Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Plasminogen activator inhibitor type I contributes to protective immunity during experimental Gram-negative sepsis (melioidosis)

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ABSTRACT

Background
Melioidosis is a frequent cause of sepsis in Southeast-Asia caused by the Gram-negative bacterium *Burkholderia (B.) pseudomallei*. Patients with melioidosis have elevated circulating levels of plasminogen activator inhibitor type-1 (PAI-1), an important regulator of inflammation and fibrinolysis. In this study we aimed to investigate the role of PAI-1 during melioidosis.

Methods
Wildtype (WT) and PAI-1 deficient (PAI-1⁻/⁻) mice were intranasally infected with *B. pseudomallei*. Mice were sacrificed after 24, 48 or 72 hours. Lungs, liver and blood were harvested to measure bacterial loads, cytokines, clinical chemistry, histopathology and coagulation parameters. Additionally, survival studies were performed.

Results
PAI-1⁻/⁻ mice demonstrated enhanced susceptibility to *B. pseudomallei* infection as evidenced by a strongly increased mortality (100% versus 58% amongst WT mice, *P* < 0.001), associated with enhanced bacterial loads in lungs, liver and blood. Additionally, PAI-1⁻/⁻ mice showed elevated levels of pro-inflammatory cytokines in lungs and plasma, accompanied by enhanced local and systemic coagulation activation (thrombin-antithrombin complexes, D-dimer), increased hepatocellular injury (plasma ASAT and ALAT) and renal failure (plasma creatinine and urea).

Conclusions
PAI-1 serves a protective role during severe Gram-negative sepsis caused by *B. pseudomallei* by limiting bacterial growth, inflammation and coagulation, and likely as a consequence thereof, distant organ injury.
INTRODUCTION

Melioidosis is an infectious disease caused by the soil-dwelling bacterium *Burkholderia (B.) pseudomallei*. This potential bioterrorism threat agent is an important cause of community acquired sepsis in Southeast Asia and Northern Australia with mortalities up to 40% despite appropriate antibiotic therapy. Melioidosis is often associated with pneumonia and bacterial dissemination to distant sites, with many possible disease manifestations, septic shock being the most severe. The high mortality and relatively poor responsiveness of *B. pseudomallei* to antibiotics emphasize the importance of understanding non-specific host defense mechanisms. Such knowledge may ultimately pave the way for new treatment options.

During severe infection a range of host defense mechanisms becomes activated, resulting in a strong inflammatory response together with increased procoagulant activity and suppression of the fibrinolytic system. The interplay between inflammation and blood coagulation is considered to be an essential part of the host defense against infectious agents. The strong pro-inflammatory response observed during severe sepsis may lead to disseminated intravascular coagulation, which contributes to multi-organ failure and high mortality rates. Plasminogen activator inhibitor type 1 (PAI-1) is considered to be one of the main inhibitors of the fibrinolytic system, together with alpha-2-antiplasmin. By inactivating both urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), PAI-1 inhibits generation of the key-enzyme plasmin and subsequent fibrin degradation. Besides its role in maintaining normal hemostasis by regulating the fibrinolytic system, PAI-1 has also been implicated in other processes and diseases that are not or only partially related to its capacity to inhibit plasminogen generation, including wound healing, atherosclerosis, metabolic diseases and tumor angiogenesis. PAI-1 may have a similar protective effect during Gram-positive pneumonia caused by *Streptococcus (S.) pneumoniae*, although the results presented are inconsistent. Our laboratory recently reported marked coagulation activation in patients with culture proven melioidosis together with unfavorable outcomes and enhanced mortality.
with downregulation of anticoagulant pathways and alterations in fibrinolytic parameters\textsuperscript{21}. Specifically, plasma concentrations of PAI-1 were strongly increased\textsuperscript{21}. In the present study we aimed to characterize the role of PAI-1 during severe pneumonia-derived sepsis caused by \textit{B. pseudomallei} using our established mouse model of melioidosis\textsuperscript{22, 23}. We here show that endogenous PAI-1 is beneficial in the host defense against experimentally induced melioidosis.

\textbf{METHODS}

\textbf{Mice}
Pathogen-free 10-week old male wild type (WT) C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands) 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. PAI-1\textsuperscript{-/-} mice on a C57BL/6 genetic background were obtained from Jackson Laboratories (Bar Harbour, ME). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

\textbf{Experimental infection and determination of bacterial growth}
Experimental melioidosis was induced by intranasal inoculation with \textit{B. pseudomallei} strain 1026b (500 colony forming units (CFU)/ 50 µL 0.9% NaCl) as previously described\textsuperscript{22, 23}. For survival experiments mice were checked every 6 hours until death occurred. Sample harvesting and processing and determination of bacterial growth were done as described\textsuperscript{22, 23}. Briefly, 24, 48 and 72 hours after induction of infection mice were sacrificed under intraperitoneal 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% CO\textsubscript{2} for 20 h before colonies were counted.

\textbf{Assays}
Assays were done in citrated plasma and lung homogenates as described\textsuperscript{22, 23}. 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) benzenesulfonylfluoride], 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 g at 4°C for 10 min. Supernatants were sterilized using 0.22 µm pore-size filters (SLGVT13 NL, Millex*-GV, Millipore, Billerica, MA) and stored at -20°C until analysis. Interleukin (IL)-6, IL-10, IL-12p70, interferon
(IFN)-γ and tumor necrosis factor (TNF)-α were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendations. Mouse PAI-1 (Kordia, Leiden, the Netherlands), D-dimer (Asserachrom D-dimer, Roche Woerden, the Netherlands) and thrombin-antithrombin complexes (TATc; Affinity Biologicals, Ancaster, Ontario, Canada) were measured with commercially available ELISA kits. Plasminogen activator activity (PAA) was measured by an amidolytic assay as described previously\textsuperscript{24, 25}. Plasma lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), urea and creatinine 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 heating for 5 minutes at 95°C, lung homogenate samples were run on a 6% SDS-PAGE gel 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). Lung protein contents were analyzed using ImageJ (version 2006.02.01, US National Institutes of Health, Bethesda, MD) and expressed as the percentage of increase in intensity compared to WT.

**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\textsuperscript{22, 23}. All slides were coded and scored blinded 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. Granulocyte stainings, using fluorescein isothiocyanate-labeled rat-anti-mouse Ly-6G mAb (BD Pharmingen, San Diego, CA) as well as staining for fibrin(ogen) were performed as described previously\textsuperscript{13, 26, 27}. Ly-6G and fibrin(ogen) 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.
Comparisons between groups were conducted using the Mann-Whitney \( U \) test. For survival studies Kaplan-Meier analyses followed by log rank test were performed. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). \( P \)-values less than 0.05 were considered statistically significant.

## RESULTS

### PAI-1 is upregulated during murine melioidosis

To obtain insight in local pulmonary concentrations of PAI-1 during experimentally induced melioidosis we measured PAI-1 protein levels in lung homogenates of uninfected WT mice and in WT mice infected with \( B. \) pseudomallei. PAI-1 was detected at levels 0.9 - 1.5 ng/mL in lung homogenates of uninfected mice. Infection with \( B. \) pseudomallei was associated with a significant increase in lung PAI-1 levels at 24, 48, and 72 hours after inoculation (\( P < 0.01 \) versus control for all time points; Figure 1).

### PAI-1\(^{-/-}\) mice show an accelerated mortality during experimental melioidosis

To investigate whether PAI-1 deficiency impacts on mortality during murine melioidosis we intranasally infected WT and PAI-1\(^{-/-}\) mice with 500 CFU of \( B. \) pseudomallei and observed them during the following 40 days (Figure 2). PAI-1\(^{-/-}\) mice showed a clear survival disadvantage when compared to WT (\( P < 0.0001 \)) indicating that endogenous PAI-1 is protective during experimental melioidosis.

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**Figure 1. Increased pulmonary PAI-1 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. PAI-1 protein concentrations were measured in lung homogenates by ELISA, \( n = 5-8 \) 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. PAI-1\(^{-/-}\) mice show an accelerated mortality during experimental melioidosis.** Wildtype (WT) and PAI-1\(^{-/-}\) mice were infected intranasally with 500 CFU of \( B. \) pseudomallei and mortality was assessed every 6 hours, \( n = 9-12 \) mice per group. Comparison between groups was done by using Kaplan-Meier analysis followed by log rank tests.
**PAI-1 deficiency facilitates bacterial growth and dissemination**

To determine whether the increased mortality of PAI-1−/− mice was associated with changes in bacterial growth, we evaluated bacterial loads in the lungs of WT mice 24, 48 and 72 hours after intranasal inoculation and compared these with bacterial loads in PAI-1−/− mice. Whereas during the first 48 hours after infection pulmonary bacterial counts were similar in PAI-1−/− and WT mice, at 72 hours PAI-1−/− mice demonstrated markedly elevated bacterial loads in their lungs (P < 0.001; Figure 3A).

To investigate the impact of PAI-1 on the dissemination of the infection we evaluated bacterial numbers in liver homogenates and blood. PAI-1−/− mice clearly showed increased bacterial loads in both liver homogenates and blood 72 hours after induction of infection (Figure 3B-C). Thus, PAI-1 deficiency facilitates both pulmonary and systemic growth of the *B. pseudomallei* bacterium, in particular at later time points.

**Figure 3. PAI-1 deficiency facilitates bacterial growth and dissemination.** Mice were inoculated with 500 CFU of *B. pseudomallei* and sacrificed after 24, 48 and 72 hours. Bacterial loads were determined in lung homogenates (A), liver homogenates (B) and blood (C). 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 PAI-1−/− mice (n = 8 mice per group for each time point). **P < 0.01 and ***P < 0.001 for the difference between WT and PAI-1−/− mice (Mann-Whitney U test).

**PAI-1−/− mice demonstrate a reduced cytokine response early after infection with *B. pseudomallei***

Since cytokines are important regulators of the host immune response during melioidosis, we measured pulmonary and systemic levels of TNF-α, IL-6, IL-10, IL-12p70 and IFN-g (Table 1). At 24 hours after infection, when bacterial loads were still similar in both mouse strains, lung levels of TNF-α, IL-12p70 and IFN-g were decreased in PAI-1−/− mice. In contrast, at 72 hours, pulmonary cytokine concentrations were higher in PAI-1−/− mice, significantly for TNF-α and IL-6 (P < 0.001). Similarly, at 24 hours post infection PAI-1−/− mice displayed lower plasma concentrations of TNF-α, IL-12p70 and IFN-g when compared with WT mice, whereas at 72 hours the plasma levels of all cytokines measured were higher in PAI-1−/− mice.
Chapter 13

PAI-1 deficiency does not impact on lung pathology after infection with *B. pseudomallei* but is associated with enhanced neutrophil influx

Our model of melioidosis is associated with profound lung pathology. Both WT and PAI-1−/− mice infected with *B. pseudomallei* showed 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; however no significant differences in histological scores of the lungs between WT and PAI-1−/− mice were observed. On the contrary, in PAI-1−/− mice significantly increased Ly-6 staining, a marker of neutrophil influx was observed 48 hours ($P < 0.01$) and 72 hours after infection ($P < 0.05$) (Figure 4D-F).

Table 1. Cytokine concentrations in lung homogenates and plasma of WT and PAI-1−/− mice during experimental melioidosis.

<table>
<thead>
<tr>
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<th>Lung homogenates</th>
<th>Plasma</th>
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<tbody>
<tr>
<td></td>
<td>$pg/mL$</td>
<td>WT</td>
</tr>
<tr>
<td>24h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>3254 (1648-4106)</td>
<td>1159 (532-2160)</td>
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<tr>
<td>IL-6</td>
<td>4380 (3196-5455)</td>
<td>4328 (2822-7840)</td>
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<tr>
<td>IL-10</td>
<td>1.5 (1.2-1.7)</td>
<td>0.85 (0.1-1.7)</td>
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<tr>
<td>IL-12p70</td>
<td>32 (28-43)</td>
<td>18 (9-24)</td>
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<tr>
<td>IFN-γ</td>
<td>28 (25-43)</td>
<td>17 (9-21)**</td>
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<td></td>
<td></td>
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<tr>
<td>48h</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>1001 (676-1180)</td>
<td>1559 (1190-1810)</td>
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<tr>
<td>IL-6</td>
<td>3234 (1938-6642)</td>
<td>5256 (5066-7241)</td>
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<tr>
<td>IL-10</td>
<td>28 (17-86)</td>
<td>71 (46-111)</td>
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<tr>
<td>IL-12p70</td>
<td>19 (10-42)</td>
<td>41 (36-59)*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3 (2-40)</td>
<td>5 (4-8)*</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72h</td>
<td></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>508 (444-631)</td>
<td>1041 (884-1561)***</td>
</tr>
<tr>
<td>IL-6</td>
<td>342 (291-963)</td>
<td>10000 (8395-10000)****$</td>
</tr>
<tr>
<td>IL-10</td>
<td>90 (48-315)</td>
<td>103 (84-253)</td>
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<tr>
<td>IL-12p70</td>
<td>52 (41-80)</td>
<td>101 (29-108)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>33 (30-39)</td>
<td>62 (27-100)</td>
</tr>
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</table>

Pulmonary and plasma cytokine levels after intranasal infection with 500 CFU of *B. pseudomallei*. Wild type (WT) and PAI-1−/− 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. IL interleukin, TNF-α tumor necrosis factor-α, IFN-γ interferon-γ. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ for WT versus PAI-1−/− mice (Mann-Whitney U test). < indicates data below detection limits. $ indicates that some data are above detection limits.
PAI-1 deficiency results in increased distant organ injury

As PAI-1<sup>−/−</sup> mice showed a markedly increased mortality and enhanced bacterial loads at multiple body sites, we wondered whether PAI-1 deficiency would affect the extent of distant organ injury. Relative to WT mice, PAI-1<sup>−/−</sup> mice showed a strong increase in plasma LDH levels (Figure 5A), a general parameter for tissue damage, as well as marked rises in plasma creatinine and urea concentrations 72 hours after induction of infection, indicative for renal failure (Figure 5B-C). Moreover, in PAI-1<sup>−/−</sup> mice plasma levels of ASAT and ALAT, both parameters for hepatocellular injury, were substantially increased after 72 hours (Figure 5D-E). Hence, PAI-1 seems to play a protective role in the development of distant organ injury during experimental melioidosis.

PAI-1 deficiency leads to activation of coagulation and fibrinolysis during infection with *B. pseudomallei*

Having established that endogenous PAI-1 plays a protective role during infection with *B. pseudomallei,*
we next wondered whether this would impact activation of coagulation and fibrinolysis. Therefore TATc, a parameter of coagulation induction, was measured in lung homogenates of WT and PAI-1−/− mice 24, 48 and 72 hours after inoculation of \textit{B. pseudomallei}. PAI-1−/− mice demonstrated increased TATc when compared to WT\textdagger ($P<0.01$; Figure 6A). Additionally, immunohistological staining of fibrin showed increased fibrin depositions 48 hours after infection in PAI-1−/− mice ($P<0.01$), while early during the infection no fibrin staining was detectable (Figure 6B-D). To obtain insight in the net fibrinolytic activity during experimental melioidosis we measured PAA. Lung PAA was markedly increased in PAI-1−/− mice when compared to WT mice ($P<0.001$; Figure 6E), indicating a net pro-fibrinolytic state. To obtain insight in activation of fibrin degradation during infection with \textit{B. pseudomallei}, fibrin degradation products were measured in lung homogenates by western blotting 72 hours after infection, and in PAI-1−/− they were markedly increased: D-dimer levels demonstrated an almost 2-fold increase (188% in PAI-1−/− mice versus 100% in WT, $P<0.05$) and fragment X levels were 2.5-fold increased (247% in PAI-1−/− mice versus 100% in WT, $P<0.05$) (Figure 6F-G). Hence, both parameters for coagulation and fibrinolysis were upregulated during infection with \textit{B. pseudomallei}, which was further enhanced in the PAI-1 deficient animals.

**Figure 5.** PAI-1−/− mice show increased distant organ injury. Mice were intranasally inoculated with 500 CFU of \textit{B. pseudomallei} and sacrificed after 24, 48 and 72 hours. PAI-1−/− mice showed enhanced generalized distant organ injury as reflected by elevated plasma lactate dehydrogenase (LDH) levels (A). PAI-1−/− mice also showed increased renal failure when compared to WT mice as reflected by elevated plasma creatinine (B) and urea levels (C). Finally, PAI-1−/− mice showed increased liver injury, as reflected by elevated plasma concentrations of ASAT (D) and ALAT (E). 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 PAI-1−/− mice ($n=8$ mice per group for each time point). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ for WT versus PAI-1−/− mice (Mann-Whitney U test).
Figure 6. PAI-1−/− mice show increased activation of coagulation and fibrinolysis during melioidosis. Mice were infected intranasally with 500 CFU of *B. pseudomallei*. After 24, 48 and 72 hours coagulation activation (TATc; A) was measured in lung homogenates. Increased fibrin depositions were seen in lungs of PAI-1−/− mice compared to WT mice 48 hours after infection (Figure 6B). Figure C and D show representative photographs of fibrin-staining of lung tissue 48 hours after infection (original magnification x400). As expected, PAI-1−/− mice demonstrated enhanced PA activity in lung homogenates (*P* < 0.001 versus WT mice, Figure 6E). In line with an increased fibrinolytic activity in PAI-1−/− mice, these animals showed increased amounts of fibrin degradation products when compared to WT as measured by western blotting on lung homogenates 72 hours after infection: in particular amounts of Fragment X (FX; F) and D-dimer (Dd; G) were significantly increased in PAI-1−/− mice. C: positive control sample (normal mouse plasma, uninfected, dilution 1:50). Fmm: fibrin-multimers. 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 PAI-1−/− mice (*n* = 8 mice per group for each time point). *P*< 0.05, **P**< 0.01, ***P***< 0.001 for WT versus PAI-1−/− mice (Mann-Whitney *U* test).
DISCUSSION

Our laboratory recently reported markedly elevated circulating levels of PAI-1 in patients with culture proven melioidosis\textsuperscript{21}. We here aimed to study the functional role of endogenous PAI-1 during this infection using our established mouse model of melioidosis. We found that PAI-1 production is enhanced during experimental melioidosis. Strikingly, PAI-1\textsuperscript{−/−} mice showed a strongly reduced survival after infection with \textit{B. pseudomallei}, which was accompanied by significantly increased bacterial growth and dissemination when compared to WT mice. Furthermore, PAI-1\textsuperscript{−/−} mice had increased pulmonary inflammation, coagulation and fibrinolysis and more severe distant organ injury. These data indicate that endogenous PAI-1 has a protective role during Gram-negative pneumonia-derived sepsis caused by \textit{B. pseudomallei}.

Several studies have documented that elevated PAI-1 concentrations are associated with a poor outcome in patients with sepsis\textsuperscript{17, 18}. In accordance, we recently reported higher PAI-1 levels in melioidosis patients who subsequently died, although the difference with survivors was not significant\textsuperscript{21}. Although these clinical data may suggest a detrimental role for PAI-1 in the pathogenesis of sepsis and melioidosis, our current results demonstrate experimental evidence for a protective role of PAI-1 during infection with \textit{B. pseudomallei}. These findings are in line with earlier studies showing that PAI-1\textsuperscript{−/−} mice have an impaired host defense during \textit{Klebsiella} or pneumococcal pneumonia, although in the latter model published results are inconsistent\textsuperscript{13, 19, 20}.

PAI-1 can impact on host defense mechanisms in multiple ways. PAI-1 is one of the main inhibitors of fibrinolysis by virtue of its capacity to inhibit the plasminogen activators uPA and tPA. Inhibition of uPA and tPA results in a strongly reduced generation of plasmin, the protease responsible for fibrin degradation. In line, uninfected PAI-1\textsuperscript{−/−} mice display a mild hyperfibrinolytic state and have a faster whole-blood clot lysis than WT mice\textsuperscript{29}. In the present study, PAI-1\textsuperscript{−/−} mice demonstrated a pro-fibrinolytic state at the primary site of infection, especially at later time points, as reflected by elevated levels of PAA, D-dimer and other fibrin degradation products in lung homogenates. This anti-fibrinolytic function of PAI-1 may play a role in the host defense against \textit{B. pseudomallei} considering that a certain amount of fibrin, in particular fibrin-multimers, likely assist in containment of the bacteria to the primary site of infection and consequently the prevention of bacterial dissemination to distant body sites. Indeed, our data show that the enhanced fibrinolysis in PAI-1\textsuperscript{−/−} mice was associated with increased bacterial loads in lungs, liver and blood. We only measured PA activity at 72 hours after infection. Previous studies have shown that PAI-1\textsuperscript{−/−} mice display enhanced fibrinolytic activity at baseline\textsuperscript{29}; in addition, PAI-1\textsuperscript{−/−} mice demonstrated enhanced fibrinolytic activity in lungs after infection with \textit{Klebsiella} at time points where bacterial loads were similar to those in WT mice\textsuperscript{19}. Notably, PAI-1 deficiency also resulted in enhanced activation of coagulation, as indicated by elevated TATc concentrations. Considering that PAI-1 does not impact on thrombin generation, this finding is most likely explained by the strongly elevated bacterial loads in PAI-1\textsuperscript{−/−} mice, providing a more potent stimulus for activation of
the coagulation system. Earlier studies already showed a role for coagulation in protection against bacterial infections\textsuperscript{30, 31}. Subcutaneous injection with surface-protease deficient plasmids of \textit{Yersinia pestis}, the causative agent of plague, increased the median lethal dose of bacteria by a millionfold, when compared to injection with non-deficient plasmids\textsuperscript{31}, indicating that inhibition of protease activity limits bacterial growth and dissemination. Furthermore, mice with a deficiency in Factor V or with a complete fibrinogen deficiency showed a markedly increased mortality after infection with group A streptococci\textsuperscript{30}, again pointing to an increased host susceptibility to a bacterial infection when thrombin generation is impaired. Notably, at later stages of disease progression clotting likely contributes to organ failure and mortality\textsuperscript{7, 8}. In order to elucidate this late role of coagulation, studies with genetically modified mice are less suitable; investigations in which anticoagulant treatment is given to WT mice together with antibiotics hours or days after induction of infection would be more appropriate to address this issue\textsuperscript{32}.

Cytokine release is an important component of the host response to \textit{B. pseudomallei}\textsuperscript{1}. Clinical studies in melioidosis patients showed elevated serum levels of TNF-\textit{\alpha}, IL-12 and IFN-\textit{\gamma}\textsuperscript{33, 34}. It is known that the pro-inflammatory cytokine IFN-\textit{\gamma}, produced by cytotoxic T-cells and natural killer cells, has an important protective role in early resistance against \textit{B. pseudomallei} infection. Inhibition of IFN-\textit{\gamma} lowered the LD\textsubscript{50} from >5*10\textsuperscript{5} CFU to ~2 CFU and was associated with marked increases in bacterial loads in the liver and spleen\textsuperscript{35}. Likewise, inhibition of IL-12, one of the predominant inducers of IFN-\textit{\gamma}, resulted in increased mortality in the same model\textsuperscript{35}. The pro-inflammatory cytokine TNF-\textit{\alpha} is also likely to be an important element of the early immune response to \textit{B. pseudomallei}, as passive immunization against this mainly macrophage derived-cytokine increased mortality in experimental murine melioidosis\textsuperscript{35}. Interestingly, in our study PAI-1 deficiency resulted in decreased local and circulating levels of TNF-\textit{\alpha}, IL-12 and IFN-\textit{\gamma} early after infection. Although a clear explanation for this observation is lacking, the reduced early production of these three protective cytokines likely at least in part contributed to the enhanced bacterial growth and dissemination in PAI-1\textsuperscript{-/-} mice at later time points.

PAI-1 may play an important role in neutrophil migration during experimental melioidosis. First, by inhibiting plasmin, PAI-1 inhibits plasmin-mediated proteolytic degradation of various extracellular matrix components, thereby diminishing plasmin-mediated leukocyte migration\textsuperscript{10, 11}. Secondly, PAI-1 can inhibit cell adhesion and migration by competing with the uPA receptor (uPAR) for binding to vitronectin, a ligand for integrins\textsuperscript{36, 37}. During critical illness, uPAR has been shown to be important in the recruitment of leukocytes towards the primary site of infection, as was described for pneumonia caused by \textit{Pseudomonas aeruginosa} or \textit{S. pneumoniae}\textsuperscript{36, 38}. Our laboratory previously demonstrated increased uPAR expression on circulating neutrophils of patients with melioidosis\textsuperscript{25}. Furthermore, uPAR\textsuperscript{-/-} mice infected with \textit{B. pseudomallei} showed diminished neutrophil recruitment towards the pulmonary compartment, which was accompanied by enhanced bacterial growth and dissemination. Our present data suggest that endogenous PAI-1 inhibits uPAR
mediated neutrophil recruitment during melioidosis, as indicated by elevated neutrophil numbers in infected lung tissue of PAI-1−/− mice. Notably, such an inhibitory function for PAI-1 on neutrophil influx into the lungs could not be demonstrated during murine pneumonia caused by either K. pneumoniae or S. pneumoniae13, 19. In line with the present results, PAI-1−/− mice had greatly enhanced influx of neutrophils to the site of inflammation in a model of turpentine-induced tissue injury, which was associated by increased edema and necrosis39. As such, PAI-1 can have different effects on neutrophil recruitment dependent on the model used.

At late stage infection, shortly before the first deaths occurred, PAI-1−/− mice displayed strongly increased bacterial loads in distant body sites and likely as a consequence thereof markedly increased end organ damage and failure, as reflected by elevated plasma levels of creatinine and urea (indicating renal failure), transaminases (indicating hepatocellular injury) and LDH (reflective of cell injury in general). Moreover, high bacterial loads at later time points were accompanied by markedly increased pro-inflammatory cytokine concentrations in particular in plasma. The extent of lung pathology did not differ between groups according to the semi-quantitative histopathology score used, in spite of increased neutrophil influx in PAI-1−/− mice. Possibly, however, this score may be not sensitive enough to detect enhanced lung injury, especially in the context of already extensive lung pathology in WT mice. Together these data suggest that PAI-1 primarily protects mice against melioidosis induced death by limiting infection and systemic inflammation and multiple organ failure. The current study is the first to report on the role of endogenous PAI-1 in melioidosis. The absence of PAI-1 led to strongly enhanced bacterial growth and dissemination relatively late in the course of B. pseudomallei infection, which was accompanied with overwhelming systemic inflammation, multiple organ failure and increased mortality. Since we only used one infectious dose, caution is warranted with regard to generalization of the data presented here. Nonetheless, the current data suggest that PAI-1 serves a protective role during melioidosis.

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