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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

Joanne L. Lomas-Neira, Daithi S. Heffernan, Alfred Ayala, and Sean F. Monaghan

¹Division of Surgical Research, Department of Surgery, Rhode Island Hospital/Alpert School of Medicine at Brown University, Providence, RI

Abstract

We have demonstrated hemorrhagic shock “priming” for the development of indirect (i)ARDS in mice following subsequent septic challenge and show pathology characteristic of patients with iARDS, including increased lung micro-vascular permeability and arterial PO_2/FIO_2 reduced to levels comparable to mild/moderate ARDS during the 48 hours following hemorrhage. Loss of endothelial cell (EC) barrier function is a major component in the development of iARDS. EC growth factors, Angiopoietin (Ang)-1 & 2, maintain vascular homeostasis via tightly regulated competitive interaction with tyrosine kinase receptor, Tie2, expressed on ECs. Ang-2/Tie2 binding, in contrast to Ang-1, is believed to produce vessel destabilization, pulmonary leakage and inflammation. Recent clinical findings from our trauma/surgical intensive care units and others have reported elevated Ang-2 in the plasma from patients that develop ARDS. We have previously described similarly elevated Ang-2 in plasma and lung tissue in our shock/sepsis model for the development of iARDS and demonstrated effective reduction in indices of inflammation and lung tissue injury following siRNA inhibition of Ang-2 protein synthesis. In this study we show that Ang-2 in lung tissue and plasma spikes following hemorrhage (priming) and remain elevated at sepsis induction. Also, that transient inhibition of Ang-2 function immediately following hemorrhage, suppressing priming, but not following sepsis, impacts the development of iARDS in our model. Our data demonstrates that selective temporal blockade of Ang-2 function following hemorrhagic shock priming, significantly improved PO_2/FIO_2 , decreased lung protein leak and indices of inflammation, and improved 10-day survival in our murine model for the development iARDS.

Keywords

Angiopoietin-2; indirect ARDS; ALI; neutrophil; endothelial cells; hemorrhage; priming; sepsis

Corresponding Author: Joanne Lomas-Neira, Ph.D., Department of Surgery, Division of Surgical Research, Rhode Island Hospital Alpert School of Medicine at Brown University, 593 Eddy Street, Aldrich 244, Providence, RI 02903, jlomasneira@lifespan.org, Tel: 401-444-7796, Fax: 401-444-3278.

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Introduction

Acute respiratory distress syndrome (ARDS) is a critical condition associated with significant mortality (1). Current published statistics for the U.S. report 74,000 deaths in 190,000 cases of ARDS and it is anticipated that as the U.S. faces the inevitability of an aging population, the annual incidence of ARDS will rise (2). Therapeutic strategies for ARDS have addressed a variety of treatments, including the use of low tidal volume mechanical ventilation, anti-inflammatory agents, fluid management, and anti-coagulants. However, evidence of ventilator induced lung injury (3,4), and a lack of significant improvement in clinical outcomes (5), have proven challenging to the progress of these treatment strategies. Adding to this challenge is the underlying pathology; ARDS is differentiated into direct and indirect, based on precipitating factors. Direct ARDS (pneumonia, aspiration and lung trauma) accounts for 57% of total cases, while indirect ARDS (iARDS), including extra-pulmonary sepsis and multisystem trauma, represents 43% (6,7). However, compared with direct ARDS, the pathophysiology of iARDS is much less well understood, possibly due to the involvement of multiple systemic factors.

In this respect, as a contributing event in the development of iARDS, shock resultant from life threatening blood loss (hemorrhage) is a known complication of traumatic injury. In experimental models of iARDS in mice, hemorrhage has been shown to predispose (prime) certain members of the immune cell population, including neutrophils, macrophage and T-cells, such that exposure to a secondary challenge (such as sepsis) elicits a dys-regulated/destructive inflammatory response, as in ARDS (8–11). We have demonstrated this hemorrhagic shock “priming” for the development of iARDS in mice following subsequent septic challenge and show pathology characteristic of patients with iARDS, including lung tissue inflammation, pulmonary edema, increased lung micro-vascular permeability and influx/sequestration of activated neutrophils into lung interstitium and alveolar space (9–11) as well as the arterial PO_2/FIO_2 , to a level comparable to mild to moderate ARDS (12) during the 48 hours following hemorrhage (see Methods section below). This data serves as further evidence supporting the clinical/translational value of our shock/sepsis murine model for the development of iARDS.

While we have described a significant role for neutrophils in the development of iARDS in our model (9,10), the contribution of pulmonary endothelial cells (ECs) in mediating the development of iARDS, is unclear.

During angiogenesis, EC function is believed to be mediated, in part, by the competitive binding of EC growth factors, Angiopoietin (Ang)-1 and Ang-2, to a shared receptor, Tie2, expressed primarily on ECs (13,14). Ang-1, produced by pericytes, smooth muscle cells and fibroblast, is found on the extracellular matrix. Ang-1/Tie2 binding is thought to promote vessel stabilization, anti-inflammatory, pro-survival and anti-permeability signaling through phosphorylation of Tie2 (15). In contrast, in an autocrine response, activated ECs rapidly release stored-preformed Ang-2. Ang-2/Tie2 binding is believed to produce vessel destabilization, pulmonary leakage and inflammation (16,17). Recent clinical findings from our trauma/surgical intensive care units and others have reported elevated EC growth factor, Ang-2, in the plasma from patients that develop ARDS (18–20). We have previously

described similarly elevated Ang-2 in the plasma and lung tissue in our shock/sepsis model for the development of iARDS and demonstrated effective reduction in indices of inflammation and lung tissue injury following siRNA inhibition of Ang-2 protein synthesis (20). While this method is useful in identifying potential target proteins for therapeutic intervention, similar usage is not currently feasible for treating ARDS in the human patient population.

We hypothesized that selective temporal blockade of Ang-2 function following the insults leading to the development of iARDS in our model (immediately following hemorrhage to suppress priming or following the induction of sepsis) will differentially alter the indices of inflammatory lung injury and decrease the mortality associated with iARDS in our model. To address this, here we determined the expression pattern of Ang-2 release as well as its de novo synthesis and, then; use transient inhibition of Ang-2 function to ascertain its impact on the development of iARDS in our model.

Materials and Methods

Mice

Male C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) 7–9 weeks of age were used in all experiments. Mice were housed in HEPA-filtered environmentally isolated caging units at Rhode Island Hospital Central Research Animal Facility. Experiments were performed in accordance with National Institutes of Health guidelines, approval from the Animal Use Committee of Rhode Island Hospital (Providence, RI; AWC# 0079-13) and with consideration to the ARRIVE guidelines developed by the National Center for the Replacement, Refinement, and Reduction of Animals in Research (21).

Reagents

Mouse Ang-2 ELISA assay kit was purchased from abcam, Cambridge, MA and Ang-1 ELISA kit was purchased from Life Sciences Advanced Technologies, Inc., St. Petersburg, FL. Mouse CBA “inflammation” cytokine assay kit, PE-Cy7 conjugated rat anti-mouse CD31 and BV421 conjugated hamster anti-mouse ICAM1 (CD54) were purchased from BD Bioscience, San Diego, CA. L1-10, Fc fusion protein Ang-2-specific inhibitor was kindly provided by Dr. Jon Oliner, Amgen, Inc., Thousand Oaks, CA. All other chemicals were analytical reagent grade and purchased from Sigma Chemical, St Louis, MO.

Experimental Model of indirect ALI

To test our hypothesis we used a mouse model of hemorrhagic shock induced “priming” followed by the induction of sepsis for the development of iARDS, a model with which we have considerable experience (9,10,20). This mouse model induces a decrease in the ratio of partial pressure arterial oxygen to fraction of inspired oxygen (arterial PO_2/FIO_2) that corresponds with the Berlin criteria for mild to moderate ARDS (Fig. 1) (12) and has a 60–70% mortality 48–72 hours following the induction of sepsis CLP (9,10).

Hemorrhage (Hem)

As previously described (9,10,20), mice were anesthetized using an isofluorane vaporizer setup, restrained in supine position and catheters were inserted into both femoral arteries. When fully awake, as determined by a mean blood pressure of ~95mmHg, the mice were bled (0.8–1.0 ml) over a 5–10 minute period to a mean blood pressure of 35mmHg ($\pm$ 5mmHg) and kept stable for 90 minutes. Immediately following hemorrhage mice were resuscitated (IV) with Ringers lactate at 4 times drawn blood volume.

Polymicrobial Sepsis (CLP)

24 hours post hemorrhage, sepsis was induced as a secondary challenge via cecal ligation and puncture as we have previously described (9,10,20). The timing of this secondary insult was based on previous findings by our laboratory that hemorrhage followed 24 hours by the induction of sepsis produced significantly higher levels of pro-inflammatory cytokines, increased MPO activity, and levels of neutrophil specific chemokine, MIP-2, than when sepsis was induced at earlier or later time points (9,10).

Fc fusion protein Ang-2 specific inhibitor/L1-10 was delivered sub-cutaneously (s.c.) in PBS, 4mg/kg body weight (~120ug/mouse/treatment) as described by Oliner et al., Tressel et al., and Yan et al. (22–24). For the experiments reported here, L1-10 was administered immediately following hemorrhage resuscitation or immediately following CLP or at both time points. Mice in Vehicle Control group received normal saline (22–24). Presence of bound/unbound LI-10 in plasma and lung tissue homogenates was assessed and no effect on Ang-2 ELISA performance was detected.

Survival Study

Fc fusion-protein Ang-2 specific inhibitor, L1-10, was administered at 4mg/kg/mouse (s.c.) (22–24). immediately following hemorrhage resuscitation or immediately following CLP (sepsis) surgery or at both time points. Normal saline was administered to control group mice. Mice were then returned to their cages, given access to food and water and assessed for survival benefit over a 10-day period. Initial surgery was performed on 6–8 mice per group. The experiment was replicated with a second set consisting of 6–8 mice and the data combined. The time frame for this study was based on previous survival studies by our laboratory (25). Statistical significance was assessed by Kaplan-Meier Survival analysis.

Arterial PO₂/FIO₂ was measured using a Stat Profile pHox blood gas analyzer (Nova BioMedical, Waltham, MA). Blood was collected, 3 mice/timepoint, at 4, 12, 20, 24, 30, 36 and 48 hours following hemorrhage resuscitation using cardiac stick method for time course (100ul/time point/mouse). For L1-10 treatment experiments, blood was collected at 48 hours for Vehicle control and L1-10 treated mice (5 mice/group).

Ang-2:Ang-1 kinetics study

For this study, mice were euthanized and lung tissue collected at hourly (H) time points following Hem resuscitation: 0H, 4H, 12H, 24H (the time point for CLP), 28H, 32H, 36H and 48H (time point of euthanasia for out model). Ang-1 and Ang-2 were measured in lung tissue lysates by ELISA assay. The ratio of Ang-2 to Ang-1 for each animal was expressed

as percent Ang-2 of total (Ang-1 plus Ang-2) measured. Naive baseline ratio is represented by horizontal dotted line.

Evan's Blue Dye (EBD) extravasation assay

Pulmonary vascular permeability was assessed by measuring the EBD in lung tissue homogenates as a percentage of total EBD intravenously administered (26). Briefly, EBD (2.5 mg in 0.5 ml 0.9N saline) was administered intravenously by tail vein injection 30 minutes prior to euthanasia. Lungs were harvested, frozen in liquid nitrogen, homogenized in phosphate buffered saline then incubated in 2ml of formamide for 18 hours at 60°C. Following centrifugation, EBD was measured in supernatant, dual wavelenths 620nm/740nm, and compared to EBD standard curve. Results are presented in lung tissue as % of total EBD administered.

Cytokine/chemokine Assays on supernatants samples (lung lysate samples) were performed as per manufacturer's protocol. IL-6 and TNF- α , MIP-2 and IL-10 were assayed using Cytometric Bead Array (CBA) on a FACSArray flow-cytometer (BD Bioscience, Inc.)

Myeloperoxidase (MPO) Activity

As an assessment of neutrophil influx to the lung, MPO activity was measured as previously described by our laboratory (10,27). Briefly, lung tissue was homogenized in 0.5ml of 50mM potassium phosphate buffer pH 7.4 and centrifuged at 40,000Xg at 4°C for 30 minutes. The supernatant was then reserved for cytokine analysis. The remaining pellet was re-suspended in 0.5 ml of 50mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice and then centrifuged at 12,000Xg at 4°C for 10 minutes. Supernatants were assayed at a 1:20 dilution in reaction buffer (530nmol/L o-dianisidine, 150 nmol/L H₂O₂ in 50 mM potassium phosphate buffer) and read at 490nm.

Intracellular adhesion molecule (ICAM)1

Endothelial cell expression of ICAM1 was assessed in mouse lung tissue. Single cell suspensions of lung tissue were prepared using a mouse lung dissociation kit and gentleMACS dissociator as per manufacturer's protocol (Miltenyi biotec, Auburn, CA). Cells were then incubated with PE-Cy7 labeled rat anti-mouse CD31 and BV421 labeled hamster anti-mouse ICAM1. Following flow cytometric analysis (BD FACSARIA), the mean fluorescence intensity (MFI) was calculated for the double positive cell population for each treatment group. Each group consisted of 3 mice with each sample run in duplicate.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical significant differences were determined using OneWay ANOVA, the post hoc test was TUKEY's. Calculations were performed using SigmaStat for Windows version 2.03. Group means were considered significantly different when p values were <0.05. Survival study statistical significance was assessed by Kaplan-Meier Survival analysis.

Results

Blockade of Ang-2 following both hemorrhage and sepsis significantly improves survival in our model

In an effort to maximize the effectiveness of our initial post-treatment approach we chose to target Ang-2 release initially following both hemorrhage and septic challenge. We felt this would eliminate the potential contribution of Ang-2 during not only the “priming” phase initiated by hypotensive shock, but ablate the subsequent “trigger” effect of the induction of sepsis (9,28). Ang-2 blockade was produced by administering 4 mg L1-10/kg b.w., s.c. immediately following hemorrhage resuscitation (to block pre-stored Ang-2 released in response to shock-induced “priming”) and immediately following CLP (24 hours post Hem) to block the sepsis-induced “trigger” event we have previously described. The inhibition of endogenous Ang-2 protein through administration of Ang-2 blockade at dual time points delayed the onset and decreased mortality significantly (35% at Day 8) as compared to Control (Fig. 2).

Kinetics of Ang-2 release shows spike in lung tissue and plasma following hemorrhage

That said; while we have previously documented that Ang-2 levels are elevated 24 hours following the combined insults of hemorrhage followed CLP and in the critically ill patients (20), we know little about the temporal changes in Ang-2 levels relative to these respective “priming” and “trigger” insults. Such information should not only let us refine our therapeutic approach, but should provide insight into the contributions of quick release/pre-stored pools of Ang-2 and/or impact of de novo synthesis of Ang-2. Here we show that Ang-2 release shows a rapid spike in lung tissue following hemorrhage (Fig. 3A) and a delayed, but similar increase in the plasma (Fig. 3B). Ang-2 remained elevated above the level measured in naïve mice (dotted horizontal line) at the time of induction of sepsis (CLP) in both lung tissue and plasma. Following CLP, Ang-2 steadily increased in lung tissue and, after a delay, began to increase in plasma (Fig. 3A/B).

Ang-2 blockade as single treatment immediately following hemorrhage, but not CLP, significantly improves survival

Given that there appear to be two somewhat distinct periods of Ang-2 release; one, initially in response to hemorrhagic shock and, a second, subsequent release in response to CLP, we chose to induce Ang-2 blockade by administering it as a single treatment either immediately following hemorrhage resuscitation or following CLP and then survival was again monitored for 10 days. Blockade of Ang-2 post hemorrhage delayed onset of mortality until Day 4 and provided a 30% improvement to survival at Day 7 compared to Control (Fig. 4A). No significant improvement in survival was observed when Ang-2 blockade was administered post CLP (Fig. 4B).

Having observed that blockade of Ang-2 post hemorrhage improved survival in our model, to begin to identify potential mechanisms, we assessed the impact of this treatment time point, compared to Ang-2 blockade post CLP, on lung tissue Ang-1 and Ang-2.

Blockade of Ang-2 as single treatment following hemorrhage priming restores Ang-1 to near Naïve control levels

Blocking Ang-2 immediately post-hemorrhage alone significantly reduced lung tissue Ang-2 (Fig. 5B) when compared to post CLP time point and restored Ang-1 to near Naïve control levels (Fig. 5A). L1-10 treatment administered at both time points, post hemorrhage and post CLP, further suppressed Ang-2, but did not increase Ang-1 above that of single post hemorrhage treatment.

Relative ratio of lung tissue Ang-2/Ang-1 following hemorrhage and CLP mirrors kinetics of Ang-2 release

To better understand the dynamics of Ang-2/Ang-1 expression and identify the optimal timing for blocking Ang-2 function, we measured lung tissue Ang-2 and Ang-1 at time points between 0–48 hours following hemorrhage. The ratio of Ang-2 to Ang-1 increased transiently following hemorrhage, remaining above Naïve control (dotted horizontal line) just prior to the induction of sepsis. The ratio then increased for the remainder of this experiment (Fig. 6).

Blocking Ang-2 post hemorrhage significantly improves indices of lung function

Arterial PO_2/FIO_2 was measured in our iARDS model as an index of lung function. Untreated Hem/CLP (Vehicle Control) mice were compared to mice that received a single dose of L1-10 either post Hem or post CLP (Fig. 7A). Blockade of Ang-2 post Hem, but not post CLP, significantly improved arterial PO_2/FIO_2 in these mice.

Pulmonary vascular permeability was assessed using Evan's Blue Dye extravasation assay. Similar to improved PO_2/FIO_2 , L1-10 blockade of Ang-2 post Hem significantly reduced lung permeability as measured by a decrease in the percentage of total EBD (delivered iv) recovered from lung tissue (Fig. 7B).

Blockade of Ang-2 impacts local/lung tissue cytokines

Blocking Ang-2 at single time-points post Hem or post CLP, as well as dual time point treatments, significantly reduced tissue IL-6 and increased local IL-10. Changes observed in lung tissue MIP-2 levels, while not statistically significant, showed reduced levels following post Hem and dual time point treatments (Fig 8). No change in TNF- α was observed.

Blockade of Ang-2 following hemorrhage decreases lung tissue MPO activity

As an assessment of neutrophil influx to the lung, MPO activity was measured in lung tissue lysates following both single L1-10 treatments (post hemorrhage or post CLP) and following dual treatment. Blockade of Ang-2 function immediately following hemorrhage, but not post CLP, significantly decreased neutrophil influx to lung when compared to Vehicle control (Fig.9). Dual time point treatments did not further reduce MPO activity (Fig. 9).

Blocking Ang-2 protein following hemorrhage priming significantly reduces adhesion molecule, ICAM1, expression on lung endothelial cells

ICAM1, expressed on stimulated ECs, is important in mediating neutrophil/EC interactions (29). Flow cytometry was used to identify a double positive CD31 (EC)/ICAM1 (CD54) population in single cell suspensions of mouse lung tissue. Mean fluorescent intensity (MFI) was measured for each fluorochrome in this double positive population and the ratio of ICAM1 MFI to CD31 MFI was used to illustrate changes in ICAM1 expression on ECs following Ang-2 blockade. Significantly, ICAM1 expression on lung ECs was decreased in mice that received single treatment Ang-2 blockade immediately following hemorrhage when compared to Vehicle control (Fig. 10).

Discussion

Loss of endothelial cell (EC) barrier function is a major component in the pathogenesis of indirect ARDS (30) where increased vascular permeability results in pulmonary edema as well as protein and inflammatory cell infiltrate. We have previously shown that Ang-2 is significantly increased at 24 hours post-Hem/CLP in our shock/sepsis model for the development of iARDS in mice and contributes to the inflammatory lung injury and loss of pulmonary vascular barrier function we observe (20). In this study, we present data showing that blockade of Ang-2 function following hemorrhage “priming” significantly decreases the loss of pulmonary function associated with neutrophil influx to lungs and EC/neutrophil interactions in our model.

Our first experiment assessed 10-day survival in mice that received Fc fusion-protein Ang-2 specific inhibitor, L1-10, following both hemorrhage and CLP. L1-10 has been shown to block the binding of Ang-2 to its receptor, Tie2, and inhibit blood vessel growth in solid tumors (22) as well as hind limb neovascularization after ischemia in mice (23). Also, in a model of lipopolysaccharide-induced direct acute lung injury, Kong et al. showed that blocking Ang-2 using L1-10 pre-treatment protected lung vascular barrier integrity in vitamin D receptor (VDR) deficient mice (31). Findings from our first experiment showed that inhibition of endogenous Ang-2 at dual time points significantly improved survival (35% at Day 8) compared to vehicle controls (Fig. 2).

To better understand the kinetics of Ang-2 release associated with the events of hemorrhage priming and subsequent induction of sepsis in our model, we measured Ang-2 in lung tissue and plasma at time points following hemorrhage. Ang-2 is stored preformed in ECs and is rapidly released upon activation (16,17). Our data reflects this response, with an early spike seen in lung tissue (Fig 3A) and delayed rise in plasma following hemorrhage (Fig. 3B). Importantly, while Ang-2 protein begins to decrease 12 hours following hemorrhage in both lung and plasma, these levels remain elevated above Naïve control (horizontal dotted line) at the time point of CLP. We speculate that this residual Ang-2 may indicate ongoing activation/priming of the endothelium that could potentiate EC response to subsequent a septic challenge.

To determine the significance of Ang-2 released in response to hemorrhage-induced priming with that released following the induction of sepsis on the mortality we see in our model, we

assessed 10-day survival following Ang-2 blockade a single time points. L1-10 was administered immediately following hemorrhage resuscitation or immediately following CLP (24 hours post Hem). Ang-2 blockade post hemorrhage provided a significant survival benefit compared to Control (30% by Day 7) (Fig. 4A). In addition, the delay in early mortality observed in the dual time-point treated mice (Fig. 2) was also seen following single post hemorrhage Ang-2 blockade (Fig. 4A). Interestingly, no significant survival benefit was seen when L1-10 was administered post CLP (Fig. 4B). These findings suggest that Ang-2 release associated with hemorrhage-induced priming, potentially impacting EC/neutrophil interactions and/or vascular integrity (20), plays a greater role in mediating the progression of iARDS in our model than release following CLP.

Ang-1 and Ang-2 functions are tightly regulated through their expression ratio (Ang-1:Ang-2) and competitive receptor (Tie2) binding (14). We next set out to address the question of how and when this tightly regulated system of vascular control, the anti-inflammatory, pro-survival, and vascular stability associated with Ang-1/Tie2 binding (15) transitions to a state of vessel destabilization and inflammation associated with Ang-2/Tie2 binding (32,33). To begin, we assessed the impact of each single and the dual time point treatments on lung tissue Ang-1 and Ang-2 (Fig. 5).

Of the single time point treatments, post hemorrhage blockade produced the greatest decrease in Ang-2; significant when compared to Vehicle (Fig 5B). As anticipated, a greater suppression of Ang-2, to near Naïve control level, occurred when Ang-2 blockade was administered at both time points (Fig 5B). The inverse was observed for Ang-1 at the same time points. Blockade of Ang-2 post hemorrhage generated an increase in lung tissue Ang-1 to the level of Naïve control (Fig. 5A).

These data suggest that, of the two single time points, blockade of pre-formed Ang-2, released following hemorrhagic shock-induced priming, produces the greatest impact on Ang-1/Ang-2 homeostatic balance.

This homeostatic balance has been shown to be critical in mediating EC function/dysfunction. Choi et al. describes elevated Ang-2:Ang-1 in association with paraquat intoxication induced lung injury (34). Ang-2:Ang-1 is effected by autocrine EC release of Ang-2 from either pre-stored sources (early) and/or EC Ang-2 de novo synthesis (late) (13–17). In as much, we found the relative ratio of Ang-2 to Ang-1 significantly increased at 4 hours post hemorrhage, and while declining by nearly 50% at 24 hours, remained elevated when compared to the Naïve control baseline level (Fig. 6). This elevated ratio at 24 hours post hemorrhage is significant as this suggests EC activation (EC priming) is sustained at a time point at which mice receive a secondary stimulus in the form of septic challenge (Fig. 6). Ang-1 tissue levels declined significantly between 4 and 12 hours post hemorrhage and began to increase at 24 hours post hemorrhage. However, in the 24 hours following CLP, lung tissue Ang-1 significantly and progressively decreased, while Ang-2 increased (Fig. 6).

We next set out to assess whether the impact we observed on Ang-1/Ang-2 homeostatic balance, induced by Ang-2 blockade following hemorrhage (Fig. 5), translated to improved pulmonary function. Arterial PO_2/FIO_2 was measured as functional assessment of oxygen

exchange in the lung and Evan's Blue Dye extravasation was assayed to assess pulmonary vascular permeability. Vehicle Control Hem/CLP mice showed significantly impaired arterial PO_2/FIO_2 (Fig. 7A) and increased pulmonary vascular permeability as measured by EBD extravasation (Fig. 7B). Ang-2 blockade post Hem, but not post CLP, significantly improved these indices of lung function in Hem/CLP mice. This data further points to hemorrhagic shock priming of EC response to subsequent septic challenge as mediating the pathogenesis of iARDS in our model.

To assess changes in the local/lung tissue inflammatory environment, we measured pro-inflammatory cytokines IL-6 and TNF- α , anti-inflammatory cytokine, IL-10, and neutrophil chemokine, MIP-2. Blockade of Ang-2 at all time points produced a significant decrease in lung tissue IL-6, and a significant increase in IL-10 (Fig. 7). However, and as would be anticipated 48 hours out from initial hemorrhagic shock challenge, the TNF- α level in treated mice was not significantly different from vehicle controls (Fig. 7). While MIP-2 levels following post Hem and dual treatments were decreased, there was no statistically significant difference from vehicle control (Fig. 7). This suggests that the changes, at least in IL-6 and IL-10, are secondary to changes in EC function mediated by Ang1:Ang-2.

We have also shown in our model that hemorrhage shock serves to prime peripheral blood neutrophils such that a secondary challenge (sepsis) elicits increased migration and influx to the lung corresponding with increased lung tissue injury (9–11). To assess the impact of Ang-2 blockade, and resultant change in endothelial cell regulation, on neutrophil influx we measured MPO activity in lung tissue following Ang-2 blockade at post hemorrhage or post CLP or at dual time points. Not surprisingly, suppression of Ang-2 following hemorrhage, the time point associated with neutrophil priming in our model, significantly reduced MPO activity/neutrophil influx to the lung when compared to Vehicle control (Fig. 8). Suppression of Ang-2 at both the post hemorrhage (early release) and post CLP time points showed similar suppression of MPO (Fig. 8).

Adhesion molecules on activated ECs play a significant role in mediating neutrophil influx from the vasculature to tissue. We assessed ICAM1 expression on lung ECs in our model and found ICAM1 to be significantly decreased on ECs from mice that received Ang-2 blockade post hemorrhage (Fig. 9). In contrast, ICAM1 expression following post CLP Ang-2 blockade resembled that of Vehicle control (Fig. 9). This finding is consistent with MPO data and strongly suggests the release of Ang-2 following hemorrhage contributes to the pathogenesis of iARDS in our model by mediating neutrophil/endothelial cell interactions.

The data presented here is consistent with our observation of the development of severe lung tissue injury and onset of mortality associated with our model at 48 hours post CLP and suggests a role for early release/pre-stored Ang-2 in hemorrhage priming for the development of iARDS in our model. And while it is not our suggestion that iARDS is the primary or sole event that accounts for mortality in this model, as numerous organ systems are affected by the combined insult of shock followed by septic challenge (35,36) and would be affected by Ang-2 blockade, we believe it is a significant and precipitating event that contributes to this morbid state. Thus, we feel the application of L1-10 and/or other novel

therapeutic approaches that target Ang-2 may represent novel treatment for the development of iARDS in the critically ill injured/shocked patient.

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Figure 1.

Arterial PO_2/FIO_2 (mmHg) decreases over 48 hours in our hemorrhage/sepsis model for the development of moderate indirect ARDS. (N=3 mice/time point)

Figure 2.

The inhibition of endogenous Ang-2 following hemorrhagic shock and sepsis significantly decreased mortality by 35% at Day 8. Fc fusion-protein Ang-2 specific inhibitor, L1-10, was administered prior to Hem resuscitation and immediately following CLP (24 hours post Hem). (N=13/L1-10, N=12/Control. * $p < 0.05$ vs. Control at Day 8)

Figure 3.

Kinetics of Ang-2 release in lung tissue and plasma over 48- hour time course. Ang-2 increases in lung tissue and plasma following hemorrhage and remains elevated above Naïve control (dotted horizontal line) at time point of induction of sepsis. (N=3 mice/time point, * $p < 0.05$ vs. 2 hours post Hem)

Figure 4.

Ang-2 blockade immediately following hemorrhage delays mortality and improves survival in mice by 30% at day 7. (A). Ang-2 blockade following CLP does not statistically improve survival (B). (N=12/group, * $p < 0.05$ vs. Control at day 7)

Figure 5.

Time point for Ang-2 blockade differentially affects Ang-1 and Ang-2 protein levels. Ang-1 is significantly decreased following Vehicle Hem/CLP as compared to Naïve control (A). Lung tissue Ang-1 increased significantly following a single post Hem Ang-2 blockade as well as following dual time point treatments (A). However, Ang-2 blockade as a single post CLP treatment did not produce a similar increase when compared to Vehicle (A). Ang-2 increased significantly in Vehicle (Hem/CLP) control mice as compared to Naïve control (B). Lung tissue Ang-2 decreased significantly following Hem Ang-2 blockade and was further reduced in mouse lung tissue following dual time point L1-10 treatments (B). This decrease not observed when Ang-2 blockade was initiated in post CLP (B). (N=4–5/group. *p<0.05 vs. Vehicle Control Hem/CLP)

Figure 6.

The ratio of Ang-2 to Ang-1 increases transiently following hemorrhage, but remains elevated (above Naïve Control shown as horizontal dotted line) during the 24 hours following Hem/CLP. (N=4–5 mice/time point, * $p < 0.05$ vs. Naïve control).

Figure 7.

Blockade of Ang-2 post hemorrhage significantly improves indices of pulmonary function. Arterial PO_2/FIO_2 (A) and pulmonary vascular leak as measured by EBD extravasation (B) were significantly improved following L1-10 administration post hemorrhage when compared to Vehicle Hem/CLP Control. No significant improvement was observed with L1-10 administration post CLP. (N=6 mice/group, *p< 0.05 vs. Vehicle Control Hem/CLP)

Figure 8.

Lung tissue levels of anti-inflammatory cytokine, IL-10, were significantly increased while tissue levels of IL-6 were significantly decreased following Ang-2 blockade at all time points. Lung tissue Mip-2 levels, while not statistically significant showed decreases following both post Hem and dual L1-10 treatments. TNF-α levels were not impacted when compared to Vehicle control following any L1-10 treatment time point. (N=5–7/group, *p<0.05 vs. Vehicle Control Hem/CLP)

Figure 9.

Blockade of Ang-2 immediately following hemorrhage, but not post CLP, significantly decreased neutrophil influx to lung as assessed by MPO activity when compared to Vehicle control. Ang-2 blockade at both time points, however, did not further reduce MPO activity. (N=5–7/group, * $p < 0.05$ vs. Vehicle Control Hem/CLP)

Figure 10.

ICAM1 expression (MFI) on lung CD31+/endothelial cells is significantly reduced following Ang-2 blockade post hemorrhage when compared to Vehicle Hem/CLP control. No reduction in ICAM1 expression was observed on endothelial cells following Ang-2 blockade post CLP. (N=3 mice/group, samples run in duplicate *p<0.05 vs Vehicle Control Hem/CLP)