Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Summary, general discussion and conclusion
SUMMARY

Tuberculosis (TB) is one of the most common causes of bacterial infection worldwide and melioidosis, caused by the Gram-negative bacterium *Burkholderia (B.) pseudomallei*, is a common cause of community-acquired sepsis in Northeast Thailand and Northern Australia. During severe infections the coagulation system plays a central role as part of the host immune response. Recent data have indicated that the coagulation system is also activated during TB and melioidosis, however, the exact role of the coagulation system during TB and melioidosis remains ill-defined. **Chapter 1** provides a general introduction on TB and melioidosis, with respect to epidemiology, clinical presentation and pathogenesis. In addition, a brief overview of the coagulation and fibrinolytic system under physiological circumstances and during inflammatory conditions is presented, followed by a summary of the current knowledge on this topic with respect to TB and melioidosis. Data on TB and melioidosis are, however, limited and mainly descriptive. The aim of this thesis therefore was to provide more extensive and in-depth analyses of the effects of the separate proteins involved in coagulation and fibrinolysis on the host response during TB and melioidosis.

In **Part I** of this thesis we evaluate the role of coagulation and fibrinolysis during pulmonary TB. **Chapter 2** describes the involvement of coagulation, anticoagulation and fibrinolysis in patients infected with *Mycobacterium (M.) tuberculosis*, proven by positive Ziehl-Neelsen stained sputum samples. These studies were performed in Chittagong, Bangladesh, an area in which TB is highly prevalent. Relative to uninfected controls, primary and recurrent TB were associated with a systemic net procoagulant state, as indicated by enhanced activation of coagulation (elevated plasma levels of thrombin-antithrombin complexes (TATc), D-dimer and fibrinogen; prolonged clotting times), together with impaired anticoagulant mechanisms (reduced plasma levels of antithrombin, protein C (PC) activity, free protein S and PC inhibitor). Moreover, TB patients demonstrated clear evidence of endothelial cell activation. Activation of coagulation only correlated with plasma concentrations of established TB biomarkers to a limited extent. Coagulation activation could not be detected in broncho-alveolar lavage fluid obtained from the primary site of infection in a subset of TB patients, indicating that pulmonary TB is mainly associated with a systemic hypercoagulable state, with little effects on local coagulation.

In the next chapters of **Part I** we investigated the role of coagulation and fibrinolysis during TB by using our well-established mouse model of pulmonary TB. **Chapter 3** evaluates the role of the endothelial PC receptor (EPCR) and activated PC (APC) during experimental TB. After intranasal inoculation with *M. tuberculosis*, mice demonstrated upregulation of lung EPCR expression. However, EPCR as well as APC only played a limited role in the host response during experimental pulmonary TB, as no significant differences in bacterial growth could be detected between wild type (WT) and EPCR-overexpressing mice. Nevertheless, EPCR overexpression was associated with decreased pulmonary coagulation activation and increased influx of macrophages in the early phase of infection. In EPCR-deficient mice coagulation activation was decreased as well 6 weeks later.
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post-infection, with little impact on other inflammation markers. Finally, APC-overexpression or treatment with anti-(A)PC antibodies displayed minimal effects during experimental TB. Chapter 4 focuses on the lectin-like domain of thrombomodulin (TM) during experimental TB. TM, a transmembrane vascular receptor, plays a pivotal role in coagulation and during inflammatory conditions\textsuperscript{10}. Its lectin-like domain on the NH\textsubscript{2}-terminal side is not involved in regulation of coagulation, but rather is responsible for a variety of anti-inflammatory properties of TM\textsuperscript{11,12}. We demonstrate, for the first time, downregulation of TM-expression in lung tissues of TB-positive patients, which was not related to changes in the amount of endothelium in infected lungs. To find out whether the lectin-like domain of TM would play a protective role in the host defense against TB, we infected mice lacking the lectin-like domain of TM (TM\textsuperscript{LeD/LeD} mice). Upon infection with \textit{M. tuberculosis}, they showed unaltered mycobacterial loads in lungs, liver and spleen as well as similar lung histopathology and cytokine levels when compared to infected WT mice. We concluded that the lectin-like domain of TM does not play an important role in the host response to \textit{M. tuberculosis} infection in mice. In chapter 5 we investigated the role of plasminogen activator inhibitor (PAI)-1 during murine TB. We demonstrated that only 5 weeks post-infection, bacterial loads in lungs of PAI-1 deficient mice were significantly higher compared to WT mice, while no differences were seen at the other time points (2 and 29 weeks). Furthermore, only at 2 weeks post-infection increased influx of macrophages and lymphocytes was observed. In conclusion, these data suggest that PAI-1 contributes to transient, non-specific changes in immunity during the early phase of murine tuberculosis.

Part II of this thesis focuses on coagulation and fibrinolysis during melioidosis. To unravel the effects of the different proteins involved in these processes we used our well-established mouse model of melioidosis, in which mice were intranasally infected with \textit{B. pseudomallei}\textsuperscript{13}. Additionally, we measured different proteins involved in coagulation and fibrinolysis in culture-proven melioidosis patients, who had been admitted to the Sapprasithiprasong Hospital in Ubon Ratchathani, Thailand. We first focused on the proteins involved in the PC system. In chapter 6 we investigated the effects of endogenous PC on the host response against \textit{B. pseudomallei} by using specific anti-(A)PC antibodies\textsuperscript{14}. Infected mice were treated with antibodies inhibiting both the anticoagulant and cytoprotective functions of APC (MPC1609) or the anticoagulant functions of APC (MAPC1591) only. We demonstrated that MPC1609, but not MAPC1591 significantly worsened survival, increased coagulation activation, facilitated bacterial growth and dissemination and enhanced the inflammatory response. The effects of MPC1609 could not be reversed by SEW2871, an agent stimulating the sphingosine-1-phosphate receptor 1 (S1P1)-pathway downstream from protease activated receptor (PAR)-1, suggesting that the S1P1-pathway does not play a major role in this model. From these data it can be concluded that the mere inhibition of the anticoagulant function of APC does not interfere with its protective role during melioidosis, which suggests a more prominent role for the cytoprotective effects of APC herein. Chapter 7 focuses on possible differences in the host response when generation of APC by the TM-thrombin complex is impaired by a mutation in the \textit{Tm} gene. Mice with this mutation (TM\textsuperscript{pro/pro} mice) displayed a worse survival upon
infection with *B. pseudomallei* when compared to control mice, accompanied by increased coagulation activation, a temporarily increased bacterial growth, enhanced lung neutrophil influx, bronchoalveolar inflammation at late time points and increased hepatocellular injury. Hence, it seems likely that impaired TM-mediated PC activation is detrimental in murine melioidosis. Chapter 8 analyzes whether high APC levels would influence the host response during melioidosis, for which we used mice endogenously overexpressing APC (APChigh mice). Obviously, plasma APC concentrations in APChigh mice were elevated and approximately in the same range as previously measured in patients treated with recombinant human APC. Surprisingly, APChigh mice demonstrated enhanced susceptibility to *B. pseudomallei* infection compared to WT mice as evidenced by a strongly increased mortality accompanied by increased bacterial loads in the lungs, blood and distant organs 48 hours after infection. Additionally, at this time point APChigh mice showed elevated levels of proinflammatory cytokines in lungs and plasma, together with increased pulmonary histopathology scores and neutrophil influx. At 72 hours post infection decreased levels of TATc, reflecting inhibition of coagulation, were measured in lungs of APChigh mice. We concluded that constitutively enhanced expression of APC impairs host defense during melioidosis. Chapter 9 investigates the role of EPCR, capable of enhancing the efficiency of converting PC into APC, during melioidosis15. Soluble EPCR-levels were higher in plasma of melioidosis patients than in healthy controls and were associated with an increased mortality. Furthermore, EPCR-overexpressing mice demonstrated enhanced bacterial growth and dissemination to distant organs during experimental melioidosis, accompanied by increased lung damage, neutrophil influx and cytokine production and attenuated coagulation activation. Interestingly, EPCR-deficient mice had an unremarkable response to *B. pseudomallei* infection as compared to WT mice, except for a decreased coagulation activation in plasma. We concluded that overexpression of EPCR and possibly the associated attenuated coagulation activation might have aggravated the outcome during melioidosis. However, endogenous EPCR did not impact on the host response. Chapter 10 describes experiments investigating whether deficiency of PAR-1 influences the host response during melioidosis. We discovered that PAR-1 deficient mice showed decreased bacterial loads in lungs, BALF and liver. In addition, 72 hours after infection, PAR-1 mice displayed a decreased cell influx in the lungs, which was due to a lower number of neutrophils. On the other hand, no differences in levels of pro-inflammatory cytokines and lung histopathology were seen between WT and PAR-1 deficient mice, neither a difference in mortality could be observed between both groups. Therefore it can be concluded that PAR-1 only plays a limited role in the inflammatory response during experimental melioidosis. Finally, chapter 11 focuses on the lectin-like domain of TM during melioidosis. Following exposure to *B. pseudomallei*, TMLeD/LeD mice showed a survival advantage, accompanied by decreased bacterial loads in the blood, lungs, liver and spleen. While lung histopathology did not differ between groups, TMLeD/LeD mice displayed strongly attenuated systemic inflammation, as reflected by lower plasma cytokine levels, maintenance of normal kidney and liver function, histologic evidence of reduced organ damage and damage to the spleen. In conclusion, this study revealed a detrimental role for the TM lectin-like domain during melioidosis.
Next, we focused on the influence of the fibrinolytic system on the host response during melioidosis. In **chapter 12** we first investigated the role of tissue-type plasminogen activator (tPA), a profibrinolytic protein. Infection with *B. pseudomallei* was associated with elevated levels of tPA in lungs of infected WT mice. During experimental melioidosis tPA deficient mice, expected to have increased fibrin depositions, were protected in comparison to WT mice as demonstrated by a strongly decreased mortality, together with decreased pulmonary bacterial loads, less severe histopathological scores. These results point to a harmful effect of endogenous tPA during melioidosis, which might be related to tPA-associated plasmin-induced fibrinolysis and/or a tPA-associated decrease in pro-inflammatory cytokine production. These data suggest that tPA-induced decreased fibrin levels are harmful during murine melioidosis. Then, in **chapter 13**, we focused on the effects of PAI-1, one of the important fibrinolysis inhibitors that also can influence the inflammatory response. We found that, in contrast to tPA-deficient mice, PAI-1 deficient mice, which are expected to have decreased fibrin depositions, had an enhanced susceptibility to *B. pseudomallei*. This was evidenced by a strongly increased mortality, associated with enhanced bacterial loads in lungs, liver and blood in PAI-1 deficient mice. Additionally, PAI-1 deficiency was associated with elevated levels of pro-inflammatory cytokines in lungs and plasma, accompanied by enhanced local and systemic coagulation activation, increased hepatocellular injury and renal failure. In addition to these data, in **chapter 14**, we analyzed the influence of the other important anti-fibrinolytic protein, alpha-2-antiplasmin (A2AP) during melioidosis. Culture-positive melioidosis patients showed elevated A2AP plasma levels in comparison healthy controls. Likewise, A2AP-levels in plasma and lung homogenates were elevated in mice infected with *B. pseudomallei*. In line with PAI-1 deficient mice, A2AP deficient mice had a strongly disturbed host response during experimental melioidosis as reflected by enhanced bacterial growth in the lungs and at distant sites. In addition, A2AP deficiency was associated with more severe lung pathology, an increased accumulation of neutrophils, higher lung cytokine levels, exaggerated systemic inflammation and coagulation, increased distant organ injury and enhanced lethality. This study was the first to identify A2AP as a protective mediator during severe infection. Altogether these data suggest that the presence of fibrin has protective effects on the host immune response during murine melioidosis.

In **Part III** of this thesis two additional studies are presented. First, in **chapter 15** we present the results of a clinical study investigating the effects of direct intrabronchial administration of APC during lipopolysaccharide (LPS)-induced pulmonary inflammation. As previous data had shown a favorable outcome of intravenous administration of APC during pulmonary infections and after local endotoxin challenge in the lungs as was evidenced by inhibition of inflammation and coagulation, in this study we aimed to find out whether direct intrabronchial administration of APC would have the same protective effects, while preventing severe side effects such as intracranial bleeding. Surprisingly, instead of being protective, we found that intrabronchially administered recombinant human (rh)APC had procoagulant and proinflammatory effects in LPS-challenged lung subsegments of healthy volunteers. These data argue against a role for intrapulmonary delivery
of rhAPC as a treatment strategy for lung inflammatory disorders in humans. Finally, in chapter 16 we describe a new murine model to study musculoskeletal TB, a severe extrapulmonary manifestation of chronic *M. tuberculosis* infection. Ten months after intranasal infection *M. tuberculosis* mycobacterial growth was detected in lung and femur homogenates, while Ziehl-Neelsen staining of paraffin-embedded femurs showed acid-fast rods in the myelum. Magnetic resonance imaging demonstrated osteomyelitis and macronodular tuberculomas. We concluded that this new murine model of musculoskeletal TB might be of value to further investigate immunologic and radiologic responses.

**GENERAL DISCUSSION & FUTURE DIRECTIONS**

Ample evidence has shown that infection-associated coagulation abnormalities mainly are associated with severe acute infections and occur concurrently with strong pro-inflammatory antibacterial responses. Limited data exist about coagulation activation during chronic infections. Our data showing overt activation of procoagulant factors, together with downregulation of anticoagulant mechanisms, during pulmonary TB therefore were remarkable. Moreover, although we were unable to calculate a full score for disseminated intravascular coagulation (DIC-score), because no data on platelet counts were available, our data almost met the DIC-criteria. Like in DIC, in TB patients we also observed prolonged prothrombin times, increased D-dimer and TATc levels and decreased antithrombin and PC levels. The DIC-score is considered a marker for damage to the microvasculature and associated organ failure and, apparently these processes also occur during TB. The clinical picture of TB, however, is not associated with fulminant organ failure. It seems likely that during this chronic disease a steady state is reached in which pro- and anticoagulant factors have reached an equilibrium during which further clinical deterioration of the host is prevented. Obviously this is area for further research.

We further investigated whether the PC system and in particular APC play a role during TB. Despite earlier observations, our current studies only demonstrated a limited role for EPCR and APC during murine TB. Moreover, the lectin-like domain of TM, known for its anti-inflammatory capacities, did not play a major role either in mice infected with *M. tuberculosis*. In addition, the fibrinolytic system had limited effects on the host response during murine TB, as was shown by our results on inflammation in mycobacteria-infected PAI-1 deficient mice. The discrepancy between our patient data, in which strong activation of coagulation and fibrinolysis was observed, and our murine studies, that did not point to a prominent role for proteins involved in the PC or fibrinolytic system, may have different explanations. First, caution is warranted to extrapolate murine data to the human situation. Recent data showed that genomic responses in mouse models may poorly mimic those in human inflammatory diseases. In murine models, the responses to proinflammatory stimuli are dependent on factors such as the mouse strain used, the route and dose of the challenge and the
(myco)bacterial strain used, whereas in humans the host is heterogeneous with regard to genetic background and comorbidity. Second, although we observed systemic coagulation disturbances in TB patients, we failed to show local, intrabronchial coagulation abnormalities. In our murine studies we mainly focused on the inflammatory host response in the lungs. However, possibly, when studied in a larger patient cohort, local abnormalities in coagulation and/or fibrinolysis do become apparent. Finally, during the chronic phase of pulmonary TB a steady state may be present, in which stimulating and inhibiting factors influencing inflammation and coagulation are delicately balanced and during which separate anticoagulant proteins have little direct immunomodulatory effects on the antimycobacterial host response. In the past, some studies showed protective effects of APC during TB\textsuperscript{23, 25}. However, strengthened by our murine data, we support the opinion that interfering in the coagulation system by anticoagulants such as recombinant APC (which might also have anti-inflammatory effects) is of limited value during chronic pulmonary TB.

During experimental melioidosis APC and the PC system seem to play a bidirectional role. On the one hand, depletion of endogenous APC, either in mice with limited APC generation (TM\textsuperscript{pro/pro} mice) or by using anti-APC antibodies, induced a detrimental phenotype, indicating that a minimal amount of APC is needed to mount a proper host response against \emph{B. pseudomallei}. Moreover, by using selective anti-PC antibodies, it seemed that the cytoprotective effects of APC are responsible for the protective phenotype. On the other hand, however, overexpression of APC was associated with a harmful phenotype during murine melioidosis, as was demonstrated by APC\textsuperscript{high} mice and mice overexpressing EPCR, which are likely to have increased amounts of APC due to enhanced availability of this receptor. It could be hypothesized that the APC-enriched environment might have favored bacterial growth and dissemination due to its strong anti-inflammatory and anticoagulant effects, which might in this case have been ‘too strong’ to contain bacteria and to mount a proper antibacterial response. It is assumable that containment of bacteria can be secured by the presence of fibrin that may serve to ‘wall off’ the primary infection thereby preventing bacterial dissemination. In line, too much anti-coagulation may slow down clot formation and decrease fibrin deposition, facilitating bacterial dissemination throughout the body, which in turn may aggravate the inflammatory response. This hypothesis is in line with our observations in mice with mutations in the proteins involved in fibrinolysis: deficiency of fibrinolysis inhibiting proteins (such as in PAI-1 and A2AP deficient mice), which is expected to be associated with decreased fibrin depositions, showed increased bacterial growth during murine melioidosis, while deficiency of tPA, associated with enhanced fibrin depositions, demonstrated reduced bacterial growth and dissemination in this same model. In line with PAI-1, A2AP however might also have, yet undiscovered, distinct anti-inflammatory effects apart from its anti-fibrinolytic effects, although our data did not show direct evidence for this suggestion.

In patients with severe pneumonia and (pneumo)sepsis interfering in the coagulation system was thought to be a promising treatment strategy. Indeed, administration of rhAPC was demonstrated
to strongly reduce mortality and activation of inflammation\textsuperscript{18}, especially in patients with lung sepsis\textsuperscript{26}, although a confirmatory trial failed to show the same effects\textsuperscript{27}. At first glance, treatment with rhAPC also seems to be attractive or at least worth investigating in patients with severe sepsis due to \textit{B. pseudomallei}. However, caution should be taken as all previously mentioned trials were performed in developed countries in Western Europe and the United States making it difficult to extrapolate the observed effects to the South-East Asian situation. Moreover, although during experimental melioidosis intact endogenous PC activation seems to be protective, our murine data showed that an APC-enriched environment has harmful effects on disease outcome. Therefore, to our opinion, treatment with rhAPC in patients with severe melioidosis should not be encouraged or, at least, the dose of intravenously administered rhAPC should be carefully titrated. Interference in the fibrinolytic system, on the other hand, seems a more promising strategy in the development of new additional therapies for melioidosis. Although highly preliminary and only investigated in murine models, we showed that inhibition of fibrinolysis was protective. Therefore, inhibition of fibrinolysis by exogenous fibrinolysis inhibitors such as tranexamic acid could be an attractive new approach that is worthwhile investigating. Obviously, this is also area for further research.

Finally, it is interesting to speculate on the similarities between TB and melioidosis. Both diseases can have comparable clinical presentations (chronic suppurative lesions unresponsive to conventional antibiotics, primarily affecting the lungs) and similar risk profiles (corticosteroid use, diabetes). Moreover, recent gene expression studies revealed that both diseases have a similar anti-bacterial host response dominated by interferon-signaling events\textsuperscript{28}, which is further supported by previous observations in IFN-\gamma deficient mice that displayed a strongly enhanced susceptibility for both \textit{M. tuberculosis}\textsuperscript{29} and \textit{B. pseudomallei}\textsuperscript{30}. Most likely this is due to their shared intracellular lifestyles, in which intracellular killing is largely regulated by IFN-\gamma. Whether these comparable anti-bacterial host responses fully explain the similarities between both pathogens remains area for further study as well as the explanation of the differences between both diseases such as their high (TB) or relatively low (melioidosis) prevalence in HIV-positive patients or the fact that melioidosis often presents as an acute fulminant sepsis whereas TB merely is a chronic pulmonary disease.

**CONCLUSIONS**

The studies presented in this thesis significantly contribute to the current knowledge on the role of proteins involved in coagulation, anticoagulation and fibrinolysis during TB and melioidosis. We showed that during TB, although a chronic disease, the coagulation system becomes activated. The physiological significance of this activation, however, still is unclear and we failed to show a clear functional role for proteins involved in the PC system. With respect to melioidosis, we here demonstrated that some (components of) proteins involved in coagulation and fibrinolysis significantly contribute to the host defense against \textit{B. pseudomallei}. This knowledge may add to further understanding of the pathophysiological processes during this severe septic disease and may serve as new targets for additional therapies.
REFERENCES

Summary, general discussion and conclusion


