HHS Public Access

Author manuscript

*Shock*. Author manuscript; available in PMC 2023 April 01.

Published in final edited form as:

*Shock*. 2022 April 01; 57(4): 608–615. doi:10.1097/SHK.0000000000001902.

# Patho-mechanisms for hemorrhage/sepsis-induced indirect acute respiratory distress syndrome (iARDS): A role for lung Tie1 and its regulation by neutrophils

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## Abstract

**Introduction:** Severe hemorrhage (Hem) has been shown to be causal for the development of extra-pulmonary/indirect acute respiratory distress syndrome (iARDS) and is associated with severe endothelial cell (EC) injury. EC growth factors, Angiopoietin (Ang)-1 and -2, maintain vascular homeostasis via tightly regulated competitive interaction with the tyrosine kinase receptor, Tie2, expressed on ECs.

**Objective:** Since it has been reported that the orphan receptor, Tie1, may be able to play a role in Ang:Tie2 signaling; we chose to examine Tie1's capacity to alter the lung Ang:Tie2 interaction in response to the sequential insults of shock/sepsis (cecal ligation and puncture [CLP]), culminating in iARDS.

**Methods:** Male mice were subjected to Hem alone or sequential Hem followed 24 hours late by CLP that induces iARDS. Changes in lung and/or plasma levels of Tie1, Tie2, Ang-1, Ang-2, various systemic cytokine/chemokines and indices of lung injury/inflammation were then determined. The role of Tie1 was established by intravenous administration of Tie1 specific or control siRNA at 1 hour post-Hem. Alternatively, the contribution of neutrophils was assessed by pre-treating mice with anti-neutrophil antibody depletion 48 hours prior to Hem.

**Results:** Lung tissue levels of Tie1 expression elevated over the 1$^{st}$ 6–24 hours post-Hem alone. Subsequently, we found that treatment of Hem/CLP mice with Tie1-specific siRNA not only decreased Tie1 expression in lung tissue compared to control siRNA, but, suppressed the rise in lung inflammatory cytokines, lung MPO and the rise in lung protein leak. Finally, much as we have previously shown that neutrophil interaction with resident pulmonary vascular ECs contribute significantly to Ang-2 release and EC dysfunction, central to the development of iARDS. Here we

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Author contributions: JZ, C-SC & JLL-N helped conduct the study; JZ, JL, C-SC, JLL-N & AA helped design this study; JZ, C-SC, JLL-N & AA helped analyze the data; and, JZ, C-SC, JLL-N & AA write the manuscript. All authors read and approved of the final manuscript.

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The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

report that depletion of neutrophils also decreased lung tissue Tie1 expression and increased Tie2 activation in Hem/CLP mice.

**Conclusion:** Together, these data imply that shock-induced increased expression of Tie1 can contribute to EC activation by inhibiting Ang:Tie2 interaction, culminating in EC dysfunction and the development of iARDS.

### Keywords

Tie1; Tie2; angiopoietin (Ang)-1 & -2; acute lung injury; hemorrhagic shock; sepsis; mice

## INTRODUCTION

Severe hemorrhage/hemorrhagic shock (Hem), a common component of severe traumatic injury, has been shown to be causal for the development of extra-pulmonary/indirect (i) acute respiratory distress syndrome (ARDS) and is associated with severe endothelial cell (EC) injury (1,2). Since increased microvascular permeability is a hallmark of iARDS, understanding the mechanisms for loss of EC barrier function is critical in the development and delivery of potential therapies. Endothelial cells are important mediators of tissue/organ fluid homeostasis, immunity and inflammation. They form a tight barrier between the pulmonary circulation and lung parenchyma, thus, are of crucial significance to maintenance of normal gas exchange. Because of their anatomical position, pulmonary ECs are the first cells to come into contact with mediators from the systemic circulation. These interactions serve to activate the endothelium (regulating vascular barrier function), up-regulating the expression of lung EC surface proteins, such as ICAM-1, tyrosine kinase receptor 2 (Tie2), etc., as well as stimulating growth factor production (3–5). Relative to endothelial growth factors, Angiopoietins-1 and -2 mediate EC activation through competitive binding to their shared receptor, Tie2 (6,7). Angiopoietin-1 binding induces Tie2 phosphorylation (P-Tie2) and signaling for downstream anti-inflammatory, anti-apoptotic protein synthesis and sustained vessel barrier integrity (8). Alternatively, Ang-2:Tie2 binding promotes increased pro-inflammatory signaling and decreased barrier integrity/increasing lung leak (9,10).

Angiopoietin-2 is significantly elevated in plasma from patients with ARDS and in our murine model of Hem induced-priming for the development of iARDS when followed by a subsequent septic challenge (11–14). We have demonstrated that EC interaction with hemorrhage-primed neutrophils (15,16) contributes to EC activation and Ang-2 release, while depletion of peripheral blood neutrophils prior to hemorrhage/sepsis restores Ang-1 production and Tie2 phosphorylation (13). Recent publications suggest that the orphan receptor, Tie1, expressed on ECs, may also play a role in modulating EC activation/function by forming a complex with Tie2 (17–19). This complex of Tie1:Tie2 is proposed to inhibit Ang-1 binding of/interaction with Tie2 (binding of/interaction with Tie2 leading to its phosphorylation [activation] typically is thought to promote EC quiescent/survival/anti-inflammatory phenotype), thus, contributing to an activated/pro-inflammatory EC phenotype (20). However, whether the orphan receptor, Tie1, plays a role in the vascular changes that occur in response to insults associated with iARDS is unknown. To begin to investigate this, we tested the hypothesis that Ang:Tie2 interactions are altered by the increased expression Tie1 following Hem, that this altered state is a result of EC interaction with activated

(shock-primed) PMN and that this change in Tie1 expression/interaction contributes to the development of iARDS.

## MATERIALS AND METHODS

### Animals and Groups:

Male C57BL/6 mice from Jackson Laboratory (Bar Harbor, ME) were utilized at 8–12 weeks old. This choice was made so as to maximize our ability to initially see an experimental difference in the iARDS response based on previous reports that male mice did poorer in response to these experimental stressors of shock (hemorrhage) and/or septic (CLP) challenge than pro-estrus stratified female (21,22). Animals obtained from our outside vendor were acclimated no less than 7 days, and often longer [maximum ~5 weeks], prior to utilizing these animals in studies done here. During this period they were housed in the Rhode Island Hospital (RIH) rodent facility (12-hours: 12-hours light/dark cycle, 23–25°C, 30–70% humidity) where they received standard care and diet (Standard rodent chow)/water *ad libitum*. All protocols were carried out in the morning (8–11AM) and were performed in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital (AWC# 0079-13 & 0040-16).

For the intial study, examining the impact of shock on lung Tie1, Tie2 and phosphorylated Tie-2 levels, mice were randomly separated into two experimental groups: 1) subjected to sham Hem + resuscitation (sham) and 2) to Hem + resuscitation (Hem), along with a group of unmanioulated (Naïve) animals to serve as a baseline comparator.

Subsequently, animals where randomly divided into three groups of animals each: 1) the control group (Sham): mice underwent sham Hem and sham CLP, surgical procedure with cecal exposure, however, the cecum was neither ligated nor punctured; 2) the Hem/CLP group: mice underwent hemorrhage and followed CLP + control siRNA treatment (Cont siRNA + Hem/CLP) (delivered intravenous 1 hour after Hem resuscitation); and 3) the Hem/CLP + Tie1 specific siRNA treatment group (Tie1 siRNA + Hem/CLP); to assess the contribution of Tie1 gene expression on changes in lung Tie2 activation/phosphorylation, Ang-1/-2 levels, inflammatory mediator burden, neutrophil influx and lung permeability changes.

Finally, to consider the possible contribution of neutrophil influx to the pulmonary expression of Tie1 in response to Hem/CLP, a small number of mice were randomly separated to receive either 1) intravenous tail vein delivery of rat anti-mouse neutrophil antibody (Gr1 ab+ Hem/CLP) or 2) control IgG (Cont ab+ Hem/CLP) 48 hours prior to subjecting to Hem/CLP. A time frame that we have previously shown leads to marked depletion, ~95%, of the circulating peripheral blood neutrophil numbers (15,16).

### Mouse model of iARDS (Hem/CLP):

Hemorrhagic shock (Hem; non-lethal, fixed pressure model [~40 mmHg for 90 minutes] [circulatory access obtained via bilateral femoral arterial catheterization under isoflurane anesthesia], resuscitated with 4X shed blood volume [typically 40%] in Lactated Ringers Solution) followed by a septic challenge-cecal ligation and puncture (CLP; dual-punture

with a 21 gauge needle following laparotomy under isoflurane anesthesia, resuscitated with 1 ml of Lactated Ringers Solution) 24 hours later was utilized to induce iARDS, as previously described (13,15). Using this model we have shown induces arterial blood $PO_2/FIO_2$ of ~150 mmHg by 24 hours-post Hem-CLP, along with evidence of protein leak, edema, morphological changes, increase lung tissue levels of pro-inflammatory cytokines/chemokines and neutrophil influx into the lungs (as assessed by immune-histochemistry & tissue myeloperoxidase [MPO] levels) (13,15).

### *In vivo* siRNA delivery:

Mice received siRNA in a liposome encapsulated preparation (DOTAP, Roche Applied Science, Indianapolis, IN) at 1 hour following shock-resuscitation via intravenous tail vein delivery (13,23). Mouse Tie1 siRNA-SMART pool sequences: 1. 5'-UGAGCAUGAACUAAUAUGU-3'; 2. 5'- GUAUCAGCUCUCAGAUUUC-3'; 3. 5'-UCUGUAGACACCAUUUAUA-3'; 4. 5'-CAUGGUGUUGGAUUGGUG-3' and scrambled (Control) sequence were from Dharmacon (Lafayette, CO). EC specificity of siRNA uptake for the intravenous delivery route has been previously described (23,24). The Tie1 siRNA construct efficacy was validated *ex vivo* using mouse lung primary EC monolayers.

### Neutrophil depletion.

Mice were depleted of resident neutrophils via intraperitoneal injection of 500 mg of rat anti-mouse neutrophil antibody, anti-Gr1 (clone RB6–8C5, rat IgG2b, Cat #: BE0075 from BioXcell, Lebannon, NH), per mouse 48 hours before hemorrhage as previously described (16,25).

### Sample acquisition:

Blood/plasma, bronchoalveolar lavage fluids (BALF) and lung tissues were collected either at various times post-Hem alone (6–24 hours) or at 24 hours post-CLP (when sequential Hem/CLP model was performed to assess Tie1/Tie2/P-Tie2, Ang-1/Ang-2, cytokine/chemokine, protein concentration (as a measurement for pulmonary vascular leak) and lung MPO as described previously (13,15,23,26–28).

### Quantification of Tie1/Tie2/P-Tie2, Ang-1/Ang-2, cytokines and chemokines:

Mouse Tie1 (Cat #: MBS764504) and Ang-1 (Cat #: DYC2816) ELISA kit were purchased from MyBiosource, Inc, San Diego, CA. Tie2 (Cat #: MTE200) and Phosphorylated-Tie2 (P-Tie2) (Cat #: DYC2816) ELISA kits were purchased from R&D Systems, Inc, Minneapolis, MN. Ang-2 (Cat #: ab171335) ELISA kit was purchased from Abcam, Cambridge, MA. Commercial ELISA kits were also employed to measure the levels of TNF-α, IL-6, IL-10 and MCP-1 (Cat #: 431304/431414/432704, respectively Biolegend, San Diego, CA); KC and MIP-2 (Cat #: DY453/MM200, respectively from R&D Systems, Minneapolis, MN) in lung tissue homogenates and plasma (29,30).

### MPO and BALF protein determination:

MPO in lung tissue homogenates and BALF protein levels were determined according to methods described previously (13,15,27).

### Western Blot Analysis:

Whole tissue lung protein samples/ lysates (30 μg total protein in each lane) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a 10% gel and then transblotted onto polyvinylidene fluoride membranes as previously described in our laboratory (31). The protein levels of phosphorylated-Tie2 (P-Tie2), and Tie2 were determined using specific antibodies (Cat #: HAF008/AF762, respectively from R&D Systems, Minneapolis, MN). The amount of protein in the blots was quantified using a densitometer and Image Lab 6.0 software (Bio-Rad Laboratories, California, USA).

### Statistical Analysis:

Data are expressed as mean±SD and analyzed using GraphPad Prism 5. We used the nonparametric Mann-Whitney U test to evaluate *p* values when testing two subgroups. For multiple groups' analysis, intergroup comparisons were performed by the Holm-Sidak test. The level of statistical significance was set at $P < 0.05$..

## RESULTS

### Tie1 expression elevated following Hemorrhagic shock

In our initial study we sought to determine if the impact of shock itself was sufficient to alter lung tissue levels of Tie1, Tie2 and P-Tie-2. To assess this, mice were randomly separated into two groups: 1) mice were subjected to sham hemorrhage + resuscitation, 2) to hemorrhage + resuscitation. Our findings show that Tie1 expression was indeed elevated early (6 hours) and remained elevated at 24 hours following hemorrhagic shock, but not following sham Hem (Figure 1). This finding is significant since sepsis (CLP), the triggering event for the development of iARDS following Hem shock priming in our model (15,16), is induced at this 24 hour post Hem time point. In contrast, P-Tie2 was decreased at 6 hours, but restored to sham Hem level by 24 hours following Hem shock (Figure 2A–C). No change in lung tissue Tie2 protein expression was observed in any of the treatment/control groups. This data supports the assertion that hemorrhagic shock, which we have documented previously as non-lethal in nature and insufficient to produced marked aspects of ARDS by itself (15,16), is sufficient to produce changes in both Tie1 and P-Tie2 levels/activity.

### Suppression of Tie1 expression following Hem-priming decreases markers of iARDS

We next sought to determine whether suppression of the Hem-induced rise of Tie1 gene expression could mitigate the development of full iARDS, a pathology that presents when shocked mice are subsequently subjected to a secondary insult (trigger) such as CLP (14–16). To assess this, Tie1 siRNA was administered 1 hour following Hem shock-resuscitation to suppress Tie1 gene expression. Administration of Tie1 siRNA following Hem resuscitation, but prior to the induction of sepsis (CLP), produced a marked decrease in Tie1 expression in lung tissue homogenates and Tie1 plasma levels when compared with control-siRNA treatment (Cont) (Figure 3A–B); supporting the ability of the siRNA construct to selectively suppress Tie1 protein levels here up to ~47 hours (23,24). While lung tissue Ang-2 was moderately decreased in Tie1 siRNA treated mice, neither Ang-2 or Ang-1 plasma nor Ang-1 tissue levels were significantly reduced (Figure 3A–B). Importantly, to the

extent that Tie1 expression may play a role in the development of iARDS in our model, we show that Tie1 siRNA treatment markedly attenuated the Cont+Hem/CLP-induced increase in brochoalveolar lavage fluid protein levels (BALF), an index of pulmonary vessel permeability (Figure 4A), as well as the Cont+Hem/CLP-induced increase in lung tissue MPO, a suroggate index for neutrophil influx (Figure 4B).

Interestingly, relative to the development of an inflammatory environment in the lung, we found the Cont+Hem/CLP mice exhibited a marked increase in the levels of inflammatory cytokines, IL-6 and MIP-2, but did not induce changes in tissue levels of INF-γ, TNF-α or IL-10 (Figure 5). Tie1 specific siRNA treatment did however produced a statistically significant decline in the Hem/CLP-induced increases in IL-6 and neutrophil chemotactic protein, MIP-2.

### Depletion of peripheral blood neutrophils alters Tie1:P-Tie2 expression

Finally, as we and others have previously shown through either neutrophil depletion or add-back/adoptive transfer studies (13,15,32–35), the influx and prolonged presence of neutrophils in the lung can adversely contribute the development of Hem/CLP-induced iARDS. As we have documented a change in lung MPO levels, Figure 4B, the next question was whether the presence of these granulocytes might alter the expression of Tie1, thus, contributing to a change in Tie1:Tie2 signalling. To determine this, animals where pre-treated with anti-Gr1 monoclonal antibody, a treatment we've previously documented reduces systemic blood neutrophil numbers in mice by ~95% (13,15). Data from this experiment shows that loss of $Gr1^+$ neutrophil not only significantly increases pulmonary P-Tie2, but also markedly decreases lung Tie1 expression levels (Figure 6 A–B).

## DISCUSSION

While a substantial amount is known about the interactions of the angiopoietins with their primary activating receptor on the endothelial cell, Tie2, in response to stress (20). It has been somewhat under-appreciated that the protein receptor tyrosine kinase family member, Tie1, might well also play a role in the regulation of Tie2 mediated signaling, and additionally, may also contribute to Tie2 activation in response to shock. This is in part because while Tie1 (who's expression appears to be primarily restricted to endothelial cells as well as some minor hemapoietic cell types (36,37)) was discovered/sequenced over two decades ago; it was largely documented to be crucial in embryonic development of vascular and lymphatic system as well as having roles in angiogenic vessel sprouting (36,37). Tie1 was not, however, thought to be a significant player in stress related/inflammatory vascular biology(38). Further, unlike Tie2, which is well-documented as interacting and responding to angiopoietins, specifically Ang-1 and Ang-2 (6,7,9,39,40), specific ligand(s) for Tie1 were until recently unknown (41). Despite this, several studies have indicated not only that Tie1 gene overexpression can potentiate a pro-inflammatory phenotype in endothelial cells (9,42), but its impact could potentially be the result of *cis* receptor interactions involving Tie1 and Tie2 (43,44), when Tie1 expression rises. However, whether Tie1 expression is affected by shock and/or sepsis, let alone, has a role is shock/sepsis mediated organ/lung dysfunction, was unknown.

In this respect, here we show that knock-down/silencing of Tie1 receptor gene expression *in vivo*, using intra-venous tail vein injection of Tie1 targeted siRNA encapsulated in DOTAP, a route/mode of delivery that appears to primarily alter vascular endothelial cell gene expression (13,23), not only significantly decreased the Hem and/or Hem/CLP-induced rise in lung Tie1 levels, but also attenuated the rise in lung tissue Ang-2, the increase in the pro-inflammatory mediators IL-6 and neutrophil chemotactic protein MIP-2, while attenuating the decline in Tie2 phosphorylation. Further, we observed that, relative to indices of the pathogenesis of iARDS, neutrophil influx into the lung (MPO) and lung leak (BALF:Plasma protein), were significantly decreased in mice that received Tie1 siRNA following Hem. Together, these findings support our hypothesis that Tie1 can contribute to the development of iARDS. However, how directly the Hem-induced increased expression of Tie1 culminates in/alters the activation/ phosphorylation of Tie2, can only be speculated on. Evidence for its direct activation comes from several studies (17–19) that indicate that Tie1 alters Tie2 dimerization through interrupting the binding of the Tie2 FNIII domain, which in turn alters the ability to phosphorylate Tie2 and its ability to drive downstream anti-inflammatory, pro-survival, growth factor (Ang-1) mediated signaling. The marked, although temporally transient decrease in Tie2-phosphorylation, would appear to support this assertion. The decrease in IL-6 and MIP-2 cytokine/chemokine levels in the lung tissue also provides indirect support to the actions of Tie1 on Tie2 in response to shock/sepsis and is in keeping with prior reports that suppression/silencing of Tie1 *in vitro* leads to reduced expression of various pro-inflammatory genes (9,10,42). That said; the incomplete nature of Tie1 gene suppression on these cytokines/chemokines suggest that this may not be the primary mode Tie1's actions in the process of lung injury seen here. Interestingly, Tie1 gene silencing seemed to have a much more substantial effect on the influx of neutrophils (as indicated by the suppression of MPO levels in the lung of the Hem/CLP mice treated with Tie1 siRNA). While not assessed here, it is tempting to speculate that the marked alteration in neutrophil influx produced by Tie1 siRNA treatment is related to the attenuation of the Hem/CLP-induced Tie1 mediated increase adhesion molecule expression; as increased expression of Tie1 has been reported in other models of vascular endothelial cell stress to stimulate ICAM-1, VCAM-1 and E-Selectin gene expression (9,10,42). However, whether or not this is occurring here remains to be tested.

Finally, it would appear that the induction of Tie1 seen during the response to hemorrhagic shock/sepsis is at least in part regulated by endothelial cell/neutrophil interactions as depleting mice of neutrophils decreased Tie1 levels, while increasing P-Tie2 levels in Hem/CLP mice. This is intriguing, as while it is appreciated that Tie1 appears to play a critical role in vascular remodeling and stabilization and is upregulated during wound healing (36), the actual stimuli for this is poorly characterized. While a study by McCarthy et al (45) indicates that hypoxia and VEGF, aspects of the shock/septic animal state, can induce the further expression of Tie1 in endothelial cells; how and what cells/signaling mediate the up-regulation of Tie1 remain poorly understood to this day. Also, while the observation (45) that the Tie1 protein inductive capacities of hypoxia and VEGF were independent of autocrine/paracrine secreted mediator expression during such an event/ exposure, thus, diminishing the role of components of the systemic pro-inflammatory soluble mediator response typically present in shock/septic mice/patients, it doesn't preclude

the impact of direct cell:cell interactions as our data suggest. Inasmuch, this is an intriguing area for future study.

Taken together, we would propose the following mode by which Tie1 might be working in our experimental model system (Figure 7A–C.) Typically, endothelial cell (EC) monolayer, under homeostatic conditions, is sustained by the presence of consitutively/secreted released levels of Ang-1, dervide primarily from pericytes, which the endothelial cell detects by their expression of the Tie2 receptor. Tie2 ligation by Ang-1 culminates in Tie2's phosphorylation (*, activation) and downstream signaling through AKT/PI(3)K pathway, therein, sustain cell survival/proliferation (Figure 7A). The insult of hemorrhagic shock (Hem) induces the systemic release of various inflammatory mediators as well as primes circulating neutrophils (PMN) that express increased levels of integrins that mediated their adhesion to the pulmonary vascular endothelium via ICAM-1 of the shocked animal cells (Figure 7B ❶). In such an environment this limited initial PMN: endothelial cell interaction appears to lead not only to the release of pre-formed Ang-2 form Weibel Palade bodies of the endothelial cells that competitively inhibits the Ang-1 binding of Tie2 and, thus, the Tie2's activation, but through the concomitant induction of endothelial cell 'pro-inflammatory signaling' that stimulates the upregulation (synthesis/expression) of Tie1 (Figure 7B ❷). The enhanced endothelial cell surface expression of Tie1 increases the interaction of Tie1 with Tie2, which in turn stabilizes Tie2's binding of Ang-2 (Figure 7C ❸). This further suppresses endothelial cell 'survival' signaling pathways while permitting, through default, cell 'apoptosis' and also potentiation of 'pro-inflammatory signaling' that results in not only reduced endothelial cell monolayer 'barrier function', but a further increase in PMN recruitment to the pulmonary microvascular endothelial cell (Figure 7C ❹). This points not only at a novel pathomechanism in shock meriting continued study, but elucidates potential therapeutic targets or biomarkers of treatment efficacy/ARDS resolution.

## Acknowledgments:

This work was supported by NIH R35-GM118097 (A.A.), NIH P20-GM103652 (J.L-N.) and a fellowship from the Chinese Society of Anesthesiologists (J.Z.).

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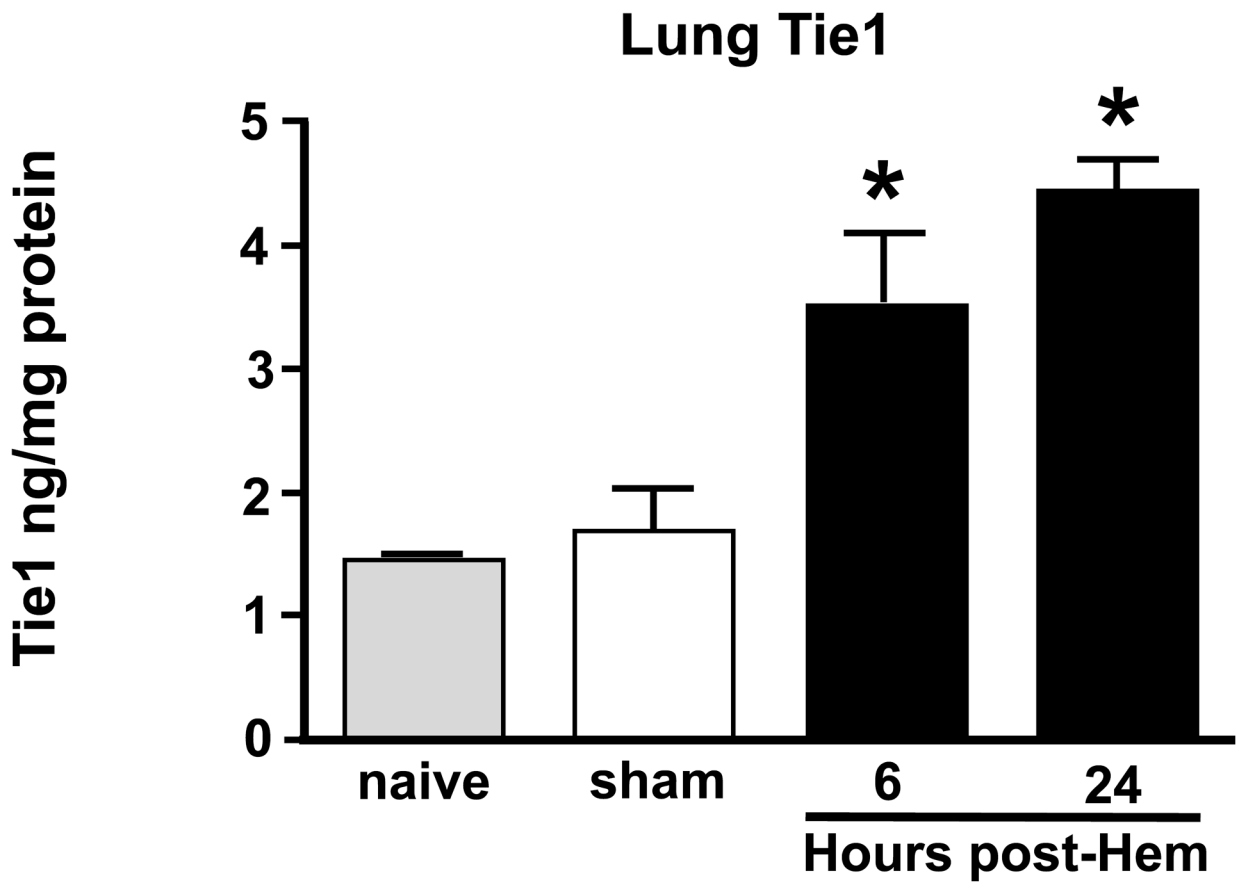


**Figure 1. Lung tissue Tie1 levels markedly increase following Hem alone.**

The lung tissue levels of Tie1, as measured by Elisa, were increased over the 1st 6–24 hours following shock with resuscitation when compared to the Sham at 6 hrs or naive animals. Data is presented as means ± standard deviation. *P<0.05, versus Sham or naïve group; n=3 mice/group.

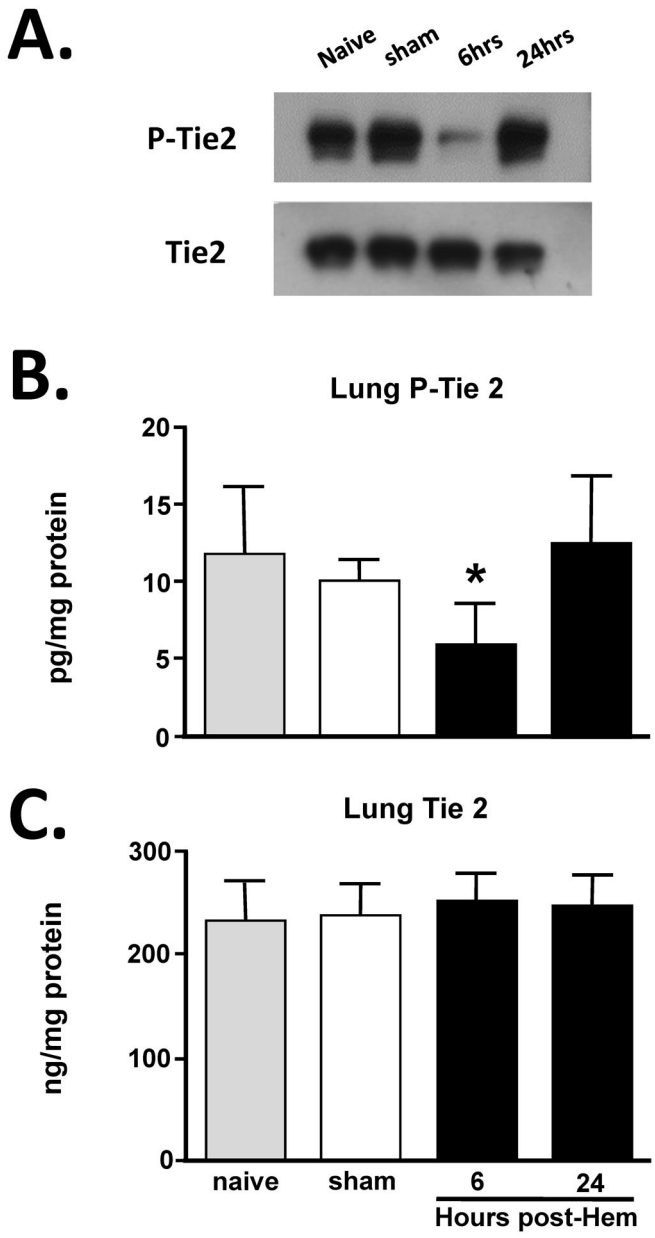


**Figure 2. Lung tissue P-Tie2, but not total Tie2, levels significantly but transiently decrease following Hem alone.**

A representative western blot of both phosphorylated-Tie2 (P-Tie2) and the unphosphorylated Tie2 (Tie2) (A.), as well as the levels of P-Tie2 (B.) and the unphosphorylated Tie2 (C.) levels as determined by ELISA. Data from cumulative samples is presented as means ± standard deviation. *P<0.05, versus Sham group; n=5–8 mice/group.

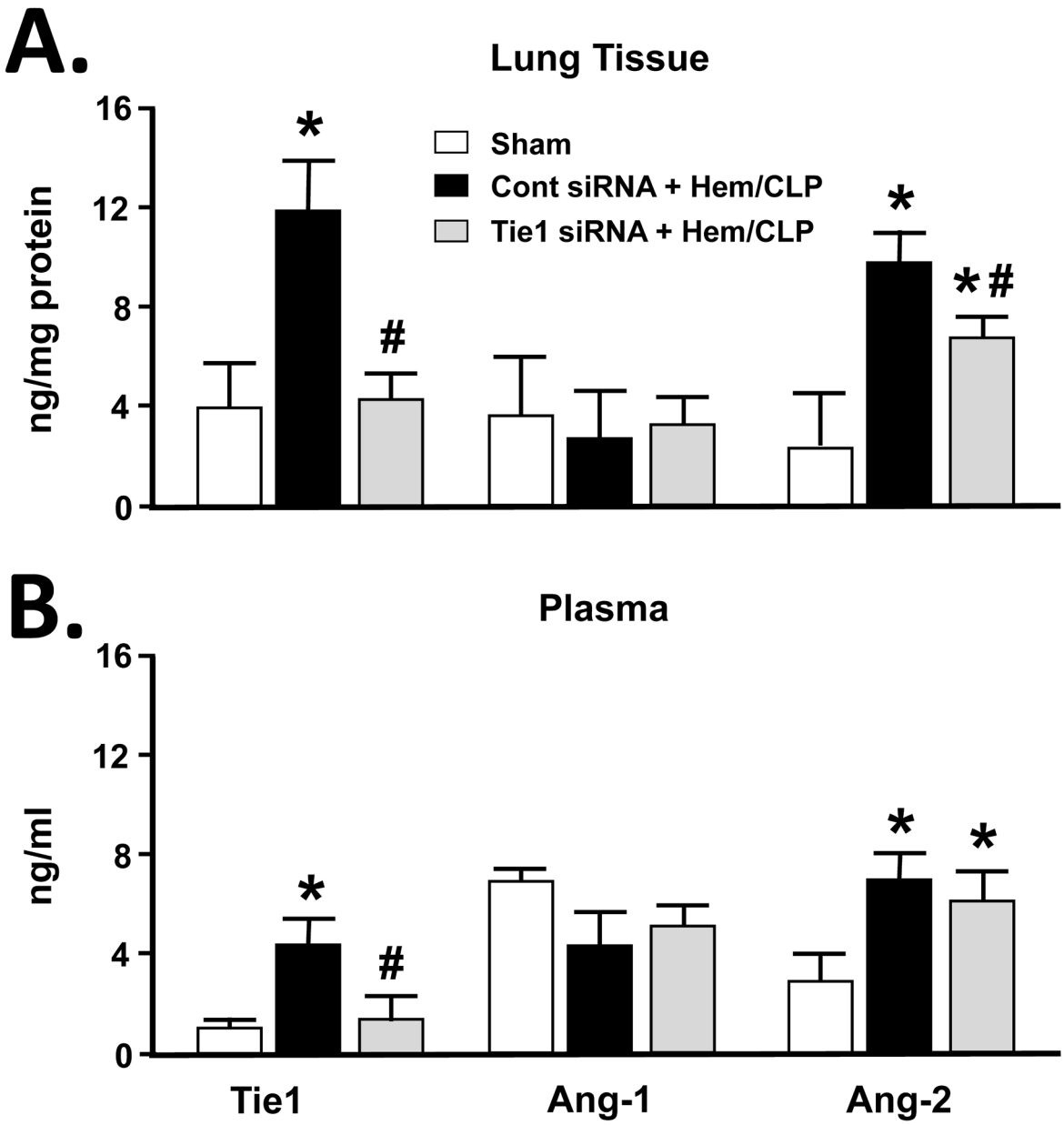


**Figure 3. Targeted Tie1 siRNA treatment markedly attenuates not only the Hem/CLP-induced rise lung/plasma Tie1 levels, but Ang-2 in mice.**

The lung tissue (A.) and plasma (B.) levels of Tie1 and Ang-2 (at 24 post-CLP) in the Control siRNA (Cont siRNA) or the Tie1 siRNA (Tie1 siRNA) treated Hem/CLP mice (siRNA administered intravenously 1 hour post-resuscitation following initial Hem), as determined by ELISA, were increased compared to the Sham animals, but not for Ang-1. However, this Hem/CLP-induced increase was markedly suppressed by Tie1 siRNA treatment for Tie1 levels and partially for lung Ang-2 levels, but not back to the concentrations seen in the Sham group. *P<0.05, versus Sham; # P<0.05, versus Cont siRNA + Hem/CLP. Data is presented as means ± standard deviation; n=4 mice/group.

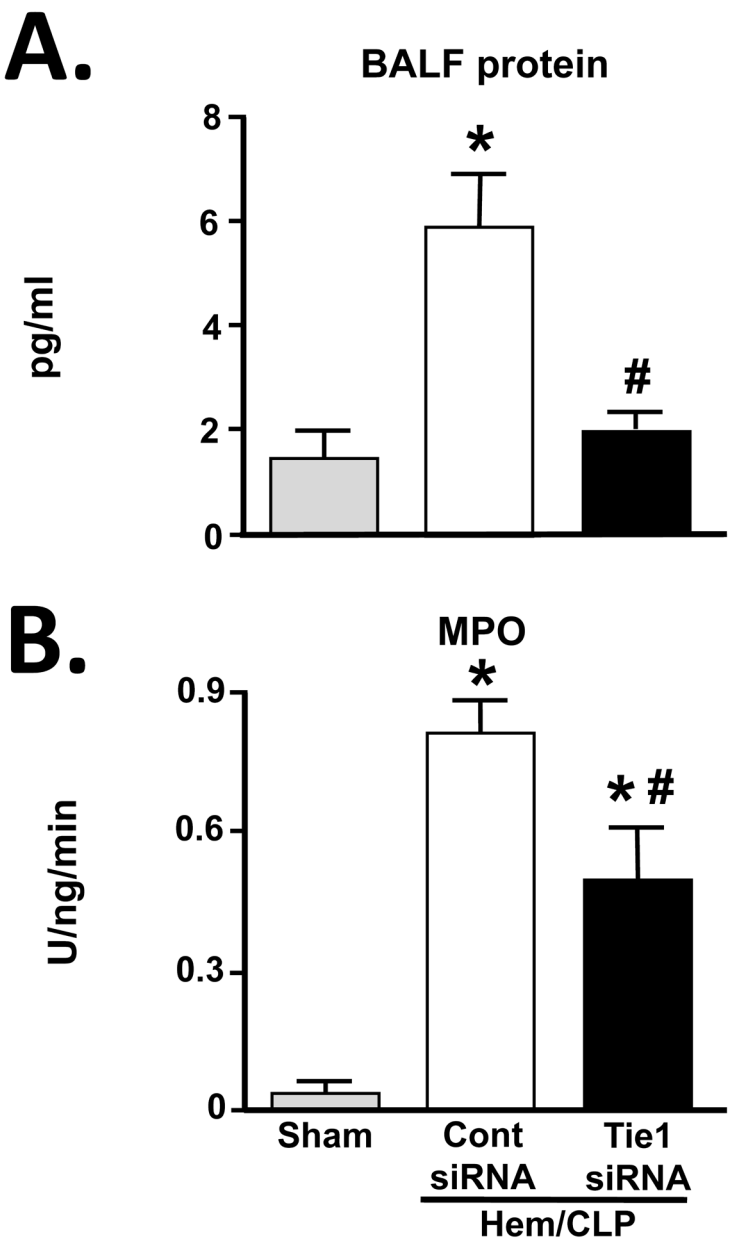


**Figure 4. Targeted Tie1 siRNA treatment markedly attenuated the Hem/CLP-induced increase lung tissue MPO levels as well as the rise in BALF protein levels in mice.**

The impact of intravenous delivery of Tie1 siRNA (Tie1) versus Control (Cont) siRNA (1 hour post-resuscitation following initial Hem) on lung protein leak/BALF protein levels (A.), as determined by protein assay, or lung tissue MPO levels (B.), as assessed by MPO assay, at 24 post-CLP or as compared to Sham treated mice. Data is presented as means ± standard deviation. *P<0.05, versus Sham group; #P<0.05, versus Cont siRNA Hem/CLP group; n=4 mice/group.

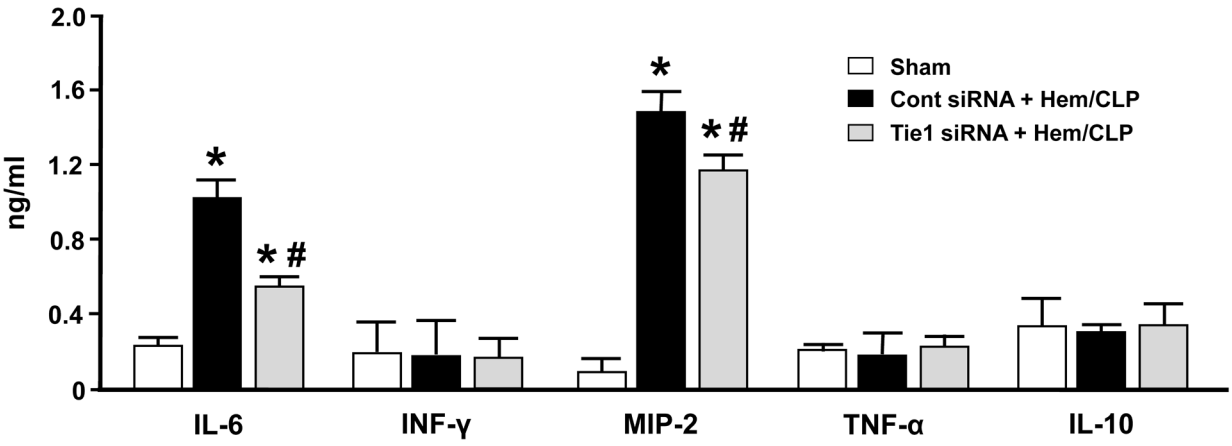


**Figure 5. Targeted Tie1 siRNA treatment markedly inhibited the Hem/CLP-induced a rise in lung tissue IL-6 and MIP2, but not TNF-α, IL-10 or INF-γ levels in mice.**

The lung tissue levels (at 24 post-CLP) of IL-6, INF-γ, MIP-2, TNF-α and IL-10 were measured by ELISA. Control (Cont) siRNA or the Tie1 siRNA (Tie1) treated Hem/CLP mice were increased compared to the Sham animals. However, this Hem/CLP-induced increase was markedly suppressed by Tie1 treatment for IL-6 and MIP-2, but not back to the levels seen in the Sham group. *P<0.05, versus Sham; # P<0.05, Cont Hem/CLP. Data is presented as means ± standard deviation. *P<0.05, versus Sham group; $^{\#}$P<0.05, versus Cont siRNA + Hem/CLP group; n=4 mice/group.

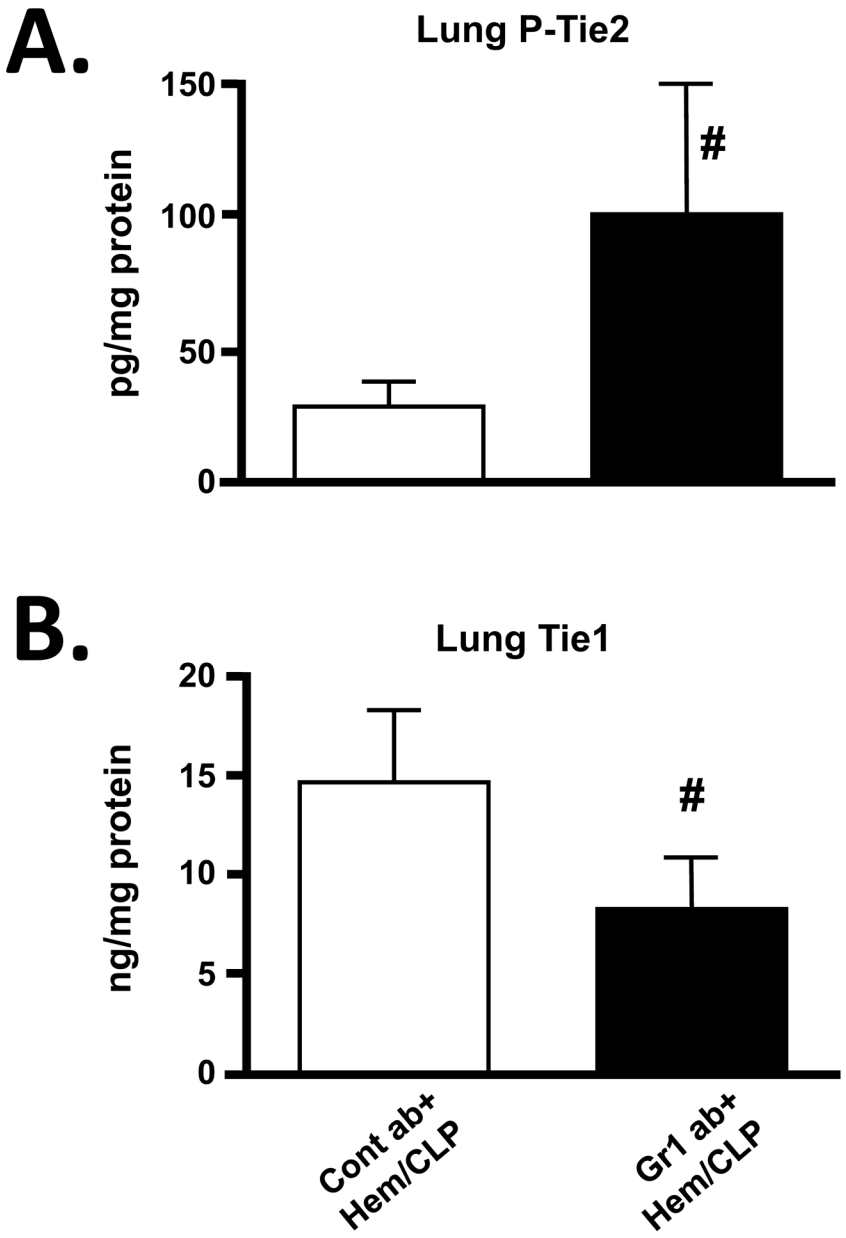


**Figure 6. The depletion of peripheral blood neutrophils prior to the induction of Hem/CLP markedly increase the lung tissue levels of P-Tie2 while decreasing Tie1.**

The lung tissue levels (at 24 post-CLP) of P-Tie2 (A.) in the neutrophil depleted (using anti-Gr1 ab [Gr1 ab+]) Hem/CLP mice, as determined by ELISA, were increased compared to the control antibody (Cont ab+) pre-treated Hem/CLP animals. Inversely, the lung tissue Tie1 levels (B.) in the neutrophil depleted (Gr1 ab+) Hem/CLP mice, again, as determined by ELISA, were decreased when compared to the Hem/CLP animals administered control antibody (Cont ab+). Data is presented as means ± standard deviation. $^{\#}P<0.05$, versus Cont ab+ Hem/CLP group; n=3 mice/group.

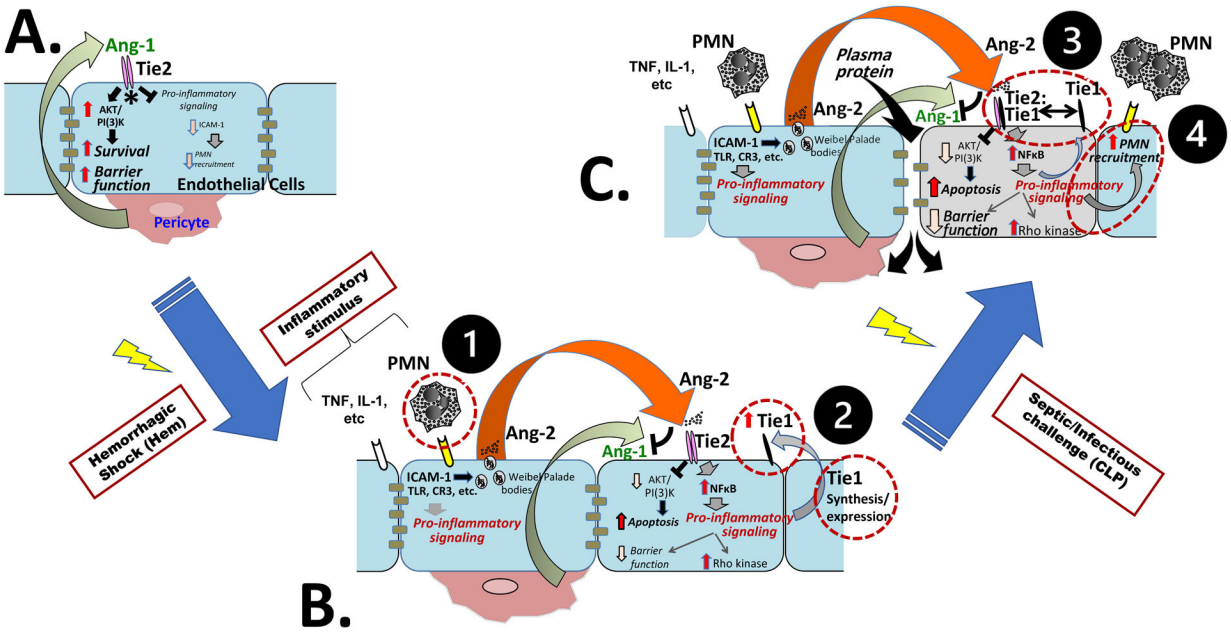


**Figure 7. Proposed mode by which shock/hemorrhage induces myelocyte up-regulation of Tie1 as well as how Tie1's interaction with Tie2 prolongs Ang-2 mediated competitive inhibitory binding of Ang-1; compromising pulmonary vascular endothelial barriers function.**

(A.) Reflects the endothelial cell (EC) monolayer in homeostatic conditions. This state is sustained by the presence of consitutively/secreted released levels of Angiopoietin-1 (Ang-1), by pericytes, for ECs expressing Tie2, which when ligated by Ang-1 culminates in Tie2's phosphorylation (*, activation) and downstream signaling through AKT/PI(3)K pathway, therein, sustain cell survival/proliferation. (B.) The insult of hemorrhagic shock (Hem) induces the systemic release of various inflammatory mediators as well as primes circulating neutrophils (PMN) that express increased levels of integrins that mediated their adhesion to the pulmonary vascular endothelium via ICAM-1 of the shocked animal (B. ❶❷❸❹). In such an environment this limited initial PMN: endothelial cell interaction appears to lead not only to the release of pre-formed Ang-2 form Weibel Palade bodies of the EC that competitively inhibits the binding and, thus, the activation of Tie2, but through EC 'pro-inflammatory signaling' stimulates the upregulation (synthesis/expression) of Tie1 (B. ❷). (C.) The enhanced expression Tie1 increases the interaction of Tie1 with Tie2, which in turn stabilizes Tie2's binding of Ang2 (C. ❸). This further suppresses cell survival signaling pathways while permitting the potentiation of 'pro-inflammatory signaling' that results in not only reduced EC monolayer barrier function but a further increase in PMN recruitment to the pulmonary microvascular ECs (C. ❹).