Nebulized recombinant human Tissue Factor Pathway Inhibitor attenuates coagulation and inflammation in rat models of direct and indirect lung injury

F.E. van den Boogaard*,†, J.J. Hofstra*+,†, X. Brands*+, M.M. Levi†, J.J.T.H. Roelofs**, S.A.J. Zaat†+, C. van ‘t Veer†+, T. van der Poll†+,§, M.J. Schultz*+

Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands:
*Laboratory of Experimental Intensive Care and Anesthesiology (L-E-I-C-A)
†Department of Intensive Care Medicine, Department of Experimental and Molecular Medicine (CEMM), Center for Experimental and Molecular Medicine (CEMM), Department of Internal Medicine, Center for Infection and Immunity Amsterdam (CINIMA), **Department of Pathology, ††Department of Medical Microbiology

(submitted)
ABSTRACT

Background: Critically ill patients are at a constant risk of direct (e.g., by pneumonia) or indirect lung injury (e.g., by sepsis). Excessive alveolar fibrin deposition is a prominent feature of lung injury, undermining pulmonary integrity and function.

Objectives: We examined the effect of local administration of recombinant human tissue factor pathway inhibitor (rh–TFPI), a natural anticoagulant, in two well-established models of lung injury in rats.

Methods: Rats received intratracheal instillation of Pseudomonas (P.) aeruginosa, causing direct lung injury, or received an intravenous injection of Escherichia coli lipopolysaccharide (LPS), causing indirect lung injury. Rats were randomized to local treatment with rh–TFPI or placebo through repeated nebulization. In addition, antibacterial effects of rh–TFPI on P. aeruginosa were studied in vitro.

Results: Challenge with P. aeruginosa or LPS was associated with increased coagulation and decreased fibrinolysis in bronchoalveolar lavage fluid (BALF) and plasma. Rh–TFPI levels in BALF increased after nebulization, while plasma rh–TFPI levels remained low and systemic TFPI activity was not affected. Nebulization of rh–TFPI attenuated pulmonary and systemic coagulation in both models, without affecting fibrinolysis. Nebulization of rh–TFPI reduced the inflammatory response and bacterial growth of P. aeruginosa in the alveolar compartment. In vitro, no antibacterial effect of rh–TFPI on P. aeruginosa was observed.

Conclusions: Local treatment with rh–TFPI does not alter systemic TFPI activity, however it attenuates both pulmonary and systemic coagulopathy. Furthermore, nebulized rh-TFPI reduces pulmonary inflammatory responses and allows increased bacterial clearance in models of direct and indirect lung injury.
INTRODUCTION

Critically ill patients, especially those who receive ventilatory support, are at a constant threat of developing lung injury, which is associated with high morbidity and mortality. Lung injury may develop either from a direct pulmonary insult such as aspiration of contaminated material from the oropharynx causing pneumonia, or from a systemic insult such as sepsis. Inflammatory responses within the pulmonary compartment coincide with increased coagulation and concomitantly compromised anticoagulant systems and fibrinolysis, resulting in a net procoagulant state. The resulting alveolar fibrin depositions may jeopardize tissue integrity and pose a serious challenge to lung function. Restoring the coagulation balance as adjunctive treatment may benefit critically ill patients who develop lung injury.

Tissue Factor (TF) is the initiator of inflammation–induced coagulation, and TF in complex with Factor (F)VIIa activates FX, which together with its cofactor FVa generates thrombin, ultimately stimulating fibrin clot formation. Tissue Factor Pathway Inhibitor (TFPI) inhibits TF–mediated initiation of the blood coagulation cascade by formation of a quaternary complex with TF–FVIIa–FXa, preventing additional FXa generation. Several studies provide evidence for a functional setback of TFPI during lung injury. TFPI in the alveolar spaces of patients with ARDS was found to be mainly truncated and inactive, rendering the endogenous anticoagulant insufficient to counterbalance the procoagulant state in the lung. In addition, ample evidence exists that blocking the TF pathway prevents inflammation-induced coagulation and preserves lung function during ARDS and pneumonia.

Systemic administration of anticoagulant agents bears the risk of at times life-threatening systemic bleedings. Local administration of anticoagulants offers an appealing treatment strategy, not only preventing the risk of additional bleedings, but also allowing higher dosages that may increase the efficacy of anticoagulants. Indeed, data from preliminary studies in humans suggest that local anticoagulant therapy with heparin has a beneficial effect on pulmonary coagulopathy and inflammation. To date, there are no data available on local treatment with TFPI as therapy for lung injury.

We here hypothesize that local administration of recombinant human (rh)–TFPI by means of nebulization exerts a local anticoagulant effect preventing systemic changes in coagulation, and that the local anticoagulant effect would affect pulmonary inflammation and host defense. Therefore, we investigated the effect of locally applied rh–TFPI on the coagulation and inflammatory response in two rat models of lung injury, one mimicking the scenario of direct lung injury, and one that resembles the scenario of indirect lung injury.
MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments. Animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care, and handling of laboratory animals. Male Sprague–Dawley rats (250 – 300 g) (Harlan, The Hague, The Netherlands) were allowed to acclimatize to laboratory conditions for at least 7 days (12:12 h day–night cycle at 22ºC) before handling.

Lung injury models

Direct lung injury was induced by intratracheal instillation of $10^8$ CFU of *P. aeruginosa* (PA01) in a total volume of 250 μL of bacterial suspension under light sedation with 5% isoflurane. For this, bacteria were cultured as described previously. In short, a 50 ml vial filled with Luria Broth (LB) was inoculated with *P. aeruginosa* from a –80°C stock and incubated overnight at 37ºC. This culture was 100-fold diluted in fresh LB medium and cultured for 6 hours at 37ºC to the mid-logarithmic growth phase (optical density (OD) of 0.5 at 620 nm wavelength). Next, *P. aeruginosa* were harvested by centrifugation at 4000 rpm for 10 minutes at 4ºC. Based on the measured OD an inoculum suspension in pyrogen-free 0.9% NaCl was prepared.

Indirect lung injury was induced by causing endotoxemia through intravenous administration of 7.5 mg/kg LPS from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO) into the penile vein under isoflurane (3%) anesthesia.

Treatment with rh-TFPI or vehicle

Rats were randomized to treatment with rh–TFPI 10 mg/kg (Tifacogin, Novartis, Emeryville, CA) or vehicle (300 mM L–arginine, 20 mM trisodium citrate dihydrate, pH 5.5) (n=7 per group). Uninfected rats were treated with nebulized vehicle (n=3) to evaluate the effect of nebulization alone. Uninfected untreated rats served as naïve controls (n=2-6). Per group rh–TFPI or vehicle was administered by nebulization in a total volume of 5 mL at 30 minutes before and every 6 hours after induction of endotoxemia or pneumonia. Rh–TFPI dosage was based on data from previous studies with rh–TFPI administered intravenously in *S. pneumoniae* pneumonia and an estimation of the efficacy provided by the nose–only exposure system, described previously. In brief, this system consists of a concentric manifold connected to the necks of bottle–like restraint tubes, in which the animals are confined with their noses adjacent to the bottlenecks and allows direct exposure of nebulized agents to the rat’s noses. At a constant oxygen flow (2 L/min) the aerosols are directed to the inhalation chamber using the AeronebPro
Micropump Nebulizer (Aerogen Ltd.). The animals were accommodated to restraint tubes at several occasions in the week before the experiments.

**Blood and tissue sampling**

At 16 hours after induction of lung injury rats were sacrificed. Blood was collected from the inferior vena cava in citrated (0.109 M) vacutainer tubes. The right lung was ligated, and the left lung was lavaged three times with 2 mL ice-cold saline, 0.3% BSA, 1 mM EDTA. The right superior lobe was fixed in 10% buffered formalin and embedded in paraffin. The remaining lung lobes were weighed and homogenized in 4 volumes (w/v) of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). For cytokine and chemokine measurements lung homogenates were diluted 1:1 in lysis buffer (containing 300 mM NaCl; 30 mM Tris; 2 mM MgCl$_2$; 2 mM CaCl$_2$; 1% Triton X-100; and pepstatin A, leupeptin, and aprotinin all from MP Biomedical; concentrations in accordance with the manufacturer’s recommendations). Total cell numbers in BALF were determined with an automated cell counter (Z2 Coulter Pariticle Counter, Beckman Coulter Corporation, Hialeah, FL). Differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

**Colony forming units in lungs and blood**

Serial ten-fold dilutions of lung homogenates, lavage fluid and whole blood from rats challenged with *P. aeruginosa* were made in sterile isotonic saline and plated onto sheep-blood agar plates. After 16 hours of incubation at 37°C in 5% CO$_2$, the numbers of colony forming units (CFU) were counted.

**Assays**

To determine the efficacy of local delivery of rh–TFPI, by measuring total rh–TFPI immunogen levels in lung lavage and plasma of rats, we developed an enzyme-linked immunosorbent assay (ELISA) using monoclonal mouse anti–human TFPI directed against the Kunitz domain 2 (Sanquin, Amsterdam, the Netherlands) as a coating antibody and polyclonal rabbit anti-human TFPI (kind gift of Dr. Joost Meijers, Amsterdam Medical Center, University of Amsterdam, The Netherlands) as a detecting antibody.

Furthermore, to determine if nebulization with rh–TFPI affected the overall systemic TFPI activity, we employed the two-stage chromogenic TFPI assay originally described by Sandset et al by measuring its inhibitory activity in a factor Xa-generation assay$^{20}$. In brief, heat–inactivated plasma was incubated with a mixture of recombinant FVIIa (Novoseven, Novo Nordisk A/S, Bagsvaerd, Denmark), a limited amount of FX (kind gift of Dr. Joost Meijers, Academic Medical Center, University of Amsterdam, The Netherlands) and relipidated recombinant TF (Innovin, Dade Behring, Surrey, UK) in order to form TF–FVIIa–FXa–TFPI complexes. To measure residual TF activity excess FX was added in the second stage and
FXa generation was determined using S2222 (Chromogenix). Standard curves were prepared by serial dilution of citrated normal rat plasma. Coagulation assays were performed in plasma and cell–free supernatants from BALF. Thrombin–antithrombin complexes (TATc) and fibrin degradation products (FDP) were measured using commercially available ELISA (TATc: Behringwerke AG, Marburg, Germany, FDP; Asserachrom D–Di, Diagnostica Stago, Asnières–sur–Seine, France); antithrombin (AT), plasminogen activator activity (PAA), and plasminogen activator inhibitor (PAI)–1 activity were measured by automated amidolytic assays21–23. Commercially available ELISA's were used to measure levels of tumor necrosis factor (TNF)–α, interleukin (IL)–6, and cytokine induced neutrophil chemoattractant (CINC)–3 (all R&D Systems, Abingdon, United Kingdom) and myeloperoxidase (MPO; HyCult biotechnology b.v., Uden, The Netherlands).

**Histopathology**
Immediately after rats were killed, lung samples were fixed in 10% buffered formalin for 24h and embedded in paraffin in a routine fashion15. Four–micrometer sections were stained with hematoxylin and eosin (H&E). All slides were coded and scored for the following parameters: infiltration, interstitial inflammation, endothelialitis, bronchitis, edema, pleuritis, thrombus formation and bleeding by a pathologist who was blinded for group identity. Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of the lung surface. The remaining parameters were rated separately on a scale from 0 (condition absent) to 4 (present in massive amounts).

**Killing assay**
In an additional experiment we aimed to test direct antibacterial or growth-inhibitory effects of rh–TFPI on *P. aeruginosa* in vitro. For this, a bacterial suspension was prepared as described above. After establishing the stability of *P. aeruginosa* in PBS for 24 hours, killing assays were performed. To investigate a direct bactericidal effect, 20 μl of increasing concentrations of rh–TFPI (based on measured rh–TFPI levels in BALF samples of rats; 20 μg/ml, 200 μg/ml, 2 mg/ml), were added to 20 μl of a suspension of 10⁶ CFU/ml *P. aeruginosa* in 160 μl PBS. Next, to investigate a potential growth-inhibiting effect of rh–TFPI on *P. aeruginosa*, 20 μl of increasing concentrations of r–TFPI were added to 20 μl of a suspension of 10⁶ CFU/ml *P. aeruginosa* in 160 μl 5% Luria Broth (LB)–medium. All suspensions were incubated in a sterile 96-well microtiter plate at 37°C. Bacterial numbers were quantified after 0, 1, 8 and 20 hours.

**Statistical analyses**
Comparisons between the experimental groups were performed using Kruskal–Wallis tests followed by Mann–Whitney U–tests where appropriate. Data are expressed as individual data or as median with interquartile ranges. Statistical analyses were per-
formed with GraphPad Prism (GraphPad Software, San Diego, CA). A $p$-value < 0.05 was considered statistically significant.

RESULT

Human TFPI levels and TFPI activity after nebulization of rh–TFPI

We used two established models of lung injury to investigate the effects of nebulized rh-TFPI on pulmonary coagulopathy and inflammation, induced by either intravenous injection of LPS (causing indirect lung injury) or by intratracheal instillation of *P. aeruginosa* (producing direct lung injury). To verify whether administration of rh–TFPI by nebulization was restricted to the alveolar compartment, we first measured levels of rh–TFPI in lavage fluid and plasma. Rh–TFPI levels were significantly increased in BALF of rats treated with nebulized rh–TFPI; no rh–TFPI was detectable in BALF of vehicle treated rats (Fig. 1A). In plasma of rh–TFPI treated rats, only minimal levels of rh–TFPI

![Graph showing changes in TFPI levels and activity](image-url)

*Figure 1. Nebulization with rh–TFPI increases levels of rh–TFPI in bronchoalveolar lavage fluid, but not substantially in plasma, without affecting systemic TFPI activity.*

Levels of rh–TFPI measured in (A) bronchoalveolar lavage fluid (BALF) and (B) plasma and TFPI activity in plasma (C) of uninfected rats (grey dots), rats treated with nebulized rh–TFPI (open dots) or vehicle (control, black dots) 16 hours after challenge with an intravenous bolus with lipopolysaccharide (LPS), or intratracheal instillation of *Pseudomonas (P) aeruginosa*. Data are represented as individual data with median. *$p$<0.05, **$p$<0.01 and ***$p$<0.001 versus controls (Mann-Whitney U test).*
were detected of 0.3 ng/ml (8 pM) (Fig. 1B). We next measured the effect of local rh–TFPI treatment on TFPI activity in plasma. Notably, TFPI activity in plasma of rats with indirect lung injury was reduced compared to normal values (1 U/ml), which is in line with reports that demonstrate loss of TFPI functionality during endotoxaemia. Importantly, nebulization with rh–TFPI did not alter systemic TFPI activity in either model (Fig. 1C). Together these findings suggest that rh–TFPI is confined within the alveolar space when nebulized.

Nebulized rh–TFPI inhibits local and systemic activation of coagulation

Lung injury is associated with a procoagulant state. Accordingly, in both direct and indirect lung injury, TATc and FDP levels were increased with a concurrent drop in AT levels in BALF (Fig. 2 A-C), and TATc levels in plasma were increased (Fig. 2D). Nebulization of rh–TFPI attenuated pulmonary coagulopathy in both models (Fig. 2A-C). However, although no difference in systemic TFPI activity was observed in rh–TFPI treated rats (Fig. 1C), nebulization with rh–TFPI attenuated TATc generation in plasma in both models (Fig. 2D), suggesting that local administration of rh–TFPI exerted systemic effects indepen-

**Figure 2. Nebulized rh–TFPI attenuates pulmonary and systemic coagulation.** Levels of (A) thrombin–antithrombin complexes (TATc), (B) antithrombin activity (AT), (C) fibrin degradation products (FDP) measured in bronchoalveolar lavage fluid (BALF) and (D) levels of TATc measured in plasma of rats treated with nebulized rh–TFPI (open bars, n=7) or vehicle (control, black bars, n=7) 16 hours after challenge with an intravenous bolus with lipopolysaccharide (LPS), or intratracheal instillation of *Pseudomonas* (P) aeruginosa and in naïve control rats (grey bars, n=6). Data are represented as median ± IQR; **p<0.01, ***p<0.001 between rh-TFPI and vehicle treated rats and ## p<0.01 versus naïve controls (Mann-Whitney U test).
dent of TFPI activity. No differences in coagulation were observed between uninfected naïve and uninfected rats treated with nebulized vehicle (Supplemental Fig. 1).

**Nebulized rh–TFPI does not affect pulmonary and systemic fibrinolysis**

During lung injury fibrinolysis is attenuated\(^2,\)\(^19\), which in our model was reflected by increased PAI-1 and decreased PAA levels in BALF and plasma (Fig. 3A-C). Nebulization of rh–TFPI did not affect fibrinolysis in rats with direct or indirect lung injury (Fig. 3C). No differences in fibrinolysis were observed between uninfected naïve and uninfected rats treated with nebulized vehicle (Supplemental Fig. 1).

**Rh–TFPI attenuates the inflammatory response**

Direct and indirect lung injury increased total cell counts in the lungs of challenged rats, mostly attributable to neutrophil influx (Table 1). Nebulization with rh–TFPI did not affect the number or composition of cells in lavage fluids. However, MPO levels

![Graphs of PAA and PAI-1 activity in BALF and Plasma](image)

**Figure 3. Nebulization with rh–TFPI does not influence local or systemic fibrinolysis.**

Plasminogen activator activity (PAA) and plasminogen activator inhibitor (PAI–1 activity in bronchoalveolar lavage fluid (BALF) (A, B) and PAA in plasma (C) in rats treated with nebulized rh–TFPI (open bars, n=7) or vehicle (control, black bars, n=7) 16 hours after challenge with an intravenous bolus with lipopolysaccharide (LPS), or intratracheal instillation of *Pseuodomonas (P) aeruginosa* and in naive control rats (grey bars, n=6). Data are represented as median ± IQR; ## p<0.01 versus naïve controls (Mann-Whitney U test).
were reduced in lung homogenates of rats with indirect lung injury and treated with nebulized rh–TFPI (Table 1). Pulmonary levels of cytokines IL–6 and TNF–α were lower

Table 1. Total cell and neutrophil count in lavage fluid and levels of myeloperoxidase.

<table>
<thead>
<tr>
<th></th>
<th>Total cells (x 10⁴/mL)</th>
<th>Neutrophils (x 10⁴/mL)</th>
<th>MPO (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>24 (19-29) (n=6)</td>
<td>0 (0-0) (n=6)</td>
<td>88 (86-89) (n=2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>direct lung injury</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>256 (245-327)</td>
<td>227 (210-298)</td>
<td>606 (543-642)</td>
</tr>
<tr>
<td>rh–TFPI</td>
<td>299 (135-395)</td>
<td>251 (131-361)</td>
<td>547 (489-599)</td>
</tr>
<tr>
<td><strong>indirect lung injury</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>47 (39-62)</td>
<td>35 (13-57)</td>
<td>287 (268-312)</td>
</tr>
<tr>
<td>rh–TFPI</td>
<td>53 (39-70)</td>
<td>29 (24-34)</td>
<td>228 (147-274)</td>
</tr>
</tbody>
</table>

Total cell and neutrophil (PMN) counts x 10⁴/mL in bronchoalveolar lavage fluid, myeloperoxidase (MPO) levels in lung homogenates, of rats treated with nebulized rh–TFPI or vehicle 16 hours induction of direct lung injury (by intratracheal instillation of *P. aeruginosa*) or indirect lung injury (by intravenous challenge with lipopolysaccharide) (n=7 per group). Controls are uninfected naïve rats (n = 2-6). Data are expressed as median (interquartile range) x 10⁴ per milliliter of bronchoalveolar lavage fluid. * p<0.05 versus vehicle (Mann Whitney U test).

Figure 4. Nebulization with rh–TFPI modulates pulmonary cytokine levels, but does not importantly affect lung histopathology. Levels of tumor necrosis factor (TNF)–α (A), interleukin (IL)–6 (B), and cytokine–induced neutrophil chemoattractant (CINC)–3 (C) in lung homogenates of uninfected, naïve rats (grey bars, n=2) or rats treated with nebulized vehicle (control, black bars, n=7) or rh–TFPI (open bars, n=7), 16 hours after challenge with an intravenous bolus with lipopolysaccharide (LPS), or intratracheal instillation of *Pseudomonas (P) aeruginosa*. Total lung histopathology scores of rats challenged with *P. aeruginosa* (D) with representative microphotographs (E, F). Data represent median ± IQR. Scale bar = 200 μm. # p<0.1, *p<0.05, ***p<0.001 versus control (Mann-Whitney U test).
Figure 5. Nebulization with rh–TFPI reduces bacterial loads in the alveolar compartment. Treatment with nebulized rh–TFPI modestly reduced bacterial numbers in bronchoalveolar lavage fluid (BALF) (A), but not in lung homogenates (B), of rats treated with nebulized rh-TFPI (open bars) compared with vehicle (control, black bars), 16 hours after intratracheal challenge with Pseudomonas aeruginosa. Data represent median ± IQR (n = 7 per group). *p<0.05 versus control (Mann-Whitney U test).

Figure 6. Rh–TFPI does not impact on Pseudomonas aeruginosa growth in vitro. Effect of increasing concentrations of rh–TFPI on Pseudomonas (P.) aeruginosa in PBS (A) and in 5% LB-medium in PBS (B) in vitro.
in rats treated with rh-TFPI in both models of lung injury (Fig. 4A and B). At 16 hours after induction of direct lung injury caused by *P. aeruginosa* pneumonia, histopathology of the lungs showed diffuse inflammatory infiltrates, mainly consisting of neutrophils. Interstitial inflammation, endothelialitis, edema and bronchitis were present to a variable extent. Intrapulmonary bleeding was observed in one vehicle treated and one rh-TFPI treated rat. Overall, lung pathology scores tended to be lower for the rh−TFPI treatment group without reaching statistical significance (Fig. 4D). No differences in cell influx or cytokines were observed between uninfected naïve and uninfected rats treated with nebulized vehicle (Supplemental Fig. 1).

**Nebulized rh−TFPI enhances bacterial clearance from the alveolar compartment**

Bacterial counts were quantified in samples of rats with *P. aeruginosa* pneumonia. In lavage fluid a modest, yet significant reduction in bacterial numbers was observed in rh-TFPI-treated rats, suggesting local treatment with rh−TFPI enhanced bacterial clearance from the alveolar compartment (Fig. 5A). Of note, bacterial numbers in lung homogenates did not differ between treatment groups (Fig. 5B). None of the rats became bacteremic (data not shown).

We next set out to investigate a potential direct bactericidal or growth-inhibitory effect of rh−TFPI in vitro. For this, we incubated viable *P. aeruginosa* with increasing concentrations of rh−TFPI in PBS (Fig. 6A) and PBS with 5% LB (Fig. 6B) for 24 hours, however, rh−TFPI did not affect CFU counts of *P. aeruginosa* in either setting. Thus, no direct antibacterial effect of rh−TFPI was observed in vitro.

**DISCUSSION**

In this study we demonstrate that local administration of rh−TFPI by nebulization reduces pulmonary coagulation, and exerts anti-inflammatory effects in two rat models of lung injury. In direct lung injury caused by *P. aeruginosa* pneumonia, rh−TFPI reduced bacterial numbers in the alveolar compartment.

Coagulation and inflammation amplify each other in response to tissue injury. TF is considered the key activator of inflammation-induced coagulation and is present in high amounts in the lung compartment. Systemically blocking the TF pathway successfully reduced pulmonary coagulopathy in several studies of lung injury. In line with these studies, we here show that nebulized rh−TFPI attenuated inflammation-induced coagulation. Notably, a recent study demonstrated that mice with low TF levels had increased alveolar haemorrhage in response to acute lung injury leading to increased lung inflammation. However, in the present study the incidence of lung haemorrhage...
was low and similar in both study groups. Local treatment with rh–TFPI also reduced plasma TATc levels, despite negligible rh–TFPI levels in plasma and unaltered systemic TFPI activity. There are several possible explanations for this systemic anticoagulant effect: in both models of lung injury, rh–TFPI exerted an anti-inflammatory effect and consequently may have provided a less potent procoagulant stimulus. The half-life of rh–TFPI is relatively short\(^6\), hence higher levels of rh–TFPI in the lung were likely reached shortly after nebulization, than measured at the time of sacrifice (i.e. 4 hours after the last nebulization). Although rh–TFPI is a relatively large molecule (45 kDa) to pass the epithelial barrier, we cannot exclude that greater amounts of rh–TFPI have crossed this barrier in these two models of lung injury, and successively attenuated coagulation systemically\(^32\). In addition, rh–TFPI is known to adhere to the endothelium, consequently plasma levels may be an underestimation of the actual levels\(^6\). Fibrinolysis was unaffected by treatment with rh-TFPI, which is in line with previous observations\(^15\).

The reported effects of rh–TFPI on the host inflammatory response are inconsistent. We found that nebulization with rh–TFPI in the lung compartment reduced TNF-α and IL-6 levels with direct lung injury and reduced MPO and TNF-α levels with indirect lung injury, while infiltration or composition of cells in lavage fluids were unaffected. In other rat models of lung injury, inhibitors of the TF pathway significantly inhibited cytokine levels in the lungs\(^11,13\), and reduced MPO levels in pneumococcal pneumonia (own observations, data not shown). However, in an experimental setting of human endotoxemia, rh–TFPI did not affect inflammatory pathways, whilst completely abrogating activation of coagulation\(^33\). Remarkably, in the same model of indirect lung injury as used in the present study, our group previously did not find any anti-inflammatory effects in the lung when rh–TFPI was administered systemically\(^10\). The current findings suggest that local administration of rh–TFPI is more effective than systemic treatment in dampening lung inflammation in lung injury.

Rats treated with nebulized rh–TFPI showed modestly, yet significantly lower bacterial numbers of \(P.\ aeruginosa\) in lavage fluid samples. Interesting in this context, recent studies have reported antimicrobial activity of C-terminal peptides of the rh–TFPI molecule against several pathogens, including \(P.\ aeruginosa\)\(^34,35\). Bacterial numbers were not altered in lung homogenates, suggesting that rh–TFPI exerted an antibacterial effect primarily in the intra-alveolar compartment, which may be attributed to relatively high alveolar concentrations of rh-TFPI due to nebulization and the fact that in the lung TF is mainly expressed by respiratory epithelial cells and alveolar macrophages\(^25\). Nonetheless, when \(P.\ aeruginosa\) was incubated with PBS with/without LB-medium, no direct antibacterial effect of rh–TFPI was observed. Possibly differences in concentrations of rh-TFPI and the absence of human plasma in our in vitro experiment, which enhanced the antibacterial effect of rh-TFPI in other in vitro studies, account for the discrepancy in findings\(^34\).
Interfering with the vicious circle of coagulation and inflammation by TF pathway inhibition was associated with favourable effects on outcome in experimental sepsis,26,36-38, and led to the OPTIMIST trial, in which adjunctive rh–TFPI treatment has been studied in human sepsis31. Although no effect on overall outcome was demonstrated, a subgroup analysis of patients with pneumonia suggested beneficial effects of the intervention. Several other observations point towards the pulmonary compartment as the major site of activity of TFPI during lung injury: mainly truncated and inactive TFPI was found in BALF of patients with ARDS7, and TFPI activity was reduced in patients with pneumonia8,14. However, the ensuing CAPTIVATE trial, specifically designed to investigate the effect of rh–TFPI in severe community-acquired pneumonia, failed to show a beneficial effect on outcome39.

In the preclinical and clinical studies mentioned above, the anticoagulant agents were administered systemically, hereby potentially increasing the risk of bleeding complications. Indeed, patients with severe sepsis treated with rh–TFPI had an increase in the incidence of adverse events with bleeding, which may have offset potentially favourable effects.31 As the pulmonary compartment is the major site of TF procoagulant activity during lung inflammation, local administration may be a more efficient and safer route of delivery, minimizing systemic adverse effects. Additionally, tissue penetration of systemically administered anticoagulant agents may be hampered during lung injury, and may be better warranted by local administration of rh–TFPI40. In previous studies in rats with lung injury, nebulization of anticoagulant agents reduced pulmonary coagulation without affecting systemic coagulation, however, nebulized danaparoid also exerted systemic effects on coagulation41,42. Recently, the first small studies of nebulized heparin treatment have been conducted in humans requiring mechanical ventilation and report inconsistent anticoagulant effects16,41,44.

Our experimental studies have several limitations. We used simplified animal models of lung injury, in which a pre-treatment strategy was used, to discern a potential effect of the study agent. This laboratory setting does not resemble the complex human situation; therefore these results should be translated to the clinical scenario with caution. Furthermore, we have based the dosage of rh–TFPI on previous studies, however the route of administration was not similar, consequently the dosage may have been sub-optimal10. In both lung injury models we found a significant local and systemic anti-coagulant effect of nebulized rh–TFPI. Lower dosages may adequately reduce pulmonary coagulopathy, whilst preventing a systemic effect on coagulation and potential adverse effects, whereas higher dosages of rh–TFPI may allow for a stronger anti-inflammatory and antibacterial effect. Lastly, we have not investigated the effect of local rh-TFPI treatment on functional endpoints, such as alveolar gas exchange.

In conclusion, coagulation imbalance in the pulmonary compartment provides a therapeutic target for local administration of anticoagulant agents. Here, nebulization
with rh–TFPI attenuated pulmonary coagulopathy, inflammation and bacterial numbers in rat models of direct and indirect lung injury. Of note, in both models systemic coagulation was affected.
REFERENCES


Supplemental Figure 1. Nebulization with rh−TFPI does not affect coagulation, fibrinolysis or cytokine levels in uninfected rats. Levels of thrombin–antithrombin complexes (TATc), antithrombin activity (AT), fibrin degradation products (FDP), plasminogen activator activity (PAA), plasminogen activator inhibitor (PAI)–1 activity measured in bronchoalveolar lavage fluid (BALF) and in plasma as indicated; levels of tumor necrosis factor (TNF)–α, interleukin (IL)–6, and cytokine–induced neutrophil chemoattractant (CINC)–3 in lung homogenates; and cell counts in BALF of uninfected untreated rats (naïve, grey bars, n=6), or treated with vehicle (control, black bars, n=3) or nebulized rh–TFPI (open bars, n=3). Data are represented as median ± IQR.