Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Overexpression of activated protein C is detrimental during severe experimental Gram-negative sepsis (melioidosis)

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ABSTRACT

Objective
The interplay between inflammation and blood coagulation is an essential part of host defense during severe pneumosepsis. Melioidosis, instigated by the Gram-negative bacterium *Burkholderia (B.) pseudomallei*, is a frequent cause of pneumosepsis in Southeast-Asia. Patients with severe pneumosepsis, including melioidosis, have decreased circulating levels of protein C. Activated protein C (APC) has anticoagulant and anti-inflammatory properties. In this study we aimed to investigate the effect of sustained elevated APC levels on the host response during melioidosis.

Design and Setting
Animal study, University research laboratory.

Subjects and Interventions
Wild type (WT) and APC-overexpressing (APC<sup>high</sup>) C57BL/6 mice. Mice were intranasally infected with viable *B. pseudomallei* and sacrificed after 24, 48 or 72 hours for harvesting of lungs, liver, spleen and blood. Additionally, survival studies were performed.

Measurements and Main Results
Plasma APC concentrations in APC<sup>high</sup>-mice (median 18.1 ng/mL) were in the same range as previously measured in patients treated with recombinant human APC. APC<sup>high</sup>-mice demonstrated enhanced susceptibility to *B. pseudomallei* infection compared to WT mice as evidenced by a strongly increased mortality accompanied by enhanced bacterial loads in the lungs, blood and distant organs 48 hours after infection. Additionally, at this time point APC<sup>high</sup>-mice showed elevated levels of proinflammatory cytokines in lungs and plasma, together with increased pulmonary histopathology scores and neutrophil influx. At 72 hours post infection decreased levels of thrombin-antithrombin complexes, reflecting inhibition of coagulation, were measured in lungs of APC<sup>high</sup>-mice.

Conclusions
Constitutively enhanced expression of APC impairs host defense during severe Gram-negative sepsis caused by *B. pseudomallei*. 
INTRODUCTION

Ample evidence has shown that severe (pneumo)sepsis is accompanied by both activation of a strong proinflammatory response on the one hand and increased procoagulant activity, blunted anticoagulant mechanisms and suppression of the fibrinolytic system on the other hand, all being part of the host defense mechanism\textsuperscript{1,2}. The interplay between inflammation and blood coagulation is considered to be an essential part of the host defense against infectious agents. The strong proinflammatory response observed during severe sepsis may lead to disseminated intravascular coagulation, which contributes to multi-organ failure and high mortality rates\textsuperscript{2}. The protein C (PC) system is an important regulator of hemostasis and the inflammatory response during infection\textsuperscript{3,4}. Initiated by complex formation of thrombin with thrombomodulin, the vitamin K-dependent zymogen PC is converted into its active form, activated protein C (APC). APC serves as one of the main inhibitors of the coagulation system via its capacity to inactivate coagulation factors Va (FV\textsubscript{a}) and VIIIa (FVIII\textsubscript{a}), together with various cofactors including protein S (PS). In addition, APC exerts cytoprotective, anti-inflammatory and anti-apoptotic properties which are mediated by the protease activated receptor-1 (PAR-1)\textsuperscript{3,4}. Many studies have shown that low PC and APC levels correlate with the occurrence of organ dysfunction and an adverse outcome in patients with sepsis\textsuperscript{3-6}. Treatment of severe sepsis patients with intravenous recombinant human APC (rhAPC) was reported to strongly reduce mortality in the PROWESS (Protein C Worldwide Evaluation in Severe Sepsis) study published in 2001\textsuperscript{7}; however, a recently completed confirmatory trial in septic shock patients (PROWESS-SHOCK) did not show any benefit from APC treatment\textsuperscript{8}, which led to the withdrawal of this compound from the market\textsuperscript{9,10}.

Melioidosis is an infectious disease caused by the soil-dwelling bacterium \textit{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\textsuperscript{11,12}. Melioidosis is often associated with pneumonia and bacterial dissemination to distant sites, with many possible disease manifestations, septic shock being the most severe \textsuperscript{12}. The high mortality and relatively poor responsiveness of \textit{B. pseudomallei} to antibiotics emphasize the importance of understanding non-specific host defense mechanisms.

Our laboratory recently reported pronounced coagulation activation in patients with culture-proven septic melioidosis together with downregulation of anticoagulant pathways\textsuperscript{13,14}. In particular, levels of PC and PS were markedly decreased, and low levels of PC correlated with a worse clinical outcome\textsuperscript{13}, a finding also reported by others\textsuperscript{15}. In the present study we investigated the effect of sustained elevated levels of APC during melioidosis, using mice overexpressing human (h) APC (APC\textsubscript{high-mice})\textsuperscript{16}. These transgenic mice express a hyperactivatable form of hAPC (D167F/D172K), which results in a gain in function with respect to APC formation, were especially generated in order to study the role of APC during severe stress\textsuperscript{16}. We show that overexpression of APC results in enhanced susceptibility to \textit{B. pseudomallei} infection as evidenced by a strongly increased
mortality, accompanied by increased bacterial loads, elevated levels of pro-inflammatory cytokines in lungs and plasma, increased histopathology scores and increased neutrophil influx in the lungs.

**MATERIALS AND METHODS**

**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. APC<sup>high</sup>-mice on a C57BL/6 genetic background were generated as described<sup>16</sup> and bred in the animal facility of the Academic Medical Center in Amsterdam. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center.

**Experimental infection and determination of bacterial growth**
Experimental melioidosis was induced by intranasal inoculation with *B. pseudomallei* strain 1026b (300 colony forming units (CFUs)/ 50 µL 0.9% NaCl) as previously described<sup>17-19</sup>. For survival experiments mice (n = 12/group) were checked every 6 hours until death occurred. Sample harvesting and processing and determination of bacterial growth were done as described<sup>17-19</sup>. For details, see Supplementary Material.

**Assays**
Lung homogenates of infected mice were diluted 1:1 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 1% Triton X-100 [pH 7.4] and protease inhibitor mix (AEBSF [4-(2-aminoethyl) benzanesulfonfluoride], EDTA-Na<sub>2</sub>, 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 (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<sup>17-19</sup>. Details are described in the Supplementary Material. Granulocyte stainings, using fluorescein isothiocyanate-labeled rat-anti-mouse Ly-6G mAb (BD Pharmingen, San Diego, CA) were done as described previously<sup>18, 20</sup>. Slides were counterstained with methylgreen (Sigma-Aldrich, St. Louis, MO). The total tissue area of the Ly-6 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 (Ly6+) 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. All variables were rank-transformed prior to analysis. Comparisons between groups were conducted using overall non-parametric ANOVA with modeled effects for strain, time and their interaction (if appropriate), followed by post-hoc Mann-Whitney U tests at the individual time points. For survival studies Kaplan-Meier analyses followed by log rank test were performed. All analyses were done using SPSS (SPSS Inc, version 18, Armonk, NY). p-values less than 0.05 were considered statistically significant.

RESULTS

APC concentrations in different organs of APC^high^-mice
Previous research on the effects of exogenous hAPC during sepsis reported hAPC levels of 44.9 ng/mL in plasma of hAPC-treated patients. To obtain insight in expression of hAPC in different organs in our study, we measured hAPC levels in plasma, lung-, kidney-, liver-, and spleen homogenates of uninfected APC^high^-mice. APC-activity was most abundant in lung- and spleen homogenates (respectively 135 ng/mL (98.9-165 ng/mL) and 108 ng/mL (70.1-185 ng/mL); Figure 1). Plasma hAPC-levels were 18.1 (14.1-30.7 ng/mL). hAPC levels in WT mice were all below detection limits.

APC^high^-mice show an accelerated mortality during experimental melioidosis
We have previously shown that our model of murine melioidosis is associated with severe sepsis and a high mortality. To investigate whether overexpression of APC impacts on mortality during murine melioidosis we intranasally infected WT and APC^high^-mice with 300 CFU of B. pseudomallei and observed them during the following 12 days (Figure 2). Surprisingly, APC^high^-mice showed a clear survival disadvantage when compared to WT mice (P < 0.05). APC^high^-mice started to die shortly beyond 72 hours of infection, eventually showing a mortality of 92% (11/12 mice).
Figure 2. APC\textsuperscript{high}-mice show an accelerated mortality during experimental melioidosis. Wild type (WT) and APC\textsuperscript{high}-mice were infected intranasally with 300 CFU of \textit{B. pseudomallei} and mortality was assessed every 6 hours, \( n = 12 \) mice per group. Comparison between groups was done by using Kaplan-Meier analysis followed by log rank tests.

Figure 3. APC-overexpression facilitates bacterial growth and dissemination. Mice were intranasally inoculated with 300 CFU of \textit{B. pseudomallei} and sacrificed after 24, 48 and 72 hours. Bacterial loads were determined in lung homogenates (A), blood (B), liver homogenates (C) and spleen homogenates (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 APC\textsuperscript{high}-mice (\( n = 8 \) mice per group for each time point). *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \) for the difference between WT and APC\textsuperscript{high}-mice (Mann-Whitney \( U \) test). BC+ denotes blood cultures positivity.
In contrast, although the initial mortality occurred simultaneously with that in APC<sup>high</sup>-mice, WT mice demonstrated an eventual mortality of only 50% (6/12 mice). These results indicate that overexpression of hAPC is detrimental during experimental melioidosis.

**APC-overexpression facilitates bacterial growth and dissemination**

To gain insight into the mechanism underlying the increased mortality of APC<sup>high</sup>-mice, we infected WT and APC<sup>high</sup>-mice with 300 CFU of *B. pseudomallei* and sacrificed them after 24, 48 or 72 hours to determine bacterial loads in lungs (the primary site of infection), blood, liver and spleen homogenates (to evaluate the extent of dissemination) (Figure 3). Relative to WT mice, APC<sup>high</sup>-mice showed markedly elevated bacterial loads in their lungs (*P* < 0.001; Figure 3A) and at distant body sites 48 hours after infection (Figure 3B-D). Seventy-two hours after infection, APC<sup>high</sup>-mice still showed increased bacterial dissemination compared to WT mice, as reflected by significantly increased bacterial loads in spleen homogenates (*P* < 0.05; Figure 3D). At this time point, bacterial loads in lungs were modestly but significantly lower in APC<sup>high</sup>-mice compared to WT mice (*P* < 0.05; Figure 3A). These data indicate that overexpression of APC facilitates the early growth and dissemination of *B. pseudomallei* during pneumonia derived sepsis.

**APC-overexpression aggravates lung pathology after infection with *B. pseudomallei* and is associated with enhanced neutrophil influx**

Our model of melioidosis is associated with profound lung pathology. Both WT and APC<sup>high</sup>-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). APC-overexpression leads to considerably lung pathology during infection as is reflected in significantly higher lung histology scores in APC<sup>high</sup>-mice when compared to WT mice 48 hours after infection (*P* < 0.05; Figure 4A-C). Neutrophils have shown to play a major role in the host defense against *B. pseudomallei*. In order to investigate the role of neutrophils in our model we measured percentage of Ly-6G staining in infected lung tissue (Figure 4D-F). Forty-eight hours after infection, APC<sup>high</sup>-mice displayed significantly increased neutrophil numbers in their lungs as compared to WT mice (*P* < 0.05; Figure 4D-F). These data were confirmed by the higher amounts of MPO, a marker for total neutrophil content, in lung homogenates of APC<sup>high</sup>-mice as compared to WT mice 48 hours after inoculation (Figure 4G). No differences in total cell counts in BALF were seen between WT and APC<sup>high</sup>-mice at any of the time points (Figure 4H). However, in APC<sup>high</sup>-mice BALF contained more neutrophils after 48 hours compared to WT mice (*P* < 0.01; Figure 4I). Together these data show a more severe proinflammatory response in the lungs of APC<sup>high</sup>-mice accompanied by increased recruitment of neutrophils.

**APC<sup>high</sup>-mice demonstrate an enhanced cytokine response after infection with *B. pseudomallei***

Since cytokines are important regulators of the host immune response during melioidosis, we measured pulmonary and plasma levels of TNF-α, IL-6, IL-10, IL-12p70, IFN-γ, MCP-1 and
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KC (Table 1). The lung and plasma levels of these mediators did not differ between mouse strains at 24 or 72 hours after infection. At 48 hours, when APChigh-mice displayed higher bacterial burdens in various body sites, these genetically modified animals showed higher concentrations of IL-6, MCP-1 and KC in lungs and higher levels of TNF-α, IL-6, IL-12p70, IFN-γ and MCP-1 in plasma when compared with WT mice.

Overexpression of APC leads to decreased coagulation activation, only at late time points

Having established that overexpression of APC plays a detrimental role during infection with B. pseudomallei, we next wondered whether this would impact activation of coagulation in the lungs and systemically during infection. Therefore TATc levels, a parameter of coagulation-induction,
Table 1. Cytokine concentrations in lung homogenates and plasma of WT and APChigh-mice during experimental melioidosis.

<table>
<thead>
<tr>
<th></th>
<th>Lung homogenates</th>
<th>Plasma</th>
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<tr>
<td></td>
<td>pg/mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>APChigh</td>
</tr>
<tr>
<td>24h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>52 (25-61)</td>
<td>28 (15-36)</td>
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<tr>
<td>IL-6</td>
<td>456 (169-557)</td>
<td>447 (167-807)</td>
</tr>
<tr>
<td>IL-10</td>
<td>47 (40-58)</td>
<td>38 (25-49)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>6.4 (5.1-10)</td>
<td>4.2 (0-8.2)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.4 (2.2-3.0)</td>
<td>1.4 (1.2-1.9)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1694 (900-1985)</td>
<td>1707 (868-2347)</td>
</tr>
<tr>
<td>KC</td>
<td>5623 (4755-7632)</td>
<td>6943 (5515-8165)</td>
</tr>
<tr>
<td>48h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>284 (136-638)</td>
<td>365 (271-549)</td>
</tr>
<tr>
<td>IL-6</td>
<td>638 (196-725)</td>
<td>1347 (926-1571)**</td>
</tr>
<tr>
<td>IL-10</td>
<td>27 (18-34)</td>
<td>24 (16-31)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>11 (8.6-17)</td>
<td>15 (8.8-15)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>9.7 (7.5-11)</td>
<td>7.7 (6.4-9.5)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2281 (1823-2539)</td>
<td>3758 (3283-4202)*</td>
</tr>
<tr>
<td>KC</td>
<td>5711 (4017-8233)</td>
<td>1899 (1454-2114)**</td>
</tr>
<tr>
<td>72h</td>
<td></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>799 (472-1637)</td>
<td>579 (414-738)</td>
</tr>
<tr>
<td>IL-6</td>
<td>4226 (3738-5804)</td>
<td>5072 (2996-5563)</td>
</tr>
<tr>
<td>IL-10</td>
<td>11 (9.1-13)</td>
<td>11 (7.1-16)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>6.9 (0-11)</td>
<td>6.4 (4.7-13)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>22 (15-34)</td>
<td>26 (20-32)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>AD</td>
<td>AD</td>
</tr>
<tr>
<td>KC</td>
<td>3409 (3087-4233)</td>
<td>3772 (2716-4298)</td>
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</table>

Pulmonary (lung homogenates and BALF) and plasma cytokine levels after intranasal infection with 300 CFU of *B. pseudomallei*. Wild type (WT) and APChigh-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. AD above detection limits, BD below detection limits, ND not determined, TNF-α tumor necrosis factor-α, IL interleukin, IFN-γ interferon-γ, MCP-1 monocyte chemotactic protein, KC keratinocyte-derived chemokine. *P < 0.05, **P < 0.01 and ***P < 0.001 for WT versus APChigh-mice (Mann-Whitney U test).
were measured in lung homogenates, BALF and plasma of WT and APC<sup>high</sup>-mice 24, 48 and 72 hours after inoculation with <i>B. pseudomallei</i>. Interestingly, at 48 hours, when marked differences in CFU counts and neutrophil influx were seen between WT and APC<sup>high</sup>-mice, no differences in TATc levels were observed in either the pulmonary or the systemic compartment (Figure 5A-C). Seventy-two hours after infection however, pulmonary TATc levels were significantly decreased in APC<sup>high</sup>-mice when compared to WT mice (P < 0.05 and P < 0.01 for lung homogenates and BALF respectively). To analyze whether overexpression of APC leads to changes in other anticoagulant factors, we measured plasma levels of antithrombin (AT), one of the most potent anticoagulants. No significant differences could be detected between WT and APC<sup>high</sup> mice at any of the time points, indicating that the observed decrease in TATc is most likely attributable to overexpression of APC (i.e. not by changes in AT levels) (Figure 5D). Finally, we performed western blots on fibrin and fibrin degradation products in lung homogenates 72 hours after infection, which revealed strongly

**Figure 5.** APC<sup>high</sup>-mice show decreased activation of coagulation during melioidosis. Mice were infected intranasally with 300 CFU of <i>B. pseudomallei</i>. After 24, 48 and 72 hours coagulation activation (TATc) was measured in lung homogenates (A), BALF (B), and citrated plasma (C). Levels of antithrombin (AT) were measured in plasma (D). Western blotting on fibrin(ogen) degradation products measured in lung homogenates 72 hours after infection demonstrated decreased fibrin(ogen) degradation products in APC<sup>high</sup>-mice compared to WT (E-G). Quantification of western blotting on fibrin(ogen) degradation products shows significantly decreased levels of fragment X (E) and D-dimer (F) in APC<sup>high</sup>-mice. Photograph of western blot on fibrin(ogen) degradation products (G). Each line represents a single mouse. Grey boxes represent WT mice, white boxes represent APC<sup>high</sup>-mice (n = 8 mice per group for each time point). *P < 0.05 and **P < 0.01, for WT versus APC<sup>high</sup>-mice (Mann-Whitney U test). C control; WT wild type.
reduced amounts of fragment X and D-dimer in APC$_{\text{high}}$ mice (fragment X: 97% vs 22%; $P < 0.05$; D-dimer: 116% vs 36%; $P < 0.01$, Figure 5E-G). Taken together, the observed impairment in host defense mechanisms during severe experimental Gram-negative sepsis as a result of enhanced APC expression concurs during decreased coagulation activation.

**DISCUSSION**

During severe pneumosepsis a shift towards increased pro-coagulant activity and inhibition of anti-coagulant activity and fibrinolysis occurs, changes that are thought to contain the infection and thereby protect the host against the detrimental effects of the infection$^{1,2}$. APC is a pleiotropic protease with anti-coagulant, pro-fibrinolytic and anti-inflammatory properties$^{3,4}$. Many clinical studies have shown reduced plasma PC-activity in patients with severe sepsis, which was associated with organ dysfunction and early death$^{5,6,23,24}$. Also in patients with *B. pseudomallei*-induced pneumosepsis low levels of PC and of its co-factor protein S were found$^{13,15}$. These data paved the way for administration of APC as an additional treatment in pneumosepsis patients and many pre-clinical and clinical studies have demonstrated its protective effects$^{7,25}$. However, due to a significant lack of benefit in sepsis patients in the recent PROWESS-SHOCK trial, rhAPC was recently withdrawn from the market$^{9,10}$. Despite this announcement, several preclinical and clinical studies provided insight into the mechanisms of action of rhAPC administration in models of endotoxemia and sepsis. These studies have shown that systemic administration of rhAPC inhibits systemic and intrabronchial coagulopathy and inflammation$^{6,7,25}$. More specifically, intravenous administration of rhAPC was able to reduce local, intrapulmonary, coagulation and inflammation in LPS-induced lung inflammation in healthy volunteers$^{26,27}$ and intrapulmonary coagulation in rodent models of bacterial pneumonia$^{28,29}$. Melioidosis is often associated with severe pneumosepsis$^{11,12}$, but data about effects of APC during this disease are limited to a single case report$^{30}$. Therefore, in this study we now tried to determine whether APC would be protective during melioidosis by comparing mice expressing high levels of hAPC with WT mice. Our data show, surprisingly, that elevated concentrations of hAPC resulted in a survival disadvantage, accompanied by significantly increased pulmonary and systemic bacterial growth. Furthermore, APC$_{\text{high}}$-mice had increased pulmonary histopathology, increased influx of neutrophils and increased release of proinflammatory cytokines in their lungs and blood. Only at late stages of infection coagulation activation was blunted in APC$_{\text{high}}$-mice. These data indicate that, in contrast to our hypothesis, overexpression of APC has a detrimental effect during severe pneumosepsis (melioidosis) caused by *B. pseudomallei*.

To answer our research question we used mice expressing a hyperactivatable form of human APC which is efficiently activated by mouse thrombin both in the absence and presence of mouse thrombomodulin$^{16}$. Before their use in the experiments, all mice were checked for transgene expression by PCR. Although one could argue that it would have been better to use mice overexpressing murine
APC instead of human APC, it is less likely that such mice would yield significantly different results since both rhAPC\(^{28,29,31}\) and recombinant murine APC (rmAPC)\(^{32,33}\) have shown (protective) effects in murine models of LPS-induced endotoxemia\(^{31}\), LPS-induced lung injury\(^{32}\), ventilator-induced lung injury\(^{33}\) and pneumococcal pneumonia\(^{28,29}\). Of note, although APC\(^{\text{high}}\)-mice respond to thrombin with increased APC production, our results cannot be extrapolated to mice with enhanced levels of the zymogen protein C.

Our results are in contrast with previous data in murine models of pneumosepsis and endotoxin-induced lung inflammation. Intravenous APC prevented LPS-induced pulmonary vascular injury by inhibiting accumulation of leukocytes\(^{34}\) and cytokine production\(^{35}\) in rats. Furthermore, inhalation of APC inhibited LPS-induced pulmonary inflammation in mice\(^{32}\). In a model of murine pneumococcal pneumonia intravenous administration of rmAPC attenuated coagulopathy and improved survival\(^{28}\) and administration of rmAPC reduced inflammation when administered early in the same model\(^{32}\). However, not all experimental models showed clear anti-inflammatory effects of APC during infection. In a model of *Pseudomonas (P.) aeruginosa* pneumonia in rats intravenous rhAPC inhibited local and systemic coagulation, but did not show any protective effects on host defense \(^{36}\). Furthermore, during *P. aeruginosa*-induced acute lung injury, intravenous rhAPC worsened oxygenation impairment, induced an increase in extracellular lung water, in lung inflammatory cell recruitment and in systemic and bronchoalveolar levels of the proinflammatory cytokines TNF-\(\alpha\) and IL-6\(^{37}\). The latter results seem to be more in line with our data, as we also demonstrated that APC induced an increased pulmonary neutrophil influx together with increased levels of pro-inflammatory cytokines.

One of the main properties of APC is its anticoagulant function via inhibition of FVa and FVIIIa\(^{3,4}\). Indeed, after 72 hours of infection decreased coagulation activation, as reflected by lower levels of TATc and fibrin degradation products, including fragment X and D-dimer, were measured in lung homogenates and bronchoalveolar lavage fluid of APC\(^{\text{high}}\)-mice. Clearly, APC-overexpression did not lead to a compensatory decrease in AT levels, indicating that the low TATc levels are most likely attributable to the anticoagulant effects of APC-overexpression. These data are in line with previous data of experimental models of pneumosepsis showing that intravenous administration of APC blunted coagulopathy\(^{28,29,36}\). Recent data from our laboratory showed that interference with fibrin deposition impacts the outcome of experimental melioidosis\(^{18,19}\). More specifically, mice deficient for plasminogen activator type 1, which are expected to have decreased fibrin depositions, showed increased bacterial growth in this model\(^{19}\), while tissue-type plasminogen activator deficient mice, which are expected to display enhanced fibrin depositions, demonstrated reduced bacterial growth and dissemination in this same model\(^{19}\). Together these data suggest that reduced coagulation in APC\(^{\text{high}}\)-mice may have contributed to impaired defense against bacterial growth and dissemination. At present, it remains to be elucidated why coagulation was not inhibited in APC\(^{\text{high}}\)-mice early after instillation of *B. pseudomallei*, although clearly the extent of coagulation activation was rela-
APC overexpression in melioidosis

tively limited before 72 hours of infection. Interestingly, recent data showed that APC also might have procoagulant properties. APC is able to shed Kunitz domain 1 from membrane-bound tissue factor pathway inhibitor (TFPI), leading to upregulation of tissue factor thereby initiating FXa-dependent coagulation. The procoagulant properties of APC might have, although speculative, counteracted the anticoagulant influences of high APC levels during the early phase of infection.

Besides its effects on coagulation, APC is also known as a protein with anti-inflammatory and cytoprotective properties. On the one hand, these effects can be mediated via binding of APC to the endothelial PC receptor, EPCR, and subsequent signalling via PAR-1. Activation of the APC-EPCR-PAR-1 axis may result in anti-inflammatory and anti-apoptotic activities, as well as stabilisation and thereby protection of the endothelial barrier. Indeed, in a model of diabetic nephropathy, overexpression of APC was protective via inhibition of endothelial and podocyte apoptosis. On the other hand, the protective effects of APC can be mediated via EPCR-independent, CD11b/CD18-PAR-1 dependent mechanisms or induced via direct binding of APC to activated \( \alpha_\beta_1 \), \( \alpha_\gamma \beta_1 \), and \( \alpha_\gamma \beta_3 \) integrins. This interaction proved to be essential for inhibition of neutrophil extravasation into the bronchoalveolar space of mice induced by rAPC. Our data show that overexpression of APC, which theoretically may have led to anti-inflammatory effects, was not protective in our model. In contrast, overexpression of APC was associated with a strong proinflammatory phenotype, as reflected by enhanced neutrophil influx, increased cytokine production and worse histopathology, particularly at 48 hours after infection. These data contrast with previous findings from our laboratory showing reduced proinflammatory cytokine levels in mice treated with APC shortly after infection with \( S. pneumoniae \) via the airways. As such, we expected APC\(^{high}\)-mice to display an attenuated early inflammatory response in the lung after infection with \( B. pseudomallei \). In light of the importance for a swift induction of an adequate innate immune response for protective immunity, this could have explained the observed enhanced bacterial growth and dissemination at 48 hours. However, with the readouts used here anti-inflammatory effects could not be detected in APC\(^{high}\)-mice, leaving the mechanism by which sustained elevation of APC levels disturbs host defense mechanisms open for speculation. Nonetheless, our results clearly demonstrate that high APC levels do not prevent excessive inflammation or death. The increased inflammatory response in APC\(^{high}\)-mice likely was the consequence of the higher bacterial loads, providing a stronger proinflammatory stimulus which could not be controlled by high APC concentrations. This likely also applies to the increased neutrophil numbers in lungs at 48 hours after infection. Although neutrophils are critical players in the host defense against \( B. pseudomallei \), previous studies have shown an association between overwhelming neutrophil recruitment and increased mortality during experimental melioidosis. These observations can be explained by the fact that neutrophil activation might acts as a 'double edged sword', on the one hand aiding in the anti-bacterial response by attacking bacteria and facilitating their killing, while on the other hand causing collateral tissue damage.
In this study we used mice overexpressing hAPC, which represents a different approach to study the effect of APC than adopted in previous studies using exogenous administration of APC via the intravenous or intraperitoneal route or via inhalation. The advantage of our model is that high levels of APC were present during the whole experiment, whereas in models of repeated exogenous administration levels of APC vary over time due to the short half-life of APC in the circulation. By measuring hAPC levels in various organs, we showed that APC is overexpressed in APCh^hp^-mice throughout the body, which is in accordance with previous studies in which these mice were used\(^\text{16}\). Moreover, the APC-levels found in our study almost paralleled those measured in the PROWESS trial (45 ng/mL), in which patients were treated with continuous intravenous administration of rhAPC\(^\text{21}\). Of note, as to mimic the human situation as much as possible, we considered continuous administration of APC in mice via the intravenous route. However, this is hard to achieve in freely moving mice. On the other hand, a limitation of our study is that it might be a challenge to extrapolate our results obtained in genetically modified mice to the human situation. Moreover, one could argue that the observed results are only applicable to this particular model. The recent failure of the PROWESS-SHOCK trial, in which humans with septic shock received rhAPC\(^\text{8}\), might be related to the fact that in this study a very heterogeneous sepsis patient population was studied with a relatively low overall mortality. The current study is the first to report on the role of overexpression of endogenous APC in melioidosis. Our data clearly show that sustained elevated levels of APC impair host defense during severe experimental Gram-negative sepsis caused by \(B.\ pseudo\text{mallei}\) (melioidosis), as reflected by increased bacterial growth and dissemination, increased pulmonary neutrophil influx, increased cytokine production and early death.

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REFERENCES

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Sample harvesting, processing and determination of bacterial growth.
Twenty-four, 48 or 72 hours after infection mice ($n = 8$ per group at each time point) 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, liver and spleen were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Bilateral bronchoalveolar lavage fluid (BALF) was obtained by exposing the trachea through a midline incision followed by cannulation with a sterile 22-gauge Abbocath-T catheter (Abott, Sligo, Ireland) and instilling and retrieving of two 0.4 mL aliquots of sterile phosphate buffered saline (PBS). CFUs were determined from serial dilutions of organ homogenates and blood that were plated on blood agar plates and incubated at 37°C in 5% CO$_2$ for 20h before colonies were counted.

Assays
Human (h)APC was measured in uninfected APChigh-mice in plasma, lung-, kidney-, liver-, and spleen homogenates containing citrate (0.105M final concentration) and benzamidin (20mM final concentration; Sigma Aldrich, St Louis, MO) using a chromogenic assay as described$^1$. In lung homogenates of infected mice, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ, monocyte chemotactic protein-1 (MCP-1) 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. Myeloperoxidase concentrations (MPO; HyCult Biotechnology, Uden, The Netherlands), keratinocyte-derived chemokine (KC; R&D Systems, Minneapolis, MN) and thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany) were measured with commercially available ELISA kits. For TATc, different concentrations of coagulated mouse plasma were used to generate a standard curve. Protein levels in BALF were measured using a Bradford-based protein assay (Bio-Rad Laboratories, Hercules, CA). Antithrombin levels were determined using the Berichrom Antithrombin kit (Siemens Healthcare Diagnostics) 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 coded and scored by a pathologist blinded for experimental groups 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 32.

**REFERENCES**