2018

Effects of Stored Red Blood Cells on the Components of the Lung-Capillary Membrane

Kaitlyn S. Ryan
University of Alabama at Birmingham

EFFECTS OF STORED RED BLOOD CELLS ON THE COMPONENTS OF THE LUNG-CAPILLARY MEMBRANE

by

KAITLYN S. RYAN

JILLIAN RICHTER, CHAIR
PALANIAPPAN SETHU
BRANT WAGENER

A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2018

EFFECTS OF STORED RED BLOOD CELLS ON THE COMPONENTS OF THE LUNG-CAPILLARY MEMBRANE

KAITLYN S. RYAN

BIOMEDICAL ENGINEERING

ABSTRACT

Trauma is the leading cause of death for Americans between the ages of 1 and 44. Implementation of massive transfusion protocols has improved early mortality due to traumatic hemorrhage; however, long-term complication rates associated with hemorrhagic shock remain high due to the development of multiple organ failure. In particular, resuscitation with large volumes of stored red blood cells **(RBC)** introduces high concentrations of free heme, which contributes to the development of acute lung injury **(ALI)** after trauma. Angiopoietin-2 **(Agpt-2)** is a mediator of trauma-related lung injury, but the effect of RBC storage-derived free heme on Agpt-2 regulation is unknown. The goal of this study was to determine (1) the effects of free heme formed during RBC storage on pulmonary microvascular endothelial cell **(PMEC)** function and Agpt-2 release and (2) the effects of PMEC-derived Agpt-2 on barrier function and inflammation of alveolar epithelial cells **(AEC)**. To test this, RBC were collected from mice and stored for 14 days, during which time free heme levels doubled. PMEC barrier function was measured following treatment with fresh or stored RBC supernatant. The effects of hemin and Agpt-2 were determined by blocking with hemopexin **(Hpx)** and an Agpt-2 neutralizing antibody. PMEC barrier damage caused by stored RBC supernatant treatment was significantly attenuated with the addition of Hpx but not with the addition of an Agpt-2 neutralizing antibody, suggesting that PMEC barrier dysfunction caused by free heme is not mediated by Agpt-2. It is unclear whether Agpt-2 release from PMEC is

mediated by heme following RBC supernatant treatment due to high variability and high background of samples via ELISA. Recombinant Agpt-2 treatment significantly upregulated inflammation in AEC; however, AEC barrier function was not affected by conditioned media from RBC-treated PMEC with low concentrations of Agpt-2, suggesting Agpt-2 is a mediator of AEC inflammation but effects on barrier function are unclear. More data are needed to determine the effects of stored blood transfusions on Agpt-2 release and the role of Agpt-2 in dysfunction of the lung-capillary barrier to develop therapies aimed at improving patient outcomes following trauma.

Keywords: stored red blood cells, lung-capillary membrane, Angiopoietin-2, heme, hemopexin

ACKNOWLEDGEMENTS

I would like to acknowledge all the people who have helped me during this process. I would like to give a special thanks to Dr. Jillian Richter for all her help, support, and guidance and for being a wonderful role model for me during my time at UAB. I would also like to thank Lisa Zheng for her help and training every day in the lab. Thank you to all my family and friends who have supported, encouraged, and believed in me when I wanted to give up. Lastly, I would like to thank the Biomedical Engineering Department at UAB and the Biomedical Engineering professors for all the resources and assistance they have provided to allow me to gather as much knowledge as possible during my time at UAB.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AEC	Alveolar epithelial cells
Agpt-1	Angiopoietin-1
Agpt-2	Angiopoietin-2
Akt	Serine/threonine kinase
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ECIS	Electrical Cell-Substrate Impedance Sensing
ELISA	Enzyme-linked immunosorbent assay
HAoECs	Human aortic endothelial cells
HO-1	Heme oxygenase 1
Hpx	Hemopexin
HSaVECs	Human dermal microvascular endothelial cells
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate-buffered saline
PMEC	Pulmonary microvascular endothelial cells
RBC	Red blood cells
TLR4	Toll-like receptor 4

INTRODUCTION

Post-Traumatic Lung Injury

Trauma is the leading cause of deaths for individuals under the age of 44 in the United States.[1] In particular, acute lung injury **(ALI)** leading to acute respiratory distress syndrome **(ARDS)** is a common cause of morbidity and late-stage mortality in trauma patients,[2] and the risk of trauma-associated ALI/ARDS increases by 6% with each unit of red blood cells **(RBC)** transfused.[3] The development of post-traumatic ALI/ARDS is driven by complex pathophysiologic processes that involve both local and systemic inflammation and breakdown of the lung-capillary barrier, leading to tissue edema and infiltration of immune/inflammatory cells.[4, 5] Loss of lung-capillary barrier function includes damage and hyperpermeability of both the pulmonary microvasculature and alveolar epithelium.[6] The mechanisms relating RBC transfusion and barrier breakdown resulting in ALI/ARDS are still unknown.

RBC Transfusion and Post-Traumatic Lung Injury

Transfusion of blood products (e.g., packed RBC, fresh frozen plasma, platelets) is the gold standard for resuscitation of trauma patients in hemorrhagic shock. However, independent of injury severity, large-volume resuscitation (>10 units of RBC in 24 hours) is associated with end-organ injury, infection, and higher risk of late-stage death.[7-9] Some studies have also shown that transfusion with RBC stored longer than 28 days can lead to

increased postinjury organ failure, infection, and other adverse clinical outcomes especially when transfusing with older blood during the first 6 hours.[5]

In the United States, RBC can be stored for up to 42 days at 4°C. Over time, RBC storage lesion occurs leading to morphological, biological, and metabolic changes as well as release of vesicles and cell-free proteins. Hemin, a cytotoxic byproduct, is formed when RBC lyse during storage and the hemoglobin inside is released and oxidized to ferric hemoglobin.[10] Levels of free heme measured in red blood during storage double between day 7 and day 35.[11]

Oxidized free heme induces an inflammatory response through Toll-like receptor 4 **(TLR4)** signaling that results in TNF-α secretion and nuclear factor kappa-light-chain-enhancer of activated B cells **(NFkB)** activation.[12-14] Administration of TAK242, a TLR4 inhibitor, or hemopexin **(Hpx)**, a regulator of free heme, significantly decreased mortality rates after transfusion of old RBC[11] implicating heme-mediated TLR4 signaling as a regulator of lung injury following resuscitation with stored RBC.

Hpx is a plasma glycoprotein that is mainly found in the liver and central nervous system and has the highest binding affinity to heme.[10] The binding of Hpx to heme induces heme oxygenase 1 **(HO-1)** accumulation which protects the cell from oxidative stress.[15] Chronic exposure to free heme induces protective levels of HO-1, however during massive transfusion, high levels of oxidized free heme are introduced into the body too quickly skewing the ratio of Hpx to heme and preventing HO-1 production.[16] Higher levels of oxidized free heme lead to neutrophil migration and inflammation.[17]

Angiopoietin-Tie2 Signaling and Lung Injury

Angiopoietins 1-4 are vascular growth factors that are involved in angiogenesis and regulation of endothelial cell-cell junctions. During homeostasis, normal barrier function is maintained through Angiopoietin-1 **(Agpt-1)** interactions with the tyrosine kinase Tie-2 receptor. Agpt-1-mediated Tie2 signaling promotes serine/threonine kinase **(Akt)** cell survival signaling and suppresses NFkB activation to prevent inflammation, stabilize junctions between endothelial cells through recruitment of cell adhesion molecules, and suppress synthesis of Angiopoitetin-2 **(Agpt-2)**.[18] Agpt-2 is a Tie2 antagonist that is upregulated during conditions of inflammation. High levels of Agpt-2, a microvascular destabilizing cytokine and regulator of lung injury**,** have been measured in plasma samples from patients with ALI and pulmonary edema.[19] Agpt-2 acts as a competitive inhibitor of Agpt-1 signaling through Tie2 receptors to cause phosphorylation of NFkB and downregulation of the PI3K/Akt pathway leading to inflammation and decreased barrier function. While Agpt-1 and Agpt-2 have been shown to bind to Tie2 receptors with similar affinity, Agpt-2 has been shown to activate Tie2 in a weaker manner than Agpt-1 binding.[20]

In animal models of trauma-associated hemorrhagic shock and sepsis, Agpt-2 has been shown to be a key mediator of vascular dysfunction and indirect lung injury.[21, 22] Preclinical in vivo mouse studies show that Agpt-2 promotes barrier dysfunction and subsequent development of ALI, while suppression of Agpt-2 decreases inflammatory processes in the lung.[21] Agpt-2 levels correlate with injury severity score and mortality rates in adult trauma patients and are elevated in patients that develop ARDS.[22, 23] Agpt-2

levels are also increased in pediatric patients following trauma and remain elevated after 24h (unpublished data).

The effect of resuscitation with stored RBC on Agpt-2-mediated post-traumatic lung injury is unknown. In particular, the effect of free heme present in stored RBC on Agpt-2 release from endothelial cells and subsequent epithelial cell dysfunction needs further investigation. We hypothesized that free heme formed in RBC during storage causes release of Agpt-2 from PMEC, which results in decreased barrier function of pulmonary microvascular endothelial cells **(PMEC)** and inflammation of alveolar epithelial cells **(AEC)**. Thus, the objectives of this study were to determine (1) whether free heme in stored RBC promotes release of Agpt-2 from PMEC and (2) if PMEC-derived Agpt-2 drives inflammation and barrier dysfunction of AEC.

EXPERIMENTAL MATERIALS AND METHODS

Cell Types

C57BL/6 Mouse lung PMEC were obtained from CellBiologics and used for 3-7 passages. Cells were cultured in complete mouse endothelial cell medium with supplement kit as directed by CellBiologics (without 0.1% Heparin). C57BL/6 Mouse AEC were obtained from CellBiologics and used for 3-9 passages. Cells were cultured in complete epithelial cell medium with supplement kit as directed by CellBiologics. All cells were cultured at 37°C and 5% CO_2 and media was changed every other day until 70% confluence was reached, then changed every day.

Reagents

Hemopexin was purchased from Athens Research and Technology (Athens, GA). Recombinant Mouse Angiopoietin-2 Protein, CF, was purchased from R&D Systems (Minneapolis, MN). Mouse/Rat Angiopoietin-2 Quantikine™ Enzyme-linked immunosorbent assay (ELISA) kit from R&D was purchased from Fisher Scientific (Hampton, NH). Thermo Scientific M-PER Mammalian Protein Extraction Reagent was purchased from Fisher Scientific (Hampton, NH). Odyssey Blocking Buffer (TBS) was purchased from LI-COR Biosciences (Lincoln, NE). Phospho-NF-kB p65 (Ser536) and NF-kB p65 (L8F6) antibodies were purchased from Cell Signaling (Danvers, MA). Monoclonal Anti-Actin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

IRDye 800CW goat anti-Rabbit IgG, IRDye 680RD Goat anti-Mouse IgG, IRDye 800CW Goat anti-Mouse IgG, and NewBlot™ Nitrocellulose 5X stripping buffer were purchased from LI-COR Biosciences (Lincoln, NE). Anti-Ang2 blocking antibody was purchased from AdipoGen (San Diego, CA). CP2D anti-coagulant and AS-3 storage buffer for blood banking are listed below in Table 1.

***Table 1*: Solutions for Blood Banking**

CP2D Anti-coagulant	**AS-3 Storage Buffer**
15.75 mL water	40 mL water
0.805 gm dextrose	0.44 gm dextrose
0.415 gm sodium citrate dehydrate	0.2352 gm sodium citrate dehydrate
0.047 gm citric acid	0.0168 gm citric acid
0.035 gm monobasic sodium phosphate	0.110 gm sodium phosphate
	0.0164 gm sodium chloride
	0.012 gm adenine

Blood Storage

Whole blood was collected by cardiac puncture from male C57BL/6 mice. Platelet-rich plasma and anti-coagulant were removed by centrifugation (100xg for 10min at 4°C then 1000xg for 10min at 4°C) and blood was resuspended in phosphate-buffered saline (PBS, 1:3) then leukoreduced using a PD-10 Desalting Column (GE Healthcare). PBS was removed from leukoreduced blood by centrifugation (1000xg for 10min at 4°C). AS-3 storage solution was added (2:9) and RBC were then either stored in 1mL syringes at 4°C with no headspace for 14 days (stored group) or used immediately (fresh group). For cell culture treatment, supernatant from the RBC was collected using a two-step centrifugation (1500xg for 5min at 22°C). Heme levels were measured in the cell-free supernatant using spectral deconvolution as described previously[24].

Barrier Function of PMEC

Electrical Cell-Substrate Impedance Sensing (ECIS) was used to monitor barrier function by measuring resistance across a monolayer over 24h. 50,000 C57BL/6 Mouse lung PMEC cells were seeded in each well of an 8W10E+ (Applied Biophysics) plate following a 15min pretreatment with cysteine solution (Applied Biophysics), 2x wash with distilled water, and a 15min gelatin coating (Cell Biologics) all at room temperature. Once the cells reached confluence, the plate was put into the ECIS incubator and cell resistance was monitored until stable. Once stable, complete media from each well was replaced with serum-free media and 30min later a 10μM Hpx pretreatment was added to the appropriate wells. After 30min, cell-free supernatant from fresh or stored RBC was added to designated wells (30%v/v) and resistance was monitored for 24h in an ECIS Model ZΘ. The same procedure was repeated following 30min serum starvation using 100ng/mL of Angiopoietin-2 blocking antibody pretreatment added to 30%v/v stored RBC supernatant prior to cell exposure. Resistance measurements were taken every 30min and normalized back to baseline values following serum starvation and pretreatment immediately prior to the addition of the RBC supernatant treatment.

Angiopoietin-2 Levels in PMEC

Mouse endothelial cells were grown to confluence in 6 well plates. Cells were washed with PBS prior to serum starvation. Serum-free media with or without 10μM Hpx was added to the cells 30min before supernatant treatment. Cells were treated with 30%v/v fresh or stored RBC supernatant in serum-free media +/- 10μM Hpx (stored

group only). Media was collected at 5min, 1, 4, 8, 12, and 24h and released Angiopoietin-2 levels were measured via ELISA.

Inflammatory Response in AEC

Mouse epithelial cells were grown to confluence on Transwell inserts (Fisher Bioreagents). Recombinant Angiopoietin-2 was dissolved in low-serum (2% FBS) media to the working concentration and added to the basolateral side after washing the top and bottom well with sterile PBS. After 24h, cells were washed with cold PBS and lysed in 75uL of protein extraction reagent with protease and phosphatase inhibitors (1:100) for 20min on ice. Cell lysates were centrifuged at 14,000xg for 20min at 4°C and the supernatants collected. The lysates were boiled at 95°C for 10min and run under electrophoresis in a 4-15% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad) at 100V. The protein was transferred to a nitrocellulose membrane on ice for 1h then blocked for 1h at room temperature with Odyssey Blocking Buffer. The nitrocellulose membrane was incubated with Phospho-NF-kB p65 (Ser536) and NF-kB p65 (L8F6) at 1:1000 in blocking buffer overnight at 4°C, washed with TBS-T, then incubated for 1h with IRDye 800CW goat anti-Rabbit IgG and IRDye 680RD Goat anti-Mouse IgG at 1:15,000 in TBS-T and imaged. The membrane was stripped for 15 minutes in 1X NewBlot Nitro Stripping Buffer and washed with PBS. It was blocked for 1h at room temperature in blocking buffer then incubated with monoclonal anti-actin antibody at 1:5000 in blocking buffer for 1h at room temperature. The membrane was washed with TBS-T then incubated with IRDye 800CW Goat anti-Mouse IgG at 1:15,000 in TBS-T

for 1h at room temperature and washed again. All protein levels were imaged and determined using LICOR.

AEC Barrier Function and Conditioned Media from RBC-treated PMEC

Mouse PMEC were grown to confluence in 6 well plates. Cells were serum starved for 30 minutes then 30%v/v stored RBC supernatant was added for 24h. Following the treatment period, conditioned media was collected and frozen. For controls, PMEC were maintained in serum-free media for 24h but not treated with RBC supernatant.

Mouse AEC were seeded in 8W10E+ (Applied Biophysics) plates that were pretreated with a cysteine solution (Applied Biophysics) for 15min, washed 2x with distilled water, and coated with a gelatin coating (Cell Biologics) for 15min all at room temperature. Once the cells reached confluence, the plate was put into the ECIS incubator and cell resistance was monitored until stable. 50%v/v of untreated conditioned media, treated conditioned media, or treated conditioned media with the addition of 100ng/mL of Agpt-2 neutralizing antibody in low-serum epithelial cell media were added and resistance was monitored for 24h in an ECIS Model ZΘ. Resistance measurements were taken every 30min and normalized back to baseline values immediately prior to the addition of the conditioned media treatment.

RESULTS

Total cell free heme levels in stored RBC supernatant are significantly higher than in fresh RBC supernatant.

Total free heme levels measured by spectral deconvolution of RBC supernatant were significantly higher in RBC stored for 14 days compared to fresh RBC (Figure 1). A breakdown of the composition of total free heme based on the average amounts of hemin, methemoglobin, oxyhemoglobin, and deoxyhemoglobin present in fresh and stored RBC supernatant are also indicated in Figure 1.

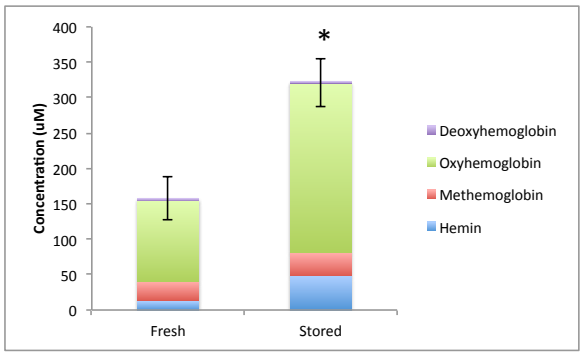

Figure 1: Total free heme levels and component breakdown of RBC supernatant.
Free heme levels were measured in fresh and stored RBC supernatant samples using spectral deconvolution. Total free heme levels in stored RBC supernatant are significantly higher than total free heme levels in fresh RBC supernatant after 14d. (*$p<0.01$) Oxyhemoglobin, methemoglobin, hemin, and deoxyhemoglobin levels are shown for component comparison. Data reported as mean ± standard error of the mean.

Free heme in stored RBC supernatant causes barrier damage in PMEC and is attenuated by Hpx.

The response of PMEC to treatment with supernatant from fresh and stored RBC as measured by changes in monolayer resistance is shown in Figure 2. Normalized resistance of the media control group significantly (*$p<0.05$) increased from baseline

levels over the 12h time period (Figure 2B). Treatment with supernatant from fresh or stored RBC did not significantly change PMEC resistance after 12h of treatment compared to starting baseline values (Figure 2B). However, compared to the normalized resistance of the control group, PMEC treated with fresh or stored RBC supernatant had significantly (*p<0.05) decreased resistances at 12h (Figure 2C). There was no significant difference in the normalized resistance of PMEC treated with stored RBC supernatant compared to fresh RBC supernatant at 12h. Blocking hemin released during RBC storage with Hpx significantly (#p<0.05) attenuated the decrease in resistance caused by stored RBC supernatant and normalized resistance was significantly (#p<0.05) higher than the fresh RBC supernatant treatment group (Figure 2C).

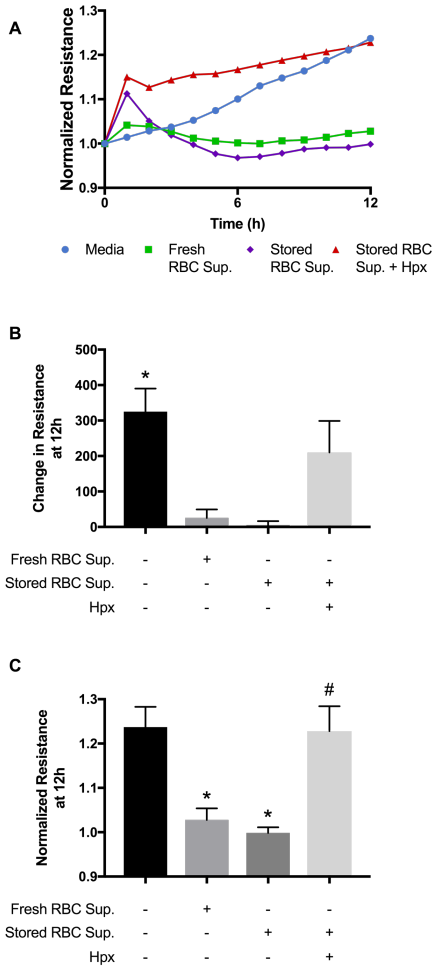

Figure 2: PMEC barrier function response to RBC supernatant with Hpx.
PMEC were grown to confluence on ECIS plates, serum starved for 1.5h, then treated with supernatant from fresh or stored RBC (30% v/v). 10μM Hpx was added 30min prior to the addition of stored RBC supernatant. A) Resistances of PMEC monolayers reported as values normalized to the individual well resistance following serum starvation and the addition of Hpx prior to treatment. B) The 12h changes in resistance for each group determined by subtracting the 12h resistance value from the pre-treatment resistance. PMEC maintained in media alone had a significant increase in resistance at 12h (*p<0.05). C) Normalized resistance values at 12h for PMEC maintained in media (control) or treated with fresh or stored RBC supernatant with and without Hpx. Treatment with RBC supernatant resulted in significantly lower resistance compared to the media control (*p<0.05). The addition of Hpx to the stored RBC treatment significantly attenuated the damage caused by stored RBC supernatant and was significantly higher the than normalized resistance of fresh RBC supernatant treatment (#p<0.05). Data reported as mean ± standard error of the mean.

Agpt-2 does not regulate PMEC barrier dysfunction caused by stored RBC.

To investigate whether Agpt-2 plays a role in endothelial hyperpermeability caused by exposure to stored RBC, PMEC were treated with stored RBC supernatant in combination with 100ng/mL of an Agpt-2 neutralizing antibody. The antibody was added to the supernatant treatment prior to cell exposure and barrier function was monitored via ECIS. The change in resistance following stored RBC supernatant treatment was not statistically significant ($p=0.07$) at 12h. However, addition of the Agpt-2 neutralizing antibody to the stored RBC treatment caused a significant ($*p<0.05$) decrease in PMEC resistance (Figure 3B). Normalized resistances for the stored RBC supernatant treatment with and without the Agpt-2 neutralizing antibody were significantly ($*p<0.05$) lower at 12h compared to the media control group (Figure 3C).

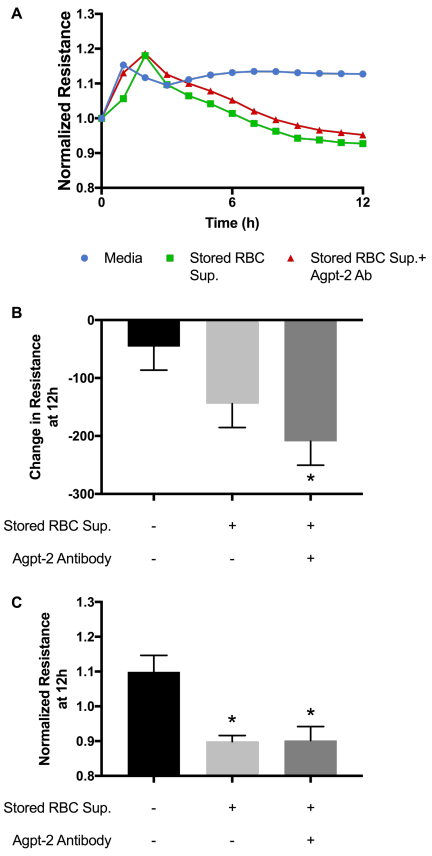

Figure 3: PMEC barrier function response to stored RBC supernatant with Agpt-2 neutralization.
PMEC were grown to confluence then serum starved for 30min. 100ng/mL of Agpt-2 neutralizing antibody was added into the 30%v/v stored RBC supernatant treatment prior to being added to the cells. A) Resistances of PMEC monolayers reported as values normalized to starting resistances following serum starvation prior to treatment. B) Changes in resistance for each group at 12h. The change in resistance at 12h following treatment with stored RBC supernatant with 100ng/mL of Agpt-2 neutralizing antibody was significant ($*p<0.05$). C) Normalized resistance for each group at 12h. The normalized resistances at 12h for the stored RBC supernatant treatment with and without the Agpt-2 neutralizing antibody were significantly lower than the resistance of the serum free media treated cells ($*p<0.05$). Data reported as mean ± standard error of the mean.

Treatment with supernatant from stored RBC causes a small amount of Agpt-2 release from PMEC independently of free heme.

To determine if hemin generated during RBC storage promotes Agpt-2 release, mouse PMEC were treated with 30% v/v of fresh RBC supernatant or stored RBC supernatant with or without the addition of 10μM Hpx, and Agpt-2 levels in the media were measured at 5min, 1, 4, 8, 12 and 24h via ELISA. Figure 4A shows the background levels of each treatment with the measured Agpt-2 concentrations at each time point. Substantial background levels were observed for each treatment, likely due to high levels of hemolysis in the RBC supernatant treatments. Furthermore, most measured concentrations of released Agpt-2 from PMEC in response to treatment with RBC supernatant did not rise above background levels until 24h. Background-subtracted concentrations of Agpt-2 at 24h are shown in Figure 4B. The level of Agpt-2 release from PMEC in response to treatment with stored supernatant was not significantly higher than the Agpt-2 release caused by treatment with fresh RBC supernatant. The addition of Hpx to PMEC treated with stored RBC supernatant did not attenuate Agpt-2 release.

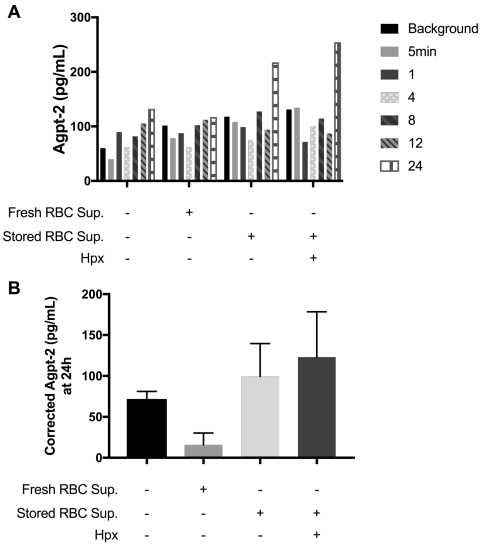

Figure 4: Agpt-2 release from PMEC in response to RBC supernatant treatment. PMEC seeded in 6 well plates were treated with fresh or stored RBC supernatant (30% v/v). 10μM Hpx was added 30min prior to treatment with stored RBC supernatant. Media was collected at 5min, 1, 4, 8, 12, and 24h and Agpt-2 levels measured via ELISA. A) Background levels of each treatment and measured concentrations of Agpt-2 release at each time point for each treatment. B) Background-subtracted Agpt-2 levels at 24h. Stored RBC treatment did not cause a statistically significant increase in Agpt-2 release compared to fresh RBC supernatant treatment. The addition of Hpx did not significantly decrease Agpt-2 levels

in the media compared to stored RBC supernatant treatment. Data reported as mean ± standard error of the mean.

Recombinant Agpt-2 increases inflammation in AEC at 24h.

While circulating Agpt-2 was not shown to regulate PMEC barrier function, the effects of Agpt-2 on AEC inflammation and barrier function required further study. To measure the inflammatory response to Agpt-2, mouse AEC were grown in 6 well plates and treated with either 5 or 500ng/mL of recombinant mouse Agpt-2. Western blot was used to measure inflammation via phosphorylation of NFkB-p65. At 24h, treatment with recombinant Agpt-2 at both doses resulted in significantly increased phosphorylation of NFkB-p65 (*p<0.05, #p<0.001).

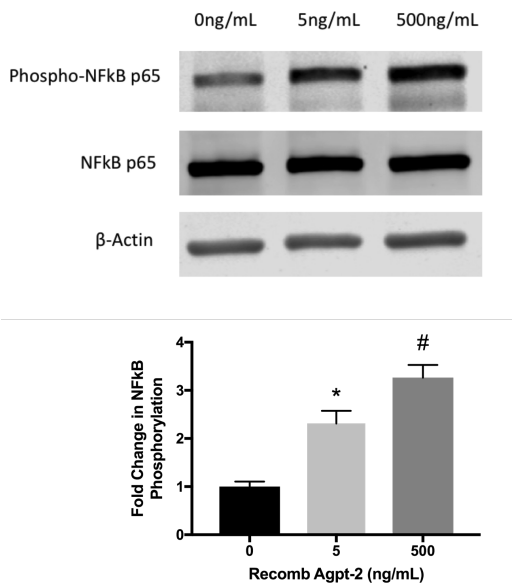

Figure 5: AEC response to recombinant Agpt-2.
Mouse AEC grown in 6 well plates were treated with either 5ng/mL or 500ng/mL of recombinant mouse Agpt-2. Cell lysate was collected and inflammation was measured via NFkB-p65 western blot. NFkB-p65 phosphorylation at 24h after 5 or 500ng/mL recombinant Agpt-2 treatment was significantly increased compared to control levels (*p<0.05, #p<0.001). Data reported as mean ± standard error of the mean.

Agpt-2 in media of RBC-treated PMEC does not affect barrier function of AEC.

To determine if RBC-mediated Agpt-2 release from PMEC regulates AEC function, AEC were treated (50%v/v) with media collected from untreated PMEC ("untreated conditioned media") or from PMEC treated with 30%v/v stored RBC supernatant

("treated conditioned media"). An Agpt-2 neutralizing antibody (100ng/mL) was added to the treated conditioned media to discern the effects of Agpt-2 on AEC function. The levels of Agpt-2 in untreated and treated conditioned media were measured via ELISA as 191.7pg/mL and 323.2pg/mL, respectively. Thus, the total amounts of Agpt-2 present in the AEC treatments were 95.9pg/mL and 161.6pg/mL. Figure 6 shows the normalized resistance values after treatment with untreated conditioned media, treated conditioned media, and treated conditioned media with 100ng/mL of Agpt-2 neutralizing antibody. The untreated conditioned media caused a significant (*$p<0.05$) change in resistance at 8h compared to initial resistance. None of the treatments caused a statistically significant change in normalized resistance at 8h compared to the media control (Figure 6C).

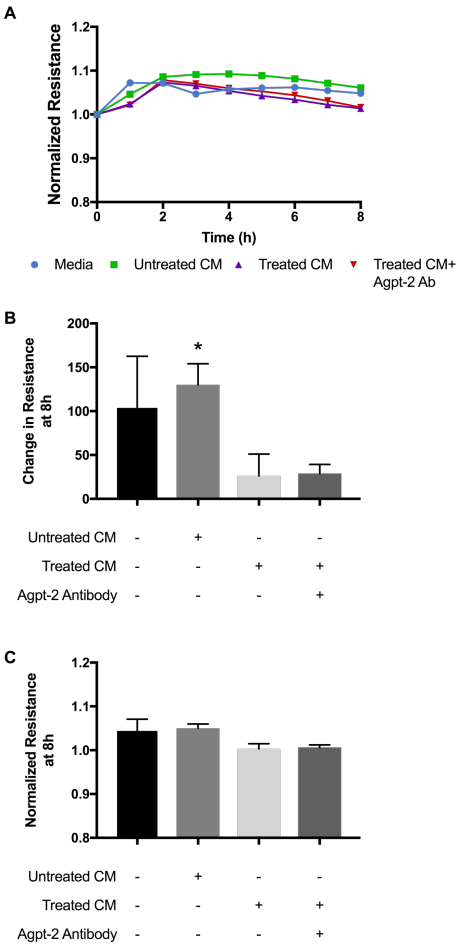

Figure 6: AEC response to conditioned media from PMEC treated with RBC supernatant. Conditioned media was collected from PMEC treated with 30%v/v stored RBC supernatant for 24h following 30min serum starvation. AEC were treated with untreated conditioned media and treated conditioned media from PMEC with the addition of 100ng/mL of Agpt-2 neutralizing antibody and monitored using ECIS. A) Resistances of PMEC monolayers reported as values normalized to starting resistances prior to treatment. B) Changes in resistance for each group at 8h. There was not a statistically significant change in barrier function following treatment with media alone or treated conditioned media treatments with and without the Agpt-2 neutralizing antibody by 8h. There was a significant (*$p<0.05$) change following treatment with the untreated conditioned media at 8h compared to initial resistance. C) Normalized resistance for each group at 8h. None of the treatments significantly altered the normalized resistance at 8h. Data reported as mean ± standard error of the mean.

DISCUSSION

Resuscitation with older RBC, containing increased amounts of free heme, has been shown to lead to poorer clinical outcomes especially when infused in the first 6h and when infusing large volumes (>10 units in 24h). Following trauma and hemorrhage, poor clinical outcomes like ALI/ARDS, edema, and multi-organ failure arise from an inflammatory response and loss of barrier function of the lung-capillary interface. Agpt-2, which is upregulated following trauma,[23] has been suggested to play a role in mediating barrier function of PMEC and AEC as well as inflammatory responses. However, the mechanisms leading to hyperpermeability and inflammation and the subsequent negative outcomes seen following early resuscitation with large amounts of older RBC are still not well understood. This work has begun to examine some possible mediators involved and their effects, testing the hypothesis that increased levels of free heme, specifically hemin, formed during blood storage contributes to PMEC hyperpermeability, Agpt-2 release, and subsequent inflammation of AEC.

Consistent with previous literature,[11] our data showed that the levels of free heme double between 0 and 14 days of RBC storage (Figure 1). The additional free heme in stored RBC supernatant causes damage to the PMEC monolayer, compared to controls, which is attenuated by blocking hemin with Hpx (Figure 2). Figures 2A and 2B show a significant increase in resistance for the media control group, suggesting the cells were still growing, but do not show a significant decrease in barrier function following

treatment with RBC supernatant compared to resistance at 0h. However, the normalized resistance at 12h was significantly lower for the fresh and stored RBC supernatant groups compared to the media control indicating the treatments caused damage to the monolayer by preventing the proliferation seen in the control group (Figure 2C). The stored RBC supernatant group caused more damage than the fresh RBC supernatant group but the difference in normalized resistance was not significant, which could be due to similar heme levels in the treatments. Blocking the hemin in the stored RBC supernatant group significantly attenuated the damage caused by the RBC supernatant suggesting hemin plays a role in regulating barrier function. It is important to note that levels of free heme measured in each sample of RBC supernatant collected for each experiment varied and that experimental treatments were based on volume. Preliminary data showed a dose response with stored RBC treatments where exposure to higher levels of free heme had more robust effects on PMEC function. Thus, future experiments should consider normalizing treatments to total cell free heme concentrations instead of using standardized volumes.

The damage from hemin on PMEC barrier function does not appear to be dependent on Agpt-2 since the negative effects of stored RBC supernatant were not ameliorated by blocking Agpt-2 with this dose of neutralizing antibody (Figure 3C). In fact, the addition of the Agpt-2 blocking antibody slightly worsened the effects of the stored RBC supernatant treatment alone when comparing the change in resistance at 12h (Figures 3A and 3B). Blocked Agpt-2 exacerbating monolayer damage suggests that Agpt-2 could be acting to promote barrier function. Agpt-2 is known as an antagonist of Agpt-1 acting on Tie2 receptors to cause damage. However, Agpt-2 has been shown to

act as an agonist in the absence of Agpt-1.[25] Therefore, actual Agpt-2 levels may not be as important as the ratio of Agpt-1 to Agpt-2 in regulating barrier function, so introducing Agpt-1 to the cell culture environment could give a more dynamic response.

It is unclear whether hemin in stored RBC supernatant mediates Agpt-2 release based on Figure 4. High variability of Agpt-2 measurements via ELISA and high background levels of treatments cloud interpretations of the data. While there were inconsistencies in the data, Figure 4B shows that stored RBC supernatant caused more Agpt-2 release than fresh RBC supernatant and the addition of Hpx did not prevent release. It is possible that the heme in the samples interfered with detection of Agpt-2 since background levels of the treatments were so high. Background levels of Agpt-2 for the media control group were higher than levels at 5min and at 24h background-subtracted media control levels were higher than the fresh RBC supernatant group. All of these measured concentrations were on the lowest end of the standard curve, which had higher O.D. values than the typical standard, so there could have been too much noise to get accurate detection. False signals and interference have been seen on slot blots, immunostaining, and heparanase activity assays suggesting that heme interferes with some colorimetric or charge based assays. There are other lesion components (e.g., microparticles, vesicles, cytokines, etc.)[26, 27] and other free heme components (e.g., oxyhemoglobin, methemoglobin) that form during blood storage that may contribute to Agpt-2 release.[28] Additionally, higher concentrations of Agpt-2 could be released from other types of endothelial cells, such as human aortic endothelial cells (HAoECs) or human dermal microvascular endothelial cells (HSaVECs), compared to mouse PMEC.[29] Strategies to reduce background levels of treatments and collect more accurate results

include removing free heme/hemoglobin from samples, increasing the dose of free heme to increase Agpt-2 release, blocking contribution of other free heme components, and measuring response of other types of endothelial cells. Each of these approaches should be considered in determining the effect of free heme on Agpt-2 release.

Figure 5 shows recombinant Agpt-2 causes a significant increase in inflammation in AEC at two different doses, as measured by NFkB-p65 phosphorylation. Based on this, we expected an inflammatory response and possible hyperpermeability of AEC when treated with conditioned media containing Agpt-2 from RBC-treated PMEC. Figure 6 shows AEC treatment with conditioned media did not significantly affect barrier function regardless of the presence of Agpt-2 in the media. Figures 6A and 6B show a significant but not substantial increase in resistance following treatment with untreated conditioned media at 8h which could indicate the cells were not fully confluent and continued to grow slightly despite being in 50% epithelial media. Figure 6C shows a slight decrease (<5%) in barrier function, compared to the media control group, following the addition of treated conditioned media and blocking Agpt-2 had no effect. None of the treatments significantly effected barrier function. However, the levels of Agpt-2 in the conditioned media were lower than recombinant Agpt-2 doses used previously, so concentrations could have been too low to have a substantial effect on barrier function. Higher doses of conditioned media from PMEC or other endothelial cells, using the strategies listed above to increase Agpt-2 release, could cause a more potent effect on barrier function in order to determine how Agpt-2 regulates hyperpermeability of AEC.

These data show that free heme causes barrier dysfunction of PMEC but the damage to the monolayer is not mediated by Agpt-2. Our studies were unable to

determine whether free heme mediates Agpt-2 release from PMEC, and future studies are needed that look at release of Agpt-2 following higher doses of treatment while inhibiting other components of stored blood in either PMEC or other types of endothelial cells. Recombinant Agpt-2 causes inflammation in AEC, however conditioned media with low levels of Agpt-2 from PMEC treated with RBC supernatant did not have an effect on barrier function of AEC. Ultimately, clinical and in vivo data demonstrate that Agpt-2 is important, but more data need to be collected to determine the factors related to trauma that may trigger or exacerbate Agpt-2 release and the effects of Agpt-2 on mediating barrier function of the lung-capillary interface.

Future Directions

This lab has created a coculture device to culture both AEC and PMEC on a membrane, which recapitulates the lung-capillary interface. Each cell type will be grown on either side of a Transwell membrane and once confluent, fresh or stored RBC supernatant will be used to treat the PMEC on the bottom of the membrane either in static culture or under perfusion. Using this model, Agpt-2 levels and overall barrier function will be measured in response to RBC supernatant treatment with inhibitors of Agpt-2, TLR4, and hemin. The membrane can also be fixed following treatment to look at inflammation in each cell type and cellular Agpt-2 levels. Experiments using this model will provide more physiologically accurate results as the two cell types are able to interact and experience more direct effects.

LIST OF REFERENCES

1. Heron M. Deaths: Leading Causes for 2014. *Natl Vital Stat Rep* 2016; **65**(5)**:** 1-96.

2. Bakowitz M, Bruns B, McCunn M. Acute lung injury and the acute respiratory distress syndrome in the injured patient. *Scand J Trauma Resusc Emerg Med* 2012; **20:** 54.

3. Chaiwat O, Lang JD, Vavilala MS, Wang J, MacKenzie EJ, Jurkovich GJ *et al.* Early packed red blood cell transfusion and acute respiratory distress syndrome after trauma. *Anesthesiology* 2009; **110**(2)**:** 351-60.

4. Stormann P, Lustenberger T, Relja B, Marzi I, Wutzler S. Role of biomarkers in acute traumatic lung injury. *Injury* 2017; **48**(11)**:** 2400-2406.

5. Koch CG, Figueroa PI, Li L, Sabik JF, 3rd, Mihaljevic T, Blackstone EH. Red blood cell storage: how long is too long? *Ann Thorac Surg* 2013; **96**(5)**:** 1894-9.

6. Martin TR, Hagimoto N, Nakamura M, Matute-Bello G. Apoptosis and epithelial injury in the lungs. *Proc Am Thorac Soc* 2005; **2**(3)**:** 214-20.

7. Johnson DJ, Scott AV, Barodka VM, Park S, Wasey JO, Ness PM *et al.* Morbidity and Mortality after High-dose Transfusion. *Anesthesiology* 2016; **124**(2)**:** 387-95.

8. Yang JC, Sun Y, Xu CX, Dang QL, Li L, Xu YG *et al.* Correlation between red blood cell transfusion volume and mortality in patients with massive blood transfusion: A large multicenter retrospective study. *Exp Ther Med* 2015; **9**(1)**:** 137-142.

9. Malone DL, Dunne J, Tracy JK, Putnam AT, Scalea TM, Napolitano LM. Blood transfusion, independent of shock severity, is associated with worse outcome in trauma. *J Trauma* 2003; **54**(5)**:** 898-905; discussion 905-7.

10. Belcher JD, Beckman JD, Balla G, Balla J, Vercellotti G. Heme degradation and vascular injury. *Antioxid Redox Signal* 2010; **12**(2)**:** 233-48.

11. Stapley R, Rodriguez C, Oh JY, Honavar J, Brandon A, Wagener BM *et al.* Red blood cell washing, nitrite therapy, and antiheme therapies prevent stored red blood cell toxicity after trauma-hemorrhage. *Free Radic Biol Med* 2015; **85:** 207-18.

12. Lin S, Yin Q, Zhong Q, Lv FL, Zhou Y, Li JQ *et al.* Heme activates TLR4-mediated inflammatory injury via MyD88/TRIF signaling pathway in intracerebral hemorrhage. *J Neuroinflammation* 2012; **9:** 46.

13. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI *et al.* Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood* 2014; **123**(3)**:** 377-90.

14. Figueiredo RT, Fernandez PL, Mourao-Sa DS, Porto BN, Dutra FF, Alves LS *et al.* Characterization of heme as activator of Toll-like receptor 4. *J Biol Chem* 2007; **282**(28)**:** 20221-9.

15. Tolosano E, Altruda F. Hemopexin: structure, function, and regulation. *DNA Cell Biol* 2002; **21**(4)**:** 297-306.

16. Gaggar A, Patel RP. There is blood in the water: hemolysis, hemoglobin, and heme in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2016; **311**(4)**:** L714-L718.

17. Andersson JA, Uddman R, Cardell LO. Hemin, a heme oxygenase substrate analog, both inhibits and enhances neutrophil random migration and chemotaxis. *Allergy* 2002; **57**(11)**:** 1008-12.

18. Makinde T, Agrawal DK. Intra and extravascular transmembrane signalling of angiopoietin-1-Tie2 receptor in health and disease. *J Cell Mol Med* 2008; **12**(3)**:** 810-28.

19. Bhandari V, Elias JA. The role of angiopoietin 2 in hyperoxia-induced acute lung injury. *Cell Cycle* 2007; **6**(9)**:** 1049-52.

20. Bogdanovic E, Nguyen VP, Dumont DJ. Activation of Tie2 by angiopoietin-1 and angiopoietin-2 results in their release and receptor internalization. *J Cell Sci* 2006; **119**(Pt 17)**:** 3551-60.

21. Lomas-Neira J, Venet F, Chung CS, Thakkar R, Heffernan D, Ayala A. Neutrophil-endothelial interactions mediate angiopoietin-2-associated pulmonary endothelial cell dysfunction in indirect acute lung injury in mice. *Am J Respir Cell Mol Biol* 2014; **50**(1)**:** 193-200.

22. Lomas-Neira JL, Heffernan DS, Ayala A, Monaghan SF. Blockade of Endothelial Growth Factor, Angiopoietin-2, Reduces Indices of Ards and Mortality in Mice

Resulting from the Dual-Insults of Hemorrhagic Shock and Sepsis. *Shock* 2016; **45**(2)**:** 157-65.

23. Ganter MT, Cohen MJ, Brohi K, Chesebro BB, Staudenmayer KL, Rahn P *et al.* Angiopoietin-2, marker and mediator of endothelial activation with prognostic significance early after trauma? *Ann Surg* 2008; **247**(2)**:** 320-6.

24. Oh JY, Hamm J, Xu X, Genschmer K, Zhong M, Lebensburger J *et al.* Absorbance and redox based approaches for measuring free heme and free hemoglobin in biological matrices. *Redox Biol* 2016; **9:** 167-177.

25. Yuan HT, Khankin EV, Karumanchi SA, Parikh SM. Angiopoietin 2 is a partial agonist/antagonist of Tie2 signaling in the endothelium. *Mol Cell Biol* 2009; **29**(8)**:** 2011-22.

26. Kim-Shapiro DB, Lee J, Gladwin MT. Storage lesion: role of red blood cell breakdown. *Transfusion* 2011; **51**(4)**:** 844-51.

27. Shukla R, Patel T, Gupte S. Release of cytokines in stored whole blood and red cell concentrate: Effect of leukoreduction. *Asian J Transfus Sci* 2015; **9**(2)**:** 145-9.

28. Kwon MS, Woo SK, Kurland DB, Yoon SH, Palmer AF, Banerjee U *et al.* Methemoglobin is an endogenous toll-like receptor 4 ligand-relevance to subarachnoid hemorrhage. *Int J Mol Sci* 2015; **16**(3)**:** 5028-46.

29. Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM *et al.* The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood* 2004; **103**(11)**:** 4150-6.