Neutrophil–Endothelial Interactions Mediate Angiopoietin-2–Associated Pulmonary Endothelial Cell Dysfunction in Indirect Acute Lung Injury in Mice

Joanne Lomas-Neira¹, Fabienne Venet², Chun-Shiang Chung¹, Rajan Thakkar¹, Daithi Heffernan¹, and Alfred Ayala¹

¹Division of Surgical Research, Department of Surgery, Rhode Island Hospital/Alpert School of Medicine at Brown University, Providence, Rhode Island; and ²Immunology Laboratories, Hospices Civils de Lyon, Lyon, France

Abstract

Unresolved inflammation in the lung is thought to elicit loss of endothelial cell (EC) barrier integrity and impaired lung function. We have shown, in a mouse model of shock/sepsis, that neutrophil interactions with resident pulmonary cells appear central to the pathogenesis of indirect acute lung injury (iALI). Normally, EC growth factors angiopoietin (Ang)-1 and Ang-2 maintain vascular homeostasis through tightly regulated interaction with the kinase receptor Tie2 expressed on ECs. Although Ang-1/Tie2 has been shown to promote vessel integrity, stimulating downstream prosurvival/antiinflammatory signaling, Ang-2, released from activated ECs, is reported to promote vessel destabilization. This mechanism of regulation, together with recent clinical findings that plasma Ang-2 levels are significantly elevated in patients who develop acute respiratory distress syndrome, has focused our investigation on the contribution of Ang-2 to the development of iALI. A murine model of hemorrhagic shock–induced priming for the development of iALI after subsequent septic challenge was used in this study. Our findings show that 1) Ang-2 is elevated in our experimental model for iALI, 2) direct EC/neutrophil interactions contribute significantly to EC Ang-2 release, and 3) suppression of Ang-2 significantly decreases

inflammatory lung injury, neutrophil influx, and lung and plasma IL-6 and TNF- α . These findings support our hypothesis and suggest that Ang-2 plays a role in the loss of pulmonary EC barrier function in the development of iALI in mice resultant from the sequential insults of hemorrhagic shock and sepsis and that this is mediated by EC interaction with activated neutrophils.

Keywords: angiopoietin-2; indirect acute lung injury; neutrophil; short interference (si)-RNA; endothelial cells

Clinical Relevance

This study contributes to a body of knowledge furthering the understanding of the cellular/protein interactions that precipitate loss of endothelial barrier function and the role that angiopoietin-2 plays in the pathological process during the development of indirect acute respiratory distress syndrome/experimental acute lung injury. In addition, this study highlights angiopoietin-2 as a potential therapeutic in the treatment of inflammatory lung injury.

Acute respiratory distress syndrome (ARDS) is a complication of trauma characterized by increased microvascular permeability, edema, inflammation, and neutrophil accumulation in the lung. Despite advances in supportive care, trauma

patients who develop ARDS face the risk of significant mortality (1, 2). Although neutrophils have been implicated as playing an important role in the pathogenesis of ARDS/experimental acute lung injury (ALI) (2–5), vascular endothelial cells (ECs),

through their exposure/responsiveness to mediators present in the systemic environment, are also critical components involved in the development of ARDS/ALI. Using a murine model of hemorrhage (shock) in combination with a subsequent

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Correspondence and requests for reprints should be addressed to 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. E-mail: jlomasneira@lifespan.org

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septic challenge, our laboratory has shown that neutrophil interactions with resident pulmonary cells are central to the development of shock-induced indirect/extrapulmonary ALI (iALI) (5–7). EC activation, as in vessel remodeling and adhesion and migration of inflammatory cells, is an adaptive response, regulating vascular homeostasis and host defense, respectively. However, the loss of EC barrier function in ARDS/iALI results in accumulation of fluid, proteins, and inflammatory cells within the lung and compromised gas exchange (8–11). This transition from adaptive activation to maladaptive/dysfunction occurs in association with unresolved inflammation (8, 12–14). Under physiological conditions, regulated expression of two EC effector proteins, angiopoietin (Ang)-1 and Ang-2, mediates blood vessel formation, remodeling, and EC interaction with inflammatory cells (15–17). Ang-1 and Ang-2 share binding affinity for a tyrosine kinase receptor, Tie2, expressed primarily on ECs (18, 19). Ang-1, produced by pericytes, smooth muscle cells, and fibroblasts, is found on the extracellular matrix. Ang-1/Tie2 binding promotes vessel integrity and downstream prosurvival and antiinflammatory signaling (20, 21). Ang-2 is stored preformed in EC Weibel-Palade bodies (storage vesicles) and is rapidly released on activation. Unlike Ang-1, binding of Ang-2 plays a role in vascular remodeling and vessel permeability (22–25). The development of mice overexpressing (16, 22) or deficient (20, 26) in Ang-1 or Ang-2 has enabled a closer examination of the relationship between these two proteins and their regulation of EC function, and Ang-1 cell therapy/overexpression has been shown to decrease LPS-induced (direct) ALI in rodents (27). How these proteins mediate or are affected by EC/neutrophil interactions *in vivo* is not known. Recent clinical findings have reported elevated EC growth factor, Ang-2, in the plasma from patients who develop ALI/inflammatory lung injury (8, 28). In our preliminary experiments, we have observed similarly elevated Ang-2 in plasma from critically ill trauma and surgical intensive care unit patients at Rhode Island Hospital and plasma and lung tissue in the local and systemic inflammatory environment produced in our shock/sepsis model for the development of iALI in mice. We have

Table 1: Demographic and Clinical Data of 12 Surgical or Trauma Intensive Care Unit Patients* with Critical Illness Due to Sepsis or Trauma

	ARDS PaO ₂ :FiO ₂ < 300 (n = 6)	No ARDS PaO ₂ :FiO ₂ > 300 (n = 6)	P Value
Age	54.3 ± 8.6	59.7 ± 6.7	0.64
Female, %	66	33	
Mean PaO ₂ /FiO ₂	215.6 ± 32.2	415 ± 30.7 [†]	0.015
Plasma Ang-2, ng/ml	4846.2 ± 1,293.3	3059.5 ± 1,235.9 [†]	0.042
Apache II	20.6 ± 1.7	17.8 ± 2.7	0.17

Definition of abbreviations: Ang-2, angiopoietin-2; ARDS, acute respiratory distress syndrome.

*The patient sample consisted of six patients with ARDS and six patients without ARDS. There was no difference between patients with ARDS and patients without ARDS regarding age (54.3 ± 8.6 vs. 59.7 ± 6.7 yr; *P* = 0.64) or APACHE II score (20.6 ± 1.7 vs. 17.8 ± 2.7; *P* = 0.17). Patients with ARDS had an average PaO₂/FiO₂ ratio of 215.6 ± 32.3. Compared with no ARDS, patients with acute lung injury had significantly higher plasma Ang-2 levels (4,846.2 ± 1,293.3 ng/ml vs. 3,059.5 ± 1,235.9 ng/ml; *P* = 0.042).

[†]*P* < 0.05 vs. PaO₂ > 300 (no ARDS).

previously shown that indices of inflammation and lung injury are significantly decreased after antibody depletion of peripheral blood neutrophils before shock (7). Together, these findings point to a yet to be explained relationship between EC growth factor, Ang-2, and possible neutrophil/EC interaction.

To investigate this, we tested the hypothesis that Ang-2 promotes the development of experimental iALI after the sequential insults of hemorrhagic shock and sepsis and that this dysfunction is associated with EC interaction with activated (shock-primed) neutrophils.

Materials and Methods

A detailed description of the methods is provided in the online supplement.

Male C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME), 7 to 9 weeks of age, were used in all experiments. Experiments were performed in accordance with National Institutes of Health guidelines and with approval from the Animal Use Committee of Rhode Island Hospital.

Human Blood

Patients who were admitted to the trauma and surgical intensive care units between June 2009 and May 2010 were enrolled in this study after obtaining direct consent or consent of their surrogate. Charts were reviewed for calculation of the APACHE II (Acute Physiology and Chronic Health Evaluation II) scores at the time of the blood draw. Criteria established by Bernard and colleagues (29) were used for the diagnosis of ARDS. Blood samples were deidentified immediately after collection. This study was

Figure 1. Angiopoietin (Ang)-2 in plasma (A) and lung tissue homogenates (B) significantly increased in mice subjected to hemorrhage and cecal ligation and puncture (Hem/CLP) compared with naive control mice. This increase was observed to a lesser extent in lung tissue homogenates (B) from mice that received a sham hemorrhage procedure (SHem). In Hem/CLP mice, plasma (C) and lung tissue homogenate (D) levels of Ang-1 significantly decreased (*n* = 5–6). **P* < 0.05 versus Hem/CLP. N.D. = not determined.

approved by Rhode Island Hospital (IRB#0132-08).

Reagents

Ang-2 short interference RNA (siRNA) constructs were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The DOTAP liposomal delivery vehicle was purchased from Roche Applied Science (Indianapolis, IN). The EasySep magnetic bead biotin selection kit for EC isolation was purchased from StemCell Technologies (Vancouver, Canada). EC culture media, MCDB-131, was purchased from VEC Technologies (Rensselaer, NY). Antibodies for Ang-1 and Ang-2 Western assays were purchased from Abcam (Cambridge, MA), and ELISA kits were purchased from BlueGene (St. Petersburg, FL). The mouse inflammation cytokine kit, the human Ang-2 kit, and anti-CD31 antibody were purchased from BD Biosciences (San Diego, CA). All other chemicals were analytical reagent grade and were purchased from Sigma Chemical (St. Louis, MO).

Model of iALI

Hemorrhage. The nonlethal, 90-minute, fix-pressure (35 ± 5 mm Hg) hemorrhage model used for these experiments has been previously described (6).

Polymicrobial sepsis/cecal ligation and puncture. Twenty-four hours after hemorrhage (or sham hemorrhage), sepsis was induced as a secondary challenge via cecal ligation and puncture (CLP) as previously described (6). Lungs and plasma were harvested 24 hours after CLP (48 h after hemorrhage or sham hemorrhage) for all *in vivo* experiments described in this study.

In vivo siRNA delivery. EC specificity of siRNA uptake for the intravenous tail vein delivery route has been previously described (30). The Ang-2 siRNA construct efficacy (target sequence 5'-GCACAAAGGAUUCGGACAA-3') was validated *ex vivo* using mouse lung primary EC monolayers.

Neutrophil depletion. Mice were depleted of resident neutrophils via intraperitoneal injection of 500 μ g of rat anti-mouse neutrophil antibody, anti-Gr1 (clone RB6-8C5, rat IgG2b), per mouse 48 hours before hemorrhage as previously described (6, 7).

Enzymatic lung digest. Lung tissue cells were isolated from PBS-perfused whole mouse lungs as previously described (6, 7).

Lung EC isolation/culture. A series of positive selections using magnetic bead antibody conjugates (CD31/CD144) (EasySep; StemCell Technologies) were used to isolate pulmonary vascular ECs (PVECs) following the manufacturer's instructions. After positive selection, cells were resuspended in EC complete media, counted using Trypan Blue Exclusion reagent, and cultured for monolayer at 37°C at 5% CO₂.

Neutrophil isolation from peripheral blood. Neutrophil isolation was performed as previously described (6).

In vitro EC/neutrophil coculture. Mouse PVEC monolayers were coincubated in Costar (Cambridge, MA) 6-well tissue culture plates (direct contact) or separated using transwell filter chambers (indirect, TransW), with PMNs isolated from peripheral blood of mice 24 hours after hemorrhage (Hem), with PMNs from naive mice, or with TNF- α (positive) or media only (negative) control mice for 4 hours. Ang-2 protein was measured in the

supernatant. Other laboratories have previously published examples of this methodology (31–33).

Cytokine assays on lung cell/tissue lysates and supernatants/plasma samples were performed as per the manufacturer's protocol.

Bronchoalveolar lavage fluid was collected to assess protein concentration as an index of pulmonary vascular permeability (injury) as previously described (5).

Western blots for Ang-1 and Ang-2 in lung lysates were processed as previously described (34–36). Integrated density value comparison (IDV Ang-2/IDV actin [control]) was performed for quantitative analysis.

Statistics

Data are expressed as means $\pm$ SEM. Statistical significant differences were determined using one-way ANOVA. Dunn's method was used in the analysis of human Ang-2. Group means were

Figure 2. Intravenous delivery of Ang-2 siRNA 2 hours after hemorrhage significantly reduced levels of Ang-2 in plasma (A) and lung tissue homogenates (B) in Hem/CLP mice compared with Hem/CLP mice that received control (Ctrl) short interference RNA (siRNA). This suppression of Ang-2 protein synthesis in siRNA-treated Hem/CLP mice was confirmed by Western blot analysis (C) and summary integrated density value (IDV) comparisons (D) of lung tissue homogenates ($n = 4-7$). [#] $P < 0.05$ versus SHem/CLP. ^{*} $P < 0.05$ versus Hem/CLP Ctrl siRNA.

considered significantly different at $P < 0.05$ using SigmaStat for Windows version 2.03.

Results

Ang-2 Is Significantly Elevated in Blood from Patients with ARDS

To initiate our investigation into the role endothelial growth factor, Ang-2, in the development of ARDS/iALI, blood was obtained from critically ill trauma or surgical intensive care unit patients at Rhode Island Hospital, Providence, Rhode Island. A $\text{PaO}_2/\text{FiO}_2$ ratio of less than 300 was used as an index of pulmonary vascular dysfunction associated with patients with ARDS. Our data show that patients with ARDS have significantly elevated Ang-2 compared with those with no ARDS (Table 1). These findings are in agreement with data reported by other investigators (8, 28).

Ang-2 Is Significantly Elevated in Shocked/Septic Mice

Our first experiment was to assess Ang-1 and Ang-2 expressions in our model. We found that, similar to patient findings, plasma (Figure 1A) and lung tissue (Figure 1B) Ang-2 were significantly elevated in our mouse model of hemorrhage priming for the development of iALI after subsequent septic shock (Hem/CLP). This increase in tissue Ang-2 was not observed in mice that were subjected to a sham hemorrhage procedure before CLP (Figures 1A and 1B). In contrast, Ang-1 plasma (Figure 1C) and lung tissue (Figure 1D) levels decreased significantly in Hem/CLP mice when compared with control mice. These data show that hemorrhagic shock serves as a nonlethal priming stimulus in our model not only for the development of iALI after subsequent septic challenge but also for increased release of Ang-2.

Elevated Ang-2 Plays a Role in the Ongoing Inflammatory Process Leading to iALI in Our Model

To assess the impact of up-regulated Ang-2 in the development of iALI after Hem/CLP, liposomal encapsulated Ang-2 siRNA was administered via intravenous tail vein injection 2 hours after hemorrhage to suppress *de novo* synthesis of Ang-2. We have previously shown, using

flowcytometric analysis and fluorescent microscopy, the selective uptake of liposomal-encapsulated Alexa-647-labeled Ang-2 siRNA by CD31+ (ECs) and a small percentage of tissue macrophages when delivered via intravenous tail vein injection (30); this route of delivery/specific targeting has been described by other laboratories (37, 38). Our results show that plasma (Figure 2A) and lung tissue (Figure 2B) Ang-2 decreased significantly in siRNA-treated Hem/CLP mice when compared with Hem/CLP mice that received control siRNA. Suppression of Ang-2 protein after siRNA treatment in Hem/CLP mice was confirmed by Western blot analysis (Figure 2C). Cytokine analysis of plasma (Figures 3A–3D) and lung lysates (Figures 3E–3H) demonstrates reduced levels of IL-6, TNF- α , and monocyte chemotactic protein-1 in Hem/CLP mice after Ang-2 protein suppression. Although plasma IL-10 levels were reduced after Ang-2 siRNA

treatment (Figure 3D), lung tissue IL-10 (Figure 3H) was not affected. These findings show that suppression of *de novo* Ang-2 synthesis results in decreased indices of iALI.

Suppression of Ang-2 Reduces Indices of Inflammatory Lung Injury

Consistent with the reduced inflammatory environment in our Ang-2 siRNA-treated mice, lung tissue histology typically showed less disruption of lung tissue architecture, septal thickening (Figure 4B), and cellular exudate (Figure 4D) after siRNA suppression of Ang-2 in Hem/CLP mice. In addition, as confirmatory physiological index of pulmonary vascular injury, assessment of bronchoalveolar lavage fluid protein showed a significant reduction in protein leakage in Hem/CLP mice that received Ang-2 siRNA (Figure 4E).

To further investigate the cellular interactions involved in Ang-2-mediated,

Figure 3. Intravenous delivery of Ang-2 siRNA 2 hours after hemorrhage significantly reduced plasma cytokines IL-6 (A), monocyte chemotactic protein (MCP)-1 (B), TNF- α (C), and IL-10 (D). A similar decrease was observed for IL-6 (E), TNF- α (F), and MCP-1 (G) in lung tissue homogenates ($n = 4-7$). * $P < 0.05$ versus SHem/CLP. * $P < 0.05$ versus Hem/CLP Ctrl siRNA.

Figure 4. Representative hematoxylin and eosin-stained lung tissue sections from Ang-2 siRNA-treated Hem/CLP mice show decreased septal thickening (B) and decreased cellular infiltrate (D) when compared with lung tissue histology from Ctrl siRNA Hem/CLP (A and C). Assessment of bronchoalveolar lavage fluid protein (E) showed a significant reduction in protein leakage in Hem/CLP mice that received Ang-2 siRNA ($n = 5-7$ mice/group). * $P < 0.05$ versus Hem/CLP Ctrl siRNA.

EC-associated lung inflammatory injury, we assessed the role of shock-primed neutrophils. Neutrophils are early responders in the inflammatory process, and EC interactions with these activated peripheral blood neutrophils could play a role in mediating EC Ang-2 release. We have previously demonstrated that the presence of shock-primed neutrophils is associated with the inflammatory lung injury observed in our model and in previous studies have shown that neutrophil depletion reduces indices of inflammation and lung tissue injury (7). On the basis of these findings, we investigated the impact of EC/neutrophil interaction on Ang-2 release.

Depletion of Peripheral Blood Neutrophils Significantly Reduces Plasma/Lung Ang-2

To assess neutrophil-associated EC Ang-2 release, anti-Gr1 antibody was used to deplete peripheral blood neutrophils before hemorrhage. Mice depleted of neutrophils (Gr1 ab Dep) showed significantly reduced plasma (Figure 5A) and lung tissue (Figure 5B) Ang-2 when compared with nondepleted (IgG Ctrl) Hem/CLP mice. Protein levels of Ang-1, the antiinflammatory/prosurvival mediating competitor of Ang-2 for Tie2 binding, were elevated in lung tissue from neutrophil-depleted mice as compared with nondepleted (IgG Ctrl) Hem/CLP mice (Figure 5C).

Having observed a decrease in Ang-2 in the absence of EC/neutrophil interactions, we determined whether direct EC/neutrophil interaction or the cytokine/chemokine environment was associated with the presence of shock-primed neutrophils mediating Ang-2 release.

Neutrophil/EC Interaction Mediates EC Activation/Ang-2 Release

In vitro coculture experiments were set up to determine the nature of neutrophils' role in mediating EC release of Ang-2. *In vitro* experiments show that 4-hours EC/neutrophil direct coculture, in contrast to Transwell/indirect stimulation, significantly increased Ang-2 release from EC monolayers (Figure 6). These experiments demonstrate that direct EC/neutrophil interaction is an important contributor to the modulation/release of Ang-2 from ECs.

Discussion

ECs modulate the propagation of the inflammatory response via interaction with proinflammatory mediators in the systemic environment. However, in the pathogenesis of ARDS/iALI, EC dysfunction and loss of pulmonary vascular barrier integrity can also contribute to inflammatory lung injury and organ failure. EC responsiveness to stimulus is mediated, in part, by angiopoietins, which are EC growth factors. Forming the Ang-Tie2 receptor system, Ang-1 and Ang-2 regulate vasculogenesis, angiogenesis, and migration and adhesion of immune cells. Ang-1 is constitutively expressed by pericytes, smooth muscle cells, and fibroblasts and maintains vascular homeostasis by acting as an antiinflammatory agent and promoting EC survival (19–21). In contrast, Ang-2 functions in an autocrine manner, enabling ECs to respond rapidly to local/systemic environmental stimuli and initiating changes that disrupt EC junction proteins leading to increased vascular permeability (22–25). Modulated expression of these two proteins provides a balance between vascular integrity and a responsive endothelium.

Gale and colleagues (19) and Thurston and colleagues (39) have previously documented the significance of Ang-Tie2 signaling in loss-of-function experiments. However, our findings of elevated Ang-2 in plasma from critically ill trauma and

Figure 5. Antibody depletion of peripheral blood neutrophils before hemorrhage significantly reduced Ang-2 protein in plasma (A) of Hem/CLP mice compared with IgG control treatment. However, a greater decrease in the levels of Ang-2 was observed in lung tissue homogenates (B) from the neutrophil depleted Hem/CLP mice. Coincident with decreased Ang-2, Western blot analysis showed increased expression of Ang-1 in lung tissue homogenates from neutrophil-depleted compared with nondepleted Hem/CLP mice (C). $^*P < 0.05$ versus Hem/CLP IgG control mice in lung tissue and plasma.

surgical ICU patients with ARDS (previously described as ALI) and similar recent findings from other laboratories (8, 28) have focused our investigation on the role of EC growth factor Ang-2 in mediating EC loss of function in the development of iALI in mice.

In this study we used a model of hemorrhagic shock followed by infectious challenge (sepsis) to produce iALI in mice. In this model, hemorrhage serves as a nonlethal priming stimulus, that, when followed 24 hours with septic challenge, produces an inflammatory response and lung injury similar to that seen in critically injured patients with ARDS (6). We

hypothesized that Ang-2, released by activated pulmonary ECs, promotes the development of iALI after the sequential insults of hemorrhagic shock and sepsis, and on the basis of our previous work in this model, that EC interaction with activated (shock primed) neutrophils serves to mediate Ang-2 associated EC dysfunction.

To validate the efficacy of our model for this study, we assessed whether hemorrhagic shock priming and subsequent septic challenge affect systemic and lung tissue levels of Ang-1 and Ang-2. Experiments by Ganta and colleagues report decreased pathology in an Ang-2-deficient mouse

model of colitis (40). Also, Nag and colleagues suggest a role for Ang-2 in the breakdown of the blood/brain barrier and EC apoptosis in a rat cortical cold injury model (41). We have previously shown that hemorrhage primes the immune responsiveness of mice such that subsequent infectious challenge elicits significantly elevated cytokine and chemokine responses in the plasma and lung tissue and lung tissue injury (6, 7).

As anticipated, Ang-2 levels significantly increased in our Hem/CLP mice, with plasma Ang-2 elevation similar to that observed in patients who develop ARDS. Ang-1 and Ang-2 in plasma and lung tissue showed a reciprocal change on both Western blot (not shown) and ELISA assays. This may be a result of the unresolved inflammatory state. Initially, EC response to inflammatory mediators, including Ang-2, increases adhesion molecule expression (42) and disrupts EC cell-cell adhesion (39), which targets immune cells to tissue beds. A mechanism for the reciprocal decrease in Ang-1 expression has recently been proposed by Kayakabe and colleagues (43), who observed suppression of synovial fibroblast Ang-1 production in the presence of IL-6 when cocultured with human umbilical vein ECs (43). The prolonged up-regulation of local/systemic IL-6 after Hem/CLP and a dysregulated inflammatory response-mediated EC Ang-2 release accounts for the changes in Ang-1/Ang-2 we observe in our model.

Having shown that Ang-2 is up-regulated in our model, we determined whether Ang-2 plays a role in the ongoing inflammatory response leading to iALI. To do this, we used intravenous delivery of liposomal encapsulated siRNA against Ang-2 to selectively and transiently suppress Ang-2 *de novo* synthesis by ECs. Although this route of siRNA delivery theoretically suppresses Ang-2 *de novo* synthesis by ECs throughout the vasculature, the sensitivity of the lung to systemic/vascular inflammation makes it a valuable model for studying the significance of EC Ang-2 release as it relates to our model of iALI. The significant reduction of indices of iALI in our model after systemic suppression of Ang-2 in the general population of vascular ECs points to Ang-2 as a significant mediator of EC dysfunction in our model for iALI.

Activated neutrophils from the peripheral blood, as early responders to sites

Figure 6. Neutrophils isolated from hemorrhaged mice significantly increase Ang-2 release from endothelial cell monolayers after direct cell versus indirect (Transwell) interaction. This was also observed, but to a lesser extent, from neutrophils isolated from Sham hemorrhaged (SHem) mice. TNF- α was used as a positive control, and media alone was used for negative control ($n = 6$). $^*P < 0.05$ versus Direct in treatment group. $^{\#}P < 0.05$ versus Direct Hem.

of inflammation, are among the initial cells interacting with the pulmonary vascular endothelium. We have previously found that depletion of peripheral blood neutrophils before hemorrhagic shock in our iALI model significantly reduces indices of inflammation and lung tissue injury after septic challenge (7). To the extent that early immune cell interactions modulate EC activation, we investigated the role of neutrophils (early responders to inflammatory mediators/chemokines released into systemic environment) in mediating EC Ang-2 release. Our experiments showed that, in the absence of activated peripheral blood neutrophils, Ang-2 in the plasma and lung tissue was significantly reduced. This was observed in concert with an increase in Ang-1 in lung tissue. These data are consistent with our previous findings of reduced cytokines, chemokines, and lung tissue injury in neutrophil depleted mice after Hem/CLP (7).

Although these findings point to a role for neutrophils in modulating EC release of

Ang-2, what is not clear from this experiment is whether it is the absence of EC/neutrophil interactions or the suppression of inflammation in the local/systemic environment in neutrophil-depleted mice that affects EC Ang-2 release. We addressed this question by coculturing PVEC monolayers with hemorrhage-primed neutrophils. In these experiments, the nature of neutrophil stimulation was controlled using direct versus indirect coculture methods. This system uses a semipermeable membrane (0.4 μ m pore) that prevents cell-to-cell contact yet allows for the exchange of soluble mediators. This designed, which allows for an assessment of the impact of soluble mediators, has previously been described by Su and colleagues, who investigated the effect of T-regulatory cells on the function of mast cells (33), and by Wang and colleagues, who examined the inhibition of T-cell proliferation by mediators released by lung epithelial cells (32). The release of Ang-2 from PVEC monolayers indicates that only

through direct EC/neutrophil interaction was Ang-2 significantly increased in assay supernatant. These findings suggest that cell-cell/adhesion molecule interactions play a role in EC dysfunction by potentiating Ang-2 release in our model.

In this study we have illuminated changes in the expression of Ang-1/Ang-2 associated with EC/neutrophil interactions that support our hypothesis that Ang-2 promotes the development of iALI in our model and that EC/neutrophil interactions play a role in the loss of EC barrier function that we see in our Hem/CLP model for the development of iALI. These data suggest that Ang-2 has both a role and is a potential therapeutic target in the treatment of ARDS. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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