Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Endogenous tissue-type plasminogen activator impairs host defense during severe experimental Gram-negative sepsis (melioidosis)

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ABSTRACT

Objective
Melioidosis is a frequent cause of severe sepsis in Southeast-Asia caused by the Gram-negative bacterium *Burkholderia (B.) pseudomallei*. Patients with melioidosis have elevated circulating levels of tissue type plasminogen activator (tPA), an important regulator of fibrinolysis. In this study we aimed to investigate the role of tPA during melioidosis.

Design and Setting
Animal study, University research laboratory

Subjects and Interventions
Wildtype (WT) and tPA deficient (tPA−/−) C57BL/6 mice. Mice were intranasally infected with viable *B. pseudomallei* and sacrificed after 24, 48 or 72 hours for harvesting of lungs, liver and blood. Additionally, survival studies were performed.

Measurements and Main Results
Experimentally induced melioidosis was associated with elevated levels of tPA in lungs of infected WT mice. During infection with *B. pseudomallei* tPA−/− mice were protected when compared to WT mice as demonstrated by a strongly decreased mortality (62% versus 100% amongst WT mice, *P* <0.0001), together with decreased pulmonary bacterial loads, less severe histopathological scores and decreased fibrinolysis. These results were accompanied with an early increase in cytokine levels in tPA−/− mice.

Conclusions
During severe Gram-negative sepsis caused by *B. pseudomallei* endogenous tPA has harmful effects with respect to survival and pulmonary bacterial growth. These effects are related to tPA-associated plasmin-induced fibrinolysis and/or a tPA-associated decrease in pro-inflammatory cytokine production.
INTRODUCTION

During severe bacterial infection a range of host defense mechanisms becomes activated, resulting in a strong inflammatory response together with increased procoagulant activity and suppression of anticoagulant mechanisms and fibrinolysis\(^1\). As a consequence, increased fibrin depositions are found in the microvasculature and extravascularly in various tissues, which is considered to contribute to organ damage during sepsis. An imbalance between coagulation and fibrinolysis during infection has especially been well documented in the lung\(^3\).

Tissue-type plasminogen activator (tPA) is one of the main activators of the fibrinolytic system by virtue of its capacity to convert plasminogen into plasmin, the main fibrin-degrading enzyme. The pro-fibrinolytic effects of tPA are counteracted by plasminogen activator inhibitor type 1 (PAI-1). Levels of tPA and PAI-1 are upregulated during clinical pneumonia and sepsis\(^3\)\(^-\)\(^5\), although for tPA results are less consistent\(^4\)\(^-\)\(^5\). Interestingly, accumulating evidence shows that mediators of fibrinolysis have functions that go beyond their “classic” role in fibrin degradation\(^2\)\(^-\)\(^8\). Our laboratory recently demonstrated a protective role for endogenous tPA in a murine model of abdominal sepsis induced by intraperitoneal injection of *Escherichia coli*, as reflected by enhanced mortality and increased bacterial dissemination in tPA deficient (tPA\(^{-/-}\)) mice\(^9\). In contrast, endogenous tPA did not influence host defense in respiratory tract infection caused by *Klebsiella pneumoniae*\(^10\).

Melioidosis, caused by the Gram-negative bacterium *Burkholderia (B.) pseudomallei*, is a disease associated with pneumonia and bacterial dissemination to distant sites, often proceeding to severe sepsis\(^11\)\(^-\)\(^13\). This potential bioterroristic agent is one of the most important causes of community acquired sepsis in Southeast Asia and Northern Australia with mortalities up to 40% despite appropriate antibiotic therapy\(^11\)\(^-\)\(^13\). Our group recently reported that patients with culture confirmed melioidosis display enhanced coagulation activation, accompanied by upregulation of fibrinolytic proteins, including tPA\(^14\). In this study we sought to unravel the functional role of endogenous tPA in the host response to melioidosis, using our well-established mouse model of lethal *B. pseudomallei* infection.

METHODS

Mice

Pathogen-free 10-week old male wild type (WT) C57BL/6 mice and tPA\(^{-/-}\) mice on a C57BL/6 genetic background were obtained from Jackson Laboratories (Bar Harbour, ME) and 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 Animal Care and Use of Committee of the University of Amsterdam approved all experiments.
Experimental infection and determination of bacterial growth

Experimental melioidosis was induced by intranasal inoculation with *B. pseudomallei* strain 1026b (500 colony forming units (CFU)/50 µL 0.9% NaCl) as previously described\textsuperscript{15-17}. For survival experiments mice were followed for 28 days, during the first 12 days of which they were checked every 6 hours until death occurred. Sample harvesting, processing and determination of bacterial growth were performed as described\textsuperscript{15-17}. For details see the Supplementary Material.

**Assays**

Lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 1% Triton X-100 [pH 7.4] and protease inhibitor mix (AEBSF [4-(2-aminoethyl)benzenesulfonfluoride], EDTA-Na\textsubscript{2}, pepstatin and leupeptin (all from MP Biomedical, Santa Ana, CA) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1730 x g at 4°C for 10 min. Supernatants were sterilized using 0.22 μm pore-size filters (Millipore, Billerica, MA) and stored at -20°C until analysis. Assays and Western blotting are described in the Supplementary Material.

**Histology and immunohistology**

Paraffin-embedded 4 μm lung sections were stained with haematoxylin and eosin (H&E) and analyzed for inflammation and tissue damage, as described previously\textsuperscript{15-17}. For details, see the Supplementary Material. Staining for granulocytes, using fluorescein isothiocyanate-labeled rat-anti-mouse Ly-6G mAb (BD Pharmingen, San Diego, CA) as well as staining for fibrinogen were performed as described previously\textsuperscript{10, 18, 19}. Slides were slightly counterstained with methyl green. Fibrinogen and Ly-6G stained slides were photographed with a microscope equipped with a digital camera (Leica CTR500, Leica Microsystems, Wetzal, Germany). Stained areas were analyzed with ImageJ (version 2006.02.01, US National Institutes of Health, Bethesda, MD) and 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. All variables were rank transformed prior to analyses. Comparisons between groups were conducted using a non-parametric ANOVA or Kruskal-Wallis test followed by pairwise Mann-Whitney *U* tests or Mann-Whitney tests only where appropriate. For survival studies Kaplan-Meier analyses followed by log rank test were performed. All analyses were done using SPSS (SPSS Inc, version 18). *P*-values < 0.05 were considered statistically significant.
RESULTS

**tPA is upregulated during murine melioidosis, while the net effect is inhibition of fibrinolysis**

Endogenous tPA was detected at levels 0.3-0.8 ng/mL in lung homogenates of uninfected mice. Experimental melioidosis was associated with a significant increase in lung tPA levels at 24, 48, and 72 hours after inoculation (overall \( P < 0.0001; P < 0.01 \) versus control for all time points; Figure 1A). Endogenous pulmonary PA activity, reflecting the net effect on fibrinolysis, in uninfected mice ranged from 97-105%. Upon infection with *B. pseudomallei* PA activity levels decreased significantly (overall \( P < 0.001; P < 0.01 \) versus control for all time points), indicating a net inhibitory effect on fibrinolysis (Figure 1B).

**tPA\(^{-}\) mice show a strongly reduced mortality during experimental melioidosis**

To investigate whether tPA deficiency impacts on mortality during murine melioidosis we intranasally infected WT and tPA\(^{-}\) mice with 500 CFU of *B. pseudomallei* and observed them during the following 4 weeks (Figure 2). Amongst WT mice the first deaths occurred after 4.5 days and all WT mice had succumbed by day 6. In sharp contrast, tPA\(^{-}\) mice showed a markedly delayed and

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**Figure 1. Increased pulmonary tPA levels during experimental melioidosis.** Wild type mice were sacrificed before (0h) and 24, 48 and 72 hours after intranasal inoculation with 500 CFU of *B. pseudomallei* to induce experimental melioidosis. Endogenous tPA protein concentrations (A) and PA activity (B) were measured in lung homogenates by ELISA and an amidolytic assay respectively, \( n = 5 \) (t = 0) or 8 (t = 24, 48 and 72) mice per group at each time point. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. **\( P < 0.01 \) for all time points versus 0h (Mann-Whitney U test).

**Figure 2. tPA-deficient mice show a strongly reduced mortality during experimental melioidosis.** Wildtype (WT, black dots) and tPA\(^{-}\) mice (white dots) were infected intranasally with 500 CFU of *B. pseudomallei* and mortality was assessed every 6 hours during the first 12 days, \( n = 12 \) in the WT and \( n = 13 \) in the tPA\(^{-}\) group. Comparison between groups was done by using Kaplan-Meier analysis followed by log rank tests.
Reduced lethality; at the end of the 28-day observation period, 38% of all tPA−/− mice was still alive (P < 0.0001 versus WT mice). Three out of 5 surviving mice still had bacteria in their lungs (median 6x10^3 CFU/mL, data not shown).

**tPA deficiency inhibits early bacterial growth at the primary site of infection**

To determine whether the reduced mortality of tPA−/− mice was accompanied by alterations in the local and systemic growth of *B. pseudomallei*, we examined bacterial loads in the lungs, liver, and blood harvested from tPA−/− and WT mice 24, 48 and 72 hours after infection. In accordance with their protected phenotype in the survival study, tPA−/− mice demonstrated reduced bacterial burdens in their lungs at 24 and 48 hours after infection (both P < 0.01); at 72 hours pulmonary bacterial loads were similar in tPA−/− and WT mice (Figure 3A). Bacterial loads at distant body sites (liver and blood) were similar (overall P-values 0.76 and 0.67, respectively) in tPA−/− and WT mice at all three time points examined (Figure 3B-C). These data suggest that endogenous tPA impairs antibacterial defense at the primary site of infection, but not at distant body sites.

**tPA−/− mice demonstrate an enhanced cytokine response in the lungs early after infection with *B. pseudomallei***

Cytokines are important regulators of the host immune response during melioidosis. To obtain a first insight into the mechanism by which tPA deficiency impacts on the growth of *B. pseudomallei* in the lungs, we measured pulmonary expression of TNF-α, IL-6, IL-10, IL-12p70, IFN-γ (Table 1). At 24 hours after infection, when bacterial loads were lower in tPA−/− mice, lung levels of TNF-α, IL-6 and IFN-γ were significantly higher in tPA−/− mice (all P<0.05 versus WT mice). At later time points, lung cytokine concentrations were similar in tPA−/− and WT mice, except for TNF-α and IFN-γ. Levels of TNF-α at 48 hours were still higher in the former mouse strain (P < 0.05 versus WT mice), while levels of IFN-γ were lower in tPA−/− mice after both 48 and 72 hours (Table 1).
tPA in melioidosis

Our model of melioidosis is associated with profound lung pathology. Both tPA-/- and WT mice infected with *B. pseudomallei* showed extensive inflammatory infiltrates in the lungs characterized by interstitial inflammation together with necrosis, endothelialitis, bronchitis, edema, thrombi and pleuritis (Figure 4A-C). Tissue damage was most prominent 72 hours after inoculation, in particular...
in WT mice; only at this late time point, tPA−/− mice displayed significantly reduced lung pathology as reflected by the semi-quantitative scoring system outlined in the Methods section (P < 0.05 versus WT mice). tPA did not influence neutrophil recruitment during *B. pseudomallei* infection, as measured by MPO concentrations in lung homogenates and semi-quantitative Ly6 staining of lung tissue slides (data not shown).

![Figure 4](image)

**Figure 4. tPA-deficient mice demonstrate lower histopathology scores.** Seventy-two hours after induction of infection lower mean histological scores as described in the Methods section of the Supplemental digital content were seen in tPA−/− mice when compared to WT mice (A). Representative photographs of WT (B) and tPA−/− (C) mice infected with 500 CFU of *B. pseudomallei* showed inflammatory infiltrates in the lungs characterized by interstitial inflammation together with necrosis, endothelialitis, bronchitis, edema, thrombi and pleuritis 72 hours after inoculation (H&E staining x100). 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 tPA−/− mice (n = 8 mice per group for each time point). **P < 0.01 for WT versus tPA−/− mice (Mann-Whitney U test).

**Table 2. Parameters of renal function and hepatocellular injury in wildtype and tPA-deficient mice during experimental melioidosis.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>tPA−/−</td>
<td>WT</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>5.5 (4.6-10)</td>
<td>6.5 (5.3-8.5)</td>
<td>7.2 (6.3-17)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>8.0 (6.0-23)</td>
<td>8.0 (5.5-11)</td>
<td>12 (9.0-36)</td>
</tr>
<tr>
<td>ASAT (U/L)</td>
<td>40 (4.0-5.5)</td>
<td>4.0 (4.0-10)</td>
<td>21 (18-45)</td>
</tr>
<tr>
<td>ALAT (U/L)</td>
<td>69 (49-85)</td>
<td>70 (59-128)</td>
<td>192 (99-261)</td>
</tr>
</tbody>
</table>

Parameters of renal and hepatocellular injury after intranasal infection with 500 CFU of *B. pseudomallei*. Wild type (WT) and tPA−/− mice were sacrificed 24, 48 and 72h after infection. Data are expressed as median (interquartile range) of n = 8 mice per group per time point. ASAT aspartate aminotranspherase, ALAT alanine aminotranspherase. *P < 0.05 for WT versus tPA−/− mice (Mann-Whitney U test).
tPA deficiency preserves renal function, but does not impact on liver injury

The melioidosis model used here is accompanied by marked distant organ injury as reflected by elevated plasma markers of renal failure (urea, creatinine) and liver injury (ASAT, ALAT), especially shortly before mortality occurs\textsuperscript{15,20}. Our data show that 48 hours post-infection tPA deficiency is associated with a moderately preserved renal function as urea levels in tPA\textsuperscript{-/-} mice were lower compared to WT mice ($P < 0.05$, Table 2). ALAT and ASAT did not differ between tPA\textsuperscript{-/-} and WT mice at 24, 48 and 72 hours post infection, indicating that tPA did not influence the extent liver injury (Table 2).

tPA deficiency reduces fibrinolysis but does not influence coagulation during infection with \textit{B. pseudomallei}

Considering its central role in fibrinolysis we evaluated the role of tPA in fibrinolysis and coagulation in the lungs during experimental melioidosis. Increased fibrin(ogen) depositions were seen in lungs of tPA\textsuperscript{-/-} mice compared to WT mice 48 hours after infection (Figure 5A-C). As expected, tPA\textsuperscript{-/-} mice demonstrated strongly reduced PA activity in lung homogenates ($P < 0.01$ versus WT mice, Figure 5D). In line with a reduced fibrinolytic activity in tPA\textsuperscript{-/-} mice, these animals showed undetectable lung D-dimer levels as determined by ELISA, whereas in WT mice D-dimer was readily detectable ($P < 0.01$, Figure 5E). In accordance, western blotting on fibrin-degradation products revealed strongly reduced amounts of fragment X (another fibrin degradation product) and D-dimer in tPA\textsuperscript{-/-} mice (D-dimer: 103% vs 39%; $P < 0.01$, fragment X: 110% vs 44%; $P < 0.05$; Figure 5G-J). tPA deficiency did not impact coagulation activation, as indicated by similar TATc concentrations in tPA\textsuperscript{-/-} and WT mice (Figure 5F). During the early time points decreased levels of uPA were measured in tPA\textsuperscript{-/-} mice, while after 72 hours no differences were seen between groups (Figure 5J). No differences in PAI-1 levels were observed in lung homogenates (Figure 5K) or plasma (Figure 5L).

DISCUSSION

Melioidosis is a severe disease associated with pneumonia and severe sepsis\textsuperscript{11-13,21}. Understanding of non-specific host-defense mechanisms, such as the coagulation and fibrinolytic system, may reveal new possible therapeutic targets in melioidosis. We here aimed to study the functional role of endogenous tPA during melioidosis using our established mouse model\textsuperscript{15-17}. A marked survival advantage was seen in tPA\textsuperscript{-/-} mice after infection with \textit{B. pseudomallei} when compared to WT mice, which was accompanied by significantly decreased pulmonary bacterial growth during the early stages of infection. Furthermore, tPA\textsuperscript{-/-} mice had decreased pulmonary inflammation after histopathologic analysis, less renal failure and decreased net fibrinolytic activity, while no differences in TATc were seen. Interestingly, at early stages tPA\textsuperscript{-/-} mice had higher pro-inflammatory cytokine levels. These data indicate that endogenous tPA plays a detrimental role during the early phase of a Gram-negative pneumonia-derived sepsis caused by \textit{B. pseudomallei}.
Many studies have demonstrated that during pulmonary inflammation and sepsis a shift towards pro-coagulant activity and inhibition of fibrinolysis occurs⁵, changes that are thought to contain the infection and thereby protect the host against the detrimental effects of infection and subsequent inflammation¹,². Although the net effect on fibrinolysis is inhibitory (primarily due to increased
tPA in melioidosis

production of PAI-1), upregulation of pro-fibrinolytic proteins, including tPA and uPA, has been described\(^3\), \(^5\), \(^7\), \(^22\), suggesting that severe infection is accompanied by enhanced synthesis and/or release of both agonistic and antagonistic mediators of fibrinolysis. In accordance, a recent study performed by our laboratory demonstrated elevated plasma concentrations of both PAI-1 and tPA in patients with culture proven melioidosis\(^14\). In these patients the ratio between activation of coagulation and fibrinolysis (TATc/PAPc (plasmin-α2-antiplasmin complexes) ratio) was increased in patients compared to healthy controls, indicating a state of decreased fibrinolysis\(^14\). Moreover, increased TATc/PAPc ratios were associated with a worse survival\(^14\), suggesting a role for the fibrinolytic system in the host defense against \textit{B. pseudomallei}. Our present results are in line with these clinical data as we also show upregulation of tPA during experimental melioidosis concurrent with a net suppression of fibrinolysis, as reflected by decreased PA activity and increased pulmonary fibrinogen deposition 48 hours after infection. We previously showed strongly elevated PAI-1 levels in lungs of mice infected with \textit{B. pseudomallei}\(^17\), which likely explains the reduced PA activity in the presence of elevated tPA protein levels. Altogether, our mouse model of melioidosis closely resembles the human situation with regard to changes in the fibrinolytic system.

Our study sought to unravel the role of endogenous tPA during severe Gram-negative sepsis caused by \textit{B. pseudomallei}. We demonstrated that deficiency of tPA is beneficial for the host with respect to survival, bacterial growth and pulmonary histopathology scores. Apparently, fibrin is an important player in the host defense against \textit{B. pseudomallei}. Interestingly, our data are in contrast with previous studies performed on Gram-negative sepsis by our laboratory and others. During \textit{E. coli}-induced abdominal sepsis our group demonstrated a protective role for endogenous tPA: tPA\(^{-/-}\) mice had an impaired host defense, as indicated by higher bacterial loads at the primary site of infection, enhanced bacterial dissemination and a reduced survival\(^9\). In addition, tPA\(^{-/-}\) mice displayed an impaired neutrophil influx into the peritoneal cavity, while adenoviral overexpression of tPA resulted in an increase in neutrophil recruitment\(^9\). Similarly, endogenous tPA also proved to be protective during \textit{E. coli}-induced pyelonephritis, as reflected by increased bacterial loads in tPA\(^{-/-}\) mice\(^23\). Finally, tPA\(^{-/-}\) mice had an unremarkable host defense in a model of severe pneumonia induced by \textit{Klebsiella pneumoniae}, as illustrated by an unaltered survival and similar bacterial burdens when compared to WT mice, indicating that endogenous tPA played an insignificant role in the immune response during this infection\(^10\). Interestingly, in this study tPA levels were decreased during the course of the infection\(^10\). A possible explanation for these differences may be caused by variations in characteristics of the different bacteria, as \textit{B. pseudomallei} mainly is an intracellular bacterium\(^11\), \(^12\), while \textit{E. coli} and \textit{K. pneumoniae} preferentially reside and replicate outside the cell. Our data show that the observed phenotype of tPA\(^{-/-}\) mice is not related to alterations in uPA or PAI-1 levels.

tPA is an important plasmin-activating serine protease, responsible for intravascular thrombolysis as tPA is mainly found in the vascular compartment\(^24\). In contrast to uPA and PAI-1 little is known
about other properties of tPA that extend beyond its role in cleaving plasminogen into plasmin. Studies involving the brain show that increased tPA-activity levels have been linked to neural damage and tPA is also believed to contribute to normal learning and synaptic plasticity (reviewed in\textsuperscript{25}).

Also during \textit{E. coli} peritonitis the observed protective effects of tPA, including a stronger neutrophil influx in tPA overexpressing mice, are thought to be independent of tPA-induced plasminogen activation, as plasminogen-deficient mice did not show any differences compared to WT mice in the same model\textsuperscript{9}. Earlier studies performed by our laboratory also supported the notion that tPA may stimulate neutrophil migration\textsuperscript{9, 18} and the generation of an oxidative burst by neutrophils. In the present study however, it is conceivable that the observed detrimental effect of tPA is mainly related to its role in fibrinolysis. We did not find any significant differences in neutrophil influx or activation at the primary site of infection, as indicated by equal results in Ly-6G staining and MPO levels respectively in tPA\textsuperscript{–/–} and WT mice. One could expect more neutrophils to migrate to the lungs of tPA\textsuperscript{–/–} mice as a consequence of enhanced local fibrin deposition; however, neutrophil recruitment occurred prior to peak fibrin generation in our model, differences between tPA\textsuperscript{–/–} and WT mice were only present at 48 hours and many other mechanisms contribute to neutrophil recruitment during pneumonia, including chemokines, complement factor and adhesion molecules\textsuperscript{26}. tPA\textsuperscript{–/–} mice showed decreased fibrinolytic activity, as reflected by decreased levels of PA activity, D-dimer and other fibrin degradation products. tPA\textsuperscript{–/–} mice also had decreased lung levels of uPA at early time points. Although the underlying mechanism remains speculative, this finding may be explained by enhanced consumption and suggest that relatively decreased uPA concentrations may further contribute to a reduced fibrinolysis in tPA\textsuperscript{–/–} mice. Interestingly, deficiency of tPA and decreased levels of uPA did not affect PAI-1 levels, suggesting that presence and production of PAI-1 is independent of the amounts of the proteins that it inhibits: tPA and uPA. In addition tPA\textsuperscript{–/–} mice demonstrated increased pulmonary fibrin depositions; although consistent with the hypo-fibrinolytic phenotype of these mice, it should be noted that the antibody used for tissue staining also detects fibrinogen. Limitation of fibrinolysis may have caused the beneficial phenotype in tPA\textsuperscript{–/–} mice in our model, considering that a certain amount of fibrin likely assists in containment of \textit{B. pseudomallei} bacteria to the primary site of infection and, possibly, facilitates clearance of these bacteria. Inhibition of fibrinolysis already is a component of the host response to infection\textsuperscript{1, 2}; tPA\textsuperscript{–/–} mice, having even less fibrinolysis, are even more protected. Of note, absence of tPA did not impact on bacterial dissemination, as no differences in bacterial loads in liver homogenates and blood were seen, suggesting that the beneficial effect of tPA deficiency is limited to the primary site of infection. In addition, our data indicate that tPA deficiency is associated with an improved antibacterial response in the lungs during early phase of the infection (when bacterial burdens are still relatively low), but is eventually not able to prevent outgrowth of \textit{B. pseudomallei} to a maximal extent during late stage infection, considering that pulmonary bacterial burdens were similar in tPA\textsuperscript{–/–} and WT mice 72 hours post infection. Notably, tPA deficiency did not result in enhanced activation of coagulation, as indicated by equal TATc concentrations between groups. In future studies it could be of interest to study the effects of exogenous (recombinant) tPA administration...
in experimental melioidosis. It should be noted, however, that such studies are difficult to perform in mice considering the short half-life of tPA. In addition, clinical implementation of recombinant tPA infusion in melioidosis seems unlikely considering the bleeding risk associated with this therapy. Our findings are in line with our recent studies in PAI-1−/− mice during experimental melioidosis, showing that PAI-1 deficiency is associated with a worse disease outcome, reflected by a worse survival, increased bacterial loads in lungs, liver and blood, increased distant organ failure and enhanced activation of fibrinolysis17. Of interest, mice with a complete fibrinogen deficiency showed a markedly increased mortality after infection with group A streptococci27, also pointing to an increased host susceptibility to a bacterial infection when clot formation is impaired. Altogether, these data suggest that a net anti-fibrinolytic state during melioidosis may improve the anti-bacterial response and disease outcome. Whether these investigations, which sought to obtain insight into host defense during melioidosis, can be of use for clinical practice remains to be established. It could be of interest to study whether inhibition of fibrinolysis could be a new strategy in the treatment of melioidosis patients.

Cytokine release is an important component of the host response to B. pseudomallei11. Clinical studies in melioidosis patients showed elevated serum levels of TNF-α, IL-6 and IFN-γ28, 29. It is known that the pro-inflammatory cytokine TNF-α is likely to be an important element of the early immune response to B. pseudomallei, as passive immunization against this mainly macrophage derived-cytokine increased mortality in experimental murine melioidosis30. In addition, the pro-inflammatory cytokine IFN-γ, produced by cytotoxic T-cells and natural killer cells, has an important protective role as well in early resistance against B. pseudomallei infection30: administration of a neutralizing monoclonal antibody against IFN-γ lowered the LD50 from > 5 · 10⁵ CFU to ~2 CFU, and was associated with marked increases in bacterial loads in the liver and spleen30. It was concluded by the authors that the rapid production of IFN-γ within the first day of infection determined whether the infection proceeds to an acute lethal outcome or becomes chronic30. Likewise, inhibition of the production of IL-12, one of the predominant inducers of IFN-γ, resulted in increased mortality in the same model30. Interestingly, in our study tPA deficiency resulted in early increased levels of pulmonary and systemic TNF-α and pulmonary IL-6 and IFN-γ. It might well be possible that these early rises in pro-inflammatory cytokines may have at least partially contributed to the protective phenotype observed in tPA−/− mice at later time points. Notably, at 48 hours post infection plasma IFN-γ levels were decreased in tPA−/− mice; this is unlikely to influence protective innate immunity, which is considered to be initiated very early after infection11. How tPA deficiency is related to increased early cytokine release remains a subject for further research. tPA deficiency did not influence IL-10 or MCP-1 levels, mediators of which the significance for host defense mechanisms in melioidosis has not been directly studied.

The current study is the first to report on the role of endogenous tPA in melioidosis. Mice deficient for tPA were protected during infection with B. pseudomallei, with respect to survival, pulmonary
bacterial loads, pulmonary histopathological scores and to a transient decrease in renal function. In addition they demonstrated increased pulmonary fibrin depositions; although consistent with the hypo-fibrinolytic phenotype of these mice, it should be noted that the antibody used for tissue staining also detects fibrinogen. A possible explanation for the observed protective effects of tPA deficiency may be related to the fact that a certain amount of fibrin is necessary to protect the host from growth and dissemination of *B. pseudomallei*. However, increased pro-inflammatory cytokine production in tPA−/− mice early during infection may contribute to the protective phenotype as well. Altogether, this study clearly shows that endogenous tissue-type plasminogen activator impairs host defense during severe experimental Gram-negative sepsis caused by *B. pseudomallei* (melioidosis).

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REFERENCES

Chapter 12


SUPPLEMENTARY MATERIAL

MATERIAL AND METHODS

Sample harvesting, processing and determination of bacterial growth.
Twenty-four, 48 and 72 hours after induction of infection mice were sacrificed under intra-peritoneal anesthesia containing ketamin (Eurovet Animal Health, Bladel, The Netherlands) and medetomidin (Pfizer Animal Health Care, Capelle aan den IJssel, The Netherlands). Blood was drawn into syringes containing sodium citrate (4:1 vol/vol). Lungs and liver were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFU were determined from serial dilutions of organ homogenates and blood that were plated on blood agar plates and incubated at 37°C 5% CO₂ for 20h before colonies were counted.

Assays
Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendations. Myeloperoxidase concentrations (MPO; HyCult Biotechnology, Uden, The Netherlands), mouse tPA (Molecular Innovations, Southfield, MI), D-dimer (Asserachrom D-dimer, Roche, Woerden, the Netherlands; lower limit of detection 10 ng/mL), thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany), mouse plasminogen activator inhibitor (PAI)-1 (Molecular Innovations, Novi, MI) and mouse urokinase plasminogen activator (uPA) (Molecular Innovations, Novi, MI) were measured with commercially available ELISA kits. Plasminogen activator (PA) activity was measured by an amidolytic assay as described previously 1, 2. Plasma urea, creatinine, alanine aminotranspherase (ALAT) and aspartate aminotranspherase (ASAT) were determined with commercial available kits (Sigma-Aldrich, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturers’ instructions.

Western Blot
After dilution with SDS sample buffer lung homogenate samples were heated for 5 minutes at 95°C, run on 6% SDS-PAGE gels under non-reducing conditions and subsequently transferred to a blotting membrane. Following blocking with 5% BSA in Tris-buffered saline containing 0.1% Tween-80 (TBS-T), the membrane was incubated overnight in 1:1000 polyclonal goat-anti-mouse-fibrinogen (Kordia, Leiden, the Netherlands) in 1% skimmed milk (Sigma-Aldrich, St. Louis, MO). Then, the membrane was incubated in 1:3000 polyclonal donkey-anti-goat IgG-HRP secondary antibody (Abcam, Cambridge, UK) in 1% BSA and imaged on a LAS3000 dark box (Fujifilm, Tokyo, Japan). Anti-fibrin(ogen) reactive bands were analyzed using ImageJ (version 2006.02.01, US National Institutes of Health, Bethesda, MD) and expressed as the percentage of intensity compared to WT mice.
Histology and immunohistology

All slides were scored in a blinded fashion by a pathologist for the following parameters: interstitial inflammation, necrosis, endothelialitis, bronchitis, edema, pleuritis, presence of thrombi and percentage of lung surface with pneumonia. All parameters were rated separately from 0 (condition absent) to 4 (most severe condition). The total histopathological score was expressed as the sum of the scores of the individual parameters, with a maximum of 24.

REFERENCES
