The tissue factor pathway in pneumonia

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CHAPTER 4

Recombinant Human Tissue Factor Pathway Inhibitor exerts anticoagulant, anti-inflammatory and antimicrobial effects in murine pneumococcal pneumonia

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ABSTRACT

Background: *Streptococcus (S.) pneumoniae* is the most common causative pathogen in community-acquired pneumonia and a major cause of sepsis. Recombinant human tissue factor pathway inhibitor (rh-TFPI) attenuates sepsis-induced coagulation and has been evaluated in clinical trials involving patients with sepsis and community-acquired pneumonia.

Objective: To examine the effect of rh-TFPI on coagulation, inflammation and bacterial outgrowth in *S. pneumoniae* pneumonia in mice, with or without concurrent antibiotic treatment.

Methods: Pneumonia was induced by intranasal inoculation with *S. pneumoniae*. Mice were treated with placebo, rh-TFPI, ceftriaxone or rh-TFPI combined with ceftriaxone. Early (8 hours) and late (24 hours) initiated treatments were evaluated. Samples were obtained 24 or 48 hours after infection, for early and late initiated treatment respectively. In vitro, placebo or rh-TFPI was added to a suspension of *S. pneumoniae*.

Results: Rh-TFPI reduced pneumonia-induced coagulation; rh-TFPI with ceftriaxone further attenuated coagulation relative to ceftriaxone alone. Rh-TFPI inhibited accumulation of neutrophils in lung tissue and reduced the levels of several cytokines and chemokines in lungs and plasma in mice not treated with antibiotics; in these animals, rh-TFPI initiated 24 hours after infection decreased pulmonary bacterial loads. In vitro, rh-TFPI also inhibited growth of *S. pneumoniae*.

Conclusions: Therapeutic rh–TFPI attenuates coagulation, inflammation and bacterial growth during pneumococcal pneumonia, whereby the latter two effects only become apparent in the absence of concurrent antibiotic treatment.
INTRODUCTION

Sepsis remains a major challenge to clinicians, with high mortality rates despite adequate use of antibacterial treatment and instillation of well-equipped intensive care units. Community-acquired pneumonia (CAP) is a common cause of sepsis, with *Streptococcus (S.) pneumoniae* as the most frequently isolated causative pathogen. As antimicrobial therapies alone are not sufficient to avert mortality in many patients with severe CAP, a need for adjunctive measures exists to further optimize treatment and improve outcome.

Pulmonary infection generally elicits a procoagulant state in the lung caused by concurrent activation of coagulation, down-regulation of anticoagulant pathways and inhibition of fibrinolysis. These local hemostatic changes may ultimately lead to intra-alveolar fibrin deposition, while abundantly present coagulation proteases can aggravate inflammation via protease-activated receptor (PAR) signalling. As such there is an intimate interaction between coagulation and inflammation within the lung upon entry of respiratory pathogens into the Airways. Tissue Factor (TF) is the main initiator of infection- and inflammation-induced activation of the coagulation cascade. TF binds to (activated) factor VII(a) (FVII(a)), forming a complex that is able to activate factor X (FX), which enables the conversion of prothrombin into thrombin. Thrombin generation via the TF pathway is controlled by TF Pathway Inhibitor (TFPI), a protein predominantly produced by endothelial cells, that prevents further FXa generation by inhibiting the TF-FVIIa complex in a FXa dependent manner.

TF has been implicated as an important mediator of excessive coagulation and lethality in sepsis. Blocking the TF pathway prevented activation of coagulation in endotoxemic humans and chimpanzees, whereas in baboons with lethal sepsis these TF directed interventions not only mitigated disseminated intravascular coagulation but also reduced mortality. These promising preclinical data led to the design and performance of clinical trials evaluating recombinant human (rh)-TFPI as a potential new therapy in patients with severe sepsis. Unfortunately, the pivotal phase III “OPTIMIST” trial, which examined the effect of rh-TFPI on 28-day all cause mortality in severe sepsis, did not show a beneficial effect on outcome in the study population as a whole. Post-hoc analysis of this trial suggested that treatment with rh-TFPI might improve survival of patients with severe CAP requiring intensive care admission in whom a causative pathogen is identified. Moreover, a negative interaction between rh-TFPI and concurrent heparin treatment was suggested. These analyses then led to the design of a new phase III clinical trial investigating the effect of rh-TFPI in patients with severe CAP in whom concurrent heparin treatment was withheld.

Our group previously investigated the effect of TF inhibition in rodent models of pneumococcal pneumonia. In these studies pretreatment with either recombinant nema-
tode anticoagulant protein c2 (rNAPc2), a selective inhibitor of the TF/FVIIa pathway, or rh-TFPI attenuated activation of coagulation in the lung without influencing pulmonary inflammation\textsuperscript{6,21}. Importantly, however, these investigations did not mimic the clinical scenario of TF inhibition during an already established respiratory tract infection and/or in the context of antibiotic therapy. In the present study we used our established mouse model of pneumococcal pneumonia\textsuperscript{6,22} to investigate the effects of rh-TFPI on bacterial outgrowth, coagulopathy and the host inflammatory response in two therapeutic settings in which rh-TFPI was initiated with or without concurrent ceftriaxone early in the time course of pneumonia (8 hours postinfection) or in a model mimicking severe CAP (24 hours postinfection).

MATERIALS AND METHODS

Animals
Specific pathogen-free C57BL/6 female mice were purchased from Harlan Sprague-Dawley (Horst, The Netherlands). All experiments were conducted with 11–week old mice. The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments.

Pharmacokinetic study
The dosing regimen of rh-TFPI (350 mg/kg by intraperitoneal injection every 8 hours: see below) was based on a previous study in which rh-TFPI given at a similar dose was found to reduce mortality in models of superantigen-induced shock and polymicrobial intra-abdominal sepsis in mice\textsuperscript{23}. To study the pharmacokinetics of this dosage of rh-TFPI in mice in vivo, we conducted pharmacokinetic studies in our laboratory. For this, naive mice were sacrificed at several time points after intraperitoneal administration of 350 mg/kg rh-TFPI (n=4 per time point) to obtain citrated (1:4) blood and lung samples.

Experimental study design
Pneumonia was induced by intranasal inoculation with S. pneumoniae serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD; \(2 \times 10^5\) colony forming units (CFU) in 50 µL, exactly as described [7;22]. Rh-TFPI (Tifacogin\textsuperscript{®}) was obtained from Novartis (East Hanover, NJ). Ceftriaxone was purchased from Pharmachemie BV (Haarlem, the Netherlands). An early and delayed initiation of treatment was studied in two separate experiments. Treatment was initiated 8 hours (early treatment) or 24 hours (late treatment) after induction of pneumonia. In both experiments four groups of mice (n = 8 per group) were treated intraperitoneally with rh-TFPI (350 mg/kg) or placebo control every 8 hours, combined with either ceftriaxone (20 mg/kg) or saline control twice daily.
Blood diluted 1:4 with citrate, bronchoalveolar lavage fluid (BALF), lungs and spleen were harvested 24 hours (early treatment) or 48 hours (late treatment) after induction of pneumonia using methods described previously. Total cell numbers in BALF were determined by an automated cell counter (Coulter Counter, Coulter Electronics, Hialeah, FL). Differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). The left lung lobe was fixed in 10% buffered formalin and embedded in paraffin. The remaining lung lobes and a part of the spleen were harvested and homogenized as previously described.

**Bacterial outgrowth**

For bacterial quantification undiluted whole blood and serial ten–fold dilutions of organ homogenates, BALF and whole blood were made in sterile isotonic saline and plated onto sheep–blood agar plates. Following 16 hours of incubation at 37°C colony forming units (CFU) were counted.

**Assays**

TFPI activity was measured in mouse plasma employing the two-stage chromogenic TFPI assay originally described by Sandset et al., with the modification that only mouse coagulation factors were used. As source of mouse TF a lysate was prepared of MLE-15 (mouse lung epithelial cell line) cells. Briefly, MLE-15 cells were scraped from the culture flask and washed extensively with PBS, the cell pellet was taken up in sterile saline (5x10⁶/ml) and a lysate was prepared by 3 freeze/thaw cycles. In the first stage of the mouse TFPI assay 30 μl of diluted plasma sample was incubated with 50 μl containing limited mouse FX (15 ng/ml) generously provided by Ryan Dorfman (Haematologic Technologies, Essex Junction, VT), 1 nM mouse FVIIa (kindly provided by Dr Lars C. Petersen, NovoNordisk, Bagsvaerd, Denmark) and 5 ul MLE-15 lysate. To measure residual TF activity excess mouse FX was added in the second stage (40 μl of 15 μg/ml) and FXa generation was determined using S2222. Standard curves were prepared by serial dilution of citrated normal mouse plasma. Rh-TFPI concentration was measured by an enzyme-linked immunosorbent assay (ELISA), using a mouse monoclonal antibody directed against the human TFPI Kunitz-2 domain (amino acids 88-160; Sanquin, Amsterdam, the Netherlands) as capture antibody, polyclonal rabbit anti-rh-TFPI (kind gift of Dr Walter Kisiel, University of New Mexico, Albuquerque, NM) as detection antibody and rh-TFPI as standard. Thrombin-antithrombin complexes (TATc) were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (TATc: Behringwerke AG, Marburg, Germany). Plasminogen activator inhibitor (PAI)-1 was measured by ELISA. Tumor necrosis factor alpha (TNF-α), interleukin (IL)–6, IL–10, monocyte chemotactic protein (MCP)–1 and interferon–gamma (IFN–γ) were determined using a
commercially available cytometric beads array multiplex assay (BD Biosciences, San Jose, CA; detection limits: 3 pg/ml, 5 pg/ml, 25 pg/ml, 20 pg/ml and 2 pg/ml respectively). Macrophage–inflammatory protein (MIP)–2, keratinocyte-derived cytokine (KC) and lipopolysaccharide-induced CXC chemokine (LIX) were measured using commercially available ELISA kits (R&D Systems, Abingdon, UK; detection limits 80 pg/ml, 10 pg/ml and 20 pg/ml respectively). Total protein was measured using Bradford Protein Assay (Bio-Rad, Hercules, CA).

**Histopathology**

Four-micrometer sections of the left lung lobe were stained with hematoxylin and eosin (H&E). All slides were coded and scored by a pathologist who was blinded for group identity for the following parameters: interstitial inflammation, endothelialitis, bronchiitis, edema, pleuritis and presence of thrombi. Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of the lung surface, the number of thrombi was counted in five non-overlapping random microscopic fields. The remaining parameters were rated separately on a scale from 0 (condition absent) to 4 (present in massive amounts). Neutrophil stainings were performed using an anti-mouse Ly-6G monoclonal antibody (BD Pharmingen, San Diego, CA), as described previously.

**Killing assay**

To test a direct effect of rh-TFPI on *S. pneumoniae* a bacterial suspension was prepared as described above. Anticoagulant-free venous blood was drawn from a healthy medication-free volunteer and clot formation was allowed at room temperature for 30 minutes. After centrifugation serum was collected and stored at -80°C. Cultures of 1300 CFU/ml of *S. pneumoniae* with 10% serum were incubated with or without 100 μg/ml rh-TFPI (both n=6 samples) in RPMI/Hepes in a humidified atmosphere at 37°C. Bacterial numbers were quantified after 20 hours.

**Statistical analysis**

Data are expressed as box-and-whiskers or (for bacterial loads) medians with individual data points. Differences between groups were analyzed by Mann–Whitney U tests, using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA). A *P*-value of < 0.05 was considered statistically significant.
RESULTS

Human TFPI levels and TFPI activity after intraperitoneal rh-TFPI administration in mice

To validate the dosage regimen used previously to study the impact of rh-TFPI on superantigen-induced shock and polymicrobial intra-abdominal sepsis in mice\textsuperscript{23}, we determined rh-TFPI antigen and TFPI activity levels in mice at various time points after intraperitoneal administration of 350 mg/kg (Figure 1). At 30 minutes after administration rh-TFPI plasma levels were 3047 [585- 3937] ng/mL, gradually decreasing to 1485 [1435 - 1705] ng/mL at 8 hours (Figure 1A). Rh-TFPI levels in lung homogenates were 11839 [9203-21369] ng/mL at 30 minutes and 836 [683-1032] ng/mL at 8 hours in lung samples (Figure 1B). Furthermore, to study the actual effect of human TFPI administration to mice on the TF inhibitory capacity reached in plasma of the treated mice, we determined TFPI activity in plasma samples with an assay containing only mouse coagulation proteins. The activity is represented relative to normal mouse plasma containing 1 unit/ml TFPI activity. Thirty minutes after administration TFPI activity was enhanced 8-fold by rh-TFPI and was still increased 4-fold at 8 hours (Fig. 1C), demonstrating the bio-activity of human TFPI in mouse in vivo.

![Figure 1](image-url)

*Fig. 1. Rh-TFPI antigen and activity levels after intraperitoneal administration.* Mice were intraperitoneally injected with 350 mg/kg rh-TFPI. At several time points the concentrations of rh-TFPI in plasma (A) and lung homogenate (B) were measured by ELISA (n=4 per time point). TFPI activity measured in plasma was enhanced ~8-fold after 30 min and gradually decreased towards 2-fold activity after 24 h (C).
Rh-TFPI does not influence the antimicrobial activity of ceftriaxone but inhibits local growth of *S. pneumoniae* during advanced pneumonia

To determine the effect of rh-TFPI on the host response to pneumococcal pneumonia in clinically relevant models, we used two distinct treatment schedules: one in which rh-TFPI was initiated 8 hours after infection (early treatment) and one in which rh-TFPI was started 24 hours post infection (late treatment). In both models rh-TFPI effects were established in the presence and the absence of concurrent antibiotic therapy. Mice were euthanized either 24 hours (early treatment) or 48 hours (late treatment) after induction of pneumonia.

First, we quantified bacterial loads at the primary site of infection (BALF and lung homogenates) and distant body sites (blood and spleen homogenates) in both models (Figure 2). As expected, ceftriaxone strongly reduced bacterial loads at all body sites tested in both the early (Figure 2, left panels) and late treatment setting (Figure 2, right panels). In the early treatment setting, ceftriaxone reduced lung bacterial counts from ~10⁶ CFU/mL (mice not treated with antibiotics) to < 10² CFU/mL (Figure 2A, P <0.001), whereas in the late treatment setting ceftriaxone reduced lung bacterial counts from ~10⁸ CFU/mL to ~10⁴ CFU/mL (Figure 2D, P<0.001). This marked local antimicrobial effect was accompanied by a strongly diminished dissemination of the infection, as reflected by prevention of bacteremia and reduced bacterial loads in spleens in all ceftriaxone treated mice (Figure 2E, F, G, H). Although a trend towards an increase in bacterial loads was seen in the late treatment setting for BALF and spleen for the rh-TFPI and ceftriaxone treatment group compared to the ceftriaxone treated mice, this did not reach statistical significance (both P = 0.07). Remarkably, in the late treatment setting rh-TFPI reduced lung bacterial loads ~10-fold in mice not treated with antibiotics (Figure 2D, P < 0.01) without influencing the dissemination of pneumococci to blood or spleen (Figure 2F, H).

Rh-TFPI inhibits local and systemic activation of coagulation

Several preclinical and clinical studies have demonstrated that TFPI is a potent inhibitor of systemic thrombin generation in sepsis. To determine the local and systemic anticoagulant properties of rh-TFPI alone or in the presence of antibiotic treatment in murine pneumococcal pneumonia, TATc levels (a marker for thrombin generation) were measured in lung homogenates and plasma (Figure 3). Ceftriaxone reduced lung and plasma TATc levels in both treatment settings, likely due to markedly lower bacterial loads in mice administered with this antibiotic (all P < 0.01 versus mice not treated with ceftriaxone). Both early and late treatment with rh-TFPI attenuated local and systemic coagulation activation in both mice treated with or without ceftriaxone, as reflected by reduced levels of TATc in lung homogenates and plasma compared to controls matched with regard to antibiotic therapy, although in the early treatment setting rh-TFPI did not further reduce lung TATc levels in mice treated with ceftriaxone to a
statistically significant extent, likely due to the fact that at this time point ceftriaxone per se already lowered lung TATc concentrations to near baseline levels.

Fig. 2. Treatment effect of rh-TFPI and/or ceftriaxone on local and systemic bacterial outgrowth in pneumococcal pneumonia. Mice were infected intranasally with *S. pneumoniae*; treatment with ceftriaxone and/or rh-TFPI was initiated after either 8 h (early treatment, with harvest of samples 24 h postinfection) or 24 h (late treatment, with harvest of samples 48 h postinfection). Graphs show the effect of treatment with placebo (open spheres), rh-TFPI (closed spheres), ceftriaxone (open quadrangles) and combined treatment (closed quadrangles) on the number of colony forming units (CFU) per millilitre bronchoalveolar lavage fluid (BALF) (A and B), lung homogenates (C and D), whole blood (E and F) and spleen homogenates (G and H) in the early (left panel) and late (right panel) treatment settings. Ceftriaxone reduced the bacterial numbers locally and systemically in both treatment settings. Rh-TFPI reduced the bacterial number ~10-fold in lung homogenate in the late treatment setting. Each symbol represents an individual mouse with horizontal lines showing medians. Dotted lines indicate detection limits. *** P<0.001, ** P<0.01, *P<0.05 compared with controls.
**Rh-TFPI does not influence PAI-1 release**

Our model of pneumococcal pneumonia is associated with inhibition of fibrinolysis due to enhanced release of PAI-1 in lungs and the circulation, which resembles changes in patients with pneumonia. Here we confirmed that *S. pneumoniae* pneumonia results in elevated levels of PAI-1 in lung homogenates and plasma (Figure 4). Both early and late treatment with ceftriaxone reversed these pneumonia induced changes in PAI-1 levels (all P <0.01 versus mice not treated with antibiotics), while rh-TFPI did not influence PAI-1 levels.

**Rh-TFPI attenuates lung inflammation**

As described earlier, pneumococcal pneumonia was associated with interstitial inflammation, endothelialitis, edema, inflammatory infiltrates and pleuritis, of which the latter was only present in the late treatment setting (Figure 5). No thrombi were found in

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**Fig. 3. Treatment effect of rh-TFPI and/or ceftriaxone on local and systemic activation of coagulation in pneumococcal pneumonia.** Mice were infected intranasally with *S. pneumoniae*; treatment with ceftriaxone and/or rh-TFPI was initiated after either 8 h (early treatment, with harvest of samples 24 h postinfection) or 24 h (late treatment, with harvest of samples 48 h postinfection). Thrombin-antithrombin complex (TATc) levels in uninfected mice (grey open box) and the effect of treatment with placebo (open box), rh-TFPI (open striped box), ceftriaxone (grey box) and combined treatment (grey striped box) on TATc levels in lung homogenate (A and B) and plasma (C and D), in the early (left panel) and late (right panel) treatment settings. Both rh-TFPI and ceftriaxone reduced TATc levels locally and systemically in either treatment setting. Rh-TFPI in combination with ceftriaxone further reduced TATc levels in plasma in the early treatment setting and in lung homogenate and plasma in the late treatment setting compared with either treatment alone. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group). *** P<0.001, ** P<0.01, *P<0.05 compared with controls. $$$ P<0.001, $$ P<0.01, $ P<0.05 compared with ceftriaxone treatment.
Recombinant human TFPI in pneumococcal pneumonia

In the early treatment setting, ceftriaxone reduced total histopathology scores (P = 0.06), which, however, became statistically significant only in combination with rh-TFPI; in the late treatment setting, ceftriaxone reduced total histopathology scores without any additional influence of rh-TFPI on the extent of lung inflammation. Late treatment with rh-TFPI alone (without antibiotics) tended to reduce the extent lung inflammation albeit not to a statistically significant extent (P = 0.13 versus control).

To further evaluate the inflammatory response in the lung, we determined the number and type of cells present in BALF (Table 1). Remarkably, although ceftriaxone diminished the extent of inflammation in lung tissue (Figure 5), this antibiotic did not influence the cellular composition of BALF, which was primarily characterized by an influx of neutrophils. However, in the late treatment setting rh-TFPI given without ceftriaxone tended to diminish neutrophil influx in BALF (P = 0.08) and significantly reduced neutrophil accumulation in lung tissue (P < 0.05, Figure 6), as did both ceftriaxone-treated groups. In addition, rh-TFPI not given with ceftriaxone reduced protein leakage, as reflected
by lower protein concentrations in BALF in the late treatment setting (Table 1). Protein concentrations in BALF of ceftriaxone-treated mice were lower too, yet this effect did not reach statistical significance.

Cytokines and chemokines play an eminent role in host defense against pneumonia \(^{30,31}\) . Therefore, we measured the levels of proinflammatory cytokines (TNF-\(\alpha\), IL-6, IFN-\(\gamma\)), an anti-inflammatory cytokine (IL-10) and chemokines (MCP-1, MIP-2, KC and LIX) in lungs and plasma (Table 2). Antibiotic treatment with ceftriaxone profoundly reduced all levels of proinflammatory cytokines and chemokines in BALF (except for TNF-\(\alpha\) and LIX levels in the early and late treatment setting respectively), lung homogenates and plasma in both treatment settings. This effect is likely to be explained by local and systemic reduction of bacterial loads and corresponded with the attenuated inflammation in the lungs of mice treated with ceftriaxone. Although no effect of rh-TFPI on lung pathology was observed, rh-TFPI exhibited significant anti-inflammatory properties, as

Fig. 5. Treatment effect of rh-TFPI and/or ceftriaxone on lung histopathology in pneumococcal pneumonia. Mice were infected intranasally with \(S.\) pneumoniae; treatment with ceftriaxone and/or rh-TFPI was initiated after either 8 h (early treatment, with harvest of samples 24 h postinfection) or 24 h (late treatment, with harvest of samples 48 h postinfection). Total lung histopathology scores (E and J) and representative microphotographs of haematoxylin and eosin stained lung sections of mice treated with placebo (A and F), rh-TFPI (B and G), ceftriaxone (C and H) and ceftriaxone combined with rh-TFPI (D and I) in the early (left panel) and late (right panel) treatment settings, respectively. In the early treatment setting ceftriaxone reduced total histopathology scores only in combination with rh-TFPI; in the late treatment setting ceftriaxone with or without rh-TFPI reduced total histopathology scores. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (\(n = 8\) per group). Magnification 200 times. ** \(P<0.01\), *\(P<0.05\) compared with controls.
reduced levels of several cytokines and chemokines both in the pulmonary and systemic compartment; in the early treatment setting rh-TFPI lowered levels of LIX in BALF and IFN-γ in plasma; in the late treatment setting IL-6, IFN-γ, LIX and MCP-1 in BALF, IL-6, TNF-α and MCP-1 in lung homogenates and IL-6 and TNF-α in plasma all were reduced by rh-TFPI. In addition, rh-TFPI increased levels of the anti-inflammatory cytokine IL-10 in BALF in both treatment settings. Furthermore, levels of IFN-γ, KC and MIP-2 in lung homogenates were also reduced by rh-TFPI in the late treatment setting; however this did not reach statistical significance (all P < 0.1). Addition of rh-TFPI to treatment with ceftriaxone did not further increase (apart from an additional reduction of LIX levels in BALF in the late treatment setting) the anti-inflammatory effects prompted by treatment with ceftriaxone alone.

**Rh-TFPI inhibits growth of S. pneumoniae in vitro**

To further investigate whether rh-TFPI affects growth of *S. pneumoniae*, we incubated *S. pneumoniae* with rh-TFPI in the presence of serum. Indeed, after overnight incubation, bacterial numbers were reduced by rh-TFPI versus control in the presence of human serum (122 [55-200] x10⁵ versus 650 [595-1130] x10⁵ CFU/ml, P=0.002, Fig 7). However, in the presence of murine serum no growth inhibition of *S. pneumoniae* by rh-TFPI was observed (data not shown).
Fig. 6. Treatment effect of rh-TFPI and/or ceftriaxone on neutrophil accumulation in lung tissue during pneumococcal pneumonia. Mice were infected intranasally with S. pneumoniae; treatment with ceftriaxone and/or rh-TFPI was initiated after either 8 h (early treatment, with harvest of samples 24 h postinfection) or 24 h (late treatment, with harvest of samples 48 h postinfection). Neutrophil accumulation in lung tissue is expressed as total Ly-6G scores (E and J) and representative slides of Ly-6G staining in mice treated with placebo (A and F), rh-TFPI (B and G), ceftriaxone (C and H) and ceftriaxone combined with rh-TFPI (D and I) in the early (left panel) and late (right panel) treatment settings, respectively. In the early treatment setting mice treated with rh-TFPI showed a modest increase in Ly-6G scores, whereas in the late treatment setting these mice showed a significant reduction in the Ly-6G score compared with controls. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group). Magnification 400 times. * indicates P<0.05 compared with controls.

Table 2. Treatment effect of rh-TFPI and ceftriaxone on levels of cytokines and chemokines in the pulmonary and systemic compartment during Streptococcus pneumoniae pneumonia.

<table>
<thead>
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<th></th>
<th>control</th>
<th>rh-TFPI</th>
<th>ceftriaxone</th>
<th>rh-TFPI and ceftriaxone</th>
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<tr>
<td></td>
<td>BALF t=24</td>
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<td></td>
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<tr>
<td>IL-6 (pg/ml)</td>
<td>49 (35-75)</td>
<td>50 (11-95)</td>
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<td>&lt; ***</td>
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<td>IL-10 (pg/ml)</td>
<td>45 (42-50)</td>
<td>64 (49-71)**</td>
<td>61 (48-64)</td>
<td>61 (52-68)**</td>
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<tr>
<td>IFNγ (pg/ml)</td>
<td>12 (5-21)</td>
<td>3 (0-12)</td>
<td>&lt; **</td>
<td>&lt; **</td>
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<tr>
<td>TNFα (pg/ml)</td>
<td>134 (111-173)</td>
<td>148 (101-194)</td>
<td>179 (125-215)</td>
<td>164 (130-186)</td>
</tr>
<tr>
<td>KC (pg/ml)</td>
<td>179 (150-211)</td>
<td>131 (66-210)</td>
<td>57 (42-72)**</td>
<td>47 (45-55)**</td>
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<tr>
<td>LIX (pg/ml)</td>
<td>744 (671-811)</td>
<td>323 (193-430)*</td>
<td>358 (172-532)**</td>
<td>81 (18-237)**</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>MIP-2 (pg/ml)</td>
<td>285 (276-305)</td>
<td>300 (197-356)</td>
<td>271 (119-331)</td>
<td>298 (264-372)</td>
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Table 2. Treatment effect of rh-TFPI and/or ceftriaxone on levels of cytokines and chemokines in the pulmonary and systemic compartment during *Streptococcus pneumoniae* pneumonia. (Continued)

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<th>Plasma t=24</th>
<th>Plasma t=48</th>
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<td><strong>IL-6 (pg/ml)</strong></td>
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<td>1046 (626-1991)</td>
<td>2099 (1500-2454)</td>
<td>147 (79-208)</td>
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<td><strong>IL-10 (pg/ml)</strong></td>
<td>39 (28-43)</td>
<td>1234 (202-1921)</td>
<td>817 (389-1154)</td>
<td>164 (88-245)</td>
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<tr>
<td><strong>IFNγ (pg/ml)</strong></td>
<td>18 (13-36)</td>
<td>7 (4-11)***</td>
<td>5 (0.8-1.2)</td>
<td>0.8 (0.0-1.2)</td>
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<td><strong>TNFα (pg/ml)</strong></td>
<td>268 (213-377)</td>
<td>151 (116-185)</td>
<td>538 (194-785)</td>
<td>44 (18-48)</td>
</tr>
<tr>
<td><strong>KC (pg/ml)</strong></td>
<td>268 (166-537)</td>
<td>89 (30-332)</td>
<td>105 (79-195)**</td>
<td>10 (6-12)</td>
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<td><strong>LIX (pg/ml)</strong></td>
<td>285 (166-343)</td>
<td>6.2 (4.4-9.2)</td>
<td>9.8 (7.9)</td>
<td>&lt; ***</td>
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<td><strong>MCP-1 (pg/ml)</strong></td>
<td>492 (278-731)</td>
<td>33 (30-124)***</td>
<td>5374 (4484-5831)</td>
<td>194 (106-244)</td>
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<td><strong>MIP-2 (pg/ml)</strong></td>
<td>440 (359-586)</td>
<td>5.2 (0.2-8.8)</td>
<td>17 (9-27)</td>
<td>184 (141-273)</td>
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<td><strong>KC (μg/ml)</strong></td>
<td>268 (213-377)</td>
<td>89 (30-332)</td>
<td>105 (79-195)**</td>
<td>10 (6-12)</td>
</tr>
<tr>
<td><strong>LIX (pg/ml)</strong></td>
<td>285 (166-343)</td>
<td>6.2 (4.4-9.2)</td>
<td>9.8 (7.9)</td>
<td>&lt; ***</td>
</tr>
<tr>
<td><strong>MCP-1 (pg/ml)</strong></td>
<td>492 (278-731)</td>
<td>33 (30-124)***</td>
<td>5374 (4484-5831)</td>
<td>194 (106-244)</td>
</tr>
<tr>
<td><strong>MIP-2 (ug/ml)</strong></td>
<td>440 (359-586)</td>
<td>5.2 (0.2-8.8)</td>
<td>17 (9-27)</td>
<td>184 (141-273)</td>
</tr>
</tbody>
</table>

Levels of cytokines and chemokines in bronchoalveolar lavage fluid (BALF), lung homogenates and plasma 24 and 48 h after induction of pneumococcal pneumonia. Data are expressed as median (interquartile range) of n = 8 per group. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; KC, keratinocyte-derived cytokine; LIX, lipopolysaccharide-induced CSC chemokine; MCP-1, monocyte chemotactic protein-1; MIP-2, Macrophage–inflammatory protein-2. *, ** and *** indicate P < 0.05, P < 0.01 and P < 0.001 vs control; $ and $$$ indicate P < 0.05 and P < 0.001 vs ceftriaxone. < Below detection limit (see Materials and methods).
DISCUSSION

The importance of the TF/FVIIa pathway as the main initiator of coagulation during severe inflammation has been well recognized. Inhibition of the TF pathway has been studied in preclinical and clinical models of endotoxemia and Gram-negative sepsis, which unanimously showed effective attenuation of inflammation-induced coagulopathy\textsuperscript{12,13,15-18,27,28,32}. In addition, blocking the TF pathway strongly inhibited activation of coagulation in the pulmonary compartment in models of sterile or infectious lung inflammation\textsuperscript{6,21,33-35}. Moreover, post-hoc analysis of the clinical phase III OPTMIST trial suggested a survival benefit from rh-TFPI therapy for severe sepsis patients with CAP in whom a causative microorganism was identified and who had not received concomitant heparin treatment\textsuperscript{18,20}, further suggesting that the lung is an important target for TF directed interventions. In the present study, we sought to mimic the clinical scenario of administration of rh-TFPI in established pneumococcal pneumonia in the context of concurrent antibiotic therapy. We here confirm the anticoagulant properties of rh-TFPI in a murine model of advanced CAP and further show that rh-TFPI exerts anti-inflammatory effects, which, however, were only demonstrable in animals not treated with antibiotics.

TF is highly expressed in the lungs by alveolar macrophages and epithelial cells and under physiological circumstances does not become exposed to blood\textsuperscript{5,8,10}. However, when TF expression is upregulated by mononuclear and/or endothelial cells in response to inflammatory or microbial mediators, or alternatively when physical tissue damage allows for direct TF-blood contact, it is able to rapidly initiate coagulation. Indeed, elevated concentrations of soluble TF, FVIIa and TATc were found in patients with unilateral pneumonia in the infected lung compared with the uninfected site\textsuperscript{6,7,36}. Of relevance for CAP, lipoteichoic acid, a major cell wall component of Gram-positive bacteria, was able
to induce activation of coagulation in the bronchoalveolar space of mice\textsuperscript{37, 38} and healthy volunteers\textsuperscript{39}. The current findings are in accordance with our previous observations of enhanced coagulation during experimental and clinical \textit{S. pneumoniae} pneumonia\textsuperscript{6, 21}, as reflected by elevated TATc levels in lungs and plasma, indicating simultaneous local and systemic activation of coagulation. These earlier investigations also pointed to a crucial role for TF in pulmonary coagulation during pneumococcal pneumonia, as indicated by enhanced TF expression in infected lung tissue and by reduced TATc levels in animals treated with rNAPc2 or rh-TFPI\textsuperscript{6, 21}. Importantly, in these studies animals were pretreated with rNAPc2 or rh-TFPI and antibiotics were withheld, leaving the question whether TF inhibition is capable of attenuating coagulation in a more clinically relevant setting (i.e. after induction of pneumonia and in the context of antibiotic therapy) unanswered. We here show that postponed rh-TFPI therapy, initiated either 8 or 24 hours after induction of \textit{S. pneumoniae} pneumonia, still exerts potent anticoagulant effects independent of concurrent antibiotic therapy. Rh-TFPI did not influence lung or plasma PAI-1 levels, which is in accordance with previous investigations\textsuperscript{12, 21}. Of note, ceftriaxone strongly inhibited the rise in TATc and PAI-1, probably due to a reduction of bacterial loads and a concurrent reduction of inflammation. Rh-TFPI treatment in combination with ceftriaxone attenuated generation of TATc more than either treatment alone, which underlines the strong coagulant response during advanced CAP.

Although consensus exists on the interconnection between coagulation and inflammation\textsuperscript{40}, the exact role of the TF/FVIIa pathway in inflammation during lung infection and injury has not been fully elucidated. The TF pathway can impact on inflammation via interaction with protease activated receptor (PAR)1 and PAR2\textsuperscript{8, 9}. Inhibition of the TF pathway in experimentally induced sepsis in baboons was associated with strongly reduced lung injury, as reflected by histopathology, protein leak and wet/dry weights, and a largely preserved lung function\textsuperscript{28, 32}. In addition, treatment with rh-TFPI or site-inactivated FVIIa reduced vascular leakage, neutrophil influx and levels of several cytokines in lungs of rats with acute lung injury\textsuperscript{34, 35}. In the present experiments rh-TFPI attenuated the host inflammatory response, as reflected by lower cytokine and chemokine levels in BALF and plasma, less neutrophil infiltration into lung tissue and less protein leakage in BALF. Lower histopathology scores were observed in the rh-TFPI treatment group (late treatment), but without reaching statistical significance ($P = 0.13$ versus controls). The effect of rh-TFPI on lung histology appears less overt than on the actual anti-inflammatory state and more reflects the tissue damage that has been caused during the preceding hours. A clear attenuation of the host inflammatory response by rh-TFPI, although in line with anti-endotoxic effects demonstrated for C-terminal TFPI peptides in a murine model of LPS-shock\textsuperscript{41}, contrasts with our previous reports on the effect of TF inhibition during experimental pneumococcal pneumonia, in which pretreatment with either rNAPc2 or rh-TFPI did not have an impact on lung inflammation in spite of a strong anticoagulant
Together these data suggest that inhibition of TF-mediated coagulation only influences inflammation during an ongoing procoagulant/proinflammatory response and not when activation of coagulation is inhibited prior to infection, suggesting redundancy of the mechanism by which inflammation is amplified. Of note, in mice with endotoxemia or polymicrobial sepsis an amplification of inflammation by coagulation could only be demonstrated in the late phase after the injury, whereas in the early phase inflammation proceeded independently of coagulation. Rh-TFPI did not affect inflammation in mice also treated with ceftriaxone, probably because antibiotic treatment per se already strongly inhibited inflammation due to a profound reduction in bacterial loads.

Rh-TFPI exerted a modest antibacterial effect in lung tissue while it did not impact on bacterial loads in BALF, nor in blood or spleen when given 24 hours after infection without antibiotics. This may in part be explained by a relatively large endothelial surface in lung tissue to which rh-TFPI attaches via its C-terminal part. Indeed, levels of rh-TFPI measured in lung homogenate were ~5-fold higher than in plasma (Fig 1). Furthermore, a lack of antibacterial effect in blood and spleen may be due to the presence of other antibacterial mechanisms in the systemic compartment, possibly obscuring the more modest antibacterial effect of rh-TFPI. Of note, by reducing the host anti-inflammatory response rh-TFPI could actually provide a more growth stimulating environment for *S. pneumoniae*, therefore the actual growth inhibiting properties of rh-TFPI in vivo may even be larger than those we measured here. Recently C-terminal TFPI peptides were shown to exert antimicrobial activity against several pathogens; C-terminal fragments were able to kill *Escherichia coli* via the complement system ex vivo and in a direct manner at higher concentrations possibly by permeabilizing bacterial membranes. C-terminal peptides also showed antimicrobial effects against another Gram-negative bacterium (*Pseudomonas aeruginosa*) and in addition against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* and certain fungi. In accordance with these findings we confirmed a growth inhibitory effect of rh-TFPI on *S. pneumoniae* in the presence of human serum in vitro; whether this effect was solely due to antibacterial properties of C-terminal TFPI fragments remains to be established. Of note, rh-TFPI did not inhibit growth of *S. pneumoniae* in the presence of murine serum. This phenomenon was also previously observed by Papareddy et al in a murine model of *E.coli* peritonitis human TFPI peptides prolonged survival, though in vitro killing of *E.coli* was not enhanced by these peptides in the presence of murine serum. Other factors in vivo than those present in murine serum ex vivo may play a role in the observed growth inhibitory effect on *S. pneumoniae* in mice in vivo. To further address this issue more research on the physiological role of (C-terminal peptides of) rh-TFPI is warranted.

Although it is possible that the modestly reduced bacterial loads in the lung (providing a less potent proinflammatory stimulus) contributed to the local inhibition of
inflammation in the late treatment group, such an effect cannot explain the systemic diminished inflammation, or the anti-inflammatory effects in the early treatment group.

The rh-TFPI dose used in the current study was based on previous investigations in which rh-TFPI was found to reduce mortality in models of superantigen-induced shock and polymicrobial intra-abdominal sepsis in mice. We here not only established that this dosing regimen resulted in rh-TFPI plasma levels that were 3-10 fold higher than those measured in sepsis patients administered with rh-TFPI by continuous intravenous infusion, but also demonstrated the effect of these levels on TFPI activity in the mouse in vivo.

Streptococcus pneumoniae is the most common causative pathogen in CAP and a frequent cause of sepsis. The current study is the first to report on the effect of TF inhibition administered during an already ongoing infection and in the context of antibiotic therapy. We show that postponed administration of rh-TFPI to mice with established pneumococcal pneumonia inhibits coagulation, inflammation and growth of S. pneumoniae whereby the anti-inflammatory and antibacterial effects only become apparent in the absence of concurrent antibiotic treatment.
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