Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
Kager, L.M.

Citation for published version (APA):
A thrombomodulin mutation that impairs active protein C generation is detrimental in severe pneumonia-derived Gram-negative sepsis (melioidosis)

Liesbeth M. Kager
W. Joost Wiersinga
Joris J. T. H. Roelofs
Onno J. de Boer
Hartmut Weiler
Cornelis van ’t Veer
Tom van der Poll

Submitted
ABSTRACT

Background
During severe (pneumo)sepsis inflammatory and coagulation pathways become activated as part of the host immune response. Thrombomodulin (TM) is involved in a range of host defense mechanisms during infection and plays a pivotal role in activation of protein C (PC) into active protein C (APC). APC has both anticoagulant and anti-inflammatory properties. In this study we investigated the effects of impaired TM-mediated APC generation during melioidosis, a common form of community-acquired Gram-negative (pneumo)sepsis in South-East Asia caused by *Burkholderia (B.) pseudomallei*.

Methods
Wildtype (WT) mice and mice with an impaired capacity to activate protein C due to a point mutation in their *Thbd* gene (*TM*<sup>pro/pro</sup> mice) were intranasally infected with *B. pseudomallei* and sacrificed after 24, 48 or 72 hours for analyses. Additionally, survival studies were performed.

Results
When compared to WT mice, *TM*<sup>pro/pro</sup> mice displayed a worse survival upon infection with *B. pseudomallei*, accompanied by increased coagulation activation, enhanced lung neutrophil influx and bronchoalveolar inflammation at late time points, together with increased hepatocellular injury. The *TM*<sup>pro/pro</sup> mutation had limited if any impact on bacterial growth and dissemination.

Conclusion
TM-mediated protein C activation contributes to protective immunity after infection with *B. pseudomallei*. These results add to a better understanding of the regulation of the inflammatory and procoagulant response during severe Gram-negative (pneumo)sepsis.
INTRODUCTION

Thrombomodulin (TM, CD141) is a multifunctional transmembrane glycoprotein receptor expressed on the surface of all vascular cells and various hematopoietic cells involved in activation of various parameters of inflammation and coagulation including protein C (PC), thrombin-activatable fibrinolysis inhibitor (TAFI), complement factors and in high mobility group box-1 (HMGB1)\(^1\), TM plays a pivotal role in the regulation of coagulation via its capacity to activate PC into active protein C (APC), mediated by high-affinity binding of thrombin to TM\(^3\), \(^4\) and further augmented via association of the endothelial protein C receptor (EPCR) to the TM-thrombin complex\(^3\), \(^4\). TM serves as an anticoagulant by inactivating coagulation factors Va and VIIIa, together with its cofactor protein S\(^3\), \(^4\). On the other hand, APC has anti-inflammatory, cytoprotective and anti-apoptotic properties through signaling via G-coupled protease activated receptors-1 (PAR-1)\(^4\). Furthermore, APC may exert anti-inflammatory effects via PAR-3\(^5\) and involvement of \(\alpha_\beta_1\), \(\alpha_\beta_3\), \(\alpha_\beta_4\) integrins\(^6\), mechanisms that are in part EPCR-independent.

Ample evidence has shown that severe (pneumo)sepsis is accompanied by both activation of a strong proinflammatory response and increased coagulation activation, inadequate anticoagulation and suppression of fibrinolysis\(^7\), \(^8\). The interplay between inflammation and blood coagulation is considered to be an essential part of host defense against pathogenic bacteria. Indeed, patients with severe sepsis displayed low levels of PC and APC, which correlated with organ dysfunction and an adverse outcome\(^9\), \(^10\). Preclinical studies investigated the role of endogenous PC during inflammation and sepsis. Mice with decreased PC levels, due heterozygous deficiency for PC, had more severe disseminated intravascular coagulation, increased fibrin depositions and higher levels of proinflammatory cytokines upon intraperitoneal injection with lipopolysaccharide (LPS)\(^11\), while reduced PC levels in mice with genetically modified (low) PC expression strongly correlated with a survival disadvantage after LPS challenge\(^12\). Furthermore, inhibition of endogenous PC increased the procoagulant response during *Escherichia coli* peritonitis\(^13\) and H1N1 influenza in mice\(^14\).

Melioidosis is an infectious disease common in Southeast-Asia and Northern-Australia and an important cause of community-acquired pneumonia and sepsis in these areas with mortalities up to 40% despite appropriate antibiotic therapy\(^15\), \(^17\). Once a patient is infected by the causative pathogen *Burkholderia (B.) pseudomallei*, this bacterium spreads rapidly throughout the body resulting in many possible disease manifestations, septic shock being the most severe\(^15\), \(^16\). Additionally, *B. pseudomallei* was recently classified as a ‘Tier 1’ disease agent considered to be an exceptional threat to security\(^18\). Previous research has demonstrated pronounced coagulation activation in patients with culture-proven septic melioidosis together with downregulation of anticoagulant pathways\(^10\), \(^19\). In particular, PC levels were markedly decreased in these patients\(^10\), \(^19\), correlating with a worse disease outcome\(^10\). In the present study, we sought to determine the role of TM and in particular its function in endogenous APC generation, in the host defense during pneumosepsis caused by *B. pseudomallei*. 
MATERIAL AND METHODS

Mice
Pathogen-free 10-week old male WT C57BL/6 mice were purchased from Charles River (Maasstricht, The Netherlands). TMpro/pro mice were generated as described\textsuperscript{20} and backcrossed eight times on a C57BL/6 background. Homozygous mutant TMpro/pro mice, due to a single amino acid substitution (Glu404Pro) in the $\text{Tthbd}$ gene, exhibit a decrease of approximately 1000-fold with respect to PC activation and approximately 100-fold with respect to binding of thrombin at physiologic levels of the enzyme\textsuperscript{20}. In addition, TMpro/pro mice produce less than 4% of APC in their alveolar space upon intratracheal administration of PC and thrombin\textsuperscript{21}. Mice were maintained at the animal care facility of the Academic Medical Center (University of Amsterdam), according to national guidelines with free access to food and water. The Committee on Use and Care of Animals of the University of Amsterdam approved all experiments.

Ethics Statement
Mice studies were carried out under the guidance of the Animal Research Institute of the Academical Medical Center in Amsterdam (ARIA). All animals were maintained at the animal care facility of the Academic Medical Center (University of Amsterdam), with free access to food and water, according to National Guidelines for the Care and Use of Laboratory Animals, which are based on the National Experiments on Animals Act (Wet op de Dierproeven (WOD)) and the Experiments on Animals Decree (Dierproevenbesluit), under the jurisdiction of the Ministry of Public Health, Welfare and Sports, the Netherlands. The Committee of Animal Care and Use (Dier Experimenten Commissie, DEC) of the University of Amsterdam approved all experiments (Permit number DIX100121-101700).

Experimental infection and determination of bacterial growth
Experimental melioidosis was induced by intranasal inoculation with $B. \text{pseudomallei}$ strain 1026b (750 colony forming units (CFU)/50$\mu$L 0.9% NaCl) as previously described\textsuperscript{22-25}. For survival experiments mice were checked every 4-6 hours until death occurred for a maximum of 15 days. Sample harvesting and processing and determination of bacterial growth were done as described\textsuperscript{22-25}.

Cell counts and flow cytometry
Bronchoalveolar lavage fluid (BALF) was obtained as described\textsuperscript{24}. Total counts of paraformaldehyde (4%)-fixed BALF cells were measured using a Coulter Counter (Beckman Coulter Inc. Brea, CA). Differential counts were determined by FACS (FACSCalibur, Becton Dickson, San Jose, CA) using directly labeled antibodies against Gr-1 (Gr-1 FITC; BD Pharmingen, San Diego, CA) and F4/80 (F4/80 APC; AbD Serotec, Oxford, UK). Neutrophilic granulocytes were defined according to their scatter pattern and Gr-1 positivity. All antibodies were used in concentrations recommended by the manufacturer.
Assays
Interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ, monocyte-chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendations. Thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany) and D-dimer (Asserachrom D-dimer, Roche Woerden, the Netherlands) were measured with commercially available ELISA kits. Protein levels in BALF were measured using a Bradford-based protein assay (Bio-Rad Laboratories, Hercules, CA). Aspartate aminotranspherase (ASAT) and alanine aminotranspherase (ALAT) were determined with commercial available kits (Sigma-Aldrich, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturers’ instructions.

Histology and immunohistochemistry
Paraffin-embedded 4 µm tissue sections were stained with haematoxylin and eosin (H&E) and analyzed for inflammation and tissue damage as described. Granulocyte stainings, using fluorescein isothiocyanate-labeled rat-anti-mouse Ly-6G mAb (BD Pharmingen, San Diego, CA) were done as described previously. Slides were counterstained with methylgreen (Sigma-Aldrich, St. Louis, MO). The total tissue area of the Ly-6G-stained slides was scanned with a slide scanner (Olympus dotSlide, Tokyo, Japan) and the obtained scans were exported in TIFF format for digital image analysis. The digital images were analyzed with ImageJ (version 2006.02.01, National Institutes of Health, Bethesda, MD) and the immunopositive (Ly6G+) area was expressed as the percentage of the total lung surface area.

Statistical analysis
Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation or as medians with interquartile ranges. Comparisons between groups were tested using the Mann-Whitney U test. For survival studies Kaplan-Meier analyses followed by Log-rank (Mantel-Cox) test were performed. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P-values < 0.05 were considered statistically significant.

RESULTS

**TMpro/pro mice have a reduced survival during murine melioidosis**
To explore whether a decreased capacity to generate APC impacts on survival during severe Gram-negative (pneumo)sepsis caused by *B. pseudomallei* we infected TMpro/pro and WT mice with 750 CFU of this bacterium and followed them for 15 days (Figure 1). TMpro/pro mice had an accelerated mortality when compared to WT mice: after 3.8 days already 7 out of 16 TMpro/pro mice (38%) had died,
**Figure 1.** T\(^{\text{pro/pro}}\) mice display a reduced survival during murine melioidosis. Mortality was assessed every 6 hours, \(n = 16-18\) mice per group. Comparison cumulative survival between groups was done by using Kaplan-Meier analysis followed by Log rank (Mantel-Cox) tests.

**Figure 2.** T\(^{\text{pro/pro}}\) mice demonstrate increased coagulation activation after infection with \(B.\) pseudomallei. Coagulation activation in lung homogenates (A) and plasma (B), as reflected by levels of TATc. Lung D-dimer levels in T\(^{\text{pro/pro}}\) mice (C). Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Grey and white boxes represent WT and T\(^{\text{pro/pro}}\) mice respectively (\(n = 8\) mice/group). *\(P < 0.05\) and **\(P < 0.01\) for the difference between WT and T\(^{\text{pro/pro}}\) mice (Mann-Whitney \(U\) test).

**Figure 3.** The T\(^{\text{pro/pro}}\) mutation has limited impact on bacterial growth and dissemination. Bacterial loads were determined in lung homogenates (A), spleen (B) and liver homogenates (C) and in whole blood (D). Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Grey boxes represent WT mice, white boxes represent T\(^{\text{pro/pro}}\) mice (\(n = 8\) mice/group). *\(P < 0.05\) for the difference between WT and T\(^{\text{pro/pro}}\) mice (Mann-Whitney \(U\) test). BC+ number of positive blood cultures/ total number of mice per group.
whereas the first WT mice did not die until 3.9 days. After the total observation period, 16 out of 18 WT mice had died (89%), while all TMpro/pro mice had passed away (100%) \((P<0.05\); Figure 1). These results indicate that a reduced capacity to generate APC renders mice more vulnerable for death during Gram-negative (pneumo)sepsis caused by \(B.\ pseudomallei\).

**TMpro/pro mice demonstrate increased coagulation activation after infection with \(B.\ pseudomallei\)**

We have previously shown that in our model of murine melioidosis severe inflammation is associated with marked coagulation activation, which is most prominent at later time points22-25. TM is involved in inhibition of coagulation, due to its capacity to augment the conversion of PC into APC by thrombin. A mutation in the EGF3/4 interdomain linker of \(Thbd\) (Glu404Pro) disrupts this co-factor activity of \(Thbd\) (which is mediated by its EGF-like repeats)1,2. We wondered whether the \(Thbd\)-Pro mutation in this domain, as is present in TMpro/pro mice, would impact on coagulation in our model of murine melioidosis. Our data show that TMpro/pro mice had increased coagulation activation both in lungs and systemically, as reflected by elevated pulmonary and plasma levels of TAT at 24 and 72 hours after infection \((P<0.05\) for the differences between WT and TMpro/pro mice, Figure 2A and B). Moreover, when compared to WT mice, TMpro/pro mice had increased lung levels of D-dimer at these time points \((P<0.01\), Figure 2C). These data show that a point mutation in the TM-gene associated with a decreased capacity to generate APC leads to enhanced coagulation activation during Gram-negative (pneumo)sepsis (melioidosis).

**The TMpro/pro mutation has limited impact on bacterial growth and dissemination**

Our model of murine melioidosis is associated with marked bacterial growth locally in lungs with subsequent spreading to distant organs22-25. In order to establish whether impaired activation of PC into APC would impact hereon, WT and TMpro/pro mice were infected with 750 CFU of \(B.\ pseudomallei\) and sacrificed after 24, 48 and 72 hours to determine bacterial loads in lungs (the primary site of infection), liver, spleen and blood (to evaluate the extent of bacterial dissemination). At 48 hours modestly increased bacterial loads were counted in lungs of TMpro/pro mice when compared to WT mice \((P<0.05\), Figure 3A). However, after 72 hours pulmonary bacterial loads of WT and TMpro/pro mice were similar. Furthermore, no differences in bacterial dissemination could be detected: WT and TMpro/pro mice had similar bacterial loads in spleen (Figure 3B), liver (Figure 3C) and blood (Figure 3D) at all time points. These data demonstrate that TM-mediated APC-generation has a modest and temporary effect on local antibacterial defense during severe Gram-negative (pneumo)sepsis.

**TMpro/pro mice exhibit increased lung tissue damage at early time points and increased neutrophil influx in the lungs**

Our murine model of melioidosis is associated with severe lung inflammation and damage22-25. To analyze whether impaired TM-mediated APC generation would impact hereon, we determined
histopathological scores of lungs after infection with *B. pseudomallei*. All mice infected with *B. pseudomallei* had inflammatory lung infiltrates characterized by interstitial inflammation together with necrosis, endothelialitis, bronchitis, edema, thrombi and pleuritis (Figure 4A-C). Twenty-four hours after infection lung histopathology was significantly increased in TM<sup>pro/pro</sup> mice when compared to WT mice (*P* < 0.05; Figure 4A-C), while at later time points no differences were seen between both mouse strains. Additionally, we analysed neutrophil recruitment to lung tissue, as it is known that neutrophils play an important role in the host response during melioidosis<sup>16, 17, 26</sup>. For this lung tissues were stained for Ly-6G. Clear neutrophilic infiltrates were seen in both WT and TM<sup>pro/pro</sup> mice, increasing over time during the course of the experiment. Seventy-two hours after infection, lung tissue of TM<sup>pro/pro</sup> mice contained significantly more neutrophils than that of WT mice (*P* < 0.01, Figure 4D-F). These data suggest that TM-mediated APC generation reduces neutrophil recruitment and lung pathology during severe Gram-negative (pneumo)sepsis.

**Impact of the TM<sup>pro/pro</sup> mutation on lung and plasma cytokine concentrations after infection with *B. pseudomallei***

Since cytokines and chemokines are important regulators of the inflammatory response to *B. pseudomallei*<sup>16, 17, 27</sup> we measured pulmonary and plasma levels of TNF-α, IL-6, IL-10, IL-12p70, IFN-γ

![Figure 4. Lung histopathology and neutrophil recruitment.](image-url)
Impaired APC generation in melioidosis

Table 1. Cytokine concentrations in lung homogenates and plasma of WT and TMpro/pro mice during murine melioidosis.

<table>
<thead>
<tr>
<th></th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>1257 (788-1441)</td>
<td>1348 (786-1650)</td>
<td>1875 (1013-2454)</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12p70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>31 (21-35)</td>
<td>18 (15-21)</td>
<td>21 (15-30)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>4015 (3489-4371)</td>
<td>3408 (3036-4222)</td>
<td>4053 (3389-5666)</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>11 (8.6-12)</td>
<td>10 (9.6-12)</td>
<td>29 (16-45)</td>
</tr>
<tr>
<td>IL-6</td>
<td>95 (92-121)</td>
<td>114 (86-131)</td>
<td>776 (206-1598)</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.9 (2.8-4.9)</td>
<td>3.4 (3.1-6.3)</td>
<td>2.0 (1.7-2.2)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>13 (11-14)</td>
<td>10 (7.9-11)</td>
<td>11 (6.3-18)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>27 (24-34)</td>
<td>15 (13-20)</td>
<td>342 (195-639)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>537 (472-659)</td>
<td>395 (376-729)</td>
<td>616 (346-662)</td>
</tr>
</tbody>
</table>

Pulmonary and plasma cytokine levels after intranasal infection with 750 CFU of *B. pseudomallei*. Mice were sacrificed 24, 48 or 72h after infection. Data are expressed as median (interquartile ranges) of *n* = 8 mice per group per time point. BD below detection limits, IFN-γ interferon-γ, IL interleukin, MCP-1 monocyte-chemoattractant protein-1, TNF-α tumor necrosis factor-α. *P* < 0.05, **P** < 0.01 and ***P*** < 0.001 for WT versus TMpro/pro mice (Mann-Whitney U test).

and MCP-1 (Supporting information; Table 1). Interestingly, early after infection (24 hours), TMpro/pro mice showed reduced IFN-γ levels in both lungs and plasma and decreased IL-12p70 levels in lung homogenates, relative to WT mice. In plasma, these differences remained present at 48 hours after infection. During the late phase of the infection (72 hours) most mediator levels were higher in TMpro/pro mice when compared with WT mice, significantly so for lung IL-12p70 and IL-6 concentrations.
**TM**<sup>pro/pro</sup> **mice display increased neutrophil influx and pro-inflammatory cytokine release in the alveolar compartment**

Many studies have demonstrated that severe pneumonia may lead to alveolar damage and subsequent alveolar leakage and release of pro-inflammatory parameters<sup>28, 29</sup>. To determine the impact of impaired APC generation on this extra-vascular, intrabronchial compartment, we determined CFU, protein leakage and parameters of inflammation in BALF 72 hours after inoculation of *B. pseudomallei*, i.e. shortly before the first deaths occurred and at a time point when lung injury is expected to be at its peak. No differences in bacterial growth (Figure 5A) or total protein content, a marker for alveolar damage (Figure 5B), could be detected in BALF of WT and TM<sup>pro/pro</sup>/

---

**Figure 5. TM**<sup>pro/pro</sup> **mice demonstrate an increased inflammatory response in their bronchoalveolar space 72 hours after infection.** Bacterial loads (A) in BALF 72 hours after infection with *B. pseudomallei* (A). Total protein content (B), total cell influx (C) and percentages of neutrophils (D) in BALF. Levels of IL-6 (E) and TNF-α (F) in BALF. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Grey boxes represent WT mice, white boxes represent TM<sup>pro/pro</sup> mice (*n* = 8 mice/group). **P < 0.01 and ***P < 0.001 for the difference between WT and TM<sup>pro/pro</sup> mice (Mann-Whitney U test). BALF bronchoalveolar lavage fluid, CFU colony forming units, IL interleukin, TNF-α tumor necrosis factor-α.
Impaired APC generation in melioidosis

pro mice, nor were there any differences in total cell influx in BALF (Figure 5C). The percentage of neutrophils in BALF of TMpro/pro mice, however, was significantly higher than in WT mice ($P < 0.01$; Figure 5D), which is in accordance with the increased neutrophil influx visualized by Ly6-staining of lung tissue. Moreover, BALF levels of the proinflammatory cytokines IL-6 (Figure 5E) and TNF-$\alpha$ (Figure 5F) were significantly increased in TMpro/pro mice when compared to WT mice ($P < 0.001$ for both cytokines). These results indicate that during severe Gram-negative (pneumosepsis) intact TM-mediated APC generation limits the proinflammatory response in the alveolar compartment.

TMpro/pro mice show enhanced hepatocellular injury

Our model of experimental melioidosis is associated with hepatocellular injury as reflected by elevated plasma levels of transaminases. To obtain insight in the possible role of TM-mediated APC generation herein, we measured ASAT and ALAT in plasma of WT and TMpro/pro mice 24, 48 and 72 hours after infection with $B.\ pseudomallei$. Indeed, when compared to WT mice, TMpro/pro mice showed strongly increased levels of plasma ASAT ($P < 0.01$ at 24 and 72 hours; Figure 6A) and ALAT ($P < 0.05$ at 72 hours post-infection; Figure 6B). Taken together, intact TM-mediated APC generation seems to protect against hepatocellular injury during experimental melioidosis.

DISCUSSION

In the present study we sought to investigate the role of TM and in particular its function in endogenous PC activation during melioidosis, a Gram-negative infection often associated with severe pneumonia and sepsis. Melioidosis, as we have demonstrated by our established mouse model, is characterized by gradual growth of bacteria from the lung followed by dissemination to distant body sites, activation of coagulation and inflammation, tissue injury and death, thereby
mimicking the clinical scenario of severe (pneumono)sepsis. Our data show that impaired TM-dependent conversion of PC into APC is associated with enhanced lethality (see comment above) during experimental melioidosis, accompanied by increased coagulation activation, bronchoalveolar inflammation and hepatocellular damage. These data indicate that the capacity to properly activate endogenous PC contributes to protective immunity during experimental melioidosis.

TM is known to play important roles in coagulation and inflammation, that are largely based on its distinct structural domains, including the lectin-like domain, EGF-like repeats, transmembrane domain and short cytoplasmic tail. The EGF-like repeats play a pivotal role in the PC-system via binding of thrombin, thereby increasing the capacity to generate APC a 100-fold. During sepsis, the expression of TM on endothelial cells is downregulated, causing impaired APC-generation that may then affect parameters of coagulation and inflammation important for the host response of the infected individual. To answer our research questions, we used genetically modified mice, TMpro/pro mice. In contrast to Thbd gene-deficient mice, which die in the embryonic stage, TMpro/pro mice develop to term and possess normal reproductive performance, but have a decreased endogenous APC synthesis ability when compared to WT mice, as was demonstrated both in the circulation and in the alveolar space. Our data showing increased coagulation activation in TMpro/pro mice, as reflected by increased levels of TATc and D-dimer, are fully in accordance with this. Interestingly, previous studies examining the impact of the TMpro/pro mutation on coagulopathy during experimental (pneumono)sepsis induced by the Gram-positive pathogen Streptococcus (S.) pneumoniae or the Gram-negative bacterium Klebsiella (K.) pneumoniae, or after intranasal administration of E. coli LPS failed to show differences in TATc in plasma or BALF between TMpro/pro and WT mice. Similarly, in a model of experimental tuberculosis no differences in lung and plasma TATc were detected between WT and TMpro/pro mice. During systemic endotoxemia TMpro/pro mice were reported to have enhanced fibrin deposition in lungs and kidneys in the presence of unaltered plasma D-dimer concentrations. Clearly, the influence of the TMpro/pro mutation on the procoagulant response depends on the type and extent of the inflammatory stimulus.

Besides its anticoagulant properties TM-activated PC also influences the host immune response during sepsis: APC may exert anti-inflammatory, anti-apoptotic and cell-protective effects by proteolytic cleavage of PAR-1. Indeed, our data demonstrate that impaired APC generation due to a mutation in the Thbd gene resulted in pro-inflammatory effects, as indicated by increased lung pathology at early time points and exaggerated bronchoalveolar inflammation and hepatocellular injury at later time points in TMpro/pro mice. Remarkably, these results are in contrast with murine models of airway inflammation induced by S. pneumoniae, K. pneumoniae or LPS, in which no differences in the abovementioned parameters for inflammation were seen between WT and TMpro/pro mice. On the other hand, TMpro/pro mice displayed enhanced diabetic nephropathy, in a model of streptozotocin-induced diabetes mellitus, accompanied by glomerular apoptosis, pointing to a detrimental phenotype when endogenous PC activation is impaired.
An important component of the host response to *B. pseudomallei* is the release of proinflammatory cytokines17, 27, 35. Clinical studies in melioidosis patients showed elevated serum levels of TNF-α, IL-6 and IFN-γ27, 35. The pro-inflammatory cytokine IFN-γ, produced by cytotoxic T-cells and natural killer cells, has an important protective role in early resistance against *B. pseudomallei* infection36: administration of a neutralizing monoclonal antibody against IFN-γ was associated with marked increases in bacterial loads in the liver and spleen, together with enhanced lethality36. Similarly, inhibition of the production of IL-12, one of the predominant inducers of IFN-γ, resulted in increased mortality in the same model36. Interestingly, we found decreased levels of IFN-γ and IL12p70 in TMpro/pro mice early after infection. Although a clear explanation for this observation is lacking, it may in part explain the modestly higher bacterial loads in the lungs of TMpro/pro mice at 48 hours post-infection.

The current study identifies TM-mediated APC generation as part of the protective host response during melioidosis and is in accordance with recent evidence from our laboratory showing that inhibition of endogenous PC by specific anti-PC antibodies converts a non-lethal model of experimental melioidosis into a lethal model, associated with increased coagulation activation, severe tissue injury and a strongly increased proinflammatory response24. Together these data emphasize the importance of adequate APC levels during melioidosis. As such, administration of recombinant human APC hypothetically could be a promising therapeutic agent in melioidosis. However, in 2012 this drug was withdrawn from the market after negative results from the PROWESS SHOCK trial in sepsis patients37. Recombinant soluble TM currently undergoes clinical evaluation as an anticoagulant and anti-inflammatory agent in patients with sepsis38, 39. It would be of interest to test the effects of soluble TM in experimental (and clinical) melioidosis.

**ACKNOWLEDGEMENTS**

The authors thank Marieke ten Brink and Joost Daalhuisen for their expert technical assistance during the animal experiments, Kamran Bakhtiari, Wil Kopatz, Marian Weijne and Lucy Leverink for performing the coagulation measurements and Regina de Beer for performing histopathological and immunohistochemical stainings.
REFERENCES


