COMBINATION THERAPY OF ANTITHROMBIN AND ALPHA-1 PROTEINASE INHIBITOR IN A MURINE MODEL OF DIRECT ACUTE LUNG INJURY

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SUMMARY

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is characterized by hypoxic respiratory failure following an either direct (e.g. pneumonia) or indirect (e.g. sepsis) pulmonary insult. Dealing with hypoxemia remains the major challenge at bedside and is currently approached with lung-protective mechanical ventilation since specific treatment targeting the pathogenesis of ARDS is lacking.

Pathological hallmarks of ARDS are uncontrolled inflammation and coagulation leading to alveolar and interstitial edema, as well as infiltration of inflammatory cells in the alveolar space. Various cytokines, proteases and their inhibitors play a pivotal role in modulation of inflammation. Recent evidence suggests that proteinase inhibitors antithrombin (AT) and α1-protease-inhibitor (A1PI), may exert anti-inflammatory, anti-coagulant and immunomodulatory properties and may even have synergistic beneficial effect in diminishing inflammation. We hypothesize that combination therapy of AT and A1PI (COMBO therapy) has beneficial effect on lung damage and inflammatory markers in a murine model of direct acute lung injury.

METHODS

Balb/c mice (n=44) received 5 mg/kg LPS (E. Coli, 0127:B8) intranasally (i.n.) simulating direct acute lung injury (T=0). One hour later (T=1) the intervention drugs AT, A1PI only and COMBO therapy were administered intraperitoneally (i.p.) and at T=6 all mice were sacrificed. State of inflammation was determined using Luminex to quantify levels of inflammatory markers TNFα, IL-1β, IL-6, KC and IL-10 in plasma and bronchoalveolar fluid (BALF), and assessing total protein levels and neutrophil influx in BALF.

RESULTS

Intranasal LPS administration induced lung injury, evidenced by markedly increased levels of TNF-α and IL-1β in BALF and increased vascular permeability in all intervention groups and the control group compared to the vehicle group ($P<0.05$, for all groups compared to vehicle). This study did not detect any differences with regards to markers of inflammation, neutrophil migration or alveolar-capillary permeability between the intervention groups compared to the control group.

CONCLUSION

This study investigated the combination therapy of AT and A1PI in a murine model of direct ARDS. No differences in markers of inflammation, neutrophil migration or alveolar-capillary permeability were detected between the intervention groups and the saline control group, not showing a beneficial effect of COMBO therapy. Future experiments are needed to test COMBO in different models, including pretreatment and an indirect lung injury model, to draw firm conclusions regarding the efficacy of COMBO.
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INTRODUCTION

Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) is characterized by hypoxemic respiratory failure with an acute onset from an either direct or indirect pulmonary insult. Direct insults include for example pneumonia, aspiration of gastric contents or mechanical ventilation, whereas indirect insults are due to for example sepsis, acute pancreatitis or drug overdose. (1-4)

First symptoms arise within a week after the initial event and the course of disease is characterized by a rapid worsening of the symptoms, such as dyspnea, cyanosis and diffuse crackles. According to the Berlin Criteria from 2012, clinical diagnosis is confirmed by a chest radiography showing bilateral alveolar infiltrates, arterial hypoxemia and severity of the syndrome is evaluated by assessing Pa02/Fi02 ratio. (5)

Inflammation and coagulation have been recognized as main pathophysiological characteristics of ARDS. Lung injury is characterized by alveolar and interstitial edema due to increased vascular permeability, as well as infiltration of macrophages, neutrophils and red blood cells into the alveolus as signs of epithelial and endothelial injury. In addition, extravascular intra-alveolar fibrin deposition – better known as hyaline membranes – are formed as product of the coagulation cascade. (6, 7) Consequently, impaired gas exchange within the lungs leads to respiratory failure in the patient.

The incidence of ARDS ranges from 10-40 per 100,000 persons/year and mortality approaches approximately 40%. (1) Dealing with severe hypoxemia remains the major challenge in the clinics and is approached with protective mechanical ventilation using low tidal volumes, if possible fractions of inspired oxygen (FiO2) below 60%, and for more severe cases prone positioning. (2, 3, 8) Nonetheless, since the first description of ARDS in the 1960s, therapy has primarily been supportive since specific treatment targeting the pathogenesis of ARDS is lacking.

Uncontrolled inflammation and coagulation in ARDS

Hallmarks of ARDS are uncontrolled inflammation and coagulation. Both mechanisms are brothers in arms playing a crucial role in the innate immune response and also in resolution of injury or infection. However, if these mechanisms are not regulated as desired, they can aggravate damage to the organs, such as in ARDS. (9-11)

Once the lungs are exposed to foreign substances, for example microbial pathogens, alveolar macrophages are activated releasing pro-inflammatory cytokines, such as TNFα, Interleukin (IL) -1, -6 and IL-8, launching the acute phase response of the innate immune system. TNFα exhibits an important role. Firstly, it causes changes in the cell-to-cell junctions leading to an increased vascular permeability. Secondly, it acts mainly on the pulmonary vascular endothelium leading to the expression of cellular adhesion molecules, stimulating adhesion of leucocytes at the site of injury. (12) Besides, activated alveolar macrophages release IL-8, a potent chemoattractant of neutrophils directing them to the site of injury. Neutrophils are part of the first-line innate immune response and in fact, can also pass naturally into the epithelial alveolar space. However, once they are activated by inflammatory cytokines or pass in large numbers due to injured vessel walls, they rather cause damage to the alveoli than combating pathogens. (3, 13, 14)
The azurophilic granules of neutrophils contain, amongst others, oxidants and serine proteases, e.g. neutrophil elastase (NE), cathepsin G and proteinase 3. (15) Under physiological circumstances, serine proteases - such as NE - play a vital role in migration of neutrophils and resolution of infections. NE usually degrades foreign molecules phagocytosed by neutrophils, but once NE is released in the extracellular compartment, it is fully active and catalyzes various proteolytic processes. For example, it can exert destructive effects on extracellular matrix proteins, such as proteoglycans, as well as important plasma proteins, such coagulation factors or fibrin, causing harmful effects on vascular permeability and regulation of coagulation. (12, 16, 17)

Direct damage to lungs or pulmonary vasculature not only leads to activation of the acute phase and immune response, but also coagulation pathways are activated resulting in hypercoagulability.

Injured pulmonary endothelium activates platelets as first step of hemostasis. Platelets accumulate at the site of injury and form a loose platelet plug. To stabilize these platelet plugs, coagulation factors are released to activate the coagulation cascade as second step of hemostasis. (18) Besides, coagulation cascade is also directly activated by release of tissue factor (TF) from various cells, such as macrophages, in response to lung injury. Activation of TF and its binding to coagulation factor VII is known as the extrinsic pathway of the coagulation cascade resulting in synthesis of fibrin via various intermediate products. (19, 20) The protease thrombin is one of the intermediates and it plays a pivotal role in modulation of various pathways and depicts the coherent connection between inflammation and coagulation. Thrombin is the major activator of the proteinase-activated receptor 1 (PAR1). (20) This receptor is highly expressed in the lungs and thereby mediates various inflammatory and coagulation responses, such as recruiting inflammatory cells, releasing pro-inflammatory cytokines, influencing microvascular permeability, coagulation cascade and increasing levels of PAI-1. (20, 21) While fulfilling its role within the coagulation cascade,
activation of thrombin results in the formation of fibrin - the main product of the coagulation cascade, strengthening the weak platelet plug with cross-linked fibrin strands.

Notably, next to intensive activation of coagulation followed by rapid consumption and diminished production of coagulation factors, inhibition of the fibrinolytic system plays an important role in hypercoagulopathy. Hence, fibrinolysis and anticoagulation are impaired, as shown by the fact that patients with ARDS show high concentrations of plasminogen activation inhibitor (PAI-1) blocking fibrinolysis and low levels of antithrombin.

Serine proteases and serine protease inhibitors: characteristics, function and role in ARDS

As described above, ARDS is characterized by a coherent interplay of excessive release of pro-inflammatory cytokines and (coagulation) proteases yielding a shift from an adequate innate immune response to a harmful inflammation and coagulopathy.

Looking on the biochemical level, serine proteases are enzymes characterized by the amino acid serine, which serves as an active site to cleave specific peptide bonds in proteins. Various groups of serine proteases exist in humans being involved in various physiological processes, such as digestion (e.g. trypsin), immune response (e.g. NE) and blood coagulation (e.g. thrombin). In order to control these enzymes, natural inhibitory systems warrant dysregulation of proteolytic processes. Serine protease inhibitors, also known as serpins, are a superfamily of proteins possessing a unique mechanism to irreversibly inhibit serine proteases acting as anti-protease. Thereby, serpins induce an unusual conformational change in the protein folding of the proteases while binding to them and being a sort of ‘suicide inhibitor’. (22, 23) Consequently, inactive protease-antiprotease complexes are formed and later cleared from the circulation. Serpins are characterized by a highly ordered tertiary structure conserved throughout evolution as these kind of proteins can also be found in animals, plants, viruses and bacteria. In humans, around 40 different types of serpins are annotated of which AT and A1PI are the ones identified early and studied most. (24)

In ARDS patients, high levels of proteases, such as NE and thrombin, contribute significantly to the intensity of the response on inflammation and coagulation. (25) In 1984 the first research has been conducted to assess the role of serine proteases in case of acute lung injury by administering mini pigs the proteases thrombin and elastase intravenously. Thereupon, they developed ‘progressive respiratory failure with an increase of pulmonary vascular resistance, a decrease in cardiac output, pulmonary leukostasis, and a disturbance of blood coagulation leading to hypercoagulability’ underlining the deleterious effects of uncontrolled proteolytic processes. (26) In 1988, administration of A1PI decreased cytotoxicity in bovine pulmonary endothelial cells suggesting proteolytic mechanisms involved in cell damage. In the same year, the antiproteases antithrombin (AT) and A1PI have been combined in an endotoxemic indirect ARDS model in sheep. They reasoned that AT, as an anticoagulant, would improve coagulopathy and that A1PI would block NE, thereby preventing inactivation of AT and decreasing lung damage by elastase itself. Importantly, they proved a beneficial synergistic effect of this combination pre-treatment in attenuation of microvascular protein permeability and transvascular protein flow, as well as a preventive effect in decrease of systemic arterial P0₂. AT alone only showed limited effect and A1PI none. (27)

Hence, under physiological circumstances serpins control proteolytic processes; unfortunately, in case of ARDS those systems seem to fail allowing release of excessive pro-inflammatory substances.
Antithrombin (AT; formerly known as antithrombin III or heparin co-factor I) is a serpin holding two important molecular segments. A ‘reactive centre’ connecting with the active serine site of the coagulation factor inhibiting its functioning and a ‘heparin-binding site’. (28) AT mainly controls the activity of thrombin (coagulation factor II), but inhibits also various other coagulation factors. Thrombin is major activator of PAR1 receptors and in case of high concentration can exert deleterious effects on endothelial cell permeability and activate various inflammatory and coagulation pathways; however, low concentrations of thrombin (<40pM) have found to be even barrier-protective. (19, 29, 30)

If heparin or heparin-like substances, such as glycosaminoglycans (GAGs), bind to the heparin binding site of AT, they are able to accelerate the action of AT a 1000-fold to a rapid coagulation inactivator. (31) Importantly, AT gets easily inhibited itself by serine proteases, such as NE. (32)

Next to inhibition of the coagulation cascade leading to decreased fibrin production, AT has proven to possess also other anti-inflammatory properties, as reviewed by Roemisch. (33) Preclinical studies testing AT in rat models have shown a significant reduction in leucocyte infiltration of the lungs and decreased neutrophil activity measured by myeloperoxidase suggesting a decrease in release of oxidants. These effects are based upon binding of AT via its heparin-binding site to an extracellular matrix protein, heparin sulfate proteoglycans (GAGs) being responsible for signal transduction. By means of binding of AT to GAG receptors on endothelial cells or on neutrophils, AT may modulate neutrophil mitigation and prevent premature neutrophil activation. (34-36)

In addition, preclinical studies have found a decrease in inflammatory parameters, such as IL-6 and TNFα, suggesting that AT can bind to various anti-inflammatory receptors via its heparin-binding site and inhibit inflammatory pathways by downregulating the inflammatory response within the pulmonary tissue. (37, 38) Notably, anti-inflammatory properties could not always be demonstrated - although anti-coagulant properties of AT are always present, suggesting that anti-coagulant and anti-inflammatory properties of AT may have different mechanism of action and therefore independent from each other. (39)

As noted earlier, activation of anti-inflammatory pathways by AT mainly happens via its heparin-binding site. Hence, one may wonder about the effects in presence of heparin. In a sheep model combination therapy of AT with heparin and also tissue plasminogen activator (TPA) improved gas exchange, increased lung compliance and reduced pulmonary edema. However, with triple therapy there is no inhibitory effect on neutrophil activation or pulmonary fluid accumulation as with AT therapy alone, suggesting that combination therapy seems to blunt the anti-inflammatory effects of AT with heparin playing a crucial role in this process. (40)

Looking at clinical evidence, in 2015 Allingstrup and his co-workers conducted a systematic review with meta-analysis and trial sequential analysis analyzing data from 30 randomized controlled trials including critically ill patients with severe sepsis and/or disseminated intravascular coagulation. All trials were with high risk of bias. Nonetheless, according to Allingstrup ‘there is insufficient evidence to support AT III substitution in any category of critically ill patients… AT III did not show impact on mortality, but increased the risk of bleeding.’ The study also looked at the heparin issue, but did not reveal any significant differences in intervention effect of AT with concomitant use of heparin or without. (41) However, there has not been a subgroup analysis with ARDS patients. Considering the very heterogeneous patient group more targeted studies are necessary to reveal which patient subgroups may benefit from AT therapy. (2)
Interestingly, a recent study showed that levels of AT are lower in patients with ARDS than compared to controls suggesting rapid consumption of the protease inhibitor in case of acute lung injury. Hence, this may be a group benefitting from AT therapy substituting a deficient protease inhibitor.

In summary, AT exerts its prominent anti-coagulant characteristics via its ‘reactive center’ binding and inhibiting various coagulation factors, whereas anti-inflammatory properties seem to be modulated via the ‘heparin-binding site’. Hence, recent evidence suggests that administration of heparin may blunt anti-inflammatory properties of AT.

**Alpha-1-proteinase-inhibitor: characteristics and function and role in ARDS**

Alpha-1-proteinase-inhibitor (A1PI, also known as alpha-1 antitrypsin) is the most abundant serpin found in plasma. The best-known function of A1PI is inhibition of neutrophil elastase (NE) accounting for 92% of its inhibition. But it also blocks other proteolytic enzymes, such as trypsin and thrombin, acting as an ‘anti-protease screen’. (42) Besides, A1PI is an acute phase protein synthesized by hepatocytes in response to inflammation. (43) Notably, A1PI is very vulnerable to oxidants, such as reactive oxygen species, causing misfolding of the protein structure leading to inactivation and loss of function. (44) Nonetheless, A1PI not only exhibits anti-proteolytic properties, but has also shown protective effects on inflammation, apoptosis and immunomodulation. (reviewed by Ehlers) (45)

Preclinical murine and rat models detected lower levels of pro-inflammatory cytokines, such as TNFα, IL-1β and IL-5, -6 and -8, and even an increase of anti-inflammatory cytokine IL-10 when A1PI was administered. (42, 46-48) Additionally, A1PI seems to reduce cell apoptosis as shown by decreased levels of caspase-3 and -8, as well as to lower oxidative stress and release of superoxides by neutrophils in vitro. (49) A decrease in neutrophil influx has been described in line with a reduction in neutrophil activity, as well as a decrease in amount of lung hemorrhage. (46, 47, 50-52) Besides, preclinical models inducing lung damage with bacteria detected significant lower concentrations bacteria in the blood in groups treated with A1PI and noticed a decrease in mortality of mice. (53)

Taking a look at the clinical perspective, no trials have determined the effect of A1PI in acute lung injury or other critically ill patients. Nonetheless, various trials have been conducted using Sivelestat, a selective neutrophil elastase inhibitor, to assess outcome of patients with acute lung injury. Outcomes of those trials are ambiguous, as some studies did not find any differences in mortality or ventilator-free days, whereas some studies showed an improved outcome of these patients. The STRIVE study was even stopped prematurely due to an increase in long-term mortality in the intervention group. (2, 54-56) However, it is important to bear in mind that these trials only give insight in inhibition of neutrophil elastase and none of the above mentioned anti-inflammatory, anti-apoptotic and immunomodulatory properties of A1PI.

Hence, preclinical studies have proven ‘serpin’ effects of A1PI, such as anti-inflammatory, anti-proteolytic, anti-apoptotic and immunomodulatory properties, suggesting a potential role in treatment of inflammatory conditions, such as ARDS, by inhibiting neutrophil serine proteases.

In summary, ARDS remains a major cause of morbidity and mortality on ICUs and recent research reveals more about pathogenesis of the syndrome. Despite all efforts to implement
pharmacological treatment modalities targeting coagulation and/or inflammation such as activated protein C (APC) or glucocorticoids to reduce lung injury. Results from trials studying these drugs are conflicting. Many others have proven to be ineffective as a therapeutic option. AT and A1PI are serine protease inhibitors proven to exert anti-coagulant and anti-inflammatory effects. Evidence suggests a potential synergistic therapeutic effect of combination treatment of these serpins in modulating inflammation and coagulation pathways in ARDS patients, thereby attenuating lung injury.

**HYPOTHESIS AND AIM OF THE STUDY**

This study aims to investigate the effect of combination therapy of AT and A1PI, named COMBO, on inflammation in a murine ARDS model. We hypothesize that combination therapy reduces inflammation and decreases vascular permeability of the lungs compared to a control group receiving normal saline as intervention.

**METHODS**

**Animals**

The experiments were conducted under protocols approved by the Animal Care and Use Committee of the Academic Medical Center. Male BALB/c mice, aged 8-12 weeks and with weights ranging from 20-25 grams, were bought from Charles River Laboratories International. The animals were used in compliance with Institutional Standards for Use of Laboratory Animals and were handled one week before experiments to diminish stress activation. The mice were housed in a specific pathogen-free facility, and had access ad libitum to food and water.

**Study groups and interventions**

The mice (n=44) were randomized into five different intervention groups (n=8 per group, see below) and one vehicle group (n=4). Experiments were spread over 7 days, each day / per experiment 6-7 mice were handled and randomly assigned to each group.

**Interventions**

<table>
<thead>
<tr>
<th>Group</th>
<th>Interventions</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Antithrombin (AT): 250U/kg in 0.5ml NaCl 0.9% (Anbinex™, Grifols)</td>
</tr>
<tr>
<td>2</td>
<td>Alpha-1 protease inhibitor (A1PI): 1.5mg/mouse in 0.5ml NaCl 0.9% (Prolastin™, Grifols)</td>
</tr>
<tr>
<td>3</td>
<td>COMBO (AT + A1PI): AT 250U/kg + A1PI 1.5mg/mouse in 0.5ml NaCl 0.9%</td>
</tr>
<tr>
<td>4</td>
<td>COMBO + heparine: AT 250U/kg + A1PI 1.5mg/mouse + Heparin 100U/kg in 0.5ml NaCl 0.9%</td>
</tr>
<tr>
<td>5</td>
<td>Hydration (0.5ml NaCl 0.9%)</td>
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Model of direct acute lung injury

At baseline, mice were weighed and labelled.
At timepoint T= -1, mice were sedated using isoflurane 2-4%, following intranasal inoculation (i.n.) of 5 mg/kg LPS (Escherichia Coli, serotype: 0127:B8, Sigma Aldrich, St. Louis, MO, USA) diluted in 50µl NaCl 0.9% or 50µl NaCl 0.9% (vehicle). Appropriate dosage of LPS to achieve an adequate lung injury model was assessed in a prior pilot trial.
One hour after inoculation of LPS or NaCl 0.9% (T=0), mice received the intervention drugs intraperitoneally (i.p.).
Five hours after administration of the intervention (T=5), mice were anesthetized using a bolus of 9.5µl KDA per gram bodyweight intraperitoneally (KDA: 1.26mL 100mg/mL Ketamine (Anesketin, EuroVetAnimal Health B.V., Bladel, The Netherlands) + 0.2mL 0.5mg/mL Dexmedetomidine (Pfizer Animal Health B.V., Capelle a/d Ijssel, The Netherlands) + 1mL 0.5mg/mL Atropine (Pharmachemie, Haarlem, The Netherlands) in 5mL 0.9% NaCl) and sacrificed by exsanguination from the carotid artery.

Whole blood was drawn using a heparin-coated syringe and subsequently centrifuged for 10 minutes at 4000rpm at 4°C (Eppendorf, microcentrifuge). Plasma was obtained and stored at -80°C for further analyses. Following exsanguination, sternum of the mouse is opened surgically and lungs with bronchi and trachea are resected. To obtain bronchoalveolar lavage fluid (BALF) the right main bronchus is clipped and a 1ml syringe is connected to the trachea. The left lung is lavaged three times with 0.5ml normal saline. BALF is centrifuged for 10 minutes at 2000 rpm at 4°C (Eppendorf, microcentrifuge) and stored at -80°C for further analyses. The lobes of the right lung are instilled with 4% paraformaldehyde and embedded in paraffin for histopathological assessment.
**Outcome measures**

a) **Inflammatory cytokines**
Inflammatory cytokines and endothelial markers (tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6, Keratinocyte-derived chemokine (KC), and Intracellular adhesion molecule (ICAM)-1) were assessed in plasma and TNF-α and IL-1β also BALF using the multi-analyte bead assay Luminex (Bio-techne, R&D systems, Minneapolis, MN, USA) according to manufacturer’s instructions. Detection limits for each biomarker are the following: TNF-α: 1.13, IL-1β: 100.55, KC: 24.02, IL-6: 18.84, ICAM-1: 210.54.

b) **Total protein count**
Total protein levels in BALF were assessed using Bradford Protein Assay Kit (OZ Biosciences, Marseille, France) according to official instructions with bovine serum albumin as standard and a detection limit of 50µg/ml.

c) **Neutrophil influx**
Differential counts were done on cytospin preparations (Shandon CytospinR 4 Cytocentrifuge; Thermo Electron Corporation) and stained with a modified Giemsa stain (DiffQuick, Dade Behring AG, Duedingen, Switzerland). Neutrophil influx is defined as the number of neutrophils according to total amount of inflammatory cells on cytospin preparations.

d) **Lung injury scoring**
The right lung is instilled with 4% paraformaldehyde and embedded in paraffin. Subsequently, slices at 4-µm thickness were stained with haematoxylin and eosin (Sigma-Aldrich Ltd) and scored by a blinded pathologist specialized in lung injury in mice. Lung injury was quantified on following items: endothelialitis, bronchitis, edema, thrombus, pleuritis and alveolar hemorrhage. Thereby a scoring system of ‘none, mild, moderate and severe’ (0, 1, 2 and 3, respectively) was used.

**Statistical analysis**
A sample size of 8 mice per group were needed, using a power of 0.8 with a chance of 0.904 (effect size 1.85) with double significance level of 0.05 to proof that observed inflammation parameters, such as cytokine levels are lower in an intervention group than in the control group.
Data are expressed as median (interquartile range) or box plot (median), as appropriate. As data were not normally distributed Kruskal-Wallis analysis was performed to allow for multiple comparisons, following uncorrected Dunn’s test for multiple comparisons – either against the saline control group or against the vehicle group. In figures, box plots were used with the lower hinge defining the 25th percentile, middle as 50th percentile, and upper hinge as the 75th percentile. Whiskers define the lowest and highest observation. P < 0.05 is considered statistically significant. Statistical analyses were performed using GraphPad Prism version 7.0a (Graphpad Software; San Diego, CA).
RESULTS

**LPS-induced acute lung injury**

All animals survived the experiment. Intranasal LPS inoculation resulted in a marked increase of inflammatory markers (TNF-α and IL-1β) in the bronchoalveolar lavage fluid of all groups compared to vehicle group, $p < 0.05$, for all. (Figure 1-B and 1-D) Besides, neutrophil count is markedly increased within the alveolar compartment, $p < 0.05$, for all interventions. (Figure 3 and 5)

![Graphs showing TNF-α and IL-1β levels in BALF and plasma](image)

*Figure 1. A-D: Markers for the acute phase response in plasma and BALF. A: TNF-α in BALF. B: TNF-α in plasma. C: IL-1β in BALF. D: IL-1β in plasma. For A - D non-parametric tests were used. (*$p < 0.05$)*
**Acute phase response**

Intranasal LPS administration resulted in markedly elevated levels of TNF-α and IL-1β in BALF of mice compared to the vehicle group, whereas plasma levels of TNF-α and IL-1β were low in all groups. (Figure 1) Statistical significant difference in BALF levels of IL-1β was reached between the group receiving COMBO and the control group. (Figure 1-D) Plasma levels of IL-6 were elevated in all groups receiving LPS i.n. compared to the vehicle group without any differences between the intervention groups compared to the saline control group. (Figure 2-A)

**Lung histopathology**

No changes in histopathology could be detected between the vehicle group and the groups receiving LPS i.n. Besides, between the intervention groups no difference could be detected.

![Figure 2](image)

**Figure 2:** Marker for inflammation (IL-6) and score for histopathological lung damage. A: IL-6 levels in plasma. B: Histopathology score for lung damage (endothelialitis, bronchitis, edema, thrombus, pleuritis and alveolar hemorrhage).

**Neutrophil activation and migration**

Plasma levels of neutrophil chemotactic factor KC were elevated in all groups receiving LPS i.n. compared to the vehicle group without any differences between the intervention groups compared to the control group. (Figure 2-B) Plasma levels of ICAM-1 were increased in all LPS i.n. groups compared to the vehicle group. The intervention group receiving A1PI only shows a trend for lower levels of ICAM-1 without reaching statistical significance compared to the control group. (Figure 2-D) Neutrophil influx in the alveolar compartment is markedly elevated in all LPS groups compared to the vehicle group. (Figure 3-B and 4)
Figure 3: Markers for neutrophil chemotaxis (KC) and endothelial activation (ICAM-1) in plasma. A: IL-6. B: KC. C: ICAM-1.

Alveolar-capillary permeability

Total levels of protein within BALF were elevated in all groups, especially in the groups receiving LPS i.n. All LPS groups showed markedly increased influx of inflammatory cells, such as neutrophils compared to the vehicle group. (Figure 4 and 5) There was no difference in alveolar-capillary permeability between the intervention groups compared to the saline control group.

Figure 4: Markers of alveolar-capillary permeability. A: Total protein levels in BALF; B: Neutrophil influx (%) in BALF.
**DISCUSSION**

This study aimed to investigate the effect of combination therapy with AT and A1PI on inflammation, neutrophil migration and alveolar-capillary permeability in a murine model of pulmonary ARDS.

The major outcome of this study is that COMBO treatment did not affect inflammation, neutrophil migration or alveolar-capillary permeability in this pulmonary ARDS model. Indeed, no differences in outcome could be identified between single therapy with AT or A1PI, COMBO therapy or COMBO therapy combined with heparin compared to the saline control group.

Notably, this present study suggests that COMBO therapy does not affect inflammation at an early, acute stage five hours after administration of the intervention when interventions are administered one hour after induction of injury. Consequently, questions remain unanswered if therapy may show effect at a later stage of the disease or if administration follows at a different time point. This study gives insight in inflammatory process of the direct ARDS model and the effect of administration of single therapy with AT, A1PI and COMBO therapy in the acute early phase of lung injury.

To evaluate the results of the study properly, strengths and limitations of the study need to be discussed within the frame of performance of animal studies. Thereby, it is essential to bear in mind that animal models are an artificial, schematic approach to simulate a clinical situation with its own limitations.

Most importantly, the model used to induce lung injury, time schedule of the experiment and intervention administration need to be addressed.
Various preclinical studies have been conducted simulating ARDS in a murine model and various different models are currently used. In this study we chose a model simulating direct pulmonary injury to investigate the effect of COMBO therapy by intranasal administration of lipopolysaccharide (LPS).

Administration of bacterial LPS, an endotoxin derived from gram-negative *E. Coli*, is a frequently used experimental model and simulates effects of a gram-negative bacteria in animals and humans. (57, 58) Importantly, Balb/c mice have been chosen for the experiments as this strain is known to be very sensitive to LPS-induced injury. (58) In the early phase, LPS instillation gives rise to various inflammatory parameters in the BALF. Also in our study intranasal administration of LPS resulted in pulmonary inflammation, as shown by the rise of inflammatory cytokines TNF-α and IL-1β in the alveolar compartment. (Figure 1, 3 and 5) Besides, LPS resulted in mild endothelial and epithelial injury, as shown by elevated total protein levels and influx of neutrophils within the alveoli in this study.

Between animal ARDS models a distinction is made between direct pulmonary ARDS models and indirect, or non-pulmonary, ones. This is based on the fact that ARDS is a syndrome caused by direct pulmonary insults, as well as indirect pulmonary insults. This present study is working with a murine pulmonary ARDS model by inducing ARDS directly instilling LPS intranasally. While administering LPS intraperitoneally followed by mechanical ventilation, a so called ‘double-hit model’ is created inducing ARDS in an indirect way. It may be hypothesized that an indirect ARDS model may achieve a greater effect of the interventions as this model may simulate a systemic state of inflammation and treatment may also be administered systemically via the peritoneum.

With regards to the time schedule of this study two major factors need to be taken into account.

First of all, this study investigates the effect of AT and A1PI within the early, acute phase of ARDS, as mice are sacrificed after five hours of treatment. With regards to AT, a preclinical study using rats did not detect any changes in inflammatory markers after sacrifice of the animals after 4 hours, which is in line with the results of this present study. (59) Besides, an increased inflammatory response has been noted, as IL-1β levels were significantly higher within the alveolar compartment in the group receiving COMBO therapy than compared to the control group, suggesting that administration of COMBO might even exert a pro-inflammatory effect instead of having a protective effect on inflammation as hypothesized. This may be partly due to the role of A1PI in the acute phase response, but A1PI administration alone did not raise markers of inflammation significantly.

Interestingly, preclinical studies finding anti-inflammatory effects of A1PI have been conducting experiments of 24 or 48 hours in total suggesting that A1PI may exert its anti-inflammatory effects in the late phase. (51, 53, 60) Additionally, one trial determining differences in survival of mice with a *P. aeruginosa* pneumonia after pre-treatment with A1PI i.p. noticed a beneficial effect of A1PI after 75 hours. (53) Nonetheless, previous studies investigating the effect of anticoagulant drugs in murine ARDS models detected significant differences between the intervention drugs after a duration of the experiment of five hours. (61)

Besides, we chose to give interventions as a treatment option one hour after induction of lung injury to simulate a clinical situation. Notably, many preclinical studies investigating the effect of AT or A1PI were testing these agents as pretreatment options or concomitant with induction of injury. (36, 39, 47, 50, 52, 62) Only few studies have studied AT and A1PI as treatment and the variation within other variables, such as sort of animal or outcome measures, poses major difficulties for comparison of the studies with the present study. (63-65)
Hence, this present study confirmed previously conducted studies with regards to the fact that there are no changes in levels of inflammation when AT is administered. Yet, it remains unclear which time point may be most appropriate to administer the interventions and which time point may be most suitable to end the experiment for determination of an effect of AT and A1PI in an ARDS model.

Furthermore, administration of the interventions is a major point of interest.

First of all, it is apparent that there are a variety of different routes of medication administration between previously conducted preclinical studies studying the effect of AT and A1PI in lung injury models. In the human setting AT and A1PI are normally administered intravenously, whereas since recently A1PI is also available as inhaled powder or nebulizer. It is important to realize that little is known about the pharmacokinetics of AT and A1PI if not administered intravenously. Interestingly, animal studies focusing on lung injury both agents have been administered intratracheally, intranasally, intravenously, intraperitoneally and using a nebulizer. (47, 51, 63, 66, 67) A1PI has been administered intraperitoneally in previously conducted animal trials showing improved survival of mice receiving 2mg A1PI after 75 hours and systemically reduced levels of TNF-α and caspase-1 and -3 compared to control groups. Nonetheless, these studies were not directed to direct lung injury, but determined the effect of A1PI in liver injury and ischemic reperfusion injury of the kidneys. (43, 68)

Secondly, drug dosing regimens are another point of discussion. As mentioned before most preclinical studies investigating the effect of AT have been conducted with rats and accordingly, drug dosages are tested within rats and not in mice. In rat models, a dosage of 250IU/kg have been proven to reduce coagulation and alveolar neutrophil infiltration, but did not have beneficial effect on inflammation. (59, 62) With regards to A1PI, the dosage in question has been used in murine models before showing significant differences between the intervention and control group. (60, 67)

Consequently, one of the study limitations is the lack of knowledge concerning pharmacodynamics and pharmacokinetics of AT and A1PI with respect to the route of administration and adequate dosing regimens.

This study did not detect any differences between the intervention groups and the control group, but as explained many factors regarding the experiment need to be taken into account to interpret the results.

With respect to future experiments it is important to differentiate between experiments optimizing details of the murine pulmonary ARDS model and experiments further investigating COMBO therapy in ARDS models.

First of all, experiments need to be conducted to gain insight in pharmacokinetics of AT and A1PI in murine models. With regards to intraperitoneal administration of AT and A1PI, concentration of the agents might be determined within the bronchoalveolar fluid to assess in which concentrations these agents actually reach the lungs and after how many time and otherwise, nebulization of the interventions needs to be examined. Accompanying investigations concerning the route of administration, various dosages of the drugs should be tested as well to ensure sensitivity in the animal used for further experiments.

Additionally, physiologic experiments might be of additional value to gain knowledge about normal and abnormal values of AT and A1PI and its substrates in the course of time. Thereby, levels of the agents should be measured systemically, as well as in bronchoalveolar fluid and both in healthy mice and in mice with acute lung injury. By this means conclusions
could be drawn about appropriate time points of intervention administration and duration of experiments.

Hence, it is important to optimize the model to be able to draw valid conclusions from the results. By learning more about the behavior of the intervention drugs in the animals and comparing healthy states with pathological ones, one may gain further insight in pathophysiology and pharmacokinetics.

Once the model is optimized and validated, future experiments could focus more extensively on the intervention drugs AT and A1PI.

As mentioned in the introduction, in previously conducted preclinical in vitro and in vivo models AT showed to have direct effects on neutrophil adhesion and migration, as well as anti-inflammatory effect. Vice versa, A1PI also proved to decrease neutrophil activation and infiltration, to modulate apoptosis by decreasing caspase-1 and caspase-3 and having long-term anti-inflammatory effects. Therefore, markers of coagulation, apoptosis and immunomodulation need to be determined to gain knowledge about the effect of AT and A1PI on these parameters. With regards to coagulation, levels of von Willebrand factor (vWF), thrombin-antithrombin complexes (TATc) and fibrin degradation products (FDP) could be identified and referring to apoptosis caspase-1 and caspase-3 are very suitable for determination of apoptosis.

This study only focused of the effect on inflammation of COMBO therapy, but in the future it is of interest to obtain a broad picture with regards to the mechanism of action of the interventions including possible effects on coagulation and apoptosis.

Moreover, it may be interesting to assess the functioning of AT and A1PI in lung injury models. Thereby, on the one hand it may be important determine concentrations of free AT and A1PI and the percentage which is bound to its substrates thrombin and neutrophil elastase. Certainly, changes of these concentrations in pathological states, such as induced lung injury, are interesting to understand underlying mechanisms of disease and to find targets to treat. Except for the percentages of free and bound AT and A1PI, it may also be of interest if AT and A1PI are possibly inactivated by oxidative stress or proteases as shown in recent in vitro studies.

**CONCLUSION**

This study investigated for the first time combination therapy of AT and A1PI in a murine model of pulmonary ARDS. No differences in markers of inflammation, neutrophil migration or alveolar-capillary permeability were detected between the intervention groups and the saline control group. Future experiments are needed to optimize the timing schedule of the murine pulmonary ARDS model and to gain insight in pharmacokinetics and – dynamics of AT and A1PI to draw valid conclusions with regards to the administered interventions.
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