

Therapeutic Recombinant Murine Activated Protein C Attenuates Pulmonary Coagulopathy and Improves Survival in Murine Pneumococcal Pneumonia

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Background. Recombinant human activated protein C (APC) improves survival of patients with severe sepsis; this beneficial effect is especially apparent in patients with pneumococcal pneumonia. The aim of this study was to determine the effect of APC treatment initiated after induction of pneumococcal pneumonia on pulmonary coagulation, inflammation, and survival, with or without concurrent antibiotic therapy.

Methods. Mice were infected intranasally with viable *Streptococcus pneumoniae* and were treated intraperitoneally after 24 h of infection with vehicle, recombinant mouse (rm) APC (125 µg), ceftriaxone (500 µg), or rm-APC plus ceftriaxone. Treatment with rm-APC or vehicle was repeated every 8 h for a maximum of 96 h. Animals were either killed 48 h after infection or were monitored in a survival study (with an extra dose of ceftriaxone given after 72 h).

Results. Rm-APC treatment inhibited pulmonary activation of coagulation, as reflected by lower levels of thrombin-antithrombin complexes and D-dimer. Rm-APC did not affect the pulmonary levels of 55 inflammatory mediators in the context of antibiotic therapy. Rm-APC added to ceftriaxone markedly improved survival, compared with ceftriaxone treatment alone.

Conclusions. Rm-APC inhibits pulmonary activation of coagulation and, when added to antibiotic therapy, improves survival in murine pneumococcal pneumonia.

Streptococcus pneumoniae is the most common causative pathogen in community-acquired pneumonia (CAP) [1]. CAP is a frequent cause of sepsis. In a recent study of sepsis, 35.6% of the patients experienced severe CAP, with *S. pneumoniae* being the most frequent cause [2]. Worldwide, *S. pneumoniae* is responsible for an

estimated 10 million deaths annually, making pneumococcal pneumonia and sepsis a major health threat [3]. The continuous increase in antibiotic resistance further adds to the clinical challenge posed by the pneumococcus. Clearly, there is an urgent need to expand the therapeutic arsenal for pneumococcal pneumonia and sepsis [4].

Severe bacterial infection results in systemic activation of coagulation and down-regulation of anticoagulant mechanisms and fibrinolysis [5]. In patients with bacterial pneumonia, a similar hemostatic imbalance has been shown in the bronchoalveolar space [6–10]. The protein C (PC) system is an important regulator of both coagulation and inflammation. The end product of this pathway, activated PC (APC), has been shown to have anticoagulant, profibrinolytic, anti-inflammatory, antiapoptotic, and other cytoprotective properties [11]. Patients with pneumonia display re-

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duced PC and APC concentrations in their bronchoalveolar lavage fluid [8, 9]. Down-regulation of the PC pathway has been correlated to disease severity and mortality associated with pneumonia and sepsis [12, 13]. Continuous intravenous infusion of recombinant human (rh) APC for 96 h, which was associated with APC-induced inhibition of coagulation and interleukin 6 (IL-6) release (a marker of systemic inflammation), improved survival among patients with severe sepsis and a high likelihood of death [14]. The beneficial effect of rh-APC in this trial seemed to be especially prominent in patients with severe sepsis due to pneumococcal pneumonia [2].

The remarkable effect of rh-APC on outcomes in patients with severe respiratory tract infection caused by *S. pneumoniae* [2] prompted our group to initiate investigations of the effect of this agent on pulmonary coagulation and inflammation in a rat model of pneumococcal pneumonia [15]. In this study, repeated intravenous bolus injections of rh-APC started 30 min before induction of infection were shown to attenuate pulmonary coagulation without influencing lung cytokine concentrations or pathology [15]. Of note, however, this investigation did not mimic the clinical scenario of APC administration during an already-established respiratory tract infection and moreover did not determine the effects of APC in the context of antibiotic therapy. Therefore, in the present study we sought to assess the effects of APC in a therapeutic setting using our well-characterized mouse model of pneumococcal pneumonia [7, 10, 16]. In this study, we initiated treatment with rm-APC 24 h after infection with *S. pneumoniae*, with or without concurrent antibiotic therapy. We examined the effects of therapeutic rm-APC in the context of antibiotic therapy on pulmonary coagulopathy and inflammation using a combination of methods, including assays for coagulation, a multiplex protein assay measuring 55 mediators implicated in inflammation, semiquantitative histopathologic and immunohistochemical evaluation, and quantitative bacterial cultures of samples from the lungs and distant body sites. Moreover, we performed a survival study.

We show here for the first time, to our knowledge, that therapeutic APC exerts combined anticoagulant and anti-inflammatory effects in the lungs in a clinically relevant mouse model of CAP due to *S. pneumoniae* and moreover that survival improves when APC is given concurrently with antibiotics, compared with when antibiotic therapy is given alone.

METHODS

Animals. Male C57BL/6 mice were purchased from Charles River. Ten-week-old mice were used in all experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Experimental infection and treatment. Pneumonia was induced by intranasal inoculation of $\sim 5 \times 10^4$ colony-forming

units of *S. pneumoniae* serotype 3 (American Type Culture Collection 6303), as described elsewhere [7, 10, 16]. Rm-APC and vehicle were obtained from Eli Lilly [17]. Rm-APC and vehicle were diluted in sterile pyrogen-free isotonic saline; ceftriaxone (Pharmachemie BV) was reconstituted with sterile pyrogen-free water and diluted likewise. In both experiments, 4 groups of mice were treated 24 h after infection with either vehicle, ceftriaxone (500 μ g), rm-APC (125 μ g), or ceftriaxone plus rm-APC. Treatment with rm-APC and vehicle was repeated every 8 h. Mice were killed 48 h after infection (8 per group) or were observed in a survival study (16 per group). In the survival study, ceftriaxone treatment was repeated after 72 h of infection. Treatment with rm-APC or vehicle was continued for a total of 96 h. Five uninfected mice were killed for establishing baseline levels of coagulation markers.

After mice were killed, citrated plasma was prepared from blood samples obtained from the vena cava inferior. Lung homogenates were prepared as described elsewhere [7, 10, 16]. In brief, the left lung was harvested and homogenized at 4°C in 4 mL of sterile saline by means of a tissue homogenizer (Biospect Products). For measurements in lung tissue samples, lung homogenates were diluted 1:2 with lysis buffer (300 mmol/L NaCl, 30 mmol/L Tris, 2 mmol/L MgCl₂, 2 mmol/L CaCl₂, and 1% [vol/vol] Triton X-100 [pH, 7.4]); protease inhibitor mix was added (AEBSF [4-(2-aminoethyl)benzeensulfonylfluoride], EDTA-NA₂, pepstatin, and leupeptin [MP Biomedical]; concentrations were in accordance with the manufacturer's recommendations), and samples were incubated on ice for 30 min, followed by centrifugation at 680 g for 10 min. Supernatants were stored at -20°C until analysis. Bacterial outgrowth was determined as described elsewhere [16, 18, 19].

Assays. Levels of thrombin-antithrombin complexes (TATc) and D-dimer in lung homogenates were measured by enzyme-linked immunosorbent assay (ELISA) (Behringwerke AG for TATc and Asserachrom D-dimer [Roche]). Myeloperoxidase (Myeloperoxidase [HyCult Biotechnology]) levels were measured by ELISA. Levels of the following cytokines, chemokines, and other inflammatory markers were measured by a multiplex assay (Rules-Based Medicine): CCL-2, -3, -4, -5, -7, -9/10, -11, -12, -19, and -21; CXCL-1, -2, -6, and -10; XCL-1; CD40; CD40L; C-reactive protein; endothelial growth factor; endothelin; factor VII; fibrinogen; fibroblast growth factor basic and 9; (granulocyte) macrophage-colony stimulating factor; glutathione S-transferase; interferon γ ; IL-1 α , -1 β , -2, -3, -4, -5, -6, -7, -10, -11, -12p70, -17, and -18; leukemia inhibitory factor; matrix metalloproteinase 9; myoglobin; oncostatin M; serum amyloid P; stem cell factor; tissue factor; tissue inhibitor of metalloproteinase 1; tumor necrosis factor α (TNF- α); thrombopoietin; vascular cell adhesion molecule 1; vascular endothelial cell growth factor; and von Willebrand factor.

Histology and immunohistochemistry. The right lung was

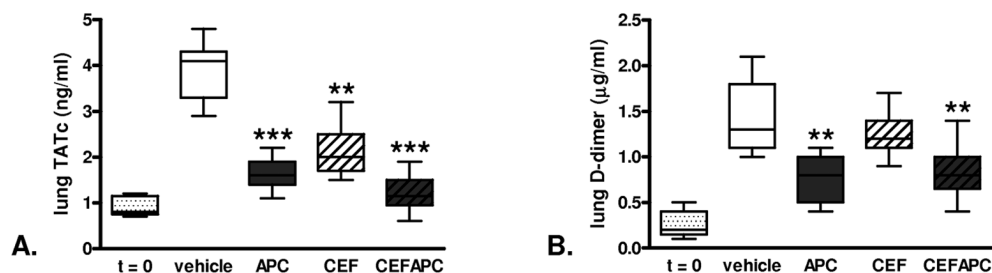


Figure 1. Attenuation of pulmonary activation of coagulation in pneumococcal pneumonia due to treatment with recombinant murine activated protein C (rm-APC) and/or ceftriaxone (CEF). Shown are lung levels of thrombin-antithrombin complexes (TATc; A) and D-dimer (B) in uninfected mice ($t = 0$; $n = 5$) and after induction of pneumococcal pneumonia in mice treated with vehicle at 48 h (white); rm-APC at 24, 32, and 40 h (gray); CEF at 24 h (striped); or rm-APC at 24, 32, and 40 h and CEF at 24 h (gray, striped). Data are for 8 mice per group at each time point. ** $P < .01$ and *** $P < .001$ for the comparison with vehicle (Mann-Whitney U test).

fixed in 10% formaline and phosphate-buffered saline at room temperature for 24 h and embedded in paraffin. Sections of 5 μm were cut, stained with hematoxylin-eosin, and analyzed by a pathologist who was blinded to the groups. To score lung inflammation and damage, the entire lung was analyzed with respect to the following parameters: bronchitis, interstitial inflammation, edema, endothelialitis, pleuritis, and thrombus formation. Each parameter was graded on a scale of 0–4 (0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe). The total histo-

pathologic score was expressed as the sum of the scores for the different parameters.

Granulocyte staining was performed using fluorescein isothiocyanate-labeled anti-mouse Ly-6G monoclonal antibody (Pharmingen), as described elsewhere [19, 20]. Ly-6G-stained slides were photographed with a microscope equipped with a digital camera (Leica CTR500; Leica Microsystems). Ten random pictures were taken per slide. Stained areas were analyzed

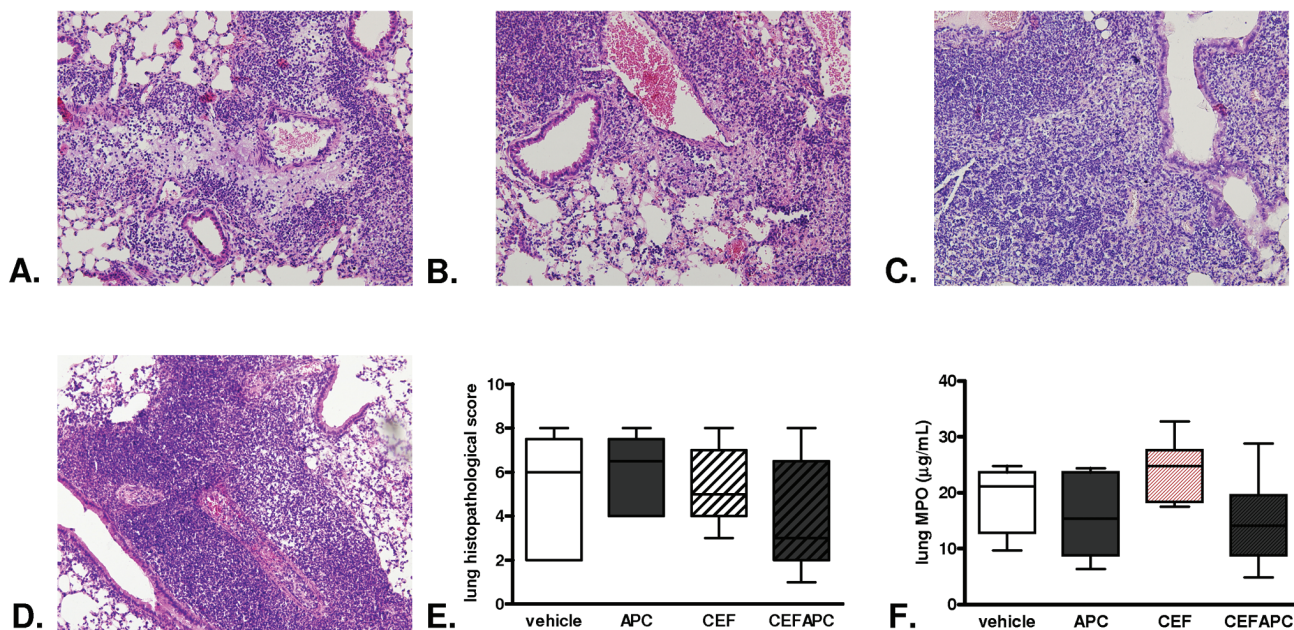


Figure 2. No influence of treatment with recombinant murine activated protein C (rm-APC) on lung histopathology and myeloperoxidase (MPO) in pneumococcal pneumonia. Shown are representative slides of lung hematoxylin-eosin stainings 48 h after induction of pneumococcal pneumonia in mice treated with vehicle (A); rm-APC at 24, 32, and 40 h (B); ceftriaxone at 24 h (C); or rm-APC at 24, 32, and 40 h and ceftriaxone (CEF) at 24 h (D). Original magnification, $\times 100$. Also shown are total pathology scores (E) and lung MPO levels (F) 48 h after induction of pneumococcal pneumonia in mice treated with vehicle (white); rm-APC at 24, 32, and 40 h (gray); CEF at 24 h (striped); or rm-APC at 24, 32, and 40 h and CEF at 24 h (gray, striped). Data are for 8 mice per group. There were no statistically significant differences between the groups (Kruskal-Wallis test).

Table 1. Effect of Recombinant Murine Activated Protein C (rm-APC), Administered With or Without Ceftriaxone, on Lung Levels of Inflammatory Mediators 48 h after Induction of Pneumococcal Pneumonia

| Inflammatory mediator | Treatment | | | |
|------------------------|------------------|-------------------------------|-------------------------------|-------------------------------|
| | Vehicle | rm-APC | Ceftriaxone | Ceftriaxone plus rm-APC |
| CCL-7, pg/mL | 6.30 (1.58–8.50) | 12.1 (10.3–15.6) ^a | 1.13 (0.77–3.27) | 0.92 (0.63–1.46) |
| CCL-11, pg/mL | 1.14 (1.05–1.20) | 1.13 (0.95–1.42) | 0.67 (0.62–0.95) ^a | 0.99 (0.89–1.22) |
| CCL-19, ng/mL | 3.19 (3.09–3.62) | 3.35 (2.89–3.43) | 2.51 (2.23–3.15) ^a | 3.04 (2.85–3.38) |
| CXCL-1, ng/mL | 3.08 (0.66–8.18) | 4.85 (2.81–5.80) | 0.31 (0.17–1.09) | 0.27 (0.17–0.49) ^a |
| CXCL-10, pg/mL | 26.3 (4.16–27.4) | 31.3 (20.4–38.6) | 2.36 (1.12–5.90) | 1.90 (1.07–3.48) ^a |
| Endothelin, pg/mL | 93.5 (90.0–114) | 93.5 (70.0–141) | 63.5 (58.5–88.0) ^a | 51.5 (47.5–107) |
| Factor VII, ng/mL | 6.70 (5.80–8.70) | 6.55 (5.60–6.95) | 5.40 (5.00–5.80) ^a | 5.30 (4.95–5.70) ^b |
| FGF-basic, ng/mL | 9.20 (6.60–13.4) | 6.00 (5.10–8.00) ^a | 5.40 (5.30–9.30) | 7.80 (6.55–9.25) |
| Fibrinogen, μ g/mL | 13.4 (5.4–16.0) | 32.8 (21.1–130) ^a | 14.8 (2.1–25.8) | 36.3 (27.4–103) ^b |
| IL-1 β , ng/mL | 9.80 (8.20–14.9) | 11.9 (9.20–16.4) | 7.40 (6.60–8.20) ^a | 6.75 (6.30–8.00) ^b |
| IL-4, pg/mL | 111 (58.0–165) | 110 (102–117) | 58.0 (39.0–83.0) | 40.0 (28.5–56) ^a |
| IL-6, pg/mL | 461 (102–1070) | 820 (399–1070) | 40.0 (20.0–143) | 42.5 (35–99) ^a |
| IL-11, pg/mL | 368 (98.0–470) | 423 (317–560) | 89.0 (39.0–193) | 65.0 (45.0–97.0) ^a |
| MMP-9, ng/mL | 0.44 (0.41–0.56) | 0.55 (0.40–0.85) | 0.20 (0.12–0.39) ^a | 0.35 (0.18–0.44) |
| Myoglobin, ng/mL | 20.6 (18.4–37.5) | 35.2 (27.5–41.3) | 27.3 (14.1–43.5) | 44.1 (31.5–62.0) ^a |
| OSM, pg/mL | 1210 (368–1540) | 1230 (925–1345) | 270 (208–615) | 238 (188–348) ^a |
| TNF- α , ng/mL | 1350 (253–1740) | 1500 (965–1660) | 290 (160–650) | 218 (158–374) ^a |
| TPO, ng/mL | 45.0 (41.6–54.6) | 47.5 (43.3–50.0) | 36.4 (31.7–39.9) ^a | 38.0 (35.4–43.0) |

NOTE. Data are median (interquartile range) lung levels. Only inflammatory markers with statistically significant differences in one of the treatment arms, compared with vehicle, are shown. All other markers showed no differences between the groups (see Methods for a complete list). FGF, fibroblast growth factor; IL, interleukin; MMP, matrix metalloproteinase; OSM, oncostatin M; TNF, tumor necrosis factor; TPO, thrombopoietin.

^a $P < .05$ for the comparison with vehicle (Mann-Whitney U test).

^b $P < .01$ for the comparison with vehicle (Mann-Whitney U test).

using Image Pro Plus (Media Cybernetics), and results were expressed as the percentage of the total surface area.

Statistical analysis. Data are expressed as box-and-whisker diagrams showing the smallest observation, lower quartile, median, upper quartile, and largest observation; as medians with interquartile ranges; or as Kaplan-Meier plots. Differences between groups were determined using the Kruskal-Wallis test, the Mann-Whitney U test, or the log rank test, as appropriate. Analyses were performed using Prism software (version 4.0; GraphPad). Differences with $P < .05$ were considered to be statistically significant.

RESULTS

Activation of coagulation. To establish the effect of rm-APC on pulmonary activation of coagulation in a clinically relevant model of pneumococcal pneumonia, we performed an analysis of treatment with rm-APC and/or ceftriaxone after 24 h of infection. Rm-APC and vehicle administration was repeated every 8 h until 48 h after infection, after which animals were killed. In this model, rm-APC attenuated pulmonary activation of coagulation, as evidenced by lower pulmonary levels of TATc and D-dimer; this effect was present in both the absence and

presence of concurrent ceftriaxone treatment (Figure 1A and 1B). Of note, TATc levels were lower in mice treated with ceftriaxone alone than in mice not treated with antibiotics.

Inflammation. Pneumococcal pneumonia was associated with pulmonary inflammation and damage, as evidenced by the occurrence of bronchitis, interstitial inflammation, edema, and endothelialitis. There were no differences in mean histopathologic scores between mice treated with vehicle, rm-APC, ceftriaxone, or rm-APC plus ceftriaxone 48 h after infection (Figure 2A–2E). Moreover, there were no differences between the treatment arms in the separate scores for bronchitis, interstitial inflammation, edema, and endothelialitis (data not shown). There were also no differences in neutrophil influx between the different treatment arms, as indicated by Ly-6G positivity scores (data not shown). In accordance with these findings, pulmonary myeloperoxidase concentrations were similar in the different treatment groups (Figure 2F).

To further investigate the effect of rm-APC treatment with or without concurrent antibiotic treatment on the pulmonary inflammatory response in pneumonia, we determined levels of 55 inflammatory markers in lung homogenates obtained 48 h after infection. After 48 h of infection, administration of rm-

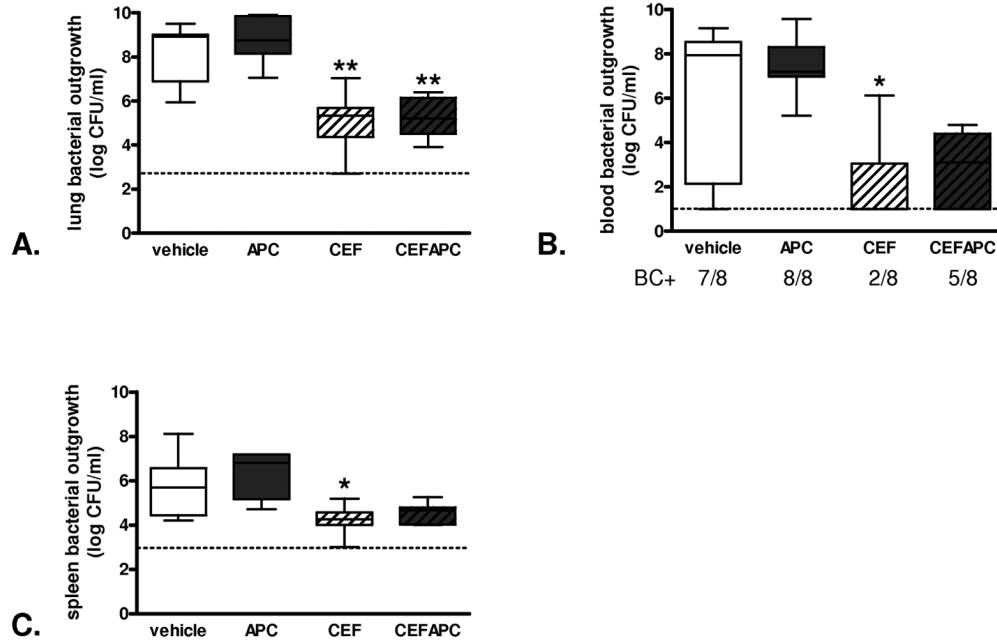


Figure 3. Decreased bacterial outgrowth in pneumococcal pneumonia due to ceftriaxone (CEF) treatment but no influence of concurrent treatment with recombinant murine activated protein C (rm-APC). Shown is bacterial outgrowth in lung (A), blood (B), and spleen (C) samples after induction of pneumococcal pneumonia in mice treated with vehicle at 48 h (white); rm-APC at 24, 32, and 40 h (gray); CEF at 24 h (striped); or rm-APC at 24, 32, and 40 h and CEF at 24 h (gray, striped). Data are for 8 mice per group. The total number of positive blood culture results per group is shown below the graph. * $P < .05$ and ** $P < .01$ for the comparison with vehicle (Mann-Whitney U test).

APC alone (ie, without antibiotics) lowered levels of basic fibroblast growth factor only and increased levels of CCL-7 and fibrinogen (Table 1). Treatment with ceftriaxone alone reduced levels of various pulmonary inflammatory markers, significantly for CCL-11, CCL-19, IL-1 β , endothelin, factor VII, matrix metalloproteinase 9, and thrombopoietin. Treatment with a combination of rm-APC and ceftriaxone decreased levels of more pulmonary inflammatory markers significantly, including CXCL-1, CXCL-10, factor VII, IL-1 β , IL-4, IL-6, IL-11, oncostatin-M, and TNF- α . Comparison between mice treated with ceftriaxone alone and mice treated with ceftriaxone and rm-APC did not yield significant differences.

Bacterial outgrowth. Rm-APC treatment did not have an effect on bacterial outgrowth in lung, blood, or spleen samples (Figure 3A–3C) 48 h after infection. As expected, ceftriaxone treatment, either in combination with rm-APC or alone, decreased bacterial loads; however, for combination treatment with rm-APC this decrease was significant only for lung samples ($P = .15$ for the comparison with blood samples; $P = .07$ for the comparison with spleen samples).

Survival. To substantiate whether treatment with rm-APC alone or in combination with ceftriaxone had an effect on survival in murine pneumococcal pneumonia, a survival study was performed (Figure 4). All animals not treated with ceftriaxone died within 4 days after infection; treatment with rm-APC failed to affect mortality in this group. As expected, sur-

vival was prolonged significantly in ceftriaxone-treated mice, compared with vehicle-treated mice ($P < .001$). Remarkably, survival was significantly prolonged with rm-APC and ceftriaxone combination treatment, compared with ceftriaxone treatment alone ($P < .01$ for the difference between these 2 groups).

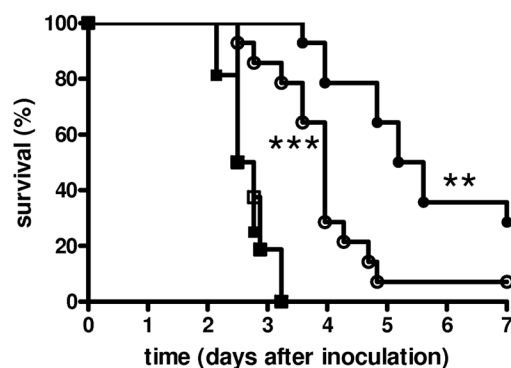


Figure 4. Prolongation of survival in mice due to ceftriaxone treatment and increased prolongation due to concurrent recombinant murine activated protein C (rm-APC) treatment. Shown is survival among mice treated with vehicle every 8 h ($n = 16$; white squares), rm-APC every 8 h ($n = 16$; black squares), ceftriaxone at 24 and 72 h ($n = 16$; white circles), or ceftriaxone at 24 and 72 h plus rm-APC every 8 h ($n = 16$; black circles), for a total of 96 h in murine pneumococcal pneumonia. ** $P < .01$ for the comparison with ceftriaxone, and *** $P < .001$ for the comparison with vehicle (log rank test).

DISCUSSION

Infusion of rh-APC has been found to reduce mortality among patients with severe sepsis [14]; this beneficial effect was especially present in patients with pneumococcal pneumonia [2]. Recently, using a rat model of *S. pneumoniae* pneumonia, we established that pretreatment with rh-APC attenuated pulmonary and systemic activation of coagulation without influencing inflammation. In the present study, we sought to determine the pulmonary effects of APC in a more clinically relevant context in which rm-APC was administered 24 h after induction of pneumonia, with or without concurrent antibiotic therapy. Moreover, we studied the effect of APC on inflammation in more detail, using a multiplex assay that concurrently measures the levels of 55 inflammatory mediators. We have shown that therapeutic rm-APC inhibits pulmonary and systemic coagulation activation without a detectable effect on lung inflammation. Moreover, we have shown that rm-APC added to ceftriaxone treatment significantly and markedly improves survival in pneumococcal pneumonia.

Systemic administration of rh-APC has been shown to inhibit systemic coagulopathy both in preclinical models of sepsis and in patients with sepsis [11, 14, 21]. In addition, previous studies have documented the capacity of intravenous APC to inhibit coagulation activation in the lungs after administration of lipopolysaccharide [22] or viable bacteria [9, 15] via the airways. Specifically, intravenous infusion of rh-APC attenuated bronchoalveolar activation of coagulation in healthy human volunteers after a bronchial or intratracheal challenge with lipopolysaccharide [22] and in rats infected with *S. pneumoniae* [15] or *Pseudomonas aeruginosa* [9]. Of note, however, intravenous rh-APC augmented *P. aeruginosa*-induced coagulation activation in the bronchoalveolar space in rats when initiated concomitantly with induction of pneumonia [23]. The present investigation differs in several ways from these earlier studies. First, in all previous studies APC infusion was started before or simultaneous with the administration of lipopolysaccharide or bacteria [9, 15, 22, 23], whereas we initiated APC 24 h after infection, more closely mimicking the clinical situation. Second, APC infusion was not combined with antibiotic therapy in any of the previous investigations [9, 15, 22, 23], whereas we evaluated the effect of APC in the context of concurrent treatment with ceftriaxone as well. Third, we used species-specific APC (rm-APC), whereas the earlier studies used rh-APC [9, 15, 22, 23]. We have confirmed here the anticoagulant properties of APC in the context of established respiratory tract infection due to *S. pneumoniae*; rm-APC inhibited activation of coagulation in the lungs when given 24 h after induction of pneumonia. Of note, whereas ceftriaxone administered 24 h after infection also modestly attenuated pulmonary coagulation (as reflected by reduced TATc concentrations in lung homogenates for antibiotic-treated mice, compared with mice not treat-

ed with antibiotics)—an effect most likely attributable to an ~1000-fold reduction in bacterial loads in the lungs—rm-APC further attenuated pulmonary coagulation in this context of antibiotic therapy.

Levels of various cytokines and chemokines were lower (significantly for CCL-11, CCL-19, IL-1 β , endothelin, factor VII, matrix metalloproteinase 9, and thrombopoietin) in ceftriaxone-treated mice than in control mice not treated with antibiotics, most likely secondary to the reduction in bacterial loads. Pulmonary levels of CXCL-1, CXCL-10, factor VII, IL-1 β , IL-4, IL-6, IL-11, oncostatin M, TNF- α , and IL-6 were lower in mice treated with ceftriaxone and rm-APC than in mice not treated with antibiotics. The administration of rm-APC did not affect mediator levels in the presence of concurrent antibiotic therapy. In previous studies, our group was unable to detect an effect of systemic rh-APC administration on the pulmonary levels of TNF- α , IL-6, or cytokine-induced neutrophil chemoattractant 3 during either *S. pneumoniae* or *P. aeruginosa* pneumonia in rats [9, 15]. Intravenous APC treatment reduced pulmonary levels of TNF- α , IL-6, and several chemokines in rodent models of sterile lung injury induced by acid aspiration [24] or intratracheal administration of elastase [25]. Additional evidence that APC can inhibit pulmonary cytokine and chemokine production was reported in a study in which intravenous APC treatment diminished levels of TNF- α , IL-6, and CCL-2 in lungs in rats with polymicrobial abdominal sepsis [26] and in a murine lung lipopolysaccharide study [27]. In this respect, it should be noted that APC can down-regulate nuclear factor κ B expression, which could have an effect on the levels of numerous cytokines [28–30]. Of note, in related unpublished experiments we observed that early treatment with rm-APC (initiated 12 h after infection) in the absence of concurrent administration of ceftriaxone reduced levels of multiple inflammatory mediators in homogenates of lungs harvested 8 h after treatment (data not shown), suggesting that during pneumococcal pneumonia potential anti-inflammatory effects of APC may depend on the timing of administration and the time point at which organs are obtained for analysis. We did not adjust for multiple comparisons when testing differences in levels of inflammatory mediator between groups; such an adjustment would result in a significance level of .0009, which is very difficult to reach with a feasible number of mice, considering the inherent variability of a biological experiment using viable bacteria and animals. However, if the differences were based solely on chance one would expect an equal distribution between higher and lower values in mice given antibiotics or not, whereas in our study there was a strong trend toward lower levels of inflammatory markers in ceftriaxone-treated mice.

Previous studies of the capacity of APC to influence neutrophil recruitment to the lungs have not produced consistent results. Intravenous APC has been reported to inhibit pul-

monary neutrophil influx in acid aspiration or elastase-induced lung injury in rodents [24, 25]; in addition, APC inhibited neutrophil influx in the bronchoalveolar space of healthy humans challenged with lipopolysaccharide via a bronchoscope [22]. In contrast, APC did not affect neutrophil counts in the bronchoalveolar lavage fluid from rats with pneumonia due to *S. pneumoniae* or *P. aeruginosa* [9, 15], whereas in one study APC augmented neutrophil influx during *P. aeruginosa* pneumonia [23]. Results of the present study that revealed no influence of rm-APC administered 24 h after induction of pneumococcal pneumonia in the presence or absence of antibiotic therapy are in line with our earlier findings in rats treated with rh-APC before induction of bacterial pneumonia [9, 15]. These earlier studies did not show an effect of pretreatment with rh-APC on lung histopathologic characteristics induced by pneumonia [9, 15], similar to the current finding with therapeutic rm-APC. Of note, we did not examine the effect of rm-APC on the recruitment of cell types other than neutrophils; as such, it would be of interest to investigate the influx of macrophages and dendritic cells during pneumococcal pneumonia in mice treated with or without rm-APC.

Remarkably, rm-APC in combination with antibiotics significantly prolonged survival, compared with antibiotics alone. To our knowledge, this is the first preclinical study in which APC has had a positive effect on survival in the clinically relevant context of concurrent antibiotic treatment. Our study does not provide a definitive explanation for this clear survival advantage other than an anticoagulant effect of rm-APC. Of note, APC was recently shown to play a role in the cleavage of extracellular histones, thereby eliminating the histone-induced toxicity in experimental sepsis [31]. It remains to be determined whether this effect of APC contributed to the survival benefit in our model of pneumococcal pneumonia.

To mimic the clinical situation, APC should be administered by continuous intravenous infusion. However, this is difficult to achieve in freely moving mice. We therefore administered rm-APC intraperitoneally every 8 h at a dose of 125 μ g (a daily dose of \sim 15 mg/kg; ie, \sim 25-fold higher than the daily dose administered to humans). This administration protocol resulted in plasma levels that were in the same range as those achieved by continuous intravenous infusion in septic patients (median levels, 154, 122, and 33 ng/mL at 1, 4, and 8 h after injection, respectively) [32]. In addition, this dose was not dissimilar to those used in previous rodent studies in which anti-inflammatory effects of recombinant APC were shown after lipopolysaccharide administration [33–36]. Therefore, studying higher APC doses would be less clinically relevant and, considering that the APC dosing schedule used here caused profound anticoagulant effects, may be associated with an increased risk for bleeding. It would be of great interest, however, to study the effects of mutant forms of APC with reduced anti-

coagulant but enhanced cytoprotective properties in models of severe pneumococcal pneumonia and sepsis [35–37].

In conclusion, we have shown that rm-APC treatment inhibits systemic and pulmonary coagulation activation in murine pneumococcal pneumonia when administered 24 h after infection. Moreover, we have demonstrated that rm-APC treatment improves survival, an effect that is dependent on the concurrent administration of antibiotics.

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