Adjunctive therapy with the Tie2 agonist Vasculotide reduces pulmonary permeability in *Streptococcus pneumoniae* infected and mechanically ventilated mice

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Community acquired pneumonia, mainly caused by *Streptococcus pneumoniae* (*S.pn.*), is a common cause of death worldwide. Despite adequate antibiotic therapy, pneumococcal pneumonia can induce pulmonary endothelial hyperpermeability leading to acute lung injury, which often requires mechanical ventilation (MV) causing ventilator-induced lung injury (VILI). Endothelial stabilization is mediated by angiopoietin-1 induced Tie2 activation. PEGylated (polyethylene glycol) Tie2-agonist Vasculotide (VT) mimics Angiopietin-1 effects. Recently, VT has been shown to reduce pulmonary hyperpermeability in murine pneumococcal pneumonia. The aim of this study was to determine whether VT reduces lung damage in *S.pn.* infected and mechanically ventilated mice. Pulmonary hyperpermeability, immune response and bacterial load were quantified in *S.pn.* infected mice treated with Ampicillin +/-VT and undergoing six hours of MV 24 h post infection. Histopathological lung changes, Tie2-expression and -phosphorylation were evaluated. VT did not alter immune response or bacterial burden, but interestingly combination treatment with ampicillin significantly reduced pulmonary hyperpermeability, histological lung damage and edema formation. Tie2-mRNA expression was reduced by *S.pn.* infection and/or MV but not restored by VT. Moreover, Tie2 phosphorylation was not affected by VT. These findings indicate that VT may be a promising adjunctive treatment option for prevention of VILI in severe pneumococcal pneumonia.

**Abbreviations**

ARDS     Acute respiratory distress syndrome  
Ang-1    Angiopoietin-1  
Ang-2    Angiopoietin-2  
BAL      Bronchoalveolar lavage  
BALF     Bronchoalveolar lavage fluid  
bw      Body weight  
CAP      Community-acquired pneumonia  
CFU      Colony-forming units  
FiO2     Fraction of inspired oxygen

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Community acquired pneumonia (CAP) is the leading infectious cause of death worldwide. Bacterial CAP is most commonly caused by Streptococcus pneumoniae (S. pn.)3. Although antibiotic resistance in CAP is rare, hospitalized patients face a short-term mortality of 10–14%, and up to 35% in severe CAP requiring mechanical ventilation (MV)4. Severe pneumonia can cause pulmonary hyperpermeability resulting in pulmonary edema and eventually acute respiratory distress syndrome (ARDS)5,6. For lung failure, only supportive therapies are available, and particularly pulmonary barrier disruption lacks therapeutic strategies7,8. In addition to antibiotics, MV is key for treating pneumonia-associated ARDS9. Yet, lifesaving MV causes further damage to the lungs, known as ventilator-induced lung injury (VILI)10,11. Despite the implementation of lung-protective ventilation strategies in clinical practice such as low tidal volume ventilation12,13, VILI still occurs particularly in pre-damaged lungs14,15.

Angiopoietins 1 and 2 (Ang-1, -2) are ligands for receptor tyrosine kinase Tie216,17. Under inflammatory conditions, Ang-1 improves endothelial integrity and reduces vascular permeability18,19 while Ang-2 acts as functional antagonist on Tie2 further aggravating inflammation and destabilizing the endothelial barrier18,20,21. Moreover, Tie2 expression is reduced in systemic inflammation further contributing to endothelial destabilization22. Ang-2 levels are significantly elevated in patients with pneumonia23 and sepsis-associated ARDS24 predicting severe or even fatal disease progression25–27, while Ang-1 levels are reduced in sepsis28 and pneumonia23.

Restoring the disturbed balance between phosphorylated and non-phosphorylated Tie2, the synthetic polyethylene glycol (PEG) clustered Tie2 agonist Vasculotide (VT)29 has been shown to reduce endothelial permeability in models of abdominal sepsis30, hemorrhagic shock31, cardiopulmonary bypass32, influenza33, and pneumococcal pneumonia34, even improving survival in influenza and abdominal sepsis30,33. In both pneumonia studies—influenza and pneumococcal pneumonia model, respectively—VT did not alter host immune response and pathogen burden30,34. As we envision that VT will primarily be developed for use in critical care patients, we investigated VT as therapy in severe murine pneumonia requiring MV. Mice with severe pneumonia were treated with antibiotics and VT, or placebo, and were mechanically ventilated. Lung permeability, immune response, bacterial load, histopathological changes and both Tie2 gene expression and phosphorylation were evaluated.

Methods
In general, the study complies with local and national guidelines and is in accordance with relevant guidelines and regulations.

Animals.  Female 8–10 weeks old C57BL/6 N mice (Charles River, Sulzfeld, Germany) weighing between 18 and 20 g were used for the experiments. All animal experiments were approved by institutional (“Tierschutzbeauftragte” (animal care taker) and “Tierschutzausschuss” of Charité–Universitätsmedizin Berlin; https://tierschutz.charite.de/tierschutz_an_der_charite/) and governmental (Landesamt für Gesundheit und Soziales Berlin; approval ID A0050/15) authorities and were in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals, which is equivalent to American ARRIVE. Therefore, our animal study meet the reporting requirements laid out in the ARRIVE guidelines. Mice were randomly assigned to the appropriate experimental groups and were kept in closed, individually ventilated cages with filter hoods (type II-L, ZOONLAB), under specific pathogen-free conditions, with free access to food and water, room temperature between 20 and 22 °C, air humidity between 50 and 65% and 12 h light/dark cycle.

Murine pneumococcal pneumonia and Vasculotide/ampicillin treatment.  Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (80 mg/kg) and xylazine (25 mg/kg) in a total volume of 60 µl followed by intranasal inoculation of 5 × 10⁶ colony-forming units (CFU) S. pn. serotype 3 (NCTC7978) in 20 µl sterile PBS. Control groups received the same volume of PBS.

In a previous study, pulmonary hyperpermeability in experimental pneumococcal pneumonia was reduced by intravenous (i.v.) administration of 500 ng/100 µl VT (Vasomune Therapeutics, Toronto, Canada)34. Accordingly, 500 ng/100 µl VT or PBS as control were applied into the lateral tail vein 22 h post infection (p.i.). At this time, a single dose of ampicillin (0.02 mg/g body weight (bw) in 200 µl) was administered i.p. Control groups received the same volume of 0.9% saline.
Mechanical ventilation. Twenty-four hours p.i., mice were anesthetized with fentanyl (0.08 mg/kg bw), midazolam (8 mg/kg bw) and medetomidine (0.8 mg/kg bw) and ventilated for 6 h via a tracheal cannula (mini-Vent, Hugo-Sachs Elektronik, March-Hugstetten). A catheter in the left carotid artery, an i.p. catheter and a transurethral catheter were placed as described. Ventilation was set to a tidal volume of 12 ml/kg bw, respiratory rate of 120/min, PEEP of 2 cm H\textsubscript{2}O and 50% fraction of inspired oxygen (Fi\textsubscript{O}\textsubscript{2}). During ventilation, mice received continuous infusion (350 µl/h) of full electrolyte solution jonosterile mixed with 0.0075 mmol/ml trometamol and 0.0005 mg/ml fentanyl. Additionally, medetomidin (0.16 mg/kg bw) and midazolam (1.6 mg/kg bw) were administered i.p. to maintain anaesthesia. Oxygen saturation (MouseOX Plus, STARRLife-Sciences, Oakmont, USA), heart rate, blood pressure and tracheal peak pressure (PULMODYN, Hugo-Sachs Elektronik) were monitored throughout MV.

At 4.5 h ventilation time, a second dose of VT or solvent mixed with 1 mg human serum albumin (HSA; Human Serum albumin solution (20%) from CSL Behring (King of Prussia, Pennsylvania, USA) diluted with 0.9% saline) was infused via the carotid catheter. Ten min prior to the end of the experiment, Fi\textsubscript{O}\textsubscript{2} was increased to 100% and anaesthesia was deepened. Five minutes later, 100 µl of heparin at a concentration of 2,500 IU/ml were administered via the carotid catheter.

After 6 h of ventilation mice were exsanguinated. The whole lung was perfused with NaCl 0.9% followed by bronchoalveolar lavage (BAL) of the right lung with proteinase inhibitor. The left lung was removed for flow cytometric analysis.

In non-ventilated control animals, only the six-h ventilation phase was omitted. Anesthesia and preparation were performed 30 h p.i., while HSA with VT or solvent was injected 1.5 h before into one of the lateral tail veins.

Pulmonary vascular leakage. Pulmonary hyperpermeability was calculated as HSA BALF/Plasma ratio with the corresponding concentrations detected by HSA ELISA (Human Albumin ELISA Kit, Bethyl Laboratories, USA) as described.

Histological analysis. Separate experiments were performed for histological analysis with 4 mice per experimental group. Mice were treated as described above but did not receive HSA with the second dose of VT. After exsanguination of the mouse the trachea was ligated, lungs and all other organs were removed immediately, fixed in 4% formaldehyde for 24–48 h, embedded in paraffin, cut into 2-µm sections and stained with hematoxylin and eosin as described. Lung damage due to pneumonia and VILI was scored by an experienced, board-certified veterinary pathologist. Lungs of all animals from the three experimental groups were examined in a blinded manner. Depicted are lungs with changes representative for each group (Fig. 2A). The degrees of VILI or pneumonia severity and of alveolar and perivascular edema formation were assessed semiquantitatively (0 = no damage, 1 = minimal damage/edema, 2 = mild damage/edema, 3 = moderate damage/edema, 4 = severe damage/edema). Three evenly distributed sections per lung were microscopically evaluated to assess edema formation.

Bacterial loads. Bacterial burden was quantified in bronchoalveolar lavage fluid (BALF) and blood by determining colony forming units (CFU) per ml as described.

Pulmonary leukocyte differentiation. To assess local immune response, leukocytes in BALF and lung tissue were discriminated by flow cytometry (Canto; BD, Heidelberg, Germany) via forward vs. side scatter characteristics and CD11b (M1/70, PE-Cy5, 0.2 mg/ml, BD, Heidelberg), CD11c (N418, Cy5, 1 mg/ml, ATCC, Manassass, Virginia, USA), CD45 (30-F11, FITC, 0.5 mg/ml, BD, Heidelberg), F4/80 (BM8, PE, 0.2 mg/ml, Thermo Fisher Scientific, Waltham, Massachusetts, USA), Ly6G (1A8, PerCP-Cy5.5, 0.2 mg/ml, BD, Heidelberg) and Siglec F (E50-2440, BV421, 0.5 mg/ml, BD, Heidelberg) staining.

Blood leukocyte differentiation. Blood leukocytes were quantified using a scil Vet abc hematology analyzer (Scil animal care company GmbH, Viernheim, Germany).

Cytokine multiplex assay. Mouse cytokines/chemokines in blood and BALF supernatant were measured using a Multiplex assay according to the manufacturer`s instructions (ProcartaPlex Multiplex Immunoassay, Thermo Fisher Scientific, USA).

Tie2 expression. Tie2 expression was analyzed by quantitative reverse-transcriptase polymerase chain reaction (qPCR Mastercycler personal, 7300 rt PCR System, Applied Biosystems, Foster City USA) using RNA extracted from the lavaged right lung. β-2-microtubulin served as housekeeping control. To ensure that an increase in gene expression is accompanied by a positive numerical value, the relative quantification (RQ) values were calculated as follows: RQ = 2\textsuperscript{ΔΔCt}. According to this, the arithmetic mean of the control group corresponded to an RQ of 1. An increase in gene expression was accompanied by RQ values > 1, while a reduction in gene expression resulted in RQ values < 1.

Tie2 phosphorylation. Tie2 phosphorylation was detected by Western Blot analysis of proteins extracted from the lavaged right lung. Snake frozen lung tissues were homogenized in RIPA buffer containing 1 mM Na\textsubscript{3}VO\textsubscript{3} (Natriummetavanadat), 1 mM NaF (Natriumfluorid) and a proteinase inhibitor (cOmplete, Mini Proteinase Inhibitor Cocktail, Roche Diagnostics GmbH, Mannheim, Germany) using a gentleMAC dissociator (Millenyi Biotec, Bergisch Gladbach, Germany). After two centrifugation steps, protein levels in the supernatant were calculated with the DC Protein Assay (BioRad Laboratories, Inc. Hercules, USA) using a 96 well-plate.
lung areas (Fig. 2E). However, treatment with ampicillin and VT considerably reduced alveolar edema formation in VILI-affected lungs (Fig. 2A,F) and perivascular edema formation in pneumonia-affected lungs (Fig. 2G) in comparison to mechanically ventilated mice without any therapy. In contrast, compared to untreated animals, neither ampicillin monotherapy nor combination with VT showed any effect. Furthermore, VT in combination with ampicillin resulted in a significant reduction of lung permeability compared to ampicillin monotherapy.

**Results**

**Combination therapy with Vasculotide and ampicillin reduced vascular hyperpermeability in mechanically ventilated mice with pneumonia.** Vascular hyperpermeability was quantified by calculating HSA BALF/plasma ratio 30 h p.i. (Fig. 1). Permeability values of non-ventilated, non-infected mice were not subject of this study. Corresponding groups only served as methodological controls, therefore no statistical testing was performed between two groups without VT therapy. p-values <0.05 were considered statistically significant.

**Ethics approval and consent to participate.** In general, the study complies with local and national guidelines and is in accordance with relevant guidelines and regulations. All animal experiments were approved by institutional (“Tierschutzbeauftragte” (animal care taker) and “Tierschutzausschuss” of Charité–Universitätsmedizin Berlin; https://tierschutz.charite.de/tierschutz_an_der_charite/) and governmental (Landesamt für Gesundheit und Soziales Berlin; approval ID A0050/15) authorities and were in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals, which is equivalent to American ARRIVE. Therefore, our animal study meets the reporting requirements laid out in the ARRIVE guidelines.

**Statistical analysis.** GraphPad Prism 6 (San Diego, California, USA) was used for the statistical analyses. Results are presented as median and interquartile range (IQR) with the respective minimum and maximum values. VT-treated groups are depicted in light grey in contrast to control groups in dark grey. Comparisons between two groups were performed using the Mann–Whitney-U-Test. Since only directly comparable experimental groups were tested as planned comparisons for the effect of VT therapy, correction for multiple testing was not applied. Effects of MV and S. pn. infection on pulmonary permeability, histological damage and immune response have already been described before and were not subject of this study. Corresponding groups only served as methodological controls, therefore no statistical testing was performed between two groups without VT therapy. p-values <0.05 were considered statistically significant.

**Vasculotide did not affect the pulmonary or systemic cellular immune response in mechanically ventilated mice with pneumonia.** To study the pulmonary and systemic immune responses, leukocytes in BALF and blood were quantified and differentiated. MV-induced sterile inflammation was associated with an increase in neutrophils in BALF (Fig. 3A), which was more pronounced after prior S. pn. infection. Recruitment of neutrophils remained unaffected by ampicillin, VT or combination of both. In contrast to BALF,
The number of neutrophilic granulocytes in the blood was reduced in infected and ventilated animals (Fig. 3B). Neither monotherapy with either VT or ampicillin, nor combination therapy improved leukocytopenia.

**Vasculotide did not modulate production of pro- or anti-inflammatory cytokines and chemokines in mechanically ventilated mice with pneumonia.** For further assessment of local and systemic inflammation, cytokines (GM-CSF, IL-1β, IL-6, IL-12p40 and IL-10) as well as chemokines (KC and MIP-2) were quantified in BALF and plasma by multiplex cytokine assay.

*S. pn.* infection and MV increased local cytokine and chemokine production measured in BALF (Fig. 3C for IL-6, Supplemental Figure S1 A–F for other cytokines/chemokines). VT in combination with ampicillin had no modulatory effect on local cytokine or chemokine concentrations. Higher concentrations of the anti-inflammatory cytokine IL-10 were observed in BALF of some animals treated with ampicillin and VT, yet results are too scattered to conclude a tendency. In systemic circulation, cytokine and chemokine concentrations were reduced by antibiotics, but this reduction was not enhanced by additional VT therapy (Fig. 3D for IL-6, Supplemental Figure S1 G–L for other cytokines/chemokines).

**Vasculotide had no effect on local or systemic bacterial burden in mechanically ventilated mice with pneumonia.** To determine the effect of Vasculotide on bacterial burden in the alveolar space and on bacteremia, we counted CFUs in BALF and blood, respectively (Fig. 4A,B). Ampicillin-therapy distinctly reduced bacterial burden in BALF and completely eradicated bacteria from systemic circulation. Bacterial burden was not affected by additional or sole VT-therapy.

**Pulmonary Tie2-receptor expression was reduced by pneumonia and MV.** Tie2-receptor expression in the lung was evaluated using qPCR (supplemental figure S2). MV alone reduced Tie2-expression compared to the control group. *S. pn.* infection with or without MV further lowered Tie2 levels. Among the groups of infected animals, no differences were found between ventilated and non-ventilated animals. VT in combination with ampicillin or the ampicillin monotherapy did not alter Tie2-expression in infected animals.

**Pulmonary Tie2 receptor phosphorylation was not increased by Vasculotide.** Analyzing protein isolated from lung homogenates, VT treatment had no significant effects on Tie2 receptor phosphorylation (Fig. 5A–D). Compared to non-ventilated, non-infected animals, the qualitative and quantitative evidence of
Figure 2. Combination treatment of VT and ampicillin improved histological lung damage. VT (500 ng/100 µl i.v.) or PBS, and ampicillin (0.4 mg/kg bw i.p.) or 0.9% saline were administered 22 h after S. pn. infection (control: PBS + hyaluronidase). The 6-h ventilation phase began 24 h p.i. A second dose of VT was administered 1.5 h prior to termination of the experiment. Histological features of pneumonia and VILI were assessed semiquantitatively by scoring (0 = no damage, 1 = minimal damage, 2 = mild damage, 3 = moderate damage, 4 = severe damage). (A) Combined treatment with VT was associated with higher lung volumes, fewer consolidated areas and diminished edema formation (asterisks in bottom panels). Representative images of centrally placed cross sections of whole lungs are shown. (B, D) Percentage of lung area affected by VILI (B) or pneumonia (D). (C, E) Severity of VILI (C) and pneumonia (E) in affected lung areas represented by an overall histological score (VILI 10 features, max. score 40; pneumonia 5 features, max. score 20). (F, G) Alveolar edema formation in VILI-affected lung areas (F) and perivascular edema formation in pneumonia-affected lung areas (G). Values are listed as median + IQR with minimum/maximum values, individual values are shown as dots (n = 4).
phospho-Tie2 decreased due to infection and/or MV. Further, we failed to observe a Tie2-activation after VT-administration.

**Discussion**

In severe murine pneumococcal pneumonia without additional ventilation, we previously observed reduction of pulmonary hyperpermeability by VT as well as by early antibiotic therapy. The present study shows that in *S. pn.* infection combined with MV neither one of the two monotherapies is effective, whereas combination therapy with ampicillin and VT significantly reduces pulmonary permeability and histological lung damage without relevantly suppressing the immune system or affecting the bacterial burden. In our model, pneumonia and ventilation synergistically enhanced inflammation and permeability. We here again observed that MV induces VILI in mice with pneumonia, which adds to pneumonia-evoked lung damage. For C57BL/6 N mice, a tidal volume of 12 ml/kg is most “protective”, as lower tidal volumes would require higher breathing frequencies resulting in even more lung damage. In this clinically relevant scenario, only the combination of both antibiotic therapy with ampicillin and adjunctive therapy with VT was efficient in reducing permeability, further supporting the notion that mechanically ventilated patients with severe pneumonia may benefit from adjunctive barrier-stabilizing therapies.

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**Figure 2.** (continued)
We assume that, in case of additional ventilation, the inflammatory lung damage is so severe that monotherapy with VT or ampicillin can no longer be effective. In our study the different mechanisms of damage seem to reinforce each other, an observation which is also supported by results of Müller-Redetzky et al. demonstrating that lung damage induced by combination of pneumonia and MV leads to a significantly higher inflammation (measured by cytokines and chemokines) as well as permeability than mere addition of these values for infection and ventilation alone. Treatment with ampicillin primarily leads to reduction of systemic inflammation and bacterial dissemination, resulting in reduced systemic cytokine concentrations and lower bacterial burden in the blood and thus stabilizing the pulmonary barrier. However, ampicillin, has no effect on sterile inflammation induced by MV. VT seams to act via direct stabilization of the endothelium without effect on inflammatory mediators and thus could be used in both sterile as well as bacterial inflammation to improve endothelial integrity. Since both agents convey their protective effects through different mechanisms it could be reasonable that a combination might be able to restore efficacy in a situation where respective monotherapies fail.

Figure 3. Pulmonary and systemic immune response was not altered by VT therapy 30 h p.i. Vasculotide (VT) (500 ng/100 µl i.v.) or PBS, and ampicillin (0.4 mg/kg bw i.p.) or 0.9% saline were administered 22 h after Streptococcus pneumonia (S. pn.) infection (control: PBS + hyaluronidase). The 6-h ventilation phase began 24 h post infection (p.i). A second dose of VT together with 1 mg HSA was administered 1.5 h prior to termination of the experiment. Immune cells in the alveolar space were isolated from BALF, stained with antibodies binding leukocyte surface markers and differentiated by flow cytometry. Blood leukocytes were analyzed with a hemocytometer. Cytokines in BALF and plasma were measured by Multiplex Cytokine assay. (A) Neutrophils (PMN) per µl BALF, (B) Neutrophils per µl blood, (C) IL-6 in BALF (pg/ml) and (D) IL-6 levels in plasma (pg/ml). Values are listed as median + IQR with minimum/maximum values, individual values are shown as dots (control n = 4, all other groups n = 7–8, Mann–Whitney-U-Test).

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The formation of pulmonary edema in pneumococcal pneumonia is primarily promoted by two detrimental factors: the disruption of blood-air barrier by bacteria and their virulence factors, and the increased endothelial permeability due to host immune response involving myeloid and non-myeloid cells, cytokines and chemokines released after activation of pathogen recognition receptors (PRRs) resulting in the recruitment of neutrophils and macrophages.
In this study, VT did not affect leukocyte recruitment, cytokine secretion or bacterial burden. Similar results have previously been published for pneumococcal and influenza virus pneumonia. However, other studies have demonstrated a reduction in neutrophil influx and cytokine concentrations. These studies have used different routes of application and prophylactic rather than therapeutic treatments, suggesting that potential immunomodulatory effects of VT may depend on the timing of its initial administration. It is further tempting to speculate that the treatment duration in our study was too short to alter the cellular immune response. Previous work by David and colleagues has shown a reduction of Tie2 expression in endotoxin-induced acute lung injury, which we also observed in MV, and even more pronounced in pneumonia and pneumonia-associated VILI. VT did not increase Tie2 expression in the previous study or the present study, suggesting that permeability reduction is not mediated by Tie2 induction but rather by increased Tie2 activation. Since VT was generated from a synthetic peptide specifically selected for its binding affinity to Tie2, this conclusion seems justified and has already been supported by different other studies. However, we were unable to detect such increased activation in vivo in this study, and a study by Wu et al. concluded that VT utilizes Tie2-independent pathways. Nonetheless, the lack of detection of Tie2 phosphorylation might also be due to methodological difficulties. Phosphorylation could have been impaired or lost to some degree during storage at −80 °C or during protein isolation. As pneumococcal infection strongly decimates Tie2 expression, Western Blot might be a method with insufficient sensitivity to detect differences. Moreover, the time point chosen for our analysis might not be ideal, however, maximum phosphorylation of Tie2 had been observed 20 to 30 min after VT-application in vitro, while David et al. found phosphorylation lasting for 72 to 96 h in vivo, suggesting that 8 h after first and 1.5 h after the last application should be a well-chosen time point. Thus, further research is needed to conclusively determine both Tie2-dependent and possible Tie2-independent VT effects at the cellular level.

Figure 4. Pulmonary and systemic bacterial burden was not altered by VT therapy 30 h p.i. Vasculotide (VT) (500 ng/100 µl i.v.) or PBS, and ampicillin (0.4 mg/kg bw i.p.) or 0.9% saline were administered 22 h after Streptococcus pneumonia (S. pn.) infection (control: PBS + hyaluronidase). The 6-h ventilation phase began 24 h post infection (p.i). A second dose of VT together with 1 mg HSA was administered 1.5 h prior to termination of the experiment. Bacterial burden in BALF and blood was quantified by plating both samples in increasing dilutions on sheep blood agar, counting CFU and thus determining concentration per µl. (A) Bacterial burden in BALF and in blood (B). Values are listed as median + IQR with minimum/maximum values, individual values are shown as dots (all groups n = 7–8, Mann–Whitney-U-Test).
Figure 5. Protein expression and phosphorylation of Tie2 receptor were not influence by VT treatment 30 h p.i. Mice were intranasally infected with *S. pneumoniae* (*S. pn*; 5 × 10⁶ colony-forming units per mouse) or sham-infected as control with phosphate buffered saline ((PBS) + hyaluronidase). Twenty-two h after infection, Vasculotide (VT) (500 ng/100 µl i.v.) or PBS, and ampicillin (0.4 mg/kg bw i.p.) or 0.9% saline were administered and 24 h post infection (p.i.) mice were ventilated for 6 h. A second dose of VT together with 1 mg human serum albumin (HSA) was administered 1.5 h prior to finishing the experiment. (A) To assess Tie2-phosphorylation, proteins were isolated from lung homogenates and Western blot was performed with antibodies for phospho-Tie2 (pTie2), Tie2 (staining after incubation with stripping buffer for detaching the pTie2 antibody from the membranes) and actin as loading control, and developed by infrared imagine. Representative blots for all groups are shown: Membrane 1 (left images) for the non-infected groups, membrane two (right images) for the infected groups. The black line separates the two membranes, dotted lines show the areas where the membranes were cut in two sections before staining. After staining both sections were developed together by infrared imagine. For quantification, the ratio between Tie2 and actin densitometry (B) and pTie2 and actin densitometry (C) was calculated, respectively. The quotient of pTie2 to Tie2 (D) was used to estimate the phosphorylation status of the receptor. Values are listed as median ± IQR with minimum/maximum values, individual values are shown as dots (control n = 4, all other groups n = 8–9; Mann–Whitney–U-Test).
**Conclusion**

In summary, VT reduced VILI in murine pneumococcal pneumonia without affecting pulmonary and systemic immune response or bacterial elimination. Particularly the formation of alveolar edema was diminished in VT-treated mice. Thus, VT may be a promising adjunctive treatment to prevent lung injury in mechanically ventilated patients with severe pneumonia.

**Data availability**

Data are available on request.

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**Figure 5.** (continued)

(B) Tie2 normalized to Actin

|          | MV | S.pn. | Ampicillin | Vasculotide |
|----------|----|-------|------------|-------------|
| S.pn.    | -  | +     | -          | +           |
| Ampicillin| -  | -     | +          | -           |
| Vasculotide| -  | +     | -          | -           |

(C) pTie2 normalized to Actin

|          | MV | S.pn. | Ampicillin | Vasculotide |
|----------|----|-------|------------|-------------|
| S.pn.    | -  | +     | -          | +           |
| Ampicillin| -  | -     | +          | -           |
| Vasculotide| -  | +     | -          | -           |

(D) pTie2/Tie2 ratio normalized to Actin

|          | MV | S.pn. | Ampicillin | Vasculotide |
|----------|----|-------|------------|-------------|
| S.pn.    | -  | +     | -          | +           |
| Ampicillin| -  | -     | +          | -           |
| Vasculotide| -  | +     | -          | -           |

LEGEND:
- solvent
- Vasculotide
References

1. Eurich, D. T., Marrie, T. J., Minhas-Sandhu, J. K. & Majumdar, S. R. Ten-year mortality after community-acquired pneumonia. A prospective cohort. Am. J. Resp. Crit. Care Med. 192, 597–604. https://doi.org/10.1164/rccm.201310-1945OC (2013).

2. Feldman, C. & Anderson, R. Epidemiology, virulence factors and management of the pneumococcus. F1000 Res. 5, 2320 (2016).

3. Feldman, C. & Anderson, R. Community-acquired pneumonia: Pathogenesis of acute cardiac events and potential adjunctive therapies. Chest 148, 523–532. https://doi.org/10.1378/chest.15-0484 (2015).

4. Ewig, S. et al. New perspectives on community-acquired pneumonia in 388 406 patients. Results from a nationwide mandatory performance measurement programme in healthcare quality. Thorax 64, 1062–1069. https://doi.org/10.1136/thx.2008.109785 (2009).

5. Goodman, R. B., Pugin, J., Lee, J. S. & Matthay, M. A. Cytokine-mediated inflammation in acute lung injury. Crit. Care Med. 34, 1947–1954. https://doi.org/10.1097/01.CCM.0000220496.48295.A9 (2006).

6. Brower, R. G. et al. Higher versus lower positive end-expiratory pressures in patients with the acute respiratory distress syndrome. N. Engl. J. Med. 351, 327–336. https://doi.org/10.1056/NEJMoa0232193 (2004).

7. Witzenrath, M. et al. Comparison of two fluid-management strategies in acute lung injury. N. Engl. J. Med. 354, 2564–2575. https://doi.org/10.1056/NEJMoa062200 (2006).

8. Ashbaugh, D. G., Bigelow, D. B., Petty, T. L. & Levine, B. E. Acute respiratory distress in adults. Lancet 2, 319–323 (1967).

9. Slutsky, A. S. Lung injury caused by mechanical ventilation. Chest 116, 95-155 (1999).

10. Webb, H. H. & Tierney, D. F. Experimental pulmonary edema due to lower tidal volumes compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. N. Engl. J. Med. 342, 1301-1308, doi:https://doi.org/10.1056/NEJM200005043421801 (2000).

11. Serpa Neto, A. et al. Association between use of lung-protective ventilation with lower tidal volumes and clinical outcomes among patients without acute respiratory distress syndrome: a meta-analysis. JAMA 308, 1651–1659. https://doi.org/10.1001/jama.2012.13730 (2012).

12. Acute Respiratory Distress Syndrome, N. et al. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury in the absence of pre-existing lung injury in healthy mice. Crit. Care 13, R1. https://doi.org/10.1186/cc7688 (2009).

13. Wiltshire, E. K. et al. Mechanical ventilation using non-invasive ventilation settings causes lung injury in the absence of pre-existing lung injury in healthy mice. Crit. Care 13, R1. https://doi.org/10.1186/cc7688 (2009).

14. Valk, C. E. et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 87, 1117–1180 (1996).

15. Maisonneuve, P. C. et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277, 55–60 (1997).

16. Gavard, J., Patel, V. & Gutkind, J. S. Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. Dev. Cell 14, 25–36. https://doi.org/10.1016/j.devcel.2007.10.019 (2008).

17. Matsuno, T. et al. Angiopoietin-1 requires p190 RhoGAP to protect against vascular leakage in vivo. J. Biol. Chem. 282, 23910–23918. https://doi.org/10.1074/jbc.M702169200 (2007).

18. Benest, A. V. et al. Angiopoietin-2 is critical for cytokine-induced vascular leakage. PLoS ONE 8, e70459. https://doi.org/10.1371/journal.pone.0070459 (2013).

19. Fiedler, U. et al. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat. Med. 12, 235–239. https://doi.org/10.1038/nm1351 (2006).

20. David, S. et al. Effects of a synthetic PEGylated Tie-2 agonist peptide on endotoxic lung injury and mortality. Am. J. Physiol. Lung Cell Mol. Physiol. 300, L851–862. https://doi.org/10.1152/ajplung.00459.2010 (2011).

21. Gutbier, B. et al. Prognostic and pathogenic role of angiopoietin-1 and -2 in pneumonia. Am. J. Respir. Crit. Care Med. 198, 220–231. https://doi.org/10.1164/rccm.201708-1733OC (2018).

22. Parikh, S. M. et al. Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. PLoS Med. 3, e46. https://doi.org/10.1371/journal.pmed.0030046 (2006).

23. Mankhambo, L. A. et al. The role of angiogenic factors in predicting clinical outcome in severe bacterial infection in Malawian children. Crit. Care 14, R91. https://doi.org/10.1186/cc9025 (2010).

24. Siner, J. M., Bhandari, V., Engle, K. M., Elias, J. A. & Siegel, M. D. Elevated serum angiopoietin 2 levels are associated with increased mortality in sepsis. Shock 31, 348–353. https://doi.org/10.1097/SHK.0b013e318186bd06 (2009).

25. Kumpers, P. et al. Excess circulating angiopoietin-2 is a strong predictor of mortality in critically ill medical patients. Crit. Care 12, R147. https://doi.org/10.1186/cc11730 (2008).

26. Ricciuto, D. R. et al. Angiopoietin-1 and angiopoietin-2 as clinically informative prognostic biomarkers of morbidity and mortality in severe sepsis. Crit. Care Med. 39, 702–710. https://doi.org/10.1097/CCM.0b013e3182b6db85 (2011).

27. Van Slyke, P. et al. Acceleration of diabetic wound healing by an angiopoietin peptide mimetic. Tissue Eng Part A 15, 1269–1280. https://doi.org/10.1089/ten.tea.2007.0400 (2009).

28. Kumpers, P. et al. The synthetic tie2 agonist peptide vasculotide protects against vascular leakage and reduces mortality in murine abdominal sepsis. Crit. Care 15, R261. https://doi.org/10.1186/cc10523 (2011).

29. Trieu, M. V. et al. Vasculotide, an angiopoietin-1 mimetic, restores microcirculatory perfusion and microvascular leakage and decreases fluid resuscitation requirements in hemorrhagic shock. Anesthesiology 128, 361–374. https://doi.org/10.1097/ALN.0000000000001967 (2018).

30. Dekker, N. A. M. et al. Vasculotide, an angiopoietin-1 mimetic, reduces pulmonary vascular leakage and preserves microcirculatory perfusion during cardiopulmonary bypass in rats. Br. J. Anaesth. 121, 1041–1051. https://doi.org/10.1093/bja/bja.2018.05.049 (2018).

31. Sugiyama, M. G. et al. The Tie2-agonist vasculotide rescues mice from influenza virus infection. Sci. Rep. 5, 11030. https://doi.org/10.1038/srep11030 (2015).

32. Gutbier, B. et al. Vasculotide reduces pulmonary hyperpermeability in experimental pneumococcal pneumonia. Crit. Care Med. 21, 274. https://doi.org/10.1097/01.CCM.000013045-017-1851-6 (2017).

33. Muller, H. C. et al. Simvastatin attenuates ventilator-induced lung injury in mice. Crit. Care 14, R143. https://doi.org/10.1186/ cc9209 (2010).

34. Muller-Redetzky, H. C. et al. Mechanical ventilation drives pneumococcal pneumonia into lung injury and sepsis in mice: Protection by adrenomedullin. Crit. Care 18, R73. https://doi.org/10.1186/cc13830 (2014).

35. Reppe, K. et al. Pulmonary immunostimulation with MALP-2 in influenza virus-infected mice increases survival after pneumococcal superinfection. Infect. Immun. 83, 4617–4629. https://doi.org/10.1128/ai.00948-15 (2015).
38. Dames, C. et al. Miniaturized bronchoscopy enables unilateral investigation, application, and sampling in mice. Am. J. Respir. Cell Mol. Biol. 51, 730–737. https://doi.org/10.1165/rcmb.2014-0052MA (2014).

39. Muller-Redetzky, H. et al. Neutralizing complement C5a protects mice with pneumococcal pulmonary sepsis. Anesthesiology 132, 795–807. https://doi.org/10.1097/ALN.0000000000003149 (2020).

40. Berger, S. et al. Delay in antibiotic therapy results in fatal disease outcome in murine pneumococcal pneumonia. Crit. Care 22, 287. https://doi.org/10.1186/s13054-018-2224-5 (2018).

41. Muller-Redetzky, H., Lienau, J., Sutorp, N. & Witzenrath, M. Therapeutic strategies in pneumonia: Going beyond antibiotics. Eur. Respir. Rev. 24, 516–524. https://doi.org/10.1183/16000617.0034-2015 (2015).

42. Muller-Redetzky, H.C., Suttorp, N. & Witzenrath, M. Dynamics of pulmonary endothelial barrier function in acute inflammation: Mechanisms and therapeutic perspectives. Cell Tissue Res. 355, 657–673. https://doi.org/10.1007/s00441-014-1821-0 (2014).

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Competing interests
Steven Chackowicz, Doug Hamilton and Paul Van Slyke are named as inventors on several patents related to the Vasculotide technology. Doug Hamilton and Steven Chackowicz are officers in Vasomune Therapeutics. All other authors declare that they have no competing interests.

Additional information
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