Angiopoietin-1 Treatment Reduces Inflammation but Does Not Prevent Ventilator-Induced Lung Injury

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Introduction

Mechanical ventilation is an important life-saving procedure. However, the procedure itself may induce or aggravate damage to lung tissue, so-called ventilator-induced lung injury (VILI) [1,2]. VILI is characterized by inflammation, enhanced alveolar-capillary membrane permeability, accumulation of protein-rich pulmonary edema and ultimately impaired gas exchange [3]. Various animal models have been used to obtain further insight into the mechanisms underlying VILI. Already in the 1980s, investigators showed that mechanical ventilation and the subsequent mechanical (over)stretch of lung tissue induces damage to the epithelial-endothelial barrier leading to impaired oxygenation [4–6]. In addition, it has been described that the mechanical forces associated with mechanical ventilation provoke an inflammatory response in the lung (biotrauma) [7,8].

Loss of integrity of epithelial and endothelial cell monolayers has been suggested to play an important role in the ventilator-induced disruption of the alveolar-capillary barrier [9]. One of the crucial systems regulating vascular cell integrity is the angiopoietin (Ang)-Tie2 system [10]. Clarifying the role of the Ang-Tie2 system in the development of lung injury has therefore become a topic of great interest [11]. However, to date little is known about the interaction of mechanical (over)stretch with the Ang-Tie2 system. It has been recognized that Ang-1 serves as a Tie2 receptor agonist by phosphorylating Tie2 on tyrosine residues. Ang-1-mediated
Tie2 signaling is required to maintain cellular integrity and quiescence of the endothelial barrier [10]. The antagonist Ang-2 is known to downregulate Tie2 signaling, thereby preparing vascular endothelial cells for enhanced responsiveness to factors that cause destabilization of the endothelial barrier [12]. However, there is also conflicting evidence that Ang-2 may cause Tie2 activation in stressed endothelial cells [13].

In a murine model of endotoxin-induced acute lung injury (ALI), Karpnaliotis et al. described that vascular permeability and pulmonary edema were accompanied by enhanced vascular endothelial growth factor (VEGF) and reduced Ang-1 levels in lung tissue [14]. The same authors proposed that changes in the balance between VEGF (pro-leakage) and Ang-1 (anti-leakage) might contribute to the pathophysiology of ALI. Protective effects of Ang-1 treatment have been shown before in experimental models of endotoxin-induced ALI [15–18]. Mei et al. demonstrated that treatment with Ang-1 attenuated vascular leakage, granulocyte infiltration and pro-inflammatory cytokine expression in lungs of endotoxin-exposed mice [17]. Consequently, the Ang-Tie2 system has been proposed as a possible therapeutic target in pulmonary diseases like ALI and its most severe form, the acute respiratory distress syndrome (ARDS) [11,19].

Vascular leakage and pulmonary inflammation are important features of VILI. Therefore, we hypothesized that Ang-1 – Tie2 signaling plays a protective role in the development of VILI. In an attempt to better reflect the human setting, we applied a relatively mild model of VILI using clinically relevant ventilator settings thereby preventing shock, metabolic acidosis and substantial damage to lung architecture [20]. The aim of present study was to investigate the influence of mechanical ventilation on the Ang-Tie2 system in lungs of healthy adult mice. Furthermore, we examined whether treatment with Ang-1, a Tie2 receptor agonist, would protect ventilated mice against important hallmarks of VILI, such as inflammation, vascular leakage and impaired gas exchange.

**Methods**

**Animals**  
Experiments were performed in accordance with international guide lines and approved by the animal care and use committees of the University Medical Center Utrecht and the Academic Medical Center Amsterdam (approval IDs: 2007.II.02.066 and DAA101607). Adult male C57Bl6 mice (n = 145; Charles River, Maastricht, the Netherlands), weighing 20 to 24 grams, were randomly assigned to different experimental groups. Mice (n = 114) were ventilated for 5 hours as described previously [20]; pressure-controlled, fractional inspired oxygen concentration (FiO₂) of 0.5, inspiration-expiration ratio of 1:1 and positive end-expiratory pressure of 2 cmH₂O. Six mice were ventilated simultaneously with either an inspiratory pressure of 10 cmH₂O (resulting in ‘low’ tidal volume (VT) ~7.5 ml/kg; LV₃) or 18 cmH₂O (resulting in ‘high’ VT ~15 ml/kg; HV₃). Respiratory rate was set at 100 and 50 breaths/min, respectively. Body temperature was kept constant between 36.5 and 37.5°C. Non-ventilated mice (n = 31) served as controls (non-ventilated controls, NVC).

**Ang-1 treatment**  
At initiation of HV₃-ventilation, recombinant human Ang-1 (carrier free; R&D systems, Minneapolis, MN) was intravenously administered (either 1 or 4 µg per animal). The dose of 1 µg has been shown to be effective in attenuating lung inflammation and injury [21]. Control HV₃-ventilated mice received the same volume of sterile saline (vehicle) intravenously.

**Hemodynamics and blood gas analysis**  
After 0, 2.5 and 5 hours, systemic blood pressure and heart rate were non-invasively monitored using a tail-cuff system (ADInstruments, Spenbach, Germany). After 5 hours, arterial blood was taken from the carotid artery for blood gas analysis (Rapidlab 865; Bayer, Mijdrecht, the Netherlands).

**Bronchoalveolar lavage**  
The right lung was lavaged by instilling 3 × 0.5 ml sterile saline. Differential counts were done on cytopsin preparations stained with Giemsa (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). Cell-free supernatant was used to measure total protein (BCA protein-assay; Pierce Biotechnology, Rockford, IL) with BSA as standard.

**Wet-to-dry ratio**  
The left lung was weighed, dried for 3 days (63°C) and weighed again.

**Histology and immunohistochemistry**  
The left lung was filled with Tissue-Tek® (Sakura Finetek, Zoeterwoude, the Netherlands), snap frozen and cut to 5 µm cryosections using a cryostat. To assess pulmonary histopathology, sections were stained with hematoxylin-eosin (H&E; Klinipath, Duiven, the Netherlands). To assess Tie2 localization, sections were stained with fluorescent antibody recognizing Tie2 (Tek4; eBioscience, San Diego, CA) or isotype control antibody (IgG1-biotin).

**Tissue homogenate preparation**  
Lung tissue of separate animals was pulverized using a liquid nitrogen-cooled mortar and pestle, and divided in several fractions allowing us to perform multiple analyses (as described below).

**Real-time RT-PCR analysis**  
PCR was performed as described previously [22]. Primer sequences: Ang-1, forward CTAGAAGGCACAAACAGCATCC, reverse CTACCCTTTAGGAAAAACACCTTC; Ang-2, forward CTTTGCGAAATCTCTAGCAG, reverse TGCCATCTTCTCgCgg-TgTT; Tie2, forward gTgTgAgTgACCAgAagg, reverse CTgAgAgCAgAgCAgCATC. Data were normalized for expression of GAPDh.

**Western blotting**  
Western blotting was performed as described previously [23]. Antibodies recognized phospho (p)-Akt (Ser473; Cell Signaling, Danvers, MA), Ang-1 or Ang-2 (both Alpha Diagnostic, San Antonio, TX). To control for equal loading, membranes were stripped when necessary and reprobed with an antibody recognizing total Akt (Akt1/PKBα; Sigma-Aldrich, Steinheim, Germany). Total Akt was chosen as a loading control since mechanical ventilation influenced expression levels of β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDh).

**ELISA**  
Tie2 protein was measured by ELISA according to manufacturer’s instructions (R&D).

**Myeloperoxidase (MPO) activity**  
MPO activity was determined as described previously [22].
Multiplex cytokine assay

125 μg protein was analyzed for keratinocyte-derived chemo-
kine (KCl), macrophage inflammatory protein (MIP)-2, monocyte
chemotactic protein (MCP)-1, interleukin (IL)-1β, IL-6, IL-10 and
VEGF by multiplex cytokine assay using a Luminex analyzer (Bio-
Rad Laboratories, Hercules, CA) according to manufacturer’s
instructions (multiplex mouse cytokine, R&D).

Statistical analysis

Data are expressed as mean ± SEM. Oxygenation variables
(LVT versus HV1) and Ang-1, Ang-2, Tie-2 protein (NVC versus
HV1) were analyzed by independent T-test. Other parameters
were analyzed by one-way ANOVA with least significant
difference (LSD) post-hoc test. P-values less than 0.05 were
considered statistically significant.

Results

Stability of the murine model of VILI

All mice survived 5 hours of LVT and HV1-ventilation after
which they were sacrificed. Analysis of systolic blood pressure and
heart rate revealed stable conditions of ventilated mice throughout
the experiment (table 1).

Table 2 illustrates that arterial oxygen tension (PaO2) was
reduced in HV1-ventilated mice when compared to LVT-
ventilated mice. Carbon dioxide tension (PaCO2), pH and base
excess (BE) remained within the physiological range during both
LVT and HV1-ventilation.

Effect of mechanical ventilation on alveolar-capillary
permeability, pulmonary edema formation and gas exchange

First, we examined whether LVT and HV1-ventilation caused
lung injury in mice without pre-existing lung injury. Total protein
levels in bronchoalveolar lavage fluid (BALF), wet-to-dry ratios of
pulmonary tissue and PaO2/FiO2 ratio in blood samples were
analyzed to evaluate the effects of mechanical ventilation on
alveolar-capillary permeability, pulmonary edema formation and
gas exchange respectively. We found that both ventilation
strategies markedly enhanced BALF protein levels and pulmonary
wet-to-dry ratios in comparison with NVC (figures 1a and b). Furthermore, PaO2/FiO2 ratios were significantly decreased when
comparing HV1-ventilated mice with LVT-ventilated mice (figure 1c).

Effect of mechanical ventilation on the Ang-Tie2 system

To assess whether 5 hours of mechanical ventilation influenced
the Ang-Tie2 system, we determined Ang-1, Ang-2 and Tie2 expression in total lung homogenates. We found that only HV1-
ventilation caused a decrease in Ang-1 and Ang-2 mRNA
compared to NVC (figures 2a and b). Both ventilation strategies
reduced Tie2 mRNA (figure 2c). Since ventilator-induced effects
on the Ang-Tie2 system, vascular leakage and oxygenation were
more pronounced after HV1-ventilation, we continued our
investigations by focusing on this specific group.

To determine if ventilator-induced reduction of Ang-1, Ang-2
and Tie2 mRNA also resulted in a reduction of Ang-1, Ang-2 and
Tie2 protein, we measured protein expression of these mediators
in total lung homogenates. We found that only HV1-
ventilated mice treated with 1 or 4 μg Ang-1 (figure 4a). At
this time point, no effect of Ang-1 treatment was observed on
Ang-2 protein expression (figure 4b). Compared to NVC, VEGF
protein expression was increased in the vehicle-treated HV1-
group (figure 5). Both doses of Ang-1 completely abolished the increase in
VEGF protein in response to HV1-ventilation.

Table 1. Hemodynamic characteristics over 5 hours of
mechanical ventilation.

<table>
<thead>
<tr>
<th></th>
<th>LVT</th>
<th>HV1</th>
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<tbody>
<tr>
<td></td>
<td>HR 0 h</td>
<td>370±11</td>
</tr>
<tr>
<td></td>
<td>HR 2.5 h</td>
<td>382±11</td>
</tr>
<tr>
<td></td>
<td>HR 5 h</td>
<td>403±12</td>
</tr>
<tr>
<td></td>
<td>BP 0 h</td>
<td>104±6</td>
</tr>
<tr>
<td></td>
<td>BP 2.5 h</td>
<td>76±6</td>
</tr>
<tr>
<td></td>
<td>BP 5 h</td>
<td>73±5</td>
</tr>
</tbody>
</table>

LVT, HV1 = mice ventilated with low or high tidal volumes; HR = heart rate in beats per minute; BP = systolic blood pressure in mmHg. Data are presented as
mean ± SEM of 11 to 12 animals per group.

Table 2. Arterial blood gas analysis after 5 hours of
mechanical ventilation.

<table>
<thead>
<tr>
<th></th>
<th>LVT</th>
<th>HV1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PaO2</td>
<td>231.6±15.8</td>
</tr>
<tr>
<td></td>
<td>PaCO2</td>
<td>32.4±3.4</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>7.51±0.03</td>
</tr>
<tr>
<td></td>
<td>BE</td>
<td>2.08±0.78</td>
</tr>
</tbody>
</table>

LVT, HV1 = mice ventilated with low or high tidal volumes; PaO2 = partial pressure of arterial oxygen in mmHg; PaCO2 = partial pressure of arterial
carbon dioxide in mmHg; BE = base excess in mmol/l. Data are presented as
mean ± SEM of 16 to 18 animals per group (*p<0.01 versus LVT).

Effect of Ang-1 on Ventilator-Induced Lung Injury

Multiplex cytokine assay

125 μg protein was analyzed for keratinocyte-derived chemo-
kine (KCl), macrophage inflammatory protein (MIP)-2, monocyte
chemotactic protein (MCP)-1, interleukin (IL)-1β, IL-6, IL-10 and
VEGF by multiplex cytokine assay using a Luminex analyzer (Bio-
Rad Laboratories, Hercules, CA) according to manufacturer’s
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To determine if ventilator-induced reduction of Ang-1, Ang-2
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Tie2 protein, we measured protein expression of these mediators
in total lung homogenates. We found that only HV1-
ventilated mice treated with 1 or 4 μg Ang-1 (figure 4a). At
this time point, no effect of Ang-1 treatment was observed on
Ang-2 protein expression (figure 4b). Compared to NVC, VEGF
protein expression was increased in the vehicle-treated HV1-
group (figure 5). Both doses of Ang-1 completely abolished the increase in
VEGF protein in response to HV1-ventilation.
Figure 1. Mechanical ventilation affects alveolar-capillary permeability, pulmonary edema formation and gas exchange. A: Alveolar-capillary permeability is represented by total protein levels in bronchoalveolar lavage fluid (BALf). B: Pulmonary edema is represented by wet-to-dry ratios of lung tissue. C: Oxygenation is represented by the ratio of partial pressure arterial oxygen and fraction inspired oxygen (PaO₂/FiO₂). Data are expressed as mean ± SEM of 9-12 (A–B) or 18–21 (C) animals for each group (* p<0.05, *** p<0.001). Ref = reference bar (PaO₂/FiO₂ ratio for mice with non-injured lungs); NVC = non-ventilated controls; LVₜ, HVₜ = mice ventilated with low or high tidal volumes. doi:10.1371/journal.pone.0015653.g001

Figure 2. Mechanical ventilation affects the angiopoietin (Ang)-Tie2 system. A–C: In total lung homogenates, mRNA expression of Ang-1, Ang-2 and Tie2 was determined by real time RT-PCR. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). No group differences in expression levels of β-actin and GAPDH were observed. D–E: In total lung homogenates, protein expression of Ang-1 and Ang-2 was determined by Western blotting. Membranes were reprobed with antibody recognizing total Akt (Akt1/PKBα) to control for equal loading. No group differences in total Akt were observed. Inset: representative Western Blot depicting immunodetectable Ang-1 and Ang-2. F: In total lung homogenates, protein expression of Tie2 was determined by ELISA. Levels were normalized for total protein concentrations. Data are depicted relative to NVC and expressed as mean ± SEM of 6–16 animals for each group (** p<0.01, *** p<0.001). G: Lung sections of non-ventilated controls were stained with fluorescent antibody recognizing Tie2 to visualize the presence of Tie2 on pulmonary cells (isotype control was negative). Magnification ×200. NVC = non-ventilated controls; LVₜ, HVₜ = mice ventilated with low or high tidal volumes. doi:10.1371/journal.pone.0015653.g002
Effect of Ang-1 treatment on ventilator-induced granulocyte infiltration in pulmonary tissue and granulocyte exudation into the alveolar space

To determine the effect of Ang-1 treatment on inflammatory activity in the lung, we first quantified granulocyte infiltration by measuring MPO activity in total lung homogenates and by counting neutrophils on BALF cytospin preparations. Compared to NVC, MPO activity and neutrophil numbers were markedly higher in lungs of HVT-ventilated mice (figures 6a and b). Administration of either 1 or 4 µg Ang-1 reduced granulocyte influx after HVT-ventilation (figure 6a). Supporting the MPO data, neutrophil numbers on BALF cytospin preparations were diminished in HV1-ventilated mice treated with 1 µg Ang-1 as compared to HV1-ventilated mice treated with vehicle (figure 6b). In addition, we stained lung sections for H&E to visualize granulocytes in pulmonary tissue. Figure 6c illustrates that both doses of Ang-1 prevented the appearance of granulocytes in lungs of HV1-ventilated mice, confirming the quantitative measures for infiltrating granulocytes.

Effect of Ang-1 treatment on ventilator-induced chemokine and cytokine expression

The effect of Ang-1 treatment on the levels of inflammatory mediators expressed by pulmonary tissue during HV1-ventilation was determined as an additional measure of inflammatory activity in the lung. HV1-ventilation induced protein expression of the chemokines KC, MIP-2 and MCP-1 in comparison with NVC (figures 7a to c). Consistent with the diminished granulocyte influx, treatment with either 1 or 4 µg Ang-1 reduced the upregulation of these chemokines after 5 hours of HV1-ventilation. Moreover, higher levels of the pro-inflammatory cytokine IL-1β were found in pulmonary tissue of HV1-ventilated mice (figure 7d). The elevated protein expression of IL-6 did not reach statistical significance (p = 0.064, figure 7e). Although Ang-1 treatment decreased the expression of IL-1β protein compared to the HV1-vehicle group, it did not influence the expression of IL-6.

Figure 3. Angiopoietin (Ang)-1 treatment induces Tie2 signaling.

In total lung homogenates, protein expression of p-Akt was determined by Western blotting as an indirect measure of Tie2 signaling. Membranes were stripped and reprobed with antibody recognizing total Akt (Akt1/PKBα) to control for equal loading. No group differences in total Akt were observed. Inset: representative Western blot depicting immunodetectable p-Akt. Data are depicted relative to NVC and expressed as mean ± SEM of 8–10 animals per group (* p<0.05, ** p<0.01). NVC = non-ventilated controls; HV1 = mice ventilated with high tidal volumes; Veh, 1 µg, 4 µg = mice intravenously treated with either vehicle (sterile saline), Ang-1 (1 µg per animal), or Ang-1 (4 µg per animal).
doi:10.1371/journal.pone.0015653.g003

Figure 4. Angiopoietin (Ang)-1 treatment reduces Ang-2 mRNA expression.

A: In total lung homogenates, mRNA expression of Ang-2 was determined by real time RT-PCR. Levels were normalized for expression of internal controls, i.e. the average value of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). No group differences in β-actin and GAPDH were observed. B: In total lung homogenates, protein expression of Ang-2 was determined by Western blotting. Membranes were reprobed with antibody recognizing total Akt (Akt1/PKBα) to control for equal loading. No group differences in total Akt were observed. Inset: representative Western blot depicting immunodetectable Ang-2. Data are depicted relative to NVC and expressed as mean ± SEM of 7–16 animals per group (** p<0.01, *** p<0.001). NVC = non-ventilated controls; HV1 = mice ventilated with high tidal volumes; Veh, 1 µg, 4 µg = mice intravenously treated with either vehicle (sterile saline), Ang-1 (1 µg per animal), or Ang-1 (4 µg per animal).
doi:10.1371/journal.pone.0015653.g004
The anti-inflammatory cytokine IL-10 was below detection level in all experimental groups.

Effect of Ang-1 treatment on ventilator-induced lung injury

Ang-1 treatment suppressed the inflammatory activity in the lung. To determine the consequences for lung injury, we evaluated whether the increase in vascular leakage and decrease in oxygenation during HV\textsubscript{T}-ventilation could be restored by Ang-1 administration. As depicted in figures 8a and b, the increased BAL\textsubscript{f} protein levels and pulmonary wet-to-dry ratios of HV\textsubscript{T}-ventilated mice were not affected by treatment with either 1 or 4 \textmu g Ang-1. Furthermore, both doses of Ang-1 did not prevent the reduction in PaO\textsubscript{2}/FiO\textsubscript{2} ratio caused by HV\textsubscript{T}-ventilation (figure 8c).  

Discussion

At the best of our knowledge this is the first report demonstrating that mechanical ventilation affects the Ang-Tie2 system in pulmonary tissue of healthy adult mice. Particularly in lungs of HV\textsubscript{T}-ventilated mice, we observed marked changes in the Ang-Tie2 system. Compared to NVC, 5 hours of HV\textsubscript{T}-ventilation resulted in downregulation of Ang-1, Ang-2 and Tie2 mRNA expression. In addition, Ang-1 and Ang-2 protein expression tended to decrease in HV\textsubscript{T}-ventilated mice at this time point. The
major finding of the present study is that treatment with Ang-1 affected only specific aspects of VILI. Ang-1 administration at initiation of ventilation diminished granulocyte infiltration, as well as chemokine (KC, MIP-2, MCP-1), cytokine (IL-1β), VEGF and Ang-2 expression in lungs of HVT-ventilated mice. However, Ang-1 treatment did not prevent the increase in BALf protein level, the increase in pulmonary wet-to-dry ratio and the reduction in PaO₂/FiO₂ ratio induced by HVT-ventilation. This result was not only observed after administration of 1 μg of Ang-1 but also after administration of 4 μg of Ang-1. In preliminary experiments we also investigated whether a higher dose of Ang-1 (i.e. 8 μg per animal) would influence alveolar-capillary permeability and pulmonary edema formation induced by mechanical ventilation. However, 8 μg of Ang-1 appeared to have no effect on ventilator-induced vascular leakage as well. In view of these data, we would like to suggest that Ang-1 treatment does not prevent the aspects of VILI driven by mechanosensitive alterations in barrier properties [24] but will only regulate the pulmonary inflammation.

In experimental studies, mechanical ventilation has been described to induce destabilization of the alveolar-capillary barrier thereby leading to enhanced pulmonary permeability and edema formation [4-6]. Most models of VILI, however, applied very high inspiratory pressures or tidal volumes when compared to those used in the human setting [25-29]. To prevent shock and metabolic acidosis due to very high inspiratory pressures or tidal volumes, we used ventilation strategies with more clinically relevant ventilator settings [20]. Even in this relatively mild model of VILI, we observed that mechanical ventilation caused a modest, but significant increase in BALf protein level and pulmonary wet-to-dry ratio.

The importance of the Ang-Tie2 system has been appreciated in the development of vascular leakage and inflammation in pulmonary diseases like ALI/ARDS [11]. In this respect, Karmpaliotis et al. proposed that changes in the balance between VEGF (pro-leakage) and Ang-1 (anti-leakage) might contribute to vascular leakage in their murine model of lipopolysaccharide (LPS)-induced ALI [14]. Here we demonstrate that HVT-ventilation as such enhanced expression of VEGF protein, decreased expression of Ang-1, Ang-2 and Tie2 mRNA and tended to reduce expression of Ang-1 and Ang-2 protein compared to NVC. Since the mice were sacrificed after 5 hours
of HV1-ventilation, we could not evaluate whether the downregulation in mRNA expression was followed by a significant decrease in protein expression at a later time point. Even so, the present study suggests that alterations in the Ang-Tie2 system are involved in the pathogenesis of VILI.

Kim et al. and Papapetropoulos et al. have demonstrated that Ang-1-mediated Tie2 signaling causes Akt phosphorylation thereby protecting the endothelial cells from apoptotic cell death [30,31]. In our study, HV1-ventilation itself caused increased p-Akt levels compared to NVC which is in agreement with previous reports [32,33]. Moreover, treatment with Ang-1 augmented p-Akt expression in lungs of HV1-ventilated mice. (Ang-1-mediated) Akt phosphorylation has been shown to inactivate the forkhead transcription factor FKH1 subsequently preventing Ang-2 expression and destabilization of the endothelial barrier [34]. This observation supports our finding that HV1-ventilation as such reduced Ang-2 mRNA and that Ang-1 treatment downregulated the transcription of Ang-2 even further. The Ang-1-induced shift towards less Ang-2 production, thus reduced Tie2 antagonism, may protect against lung inflammation and injury during HV1-ventilation.

In agreement with previous reports [27-29], we observed that ventilator-induced lung injury was accompanied by enhanced pro-inflammatory. In LPS-challenged animals, treatment with Ang-1 has already been shown to decrease leukocyte trafficking by reducing chemotactic, adhesive and pro-inflammatory mediators [17]. Our study is the first to show that Ang-1 administration downregulated infiltration of granulocytes and expression of the chemokines KC, MIP-2 and MCP-1 in an experimental model of VILI. Furthermore, we observed that administration of Ang-1 prevented the increase in the pro-inflammatory cytokine IL-1β in lungs of HV1-ventilated mice. Our data may suggest that the role of IL-1β might be less important in the development of vascular leakage during HV1-ventilation. Interestingly, IL-6 protein levels remained high in lungs of HV1-ventilated mice despite Ang-1 treatment. In pulmonary inflammation, IL-1β is primarily produced by activated alveolar macrophages whereas IL-6 may be derived from a wide variety of pulmonary cell types [35]. Since Tie2 is expressed on endothelial cells, neutrophils and macrophages [36,37], our observations may imply that IL-6 is also derived from cells that do not express the Tie2 receptor and consequently do not respond to Ang-1. With respect to the clinical situation, it would be of interest to evaluate whether Ang-1 treatment would also be effective in attenuating lung inflammation in ventilated animals with pre-existing lung inflammation.

The angiogenic growth factor VEGF has been shown to increase capillary permeability and edema formation in various experimental models of pulmonary injury, including VILI [14,38,39]. Thurston et al. described that vascular leakage induced by VEGF may be counteracted by Ang-1 [40,41]. In line with this notion, we observed that Ang-1 treatment completely abolished the increase in VEGF protein in lungs of HV1-ventilated mice. Nonetheless, it should be noted that Ang-1 treatment did not prevent alveolar-capillary permeability, pulmonary edema (i.e. vascular leakage) and impaired gas exchange induced by HV1-ventilation. These data are in apparent contrast with previously described protective effects of Ang-1 on vascular leakage in endotoxin-challenged animals [15-18] underlining that the pathways involved in endotoxin- and ventilator-induced lung injury are different. An explanation for this discrepancy might be that the enhanced inflammation is not the primary inducer of vascular leakage and impaired gas exchange during HV1-ventilation, as is the case in the induction of lung injury by LPS. It has been demonstrated that ventilator-induced mechanical stretch may also lead to the destabilization of alveolar-epithelial and capillary-endothelial barriers thereby resulting in increased vascular permeability and pulmonary edema [4-6]. Ang-1 administration will probably influence the capillary-endothelial but not the alveolar-epithelial barrier since the Tie2 receptor is mainly expressed on endothelial cells. Thus, the possibility remains that Ang-1 treatment is not capable of restoring lung injury induced by HV1-ventilation as it only modulates endothelial inflammation. The fact that Ang-1 prevents pulmonary vascular leakage in animals exposed to LPS, which induces a generalized inflammation primarily in the endothelial cells of the lung [42], supports this hypothesis.

Taken together, our data indicate that treatment with Ang-1 inhibits various aspects of VILI such as granulocyte infiltration, chemokine/cytokine and VEGF expression. However, Ang-1
treatment did not protect HV1-ventilated mice against the more crude parameters of VILI (i.e. vascular leakage and impaired gas exchange). In this respect, it is of interest that the TNF-α inhibitor Etanercept diminished inflammation and coagulation in the lungs of ventilated mice without influencing alveolar-capillary permeability and pulmonary edema, which is in line with our present results [43]. We propose that Ang-1 should not be applied to combat the mechanosensitive aspects of ventilator-induced lung injury in critically ill patients. Nonetheless, treatment with Ang-1 may well be considered as an anti-inflammatory therapy when inflammation is the primary inducer of lung injury, like in non-ventilated patients diagnosed with ALI/ARDS.

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Author Contributions

Conceived and designed the experiments: PMC NJJ MvM CJH. Performed the experiments: MAH MPH. Analyzed the data: MAH. Wrote the paper: MAH MPH MM PMC AK MJ SJ CJH. Involved in study design: AK MJ SJ CJH. Critical review manuscript: MPH MM AK MS GM.

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