Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Endogenous alpha-2-antiplasmin is protective during severe Gram-negative sepsis (melioidosis)

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Chapter 14

ABSTRACT

Rationale
Alpha-2-antiplasmin (A2AP) is a major inhibitor of fibrinolysis by virtue of its capacity to inhibit plasmin. Although the fibrinolytic system is strongly affected by infection, the functional role of A2AP in the host response to sepsis is unknown.

Objectives
To study the role of A2AP in melioidosis, a common form of community-acquired sepsis in Southeast-Asia and Northern-Australia caused by the Gram-negative bacterium Burkholderia (B.) pseudomallei.

Methods
In a single-center observational study A2AP was measured in patients with culture-proven septic melioidosis. WT and A2AP-deficient (A2AP−/−) mice were intranasally infected with B. pseudomallei to induce severe pneumosepsis (melioidosis). Parameters of inflammation and coagulation were measured and survival studies were performed.

Measurements and Main Results
Melioidosis patients showed elevated A2AP plasma levels. Likewise, A2AP-levels in plasma and lung homogenates were elevated in mice infected with B. pseudomallei. A2AP-deficient (A2AP−/−) mice had a strongly disturbed host response during experimental melioidosis as reflected by enhanced bacterial growth at the primary site of infection accompanied by increased dissemination to distant organs. In addition, A2AP−/− mice showed more severe lung pathology and injury together with an increased accumulation of neutrophils and higher cytokine levels in lung tissue. A2AP-deficiency further was associated with exaggerated systemic inflammation and coagulation, increased distant organ injury and enhanced lethality.

Conclusions
This study is the first to identify A2AP as a protective mediator during Gram-negative (pneumo) sepsis by limiting bacterial growth, inflammation, tissue injury and coagulation.
INTRODUCTION

During severe infection and sepsis a range of host defense mechanisms becomes activated, resulting in a strong pro-inflammatory response together with hemostatic alterations. These changes, characterized by activation of coagulation and inhibition of fibrinolysis, can be regarded as host-protective in containing the causative microorganisms and the associated inflammation to the site of the infection, rendering this relationship physiologically effective. Too much procoagulant activity, however, can also be disadvantageous due to excess fibrin formation, which may block capillary flow and consequently induce organ failure, and by influencing inflammatory activity, leading to disproportionate inflammation in for example the alveolar compartment during pneumonia. Increased inflammation and the accompanying disseminated intravascular coagulation are important causes of multi-organ failure and mortality during sepsis.

The serpin alpha-2-antiplasmin (A2AP) is considered one of the main inhibitors of fibrinolysis. The regulatory role of A2AP in fibrinolysis consists of formation of irreversible complexes with plasmin (PAP-complexes (PAPc)), formation of reversible complexes with plasminogen that inhibit adsorption of plasminogen to fibrin, and making fibrin more resistant to local plasmin activity through cross-linking via factor XIIIa (FXIIIa). Besides its role in fibrinolysis, no other clear functions for A2AP are known. Plasma A2AP levels were found elevated in patients with acute stroke, myocardial infarction, unstable angina and atrial fibrillation, suggesting a self-defense system against complications of ischemic events. Patients with severe sepsis showed unaltered, elevated or decreased plasma A2AP concentrations when compared with healthy controls. Several studies have consistently documented elevated circulating PAPc levels in sepsis patients, suggestive for a functional role of A2AP in inhibiting fibrinolysis during severe infection. Notably, the plasma levels of plasminogen activator inhibitor type I (PAI-1), the other main inhibitor of fibrinolysis, were elevated in patients with severe pneumonia and sepsis, which in experimental pneumosepsis models in mice contributed to protective immunity by mechanisms that at least in part were unrelated to its inhibitory effects on fibrinolysis. In contrast, to the best of our knowledge the functional role of A2AP in sepsis has not been studied previously.

In the present study we sought to identify the role of A2AP during melioidosis, a severe septic disease caused by the Gram-negative bacillus Burkholderia (B.) pseudomallei. Melioidosis is characterized by pneumonia and rapid bacterial dissemination to distant body sites, with many possible disease manifestations, septic shock being the most severe. Melioidosis is an important cause of community-acquired sepsis in Southeast-Asia and Northern-Australia with mortalities up to 40% despite appropriate antibiotic therapy. Additionally, B. pseudomallei is recently classified as a ‘Tier 1’ disease agent considered to be an exceptional threat to security. Recently, we revealed that during melioidosis fibrinolysis might hamper the antibacterial host response: tissue-type plasminogen activator (tPA)-deficiency was associated with host protective effects, while PAI-1-deficiency led to a detrimental phenotype. We here identify A2AP as a possible protective mediator during Gram-negative (pneumo)sepsis caused by B. pseudomallei.
MATERIALS AND METHODS

Patients
Thirty-four patients (mean age 52 y; range 18-86 y) with sepsis caused by *B. pseudomallei* and 32 healthy controls (mean age 41 y; range 21-59 y) from the same area were studied. All subjects were recruited prospectively at Sapprasithiprasong Hospital, Ubon Ratchathani, Thailand. Sepsis due to melioidosis was defined as culture-positivity for *B. pseudomallei* from any clinical sample plus a systemic inflammatory response syndrome\(^2\), \(^29\). Study design and subjects have been described in detail\(^29\). The study was approved by the Ministry of Public Health, Royal Government of Thailand and the Oxford Tropical Research Ethics Committee, University of Oxford, Oxford, UK. Written informed consent was obtained from all subjects before the study.

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. A2AP\(^{-/-}\)-mice\(^3\) were a kind gift from Dr. H. R. Lijnen (Center for Molecular and Vascular Biology, KU Leuven, Belgium) and were backcrossed for at least 8 times on a C57BL/6 genetic background. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center, Amsterdam, the Netherlands.

Experimental infection and determination of bacterial growth
Experimental melioidosis was induced by intranasal inoculation with *B. pseudomallei* strain 1026b (300 colony forming units (CFU)/ 50 µL 0.9%NaCl) as previously described\(^22\), \(^28\), \(^29\). For survival experiments mice were checked every 6 hours until death occurred. Sample harvesting, determination of bacterial growth and assays including western blot were described in the online data supplement.

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

Elevated plasma A2AP concentrations in patients with severe melioidosis.
To obtain insight into A2AP expression during melioidosis, we measured A2AP protein levels in plasma from 34 septic patients with culture proven B. pseudomallei infection and 32 local healthy controls. Fourteen (41%) patients with melioidosis died in-hospital. A2AP was elevated in melioidosis patients with median plasma concentrations approximately 1.4-fold higher than in healthy subjects (Figure 1A; median 81% in controls versus 113% in patients, \( P < 0.0001 \)). No differences in A2AP were found between survivors and non-survivors (data not shown).

A2AP is upregulated during murine melioidosis.
To study the role of A2AP during melioidosis at tissue level, mice were intranasally inoculated with live B. pseudomallei to induce pneumonia-derived melioidosis and sacrificed after 24, 48 and 72 hours. Infection with B. pseudomallei was associated with a significant increase in plasma A2AP levels at 24, 48, and 72 hours after inoculation (\( P < 0.05 \) versus baseline 24 and 48 hours after inoculation, \( P < 0.01 \) 72 hours after inoculation; Figure 1B) and a significant increase in lung A2AP levels 24 and 48 hours after inoculation (\( P < 0.05 \) at 24 hours and \( P < 0.001 \) at 48 hours after inoculation; Figure 1C).

A2AP-deficiency is associated with increased pulmonary bacterial loads and enhanced bacterial dissemination during experimental melioidosis.
To investigate whether A2AP-deficiency impacts on pulmonary bacterial growth, we determined bacterial loads in lung homogenates. Whereas during the early stage of infection pulmonary bacterial counts were similar in A2AP\(^{-/-}\)- and WT mice, 48 and 72 hours after inoculation A2AP\(^{-/-}\)-mice demonstrated markedly elevated bacterial loads in their lungs (\( P < 0.01 \) after 48 hours, \( P < 0.001 \).

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Figure 1. A2AP concentrations during clinical and experimental melioidosis. Plasma A2AP levels in patients with culture proven melioidosis (A). Increased levels of plasma A2AP in septic patients (\( n = 34 \), open squares) compared to healthy controls (\( n = 32 \), black squares). Data are expressed as scatter dot plots showing all observations and the median. **\( P < 0.0001 \) for patients versus controls (Mann-Whitney U test). Endogenous A2AP levels during experimental melioidosis. Wild type (WT) mice were sacrificed before (uninfected), 24, 48 and 72 hours after intranasal inoculation with B. pseudomallei. A2AP protein concentrations were measured in plasma (B) and lung homogenates (C), \( n = 8 \) 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. *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \) versus uninfected (Mann-Whitney U test).
after 72 hours; Figure 2A). In order to determine whether A2AP-deficiency impacts on bacterial dissemination, we measured bacterial loads in liver and spleen homogenates and blood in A2AP⁻/⁻-mice and compared them to WT mice (Figure 2B-D). While after 24 hours bacterial loads were similar at distant body sites of A2AP⁻/⁻- and WT mice, after 48 and 72 hours an increased number of A2AP⁻/⁻-mice had become bacteremic when compared to WT mice: after 48 hours 100% of A2AP⁻/⁻-mice versus 50% of WT mice had positive blood cultures, while after 72 hours 88% of A2AP⁻/⁻-mice versus 50% of WT mice had positive blood cultures. When compared to WT mice, bacterial loads in blood were higher as well of the A2AP⁻/⁻-mice (P < 0.05 and P < 0.01 after 48 and 72 hours respectively; Figure 2B). Bacterial loads in liver and spleen homogenates were reflective of those in blood: A2AP⁻/⁻-mice displayed an increased bacterial dissemination to liver and spleen when compared to WT mice (P < 0.05 and P < 0.001 for bacterial loads in both liver and spleen homogenates after 48 and 72 hours respectively; Figure 2C-D). Thus, A2AP-deficiency facilitates growth (at the primary site of infection) and dissemination of *B. pseudomallei*, in particular at later time points.

Figure 2. A2AP-deficiency is associated with increased pulmonary bacterial growth and dissemination. Mice were inoculated with 300 CFU of *B. pseudomallei* and sacrificed after 24, 48 or 72 hours. Colony forming units (CFU) were determined in lung homogenates (A), blood (B), liver (C) and spleen (D) homogenates. 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 A2AP⁻/⁻-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 A2AP⁻/⁻-mice (Mann-Whitney U test). BC+ number of positive blood cultures.
A2AP-deficiency is associated with increased lung inflammation.

Both WT and A2AP−/− 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 3A-C). At all time points, the extent of lung inflammation was significantly greater in A2AP−/− mice when compared to controls (*P* < 0.01 for 24 and 72 hours after infection; *P* < 0.05 48 hours after infection; Figure 3A). Additionally, 24 and 48 hours after infection A2AP−/− mice displayed significantly higher granulocyte counts in their lungs when compared to WT mice (*P* < 0.05 at 24 hours post-infection; *P* < 0.001 at 48 hours post-infection; Figure 3D-F). Enhanced neutrophil influx into lung tissue of A2AP−/− mice was confirmed by elevated lung MPO levels in A2AP−/− mice (Figure 3G). In accordance with enhanced pulmonary inflammation, A2AP−/− mice displayed strongly elevated concentrations of cytokines (TNF-α, IL-6, IL-10, IFN-γ) and the CXC chemokine KC in whole lung homogenates (Table 1), especially at later time points after infection. As in vitro studies have shown that A2AP induces TGF-β1

![Figure 3. A2AP-deficiency exaggerates lung pathology and neutrophil accumulation after infection with *B. pseudomallei*.](image)

A2AP−/− mice infected with *B. pseudomallei* showed increased histopathology scores at 24, 48 and 72h characterized by interstitial inflammation together with necrosis, endothelialitis, bronchitis, edema, thrombi and pleuritis (A). Representative photographs of lungs at 72 hours post-inoculation from WT (B) and A2AP−/− mice (C) (H&E staining x100). Increased granulocyte influx, as reflected by the intensity of the Ly-6G immunostaining of histopathological slides, after 24 and 48 hours in A2AP−/− mice infected with *B. pseudomallei* (D). The intensity of the Ly-6G staining is expressed as the percentage of the total lung surface area. Representative photographs of Ly-6G immunostaining (original magnification x100) for granulocytes of WT (E) and A2AP−/− (F) mice showing significantly increased neutrophil influx 48 hours post-inoculation. Increased levels of pulmonary MPO (G), reflecting the total pulmonary neutrophil content, were seen 48 and 72 hours after infection in the lungs of A2AP−/− mice compared to WT mice. 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 A2AP−/− mice (*n* = 8 mice per group for each time point). *P* < 0.05, **P* < 0.01 and ***P* < 0.001 for WT versus A2AP−/− mice (Mann-Whitney U test).
Table 1. Cytokine concentrations in lung homogenates and plasma of WT and A2AP<sup>−/−</sup> mice during melioidosis.

<table>
<thead>
<tr>
<th></th>
<th>Lung homogenates&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Plasma&lt;sup&gt;+&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>pg/ml</td>
<td>pg/ml</td>
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<tr>
<td></td>
<td>WT</td>
<td>A2AP&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td><strong>24h</strong></td>
<td></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>1183 (680-1563)</td>
<td>1083 (1182-2174)</td>
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<tr>
<td>IFN-γ</td>
<td>3.6 (1.8-6.6)</td>
<td>0.2 (0.9-14)*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1227 (783-2728)</td>
<td>3781 (1727-6372)*</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.8 (1.8-3.4)</td>
<td>2.8 (2.0-3.7)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>3.1 (2.1-6.00)</td>
<td>7.0 (5.9-11.4)</td>
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<tr>
<td>KC [ng/mL]</td>
<td>18 (12-23)</td>
<td>18 (11-22)</td>
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<tr>
<td>aTGF-β&lt;sub&gt;1&lt;/sub&gt; [ng/mL]</td>
<td>2.5 (1.7-3.3)</td>
<td>1.6 (1.5-2.0)*</td>
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<tr>
<td><strong>48h</strong></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>3494 (2000-41104)</td>
<td>6884 (5150-6424)**</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>24 (12-33)</td>
<td>22 (14-45)</td>
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<tr>
<td>IL-6</td>
<td>628 (274-628)</td>
<td>1552 (629-2030)**</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.2 (1.7-3.4)</td>
<td>8.7 (6.7-12.2)*</td>
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<tr>
<td>IL-12p70</td>
<td>36 (17-30)</td>
<td>27 (18-50)</td>
</tr>
<tr>
<td>KC [ng/mL]</td>
<td>5.8 (3.6-7.0)</td>
<td>11 (7.8-11)*</td>
</tr>
<tr>
<td>aTGF-β&lt;sub&gt;1&lt;/sub&gt; [ng/mL]</td>
<td>1.4 (1.4-3.6)</td>
<td>1.8 (1.2-1.9)</td>
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<tr>
<td><strong>72h</strong></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>2336 (1374-4810)</td>
<td>6271 (9881-12006)**</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.3 (5.9-11)</td>
<td>21 (14-23)**</td>
</tr>
<tr>
<td>IL-6</td>
<td>175 (93-651)</td>
<td>5850 (3356-8457)**</td>
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<tr>
<td>IL-10</td>
<td>11 (3-21)</td>
<td>225 (109-281)**</td>
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<tr>
<td>IL-12p70</td>
<td>8 (7-12)</td>
<td>11 (9-15)</td>
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<tr>
<td>KC [ng/mL]</td>
<td>5.5 (3.4-9.7)</td>
<td>27 (22-48)**</td>
</tr>
<tr>
<td>aTGF-β&lt;sub&gt;1&lt;/sub&gt; [ng/mL]</td>
<td>2.1 (1.8-2.3)</td>
<td>1.0 (0.5-1.3)**</td>
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<sup>+</sup>Data are expressed as median (interquartile range) of n = 8 mice per group per time point. A2AP<sup>−/−</sup> alpha-2-antiplasmin knockout, IFN-γ interferon-γ, IL interleukin, KC keratinocyte-derived chemokine, ND not determined, aTGF-β, active transforming growth factor-β, TNF-α tumor necrosis factor-α, WT wild-type. *P < 0.05, **P < 0.01 and ***P < 0.001 for WT versus A2AP<sup>−/−</sup> mice (Mann-Whitney U test). < indicates data below detection limits.
we wondered how A2AP-deficiency would impact on αTGF-β1 levels during infection with *B. pseudomallei*. Therefore, we determined αTGF-β1 levels in lung homogenates 24, 48 and 72 hours after infection. Clearly, after 24 and 72 hours of infection significantly lower αTGF-β1 levels were measured in A2AP−/− mice compared to WT (*P* < 0.05 and < 0.01 respectively; Table 1). Since the most prominent differences between mouse strains were seen after 72 hours of infection, we performed a bronchoalveolar lavage (BAL) at this time point in an independent experiment, in order to determine whether the proinflammatory and lung injurious effects could also be detected in the alveolar compartment. Indeed, our data showed a similar increase in bacterial loads in BALF of A2AP−/−-mice when compared to WT mice (*P* < 0.001; Figure 4A), which was accompanied by elevated protein levels, reflecting protein leakage across the bronchoalveolar barrier (*P* < 0.001; Figure 4B) and higher levels of LDH, a general parameter for cell damage (*P* < 0.001; Figure 4C). In accordance, BALF cytokine and KC levels were higher in A2AP−/−-mice compared to WT mice (Supplemental Table 1).

![Box plots showing bacterial loads and protein levels](image1)

**Figure 4.** A2AP-deficiency induces increased bacterial growth in the intra-alveolar compartment accompanied by lung injury. Bacterial loads in bronchoalveolar lavage fluid (BALF) (A) and levels of total protein (B) and LDH (C) and KC (D) were increased in BALF 72 hours after intranasal inoculation with 300 CFU of *B. pseudomallei* in A2AP−/− mice compared to WT mice. 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 A2AP−/− mice (*n* = 8 mice per group for each time point). ***P* < 0.001 for WT versus A2AP−/− mice (Mann-Whitney *U* test).
Figure 5. A2AP-deficiency does not influence bacterial killing or cytokine release from macrophages. Bacterial killing by bone marrow-derived macrophages (BMDM). No differences between WT (grey dots; n = 4) and A2AP−/− mice (white dots; n = 4) in bacterial killing 1 and 6 hours after infection (A). Data are expressed as medians with interquartile ranges. TNF-α release from alveolar macrophages (B) and BMDM (C) after stimulation with heat-killed *B. pseudomallei* (1*10^7/mL) while exogenous A2AP (70 μg/mL) or RPMI1640 was added. After 20h of stimulation, no differences in TNF-α release were observed between macrophages of WT mice (grey boxes), WT mice to which exogenous A2AP was added (grey striped boxes) and A2AP−/− mice (white boxes). Data are expressed as box and whisker plots with medians, n = 8 mice per strain.

Figure 6. A2AP-deficiency is associated with increased systemic inflammation, (hepato)cellular injury and lethality during melioidosis. Mice were intranasally inoculated with 300 CFU of *B. pseudomallei* and sacrificed after 24, 48 or 72 hours. When compared to WT mice, A2AP−/− mice showed enhanced distant organ injury as reflected by increased levels of aspartate aminotransferase (ASAT; C), alanine aminotransferase (ALAT; D). No significant differences in plasma creatinine, a parameter for renal insufficiency, were seen in A2AP−/− mice when compared to WT mice (B). 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 A2AP−/− mice (n = 8 mice per group for each time point). **P < 0.01 and ***P < 0.001 for the difference between WT and A2AP−/− mice (Mann-Whitney U test). A2AP−/− mice showed an accelerated and increased mortality during experimental melioidosis (E). Mortality was assessed every 6 hours, n = 15-16 mice per group. Comparison between groups was done by Kaplan-Meier analysis followed by log rank tests.
**A2AP-deficiency does not influence bacterial killing or cytokine release from macrophages.**

Despite their robust inflammatory response (Table 1 and Figures 3 and 4) A2AP/− mice exhibited increased bacteremia. Therefore, we performed a killing assay to find out whether defects in bacterial killing by infected macrophages would be responsible for these observations. Our results show no differences in bacterial killing by A2AP/− and WT macrophages (Figure 5A). In accordance, recombinant A2AP did not have a direct growth inhibitory effect on *B. pseudomallei* in vitro (data not shown). Since cytokine data suggest that deficiency or the presence of A2AP might have an effect on inflammatory mediator generation by lung immune cells, we stimulated A2AP/− and WT lung and bone marrow-derived macrophages *ex vivo* with heat-killed *B. pseudomallei* and in addition determined the impact of recombinant A2AP (Figure 5B-C). Our data show that neither deficiency nor addition of A2AP influenced TNF-α release by macrophages.

**A2AP-deficiency results in increased systemic inflammation and is associated with a worse survival.**

As A2AP/−-mice showed increased bacterial dissemination to blood, liver and spleen, we wondered whether this would result in increased systemic inflammation and distant organ injury. Indeed, plasma levels of cytokines were much higher in A2AP/− than in WT mice (Table 1). In addition, the plasma levels of LDH, a marker for general cellular injury, were elevated in A2AP/−-mice relative to WT mice at 72 hours after infection with *B. pseudomallei* (*P* < 0.001; Figure 6A). At this stage of severe sepsis, A2AP/−-mice also showed increased plasma levels of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), markers of hepatocellular injury (*P* < 0.001 for ASAT, *P* < 0.01 for ALAT; Figure 6C and D respectively). No differences in creatinine (Figure 6B) or urea (data not shown), markers for renal failure, were seen between A2AP/− and WT mice at any of the time points. Taken together, A2AP seems to play a protective role in the development of distant organ injury during experimental melioidosis. Finally, as A2AP/−-mice showed a markedly increased pulmonary and distant bacterial growth and tissue damage, we investigated whether this would influence mortality during murine melioidosis. Clearly, A2AP/−-mice demonstrated an accelerated and enhanced mortality when compared to WT mice (*P* < 0.01) indicating that endogenous A2AP is protective during experimental melioidosis (Figure 6E).

**A2AP-deficiency leads to activation of coagulation and fibrinolysis during infection with *B. pseudomallei*.**

TATc, a parameter of coagulation-induction, was measured in plasma, lung homogenates and BALF of WT and A2AP/−-mice 24, 48 and 72 hours after inoculation of *B. pseudomallei*. At the later time points, A2AP/−-mice demonstrated increased plasma TATc when compared to WT mice (*P* < 0.01 for both 48 and 72 hours after inoculation; Figure 7A). No differences were seen in TATc concentrations in lung homogenates (Figure 7B), however, in BALF 72 hours after infection TATc was markedly increased (*P* < 0.001; Figure 7C). To obtain insight in activation of fibrin degradation during infection with *B. pseudomallei*, fibrin degradation products (FDP), including D-dimer and
Figure 7. A2AP-deficiency results in enhanced coagulation and fibrinolysis during melioidosis. Mice were infected intranasally with 300 CFU B. pseudomallei to induce melioidosis. After 48 and 72 hours coagulation activation as reflected by levels of TATc were significantly increased in A2AP−/− mice compared to WT mice in plasma (A) and BALF (C), but not in lung homogenates (B). Western blotting on fibrin(ogen) degradation products including fragment X (240-260 kD) and D-dimer (200 kD) in lung homogenates and BALF 24, 48 and 72 hours after infection (D-G). When compared to WT mice, increased fibrin(ogen) degradation products were seen in A2AP−/− mice in lung homogenates 48 (E) and 72 hours (F) after infection and in BALF 72 hours after infection (G). Semi-quantitative analysis of the blots showed that after 48 and 72 hours D-dimer levels were 2.7 and 2.8-fold increased respectively in lung homogenates and 7.5-fold increased in BALF (H). Levels of fragment X in lung homogenates showed 2.8 and 3.8-fold increases after 48 and 72 hours respectively and a 7-fold increase in BALF after 72 hours (I). C positive control sample (fully clotted mouse plasma, uninfected, dilution 1:50). Data are expressed as box and whisker plots (A-C) showing the smallest observation, lower quartile, median, upper quartile and largest observation and as bars (H-I), showing medians with interquartile ranges. Grey boxes and bars represent WT mice, white boxes and bars represent A2AP−/− mice (n = 8-9 mice per group for each time point). In panels D-G every lane represents one mouse sample (n = 6-7 mice per group for each time point). **P < 0.01 and ***P < 0.001 for the difference between WT and A2AP−/− mice (Mann-Whitney U test).
fragment X levels were measured by western blotting. After 24 hours, no differences in FDP were seen between WT and A2AP-/--mice (Figure 7D). However, after 48 and 72 hours FDP were markedly increased in A2AP-/--mice both in lung homogenates and in BALF (Figure 7E-G). At these time points, D-dimer levels in lung homogenates of A2AP-/--mice demonstrated a 2.6 and 2.7-fold increase respectively when compared to WT mice (Figure 7H; both \( P < 0.01 \)), while fragment X levels demonstrated a 2.8 and 3.8-fold increase respectively (Figure 7I; both \( P < 0.01 \)). In BALF, 72 hours after infection D-dimer and fragment X levels showed a 7.5 and 7-fold increase in A2AP-/--mice respectively (Figure 7H-I; both \( P < 0.01 \)).

**DISCUSSION**

Although several studies have described altered circulating A2AP levels in patients with severe infection\(^9\)-\(^12\), the functional role of this major fibrinolysis inhibitor in the host response to sepsis has not been investigated before. Our data clearly show that endogenous A2AP is protective during experimental Gram-negative (pneumo)sepsis caused by *B. pseudomallei*.

A2AP is considered the principal inhibitor of plasmin and thereby one of the major inhibitors of fibrinolysis\(^5\)-\(^7\). Patients with severe pneumonia, sepsis or acute respiratory distress syndrome were reported to have increased plasma concentrations of A2AP\(^9\),\(^15\), which is in line with our data. Notably, however, some investigations failed to show higher A2AP levels in sepsis patients\(^10\)-\(^12\), in spite of the fact that severe infection, including melioidosis, invariably is associated with elevated plasma levels of PAPc, in which A2AP is irreversibly bound to plasmin\(^11\),\(^12\),\(^32\),\(^33\). Infection of WT mice with *B. pseudomallei* resulted in an increase in both plasma and lung A2AP levels, comparable to the human disease. While these observational data on plasma A2AP concentrations in patients and mice with Gram-negative sepsis provide a further rationale to study the involvement of A2AP in severe infection, they do not offer insight into a possible functional role, either protective or detrimental, in the host response. For this we used A2AP-/--mice. These animals have a normal phenotype, with no effect on fertility, growth, development or post-traumatic bleeding; as expected, they do exhibit enhanced endogenous fibrinolytic activity\(^30\).

Ample evidence has demonstrated an altered hemostatic balance during severe (pneumo)sepsis both in the lungs and the circulation with increased procoagulant activity and inhibition of fibrinolysis\(^1\)-\(^34\). It is considered that a disturbed fibrin turnover may promote extravascular alveolar fibrin deposition in the lung and contribute to disseminated intravascular coagulation and subsequently to respiratory distress and multiorgan failure. Also in melioidosis patients concurrent activation and inhibition of fibrinolysis have been described, indicated by elevated plasma levels of tPA and PAI-1 respectively, with a net state of decreased fibrinolysis, as reflected by an increased ratio between coagulation and fibrinolysis (TATc/PAPc ratio)\(^33\). In these patients, increased TATc/ PAPc ratios were also associated with a higher mortality\(^33\), suggesting that fibrinolysis plays a role.
in the host defense against *B. pseudomallei*. In addition to these clinical data, the protective effect of fibrin during melioidosis was supported by recently published data from our laboratory using the experimental model of infection with *B. pseudomallei* in mice. PAI-1−/−-mice, which are expected to have decreased fibrin depositions, showed a pro-fibrinolytic state with increased bacterial growth and inflammation, and an increased lethality, while tPA−/−-mice, which are expected to have increased fibrin depositions, demonstrated lower FDP levels together with reduced bacterial growth and dissemination, less inflammation and a lower mortality. Our present data are fully in line with these observations. We here demonstrate that during experimental melioidosis A2AP-deficiency is associated with a pro-fibrinolytic state, as reflected by increased levels of FDP, including D-dimer and fragment X. This pro-fibrinolytic state, which was most prominent after 48 and 72 hours of infection, was associated with increased release of pro-inflammatory cytokines, increased amounts of neutrophils in the lungs and increased organ failure. Clearly, all these results point to a protective role for fibrin in the host response against *B. pseudomallei*. Fibrin may serve to ‘wall off’ the primary infection and to prevent bacterial dissemination which in turn may slow down progression of inflammation. In the absence of inhibitors of fibrinolysis, such as in A2AP−/− or PAI-1−/−-mice, bacteria are less limited in multiplication and dissemination, which consequently may aggravate the course of infection in terms of inflammation and injury. Interestingly, A2AP-deficiency also resulted in enhanced activation of coagulation, as reflected by increased levels of plasma and lung TATc. Taking into account that A2AP does not influence thrombin generation, these observations are most likely explained by the strongly increased bacterial loads in A2AP−/−-mice, resulting in a more potent stimulus for the coagulation system. At later stages of infection progressive clotting might have further contributed to multi-organ failure and increased mortality, as has been described in sepsis patients.

Previous experiments with A2AP−/−-mice focussed on the role of A2AP in production of TGF-β and fibrosis and systemic sclerosis. TGF-β, a pleiotropic cytokine, is regarded as a key regulator of the immune response, but also as the major inducer of fibrosis. In a model of bleomycin-induced dermal fibrosis in mice, A2AP-deficiency attenuated TGF-β1 production, hampered the induction of myofibroblast differentiation and diminished fibrosis. Moreover, it was found that A2AP specifically induced the production of TGF-β1 in fibroblasts. Our data showing decreased levels of TGF-β1 in lung homogenates of A2AP−/−-mice are in line with these observations. Interestingly, a recent study from our laboratory showed that mice infected with *B. pseudomallei* and treated with anti-TGF-β antibodies displayed a subtle, but protective phenotype, with diminished bacterial loads in lungs and spleen and less distant organ failure; anti-TGF-β treatment did not impact on mortality. Thus, the effect of A2AP on TGF-β levels is unlikely to contribute to its protective effect in experimental melioidosis.

Once the immune system is activated during melioidosis neutrophils are recruited to the site of infection. Neutrophils play an important role in the host defense against *B. pseudomallei* as they are involved in early bacterial containment. On the other hand, exaggerated neutrophil recruit-
ment and subsequent release of proinflammatory mediators may be detrimental, illustrating the ‘double-edged sword’ character of innate immunity. Our data show that the early influx of neutrophils into lungs of A2AP+/−-mice was similar to that in WT mice, arguing against a direct involvement of A2AP herein. At later time points, A2AP+/−-mice displayed higher neutrophil numbers, most likely as a consequence of the higher bacterial loads. In support of this notion, A2AP+/− macrophages showed unaltered TNF-α release upon exposure to *Burkholderia in vitro* and recombinant A2AP did not influence macrophage responsiveness, indicating that A2AP does not directly impact on inflammatory responses elicited by *B. pseudomallei*. This aggravated neutrophil influx, together with the release of neutrophil-derived proteases and proinflammatory cytokines, probably at least in part was responsible for the enhanced lung injury in A2AP+/−-mice, as reflected by lung histopathology scores and elevated protein and LDH concentrations in BALF. A2AP−/− mice in addition showed increased distant organ damage, including hepatocellular injury, reflected by elevated plasma transaminase concentrations and cellular injury in general, indicated by elevated plasma LDH levels. The exaggerated pro-inflammatory and injurious response in A2AP−/−-mice most likely explains their accelerated mortality.

It remains to be determined whether the observed effects of A2AP-deficiency during infection with *B. pseudomallei* also apply to infections with other Gram-negative bacteria. Published studies from our group indicate that PAI-1 (like A2AP an inhibitor of fibrinolysis) has similar protective roles in pneumonia derived sepsis caused by either *B. pseudomallei* or *Klebsiella pneumoniae*. Although these previous data cannot be directly extrapolated to A2AP−/−-mice, they suggest that the current results may also be applicable to other Gram-negative pathogens. Clearly independent experiments are required to establish this.

Caution is needed when extrapolating data from mouse experiments to human disease. Murine models like the one used here make use of a homogenous group of experimental animals with identical genotype, sex and (relatively young) age exposed to a well-controlled bacterial challenge, while patients form a heterogeneous group in which multiple factors modify disease outcome including extent of pathogen exposure, older age, co-morbidities, co-medication and genetic composition. Taking these precautions into mind, the present study is the first to describe the possible protective role of A2AP in a clinically relevant model of severe Gram-negative sepsis.

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REFERENCES

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Experimental infection and determination of bacterial growth
Sample harvesting, processing and determination of bacterial growth were done as described\(^1-3\). Briefly, 24, 48 and 72 hours after 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, 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 canulation with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland), instilling and retrieving of two 0.5 mL aliquots of sterile phosphate buffered saline (PBS). CFU were determined from serial dilutions of organ homogenates, blood and BALF that were plated on blood agar plates and incubated at 37°C 5% CO\(_2\) for 20 h before colonies were counted.

Assays
Human and mouse A2AP were measured using an automated blood coagulation analyzer (BCS® XP, Siemens Health Care Diagnostics, Marburg, Germany) and expressed as percentages of the level in normal pooled human plasma (pool of more than 200 healthy volunteers). For cytokines and A2AP measurements, lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 1% Triton X-100 and protease inhibitor cocktail (Roche, Indianapolis, IN) 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 \(\mu\)m pore-size filters (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. Myeloperoxidase (MPO; HyCult Biotechnology, Uden, The Netherlands), keratinocyte-derived chemokine (KC; R&D Systems, Minneapolis, MN), active transforming growth factor-β\(_1\) (αTGF-β\(_1\); R&D systems) and thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany) were measured with commercially available ELISA kits. Protein levels in BALF were measured using a Bradford-based protein assay (Bio-Rad Laboratories, Hercules, CA). Lactate dehydrogenase (LDH), aspartate aminotranspherase (ASA T), alanine aminotranspherase (ALA T), 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.
Bacterial killing assay

Bacterial killing was determined as described elsewhere. In brief, bone marrow-derived macrophages (BMM) were harvested from WT and A2AP−/− mice (4 mice per strain) and were grown and differentiated in serum-free culture medium RPMI 1640 (Gibco, Life technologies, Bleiswijk, The Netherlands) containing 5% Panexin BMM (Pan Biotech, Aidenbach, Germany), 1% L-glutamine (Gibco) and 2 ng/mL recombinant murine granulocyte macrophage colony stimulating factor (Invitrogen, Merelbeke, Belgium). One day before infection, BMM were seeded into 24-well plates at a density of 2*10^5/well (final concentration 4*10^5/mL) and pre-treated with 100 ng/mL *E. coli* lipopolysaccharide O111:B4 (LPS; Sigma-Aldrich, St. Louis, MO) for 16 hours as described before. *B. pseudomallei* strain 1026b was freshly thawed from a frozen stock and grown on blood agar for 20h at 37°C, harvested and diluted in cell culture medium. Prior to infection, each well was washed twice with PBS and infected with a multiplicity of infection (MOI) of 30 for 30 min at 37°C as indicated. The medium was then removed and exchanged for medium containing 250 μg/mL kanamycin (Invitrogen) for 30 min (this point was taken as time zero), hereafter cells were incubated in medium containing 250 μg/mL kanamycin during further incubation to eliminate extracellular bacteria. To evaluate the number of intracellular bacteria at indicated time points, cells were washed twice with PBS and subsequently lysed using 500 μL PBS containing 0.1% Triton-X (Sigma-Aldrich) per well. Appropriate dilutions of these suspensions were plated onto blood agar plates and incubated at 37°C for 24h before colonies were counted. Bacterial killing was expressed as the percentage of killed bacteria in relation to \( t = 0 \).

Ex vivo stimulations

Alveolar macrophages (AM) and BMDM were harvested from WT and A2AP−/− mice by respectively bronchoalveolar lavage and by flushing tibia and femurs (n = 8/strain) as described\(^1,4\). Adherent monolayer cells of AM and BMDM were stimulated with heat-killed *B. pseudomallei* (2*10^7/mL) for 20h, while mouse recombinant A2AP (70 µg/mL; R&D Systems, catalogue number 1239-PI) or RMPI1640 (control) was added. For the preparation of heat-killed *B. pseudomallei*, bacteria were incubated in a water bath of 70°C for 30 minutes. Supernatants were collected and stored at -20°C until assayed for TNF-α release.

Measurement of the effect of A2AP on bacterial growth

Human A2AP (Abcam, Cambridge, UK, catalogue number ab90921) was dissolved in sterile dH\(_2\)O and assayed in doubling dilutions from 2.2 to 70 μg/mL. We chose for human A2AP as we considered this more relevant to the clinical situation. *B. pseudomallei* was grown to a final concentration of 5*10^5 CFU/mL in LB medium. A2AP 50 μL/well or buffer control (200 mM NaCl, 20 mM bis Tris, pH 7.4) 50 μL/well were pipetted into a flat-bottom 96-wells plate and at \( t = 0 \) bacterial suspension 50 μL/well was added. Cultures were incubated at 37°C 5% CO\(_2\) for 6 and 24 hours. Growth was defined both by turbidity and after plating serial dilutions on blood agar plates. Colonies were counted after incubation at 37°C for 24h. Data are expressed as % CFU of buffer control.
**Western Blot**

Western blotting was done as described previously\(^2\)\(^-\)\(^3\). In brief, 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). Then, the membrane was incubated in 1:3000 polyclonal donkey-anti-goat IgG-HRP secondary antibody (Abcam) in 1% BSA and imaged on a LAS3000 dark box (Fujifilm, Tokyo, Japan). Anti-fibrinogen reactive bands of fibrin degradation products (including fragment-X and D-dimer) were analyzed using ImageJ (version 2006.02.01, US National Institutes of Health, Bethesda, MD). Densities were expressed as -fold increase compared to the mean density of the WT-group.

**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\(^1\)\(^-\)\(^3\). 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. Granulocyte stainings, using fluorescein isothiocyanate-labeled rat-anti-mouse Ly-6G mAb (BD Pharmingen, San Diego, CA) were done as described previously\(^2\)\(^-\)\(^3\). Slides were counterstained with methylgreen (Sigma-Aldrich). The total tissue area of the Ly-6G-stained slides was scanned with a slide scanner (Olympus dotSlide, Tokyo, Japan) and the obtained scans were exported in TIFF format for digital image analysis. The digital images were analyzed with ImageJ (version 2006.02.01, National Institutes of Health, Bethesda, MD) and the immunopositive (Ly6G+) area was expressed as the percentage of the total lung surface area.

**REFERENCES**

**Tables**

**Supplemental Table E1.** Cytokine concentrations in bronchoalveolar lavage fluid 72 hours after infection with *B. pseudomallei.*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>BALF*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pg/ml</strong></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>483 (255-776)</td>
</tr>
<tr>
<td>IL-6</td>
<td>277 9106-3350</td>
</tr>
<tr>
<td>IL-10</td>
<td>0 (0-1.3)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>35 (29-38)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>9.2 (6.1-16)</td>
</tr>
</tbody>
</table>

*Data are expressed as median (interquartile range) of *n* = 8 mice per group per time point. A2AP^-/- alpha-2-antiplasmin knockout, BALF bronchoalveolar lavage fluid, IFN-γ interferon-γ, IL interleukin, TNF-α tumor necrosis factor-α, WT wildtype. *P < 0.05, **P < 0.01 and ***P < 0.001 for WT versus A2AP^-/- mice (Mann-Whitney U test).*