Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
Kager, L.M.

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The endothelial protein C receptor and activated protein C play a limited role in host defense during experimental tuberculosis

Liesbeth M. Kager
Joris J. T. H. Roelofs
Alex F. de Vos
Catharina W. Wieland
Marcel Schouten
Joost C. M. Meijers
Berend Isermann
Cornelis van ’t Veer
Charles T. Esmon
Tom van der Poll

ABSTRACT

Background and objective
The protein C (PC) system is an important regulator of both coagulation and inflammation. Activated PC (APC), together with its receptor the endothelial protein C receptor (EPCR), has anticoagulant and anti-inflammatory properties. During tuberculosis (TB), a devastating chronic pulmonary disease caused by Mycobacterium (M.) tuberculosis, both a local inflammatory reaction characterized by the recruitment of mainly mononuclear cells and the formation of pulmonary granulomas as well as activation of coagulation occurs as part of the host immune response.

Methods
We investigated the role of EPCR and APC in a mouse model of TB using mice overexpressing EPCR (Tie2-EPCR), mice deficient for EPCR (EPCR^−/−), mice treated with APC-inhibiting antibodies and mice overexpressing APC (APC^{high}) and compared them with wild type (WT) mice. Blood and organs were harvested to quantify bacterial loads, cellular influxes, cytokines, histopathology and coagulation parameters. Additionally observation studies were performed.

Results
Lung EPCR expression was upregulated during experimental TB. No significant differences in bacterial growth were seen between WT and Tie2-EPCR mice. However, Tie2-EPCR mice had decreased pulmonary coagulation activation, displayed an increased influx of macrophages 2 and 6 weeks after infection, but no increase in other proinflammatory markers. On the other hand, in EPCR^−/−-mice coagulation activation was decreased 6 weeks post-infection, with little impact on other inflammation markers. APC-overexpression or treatment with anti-(A)PC antibodies displayed minimal effects during experimental TB.

Conclusions
EPCR and APC play a limited role in the host response during experimental pulmonary TB.
INTRODUCTION

Tuberculosis (TB) is one of the most widespread infectious diseases worldwide, with one-third of the world population being infected with the causative agent *Mycobacterium (M.) tuberculosis*\(^1\,2\). In 2011, the incidence of TB was 8.8 million worldwide and 1.5 million people died from this disease\(^1\). Most infected individuals fail to progress to clinical disease because, due to a strong protective T helper -1 (T\(_{H1}\)) response, the tubercle bacilli remain dormant inside granuloma, tissue nodules consisting of infected macrophages surrounded by lymphocytes and a fibrotic capsule\(^3\). *M. tuberculosis* bacilli that are not fully eradicated from the lungs remain a potential danger to the infected individual\(^2\,3\). Hence, there is a clear need to increase our understanding of host defense mechanisms during TB.

Ample evidence exists about the close relationship between inflammation and coagulation\(^4\). The pro-inflammatory response may evoke coagulation activation, while coagulation factors can act pro-inflammatory. Activated protein C (APC), one of the major endogenous anticoagulants by virtue of its capacity to inactivate coagulation factors Va and VIIIa, has drawn a lot of attention due to its concurrent anti-inflammatory, cytoprotective properties\(^5\). APC results from protein C (PC) activation by thrombin in a thrombomodulin (TM)-dependent way, a reaction that is accelerated dramatically when PC binds to the endothelial protein C receptor (EPCR)\(^6\,7\). When bound to EPCR and protease activated receptor (PAR)-1, APC can exert anti-inflammatory, anti-apoptotic and barrier protective signals in endothelial cells\(^5\). EPCR was originally identified as a transmembrane endothelial receptor, but has also been detected in a number of other cell types including neutrophils, monocytes, eosinophils, vascular smooth muscle cells and renal tubular epithelial cells\(^7\,9\). Previous studies have provided evidence for a role for EPCR during sepsis. Inhibition of EPCR-binding of PC and APC with EPCR-blocking antibodies was found to exacerbate the septic response in baboons\(^10\,11\). Conditional EPCR-gene deletion, resulting in absent EPCR on vascular membranes, exaggerated the host responses to LPS, reflected by more thrombin and cytokine generation, neutrophil sequestration in the lung and a higher mortality rate, which was primarily due to deficiency of EPCR on non-hematopoietic cells\(^12\). On the contrary, mice overexpressing EPCR that were consequently generating more APC in response to thrombin, were protected against LPS challenge\(^13\).

Previous research in pulmonary TB patients has demonstrated a hypercoaguable state during this disease, with elevated plasma fibrinogen levels and increased platelet aggregation\(^14\,15\), activation of fibrinolysis as reflected by increased levels of tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1)\(^14\) and downregulation of anticoagulant factors such as antithrombin and PC\(^15\). Furthermore, *in vitro* studies demonstrated that APC may play an important role during pulmonary TB. Mice with a mutation in the TM-gene resulting in minimal capacity for APC generation (TM\(^{pro/pro}\) mice) demonstrated uncontrolled lung inflammation, including higher lung weights, a diminished capacity to form well-shaped granulomas and elevated levels of pro-inflammatory cytokines, which was accompanied by a reduced survival\(^16\). Of note,
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TMpro/mice have an intact lectin-like domain of thrombomodulin\textsuperscript{17}, of which host-protective, anti-inflammatory effects have been described as well\textsuperscript{18, 19}. Little knowledge exists about the possible direct role of APC and its receptor EPCR during pulmonary TB. In the present study we analyzed coagulant and inflammatory responses in the lungs of EPCR-overexpressing mice after intranasal infection with live virulent \textit{M. tuberculosis} and compared these with reactions in normal wild type (WT) mice. Additionally, we analyzed host responses in EPCR-deficient mice, mice treated with APC inhibiting antibodies, inhibiting both the anticoagulant and inflammatory functions of APC or only the anticoagulant function of APC, and mice overexpressing APC.

**MATERIALS AND METHODS**

**Patients**
Lung tissues of pulmonary TB-patients (N = 8; mean age 62 years, range 32-85y; 38% male) were obtained after partial lobectomy (N = 2) or from post-mortem samples (N = 6) of patients who died from (disseminated) TB or TB-related causes. TB infection was confirmed in these tissues by Ziehl-Neelsen (ZN) positive staining for acid-fast bacilli. Control tissues (N = 10; mean age 57y, range 18-77y; 50% male) were obtained from patients after (partial) lobectomy because of (suspicion of) lung cancer or pulmonary metastases. Control tissues were free of signs of pulmonary infection or cancer, as confirmed by reviewing all cases by a qualified histopathologist.

**Mice**
Pathogen-free 8-10-week old female WT C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands). Mice overexpressing EPCR (Tie2-EPCR) and mice conditionally knockout for the EPCR-gene (EPCR\textsuperscript{-/-}) were generated as described\textsuperscript{13, 20}. Overexpression of EPCR was achieved by placing the expression of the EPCR-gene under a Tie-2 promoter\textsuperscript{13}. Meox2\textsuperscript{+/+}cre Procr\textsuperscript{-/-} mice (abbreviated as EPCR\textsuperscript{-/-} mice) were generated as described\textsuperscript{20}. Briefly, via a conditional knockout system (cre-Lox) the EPCR-gene was deleted in embryos while the necessary EPCR-expression on placental giant trophoblasts was left intact, enabling the embryos to be carried to term and to develop normally\textsuperscript{20}. APC\textsuperscript{high} mice were generated as described\textsuperscript{21}. All strains were backcrossed on a C57BL/6 background for at least 6 times. Mice were 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.

**Monoclonal antibodies**
Endogenous (A)PC was blocked with the use of the rat monoclonal antibody (mAb) MPC1609 and mAb MAPC1591 as previously described\textsuperscript{22, 23}. The class-matched antibody MCO1716,
targeted against the keyhole limpet hemocyanin protein, was used as control treatment. Both MPC1609 and MAPC1591 inhibit APC anticoagulant activity in vivo, whereas MPC1609 also inhibits APC signaling effects\textsuperscript{22, 23}. Antibodies were dialyzed using a 3.5kDa Slide-A-Lyzer dialysis cassette (Thermo Scientific, Rockford, IL) to remove buffer components. Antibodies were injected intraperitoneally (200 µg in 200 µL NaCl0.9%) 30 minutes before infection which was repeated three times a week during the course of the experiment.

**Experimental infection**

A virulent laboratory strain of \textit{M. tuberculosis} (Erdman) was grown in liquid Dubos medium containing 0.01% Tween-80. A replicate culture was incubated at 37°C, harvested at mid-log phase and stored in aliquots at -70°C. For each experiment, a vial was thawed and washed with sterile NaCl0.9%. TB was induced as described previously \textsuperscript{24, 25}. Briefly, mice were anesthetized by isoflurane inhalation (Abbott Laboratories, Kent, UK) and infected intranasally with 150 CFU of viable \textit{M. tuberculosis} in 50 µL NaCl0.9%. The inoculum was placed on Middlebrook 7H11-plates immediately after inoculation to determine viable counts. Groups of 8 mice per time-point were sacrificed 2 or 6 weeks after infection. In addition, survival studies were performed with an observation period of 46 weeks. After this period, surviving mice were euthanized to obtain insight in the role of EPCR in chronic infection. Organs were processed as described previously\textsuperscript{16, 25, 26}. Briefly, lungs and liver were removed aseptically and homogenized in 5 volumes of sterile NaCl0.9%. Ten-fold dilutions were plated on Middlebrook 7H11-plates to determine bacterial loads. Colonies were counted after 21 days incubation at 37°C. Numbers of CFU were provided per mL of lung or liver homogenate.

**Lung histology and immunohistology of patients and mice**

Lung tissues of TB-positive patients and controls were removed, fixed in 10% formalin for 24h and embedded in paraffin. Lungs of mice infected with \textit{M. tuberculosis} were removed 2, 6 or 46 weeks after infection, fixed in 10% formalin for 24h and embedded in paraffin. Of both human and mouse lung tissues 4 µm-thick sections were stained with hematoxylin and eosin (H&E) and ZN. For mouse studies H&E-stained slides were coded and scored from 0 (absent) to 4 (most severe) for the following parameters: interstitial inflammation, endothelialitis, peri-bronchitis, edema, granuloma formation and pleuritis by a pathologist without knowledge of the genotype of mice or treatment\textsuperscript{16, 25, 26}. The total ‘lung inflammation score’ was expressed as the sum of the scores for each parameter, the maximum being 24. Confluent (diffuse) inflammatory infiltrate was quantified separately and expressed as percentage of the lung area. Immunohistochemical detection of membrane-bound (m) EPCR on human and mouse tissues was done as described\textsuperscript{27-29}. Briefly, for human stainings after quenching endogenous peroxidase activity and blocking nonspecific binding, slides were incubated with a monoclonal goat-anti-human EPCR antibody (mAb1489) as primary antibody, followed by polymer mouse-anti-goat-HRP (Immunologic, Duiven, The Netherlands) as secondary antibody. Slides were developed using 3,3’-diaminobenzidine-tetrahydrochloride (DAB; Sigma, St Louis, MO). For stainings of mouse tissue, slides were incubated with a goat anti-mouse EPCR poly-
clonal antibody (GT262) as primary antibody, followed by rabbit-anti-goat IgG (SouthernBiotec, Birmingham, AL) as secondary antibody and polymer anti-rabbit-HRP (Immunologic, Duiven, The Netherlands) as a tertiary antibody. Slides were counterstained with methylgreen (Sigma-Aldrich, St. Louis, MO). The total tissue area of the mEPCR-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, NIH, Bethesda, MD) and the immunopositive (EPCR+) area was expressed as the percentage of the total lung surface area.

Flow cytometry
Lung cell suspensions were obtained by crushing lungs through a 40-μm pore-size cell strainer (BD, San Jose, CA) as described previously16, 25, 26. Erythrocytes were lysed using ACK lysing buffer (BioWhittaker, Verviers, Belgium); the remaining cells were washed twice with FACS-buffer (phosphate buffered saline (PBS) supplemented with 0.5% BSA, 0.01% Na₃, and 0.35 mM EDTA) and counted using a haemocytometer. Cells were brought to a concentration of 1 x 10⁷ cells/mL in FACS-buffer. Immunostaining for cell-surface molecules was performed for 30 minutes at 4°C using directly labeled antibodies against CD3 (CD3-phycoerythrin), CD4 (CD4-allophycocyanin), CD8 (CD8-peridinin chlorophyl protein) or GR-1 (GR-1-fluorescein isothiocyanate (FITC)). All antibodies were used in concentrations recommended by the manufacturer (BD Pharmingen, San Diego, CA). After staining, cells were fixed in 2% paraformaldehyde and the percentages of cells were determined by flow cytometric analysis using a fluorescence-activated cell sorter (FACS Calibur; Becton Dickinson Immunocytometry Systems, San Jose, CA). The percentages of polymorphonuclear cells (PMNs), macrophages and lymphocytes were determined using GR-1 expression (GR-1 high, intermediate and low, respectively) and T-cell surface proteins were analyzed on CD3+ cells within the lymphocyte gate.

Assays
For cytokine measurements, organ homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100 and protease inhibitor cocktail (Roche, Indianapolis, IN) and incubated on ice for 30 min. Homogenates were centrifuged at 1500g at 4°C for 15 min and supernatants were sterilized using a 0.22μm pore-size filter (Corning Inc., Corning, NY) and stored at -20°C until analysis. Mouse lung EPCR-levels were measured as described30, using monoclonal rat-anti-mouse EPCR (Mab1560) as capture antibody and polyclonal goat-anti-mouse EPCR (GT262) as detection antibody. Levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1b, IL-4, IL-6, IL-10, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 were measured by ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MO). Thrombin-antithrombin complexes were assayed with an ELISA from Siemens Healthcare Diagnostics (Marburg, Germany).
Statistical analysis
All data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Comparisons between groups were performed using a Mann-Whitney U test. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P-values < 0.05 were considered statistically significant.

RESULTS

Tissue EPCR expression during human pulmonary TB
The lungs are the primary site of infection during human TB³,³¹, although also systemic effects on inflammation and coagulation can be observed in these patients²,³,¹⁴,¹⁵,³¹,³². In order to investigate the effects of EPCR during human pulmonary TB, we measured cell-associated mEPCR expression in lung tissues of proven TB-infected patients and compared them with control lung tissues. Overall, expression of mEPCR in lung tissues of TB-infected patients was similar to controls (Figure 1A). However, TB-infected lungs showed markedly diminished mEPCR inside granuloma. This was present in all patients studied. Figure 1B-G show representative photographs of respectively EPCR, H&E and ZN-stained slides (original magnification x100) of control (B-D) and TB-infected (E-G) lung tissues.

EPCR-expression is upregulated in murine TB
Considering that it is not feasible to study the role of EPCR at tissue level during human TB, we used our well established mouse model of intranasal inoculation with live M. tuberculosis to induce pulmonary TB¹⁶,²⁵,²⁶. Mice were sacrificed after 2, 6 and 46 weeks and EPCR protein levels in whole lung homogenates, indicative of the sum of m- and sEPCR in the pulmonary compartment, were measured. Pulmonary TB was associated with a significant increase in EPCR levels in lung homogenates at 6 and 46 weeks after inoculation (for both time points P < 0.05 versus uninfected mice; Figure 2A). Next, we assessed expression of mEPCR in lung tissue slides of WT and Tie2-EPCR mice 2, 6 and 46 weeks after inoculation and compared them with uninfected WT and Tie2-EPCR mice respectively. Compared to uninfected mice, both infected WT and Tie2-EPCR mice displayed significantly increased lung mEPCR-expression (Figure 2B). Figure 2C-J show representative photographs of lung tissues stained for mEPCR (original magnification x100) of uninfected mice (2C, G) and mice with pulmonary TB (all other). WT mice (2C-F) were compared with Tie2-EPCR mice (2G-J), 2, 6 and 46 weeks after infection. Clearly, mEPCR was abundantly present in lung tissue of Tie2-EPCR mice, showing mEPCR-positive staining not only in small arterioles but also in lung capillaries (2G-J). Together, these data indicate that during murine pulmonary TB EPCR is upregulated, suggesting an important role for this receptor in the host defense against this pathogen.
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Overexpression of EPCR has limited effects on mycobacterial growth and dissemination

Our experimental model of TB is associated with marked mycobacterial growth in the lungs and with bacterial dissemination to distant sites, such as the liver\textsuperscript{16, 25, 26}. To investigate whether overexpression of EPCR impacts on pulmonary bacterial growth we infected WT and Tie2-EPCR mice with viable \textit{M. tuberculosis} and determined mycobacterial loads in the lungs 2 and 6 weeks after infection. No significant differences were seen in bacterial loads in the lungs between WT and Tie2-EPCR mice at either time point (Figure 3A). To study the dissemination of \textit{M. tuberculosis}, mycobacterial loads were measured in liver homogenates. No differences were seen between WT and Tie2-EPCR mice (Figure 3B). Finally, to examine a possible role for EPCR in chronic infection and to find out whether overexpression of EPCR impacted on survival, mice were followed during a 46 weeks observation period. During this period, 8 out of 18 animals died in the WT group, while 12

Figure 1. EPCR in human lung tissues. Human EPCR-staining of lung tissues of TB-positive patients (N = 8) compared with control lung tissues of uninfected patients (N = 10), as described in the Methods section (A). Stained areas are presented as the percentage of the total lung surface area. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (Mann-Whitney \textit{U} test). Representative photographs of uninfected control tissues (B-D) and TB-infected tissues (E-G), clearly showing well-defined granuloma, stained for human mEPCR (B and E respectively), H&E (C, F) and Ziehl-Neelsen (D, G) (original magnification 100x, inset 400x).
out of 19 Tie2-EPCR mice died ($P=0.24$). In the remaining mice mycobacterial loads were similar in lungs (Figure 3A) and liver (Figure 3B) of both mouse strains. These data indicate that overexpression of EPCR has little impact on bacterial growth and host survival during experimental TB.

**EPCR-overexpression reduces lung coagulation activation during pulmonary TB**

Considering the important role for EPCR in the regulation of hemostasis, we next wondered whether EPCR-overexpression would impact on activation of coagulation. Therefore TATc, a parameter of coagulation-activation, was measured in lung homogenates and plasma of WT and Tie2-
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EPCR mice 2, 6 and 46 weeks after inoculation with *M. tuberculosis* (Figure 4A-B). Clearly, after 6 weeks of infection, EPCR-overexpression reduced coagulation activation as reflected by decreased levels of TATc, in lung homogenates of Tie2-EPCR mice (median 9.7 versus 7.0 in WT; *P* < 0.01; Figure 4A). Furthermore, in plasma TATc levels both in WT and Tie2-EPCR mice did not rise until the chronic phase of disease, after 46 weeks of infection (Figure 4B).

**EPCR-overexpression does not impact on lung histopathology**

To investigate whether overexpression of EPCR was accompanied by altered lung inflammation during murine TB, we performed histopathologic analyses of lung tissue slides prepared from WT and Tie2-EPCR mice 2, 6 and 46 weeks after infection. At all time-points, both groups displayed equal levels of granulomatous inflammation, which was hardly visible after 2 weeks of infection, but...
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Increased in extent and severity after 6 and 46 weeks of infection. In line, total pathology scores were also similar after 2 and 6 weeks of infection, while after 46 weeks the pulmonary inflammation was so extensive that it was impossible to reliably determine pathology scores (Figure 5A). The percentages of inflamed lung area did not differ significantly between WT mice and Tie2-EPCR mice either (Figure 5B). Figure 5C-H show representative photographs of lung histology of WT (5C-E) and Tie2-EPCR mice (5F-H) 2, 6 and 46 weeks after infection with M. tuberculosis.

Cellular composition of lung infiltrates in WT and Tie2-EPCR mice

To obtain more insight into the cellular composition of the pulmonary infiltrates in WT and Tie2-EPCR mice, we prepared whole lung cell suspensions at 2 and 6 weeks after infection and determined subsets of inflammatory cells by FACS-analysis (Table 1). For all time points, total leukocyte counts were similar in both WT and Tie2-EPCR mice. After 2 and 6 weeks of infection with M. tuberculosis Tie2-EPCR mice had significantly increased percentages of macrophages in their lungs as compared to WT mice (P < 0.001 and P < 0.05 for 2 and 6 weeks respectively). Percentages of PMNs and lymphocytes were similar after 2 and 6 weeks of infection in both WT and Tie2-EPCR mice. As CD4+ and CD8+ lymphocytes are important cells in the host response during TB, we analyzed whole-lung CD3+ lymphocytes with respect to expression of CD4 and CD8. The percentages of CD3+/CD4+ and CD3+/CD4+ lymphocytes were similar in both groups at all time points (Table 1).

Cytokine and chemokine response during experimental TB

Cytokines and chemokines play a pivotal role in the regulation of the immune response to TB. Therefore we measured the concentrations of TH1 cytokines (IFN-γ, TNF-α), TH2 cytokines (IL-4, IL-6, IL-10), IL-1β and chemokines (MIP-2, KC) in lung homogenates obtained 2, 6 and 46 weeks after infection. The concentrations of all mediators were similar in lungs of WT and Tie2-EPCR mice at all time points (Table 2).

Deficiency of EPCR, APC-overexpression or selective inhibition of (A)PC has limited impact on bacterial growth, inflammation and coagulation during experimental pulmonary TB

Previous studies using mice with limited APC generation (TMpro/pro-mice) demonstrated that APC may contribute to a protective phenotype with respect to bacterial loads in lung and liver homogenates, lung inflammation and levels of pro-inflammatory cytokines. By using EPCR−/− mice, APChigh-mice and mice treated with (A)PC blocking antibodies we aimed to further investigate the impact of EPCR and APC on experimental pulmonary TB. After intranasal inoculation, mice were sacrificed after 6 weeks and bacterial loads were counted in lung and liver homogenates (Figure 6A-B). Only in EPCR−/−mice bacterial loads in liver homogenates were significantly higher when compared to WT mice (Figure 6B; P < 0.01). Lung bacterial loads were similar in WT mice when compared to EPCR−/−mice, APChigh-mice or mice treated with anti-(A)PC antibodies (Figure 6A). Next, to find out whether deficiency of EPCR, overexpression of APC or inhibition
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of (A)PC would impact on lung histology, we measured mean lung histology scores and percentages of lung inflammation. No differences in mean lung histology score were seen between WT and mutant mice (Figure 6C). However, EPCR−/− mice and mice in which both the anticoagulant and cytoprotective function of APC were blocked with a monoclonal antibody (MPC1609) displayed a significantly enhanced percentage of lung inflammation when compared to WT and control mice respectively (Figure 6D; \( P < 0.05 \) for both comparisons). Then, to show whether EPCR-deficiency, APC-overexpression or blockage of (A)PC would impact on lung coagulation, we measured TATc in lung homogenates. Only EPCR−/− mice had significantly decreased TATc levels, when compared with WT mice (Figure 6E; \( P < 0.01 \)). Finally, we compared total numbers

Figure 5. EPCR-overexpression does not impact on lung histopathology. Similar mean histological scores (A) and percentages of inflammation (B) between WT (grey boxes) and Tie2-EPCR mice (white boxes), 2, 6 and 46 weeks after inoculation with *M. tuberculosis*. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation (Mann-Whitney *U* test). N = 8 (2 and 6 weeks time point) or 7-9 (46 weeks time point) mice per group. N.D. not determinable. Representative histological sections of lungs of WT (C-E) and Tie2-EPCR mice (F-H), infected with 150 CFU of *M. tuberculosis* 2, 6 and 46 weeks earlier (H&E staining, magnification 100x).
Table 1. Effects of EPCR-overexpression on total and differential lung cell counts

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<th>PMNs %</th>
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Total leukocyte counts and differential cell counts in lungs of WT and Tie2-EPCR 2 and 6 weeks after intranasal infection with 150 CFU of M. tuberculosis. Percentages of polymorphonuclear cells (PMNs) macrophages (Mφs) and lymphocytes were determined on Gr-1 expression (Gr-1 high, intermediate and low respectively) and T-cell subsets (CD4+ and CD8+) are presented as the percentage of positive cells in the CD3+ gate. Data are expressed as medians ± interquartile ranges of 8 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 versus WT (Mann-Whitney U test).

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of leukocytes in lungs of TB-infected mice. Figure 6F shows that only mice treated with MPC1609 had significantly increased numbers of total leukocytes compare to mice treated with control antibodies (P < 0.01), while other groups did not show any differences (Figure 6F). With respect to differential cell counts, none of the experiments showed significant differences in percentages of lymphocytes, macrophages or polymorphonuclear neutrophils between the experimental groups (Table 3). However, EPCR−/−-mice did show significantly decreased percentages of CD4+-lymphocytes, while APChigh-mice showed decreased numbers of CD8+-lymphocytes (Table 3; P < 0.05 for both comparisons). Finally, we measured cytokine-levels in EPCR−/−-mice, APChigh-mice and in mice treated with (A)PC-antibodies. EPCR−/−-mice displayed significantly decreased levels of lung TNF-α (Supplementary material; Figure 1, P < 0.05). APChigh-mice and mice treated with anti-(A)PC antibodies did not show any differences in lung cytokines levels when compared to WT mice (Supplementary Material; Figure 1).
Table 2. Effects of EPCR-overexpression on pulmonary cytokine and chemokine levels.

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</tr>
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<td>KC</td>
<td>297 (228-363)</td>
<td>215 (184-223)</td>
<td>727 (637-969)</td>
</tr>
<tr>
<td>MIP-2</td>
<td>377 (325-447)</td>
<td>350 (261-430)</td>
<td>812 (764-869)</td>
</tr>
</tbody>
</table>

Cytokine and chemokine concentrations in lung homogenates of WT and Tie2-EPCR mice 2, 6 and 46 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. IFN-γ interferon-γ; IL interleukin; KC keratinocyte-derived chemokine; MIP-2 macrophage inflammatory protein-2; TNF-α tumor necrosis factor-α. Data are expressed as medians ± interquartile ranges of 8 (2 and 6 weeks time point) or 7-9 (46 weeks time point) mice per group.

Table 3. Effects of EPCR deficiency, APC overexpression and anti-(A)PC antibodies on total and differential lung cell counts.

<table>
<thead>
<tr>
<th></th>
<th>Leukocytes x 10⁶/mL</th>
<th>Mφs %</th>
<th>PMNs %</th>
<th>Lymphocytes %</th>
<th>CD4+ % of total CD3+ lymphocytes</th>
<th>CD8+ % of total CD3+ lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>All 6w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>31 (27-47)</td>
<td>32 (28-33)</td>
<td>2.6 (2.1-2.8)</td>
<td>65 (63-69)</td>
<td>57 (55-59)</td>
<td>43 (41-45)</td>
</tr>
<tr>
<td>EPCR+</td>
<td>42 (28-63)</td>
<td>38 (34-43)*</td>
<td>2.8 (1.6-3.3)</td>
<td>60 (52-62)</td>
<td>47 (41-56)*</td>
<td>53 (44-59)*</td>
</tr>
<tr>
<td>WT</td>
<td>36 (29-50)</td>
<td>27 (22-27)</td>
<td>1.6 (1.5-1.7)</td>
<td>71 (71-76)</td>
<td>61 (58-63)</td>
<td>39 (37-42)</td>
</tr>
<tr>
<td>APC+</td>
<td>46 (33-76)</td>
<td>26 (24-29)</td>
<td>1.7 (1.6-2.2)</td>
<td>72 (69-74)</td>
<td>66 (62-69)*</td>
<td>34 (31-38)*</td>
</tr>
<tr>
<td>MCO1716</td>
<td>14 (10-16)</td>
<td>37 (36-39)</td>
<td>4.3 (3.6-5.4)</td>
<td>57 (54-59)</td>
<td>59 (52-66)</td>
<td>41 (34-48)</td>
</tr>
<tr>
<td>MPC1609</td>
<td>21 (19-27)*</td>
<td>37 (33-39)</td>
<td>4.2 (3.2-5.7)</td>
<td>56 (55-61)</td>
<td>63 (61-65)</td>
<td>37 (36-39)</td>
</tr>
<tr>
<td>MAPC1591</td>
<td>17 (13-24)</td>
<td>36 (34-40)</td>
<td>4.5 (3.5-5.5)</td>
<td>60 (53-60)</td>
<td>65 (57-67)</td>
<td>35 (33-43)</td>
</tr>
</tbody>
</table>

Total leukocyte counts and differential cell counts in lungs of WT mice compared to mice deficient for EPCR (EPCR+*) mice, mice overexpressing APC (APC+*) mice and mice treated with control antibodies (MCO1716), antibodies blocking both the anticoagulant and cytoprotective function of (A)PC (MPC1609) and antibodies blocking only the anticoagulant function of (A)PC (MAPC1591), 6 weeks after intranasal infection with 150 CFU of *M. tuberculosis*. Percentages of polymorphonuclear cells (PMNs) macrophages (Mφs) and lymphocytes were determined on Gr-1 expression (Gr-1 high, intermediate and low respectively) and T-cell subsets (CD4+ and CD8+) are presented as the percentage of positive cells in the CD3+ gate. Data are expressed as medians ± interquartile ranges of 8 mice per group. *P < 0.05, **P < 0.01 versus WT (Mann-Whitney U test).
Figure 6. Deficiency of EPCR, APC-overexpression or selective inhibition of (A)PC has limited impact on bacterial growth, inflammation and coagulation during experimental pulmonary TB.

WT (grey boxes), EPCR-deficient (EPCR⁻/⁻) mice (white boxes), APC-overexpressing (APC[src]) (white dotted boxes) and mice treated with control (MCP1716; grey horizontally stiped boxes) antibodies, antibodies that inhibit both the anticoagulant and cytoprotective function of (A)PC (MPC1609; white horizontally striped boxes) and with antibodies that only inhibit the anticoagulant function of PC (MAPC1591; white vertically striped boxes) were inoculated with 150 CFU of *M. tuberculosis* (Erdman strain) and sacrificed after 6 weeks. Bacterial loads in lung (A) and liver (B) homogenates were measured as well as lung mean histological scores (C) and percentages of lung inflammation (D). Finally, lung TATc levels (E) and total numbers of leukocytes in the lungs (F) were measured. CFU colony forming unit; N number; TATc thrombin-antithrombin complex. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. N = 8 mice per group. *P < 0.05 and **P < 0.01 for WT versus EPCR⁻/⁻ or APCR[src] mice or for MCO1716 treated versus MPC1609-treated mice (Mann-Whitney U test).
DISCUSSION

Ample evidence has indicated that during infection coagulation is activated with inhibition of anticoagulant pathways, responses that are considered to contain the infection and to prevent further dissemination. EPCR enhances anticoagulation by accelerating activation of PC to APC and in addition mediates anti-inflammatory, anti-apoptotic and cytoprotective effects by facilitating APC-mediated signaling via PAR-1. In this study we aimed to investigate the role of EPCR and APC on the host response during TB by analyzing TB-infected human lung tissues and by using our established mouse model of pulmonary TB in which we compared WT mice with mice over-expressing EPCR. EPCR-overexpression in these mice leads to a more rapid conversion of PC into APC. In theory, increased availability of APC could affect the inflammatory response to TB in two ways. Firstly, via deactivation of FVa and FVIIIa, APC provides an anticoagulant environment, resulting in fewer microthrombi and, possibly, formation of less well organized granulomas, since granulomas are surrounded by an extensive fibrotic capsule which is constructed along a scaffold of fibrin, the end product of the coagulation cascade. Secondly, in accordance with its anti-inflammatory, cytoprotective effects, more APC could result in a decreased proinflammatory reaction. In this study we demonstrate that EPCR-overexpressing mice displayed decreased activation of coagulation 6 weeks after infection, which, however, was associated with only small, subtle decreases in the inflammatory response during infection with \textit{M. tuberculosis}.

Previous clinical and preclinical studies have provided evidence for a role for EPCR during infections, including (pneumo)sepsis. It has been hypothesised that during these conditions EPCR is downregulated on injured endothelium, which consequently impairs the ability to generate APC. For example, in children with meningococcal sepsis endothelial mEPCR levels were lower than those in controls. Downregulation of mEPCR may occur both via inhibition of EPCR gene transcription or via protease-mediated shedding from the endothelial cell surface, releasing the soluble form of EPCR (sEPCR) in the circulation. In this study we compared human mEPCR expression on lung tissues of TB-infected patients with uninfected controls. At first glance, lung tissues of TB-patients and in particular granulomatous areas of these tissues appeared to be less vascularised and seemed to contain fewer 'larger vessels' such as arterioles and arteries. This would be suggestive for a decrease in mEPCR staining, as EPCR normally only occurs on endothelium of larger vessels, but not on capillaries. However, after comparison of percentages of EPCR positive-stained tissue, we were not able to show any differences in lung mEPCR expression between the two groups. Our mouse studies showed upregulation of both mEPCR in TB-infected lung tissues and of total EPCR levels in whole lung homogenates. In murine TB, upregulation of EPCR is possibly due to increased vascularisation of TB-infected lungs, which could be related to the fact that mice, contrary to humans, are unable to form well-organized granuloma upon TB-infection. Furthermore, rodent models showed that EPCR mRNA is upregulated potently by thrombin and the mouse EPCR-gene contains a thrombin response element. So increased EPCR levels could also be a result of
the coagulation activation in plasma we observed after 46 weeks of infection. On the other hand, upregulation of EPCR could also be a result of influx of other cell types such as neutrophils or monocytes, of which is known that they may express EPCR as well7,8.

Clinical studies described activation of the coagulation system in TB-infected patients, together with downregulation of major anticoagulant proteins such as antithrombin and PC14,15. In this study, by using EPCR-overexpressing mice, we aimed to influence the procoagulant response and/or change the associated proinflammatory phenotype elicited by M. tuberculosis. Our results show that EPCR-overexpression indeed induce decreased coagulation activation, as reflected by decreased TATc levels in lung homogenates of Tie2-EPCR mice 6 weeks after infection. These data are in line with previous studies showing that Tie2-EPCR mice had decreased coagulation activation during ventilator-induced lung injury41 or after FXa administration13. However, our observation that EPCR−/−mice showed similar effects on coagulation, 6 weeks after infection, is an interesting finding for which we do not have a clear explanation. One explanation could be that in EPCR-deficient mice APC cannot bind to EPCR on the endothelium, leading to relatively higher amounts of ‘free’ APC in the circulation, which the might favor an anticoagulant environment. Of note, no differences in TATc levels were seen between WT and Tie2-EPCR mice 2 and 46 weeks after infection. Interestingly, both in ‘Tie2-EPCR and EPCR−/−mice the differences in coagulation activation were not or only to a limited extent associated with other changes in inflammation. Only a mild increased influx of macrophages in Tie2-EPCR mice compared to WT mice 2 and 6 weeks after infection could be observed, together with enhanced bacterial loads in liver homogenates and increased histopathological scores 2 weeks after infection in EPCR−/−mice when compared to WT mice. Overexpression or deficiency of EPCR did not impact on survival either. These data are in contrast with previous studies in murine TB16. Mice with impaired APC generation as a consequence of a mutation in the APC-generating domain of TM (TMpro/pro-mice), failed to show any significant differences in TATc when compared to WT16. However, TMpro/pro-mice did demonstrate uncontrolled lung inflammation, including higher lung weights and elevated levels of pro-inflammatory cytokines, indicating that at least in this study design the anti-inflammatory properties of APC played a more important role than its anticoagulant effects. Of importance, however, the role of TM in murine TB was studied using a different M. tuberculosis strain (H37Rv versus the more virulent Erdman strain used here) given at a much higher dose (10⁵ versus 150 CFU in the present study)16.

In order to further investigate the impact of the protein C system on the host response against M. tuberculosis, we used APChigh-mice21 and treated mice with antibodies that inhibit both the anticoagulant and cytoprotective function of (A)PC or only the anticoagulant function of PC22. Our data show that overexpression of APC did not impact on coagulation or inflammation during murine TB. This is in contrast with earlier studies demonstrating protective effects of APC overexpression against diabetic nephropathy21 or with studies from our own laboratory demonstrating detrimental effects of APC-overexpression during murine melioidosis with respect to bacterial growth,
neutrophil influx and release of proinflammatory cytokines\cite{42}. Furthermore, treatment with antibodies inhibiting both the (endogenous) anticoagulant and cytoprotective function of APC (MPC1609) showed subtle differences in inflammatory markers when compared with treatment with control antibodies. In particular, MPC1609-treated mice displayed increased lung inflammation scores 6 weeks after infection and had higher influxes in total numbers of leukocytes in their lungs when compared to mice treated with control antibodies. These results are in accordance with recent data from our own laboratory showing increased levels of the pro-inflammatory cytokine IL-6 and increased bacterial loads in MPC1609-treated mice during \textit{E. coli} peritonitis\cite{43}. Our results fail to show a phenotype for MAPC1591-treated mice in which only the anticoagulant function is inhibited, indicating that mainly the cytoprotective and not the anticoagulant activity of (A)PC is involved in the pro-inflammatory changes in these mice. Both in MPC1609 and MAPC1591-treated mice no differences in coagulation activation were seen when compared to treatment with control antibodies. As it is known that inflammation stimulates coagulation and vice versa\cite{4}, the proinflammatory stimulus was apparently too low to induce measurable and significant differences. Of note, one could argue that the lack of any effect could be the result of a short half-life of the used antibodies. However, in a model of LPS-induced sepsis MPC1609 and MAPC1591 were applied only once, while a progressive effect on mortality could still be measured after 36 hours\cite{22}, while in a model of cancer cell extravasation these antibodies were given after 48 and 96 hours, resulting in a sustained detrimental effect on cancer cell extravasation for MPC1609\cite{23}. Therefore, to our opinion, both models clearly illustrate, that a dosage frequency of once every 2 to 3 days is sufficient for modulation of APC-induced effects.

An important component of the host immune response against \textit{M. tuberculosis} is cytokine release\cite{2,3,31}. Mycobacteria mainly grow and survive in phagosomes in alveolar macrophages. Once infected the macrophage elicits a strong proinflammatory response driven by release of various cytokines and chemokines, of which TNF-\(\alpha\) is most important, resulting in recruitment of a range of inflammatory cells, including natural killer cells and lymphocytes\cite{2,31}. This proinflammatory reaction is regulated by production of IFN-\(\gamma\), which also has anti-mycobacterial effects\cite{2,31}. Our data fail to show any differences in cytokine and chemokine release in the lungs between WT and Tie2-EPCR mice. Except for a slight decrease in TNF-\(\alpha\) 2 weeks after infection, no differences were seen between WT and EPCR\(-/-\) mice either. In conclusion, in this study we demonstrated that overexpression or deficiency of EPCR modestly influences local coagulation activation during infection with \textit{M. tuberculosis} without significant changes in inflammatory parameters. Additionally, overexpression of APC or treatment with anti (A)PC antibodies did not have a strong impact on the inflammatory response induced by \textit{M. tuberculosis}. These data suggest that the protein C system plays a limited role in host defense during lung TB.
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Chapter 3

REFERENCES


Supplementary Figure 1. Cytokine-levels in EPCR<sup>+/−</sup>-mice, APC<sup>high</sup>-mice and in mice treated with (A)PC-antibodies. WT (grey boxes), EPCR-deficient (EPCR<sup>+/−</sup>) mice (white boxes), APC-overexpressing (APC<sup>high</sup>) (white dotted boxes) and mice treated with control (MCP1716; grey horizontally striped boxes) antibodies, antibodies that inhibit both the anticoagulant and cytoprotective function of (A)PC (MPC1609; white horizontally striped boxes) and with antibodies that only inhibit the anticoagulant function of PC (MAPC1591; white vertically striped boxes) were inoculated with 150 CFU of *M. tuberculosis* (Erdman strain) and sacrificed after 6 weeks. Cytokine and chemokine concentrations were measured in lung homogenates. IFN-γ interferon-γ; IL interleukin; KC keratinocyte-derived chemokine; MIP-2 macrophage inflammatory protein-2; TNF-α tumor necrosis factor-α. Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. *N* = 8 mice per group. *P* < 0.05 for WT versus EPCR<sup>+/−</sup> mice (Mann-Whitney *U* test).