The tissue factor pathway in pneumonia

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CHAPTER 3

Endogenous Tissue Factor Pathway Inhibitor has a limited effect on host defence in murine pneumococcal pneumonia

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ABSTRACT

Introduction: Streptococcus (S.) pneumoniae is the most common causative pathogen in community-acquired pneumonia. Coagulation and inflammation interact in the host response to infection. Tissue Factor Pathway Inhibitor (TFPI) is a natural anticoagulant protein that inhibits Tissue Factor (TF), the main activator of inflammation-induced coagulation.

Objective: To investigate the effect of endogenous TFPI levels on coagulation, inflammation and bacterial growth during S. pneumoniae pneumonia in mice.

Methods: The effect of low endogenous TFPI levels was studied by administration of a neutralizing anti-TFPI antibody to wild-type mice, and by using genetically modified mice expressing low levels of TFPI, due to a genetic deletion of the first Kunitz domain of TFPI (TFPI<sub>K1</sub>(/-)) rescued with a human TFPI transgene. Pneumonia was induced by intranasal inoculation with S. pneumoniae and samples were obtained at 6, 24 and 48 hours after infection.

Results: Anti-TFPI reduced TFPI activity by ~50%. Homozygous lowTFPI mice and heterozygous controls had ~10% and ~50% of normal TFPI activity respectively. TFPI levels did not influence bacterial growth or dissemination. Whereas lung pathology was unaffected in all groups, mice with ~10% (but not with ~50%) of TFPI levels displayed elevated lung cytokine and chemokine concentrations 24 hours after infection. None of the groups with low TFPI levels showed an altered procoagulant response in lungs or plasma during pneumonia.

Conclusions: These data argue against an important role for endogenous TFPI in the antibacterial, inflammatory and procoagulant response during pneumococcal pneumonia.
INTRODUCTION

Streptococcus (S.) pneumoniae is the leading causative pathogen in community-acquired pneumonia (CAP), which frequently progresses into sepsis and is held responsible for an estimated 10 million deaths worldwide annually\(^1\)-\(^3\). Despite the availability of wide-ranging antibiotic resources, outcome has not improved over the past decades and urges us to expand our knowledge of the host defence mechanisms that influence the outcome of pneumococcal pneumonia and sepsis.

Pneumonia is associated with a local procoagulant state due to enhanced activation of coagulation and downregulation of anticoagulant mechanisms and fibrinolysis in the alveolar compartment, as has been shown in the lung compartment of patients and experimental animals with pneumococcal pneumonia and sepsis\(^4\)-\(^8\). The resulting local haemostatic misbalance favours intrapulmonary fibrin deposition and lung injury, which compromises tissue integrity and poses a serious challenge to lung function\(^9\).

Tissue Factor (TF) is the main initiator of infection- and inflammation-induced activation of the coagulation cascade\(^10\). TF in complex with Factor (F)VIIa activates FX, which together with its cofactor FVa generates thrombin, ultimately stimulating fibrin clot formation. The natural occurring protein Tissue Factor Pathway Inhibitor (TFPI), controls thrombin generation via the TF pathway by initial binding to FXa, and the resulting TFPI-FXa complex inhibits TF-FVIIa by formation of the quaternary TF-FVIIa-TFPI-FXa complex, preventing additional FXa generation\(^11\).

In the alveolar spaces of patients with lung injury, TFPI was found to be present mainly in a truncated and inactive form\(^12\). This functional setback of TFPI results in insufficiency to counterbalance the procoagulant state in the lung during pneumonia\(^12\)-\(^14\), and may deregulate the local inflammatory response. Conversely, enhanced coagulation may be an important element of the host response to prevent bacterial dissemination of an invading pathogen\(^15\),\(^16\). Several experimental and clinical studies have been undertaken to evaluate the effect of restoring the coagulation imbalance by blocking the TF pathway as adjunctive treatment during lung inflammation and infection. In these studies coagulation was effectively attenuated, but the effect on pulmonary inflammation and outcome has been inconsistent\(^4\),\(^7\),\(^17\)-\(^22\). However, to date, the role of endogenous TFPI during the course of pneumonia remains unclear.

In the present study we used two complementary ways to investigate the role of endogenous TFPI on coagulopathy, the host inflammatory response, bacterial loads and dissemination in pneumonia. For this, we intranasally instilled viable S. pneumoniae in wild-type mice treated with an anti-mouse TFPI antibody and in genetically modified mice with low TFPI expression.
MATERIALS AND METHODS

Animals
TFPI\(_{K1}(+/-)\) deficient mice on a C57BL/6 background\(^{23}\), which have a deletion of the first Kunitz domain of TFPI in the mutant gene, were intercrossed with mice bearing a human TFPI\(α\) transgene under control of a Tie2 promoter. Generation of the Tie2-hTFPI transgenic mice will be described in more detail elsewhere. Intercrossing of TFPI\(_{K1}(+/-)\) mice with TFPI\(_{K1}(+/-)/hTFPI^{+}\) mice resulted in TFPI\(_{K1}(-/-)/hTFPI^{+}\)
offspring, indicating that the hTFPI transgene rescued the described embryonic lethality of homozygous TFPI\(_{K1}(-/-)\) mice\(^{23}\). TFPI\(_{K1}(-/-)/hTFPI^{+}\) mice (from here on referred to as lowTFPI mice) have circulating hTFPI\(α\) concentrations of 0.038 nM, increasing to 0.57 nM after heparin treatment in vivo. Mice were bred at the animal care facility of the Academic Medical Centre. Experiments were conducted with 10-12 week old TFPI\(_{K1}(-/-)\) homozygous and TFPI\(_{K1}(+/-)\) heterozygous offspring bearing the hTFPI transgene and specific pathogen-free C57BL/6 mice (WT) purchased from Charles River (Maastricht, the Netherlands). The Institutional Animal Care and Use Committee of the Academic Medical Centre approved all experiments.

Study design
S. pneumoniae serotype 3 (American Type Culture Collection, ATCC 6303, Rockville, MD) was used to induce pneumonia. Bacteria were grown as described\(^{7, 20, 24}\) and \(\sim 5 \times 10^{4}\) colony-forming units (CFU) in 50 \(\mu\)L were inoculated intranasally. In separate experiments WT mice were treated with inhibitory polyclonal rabbit anti-murine TFPI IgG (anti-TFPI) obtained by immunizing rabbits with murine TFPI (residue 1-160) produced in Escherichia coli, or control rabbit IgG (100 \(\mu\)g in 200 \(\mu\)l) intravenously at time of infection, and every 24 hours. At predefined time points in the early (6 hours), intermediate (24 hours) or late phase (48 hours, just before the first deaths are expected to occur\(^{25}\)) of infection, blood diluted 4:1 with citrate, lungs and spleen were harvested using methods described previously\(^{7, 26}\). 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\(^{20}\).

Bacterial quantification
For bacterial quantification undiluted whole blood and serial ten-fold dilutions of organ homogenates and blood were made in sterile isotonic saline and plated onto sheep-blood agar plates. Following 16 hours of incubation at 37°C CFU were counted.
Assays
TFPI activity was measured in mouse plasma using a two-stage chromogenic TFPI assay as described previously. Standard curves were prepared by serial dilution of citrated normal mouse plasma and the mean plasma TFPI activity of WT mice was arbitrarily assigned a value of 1 U/ml. Thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany), D-dimer (Asserachrom D-dimer, Roche, Woerden, the Netherlands), macrophage–inflammatory protein (MIP)–2, keratinocyte-derived cytokine (KC), interleukin (IL)-1β (R&D Systems, Abingdon, UK) and myeloperoxidase (MPO; HyCult Biotechnology, Uden, The Netherlands) were measured using commercially available ELISA kits. Tumour necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendations.

Histopathology
Immediately after mice had been sacrificed, the left lobe was fixed in 10% buffered formalin for 24 hours and embedded in paraffin in a routine fashion. Four-micrometre sections were stained with hematoxylin and eosin (H&E). A pathologist scored all slides in a blinded fashion for the following parameters: interstitial inflammation, endothelialitis, bronchitis, oedema, pleuritis, thrombus formation and the proportion of the slide surface that showed confluent inflammation (pneumonia). All parameters were rated separately from 0 (condition absent) to 4 (most severe condition) and the total histopathological score was expressed as the sum of the scores of the individual parameters. The number of thrombi was counted in 5 random microscopic fields.

Statistical analysis
Data are expressed as box-and-whiskers diagrams. Differences between groups were analysed by Mann–Whitney U tests, using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A p-value of < 0.05 was considered statistically significant.

RESULTS
Low TFPI levels do not influence bacterial growth or dissemination in pneumococcal pneumonia
Pneumonia was associated with a gradual decline in plasma TFPI activity in WT mice (Figure 1). To obtain a first insight into the role of endogenous TFPI in the host response to pneumonia, we treated WT mice with a neutralizing anti-mouse TFPI antibody. Anti-TFPI reduced TFPI activity in plasma of uninfected mice by approximately 50% (p=0.029
versus mice treated with control antibody). TFPI activity remained significantly lower in anti-TFPI treated mice throughout the course of the infection (Figure 1A).

Considering that the extent of TFPI activity inhibition produced by the anti-TFPI antibody might not be sufficient to reveal a role of endogenous TFPI, we also made use of genetically modified mice with low (human) TFPI expression, generated by introduction of a human TFPI transgene on a TFPI<sub>K1</sub> deficient background. Notably, human TFPI transgene expression did not influence TFPI activity in TFPI<sub>K1</sub>(+/-) mice relative to control WT mice (Figure 1B). Uninfected TFPI<sub>K1</sub>(+/-)/hTFPI+ mice showed an approximately 50%
reduction in endogenous TFPI activity (p=0.012 versus WT mice, Figure 1B), and at 48 hours after infection with *S. pneumoniae*, which resembled the extent of TFPI activity reduction in anti-TFPI treated WT mice. However, in lowTFPI mice TFPI activity was reduced to ~10% before and at 24 and 48 hours infection (p<0.001 versus WT mice, Figure 1B).

TFPI has been reported to exert antibacterial effects. To investigate the effect of endogenous TFPI levels on bacterial multiplication and dissemination in pneumococcal pneumonia, we determined bacterial loads in lung, spleen and blood at various time points after infection. Neither anti-TFPI treated mice (Figure 2A-C), nor lowTFPI mice (Figure 2D-F) showed differences in bacterial loads when compared to their respective controls in any body site tested.

**Figure 2. Low TFPI levels do not impact on bacterial loads in pneumococcal pneumonia.** Graphs show the number of colony forming units (CFU) per ml lung homogenate (A, D), spleen homogenate (B, E), and whole blood (C, F) of wild-type (WT) mice treated with anti-mouse TFPI (anti-TFPI, open boxes) or control (grey boxes) antibody (A-C) 6, 24 and 48 hours after infection, and of WT (grey boxes), lowTFPI mice (TFPI(-/-), open boxes) and heterozygous littermates (TFPI(+/-), striped boxes) (D-F) 24 and 48 hours after intranasal infection with *S. pneumoniae*. Heterozygous littermates were not studies at 24 hours. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n = 8 per group). n.d, not detected.

**Low TFPI levels do not influence pulmonary or systemic inflammation**

Considering that the TF pathway can exert a variety of proinflammatory effects, we next set out to investigate the impact of low TFPI levels on the host inflammatory response during pneumococcal pneumonia. Pneumonia was associated with pulmonary inflammation as evidenced by the occurrence of bronchitis, interstitial inflammation, oedema and endothelialitis at 24 hours and 48 hours after infection with *S. pneumoniae* in all mice. Anti-TFPI treatment did not influence the extent of lung inflammation at either 24 or 48 hours infection, as reflected by similar pathology scores determined using
the semi-quantitative scoring system described in the Methods (Figure 3A and B). The extent of lung pathology tended to be lower in lowTFPI compared with WT mice, however this difference did not reach significance (p=0.09) (Figure 3C and D). Furthermore, MPO concentrations in whole lung homogenates, reflecting the number of neutrophils in lung tissue, did not differ between mice treated with anti-TFPI or control antibody or between lowTFPI and WT mice (data not shown).

Very low TFPI levels result in a transient increase in lung cytokine levels
To further evaluate the impact of TFPI levels on pulmonary inflammation during pneumococcal pneumonia, we measured the levels of various cytokines (TNF-α, IL-6, IL-1β) and chemokines (KC, MIP-2) in lung homogenates obtained 24 and 48 hours after infection. Whereas anti-TFPI treatment did not influence the levels of these mediators (data not shown), lowTFPI mice demonstrated increased lung concentrations relative to WT mice at 24 hours after infection (TNF-α, IL-6, IL-1β, MIP-2); these differences had subsided at 48 hours (Table 1). Plasma TNF-α, IL-6, IFNγ and MCP-1 concentrations did

Figure 3. Lung pathology is not affected by low TFPI levels during pneumococcal pneumonia.
Mice were intranasally infected with S. pneumoniae and samples obtained 24 and 48 hours after infection. Total lung histopathology scores (PA) in wild-type (WT) mice treated with anti-mouse TFPI (anti-TFPI, open boxes) or control (grey boxes) antibody (A), with representative photomicrographs of lung hematoxylin and eosin (H&E) staining 48 hours after infection (B). Total PA scores in WT (grey boxes) and lowTFPI mice (TFPI(-/-), open boxes) (C), with representative photomicrographs of lung hematoxylin and eosin (H&E) staining 48 hours after infection (D). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n=8 per group). Scale bar = 200 μm. # p=0.09.
not differ between mice treated with anti-TFPI or control antibody (data not shown) or between lowTFPI and WT mice (Table 2).

**Low TFPI levels do not influence the procoagulant response to pneumonia**

Infection with *S. pneumoniae* via the airways resulted in local and systemic activation of the coagulation system, as reflected by an increase in lung (Figure 4A) and plasma TATc concentrations (Figure 4B), which was especially apparent at 48 hours post infection. Anti-TFPI treatment did not influence lung or plasma TATc levels in either uninfected mice, or at any time point after induction of pneumonia.

Neither lung (Figure 4C) nor plasma TATc levels (Figure 4D) differed between lowTFPI mice, heterozygous and WT controls. We confirmed the absence of an effect on coagulation of low TFPI levels by measuring D-dimer in lungs and plasma of lowTFPI mice, heterozygous and WT controls before and 48 hours after infection. While pneumonia

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Levels of cytokines and chemokines in lung homogenates in wild-type (WT) and homozygous lowTFPI mice, 24 and 48 hours after induction of pneumococcal pneumonia (n=8 per group). Data are expressed as median (interquartile ranges). TNF, tumour necrosis factor; IL, interleukin; KC, keratinocyte-derived cytokine; MIP-2, Macrophage–inflammatory protein–2. * and ** indicate p<0.05 and p<0.01 compared with WT.

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Levels of cytokines and chemokines in plasma of wild-type (WT) and homozygous lowTFPI mice, 24 and 48 hours after induction of pneumococcal pneumonia (n=8 per group). Data are expressed as median (interquartile ranges). TNF, tumour necrosis factor; IL, interleukin; IFN, interferon; MCP-1, monocyte chemotactic protein.
clearly was associated with a rise in lung and plasma D-dimer concentrations, no differences were found between mouse strains (Supplementary Figure 1).

**DISCUSSION**

Activation of the coagulation system via the TF pathway is a hallmark of the host inflammatory reaction to tissue injury. The role of TF-induced coagulation during inflammation has been investigated in numerous experimental and clinical studies, mainly by administration of exogenous inhibitors of this pathway, resulting in attenuation of inflammation-induced coagulopathy. Moreover, effective blockade of the activity of TF prevents local activation of coagulation in pneumonia. At the same time, pulmonary levels of endogenous TFPI increase during lung inflammation, while its activity becomes compromised due to truncation and inactivation by serine proteases. In

![Figure 4. Low TFPI levels do not impact on coagulation during pneumococcal pneumonia.](image)

Thrombin-antithrombin complexes (TATc) levels in lung homogenates (A, C) and plasma (B, D) of wild-type (WT) mice treated with anti-mouse-TFPI (anti-TFPI, open boxes) or control antibody (grey boxes) uninfected and 6, 24 and 48 hours after infection with *S. pneumoniae* (A, B), and of WT mice (WT, grey boxes), lowTFPI mice (TFPI(-/-), open boxes) and heterozygous littermates (TFPI(+/-), striped boxes) 24 and 48 hours after intranasal infection (C, D). Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n=4 per uninfected group, n=8 per infected group).
the present study we aimed to evaluate the role of endogenous TFPI during pneumonia on local and systemic coagulation, inflammation and bacterial loads. We demonstrate that a reduction of endogenous TFPI levels, with concurrently decreased TFPI activity, does not affect bacterial numbers and modestly influences the host inflammatory response, while leaving inflammation-induced coagulation unaffected in a murine model of pneumococcal pneumonia.

TF is abundantly expressed in the lung by alveolar epithelial cells and macrophages, where it has a major role in activating coagulation upon tissue injury or stimulation by inflammatory mediators\(^3\). Indeed, enhanced levels of TF, FVIIa and TATc were found in lavage fluid from the affected lung of healthy volunteers challenged with lipoteichoic acid, a major cell wall component of Gram-positive bacteria, and of patients with pneumonia\(^5\), \(^7\), \(^37\)-\(^40\). Further evidence for a key role of TF in pulmonary coagulopathy during pneumonia, is provided by animal studies, in which administration of TF pathway blocking agents attenuated procoagulant changes\(^4\), \(^7\), \(^20\). In line with these reports, in the present study, we found enhanced pulmonary and systemic coagulation in infected animals, as reflected by increasing TATc levels in lung homogenates and plasma in the course of pneumonia.

TFPI is the only known endogenous regulator of the TF-dependent pathway of coagulation and is of crucial physiological importance, clearly demonstrated by the fact that TFPI-null mouse embryos lacking the Kunitz-1 domain do not survive embryogenesis\(^23\). In addition, to date, no patients with TFPI-deficiency have been identified. Reportedly, from various organs, the human lung expresses the highest quantities of TFPI mRNA\(^41\). In the lung, TFPI is present along alveolar septae and in the alveolar epithelium, suggesting that TFPI may be important in the setting of lung injury, when it can be released into the alveolar space\(^12\). Indeed, elevated levels of TFPI were measured in lavage fluid of patients suffering from the acute respiratory distress syndrome (ARDs) or pneumonia\(^5\), \(^12\), \(^37\). In line with previous reports in patients suggesting a decline in TFPI activity in lung inflammation\(^12\)-\(^14\), we observed diminished TFPI activity in the course of pneumonia. We sought to obtain more insight into the functional role of endogenous TFPI during pneumonia, using two complementary experimental strategies. First, we treated WT mice with a TFPI inhibiting antibody, using a dosing regimen that previously achieved total inhibition of plasma TFPI activity\(^42\). In contrast, in our studies TFPI activity was inhibited to an extent of ~50% by this treatment. In a second set of experiments, we used genetically modified lowTFPI mice with a Tie2 promoter containing hTFPI transgene. TFPI\(^{K1}(+)/hTFPI+\) mice, similar to TFPI\(^{K1}(+)/hTFPI+\) mice lacking the human TFPI transgene\(^43\), had endogenous TFPI activity ~50% of normal levels, while lowTFPI mice demonstrated ~10% of WT TFPI activity. Even in mice with a 90% reduction in TFPI activity, coagulation activation was not affected, as reflected by unaltered TATc and D-dimer levels in lung and plasma. Together these results suggest that either very low levels of TFPI activity are sufficient to attenuate
inflammation-induced coagulation during pneumococcal pneumonia, or in contrast, that endogenous TFPI does not play a key role herein.

Coagulation and inflammation interact via the TF pathway, at least in part mediated by protease-activated receptor (PAR)1 and PAR2. An important contribution of TF to the host inflammatory response was implicated by early studies, showing reduced lung injury with preserved lung function and improved outcome in septic baboons treated with TF blocking agents. In models of direct lung injury, inhibition of the TF pathway has yielded inconsistent effects on inflammation. In rats with acute lung injury, blocking the TF pathway attenuated vascular leakage, neutrophil influx and levels of cytokines and chemokines. In studies from our group, these parameters were only affected by treatment with recombinant human (rh)-TFPI during already ongoing infection in murine pneumococcal pneumonia, but not when animals were pre-treated with rh-TFPI or other TF blocking agents. Of clinical importance, rh-TFPI failed to show a beneficial effect in patients with severe CAP. However, these aforementioned studies investigated the effect of exogenous inhibitors of the TF pathway. In the present study, we found increased levels of cytokines and chemokines in lungs of mice expressing ~10% of normal TFPI levels 24 hours after infection, but not in mice with less extensive TFPI inhibition, suggestive of a temporary proinflammatory effect of the TF pathway during pneumococcal pneumonia that becomes apparent only at very low endogenous TFPI concentrations. These data suggest that TFPI dampens TF-mediated proinflammatory effects early in the course of the infection; while its contribution to the inflammatory response in the advanced phase of pneumonia may be obscured by other inflammatory mechanisms. Notably, the proinflammatory effect of low TFPI levels only becomes apparent in the lung compartment, which is the predominant site of TF expression. The modulating effect on inflammation appears to be mediated independently of coagulation, and may be attributed to TF-PAR2-signalling. PAR2 is widely expressed in the airways and is a ligand for TF/FVIIa and FXa in the ternary TF-FVIIa-X complex.

Containment of invading pathogens at the site of entry may be an important role played by the coagulation system in the initial host response. Recently, it was shown that neutrophil serine protease–induced TFPI cleavage supports coagulation during systemic infection, contributing to the retention of bacteria inside microvessels. Studies of mice infected with streptococci showed that fibrin deposition limited the survival and dissemination of bacteria. In addition, recent in vivo and in vitro studies have revealed antibacterial properties of the carboxy-terminal peptides of the rh–TFPI molecule. However, in vivo only about 10% of plasma TFPI circulates in a full-length free form, and truncated forms of TFPI lack most of their C-terminal. This, together with unaltered coagulation in mice with low TFPI activity, may explain for the current observation, that reduced TFPI levels exhibited no effect on bacterial loads or dissemination in pneumococcal pneumonia.
In conclusion, we used WT mice treated with an anti-TFPI antibody and genetically modified lowTFPI mice rescued by a human TFPI transgene to evaluate the role of endogenous TFPI in pneumococcal pneumonia. The data presented argue against an important role for endogenous TFPI in bacterial growth and dissemination, or in attenuation of local or systemic coagulation activation during respiratory tract infection caused by *S. pneumoniae*. 
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Endogenous TFPI in pneumococcal pneumonia


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Supplementary Figure 1. Pulmonary and systemic levels of D-dimer in lowTFPI mice during pneumococcal pneumonia. D-dimer levels in lung homogenates (A) and plasma (B) of wild-type (WT, grey boxes), heterozygous (TFPI(+/-), striped boxes) and homozygous (TFPI(-/-), open boxes) lowTFPI mice uninfected and 48 hours after intranasal infection with *S. pneumoniae*. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (n=4 per uninfected group, n = 8 per infected group).