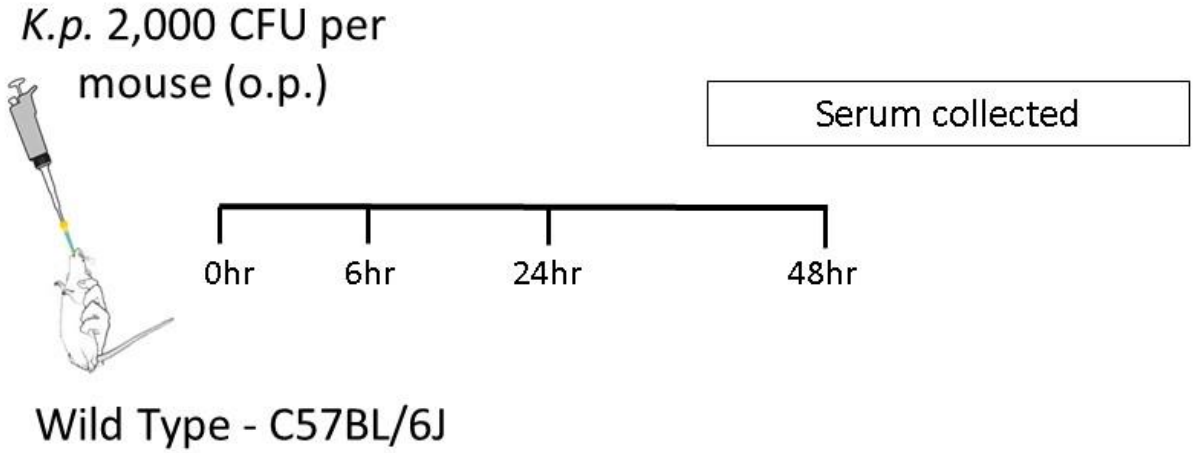

A)
7 hours
16 hours
PHB1 (ng/mL)
10
8
6
4
2
0
*
***
***
Vehicle
rPHB1
LPS
LPS+rPHB1
LPS - - + + - + +
rPHB1 - + - + + - +
B)
Kidney
PHB1 mRNA (AUs)
1.5
1.0
0.5
0.0
**
**
**
Liver
PHB1 mRNA (AUs)
1.5
1.0
0.5
0.0
*
**
Lung
PHB1 mRNA (AUs)
20
15
10
5
0
**

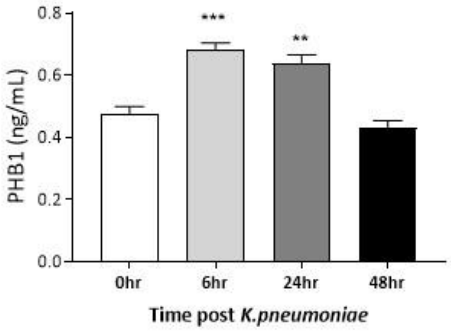
Figure 2.8: Serum PHB1 is elevated during murine *Klebsiella pneumoniae* model of sepsis.

A) Experimental design of *Klebsiella pneumoniae* (*K.p.*) model of sepsis. C57Bl/6J male, 8-10-week-old mice were instilled oropharyngeally (o.p.) with 2,000 colony forming units (CFU) of *K.p*. Mice were euthanized 6, 24, or 48 hr post treatment. 0hr = untreated control. **B)** Blood was drawn via cardiac puncture and serum PHB1 protein levels were measured by ELISA. All data are expressed as mean +/- SEM. n=5/group. **, $P<0.01$; ***, $P<0.001$.

A)


K.p. 2,000 CFU per mouse (o.p.)
Serum collected
0hr
6hr
24hr
48hr
Wild Type - C57BL/6J

B)


PHB1 (ng/mL)
0.8
0.6
0.4
0.2
0.0
***
**
0hr
6hr
24hr
48hr
Time post *K.pneumoniae*

2.6 DISCUSSION

Sepsis is a condition by which the host's immune response becomes dysregulated, leading to overproduction of cytokines/chemokines and excessive organ injury. Damaged mitochondria contribute to systemic inflammation by producing ROS, cytochrome c, and mtDNA (35, 36). Hence, reducing cytokine/chemokine production and preserving mitochondrial function represent novel therapeutic targets to dampen systemic inflammation and mitigate tissue damage during severe sepsis. PHB is a ubiquitous protein found in lipid-rich cellular compartments, particularly in the mitochondrial inner membrane, where it has multiple roles maintaining mitochondrial structure and regulating metabolism. The study presented here focuses on PHB's known characteristics as an anti-inflammatory and cytoprotective protein and investigates PHB's role in systemic inflammation. We found that mice i.p. injected with LPS have increased serum PHB1, and injection of rPHB1 post LPS decreases inflammation, modulates circulating immune cells, and decreases organ injury. Herein, we introduce PHB1 as a novel signaling molecule during sepsis that dampens systemic inflammation.

Our project utilizes an LPS-induced model of sepsis by which LPS binds to Toll-Like Receptors (TLRs), specifically TLR4, to activate the NF-κB inflammatory signaling cascade and produce cytokines/chemokines, such as TNF-α, IL-1β, IL-6, and IL-10 (12). Notably, rPHB1 treatment blunted IL-6 production systemically, as well as in peripheral tissues including the kidney, lung, and liver. However, rPHB1 did not alter the expression of pro-inflammatory cytokine IL-1β. This could indicate PHB does not interact specifically with NLRP3 inflammasome signaling, which is known to regulate and induce IL-1β production (45). Moreover, rPHB1 treatment also had varying effects on the anti-inflammatory cytokine IL-10 expression within the kidney, lung, and liver tissues. rPHB1 post LPS augmented IL-10

expression in the lung which may suggest an interaction between PHB1 and IL-10 in the context of sepsis. Recent studies have suggested IL-10 treatment, in a murine colitis model, upregulates PHB and dampens intestinal inflammation and fibrosis (46). Future studies are needed to determine how PHB1 mechanistically upregulates IL-10 during models of inflammation while suppressing other NF-κB induced cytokines.

Previous studies have demonstrated that endogenous PHB1 and PHB2 are downregulated by various small non-coding RNAs (17, 47, 48), as well as PHBs have feedback loops with androgen and estrogen receptor-mediated transcription (49, 50). Although PHB regulation has been identified intracellularly, no reports have examined exogenous PHB. In this study, we show that septic mice have significantly increased serum PHB1. This was confirmed in two models of sepsis, as mice infected with *K.p.* or injected with LPS had increased serum PHB1. To our understanding, this is the first study that reveals PHB1 is shed into circulation during sepsis. Although PHB has been observed in serum of cancer patients (51, 52), it is unclear how PHB1 may function in circulation, though conceivably as a signaling molecule during inflammatory stress to modulate function of immune cell in circulation.

PHBs have recently been implicated in immune cell function and inflammation. It has been reported that PHB1/2 mediate B lymphocyte receptor signaling to ultimately activate the NF-κB pathway (33). Furthermore, PHBs have been reported to regulate mast cell degranulation and FcεRI signaling important in the allergic response (34), as well as modulate T cell maturation and metabolism (53). Although these studies identify PHBs as important regulators of immune cell function, there remains limited information regarding PHB's role in the context of immune cell-driven systemic inflammation. Our data presented here provide evidence that exogenous PHB1 alters the phenotypes of circulating immune cells. We found that mice treated

with rPHB1 had decreased CD11b cell surface display on circulating neutrophils but had increased percentage of inflammatory monocytes. CD11b is an integrin responsible for extravasation, or the adhesion and migration of immune cells out of circulation and into target tissues (54). CD11b expression has been shown to be cholesterol-dependent (55), which may explain its interaction with PHB, a cholesterol-dependent lipid raft scaffold. Because rPHB1 appears to limit CD11b cell surface display but promote pro-inflammatory polarization in monocytes, this may suggest PHB1 not only has cell-specific functions, but also augments immune cell activation. Decreasing CD11b expression could signify PHB1's protective effects in mitigating neutrophil accumulation in organs and the subsequent tissue damage while still promoting activated monocytes to combat pathogens in circulation.

The focus of this study was limited to investigating solely the effects of PHB1 on systemic inflammation. However, previous studies have indicated that PHB1 and PHB2 are interdependent homologs which interact to form a large, ring-like protein complex, and deletion of an individual PHB gene leads to loss of both (19-23). Future studies are necessary to evaluate PHB2's effect on systemic inflammation and determine whether recombinant PHB2 can too be used as a treatment to decrease systemic inflammation. Furthermore, experiments designed to delve into PHB's role in the lung, regulating IL-10 and modulating immune cell populations, would expand upon our understanding of PHB's effects on inflammation, particularly infectious and inflammatory pulmonary diseases.

In summary, previous studies have shown that PHB has many diverse functional characteristics including its inert anti-inflammatory, antioxidant, and cytoprotective capabilities. However, the systemic effects of rPHB1 in sepsis had been unidentified. Herein, we provide evidence suggesting rPHB1is a potential regulator of the widespread inflammation present

during sepsis. We show that LPS or *K.p.* murine models of sepsis increase serum PHB1 protein levels, and rPHB1 treatment post LPS mitigates serum pro-inflammatory cytokines and LDH activity. Additionally, rPHB1 blunts LPS-induced IL-6 and MIP-2 expression in liver, lung, and kidney tissues. Furthermore, rPHB1 decreases CD11b expression on circulating neutrophils. Collectively, these data suggest PHB1 has an important role in regulating systemic inflammation and mitigating tissue injury during sepsis. Our novel findings provide a basis for determining whether PHB is an effective therapeutic target in inflammatory diseases.

CHAPTER THREE: THE SCAFFOLD PROTEIN PROHIBITIN IS A NOVEL REGULATOR OF MACROPHAGE LIPID RAFTS AND INFLAMMATORY SIGNAL TRANSDUCTION

3.1 ABSTRACT

Prohibitins (PHB1 and PHB2) are ubiquitously expressed proteins with critical roles in multiple biological processes including cell proliferation, apoptosis, transcriptional control and cell signaling. PHB1/PHB2 are interdependent homologs that are both required to form the ring-like PHB protein complex found in lipid-rich cellular compartments. Recent studies have implicated PHB as a regulator of immune cell signaling, including PHB acting as a scaffold to mediate signal transduction of B lymphocytes and mast cells. Moreover, PHB has been identified in the plasma membrane lipid rafts of macrophages, which are integral drivers of inflammation and innate immunity. However, the specific role of PHB in the receptor-mediated innate immune response of macrophages is currently unknown. Herein, we hypothesize that PHB is a novel regulator of lipid raft-dependent inflammatory signaling in macrophages. To evaluate the role of PHB in macrophage signal transduction, RAW 264.7 macrophages with shRNA against Prohibitin 2 (PHB2) or scrambled control (Scr) were stimulated with lipopolysaccharide (LPS) or recombinant tumor necrosis factor-alpha (TNF-α), which activate receptors known to be dependent on lipid raft assembly (CD14/TLR4 and TNFR1 respectively). Silencing PHB2 attenuated nuclear factor-kappa-B (NF-κB) activation and decreased expression and production of cytokines/chemokines. PHB2 deficient macrophages had decreased cell surface display of TNFR1, CD14, and TLR4 receptors and lipid raft marker ganglioside GM1 at baseline and post stimuli. Furthermore, PHB2 deficient macrophages stimulated with LPS had decreased membrane packing providing additional evidence of PHB's importance in lipid raft formation.

Taken together, these data suggest a novel role for PHB in regulating macrophage inflammatory signaling cascades via maintenance of the lipid raft structure. These findings reveal PHB as a potential molecular target for modulating inflammatory signal transduction.

3.2 INTRODUCTION

Macrophages are integral drivers of inflammation and the innate immune response. Macrophages initiate the innate immune response through the activation of surface or intracellular pattern recognition receptors (PRRs) that initiate signaling cascades (i.e. nuclear factor (NF)-κB activation) and subsequent production of inflammatory cytokines and chemokines. This macrophage-driven process of inflammatory signaling is largely dependent on microdomains of the plasma membrane enriched in cholesterol, sphingolipids and glycoproteins, termed 'lipid rafts' (56). The assembly of these lipid rafts creates a highly ordered structure that can act as a platform for receptor trafficking, including PRRs, and regulate signal transduction (31). Furthermore, it is evident that altering the abundance of cholesterol and proteins in macrophage lipid rafts can consequently affect PRR expression and downstream signaling (32, 57-61). Toll-like receptor 4 (TLR4) is a PRR known to traffic to lipid rafts and initiate inflammation (62-64), yet depletion of cholesterol and/or disruption of lipid rafts decreases TLR4 signaling (63, 65). Given that macrophages are critical for the initiation of TLR4-driven inflammation, understanding the assembly and composition of lipid rafts could reveal novel therapeutic targets for modulating inflammatory signal transduction.

Prohibitins (PHBs) are ubiquitously expressed proteins that belong to the SPFH-family (stomatin/prohibitin/flotillin/HflK), a group of proteins localized to lipid-rich cellular compartments, including plasma membrane lipid rafts (29). The active form of PHB is a large ring-like protein complex comprised of interdependent homologs PHB1 and PHB2 (18). Studies have indicated that deletion of an individual PHB1 or PHB2 gene leads to a loss of both protein subunits, and degradation of one protein prevents assembly of the complex (19-23). PHBs have critical roles in multiple biological processes including cell proliferation, apoptosis (23),

oxidative stress (24), signal transduction (25, 26), and metabolism (27). These diverse functions are largely attributed to PHB's post-translational modifications and its ability to traffic to numerous cellular components (i.e. mitochondria, plasma membrane, Golgi, etc.).

PHBs are highly expressed in immune cells (30) and proteomic analyses have identified PHBs in the lipid rafts of mast cells, macrophages, and lymphocytes (31-33). Recent studies have indicated PHB influences immune cell function by regulating CD86 phosphorylation of IκBα in B lymphocytes (33) and promoting mast cell activation and degranulation (34). Moreover, studies have proposed that since PHBs are upregulated in lipid rafts upon immune cell stimulation, PHBs could be interacting with various receptors known to modulate signaling cascades (66, 67). However, the specific role of PHB in receptor-mediated innate immune responses has yet to be determined.

Herein, we present data suggesting PHB is a novel regulator of lipid raft-dependent inflammatory signaling in macrophages. Our data indicate that silencing PHB2 attenuates cytokine and chemokine expression/production by various inflammatory ligands (LPS and TNF-α) which activate receptors known to be dependent on lipid raft assembly (CD14/TLR4 and TNFR1 respectively). Additionally, silencing PHB2 decreases lipid raft formation and subsequent cell surface display of receptors CD14/TLR4 and TNFR1, thus mitigating downstream signaling. Collectively, these data provide novel evidence characterizing PHB's role in maintaining macrophage lipid raft composition and add to a growing field that PHBs influence immune cell function.

3.3 MATERIALS AND METHODS

3.3.1 Reagents

Escherichia coli 0111:B4 Lipopolysaccharide was purchased from Sigma-Aldrich (St. Louis, MO); Dulbecco's Modified Eagle Media, Fetal Bovine Serum, penicillin, and streptomycin purchased from Thermo Fisher Scientific (Waltham, MA); Dimethyl sulfoxide from American Type Culture Collection (Manassas, VA); and Recombinant TNF-alpha was purchased from R&D Systems (Minneapolis, MN).

3.3.2 Isolation of primary peritoneal macrophages

Primary peritoneal macrophages were isolated from adult male C57Bl/6J mice (Jackson Laboratory; Bar Harbor, ME). Briefly, mice were intraperitoneally (i.p.) injected with 2ml of 4% Brewers thioglycolate (Sigma-Aldrich; St. Louis, MO) and euthanized 96 hours later via CO_2. To isolate peritoneal macrophages, the abdominal cavity was flushed with 10ml sterile PBS. Peritoneal lavage was then centrifuged at 1,000 RPM for 6 min and supernatant discarded. Peritoneal cells were then washed twice with PBS-CMF and resuspended in DMEM supplemented with 0.1% FBS, 100 U/ml penicillin, and 100µg/ml streptomycin. Cells were counted and seeded at $1.5x10^6$ cells/ml in 12-well plates. Media was changed 2 hours after plating and cells were allowed to adhere overnight. 24 hours after plating, cells were stimulated with 3ng/ml LPS for 2, 6, 12, or 24 hours. To ascertain purity, an aliquot of cells was centrifuged onto slides using a Cytospin 4 (Thermo Fisher Scientific; Waltham, MA), stained with Diff Quik solution (Thermo Fisher) and differential cell counts quantified. Peritoneal cells yielded were >95% macrophages.

3.3.3 Transduction and selection of RAW 264.7 macrophages

Stable macrophage lines were produced using a set of five lentiviral shRNAs against murine Prohibitin2 from Open Biosystems/Thermo-Fisher (Huntsville, AL). Lentiviral packaging was achieved by using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) to transiently transfect HEK293T/17 cells (American Type Culture Collection; Manassas, VA) with shRNA in a pLKO.1 vector together with vesicular stomatitis virus G glycoprotein and packaging plasmids. Supernatant was collected 48 hours post-infection and concentrated by centrifugation (50,000x g, 2 hours). Pellets were resuspended in PBS and used for infection. Titers were determined by performing quantitative PCR to measure the number of lentiviral particles that integrated into the host genome. In addition, biological titration of viruses that co-expressed fluorescent moieties was determined by flow cytometry. RAW 264.7 macrophages (ATCC) were infected at MOI of 100 and selected 48 hours post-infection with 10 μg/ml puromycin (Calbiochem; San Diego, CA) for ~9 days. The puromycin-selected stable cells (passage 10-16) were used for all further experiments after re-plating in puromycin-free media. Silencing was assessed by immunoblotting. One of the five shRNA was determined to be most effective; the target sequences of this shRNA and the sequence for the negative control (scrambled shRNA) are: (i) PHB2-D (TRCN0000054431) CTGAGCAAGAATCCTGGCTAT and (ii) Scrambled shRNA, CCTAAGGTTAAG GAGCGAGGGCGAC- TTAACCTTAGG.

3.3.4 Cell culture

RAW 264.7 macrophages (ATCC; Manassas, VA; ATCC TIB-71) or primary peritoneal macrophages (as described above) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100μg/ml streptomycin, and 1mM sodium pyruvate at 37°C, 95% humidity, and 5%

CO_2. Cells were plated in 12-well dishes ($1.5x10^6$ cells/ml) and cultured overnight to allow for adherence. For LPS-stimulation experiments, cells were treated with PBS control or 3ng/ml LPS and incubated for 2, 6, 12, or 24 hours post treatment. For TNF-alpha-stimulation experiments, cells were treated with PBS or 30ng/ml recombinant TNF-alpha and incubated for 30 minutes or 2 hours post treatment. Supernatant was collected for ELISA. Cells were rinsed twice with PBS and then lysed with RIPA buffer for Western blotting or with RLT buffer (+ β-mercaptoethanol) (Qiagen; Hilden, Germany) for qRT-PCR.

3.3.5 RNA isolation, cDNA, and Polymerase chain reaction

RNA was isolated from cells via RNeasy kit (Qiagen; Hilden, Germany). RNA was transcribed into cDNA using a high Capacity cDNA Reverse Transcription kit from Applied Biosystems (Thermo Fisher Scientific; Waltham, MA) according to manufacturer's protocol. Real-time PCR was performed in duplicate for PHB1 (Mm01627033_g1), PHB2 (Mm00476104_m1), TNF-α (Mm00443258_m1), IL-6 (Mm00446190_m1), IL-1β (Mm00434228_m1), IL-10 (Mm00439614_m1), chemokine ligand-1 (Cxcl1) (Mm04207460_m1), and chemokine ligand-2 (Cxcl2) (Mm00436450_m1). All primer/probes were purchased from Applied Biosystems (Foster City, CA). Gene expression was normalized to the house keeping gene 18S (Hs99999901_s1), and expression levels were normalized to saline-treated controls using the relative quantification method (Boulter 2016).

3.3.6 Western blotting

Cells were lysed with RIPA buffer and protein concentration of cell lysates was determined according to Pierce BCA Assay kit (Thermo Fisher Scientific; Waltham, MA). 20µg of total protein from each sample were separated by Bolt Bis-Tris Plus polyacrylamide gel

electrophoresis and protein was transferred to PVDF membrane using iBlot 2 Gel Transfer Device (Thermo Fisher). Membranes were blocked with 5% milk/TBS, washed, and probed with anti-PHB1 (1:1,000) (Abcam; Cambridge, UK) or anti-PHB2 (1:500) (BioLegend; San Diego, CA) antibodies and β-actin control antibody (1:1,000) (Thermo Fisher). Signal was detected by goat anti-rabbit IR Dye 800CW secondary antibody (1:10,000) and visualized using the Li-cor system (Lincoln, NE). Images were analyzed via ImageJ software.

3.3.7 NF-κB luciferase assay

Fugene HD (Promega, Madison WI) transfection reagent was used (4.4 uls per ug DNA) to co-transfect RAW264.7 cells with reporter plasmid pNFκB-Luc (Clontech, Mountain View, CA) and with pRL-TK (Promega, Madison WI) as transfection normalization control. 24 hours post-transfection, cells were washed, fresh media added, and either left untreated or treated with LPS for 2 or 6 hours. Cells were washed, lysed with passive lysis buffer and luciferase activity assessed using dual luciferase assay (Promega, Madison WI) measured on an Infinite 200 PRO plate reader (Tecan, Mannedorf, Switzerland).

3.3.8 p65 NF-κB activation assay

Whole cell extracts of equal protein (Pierce BCA assay) were analyzed with the TransAM NF-κB p65 kit (Active Motif, Carlsbad, CA) per manufacturer's conditions. Briefly, Complete Binding Buffer was added to each well to be used of the supplied plate. Then 40µg protein of sample whole cell extract was added per well and incubated at room temperature for 1 hour. Plate was washed and incubated with p65 NF-κB primary antibody (1:1,000). Plate was washed and incubated with HRP-conjugated secondary antibody (1:1,000). Plate was washed and Developing Solution added protected from light to all wells. Stop Solution was added, and

absorbance read at 450nm. OD values were normalized to PBS-control samples set as a value of 1.0.

3.3.9 Flow cytometry analysis of CD14, TLR4, and TNFR1

After treatment with ligand as previously described, cells were processed for flow cytometry. Briefly, cells were rinsed with PBS twice, lifted, and centrifuged at 100xG for 5 min. Pellets of each sample were resuspended in Block Buffer (5% normal mouse serum, 5% normal rat serum, 1% FCR block, 89% FACS buffer) and incubated at room temperature for 15 min. Then samples were incubated with antibody (1:100), washed, and analyzed. Anti-mouse CD14 PE (cat# 12-0141), CD284 (TLR4) PE (cat# 12-9041) and isotype control antibodies were from eBioscience (San Diego, CA). Anti-mouse CD120a (TNFR1) APC (cat# 113006) and isotype control antibodies were from BioLegend (San Diego, CA). Flow cytometry was performed using an LSR II (BD Biosciences, Ontario, Canada) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

3.3.10 Lipid raft imaging

Cells were stained using the Vybrant AlexaFluor 488 Lipid raft labeling kit (Invitrogen, Carlsbad, VA) per manufacturer's conditions. Briefly, cells were incubated with fluorescent Ct-B conjugate and then anti-Ct-B antibody at 4°C, each with appropriate wash steps using chilled sterile PBS. Cells were fixed using 4% formaldehyde then washed several times with chilled PBS. Media was replaced without serum or phenol red. Olympus FV1000 microscope (Center Valley, PA) was used; oil immersion, 60x objective, 488 excitation channel. Images were analyzed using Fiji/ImageJ (NIH).

3.3.11 Laurdan labeling and two-photon microscopy

After 6 hours LPS treatment or PBS control, cells were stained with Laurdan (Cayman Chemical, Ann Arbor, MI) for lipid raft visualization. Briefly, 5 µmol/L Laurdan was prepared in serum-free DMEM medium and incubated with $2x10^6$ cells/mL for 30 min at 37°C. Cells were subsequently washed and fixed using 4% formaldehyde. To evaluate the effect of cholesterol depletion on lipid raft formation, cells were pretreated with 10 mmol/L methyl-β-cyclodextrin (Cayman Chemical) in DMEM media containing 0.1% BSA for 3 min prior to Laurdan labeling. Laurdan was measured by two-photon microscopy (Olympus FV 1000, oil immersion, 60x objective) at 400-460 nm and 470-530 nm to calculate generalized polarization (GP)-values, which indicate ordered and disordered states of the membrane, respectively.

Two-photon microscopy images of Laurdan labeled cells were captured as 16-bit/channel TIFF format and were processed using Fiji/ImageJ (NIH). The mean intensities of red and green channels were measured of each whole cell by drawing an oval around the entire cell. GP-values were obtained by the formula, $GP= (I_{400-600}-I_{470-530}) \div (I_{400-460}+I_{470-530})$, where I stands for intensities of each region of interest for the respective channels (77).

3.3.12 Statistical analysis

Analysis was performed using GraphPad Prism software (San Diego, CA). Data are represented as mean ± SEM. Two-tailed student's t test was applied for comparisons of two groups, and ANOVA for comparisons of >2 groups. For all tests, $p<0.05$ was considered significant.

3.4 RESULTS

3.4.1 Lipopolysaccharide reduces macrophage PHB1 and PHB2 expression.

PHBs have been reported to exhibit anti-inflammatory, antioxidant, and cytoprotective roles in the context of health and disease (13-17). However, it is currently unknown if PHBs have a role in immune-driven inflammation. Additionally, PHB function in macrophages, a cellular component of the innate immune response known to have a significant role in inflammation, is currently unknown. Based on data indicating PHB protein levels are modulated in response to cellular stress (33) and emerging evidence of PHBs regulating various kinase signal transduction pathways (25), we hypothesized that PHBs regulate macrophage inflammatory signaling.

To test this hypothesis, RAW 264.7 murine macrophages were stimulated with 3ng/ml LPS or PBS control for 2, 6, 12, or 24 hours, which are key timepoints when various cyto/chemokines are significantly increased (Appendix 1). After LPS stimulation, macrophage PHB1 and PHB2 mRNA expression was significantly decreased 12 hours post LPS and remained downregulated even at 24 hours (**Figure 3.1A**). To validate these results in primary macrophages, murine peritoneal macrophages were harvested after thioglycolate elicitation and simulated with LPS; PHB1 and PHB2 mRNA was measured. PHB1/2 expression in peritoneal macrophages was also decreased after LPS stimulation (**Supplemental Figure 3.1B**).

To further confirm our cell-based results, C57Bl/6J mice were injected intraperitoneally (i.p.) with 12mg/kg LPS and liver tissue was harvested 16 hours post injection, which is a timepoint when LPS has caused systemic inflammation. PHB1 and PHB2 mRNA expression was measured by qRT-PCR. Liver mRNA expression of PHB1 and PHB2 was decreased 16 hours

post LPS injection (**Figure 3.1B**). Taken together, these data suggest LPS regulates PHB expression.

3.4.2 PHB2 deficiency in macrophages attenuates LPS induction of cytokines and chemokines.

To determine the molecular mechanism of PHBs in macrophage inflammatory signaling, we attempted to knock down both PHB1 and PHB2 homologs in RAW 264.7 macrophage lines using shRNA hush plasmid for PHB1 (PHB1kd), PHB2 (PHB2kd), or scrambled control shRNA (Scr). The PHB1kd cell line was not viable (data not shown); however, we were able to generate a stable, viable PHB2kd cell line with approximately 30% knockdown of PHB2 (**Figure 3.2A**). Interestingly, the PHB2kd stable cell line also had a 20% knockdown of PHB1 protein compared to Scr (**Figure 3.2A**). In addition to the reductions seen in protein levels, the PHB2kd stable cell line had a 50-70% reduction in mRNA expression of both PHB1 and PHB2 (**Figure 3.2B**). These findings are consistent with previous literature suggesting PHB subunits are dependent on each other, and knockdown of either PHB leads to a concomitant decrease, although to a lesser degree, of the other subunit (68).

To investigate if the deficiency of PHB2 in macrophages alters the inflammatory response, PHB2kd and Scr macrophages were stimulated with 3ng/ml LPS for 2 or 6 hours. Confirming a role for PHB2 in inflammatory signaling, PHB2kd macrophages stimulated with LPS had a significant decrease in the mRNA expression of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β (**Figure 3.3A**) and anti-inflammatory cytokine IL-10 (**Figure 3.3B**). Additionally, chemokines MIP-2 and KC mRNA expression were also decreased in PHB2kd compared to Scr post LPS stimulation (**Figure 3.3C**). As shown in **Figure 3.3D**, PHB1 and PHB2 mRNA expression remained decreased in PHB2kd macrophages stimulated with LPS

compared with Scr. These data suggest that PHB2 is important in the macrophage expression of LPS-induced cyto/chemokines.

3.4.3 Macrophage PHB2 deficiency decreases LPS induced nuclear-factor-kappa-B activation.

LPS activates the inflammatory response via binding to cell-surface CD14 and TLR4 co-receptors (69). This ligation initiates down-stream signal transduction leading to activation and translocation of nuclear factor kappa-B (NF-κB). Activation of NF-κB leads to the transcription of cytokines and chemokines, both pro-inflammatory and anti-inflammatory in nature (70). In order to understand where in this LPS-induced inflammatory signaling pathway PHB2 may be acting, we next investigated whether PHB2 deficiency affects NF-κB activation. PHB2kd and Scr macrophages were transiently transfected with NF-κB driven firefly luciferase and renilla luciferase plasmids and then were stimulated with 3ng/ml LPS for 2 or 6 hours. As shown in **Figure 3.4A**, PHB2kd macrophages displayed decreased NF-κB activation compared to Scr at both time points post LPS. Additionally, p65 activation was also attenuated in PHB2kd macrophages 15- and 30-minutes post LPS (**Figure 3.4B**). Taken together, these data indicate PHB2 deficiency also decreases LPS-induced NF-κB activation.

3.4.4 PHB2 deficiency in macrophages attenuates LPS induction of MyD88 independent cytokines and chemokines.

LPS ligation to CD14 and TLR4 co-receptors initiates a bifurcation of the signal into two separate arms of the cascade: the myeloid differentiation primary response 88 (MyD88) pathway and the MyD88-independent TRIF pathway. Signaling through the TRIF pathway leads to activation and translocation of interferon regulatory factor 3 (IRF3). Activation of IRF3 leads to

the transcription of distinct cytokines interferon beta (IFN-β) and interferon gamma-induced protein 10 (IP-10) (71). Given that PHB2kd macrophages have decreased MyD88 dependent cytokine expression (**Figure 3.3**) and NF-κB activation (**Figure 3.4**), we sought to determine if MyD88-independent pathways were also suppressed. To determine PHB2's effects on the MyD88-independent pathway, we analyzed mRNA expression of IFNβ and IP-10. Similar to MyD88 dependent pathways/cytokines, PHB2kd macrophages had attenuated expression of IP-10 and IFN-β compared to Scr at both 2 and 6 hours post LPS (**Figure 3.5A**). These data confirm the role of PHB2 in the inflammatory signaling cascade is up-stream of the signal transduction bifurcation.

3.4.5 PHB2 regulates cell surface levels of CD14 and TLR4.

Next, we identified whether PHB2 plays a role in CD14 and TLR4 co-receptor production. PHB2kd and Scr macrophages were exposed to 3ng/ml LPS or PBS control for 2 or 6 hours, and cell surface display of CD14 and TLR4 was quantified by flow cytometry. At baseline, PHB2kd macrophages had decreased mean fluorescence intensity (MFI) of CD14 (**Figure 3.6A**) which remained decreased at 2 and 6 hours post LPS compared to Scr. **Figures 3.6B** and **3.6C** show histograms representative of CD14 flow cytometry data. Similarly, TLR4 levels in PHB2kd macrophages were decreased at baseline, 2 and 6 hours post LPS (**Figure 3.6D**). These data suggest that PHB2 is required for macrophage cell surface expression of CD14 and TLR4 co-receptors.

3.4.6 PHB2 regulates tumor necrosis factor signaling pathway.

In order to determine whether PHB2 solely alters LPS signaling through CD14/TLR4 receptors, we next investigated PHB2 in the tumor necrosis factor (TNF) signaling pathway.

TNF-α acts through the TNF receptor (TNFR1) which is ubiquitously expressed and mediates inflammatory signaling (72). PHB2kd and Scr macrophages were stimulated with recombinant TNF-α for 30 minutes or 2 hours. As shown in **Figure 3.7A**, macrophages deficient in PHB2 had decreased mRNA expression of TNF-α compared to Scr at both time points. Furthermore, similar results were found for anti-inflammatory cytokine IL-10 (**Figure 3.7B**).

Given that PHB2kd macrophages had decreased cell surface expression of TLR4/CD14 at baseline and post LPS stimulation, we examined if the subsequent decrease of TNF signaling was a result of decreased TNF receptor (TNFR1) expression. Cell surface display of TNFR1 was measured by flow cytometry in PHB2kd and Scr macrophages at baseline and post stimulation with recombinant TNF-α (**Figure 3.7D**). The mean fluorescence intensity of TNFR1 decreased with recombinant TNF-α stimulation in both cell lines over time (**Figure 3.7D**). This decrease in cell surface TNFR1 could indicate macrophage TNFR1 is being internalized which has been reported to happen after stimulation (73). Furthermore, PHB2kd macrophages continued to have decreased TNFR1 levels compared to Scr at baseline, 2 and 6 hours post TNF-α. These results indicate PHB2 is important, not only in CD14/TLR4 signaling, but also in the TNF signal transduction pathway. To determine how PHB2 may be influencing two separate signal cascades, we next focused on PHB's role in lipid rafts, as CD14, TLR4, and TNFR1 are all lipid raft receptors (74).

3.4.7 Silencing PHB2 decreases formation of lipid rafts post LPS stimulation.

Lipid rafts are microdomains of the plasma membrane that are required for trafficking of receptors and operation of central biological processes (75). Given previous studies have noted that PHBs translocate to the lipid rafts under cellular stress (76), we hypothesized PHB is an important regulator of plasma membrane lipid raft formation. To test this, PHB2kd and Scr

macrophages were stained with cholera toxin subunit B (a lipid raft marker which binds to raft ganglioside GM1) at baseline or 6 hours post LPS stimulation. As observed in **Figure 3.8A**, PHB2kd macrophages have decreased lipid raft fluorescence intensity at both baseline and 6 hours post LPS compared to Scr. **Figure 3.8B** is a quantitative representation of the fluorescent images in figure 8A. Because PHB2kd macrophages exhibit decreased lipid raft formation, this could suggest a role for PHB2 in maintaining lipid raft structure which is required for trafficking of TNFR, CD14, and TLR4 receptors to the plasma membrane and subsequent down-stream inflammatory signaling.

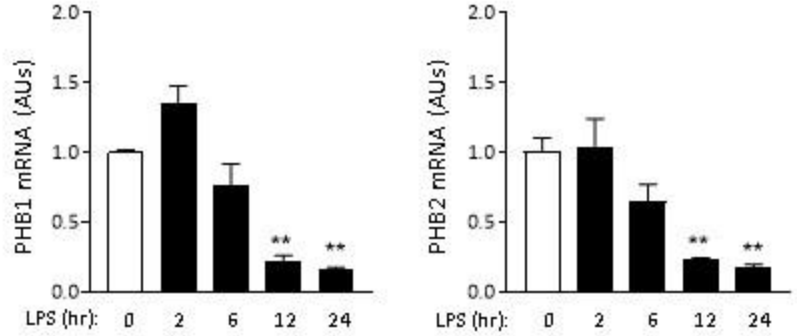
Clustering of essential cholesterols, sphingolipids, and proteins within the plasma membrane is crucial for lipid raft formation; and maintaining these raft structures is important for innate immune responses (74). To determine whether PHB2 acts as a scaffold to maintain lipid raft structure, we next examined membrane polarity as a marker for plasma membrane integrity. Laurdan is a lipophilic probe that is sensitive to the physical state of surrounding phospholipids and emits fluorescence spectra depending on the polarity of these surrounding molecules. The Laurdan probe allows for quantification of the generalized polarization (GP value) of the plasma membrane. GP values closer to +1 indicate ordered phase or a more lipid-rich plasma membrane, whereas GP values closer to -1 indicate disordered phase or a fluid microenvironment (77). PHB2kd and Scr macrophages were stained with Laurdan dye at baseline or 6 hours post LPS stimulation. **Figure 3.9A** displays representative images of 2-photon microscopy. As shown in **Figure 3.9B**, quantitative results indicate PHB2kd macrophages stimulated with LPS have decreased generalized polarization compared to Scr. These data provide further evidence of the role of PHB2 in maintaining lipid microdomains and allowing subsequent receptor trafficking and signal transduction.

3.5 FIGURES

Figure 3.1: LPS reduces macrophage PHB1 and PHB2 expression.

RAW 264.7 murine macrophages were exposed to LPS (3ng/ml) or PBS control for 2, 6, 12, or 24hr. **A)** Expression of PHB1 and PHB2 was profiled by quantitative real time-PCR normalized to 18S as arbitrary units (AUs). **B)** C57Bl6J mice were treated intraperitoneally (i.p.) with LPS. Liver tissue was harvested 16hr post treatment. Tissue expression of PHB1and PHB2 was profiled by qRT-PCR normalized to 18S (AUs). All data are mean +/- SEM and derive from at least 2 independent experiments. **, $P<0.01$; ***, $P<0.001$.

A)


PHB1 mRNA (AUs)
2.0
1.5
1.0
0.5
0.0
**
**
LPS (hr): 0 2 6 12 24
PHB2 mRNA (AUs)
2.0
1.5
1.0
0.5
0.0
**
**
LPS (hr): 0 2 6 12 24

B)

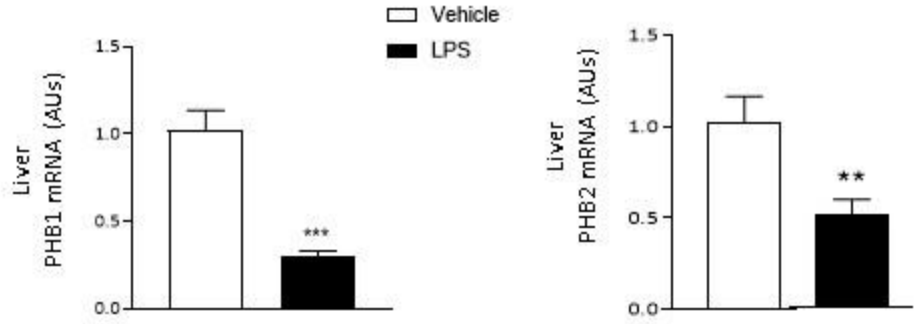

Vehicle
LPS
Liver
PHB1 mRNA (AUs)
1.5
1.0
0.5
0.0
***
Liver
PHB2 mRNA (AUs)
1.5
1.0
0.5
0.0
**

Figure 3.2: Development of macrophage cell line deficient in PHB1 and PHB2 expression and production.

Stable RAW 264.7 macrophages with PHB2 shRNA (PHB2kd) or scrambled control (Scr)**. A)** Example immunoblots for PHB1 and PHB2 in PHB2kd and Scr macrophages (left) with graphs quantifying densitometry compared to β-actin (right) in relative arbitrary units (AUs). **B)** Silencing efficiency of PHB1 and PHB2 evaluated by qRT-PCR in comparison to Scr control plasmid shRNA (AUs). All data are mean +/- SEM and derive from at least 2 independent experiments. *, $P<0.05$; **, $P<0.01$.

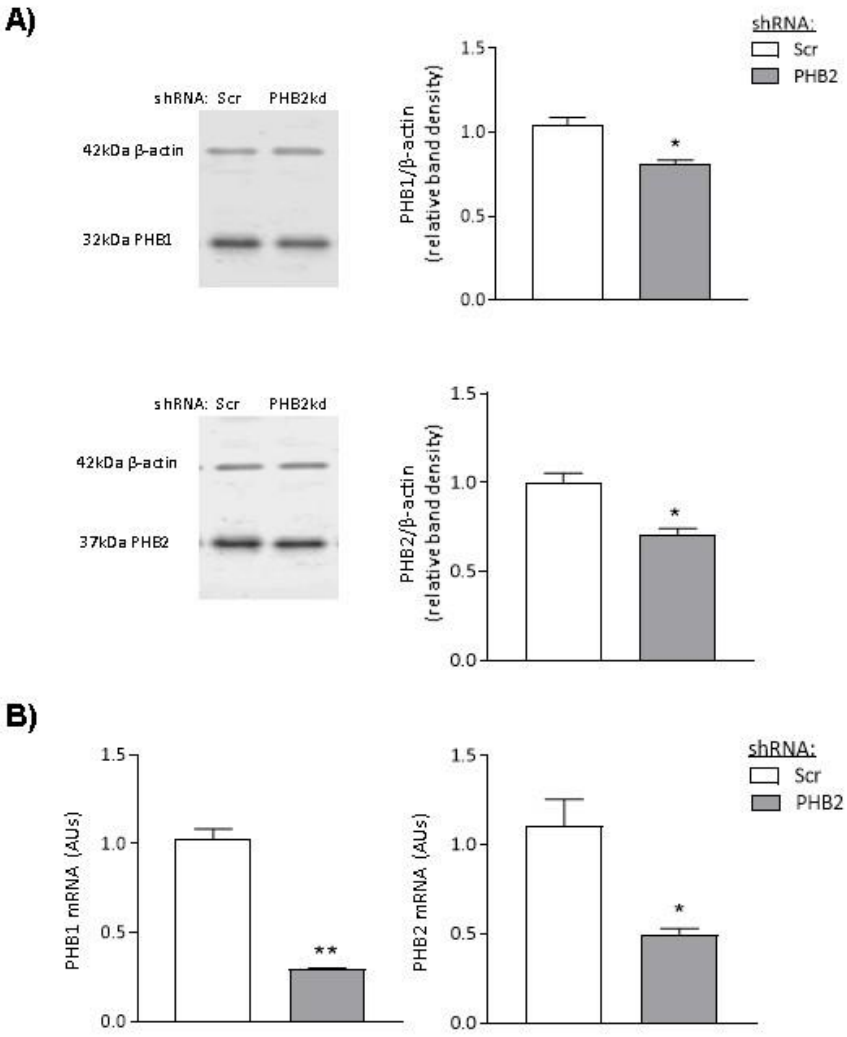

A)
shRNA: Scr PHB2kd
42kDa β-actin
32kDa PHB1
PHB1/β-actin (relative band density)
shRNA:
Scr
PHB2
1.5
1.0
0.5
0.0
*
shRNA: Scr PHB2kd
42kDa β-actin
37kDa PHB2
PHB2/β-actin (relative band density)
B)
PHB1 mRNA (AUs)
**
PHB2 mRNA (AUs)
*

Figure 3.3: PHB2 deficiency in macrophages attenuates LPS induction of cytokines and chemokines.

Stable RAW 264.7 macrophages with PHB2 shRNA knockdown or Scr control shRNA were exposed to control buffer or 3ng/ml LPS for 2 or 6hr. Macrophages were assayed by qRT-PCR for pro-inflammatory cytokines **A)** TNF-α, IL-6, and IL-1β; anti-inflammatory cytokine **B)** IL-10; **C)** chemokines KC and MIP-2.; and **D)** PHB1 and PHB2. Transcript abundance was normalized to 18S as AUs. All data are mean +/- SEM and derive from at least 2 independent experiments. *, $P<0.05$; ***, $P<0.001$.

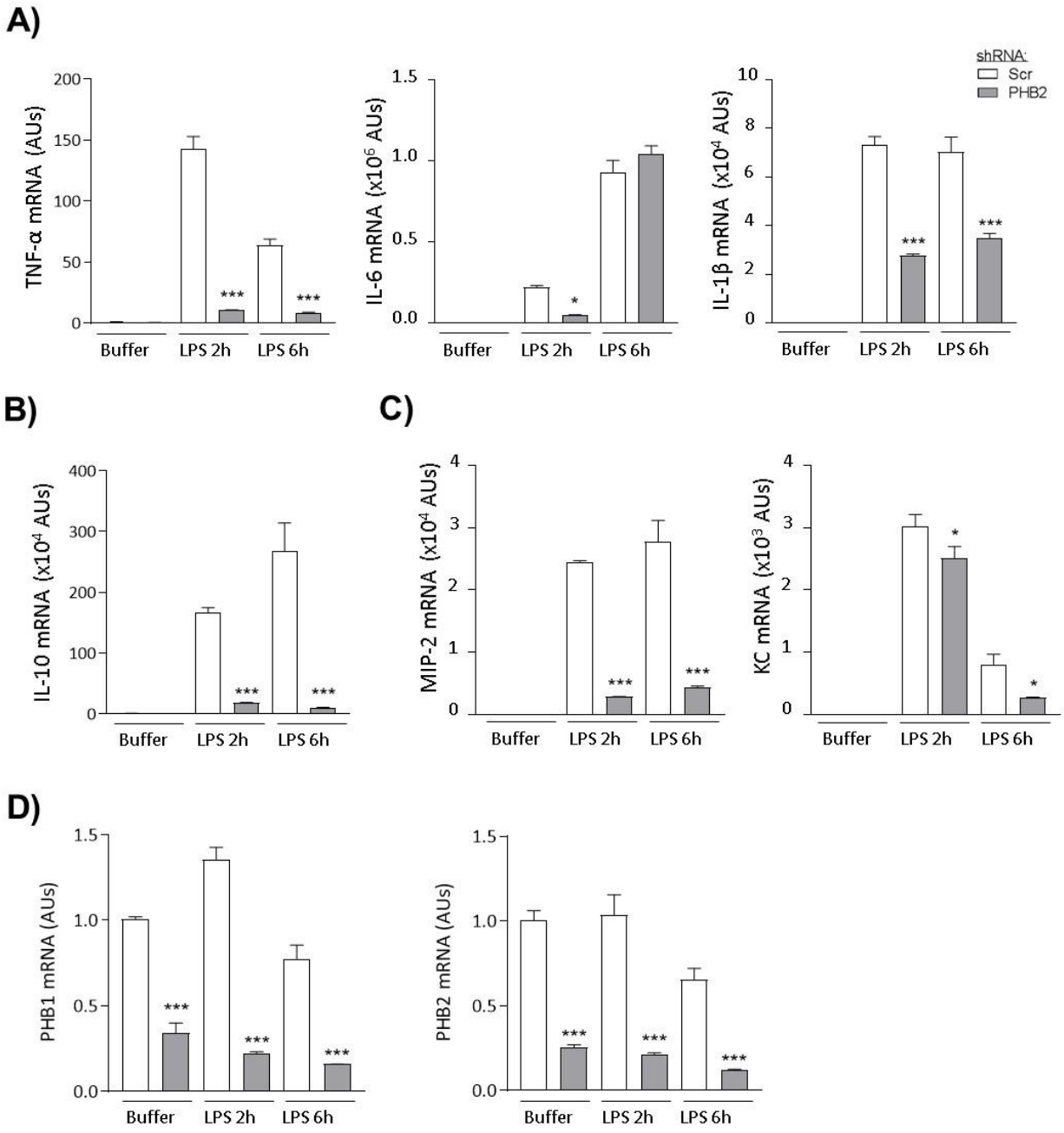

A)
TNF-α mRNA (AUs)
IL-6 mRNA (x10^6 AUs)
IL-1β mRNA (x10^4 AUs)
shRNA:
Scr
PHB2
Buffer
LPS 2h
LPS 6h
B)
IL-10 mRNA (x10^4 AUs)
C)
MIP-2 mRNA (x10^4 AUs)
KC mRNA (x10^3 AUs)
D)
PHB1 mRNA (AUs)
PHB2 mRNA (AUs)

Figure 3.4: Macrophage PHB2 deficiency decreases LPS induced nuclear-factor-kappa-B activation.

A) PHB2 shRNA or Scr control shRNA macrophages were transiently transfected with NF-κB driven firefly luciferase and renilla luciferase plasmids and exposed to control buffer or 3ng/ml LPS for 2 or 6hr. Relative luciferase units (RLUs) were then quantified by luminometry in cell lysates. **B)** PHB2 shRNA or Scr control shRNA stable macrophages were exposed to buffer or LPS for 15, 30, or 60min and were assayed by ELISA for activated p65 NF-κB using equal protein aliquots of cell lysates (AUs). All data are mean +/- SEM and derive from at least 2 independent experiments. *, $P<0.05$; ***, $P<0.001$.

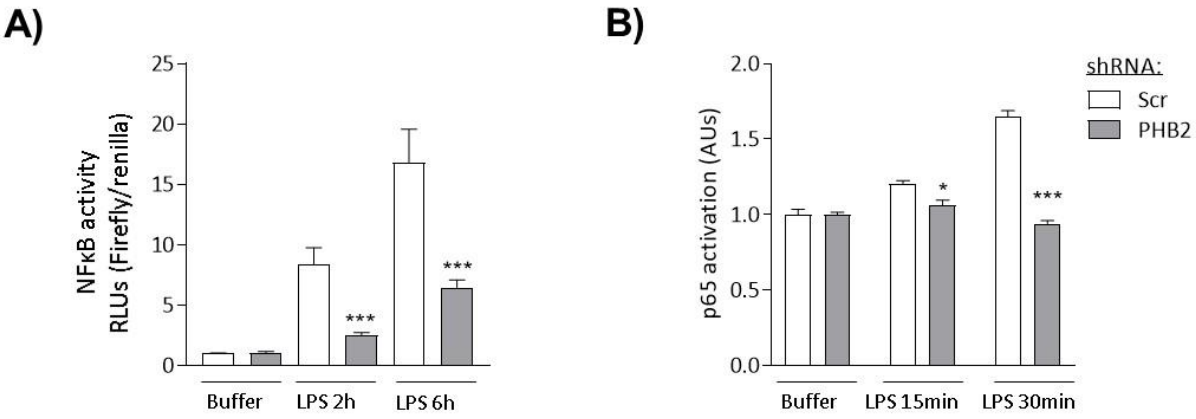

A)
NFκB activity
RLUs (Firefly/renilla)
25
20
15
10
5
0
***
***
Buffer
LPS 2h
LPS 6h
B)
p65 activation (AUs)
2.0
1.5
1.0
0.5
0.0
*
***
shRNA:
Scr
PHB2
Buffer
LPS 15min
LPS 30min

Figure 3.5: PHB2 deficiency in macrophages attenuates LPS induction of MyD88 independent cytokines and chemokines.

Stable RAW 264.7 macrophages with PHB2 shRNA knockdown or Scr control shRNA were exposed to control buffer or 3ng/ml LPS for 2 or 6hr. **A)** Macrophages were assayed by qRT-PCR for MyD88 independent chemokine IP-10 and cytokine IFN-β. Transcript abundance was normalized to 18S as AUs. All data are mean +/- SEM and derive from at least 2 independent experiments. ***, $P<0.001$.

A)

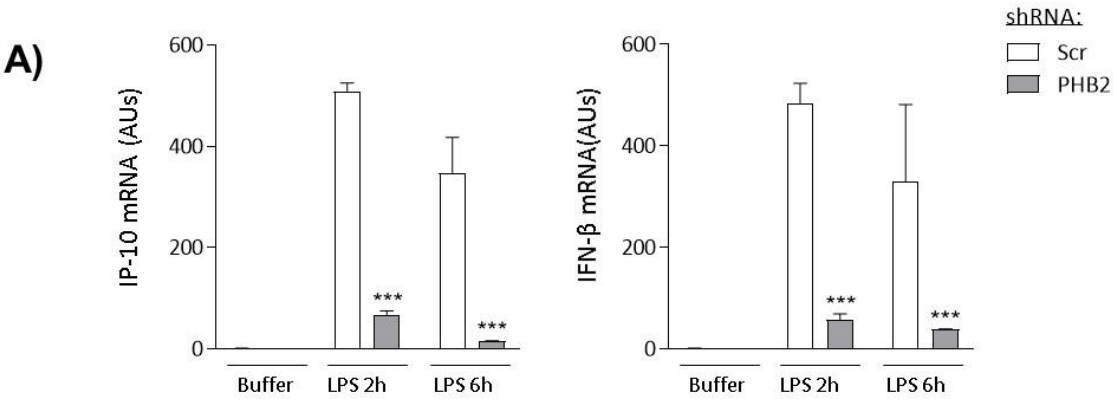

IP-10 mRNA (AUs)
600
400
200
0
***
***
Buffer
LPS 2h
LPS 6h
IFN-β mRNA(AUs)
600
400
200
0
***
***
Buffer
LPS 2h
LPS 6h
shRNA:
Scr
PHB2

Figure 3.6: PHB2 regulates cell surface levels of CD14 and TLR4.

Stable RAW 264.7 macrophages with PHB2 shRNA or Scr control shRNA were exposed to control buffer or 3ng/ml LPS for 2 or 6hr. **A)** Cell surface display of CD14 was quantified by flow cytometry; MFI= mean fluorescence intensity. **B)** Histogram is representative of CD14 levels for PHB2kd and Scr macrophages at baseline buffer control treatment. **C)** Histogram is representative of CD14 levels for PHB2kd and Scr macrophages 2 hours post LPS treatment. **D)** Cell surface display of TLR4 was quantified by flow cytometry (MFI). All data are mean +/- SEM and derive from at least 3 independent experiments. **, $P<0.01$; ***, $P<0.001$. AU= arbitrary units.

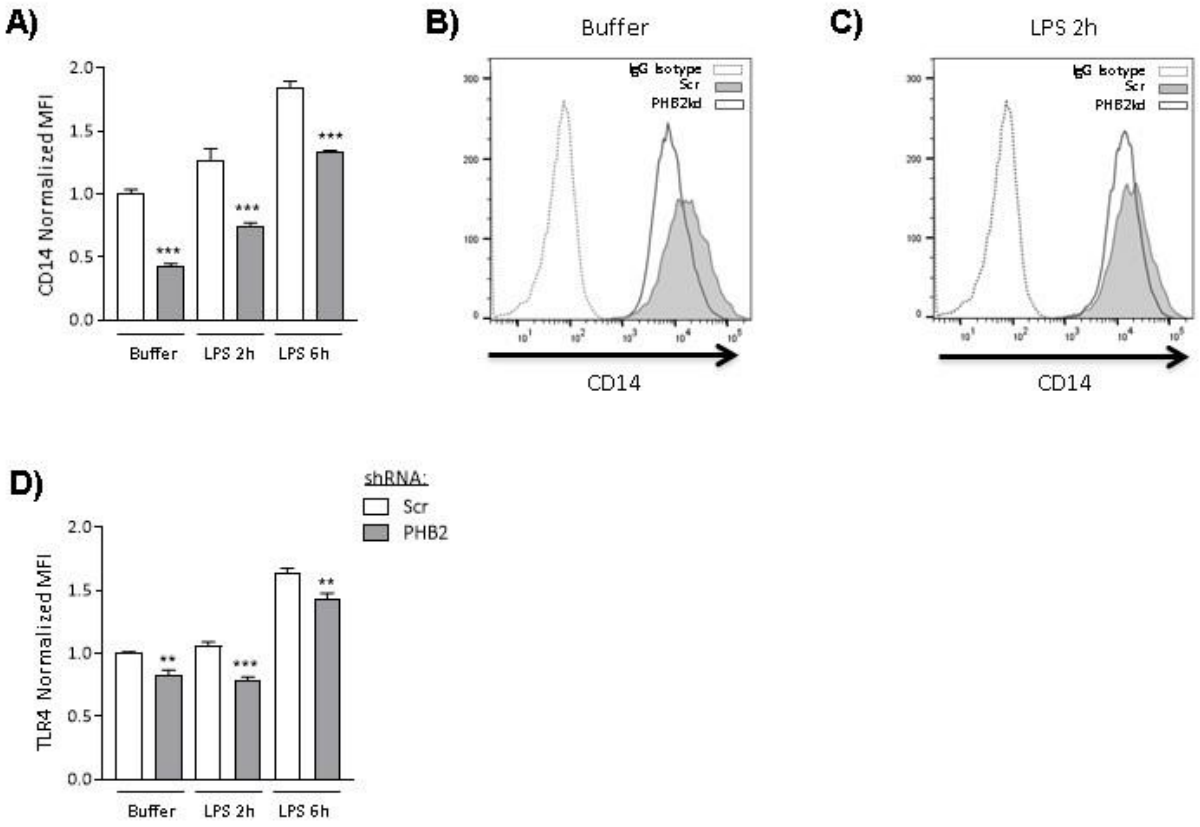

A)
CD14 Normalized MFI
2.0
1.5
1.0
0.5
0.0
***
***
***
Buffer
LPS 2h
LPS 6h
B)
Buffer
IgG Isotype
Scr
PHB2kd
CD14
C)
LPS 2h
IgG Isotype
Scr
PHB2kd
CD14
D)
shRNA:
Scr
PHB2
TLR4 Normalized MFI
2.0
1.5
1.0
0.5
0.0
**
***
**
Buffer
LPS 2h
LPS 6h

Figure 3.7: PHB2 regulates tumor necrosis factor signaling pathway.

Stable RAW 264.7 macrophages with PHB2 shRNA or Scr control shRNA were exposed to control buffer or recombinant TNF-α (30ng/ml) for 30min or 2hr. Transcript abundance (normalized to 18S) was quantified by qRT-PCR for cytokines **A)** TNF-α, **B)** IL-10, **C)** IL-6 and IL-1β. **D)** Stable RAW 264.7 macrophages with PHB2 shRNA or Scr control shRNA were exposed to control buffer or recombinant TNF-α (30ng/ml) for 2hr or 6hr and cell surface display of TNFR1 was quantified; MFI= mean fluorescence intensity. **E)** Histogram representative of baseline TNFR1 levels for PHB2kd and Scr macrophages. All data are mean +/- SEM and derive from at least 3 independent experiments. ***, $P<0.001$. AU= arbitrary units.

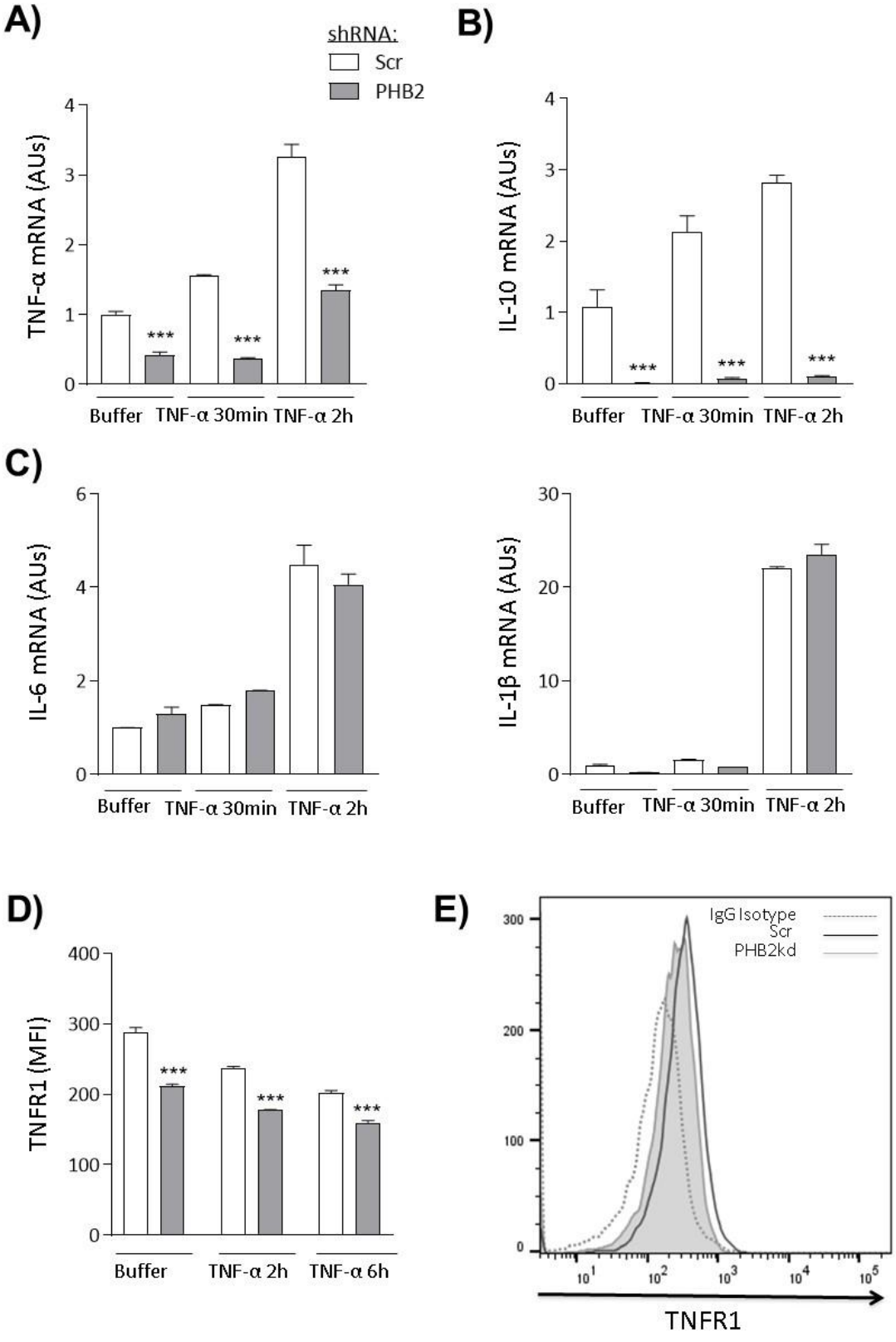

A)
shRNA:
Scr
PHB2
TNF-α mRNA (AUs)
Buffer
TNF-α 30min
TNF-α 2h
B)
IL-10 mRNA (AUs)
C)
IL-6 mRNA (AUs)
IL-1β mRNA (AUs)
D)
TNFR1 (MFI)
TNF-α 6h
E)
IgG Isotype
Scr
PHB2kd
TNFR1

Figure 3.8: Silencing PHB2 decreases formation of lipid rafts post LPS stimulation.

Stable RAW 264.7 macrophages with PHB2 shRNA or Scr control shRNA were exposed to control buffer or 3ng/ml LPS for 2 or 6hr. **A)** Cells were stained with fluorescent cholera toxin B label as described in methods. Representative z-stack 8-bit TIFF images were **B)** quantified for corrected total cell fluorescence (AUs). Each group represents data from at least 3 independent experiments with measurements of at least 120 individual cells. Scale bar, 5μm. *, $P<0.05$; ***, $P<0.001$.

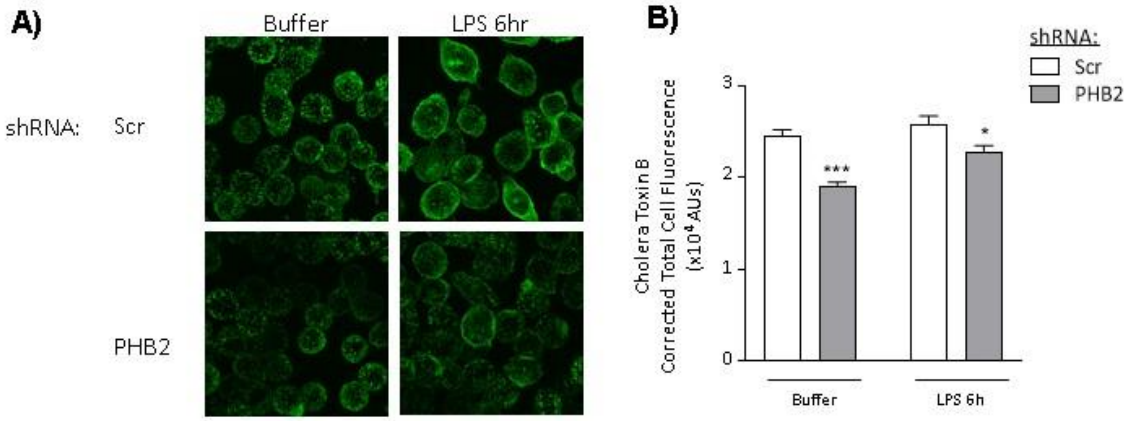
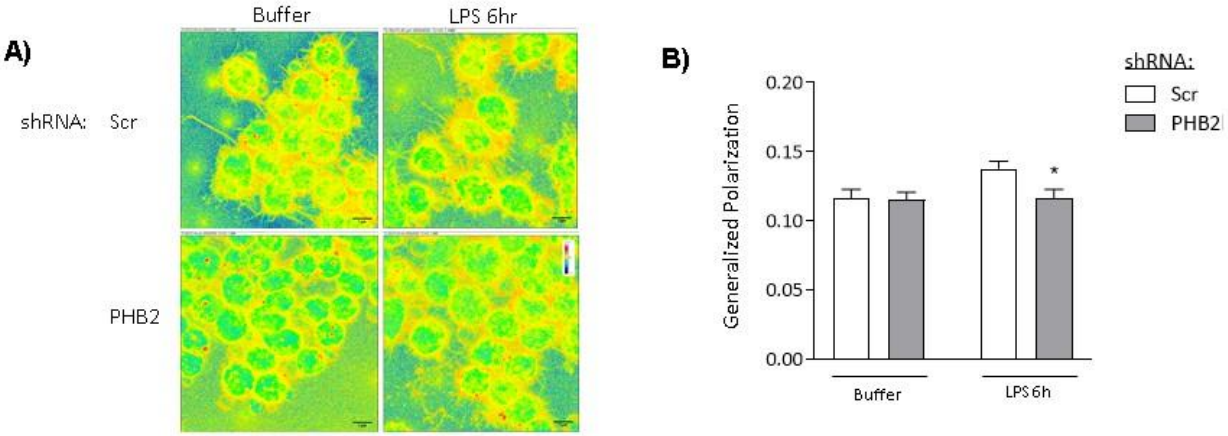

A)
Buffer
LPS 6hr
shRNA:
Scr
PHB2
B)
shRNA:
Scr
PHB2
Cholera Toxin B
Corrected Total Cell Fluorescence
(x10⁴ AUs)
3
2
1
0
***
*
Buffer
LPS 6h

Figure 3.9: Silencing PHB2 decreases macrophage membrane polarity

Stable RAW 264.7 macrophages with PHB2 shRNA or Scr control shRNA were exposed to control buffer or 3ng/ml LPS for 2 or 6hr. **A)** Representative two-photon microscopy images of Laurdan labeled macrophages exposed as shown. 16-bit TIFF format captured images were analyzed in RBG, Red, and green channels, respectively. Cells were selected by drawing an oval around the entire cell and mean intensities from red and green channels were recorded to calculate generalized polarization (GP)-values as described in the methods. Scale bar, 5μm. **B)** Quantification of GP values from microscopy images (77). All data are mean +/- SEM and are derived from at least 3 independent experiments with measurements of at least 40 individual cells. *, $P<0.05$.


A)
Buffer
LPS 6hr
shRNA: Scr
PHB2
B)
shRNA:
Scr
PHB2
Generalized Polarization
0.20
0.15
0.10
0.05
0.00
*
Buffer
LPS 6h

3.6 DISCUSSION

PHBs are pleiotropic proteins with key roles in cell proliferation, apoptosis, oxidative stress, signal transduction, and metabolism. Numerous studies have illustrated how PHB's diverse effects are pertinent to a wide range of inflammatory diseases, such as colitis and diabetes. However, PHB is understudied in the context of immune cell function and the innate immune response that drives inflammation. Here, we introduce PHB2 as a novel regulator of the macrophage innate immune response by stabilizing lipid raft formation and facilitating the recruitment of receptors (i.e. CD14/TLR, TNFR) to promote downstream inflammatory signal transduction.

In this study, we determine that LPS stimulation significantly decreases PHB1/PHB2 expression in RAW 264.7 macrophages and primary peritoneal macrophages. This downregulation is also evident *in vivo* as C57Bl/6J mice injected with LPS have decreased liver PHB1 expression. Multiple cellular pathways have been reported to regulate the expression and production of PHBs. For instance, small non-coding RNAs, including miR-128 and miR-539, have been identified to regulate PHB expression and production in the context of apoptosis (17, 78). Additionally, excess miR-27a decreases PHB expression leading to reduced mitochondrial biogenesis and/or increased reactive oxygen species (79). Although we do not verify in this study whether the decreased macrophage PHB1/2 expression post LPS is a regulated by miRNAs, it has been previously shown that certain miRNAs are induced in macrophages during inflammation and promote TLR4 signal transduction (80). These findings could suggest miRNAs as a mechanism responsible for PHB's role in regulating the macrophage innate immune response.

Our attempts to develop a macrophage cell line deficient in PHB1 were unsuccessful as there were issues with viability (data not shown). However, we were able to generate a stable and viable PHB2 knockdown macrophage line using an shRNA target sequence specifically for PHB2 (CTGAGCAAGAATCCTGGCTAT). When confirming knockdown of protein levels and mRNA expression, we did observe that both PHB1 and PHB2 were significantly reduced. It has been shown previously that PHBs are interdependent homologs that interact with each other to form a large, ring-like protein complex, and deletion of an individual PHB gene leads to loss of both (19-23). Our data presented here confirm that macrophage PHB1 expression and production are dependent on PHB2. Since both PHB1 and PHB2 were reduced in our stable macrophage cell line, we cannot definitively conclude our findings represent the sole functions of PHB2. Additionally, individual PHBs can operate separately from one another and have distinct functions in various cellular compartments (49, 50). However, our findings do represent that altered levels of both PHBs leads to decreased macrophage inflammatory responses.

PHBs have recently been implicated as regulators of immune cell signaling. It has been reported that PHB1/2 acts as a scaffold at the plasma membrane of B lymphocytes, binding to CD86 and mediating signal transduction to ultimately activate NF-κB p50/p65 (33). This study supports our findings presented here, as CD86 is a protein that translocates to the lipid rafts to initiate signal transduction (81). Furthermore, it has also been reported that PHB's scaffolding function in the lipid membrane of mast cells regulates degranulation and FcεRI signaling (34). Although these studies identify PHB as a scaffold to enable signal transduction, the mechanism of PHB at the lipid raft domains was not examined. Herein, we provide evidence of PHB acting as a scaffold for lipid raft formation in macrophages, which then provides the platforms essential for receptor presence and assembly. We found that PHB2kd macrophages have decreased cell

surface display of innate immune receptors CD14/TLR4 and TNFR1 at baseline and post LPS stimulation, as well as decreased lipid raft formation, as quantified via lipid raft marker ganglioside GM1. These data suggest PHB2 is an important regulator of macrophage inflammatory signal transduction by modulating and maintaining structure of plasma membrane lipid rafts.

To provide additional evidence of PHB2's role in regulating lipid domains, we utilized Laurdan stain to determine PHB2's effects on the physical state of the plasma membrane. We found macrophage generalized polarization (GP) values increased with LPS stimulation suggesting clustering of lipids and/or formation of lipid rafts. However, PHB2kd macrophages did not have an LPS-induced increase in GP. Interestingly, despite our prior findings indicating baseline differences between the PHB2-sufficient and -deficient cell lines (GM1 lipid raft marker and cell surface display of receptors), Laurdan stain did not yield baseline differences in PHB2kd macrophages. Previous studies have suggested that ganglioside GM1, the marker we used to identify lipid raft formation, may be a better indicator of functional changes (i.e. cholesterol recruitment and/or organization) in lipid rafts (82, 83), whereas Laurdan displays overall physical state of the plasma membrane. Taken together, these conflicting baseline data may represent PHB2's differential effects on structural and functional changes to the macrophage lipid rafts.

We acknowledge that the results of this study are methodologically limited to the use of RAW 264.7 immortalized macrophage cell line. However, we did find comparable results with LPS-stimulation experiments on primary peritoneal macrophages. Another limitation is that we did not fully assess the compositional structure of the lipid raft; however, future studies will include the isolation of macrophage lipid rafts to determine molecular composition via mass

spectrometry and the creation of a murine strain that is deficient in PHBs specifically in myeloid cells.

In summary, the data presented here indicate that PHB acts as a scaffold for macrophage lipid raft structures, thus regulating receptor trafficking and subsequent signal transduction. We provide evidence that PHB2 deficient macrophages have decreased lipid raft formation, cell surface display of CD14/TLR4 and TNFR1 receptors, NF-κB/p65 activation, and cytokine/chemokine expression and production. Collectively, these data provide novel evidence for PHB's critical role in regulating macrophage inflammatory signaling and add to a growing field that PHBs influence immune cell function.

CHAPTER FOUR: GENERAL DISCUSSION AND SUMMARY

The objective of this dissertation project was to investigate the role of PHB in immune-driven inflammation. By analyzing PHB in the context of sepsis, we first identified PHB as a novel regulator of systemic inflammation via production of cytokines/chemokines and via modulation of the phenotypes of circulating immune cells (aim 1). This led us to further delve into the molecular mechanism of PHB in macrophage inflammatory signal transduction (aim 2). Interestingly, we found that PHB in macrophages is necessary to support formation of plasma membrane lipid rafts and subsequent trafficking of receptors important for the innate immune response. This is the first study to reveal PHB's functions in systemic inflammation and macrophage inflammatory signaling cascades. In this report, we provide insight into the diverse yet complementary roles of PHB in regulating various aspects of inflammation.

Aim 1 of this project focuses on the inflammation-driven disease, sepsis, which represents a substantial healthcare and economic burden to the United States. The mortality rate in septic patients is exceedingly high, due in large part to the organ failure that accompanies the dysregulated inflammatory response to infection (1-3). Therapeutic interventions include administration of antibiotics, crystalloid fluids and vasopressors for hypotension, and oxygen for tissue hypoxia (10). However, innovative approaches that both combat the systemic inflammation and mitigate the tissue/organ damage associated with sepsis are necessary to improve patient outcomes. Given that PHBs have been shown to have anti-inflammatory, antioxidant, and cytoprotective roles in multiple disease states, we hypothesized that PHB is a regulator of the systemic inflammation present in sepsis. To test this, we utilized the LPS murine model of sepsis and determined whether injection of rPHB1 mitigates inflammation and tissue injury. We found that rPHB1 treatment dampened LPS-induced serum and organ IL-6

production/expression. Moreover, rPHB1 abrogated serum LDH, which is continuously monitored in a clinical setting as increased serum lactate implies hypoperfusion and progression of organ dysfunction within septic patients (10). These data suggest rPHB1 has therapeutic effects in attenuating systemic inflammation and tissue/organ damage.

In this report, we demonstrated that two different *in vivo* models of sepsis yielded increased serum PHB1 levels. Mice i.p. injected with LPS had significantly increased serum PHB1 at both the 7- and 16-hour time points, and mice infected with *Klebsiella pneumoniae* o.p. had increased serum PHB1 that was significantly elevated at 6- and 24-hours post infection. To our understanding, this is the first study that reveals PHB1 is shed into circulation during sepsis. Previously, PHB has been observed in serum of cancer patients (51, 52), but it remains unclear how PHB1 may function in circulation. We propose that perhaps PHB1 acts as a signaling molecule, since we did find that rPHB1 treatment modulated blood immune cell populations. rPHB1 attenuated cell surface display of the adhesion molecule CD11b on circulating PMNs but increased percentage of inflammatory monocytes in the blood. These findings have clinical implications as mitigating PMN recruitment to the periphery could lessen host tissue damage (9). Furthermore, the altered monocyte phenotypes may indicate that rPHB1 promotes monocyte activation to combat pathogens in circulation.

Future studies are needed to determine how PHB is systemically shed into circulation. While there are many possible mechanisms of this occurrence, perhaps the most likely is that PHB is shed directly into the bloodstream through endocrine processes of the liver. This is a plausible channel as PHB is highly expressed in the liver (30). These future directions could reveal a novel designation of PHB as a hormone, especially since we found PHB may be specifically targeting and modulating immune cells in circulation. Additionally, we found that

PHB promotes pro-inflammatory monocyte/macrophages, whereas previous studies almost exclusively indicate PHB to be anti-inflammatory in other cell types and in a variety of disease models. However, none of these studies focused on the role of PHB in monocytes/macrophages in the context of inflammatory signaling. Hence, data from this dissertation project are the first to reveal a pro-inflammatory role for PHB. Based on our findings in aim 2 (discussed in more detail below), we propose PHB interacts at the lipid raft microdomains of immune cells to enhance display of receptors such as pattern recognition receptors (PRRs) and cytokine receptors, which are highly expressed in monocytes/macrophages (**Figure 4.1)**. This could potentially explain how PHB is pro-inflammatory in monocytes/macrophages but is anti-inflammatory in other cell types. Future studies needed to track PHB's movement and determine whether exogenous PHB is able to incorporate into the plasma membrane lipid rafts and act as a scaffold to augment immune signaling. The data collected from this dissertation project unlock many interesting potential directions to unearth PHB's importance in inflammatory signaling.

Aim 2 of this project focuses on immune-mediated inflammation. To better characterize the role of PHB in immune cells, we developed RAW 264.7 murine macrophage cell lines deficient in PHB1 or PHB2 using lentiviral shRNAs. Although our attempts at knocking down PHB1 proved to be unsuccessful, the PHB2 knockdown macrophage line we developed was viable and stable. However, when confirming knockdown of protein levels and mRNA expression, we did observe that both PHB1 and PHB2 were significantly reduced. This interdependence of gene expression and protein production has been supported by previous studies (19-23). Since our shRNA target sequence was specifically for PHB2, we referred to the cell line as "PHB2kd" macrophages.

In this study, we indicated that silencing PHB2 attenuates cytokine and chemokine expression/production by two inflammatory ligands, LPS or TNF-α, that are known activators of receptors dependent on lipid raft assembly (CD14/TLR4 and TNFR1 respectively). Conclusively, PHB2kd macrophages had decreased lipid raft formation and subsequent cell surface display of receptors CD14/TLR4 and TNFR1. Downstream signaling was mitigated, including NF-κB/p65 activation. Thus, we propose that PHB acts as a scaffolding protein at the plasma membrane to maintain lipid raft structure, enabling receptors to traffic to the rafts and regulating signal transduction. Collectively, these data provide novel evidence characterizing PHB's role in regulating lipid raft-dependent inflammatory signaling in macrophages.

This project is mechanistically limited as aim 1 solely focuses on rPHB1 treatment and aim 2 targeted PHB2. Although we found that PHB2kd macrophages had reduced PHB1 levels in addition to reduced PHB2, future studies are needed to determine if shRNA for PHB1 has similar effects. In addition, overexpression of PHB1 and/or PHB2 in cultured macrophages would be beneficial to confirm our results. Moreover, studies evaluating the role of rPHB2 as an *in vivo* regulator of systemic inflammation, as well as rPHB1/rPHB2 combination treatment, would be beneficial to completely characterizing PHB in inflammatory signaling. Although PHB1 and PHB2 are known to create a heterooligomeric complex in the mitochondrial inner membrane, individual PHBs can operate separately from one another and have distinct functions in various cellular compartments (49, 50). Perhaps solely treating mice with rPHB1 in aim yields a different response than combination or rPHB2 alone. Furthermore, future studies will include the creation of a murine strain that is deficient in PHBs specifically in myeloid cells.

All in all, these two separate aims of the dissertation project reveal PHBs have diverse functions in systemic versus macrophage-specific inflammation. First, we determined that PHB

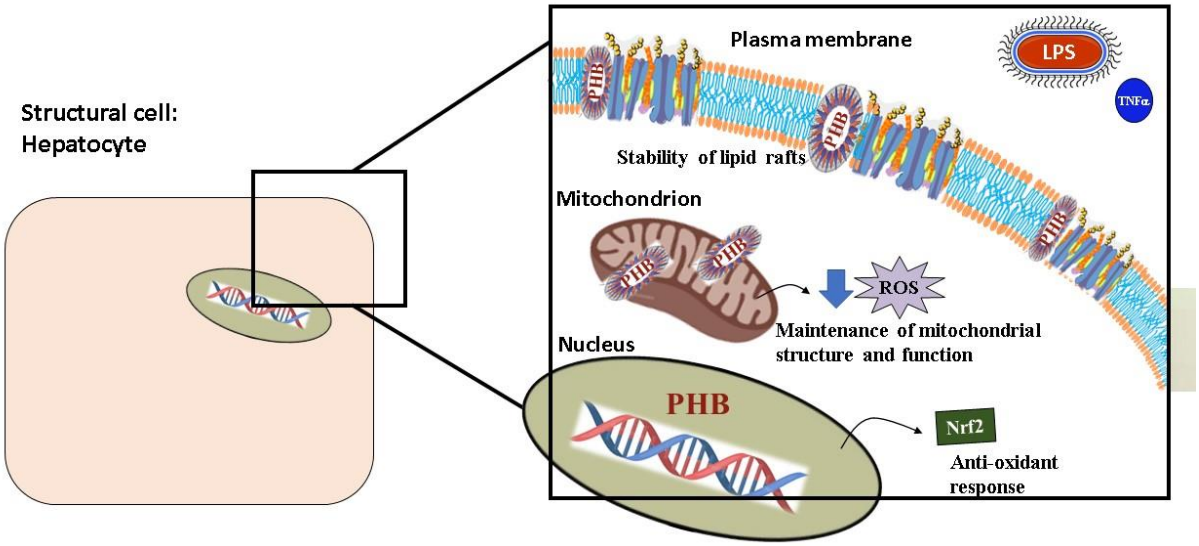
attenuates systemic inflammation and tissue/organ damage via decreasing pro-inflammatory cytokines and chemokines, as well as decreasing serum presence of LDH. However, when investigating the role of PHB in monocytes/macrophages, we found that PHB not only increases populations of pro-inflammatory monocytes *in vivo*, but also regulates vital macrophage inflammatory signaling (as shown *in vitro*). We resolved this mechanism to be lipid-raft regulated, and perhaps this gives us insight into the differences of PHB's cell-specific functions. Lipid rafts act as platforms for PRRs and cytokine receptors to translocate and become expressed at the cell surface. Activation of these receptors leads to downstream signaling cascades and production of inflammatory mediators. Our claim is that, since monocytes/macrophages have exceedingly high gene expression of PRRs and cytokine receptors compared to other cell types, the function of PHB in regulating lipid raft formation and inflammatory signaling would mechanistically greater impact monocytes and macrophages (30, 84). Therefore, these data suggest PHB has a compartment- and cell-specific role in mediating the monocyte/macrophage innate immune response. Perhaps the membrane-bound PHB acts as a scaffold for lipid raft formation and cell surface display of macrophage PRRs and cytokine receptors, regulating innate immune inflammatory signal transduction; whereas PHB in the nucleus and mitochondrial inner membrane supports antioxidant pathways, having overall anti-inflammatory effects in cells with low PRR/cytokine receptor expression (**Figure 4.1**).

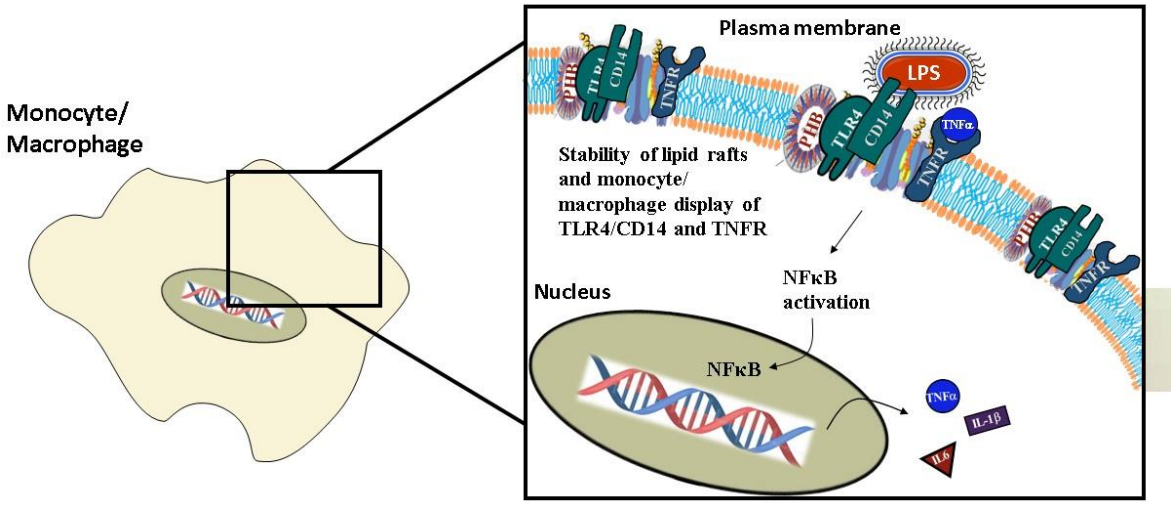
Future studies are needed to expand upon these divergent mechanistic functions of PHB. However, we are the first team to introduce this novel pro-inflammatory element of PHB's roles in inflammation, and our results would suggest PHB promotes monocyte/macrophage response to infection while protecting structural cells from inflammatory damage. In diseases of systemic inflammation, particularly infectious diseases, PHB constitutes a significant therapeutic target to

regulate inflammatory cell dynamics. These novel findings provide key information to advance our understanding of PHB and have potentially wide-ranging implications across numerous inflammatory diseases.

Figure 4.1: Proposed mechanism of PHB at plasma membrane lipid rafts.

In structural cells, it is known that PHB at the nucleus plays a role in the Nrf2 antioxidant response and PHB at the mitochondrial inner membrane maintains structure and function. However, we propose the PHB complex also provides stability at the plasma membrane lipid rafts, allowing for receptor display and subsequent downstream signaling. In the diagram, receptors such as TLR4/CD14 and TNFR are present at the cell surface of monocytes/macrophages where they can bind to LPS or TNF-α, respectively. This ligation allows for NF-κB to become activated where it then translocates to the nucleus and promotes the transcription of cytokines/chemokines. Perhaps this pro-inflammatory mechanism outweighs previously studied anti-inflammatory/antioxidant mechanisms of PHB (roles in the nucleus and mitochondria) in monocytes/macrophages specifically, because PRRs are highly expressed in monocytes/macrophages compared to other cell types. Therefore, despite PHB's anti-inflammatory/antioxidant effects in structural cells during LPS-induced inflammation, PHB's role in providing stability to the lipid rafts allows for monocyte/macrophage cell surface display of PRRs and cytokine receptors, recognition of PAMPs and DAMPs, and the subsequent pro-inflammatory signaling cascades to commence.


Structural cell:
Hepatocyte
Plasma membrane
LPS
TNFα
PHB
Stability of lipid rafts
Mitochondrion
ROS
Maintenance of mitochondrial
structure and function
Nucleus
PHB
Nrf2
Anti-oxidant
response


Monocyte/
Macrophage
Plasma membrane
LPS
TLR4
CD14
TNFR
TNFα
PHB
Stability of lipid rafts
and monocyte/
macrophage display of
TLR4/CD14 and TNFR
NFκB
activation
Nucleus
NFκB
TNFα
IL-1β
IL6

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East Carolina University
Tomorrow starts here.

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

February 13, 2018

Kym Gowdy, Ph.D.
Department of Pharmacology
EW Life Sciences Building
East Carolina University

Dear Dr. Gowdy:

Your Animal Use Protocol entitled, "The Role of Scavenger Receptor B11 in Pulmonary Bacterial and Viral Infections in Mice" (AUP #W243a) was reviewed by this institution's Animal Care and Use Committee on February 13, 2018. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

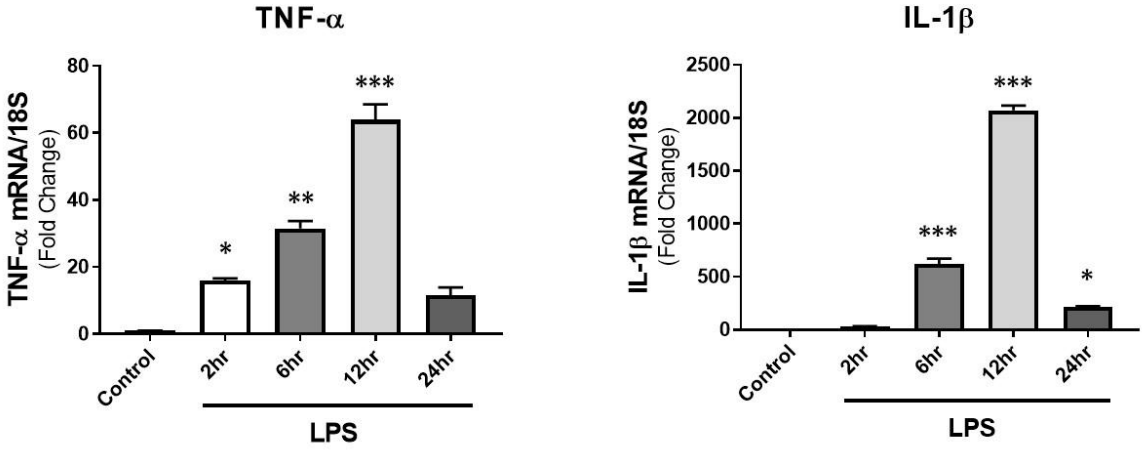
S McRae

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

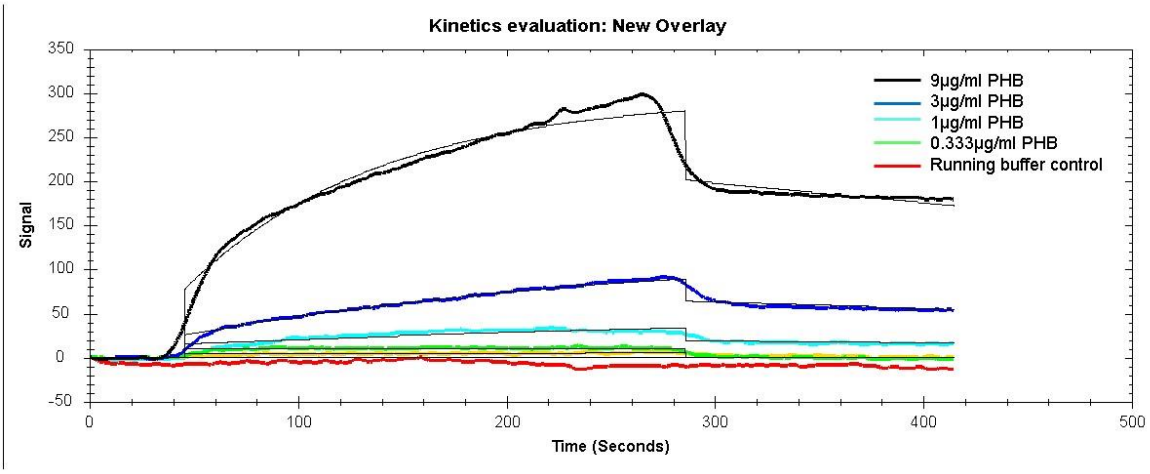
APPENDIX B

RAW 264.7 macrophages have pro-inflammatory response to LPS



APPENDIX C

Surface Plasmon Resonance experiment: rPHB1 binds to LPS in a concentration-dependent manner



APPENDIX D

Macrophages treated with rPHB1 and LPS have increased pro-inflammatory cytokine expression