The tissue factor pathway in pneumonia

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CHAPTER 11

Summary and general discussion
Infectious diseases are a major cause of morbidity and mortality worldwide. Particularly the lung is at a constant risk to become infected by invading microorganisms as this organ is directly exposed to the environment. Community-acquired pneumonia (CAP) is a common illness throughout the world with Streptococcus (S.) pneumoniae, the pneumococcus, as the most frequently isolated causative pathogen. As mortality rates have stagnated over the past years, expanding our current knowledge on the innate immune system is required in the quest to develop adjunctive treatment options and improve overall outcome of pneumonia.

Activation of the coagulation system is a hallmark of the host response during infection, and may be considered as an attempt to contain invading pathogens and the subsequent inflammatory response. In addition, it has become increasingly clear that components of the coagulation system can influence inflammation, vice versa facilitating an adequate host response against infectious agents. However, an exaggerated inflammatory response with collateral tissue damage is deleterious to the host. In this instance, the coagulation response itself may contribute to disease. **Chapter 1** is a general introduction that describes coagulation initiated by the Tissue Factor (TF) pathway and platelets under physiological circumstances and during lung infection, and the role of Protease-Activated Receptor (PAR)2 in the crosstalk between coagulation and inflammation. In this thesis we used rodent models to study the role of coagulation during lung infection, with a special focus on the TF pathway and PAR2-signalling in pneumococcal pneumonia.

In **Part I** of this thesis we evaluated the role of TF-induced coagulation in pneumonia. In **chapter 2** we studied the role of endogenous TF levels on host defense in pneumococcal pneumonia. Mice expressing ~1% of normal TF levels were intranasally infected with *S. pneumoniae*. Infection-induced coagulation was attenuated in low TF mice in the early and late phase of infection. In between these phases the attenuation of coagulation in low TF mice was abolished, and this coincided with a transient pro-inflammatory response in the lungs. During the course of pneumonia low TF mice did not have altered bacterial loads. We conclude that reduced TF levels during pneumococcal pneumonia coincide with attenuated infection-induced coagulation with limited impact on lung inflammation or antibacterial defense. Vice versa, in **chapter 3**, the role of the endogenous TF pathway inhibitor (TFPI) in pneumococcal pneumonia was studied in mice treated with a neutralizing anti-TFPI antibody, and genetically modified mice with low TFPI expression. In the course of pneumonia plasma TFPI activity was diminished. Although TFPI activity in mice was reduced to ~50% by anti-TFPI antibodies and ~10% in low TFPI mice, none of these mice showed an altered procoagulant response in lungs or plasma.
during infection. Mice with ~10% (but not with ~50%) of TFPI levels displayed transient elevated lung cytokine and chemokine concentrations, however lung pathology was unaffected. TFPI levels did not influence bacterial growth or dissemination. These data argue against an important role for endogenous TFPI in the procoagulant, inflammatory and antibacterial response during pneumococcal pneumonia.

In chapter 4 we investigated the effect of early and late initiated treatment with recombinant human (rh)-TFPI, either or not as an add-on to antibiotic therapy in ongoing pneumococcal pneumonia. Besides reducing pneumonia-induced coagulation, rh-TFPI also attenuated the host inflammatory response, as reflected by lower cytokine and chemokine levels in lungs and plasma, less neutrophil infiltration into lung tissue and less protein leakage in bronchoalveolar lavage fluid. Notably, rh-TFPI exerted a modest antibacterial effect in lung tissue when administered in the late phase of infection and we confirmed a growth inhibitory effect of rh-TFPI on *S. pneumoniae* in vitro. The anti-inflammatory and antibacterial effects did not become apparent in mice concomitantly treated with ceftriaxone. We conclude that therapeutic rh-TFPI in established pneumococcal pneumonia inhibits coagulation, inflammation and pneumococcal growth.

One important drawback of systemic administration of anticoagulants is the increased risk of bleeding complications. In addition, the ability of systemically administered drugs to concentrate in sites of infection is uncertain. To overcome these potential flaws of systemic administration of rh-TFPI, in chapter 5 we studied the feasibility and effect of intra-alveolar administration of rh-TFPI by nebulization in a rat model of pneumococcal pneumonia. Local treatment with rh-TFPI attenuated local coagulation, while systemic TFPI activity and thrombin-antithrombin complex (TATc) levels remained unaffected. No lung haemorrhage was observed after intra-alveolar administration of rh-TFPI. Nebulized rh-TFPI did not impact on lung tissue injury or bacterial loads in lung and blood. We report that nebulization with rh-TFPI has anticoagulant effects restricted to the lung compartment without local haemorrhagic adverse effects and thus seems feasible and safe in rat pneumonia. We pursued to study local treatment with rh-TFPI in two rat models of lung injury, described in chapter 6. Rats received an intratracheal challenge with *Pseudomonas aeruginosa*, a Gram-negative pathogen causing direct lung injury, or received an intravenous injection of lipopolysaccharide (LPS), a membrane constituent of Gram-negative bacteria, causing indirect lung injury. Both models were associated with increased pulmonary and systemic coagulation activity. Nebulization with rh-TFPI did not affect systemic TFPI activity. However, in contrast with the observations in chapter 5, nebulization with rh-TFPI attenuated both pulmonary and systemic coagulation in these models of lung injury. Nebulized rh-TFPI also reduced the inflammatory response, which may have resulted in a less potent procoagulant stimulus, explaining for attenuation of systemic coagulation despite unaltered systemic TFPI activity. Furthermore, nebulization with rh-TFPI reduced bacterial growth of *P. aeruginosa* in the alveolar compartment.
Mounting evidence points towards a significant role for coagulation in the enhancement of inflammation as an essential part of host defense during infection. It has become clear that components of the coagulation system are able to signal via PARs. In Part II of this thesis we focus on the role of PAR2, which is a substrate for proteases derived from the TF pathway, during pneumococcal pneumonia. In chapter 7 we investigated the role of PAR2 in pneumococcal pneumonia. In mice intranasally infected with S. pneumoniae, the absence of PAR2 resulted in an improved host defense, as reflected by lower bacterial loads in lungs, largely preserved lung barrier integrity with markedly reduced dissemination of pneumococci, ultimately resulting in reduced mortality. Notably, PAR2 deficiency did not influence bacterial loads after intravenous infection. Inhibition of the archetypical PAR2 activating proteases did not impact on bacterial loads. Furthermore, we were unable to show direct activation of PAR2 by S. pneumoniae in vitro. Taken together, these data demonstrate that S. pneumoniae misuses PAR2 in the airways to cause systemic dissemination. We continued to investigate the role of potential PAR2 activating proteases, such as tryptase and granzyme A in the next chapters. Chapter 8 describes the role of mast cells, as an important source of tryptase in the airways, in pneumococcal pneumonia. In lung tissue obtained from pneumonia patients, the constitutive presence of tryptase positive mast cells was reduced. Mast cell deficient mice showed reduced bacterial counts with less systemic bacterial dissemination, less inflammation, and a prolonged survival in the first days after infection, although overall mortality was not affected. Mast cell stabilizing agents did not influence antibacterial defense or inflammation. We conclude that mast cells exhibit an unfavorable role in host defense during pneumococcal pneumonia by a mechanism independent of their degranulation. In chapter 9 we show that levels of granzyme A, a trypsin-like protease, are elevated in BALF from the infected lung from CAP patients. We observed constitutive granzyme A expression by resident and non-resident cells in human lung tissue. Mice deficient of granzyme A showed better survival and lower bacterial counts in bronchoalveolar lavage fluid and distant organ sites after intranasal infection with S. pneumoniae. Natural Killer (NK) cells show strong granzyme A expression, however NK cell depletion did not influence bacterial loads. Taken together these data demonstrate that granzyme A plays an unfavorable role in host defense during pneumococcal pneumonia by a mechanism that does not depend on NK cells.

Platelets are chief effectors of primary hemostasis and provide a suitable surface for the activation of coagulation factors. In critically ill patients thrombocytopenia is an independent predictor of mortality. Part III describes the role of platelets in pneumonia caused by S. pneumoniae. In chapter 10 we depleted mice from platelets by anti-mouse thrombocyte serum, and in a different set of experiments we inhibited aggregation and secondary activation of platelets with clopidogrel, an irreversible inhibitor of the
P2Y₁₂ receptor for adenosine diphosphate on platelets. Thrombocytopenic mice demonstrated reduced survival during pneumococcal pneumonia, associated with higher bacterial loads in lungs and increased systemic bacterial dissemination. These mice also showed increased systemic activation of coagulation and cytokine levels. Clopidogrel treatment strongly prolonged the bleeding time, but did not impact on bacterial loads during infection. We conclude that platelets are not requisite for coagulation activation and play a protective role during pneumococcal pneumonia independent of their aggregation.

GENERAL DISCUSSION

With the studies described in this thesis, we aimed to obtain further insight into the role of coagulation on host defense during pneumonia, with a special focus on the TF pathway. Enhanced levels of TF, FVIIa and TATc have been demonstrated in bronchoalveolar lavage fluid from the affected lung of healthy volunteers challenged with bacterial cell wall components, and of patients with pneumonia. It is generally accepted that TF is responsible for activation of the coagulation cascade in the lung during pneumonia, evidenced by attenuation of coagulation by inhibition of the TF pathway during lung infection. At the same time, TFPI appears to be inactivated due to truncation and its increased production in response to lung infection is not sufficient to counterbalance increased TF procoagulant activity. Accordingly, coagulation was enhanced with a concurrent decline in TFPI activity during the course of CAP induced by S. pneumoniae and (in)direct lung injury reported in this thesis. Moreover, we confirmed the essential role of TF for induction of coagulation in pneumonia in mice expressing low endogenous TF levels. Remarkably, low endogenous TFPI levels did not alter the procoagulant response during lung infection, which suggest that either low (<10%) levels of TFPI activity are sufficient in attenuating infection-induced coagulation or that endogenous TFPI plays a negligible role herein. However, in line with previous reports, administration of rh-TFPI effectively reduced inflammation-induced coagulation.

Ample evidence points towards a mutual interaction between coagulation and inflammation. Previous studies evaluating the effect of TF-induced coagulation on inflammation have reported inconsistent results. Early studies in septic baboons treated with TF blocking agents showed reduced lung injury with preserved lung function and improved outcome. However, in an experimental setting of human endotoxemia, rh–TFPI did not affect inflammatory pathways, whilst completely abrogating activation of coagulation. In rat models of direct lung injury blocking the TF pathway attenuated vascular leakage, neutrophil influx and levels of cytokines and chemokines. However, when mice were treated with TF blocking agents these parameters were not affected.
In our pneumococcal pneumonia studies we unexpectedly observed a transient pro-inflammatory effect in low TF mice. This could at least in part be attributed to attenuated mitogen-activated protein kinase phosphatase (MPK)-1 expression, which is an important inhibitor of inflammation\textsuperscript{24} and suggests that under normal circumstances TF-induced inflammation is regulated by this feedback mechanism. Notably, low TFPI mice also had a transient pro-inflammatory effect, mediated independently of coagulation, considering the unaltered coagulant response, pointing towards a role for TFPI to blunt TF-mediated early inflammation during pneumonia. However, previous studies report no impact of treatment with TF-inhibiting agents on lung inflammation when initiated at the time of infection\textsuperscript{6,12}. In contrast, in chapter 4 we demonstrate that, when administered in a clinically more relevant situation of already established CAP, rh-TFPI exerted clear anti-inflammatory effects. These data suggest that inhibition of TF-mediated coagulation only influences inflammation during an ongoing procoagulant and proinflammatory response, suggesting redundancy of the mechanism by which inflammation is amplified when activation of coagulation is inhibited prior to infection. This hypothesis is further supported by the observation that 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\textsuperscript{25}. Local pre-treatment with rh-TFPI by nebulization did not impact on inflammation during pneumococcal pneumonia, whereas it diminished inflammation in rats with indirect lung injury due to sepsis and pneumonia caused by \textit{P. aeruginosa}. Remarkably, systemic administration of rh–TFPI in a similar model of indirect lung injury previously did not show anti-inflammatory effects\textsuperscript{26}, suggesting that local administration of rh–TFPI is more effective than systemic treatment in dampening lung inflammation in this setting. Taken together, the effect of blocking the TF pathway on inflammation appears to depend on the site and timing of TF inhibition, and furthermore varies between infecting pathogens and the host species.

In experimental Gram-negative infection, inactivation of TFPI by neutrophil serine proteases supported coagulation during systemic infection, contributing to the retention of bacteria inside microvessels and suppression of pathogen dissemination\textsuperscript{13}. In our studies neither mice with low TF or low TFPI levels had altered bacterial numbers or bacterial dissemination, arguing against a direct role of the endogenous TF pathway in the antimicrobial response against \textit{S. pneumoniae}. Recently, studies have reported antimicrobial activity of C-terminal peptides of the rh–TFPI molecule against several pathogens, including \textit{P. aeruginosa}\textsuperscript{27,28}. Indeed we observed antimicrobial effects of rh-TFPI in bronchoalveolar lavage fluids of rats pre-treated with nebulized rh-TFPI in \textit{P. aeruginosa} pneumonia, and similar effects during pneumococcal pneumonia in the lungs of mice with established pneumonia after systemic administration. Of note, the lower bacterial counts may have accounted for a less strong pro-inflammatory stimulus.
in respective animals. However, no antimicrobial effects were observed during pneumococcal pneumonia in rats pretreated with local rh-TFPI. Differences between the anti-inflammatory and antibacterial effects of rh-TFPI during various models of lung injury add to the notion that host defense pathways against different pathogens, although all cause (infectious) lung injury, rely on distinct mechanisms. These respective lung injurious circumstances may each require further dosage optimization of (nebulized) rh-TFPI to yield local anti-inflammatory or antibacterial effects.

Initial promising preclinical results of TF blocking agents have prompted the performance of the OPTIMIST trial, investigating rh-TFPI in sepsis patients, which did not show an overall improved outcome, but suggested a protective effect from rh-TFPI in a subgroup of CAP patients. The ensuing CAPTIVATE trial, specifically designed to investigate the effect of rh-TFPI in severe CAP however, failed to show a beneficial effect on outcome. Obviously, patients are concurrently treated with antibiotics and this may at least partially explain the negative results of these trials. Of interest in this respect, we did not observe any anti-inflammatory or antibacterial effects in mice also treated with ceftriaxone, probably because antibiotic treatment per se already profoundly reduced bacterial loads and inflammation. Additionally, in these trials anticoagulant agents were administered systemically, consequently increasing the incidence of bleeding complications. Indeed, the OPTIMIST trial reported more adverse events with bleeding in patients treated with rh-TFPI than in placebo treated patients (24% versus 19%), which may have counterweighed potentially favorable effects. In this thesis, we show that local delivery of rh-TFPI is feasible and nebulized rh-TFPI was not associated with increased pulmonary hemorrhage. Future studies have to assess its effectiveness in clinical practice.

Protease-activated receptors (PARs) have been shown to play a key role in the regulation of inflammation in the lungs. In the second part of this thesis, we looked at the role of PAR2, as a substrate for TF pathway derived proteases, in host defense during pneumonia. From our observations a clear detrimental effect of PAR2 for the host was demonstrated, which was caused by loss of the lung barrier integrity, with subsequent enhanced dissemination and increased mortality. These findings contrast with previous reports in which PAR2 protected mice from lung injury and lethality from influenza A and in pneumonia caused by *P. aeruginosa* and once again stress the heterogeneity of the host defence in response to different pathogens.

We aimed to identify which serine protease is responsible for the PAR2-mediated unfavorable phenotype in pneumococcal pneumonia. Firstly, by blocking the TF pathway in both wild-type and PAR2 deficient mice, we showed that TF pathway derived proteases do not contribute to the observed phenotype, which is in line with the fact that low TF mice are not protected during pneumococcal pneumonia. We proceeded to evaluate other endogenous candidate PAR2 activating proteases. Interestingly, both mast cell
deficient mice, thus lacking the main source of tryptase, and granzyme A deficient mice, demonstrated similar improved host defense to PAR2 deficient mice. However, we were unable to yield the same effects by pharmacological inhibition of tryptase, inhibition of mast cell degranulation, and other endogenous protease inhibitors in this model. Non-selective inhibition of these agents and redundant mechanisms are possible explanations for these observations; however, mast cells and granzyme A have immunomodulatory effects that are mediated independent of PAR2. Finally we were unable to show PAR2 activating properties of proteases derived from *S. pneumoniae* in vitro. Undoubtedly, performing experiments with live pneumococci may have hampered the experimental set-up and more research using *S. pneumoniae* derived proteases would provide more definitive answers about the ability of the pneumococcus to directly misuse PAR2 in its advantage to cause invasive disease.

Next to TF-initiated coagulation, platelets are other key effectors of an initial rapid coagulation response. Thrombocytopenia is a common finding in critically ill patients and an independent predictor of mortality of ICU patients with CAP. Indeed, we demonstrate that lack of platelets promotes bacterial dissemination and results in increased mortality. Remarkably, we found that thrombocytopenia is associated with enhanced rather than reduced coagulation, likely due to increased bacterial loads and a pro-inflammatory state. Our findings demonstrate that platelets are not requisite for infection-induced coagulation.

**CONCLUSIONS**

From our studies presented in this thesis, it becomes clear that the role of the TF pathway in host defense during pneumonia is not univocal. Low levels of endogenous TF or TFPI had limited impact on inflammation and bacterial loads, although one must bear in mind that TF- and TFPI-deficient mice are not viable and that these mice are rescued by a human transgene, which implies that this transgene provides essential levels of these proteins. Our data support an anti-inflammatory and antibacterial effect of rh-TFPI in pneumonia, which depends on the timing and route of administration and on the causative pathogen. However these effects may not become apparent with concurrent antibiotic treatment, which is common practice in the clinical situation. Overall, extrapolation of these experimental results to the complex and heterogeneous human setting should be done with caution. We furthermore demonstrate that *S. pneumoniae* misuses PAR2 in its advantage to cause invasive disease, however we failed to identify the protease(s) responsible for PAR2 activation in pneumonia. Nonetheless, this obser-
vation may provide new therapeutic strategies to improve outcome of pneumococcal pneumonia.
## REFERENCES


