Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Endogenous protein C has a protective role during Gram-negative pneumosepsis (melioidosis)

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ABSTRACT

Background
Activated protein C (APC) exerts anticoagulant effects via inactivation of factors Va and VIIIa and cytoprotective effects via protease activated receptor (PAR)-1. Inhibition of endogenous APC in endotoxemia and sepsis results in exacerbation of coagulation and inflammation, with consequent enhanced lethality. We here sought to dissect the distinct roles of the anticoagulant and cytoprotective functions of endogenous APC in severe Gram-negative pneumonia derived sepsis (melioidosis).

Methods
We infected wildtype (WT) mice with *Burkholderia pseudomallei*, a common sepsis pathogen in Southeast-Asia, and treated them with antibodies inhibiting both the anticoagulant and cytoprotective functions of APC (MPC1609) or the anticoagulant functions of APC (MAPC1591) only. Additionally, we administered SEW2871 (stimulating the S1P1-pathway downstream from PAR-1) to control and MPC1609-treated mice.

Results
MPC1609, but not MAPC1591 significantly worsened survival, increased coagulation activation, facilitated bacterial growth and dissemination and enhanced the inflammatory response. The effects of MPC1609 could not be reversed by SEW2871 suggesting that S1P1 does not play a major role in this model.

Conclusions
These results suggest that the mere inhibition of the anticoagulant function of APC does not interfere with its protective role during Gram-negative pneumosepsis, suggesting a more prominent role for cytoprotective effects of APC herein.
INTRODUCTION

The protein C (PC) system is an important regulator of hemostasis and the inflammatory response during infection. Initiated by complex formation of thrombin with the endothelial receptor thrombomodulin, the zymogen PC is converted into its active form, activated protein C (APC), a process that is strongly facilitated by the endothelial protein C receptor (EPCR). APC serves as one of the main inhibitors of the coagulation system via its capacity to proteolytically degrade coagulation factors Va and VIIIa. In addition, APC exerts cytoprotective, anti-inflammatory and anti-apoptotic properties, which are mediated by protease activated receptor 1 (PAR-1). For example, APC restores vascular barrier disruptions through activation of the sphingosine-1-phosphate receptor-1 (S1P1) pathway, a process also mediated via activation of the EPCR and PAR-1. This barrier-protective effect has been found to be of great importance during inflammation and infection induced by lipopolysaccharide (LPS) as endothelial barrier disruption and subsequent fluid leakage were reduced upon administration of S1P, the ligand for S1P1. Moreover, APC is capable of cleaving histones, major mediators of death in sepsis, thereby reducing cytotoxicity. These differential properties of APC likely all contribute to its protective effects in preclinical models of systemic inflammation and infection.

Low PC and APC levels correlated with the occurrence of organ dysfunction and an adverse outcome in patients with sepsis. Previous studies have tried to unravel the role of endogenous PC during inflammation and sepsis. Mice, heterozygously deficient of PC, had more severe disseminated intravascular coagulation, increased fibrin depositions and higher levels of proinflammatory cytokines upon intraperitoneal injection with LPS and reduced PC levels in mouse strains with genetically modified (low) PC expression strongly correlated with survival outcomes following LPS challenge. Inhibition of endogenous PC increased the procoagulant response during Escherichia coli peritonitis and H1N1 influenza in mice. Studies using antibodies capable of blocking both the cytoprotective and the anticoagulant function of (A)PC or blocking only the anticoagulant function of APC further expanded the knowledge on the role of endogenous (A)PC during inflammation: mainly the cytoprotective and not the anticoagulant activity of (A)PC seemed to be required for protection against LPS-induced toxicity in mice.

Knowledge on the distinct anticoagulant and cytoprotective functions of endogenous (A)PC during infection and sepsis is limited, in particular in the clinically relevant setting of a gradually growing bacterial load spreading from an initially localized infectious source. Therefore, in the present study we aimed to characterize the role of the endogenous PC system during severe pneumonia-derived sepsis. For this we used our established model of Gram-negative pneumosepsis caused by Burkholderia (B.) pseudomallei, the causative agent of melioidosis, an important cause of community-acquired sepsis in Southeast Asia. We and others recently reported pronounced coagulation activation in patients with culture-proven septic melioidosis together with downregulation of anticoagulant pathways. In particular, levels of PC were markedly decreased, and low
levels of PC correlated with a worse clinical outcome\textsuperscript{26, 27}. We here show that blocking both the anticoagulant and anti-inflammatory functions of endogenous (A)PC, but not inhibition of the anticoagulant PC function alone, strongly worsens the outcome of experimentally induced severe pneumonia derived Gram-negative sepsis caused by \textit{B. pseudomallei}.

\section*{MATERIALS AND METHODS}

\subsection*{Mice}
Pathogen-free 10-week old female 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. The Animal Care and Use of Committee of the University of Amsterdam approved all experiments.

\subsection*{Experimental infection and determination of bacterial growth}
Pneumosepsis was induced by intranasal inoculation with \textit{B. pseudomallei} strain 1026b (250 or 750 colony forming units (CFUs) in 50 μL NaCl0.9%) as previously described\textsuperscript{20-22}; these infectious doses represent an approximate LD65 and LD100 respectively. Mice were euthanized 24, 48 or 72 hours after infection, or survival studies were performed. For survival experiments mice were checked every 4-6 hours until death occurred for a maximum of 16 days. Sample harvesting and processing and determination of bacterial growth were done as described\textsuperscript{20-22}. Briefly, mice received an intraperitoneal injection containing ketamin (Eurovet Animal Health, Bladel, The Netherlands) and medetomidin (Pfizer Animal Health Care, Capelle aan den IJssel, The Netherlands). The abdomen was opened and blood from the vena cava inferior was drawn into syringes containing sodium citrate (4:1 vol/vol). Bronchoalveolar lavage fluid (BALF) was harvested by unilateral lavage of the right lung with two aliquots of 400 μL sterile phosphate-buffered saline. Lungs and liver were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of organ homogenates and blood plated on blood agar plates and incubated at 37°C 5% CO\textsubscript{2} for 20h before colonies were counted.

\subsection*{Monoclonal antibodies and S1P1 agonist}
Endogenous (A)PC was blocked with the use of the rat monoclonal antibody (mAb) MPC1609 and mAb MAPC1591 as previously described\textsuperscript{19, 28}. The class-matched mAb MCO1716, targeted against the keyhole limpet hemocyanin protein, was used as control treatment. MPC1609 inhibits both APC-generation and thereby APC anticoagulant and cytoprotective signaling pathways, whereas MAPC1591 inhibits APC anticoagulant activity\textsuperscript{19, 28}. More in detail, MPC1609 inhibits PC and APC binding to the endothelium or phospholipids by masking the Gla-domain of PC or
APC which was responsible for the binding of PC or APC. MAPC1591 recognized APC, but not PC through interacting with an epitope that still allowed APC to bind cell surfaces and phospholipids, but blocked APC anticoagulant activity presumably by preventing APC interaction of Fvα. Previous in vivo studies, involving a model of lethal H1N1-influenza and a model of E. coli peritonitis have described that treatment with MPC1609 reduced PC levels during the course of infection. Antibodies were injected intraperitoneally (200 µg in 200 µl NaCl0.9%) 30 minutes before infection, which was repeated once daily during the course of the experiment. SEW-2871, a selective S1P1 agonist (BIOMOL International, Plymouth Meeting, PA), was administered intraperitoneally (10 mg/kg body weight) 4 hours before inoculation, which was repeated once daily during the course of the experiment; normal saline served as a control.

Assays
Lung homogenates were prepared as described. Interleukin (IL)-6, IL-10 and monocyte-chemoattractant protein (MCP)-1 were measured by cytometric bead array (CBA) multiplex assay (BD Biosciences, San Jose, CA). Tumor necrosis factor (TNF)-α (R&D systems, Minneapolis, MN), keratinocyte-derived chemokine (KC; R&D Systems, Minneapolis, MN), myeloperoxidase (MPO; HyCult-Biotechnology, Uden, The Netherlands), thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany) and D-dimer (Asserachrom D-dimer, Roche Woerden, the Netherlands) were measured with commercially available ELISA kits. Total protein in BALF was measured using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Nucleosomes in BALF were measured by an ELISA as described.

Cell counts and flow cytometry
Total counts of paraformaldehyde (4%)-fixed BALF cells were measured using a Coulter Counter (Beckman Coulter Inc. Brea, CA). Differential counts were determined by FACS (FACSCalibur, Becton Dickson, San Jose, CA) using directly labeled antibodies against GR-1 (GR-1 FITC; BD Pharmingen, San Diego, CA) and F4/80 (F4/80 APC; AbD Serotec, Oxford, UK). The GR-1 FITC antibody detects the myeloid differentiation antigen Gr-1, also known as Ly-6G, a 21-25-kDa GPI-anchored protein which is present on granulocytes, including neutrophils and eosinophils. The F4/80 antibody recognizes the murine F4/80 antigen, a 160 kD cell surface glycoprotein, expressed on a wide range of mature tissue macrophages including macrophages located in the lung. All antibodies were used in concentrations recommended by the manufacturer. Granulocytes were defined according to their scatter pattern and GR-1 positivity, macrophages according to their scatter-pattern and F4/80 positivity.

Histology and immunohistology
Paraffin-embedded 4 µm lung sections were stained with haematoxylin and eosin (H&E) and analyzed for inflammation and tissue damage, as described previously. All slides were scored by a pathologist blinded for treatment 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 histopathology 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 previously20, 21. The Ly6G antibody detects Ly6G, formerly known as myeloid differentiation antigen Gr-1, a 21-25-kDa GPI-anchored protein which is present on granulocytes, including neutrophils and eosinophils. 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 means with standard error of the means (SEM). 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 < 0.05 were considered statistically significant.

RESULTS

Inhibition of both the signalling and the anticoagulant function of PC increases lethality
To gain insight into the role of endogenous PC and APC during severe pneumosepsis we infected WT mice with 750 CFU (LD100) or 250 CFU (LD65) of B. pseudomallei and performed survival studies. Mice were treated with distinct antibodies to identify the different functions of endogenous PC19, 28: MPC1609, inhibiting both anticoagulant and signalling effects of the activated form of PC, or MAPC1591, blocking only the anticoagulant effects of APC; the control group was injected with MCO1716, an irrelevant control antibody. Treatment with MPC1609 significantly shortened survival after a lethal dose (LD100) of B. pseudomallei (P < 0.01) whereas only inhibiting the anticoagulant effects of APC with MAPC1591 did not result in a difference when compared to the MCO1716 control group (Figure 1A). Strikingly, intranasal inoculation with a LD65 dose of B. pseudomallei resulted universally and rapidly in death of all MPC1609 injected mice, while lethality in MAPC1591 and MCO1716 treated mice was similar (P < 0.0001 for the difference between MPC1609 versus other groups; Figure 1B). These results might indicate that the combined cytoprotective and anticoagulant function of (A)PC is are important for protection against mortality in this model.
The cytoprotective properties of endogenous APC contribute to its anticoagulant effects during severe sepsis

The model of severe Gram-negative sepsis (melioidosis) used here is associated with profound activation of coagulation\textsuperscript{20,21}. In order to determine the role of endogenous (A)PC in the regulation of the procoagulant response during pneumosepsis WT mice, treated with either MPC1609, MAPC1591 or MCO1716 were infected with 750 or 250 CFU of \textit{B. pseudomallei} and sacrificed after 24, 48 and 72 hours to determine TATc and D-dimer levels in lung homogenates (the primary site of infection). Treatment with MPC1609 induced increased TATc levels in lungs 48 hours after inoculation when compared to treatment with control MCO1716 ($P < 0.001$), whereas treatment with MAPC1591 did not induce any differences at this time-point (Figure 2A). These same results were observed after 72 hours, when a low, not fully lethal inoculum (LD65) was used ($P < 0.05$). Interestingly, after 72 hours, when using a lethal inoculum (LD100), coagulation activation was significantly increased in the MAPC1591 treated group, when compared to controls ($P < 0.01$; Figure 2A), whereas in the MPC1609 group no measurements could be performed as 6 out of 8 animals had already died at this time-point, precluding an adequate analysis of the effects of this antibody during late stage infection. Similarly, lung D-dimer levels were markedly increased in MPC1609 treated mice 24 ($P < 0.01$) and 48 hours ($P < 0.05$) after high dose infection and 72 hours after low dose infection ($P < 0.01$) (Figure 2B). D-dimer was also measured in plasma harvested 24 or 48 hours after high dose infection; in accordance with results obtained in lungs, MPC1609 but not MAPC1591 enhanced plasma D-dimer levels relative to MCO1716 control treatment (data not shown). These results clearly indicate that inhibiting both the anti-inflammatory and anticoagulant functions of (A)PC exacerbates activation of coagulation; selective inhibition of the anticoagulant function of (A)PC only enhanced activation of coagulation during late stage high dose infection.
Figure 2. Inhibition of both the anti-coagulant and cytoprotective function of (A)PC enhances coagulation activation. Mice were treated with MPC1609 (inhibiting both the anticoagulant and cytoprotective functions of PC; white bars), MAPC1591 (inhibiting only the anticoagulant functions of PC; striped bars) or MCO1716 (control; black bars) and infected intranasally with 750 CFU (LD100) or 250 CFU (LD65) of *B. pseudomallei*. Mice were sacrificed after 24, 48 or 72 hours and TATc (A) and D-dimer (B) were measured in lung homogenates. Data are expressed as bars with means ± SEM; n = 7-8 mice per group for each time point. *P < 0.05, **P < 0.01 and ***P < 0.001 versus MCO1716 (Mann-Whitney U test). ND not determined; TATc, thrombin-antithrombin complexes.

Figure 3. Inhibition of both the anti-coagulant and cytoprotective function of (A)PC facilitates bacterial growth and dissemination. Mice were treated with MPC1609 (inhibiting both the anticoagulant and cytoprotective functions of PC; white bars), MAPC1591 (inhibiting only the anticoagulant functions of PC; striped bars) or MCO1716 (control; black bars) and infected intranasally with 750 CFU (LD100) or 250 CFU (LD65) of *B. pseudomallei*. Mice were sacrificed after 24, 48 or 72 hours and bacterial loads were measured in lung homogenates (A), BALF (B), whole blood (C) and liver homogenates (D). Data are expressed as bars with means ± SEM; n = 7-8 mice per group for each time point. *P < 0.05, **P < 0.01 and ***P < 0.001 versus MCO1716 (Mann-Whitney U test). BALF bronchoalveolar lavage fluid; ND not determined.
Inhibition of both the cytoprotective and anticoagulant properties of endogenous PC facilitates bacterial growth and dissemination

Our model of pneumosepsis is associated with marked bacterial growth locally in lung tissue and BALF, but also systemically in blood and liver. In order to establish whether endogenous PC impacts on bacterial growth and dissemination, WT mice, treated with either MPC1609, MAPC1591 or MCO1716, were infected with 750 or 250 CFU of *B. pseudomallei* and sacrificed after 24, 48 and 72 hours to determine bacterial loads in lungs and BALF (the primary site of infection), liver and blood (to evaluate the extent of bacterial dissemination). At 24 hours post infection, bacterial burdens were similar in all treatment groups in all body sites examined (Figure 3). At 48 hours, however, mice administered with MPC1609 had significantly increased bacterial loads when compared with MCO1716 treated animals, both at the primary site of infection, in lung homogenates and BALF (both *P* < 0.001; Figure 3A-B), and in a distant body site, the liver (*P* < 0.05; Figure 3D); at this time point bacterial counts in blood tended to be higher in MPC1609 treated mice (*P* = 0.12 versus MCO1716; Figure 3C). Seventy-two hours after LD65 infection MPC1609 treatment was associated with significantly higher bacterial loads locally in lung homogenates and BALF and systemically in blood and liver homogenates (all *P* < 0.01; Figure 3A-D). In addition, at this time point, all mice had positive blood cultures in the MPC1609 group, while in the control group (MCO1716) only 1 out of 8 blood cultures was positive (Figure 3C). Importantly, treatment with MAPC1591 did not influence bacterial growth or dissemination after infection with either 750 or 250 CFU of *B. pseudomallei*, when compared to MCO1716 administration. Hence, both the cytoprotective and the anticoagulant function of (A)PC, but not the anticoagulant effects only, are important in antibacterial defense in this model of severe Gram-negative pneumosepsis.

Inhibition of both the cytoprotective and anticoagulant properties of endogenous (A)PC exaggerates lung inflammation during pneumonia derived sepsis

The current model of pneumosepsis is associated with profound lung pathology, inflammatory cell recruitment and local inflammation. In the present study, mice in all study groups 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-D). During the first 48 hours after infection with the LD100 dose, the extent of lung pathology, quantified by the semi-quantitative scoring system described in the Methods section, was similar in all treatment groups; 72 hours after LD100 infection the impact of MPC1609 could not be evaluated due to early mortality, whereas MAPC1591 administration did not alter lung pathology relative to MCO1718. At 72 hours after LD65 infection, however, MPC1609 treated mice displayed enhanced lung pathology, when compared with MCO1718 administered animals (*P* < 0.05). Figures 4B (MCO1716), C (MPC1609) and D (MAPC1591) show representative H&E-stainings of lung tissue obtained 72 hours after intranasal inoculation (LD65) with *B. pseudomallei*. In concordance with histology scores, the number of Ly6G-positive neutrophils in lung tissue slides was higher MPC1609 treated mice 72 hours after LD65 infection (*P* < 0.05 versus
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Figure 4. Lung pathology. Mice were treated with MPC1609 (inhibiting both the anticoagulant and cytoprotective functions of PC; white bars), MAPC1591 (inhibiting only the anticoagulant functions of PC; striped bars) or MCO1716 (control; black bars) and infected intranasally with 750 CFU (LD100) or 250 CFU (LD65) of *B. pseudomallei*. Mice were sacrificed after 24, 48 or 72 hours. (A) Lung histopathology scores determined as described in the Methods section. Representative lung tissue slides (H&E staining x100) 72 hours after LD65 infection in mice treated with MCO1716 (B), MPC1609 (C) or MAPC1591 (D). Ly6G-positivity (expressed as % of total lung surface) (E). Representative photographs of Ly6G-immunostaining (original magnification x100) for granulocytes of mice treated with MCO1716 (F), MPC1609 (G) and MAPC1591 (H). Lung MPO (I), Total leukocyte number in BALF (J), Neutrophil number in BALF (K), Total protein concentration in BALF (L), Nucleosome levels in BALF (M). Data are expressed as bars with means ± SEM; n = 7-8 mice per group for each time point. *P < 0.05, **P < 0.01 and ***P < 0.001 versus MCO1716 (Mann-Whitney U test). BALF bronchoalveolar lavage fluid; MPO myeloperoxidase; ND not determined.

MCO1716; Figure 4E; Figures 4F (MCO1716), G (MPC1609) and H (MAPC1591) show representative photographs of Ly-6G stainings of lung tissue harvested at this time point). The inhibitory effect of endogenous PC on lung inflammation and pathology was further confirmed by elevated MPO concentrations (indicative for neutrophil numbers and activation) in lung homogenates harvested from the MPC1609 treated group 48 hours after LD100 infection (*P < 0.05 versus MCO1716) and 72 hours after LD65 infection (*P < 0.01 versus MCO1716; Figure 4I). Moreover, BALF obtained from MPC1609-treated mice contained significantly more cells than BALF from MCO1716 administered animals 48 hours after LD100 infection (*P < 0.001; Figure 4J). These
differences were mainly caused by enhanced numbers of neutrophils in the MPC1609-treated mice ($P < 0.001$; Figure 4K). The protective effect of endogenous PC on the development of lung injury was further supported by total protein concentrations in BALF (indicative for vascular leak): MPC1609 administration was accompanied by elevated protein levels in BALF harvested 48 hours after LD100 infection ($P < 0.01$ versus MCO1716) or 72 hours after LD65 infection ($P < 0.05$ versus MCO1716; Figure 4L). Administration of MAPC1591 did not influence any of these parameters. Together these data suggest that the combined cytoprotective and anticoagulant properties of endogenous (A)PC exert lung protective effects in this model of severe pneumonia derived sepsis.

**Endogenous (A)PC inhibits the release of nucleosomes in the bronchoalveolar space during sepsis induced lung injury**

Recently, APC was demonstrated to be capable of cleaving histones, major mediators of death in sepsis, thereby reducing cytotoxicity\(^\dagger\). Our laboratory identified nucleosomes (complexes containing DNA and histones) in BALF as damage markers during hyperoxia induced lung injury\(^{31}\). Therefore, we considered it of interest to study the possible role of endogenous APC in the release of nucleosomes into the bronchoalveolar space during sepsis induced lung injury. BALF nucleosome levels increased during the course of the infection, becoming detectable 48 hours after infection and reaching peak values after 72 hours (Figure 4M). At 48 hours after LD100 infection, all three treatment groups had similar nucleosome concentrations; at 72 hours levels were similar in MAPC1591 and MCO1716 treated animals. At 72 hours after LD65 infection, however, treatment with either MPC1609 or MAPC1591 was associated with elevated nucleosome levels in BALF (both $P < 0.05$ versus MCO1716).

**Inhibition of both the cytoprotective and anticoagulant effects of endogenous (A)PC enhances local and systemic cytokine release during sepsis**

In order to examine the impact of endogenous (A)PC on cytokine and chemokine release during pneumonia derived sepsis, we measured TNF-$\alpha$, IL-6, IL-10, MCP-1 and KC levels in lung homogenates and plasma 24, 48 and 72 hours after infection. At 24 hours after inoculation all cytokine levels were low and did not differ between the treatment groups in either lung homogenates or plasma (data not shown). Additionally, 72 hours after LD100, lung or plasma cytokine levels did not differ between MAPC1591 and MCO1716 treated animals (data not shown). However, 48 hours after LD100 infection and 72 hours after LD65 infection, almost all cytokine and chemokine levels were higher in lung homogenates and plasma of MPC1609 treated (but not of MAPC1591 administered) animals (Table 1). These results suggest that the combined cytoprotective and anticoagulant function of endogenous (A)PC limits local and systemic cytokine release during severe pneumonia derived sepsis.
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Treatment with an S1P1 agonist does not reverse the effect of MPC1609

One of the cytoprotective functions of APC involves protection of the vascular barrier through activation of the S1P1 pathway, which is mediated via activation of PAR-1. In order to determine whether the observed detrimental effects of MPC1609 were in part mediated via the APC-PAR-1-S1P1-pathway, we repeated the experiment in which the effect of MPC1609 was investigated 48 hours after intranasal infection with *B. pseudomallei*. Wild type (WT) mice were treated with antibodies to inhibit both anticoagulant and signalling effects of APC (MPC1609) or only the anticoagulant effects of APC (MAPC1591) or an irrelevant control antibody (MCO1716). Mice were sacrificed 24, 48 or 72 hours after LD100 infection or 72 hours after LD65 infection. Only data obtained 48 hours after LD100 and 72 hours after LD65 infection are shown; at 24 hours after LD100 infection no differences between groups were detected (not shown), whereas 72 hours after LD100 infection early mortality in MPC1609 treated mice precluded adequate comparisons (not shown). Data are expressed as median (interquartile ranges) of *n* = 7-8 mice per group per time point. IL interleukin, TNF-α tumor necrosis factor-α, MCP-1 monocyte-chemoattractant protein-1. BD below detection limits; NEM not enough material. *P* < 0.05, **P < 0.01 and ***P < 0.001 for difference with MCO1716 control treatment (Mann-Whitney U test).

Table 1. Inhibition of both the cytoprotective and anticoagulant function of endogenous APC enhances local and systemic cytokine release during Gram-negative pneumosepsis.

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Pulmonary and plasma cytokine levels after intranasal infection with *B. pseudomallei*. Wild type (WT) mice were treated with antibodies to inhibit both anticoagulant and signalling effects of APC (MPC1609) or only the anticoagulant effects of APC (MAPC1591) or an irrelevant control antibody (MCO1716). Mice were sacrificed 24, 48 or 72 hours after LD100 infection or 72 hours after LD65 infection. Only data obtained 48 hours after LD100 and 72 hours after LD65 infection are shown; at 24 hours after LD100 infection no differences between groups were detected (not shown), whereas 72 hours after LD100 infection early mortality in MPC1609 treated mice precluded adequate comparisons (not shown). Data are expressed as median (interquartile ranges) of *n* = 7-8 mice per group per time point. IL interleukin, TNF-α tumor necrosis factor-α, MCP-1 monocyte-chemoattractant protein-1. BD below detection limits; NEM not enough material. *P* < 0.05, **P < 0.01 and ***P < 0.001 for difference with MCO1716 control treatment (Mann-Whitney U test).
Endogenous PC in melioidosis

After LD100 infection, now in the presence or absence of co-treatment with a downstream S1P1 agonist, SEW2871, a selective sphingosine-1-phosphate receptor-1 (S1P1) agonist, does not reverse MPC1609 effects. Mice were treated with MPC1609 (inhibiting both the anticoagulant and cytoprotective functions of PC; white bars) or MCO1716 (control; black bars) with or without the selective S1P1 agonist SEW2871 (with MPC1609: white-hatched bars; with MCO1716: grey-hatched bars). All mice were infected intranasally with 750 CFU (LD100) of *B. pseudomallei* and sacrificed after 48 hours. Bacterial loads in lungs (A), BALF (B), blood (C) and liver (D), Thrombin-antithrombin complexes (TATc) in lung homogenates (E), Total protein in BALF (F) and lung G) and plasma (H) IL-6, a proinflammatory cytokine, while, again, no differences were seen between mice treated with SEW2871 and control mice. Data are expressed as bars with means ± SEM; n = 8 mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 versus MCO1716, ΔP < 0.05 versus MPC1609 (Mann-Whitney U test). BALF bronchoalveolar lavage fluid; ND not determined; S1P1 sphingosine-1-phosphate receptor-1; TATc thrombin-antithrombin complexes.

DISCUSSION

APC is a pleiotropic protease with multiple inhibitory effects on coagulation and inflammation\(^1^,\(^3\). While the endogenous PC system has been implicated to play a protective role in sepsis, knowledge on the relative contribution of the anticoagulant and cytoprotective effects of endogenous PC in sepsis pathogenesis is limited. In this study we investigated the role of endogenous PC during Gram-negative pneumosepsis using our well-established mouse model of severe *B. pseudomallei*
infection, characterized by gradual growth of bacteria from the lung with subsequent dissemination to distant body sites, activation of procoagulant and inflammatory pathways, tissue injury and death, thereby mimicking the clinical scenario of severe sepsis\textsuperscript{20-22}. We show that inhibition of both the anticoagulant and cytoprotective functions of PC (with MPC1609) significantly worsened survival after either a LD100 or LD65 dose of \textit{B. pseudomallei} whereas selective inhibition of the anticoagulant effects of APC (with MAPC1591) was without effect. Moreover, treatment with MPC1609 enhanced coagulation activation, facilitated bacterial growth at the primary site of infection and at distant body sites and enhanced the inflammatory response (leukocyte recruitment, cytokine levels and nucleosome release). In contrast, MAPC1591 administration did not impact on the antibacterial or inflammatory response. The effects of MPC1609 could not be reversed by SEW2871 (stimulating the S1P1 pathway downstream from PAR1), suggesting that S1P1 does not play a major role in this model. These results suggest that the combined anti-inflammatory and anticoagulant function of the protein C system contributes to adequate host defense during pneumosepsis caused by \textit{B. pseudomallei}.

Previous research has shown that MPC1609 blocks all known functions of PC and APC, including its anticoagulant activities and protection of the endothelial barrier, via blockage of binding of APC to the endothelium\textsuperscript{19}. MAPC1591, on the other hand, enhances APC binding to the endothelium and does not block the protective effects of APC on the endothelial barrier. Similar to MPC1609, MAPC1591 completely inhibited APC anticoagulant activity \textit{in vitro}. As such, MAPC1591 is a selective inhibitor of the anticoagulant properties of APC\textsuperscript{19}. Our results show that MPC1609 clearly enhanced activation of coagulation at the primary site of infection and in the circulation. MAPC1591 had a more modest effect on coagulation: only when using a high inoculum (LD100) and at a late time point increased TATc levels were observed in MAPC1591 treated animals. The more profound effect of MPC1609 on local and systemic coagulation suggests that the anti-inflammatory and cytoprotective properties of APC contribute to its anticoagulant potency during the severe inflammatory response syndrome caused by sepsis. In accordance, in mice challenged with LPS intravenously MPC1609 administration resulted in higher plasma TATc levels than MAPC1591 injection, accompanied by much higher plasma IL-6 concentrations in the former group\textsuperscript{19}.

Besides impacting on coagulation, MPC1609, but not MAPC1591, caused profound alterations in many host responses examined during the course of \textit{B. pseudomallei}-induced sepsis. The effects of MPC1609 became apparent beyond the first 24 hours and included increased bacterial growth and dissemination. In accordance, our laboratory recently reported transiently increased bacterial burdens in MPC1609 treated mice with \textit{E. coli} peritonitis\textsuperscript{87}. These findings are remarkable, considering that (A)PC is not known to directly influence antibacterial effector mechanisms. Possibly, enhanced fibrin formation in the context of MPC1609 treatment may provide bacteria with a niche that protects them from eradication by immune cells. This is unlikely to explain the effect of MPC1609 in full, however: recent data from our laboratory showed that tissue-type plasminogen activator deficient mice, which like MPC1609 treated mice are expected to display enhanced fibrin
depositions albeit through a different mechanism (i.e. reduced fibrinolysis), demonstrated reduced bacterial growth and dissemination in this same model of *B. pseudomallei*-induced pneumosepsis\(^{21}\). Moreover, mice deficient for plasminogen-activator inhibitor type-1, which are expected to have to decreased fibrin depositions, showed increased bacterial growth in this model\(^{20}\), further arguing against a role for fibrin in shielding bacteria from the host immune system. Furthermore, MPC1609, but not MAPC1591, exaggerated lung damage, associated with stronger neutrophil influx and higher cytokine levels, and reflected by increased histopathology and higher protein contents of BALF. Together, these data suggest that the combined anticoagulant and cytoprotective function of endogenous (A)PC contributes to the integrity of host defense against *B. pseudomallei*. Alternatively, the cytoprotective properties of (A)PC alone could be sufficient for this beneficial effect. Our results obtained with SEW2871 argue against an important role for the S1P1 pathway. APC may restore vascular barrier disruption at least in part through a process mediated via APC-EP-CR co-localization on lipid rafts, activation of PAR-1 and subsequent APC-EPCR-PAR-1 cross-activation of S1P1 signalling pathways\(^{4,7,32}\). In accordance with a role for S1P1 in protection against endothelial barrier disruption, intravenous administration of its ligand S1P reduced vascular leakage in murine models of LPS-mediated or high-tidal mechanical ventilation induced acute lung injury\(^{8,9}\). In the present study we tried to restore the detrimental effects of treatment with MPC1609 by using a S1P1-agonist, SEW2871, seeking to bypass the MPC1609 induced inhibition of the APC-PAR-1 interaction by direct activation of S1P1 downstream from PAR-1. In an earlier study SEW2871 reversed MPC1609 induced vascular leakage and growth of metastasis in a model of cancer cell extravasation of melanoma cells in mouse lungs\(^{28}\); moreover, SEW2871 significantly reduced ischemia-reperfusion injury in the mouse kidney\(^{29}\), together providing evidence for the biological activity of this compound. As such, the lack of an effect of SEW2871 in our study suggests that during pneumosepsis the protective effects of endogenous (A)PC are not fully driven by the S1P1 pathway. Notably, recent evidence has demonstrated that APC may exert cytoprotective effects by a pathway that does not rely on PAR-1\(^{13}\). Moreover, APC-PAR-1 signaling might exert S1P1 independent effects on endothelial barrier function (e.g. via the angiopoietin/tie2 axis) and additionally APC-PAR-1 signaling can result in cytoprotective effects (antiapoptotic and anti-inflammatory effects) for which S1P1 has not been implicated. Hence, our experiments using SEW2871 do not fully address the contribution of the complete spectrum of cytoprotective effects of endogenous APC in the phenotype of MPC1609 treated mice. Experiments with an antibody inhibiting only the cytoprotective functions of APC are required to test this possibility; however, to the best of our knowledge such antibodies have not been generated thus far.

Our data obtained with MPC1609 are in line with previous investigations that used partially PC-deficient mice to show the protective anticoagulant and anti-inflammatory role of endogenous PC during endotoxemia\(^{15,16}\). In accordance, MPC1609 exaggerated coagulation and IL-6 release after intravenous injection of LPS\(^{19}\) and intraperitoneal administration of *E. coli* in mice\(^{17}\), which in the LPS model was accompanied by increased lethality\(^{19}\). Additionally, in lethal H1N1 influenza in mice treatment with MPC1609 aggravated lung coagulation and lung histopathology\(^{18}\). However, in
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this model, MPC1609 treatment lowered bronchoalveolar neutrophil influx and total protein levels and delayed mortality\(^{18}\), which is in contrast with our present findings. A possible explanation for these discrepancies may be that the course of a viral infection strongly differs from overwhelming bacterial (pneumo)sepsis.

Recent data show that extracellular histones released in response to an inflammatory challenge contribute to endothelial dysfunction, organ failure and death during sepsis\(^{10}, 34\). Extracellular histones were found to be cytotoxic toward endothelium *in vitro* and lethal in mice\(^{10}\). APC cleaves histones and thereby reduces their cytotoxicity; blockage of PC activation by MPC1609 exacerbated a sublethal LPS challenge into lethality, which was reversed by treatment with antibody to histone \(^4\)\(^{10}\). Our data are in line with these observations. The concentrations of nucleosomes, structural units containing a segment of DNA wound around a histone protein core, increased in BALF after infection, resembling previous findings during hypoxia-induced lung injury\(^{31}\). Remarkably, unlike many other responses examined in this study, both MPC1609 and MAPC1591 administration led to significantly higher nucleosome levels compared to MCO1716 control treatment during late stage LD65 infection, suggesting that the protease activity mediating APC’s anticoagulant function is responsible for this effect.

Recently, recombinant human APC was withdrawn from the market as a therapeutic for sepsis after the negative results from the PROWESS shock trial\(^{35}\). Nonetheless, several preclinical studies have provided insight into the mechanisms of action of recombinant APC administration in models of endotoxemia and sepsis\(^{36}\). The effects of recombinant APC cannot be directly extrapolated to the function of the endogenous PC-system\(^{37}\); these studies made use of recombinant APC mutants with selective anticoagulant properties, showing that inhibition of coagulation may not contribute to the protective effects of exogenous APC during endotoxemia and sepsis\(^{38}\). In conclusion, our study is the first to investigate the distinct roles of the anticoagulant and cytoprotective functions of the endogenous PC system in host defense during Gram-negative sepsis. Hence, both previous investigations using recombinant APC and the current studies examining the function of endogenous APC indicate that the mere anticoagulant effects of APC do not play a major role in sepsis outcome. Although the current results suggest that the cytoprotective effects of endogenous APC are most important for its protective role during severe Gram-negative pneumosepsis, experiments with an antibody that selectively targets the cytoprotective properties of APC are warranted to firmly conclude this.

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