

**AGGRAVATION OF ENDOTOXIN-INDUCED DISSEMINATED
INTRAVASCULAR COAGULATION AND CYTOKINE ACTIVATION
IN HETEROZYGOUS PROTEIN C DEFICIENT MICE**

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ABSTRACT

In the pathogenesis of sepsis and disseminated intravascular coagulation (DIC) dysfunctional anticoagulant pathways are important. The function of the protein C system in DIC is impaired due to low levels of protein C and downregulation of thrombomodulin. Administration of (activated) protein C results in an improved outcome in experimental and clinical studies of DIC. It is not known whether congenital deficiencies in the protein C system are associated with more severe DIC. The aim of the present study was to investigate the effect of a heterozygous deficiency of protein C on experimental DIC in mice.

Mice with a one allele targeted disruption of the protein C gene (PC +/- mice) and wild-type littermates (PC +/+) were injected with *E. coli* endotoxin (50 mg/kg) i.p.. PC+/- mice had more severe DIC, as evidenced by a greater decrease in fibrinogen level and a larger drop in platelet count. Histology showed more fibrin deposition in lungs, kidneys and liver in mice with a heterozygous deficiency of protein C. Interestingly, PC+/- mice had significantly higher levels of pro-inflammatory cytokines TNF- α , IL-6 and IL-1 β , indicating an interaction between the protein C system and the inflammatory response. There was a lower survival at 12 and 24 hrs after endotoxin in the PC +/- mice.

These results confirm the important role of the protein C system in the coagulative-inflammatory response upon endotoxemia and may suggest that congenital deficiencies in the protein C system are associated with more severe DIC and adverse outcome in sepsis.

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INTRODUCTION

Severe infection or sepsis is often associated with hemostatic abnormalities, in its most severe form manifested as disseminated intravascular coagulation.(DIC).¹. Several pathways contribute to the prothrombotic state in patients with a systemic inflammatory response to severe infection or sepsis.² Beside tissue factor-induced activation of coagulation and a PAI-1-mediated inhibited fibrinolysis, dysfunctional physiological anticoagulant pathways represent important mechanisms in the pathogenesis of the hemostatic derangement. One of the most important physiological anticoagulant pathways that is capable of modulating activation of coagulation is the protein C system.^{3,4} There is indeed ample evidence for an impaired function of the protein C system, with relevance for hemostatic activation, morbidity, and mortality, during severe sepsis.⁵ Firstly, plasma levels of protein C are markedly reduced in patients with sepsis and low levels of protein C have been shown to be closely associated with morbidity and mortality.^{6,7} Secondly, activation of protein C may be severely compromised by downregulation of the endothelial surface receptor thrombomodulin by pro-inflammatory cytokines.⁸ Recently, these *in vitro* findings have been corroborated by the observation of downregulated thrombomodulin on endothelium in skin biopsies from patients with severe Gram negative septicemia, associated with decreased activated protein C.⁹ Thirdly, administration of activated protein C has been shown to reduce mortality and morbidity in experimental models of bacteremia and in a recently published randomised controlled trial in patients with sepsis.^{10,11} Besides the coagulation-modulating properties of activated protein C in sepsis, *in vitro* and *in vivo* studies point to an additional anti-inflammatory effect.¹²⁻¹⁴

Also, it is not known whether individuals with a congenital heterozygous deficiency of protein C have a different hemostatic or inflammatory response to severe infection or sepsis. Mice with a one-allele targeted disruption of the protein C gene, resulting in a heterozygous deficiency of protein C, may serve as a tool to study the effect of such a deficiency on the endotoxin-induced hemostatic derangement and to more precisely analyze the inflammatory response during protein C deficiency. In this study we show that heterozygous deficiency of protein C considerably affects both the hemostatic and inflammatory response to endotoxin in mice.

METHODS

mice

The experiments were approved by the Institutional Review Board and were conducted according to the guidelines for animal experiments of the NIH. Mice with a heterozygous deficiency of protein C were generated as described previously.¹⁵ Briefly, a targeting vector was introduced by homologous recombination into R1 embryonic stem (ES) cells. This targeting vector contained a 6.5 kb 5' flanking region of the protein C gene, ranging from a 5' XbaI site to an XhoI site 456 basepairs upstream of exon 2 (the first translated exon). The 2.8 kb 3' flanking region of the protein C gene ranged from a HindIII site 390 basepairs further than the stop codon to a EcoRI site. Aggregation of recombinant R1 ES cells with morula stage embryos led to the generation of chimeric mice, from which a germ line transmitting mouse and subsequently heterozygous protein C deficient mice were generated. The mice were backcrossed to the F4 generation in C57BL/6J mice. Deficiency of protein C was confirmed by Northern blot analysis of RNA and measurement of protein C in plasma. As described previously, mice with a heterozygous deficiency of protein C were apparently healthy, had a normal life span and were capable of delivering following generations of mice.¹⁵ Genotypic screening from the offspring of protein C +/- mice was performed by Southern blot hybridization of DNA extracted from mouse tails, using a 0.9 kb EcoRI/XbaI 5'external probe, as described previously.¹⁵ EcoRI digests of genomic DNA yielded a differential restriction pattern of 11 kb for the mice with a wild type protein C gene and a 7.5 kb band for recombinant alleles. Wild type (protein C +/+ mice) littermates were used as controls in our experiments.

experimental design

Protein C +/- mice and their wild type littermates, weighing 25-30 grams, were injected intraperitoneally with *E coli* endotoxin (serotype O111:B4) at a dose of 50 mg/kg (Sigma, St Louis, MO) or saline (control). Blood for the assay of platelet count, coagulation factors, cytokines, and clinical chemistry was collected at 4, 8, and 12 hours after injection of endotoxin from the inferior caval vein of anaesthetised mice and anticoagulated with EDTA (10 mmol/l) or citrate (3.2% final concentration). Each time point consisted of observations in 6 mice from each experimental group. Histological studies were performed at 8 and 12

hours after injection of endotoxin. Survival was investigated in groups of 16 mice at 12 and 24 hours after the administration of endotoxin.

assays

Protein C plasma activity levels were measured as previously described with an amidolytic assay using chromogenic substrate S2366 (Chromogenix, Milan, Italy). Platelets were measured using an automated cell counter (Abbott Cell-Dyn 1330 system, Abbott Park, IL). Fibrinogen was assayed according to Clauss and plasma levels of coagulation factor activity by a one-stage clotting assay on an automated clotting analyzer (STA-R, Roche Diagnostics, Netherlands) using pooled mice plasma as reference material. PAI-1 was measured with an ELISA as previously described.¹⁶ Circulating levels of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β and IL-10 were measured with their respective ELISA's for murine plasma (TNF- α : Genzyme, Cambridge, MA, IL-1 β : R&D Systems, Minneapolis, MN, IL-6 and IL-10: Pharmingen, San Diego, CA), as previously described.^{17,18} Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and urea were determined with commercially available kits (Sigma) using a Hitachi analyser (Boehringer Mannheim, Germany).

histology

A cannula was introduced in the right atrium and mice were perfused with 1% phosphate buffered paraformaldehyde at 100 cm H₂O pressure for 5 minutes. Subsequently, the trachea was cannulated and 1% phosphate buffered paraformaldehyde was perfused at 30 cm H₂O through the airways. The heart and lungs were removed en bloc and the heart was separated from the lungs and the large vessels. Furthermore, the kidneys, liver and brain were removed. The samples were cryoembedded or postfixed for 24 hours in 1% phosphate buffered paraformaldehyde, dehydrated and embedded in paraffin. Four μ m sections were immunostained with a monoclonal antibody directed towards fibrinogen. For granulocyte staining, slides were deparaffinized and rehydrated. Slides were incubated in 10% normal goat serum (DAKO, Glostrup, Denmark) and then exposed to FITC-labeled anti-mouse Ly-6-G monoclonal antibody (BD Pharmingen, San Diego, CA). Secondary incubation with rabbit anti-FITC antibody (DAKO), with a biotinylated swine anti-rabbit antibody (DAKO),

and ultimately with a streptavidin-ABC solution (DAKO) was performed and subsequently the slides were developed using 1% H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in Tris-HCl.

statistics

Results are presented as mean values \pm SD. Statistical analysis was performed by ANOVA and subsequent Newman-Keuls test. A p-value <0.05 was considered statistically significant.

RESULTS

protein C, platelets and coagulation factors

Base-line protein C activity levels were $63 \pm 5\%$ in protein C +/- mice as compared to $98 \pm 4\%$ in wild-type littermates (n=6), which is consistent with earlier observations.¹³ After injection of endotoxin, protein C levels dropped in both groups, reaching levels as low as $17 \pm 5\%$ in the heterozygous protein C deficient group and $35 \pm 6\%$ in the wild-type mice at 12 hours after endotoxin administration (figure 1) ($p=0.01$ for protein C +/- compared with protein C +/+ mice). Protein C deficient mice that were injected with saline had stable protein C activity over time (data not shown).

As shown in figure 2, platelet count dropped from $489 \pm 21 \times 10^9/l$ to $171 \pm 28 \times 10^9/l$ at 12 hours after the injection of endotoxin in wild type mice. In mice with a heterozygous protein C deficiency, the drop in platelet count was significantly more profound, reaching levels as low as $32 \pm 21 \times 10^9/l$ at 12 hours after endotoxin administration (n=6, $p<0.001$). Fibrinogen plasma levels followed a similar trend with significantly lower levels in the protein C +/- mice as compared with wild type mice (figure 2). The fibrinogen plasma level at 12 hours after endotoxin injection was 0.6 ± 0.2 g/l in the protein C +/- group as compared with 2.7 ± 0.3 g/l in the protein C +/+ group (n=6, $p=0.01$). Also plasma levels of factor VII and factor V activity were lower in the protein C deficient mice as compared with wild type mice (table 1). PAI-1 plasma levels increased upon the administration of endotoxin from 2.5 ± 1.1 ng/ml to 12.9 ± 2.7 ng/ml but this increase was not different between the two groups of mice studied. Protein C +/+ and +/- mice that received saline had stable levels of platelets and coagulation factors at subsequent time points during the experiment.

fibrin deposition and granulocyte invasion

Histologic studies showed more extensive fibrin deposition in various organs at 12 hours after endotoxin administration in mice with a heterozygous deficiency of protein C as compared to wild-type mice. Fibrin deposition was most pronounced in small and mid-size vessels in kidneys, small pulmonary arterioles and in liver vasculature. Figure 3 demonstrates the difference in fibrin deposition in lung, kidney, and liver at 12 hours after endotoxin administration between protein C +/- mice and protein C +/+ mice. In general,

intravascular fibrin deposition was associated with signs of inflammation, evidenced by small infiltrates of granulocytes, in particular in lung and liver (figure 4). Quantitative analysis revealed a 2.1-fold and 1.8-fold increase in the number of granulocytes in lung and liver of protein C deficient mice as compared with wild type mice, respectively.

inflammatory mediators

Interestingly, circulating levels of inflammatory cytokines upon endotoxemia were significantly higher in the protein C deficient mice as compared to wild-type mice, suggesting that protein C may indeed modulate inflammatory responses to endotoxin (table 2). Peak plasma levels of TNF- α were approximately fourfold higher in the protein C +/- group as compared to the wild type group at 4 hours after endotoxin administration (842 ± 42 pg/ml versus 231 ± 31 pg/ml, $n=6$, $p=0.01$) and remained significantly higher in the protein C deficient group at 8 hours. Also levels of IL-6 and IL-1 β were significantly higher in the protein C +/- mice at 4 hours after endotoxin administration. At 12 hours after endotoxin administration, there was no difference in levels of TNF- α , IL-6 and IL-1 β between the two groups (data not shown). Levels of the anti-inflammatory cytokine IL-10 showed a trend towards higher values in the protein C deficient group in comparison to wild type mice, but this did not reach statistical significance.

organ failure and mortality

Liver damage was assessed by measuring liver enzymes (ALT and AST), whereas as an estimate of kidney failure blood urea nitrogen (BUN) was measured. Protein C +/- mice had higher ALT and AST levels at 12 hrs as compared with wild type mice. Peak ALT and AST levels in protein C +/- mice were 362 ± 41 U/l and 291 ± 38 U/l as compared with 275 ± 46 and 196 ± 23 U/l in wild type mice, respectively ($p=0.01$ for both ALT and AST). BUN increased from 30 ± 4 mg/dl to 52 ± 7 mg/dl at 12 hrs after endotoxin administration in wild type mice and was also more elevated in protein C +/- mice (63 ± 6 mg/dl, $p=0.03$). As shown in figure 5, there was a significantly lower mortality in protein C ++ mice as compared to protein C +/- mice at 12 and 24 hrs after the administration of endotoxin ($p=0.02$ and 0.04 , respectively).

DISCUSSION

The protein C system plays a fundamental role in regulating the balance between adequate hemostasis and maintaining blood fluidity as an endothelial-cell associated anticoagulant system.³ For activation of protein C endothelial cell-bound thrombomodulin, which is abundant in the microcirculation, is essential, which renders the system of pivotal importance in the maintenance of microvascular patency. In a situation of systemic endothelial cell perturbation, as exemplified by the systemic inflammatory response in sepsis, a dysfunctional protein C system is likely to contribute to microvascular failure and may contribute to the ensuing organ failure. There is indeed ample experimental and clinical evidence demonstrating the importance of the protein C system in sepsis.^{5,13} Many studies show dysfunction of the protein C system in sepsis and the extent of the defect appears to be directly related to the severity of the disease.^{19,20} In addition, restoration of the protein C pathway (for example by administration of activated protein C) results in an improved outcome in experimental bacteremia or clinical sepsis.^{10,11}

The present observation adds to that notion, demonstrating that mice with a one-allele targeted disruption of the protein C gene, leading to a heterozygous deficiency of protein C, develop more severe signs of DIC. Presumably, the much lower level of zymogen protein C in combination with the endotoxin-induced downregulation of thrombomodulin results in less activated protein C and thereby less inhibition of thrombin generation.⁸ As a consequence, these mice have more extensive fibrin deposition in the vasculature of various organs in comparison with wild-type littermates. Protein C deficient mice demonstrate more extensive organ damage upon the injection of endotoxin and a trend towards a lower survival rate in mice with a heterozygous deficiency of protein C was observed. These findings underline the pivotal importance of the protein C system in the development of endotoxin-induced activation of coagulation, fibrin deposition and organ failure and the delicate role of the protein C plasma concentration in the severity of the response.

Interestingly, there was not only a difference in the coagulation response between the protein C deficient mice and the wild-type mice but also significant differences in inflammatory responses upon endotoxin administration were demonstrable, as shown by circulating levels of pro-inflammatory cytokines. This observation is in agreement with previous studies, pointing at a role of the protein C system in modulating the systemic inflammatory response

in sepsis or endotoxemia.^{13,14} Indeed, activated protein C has been found to inhibit endotoxin-induced production of TNF- α , IL-1 β , IL-6 and IL-8 by cultured monocytes/macrophages.^{21,22} Further, activated protein C also abrogated endotoxin-induced cytokine release and leucocyte activation in rats *in vivo*.²³ Experiments in which the protein C pathway was blocked in septic baboons exacerbated the inflammatory response.²⁴ Conversely, administration of activated protein C ameliorated the inflammatory activation upon the intravenous infusion of *E. coli*.²⁵ Similar experiments in rodents showed identical results and demonstrated a beneficial effect on inflammatory effects in various tissues.²³ It was demonstrated *in vitro* that monocytes bear an activated protein C binding site that may mediate downstream inflammatory processes,²⁶ and that activated protein C can block NF κ B nuclear translocation, which is a prerequisite for increases in pro-inflammatory cytokines and adhesion molecules.²⁷ Binding of activated protein C to the endothelial protein C receptor may mediate another pathway by which activated protein C modulates inflammation.¹³ This binding was shown to affect gene expression profiles of cells expressing the protein C receptor. Recent experiments demonstrated that binding of activated protein C to the protein C receptor (that can also be detected on mononuclear cells) inhibited endotoxin-induced tissue factor expression on monocytes. Finally, septic patients receiving recombinant human activated protein C had lower IL-6 levels than placebo-treated controls.¹¹ Taken together, there is compelling evidence that activated protein C may act as an inflammatory mediator, which is supported by our present observations.

Human subjects with a heterozygous protein C deficiency have a higher risk of developing venous thromboembolism. Also other defects involving the protein C system, such as protein S deficiency or resistance towards activated protein C (most frequently caused by the factor V Leiden mutation), are classified as thrombophilia. It is not known whether these patients have a higher risk of disseminated intravascular coagulation during sepsis or whether their deficiency contributes to an adverse outcome. A low level of protein C on admission is an independent predictor of mortality in sepsis but it is unclear to what extent the pre-existing protein C plasma affects the protein C level on admission.⁷ Our experimental results may indicate that patients with a protein C deficiency have more extensive coagulation activation, potentially leading to increased microvascular fibrin deposition, as compared with patients with normal protein C levels. However, additional analyses from clinical studies are required

to attain a more solid clinical basis for this hypothesis. Interestingly, a recent study in mice with a deficiency of antithrombin also showed more severe coagulation activation upon endotoxemia.²⁸ In line with that observation, thrombophilia in general may hypothetically be associated with a more severe coagulopathy and an adverse outcome in sepsis.

In conclusion, mice with a one allele targeted disruption of the protein C gene, leading to heterozygous protein C deficiency, have a more severe derangement of coagulation upon endotoxemia, leading to more extensive intravascular fibrin deposition and organ failure. In addition, protein C deficient mice have higher levels of circulating pro-inflammatory cytokines, suggesting that the protein C system is indeed involved in the modulation of the inflammatory response in sepsis. Whether these findings are clinically relevant for patients with a protein C deficiency or other thrombophilic defects involving the protein C system needs further study.

table 1

	protein C +/-	protein C +/+	p
factor V (%)			
- baseline	105 ± 7	101 ± 8	0.3
- 4 hr	52 ± 12	71 ± 9	0.04
- 12 hr	36 ± 9	53 ± 11	0.01
factor VII (%)			
- baseline	98 ± 3	107 ± 4	0.6
- 4 hr	48 ± 7	56 ± 6	0.05
- 12 hr	27 ± 5	42 ± 5	0.005
PAI-1 (ng/ml)			
- baseline	2.5 ± 1.1	2.8 ± 1.2	0.5
- 4 hr	7.5 ± 2.4	5.9 ± 2.9	0.09
- 12 hr	12.9 ± 2.7	10.1 ± 2.2	0.1

Plasma levels of factor V and factor VII clotting activity and of plasminogen activator inhibitor, type 1 (PAI-1) before and after administration of endotoxin to protein C +/- mice and wild-type littermates (protein C +/+). N=6 at each timepoint in both groups. Mean values ± SD are given.

table 2

	protein C +/+	protein C +/-	p
TNF- α	231 \pm 31	841 \pm 42	0.01
IL-6	1140 \pm 192	1754 \pm 214	0.04
IL-1 β	1244 \pm 97	1432 \pm 85	0.05
IL-10	1071 \pm 231	1196 \pm 276	0.1

Circulating levels of cytokines (pg/ml) in protein C deficient mice and wild-type littermates at 4 hours after administration of endotoxin. N=6 at each timepoint in both groups. Mean values \pm SD are given.

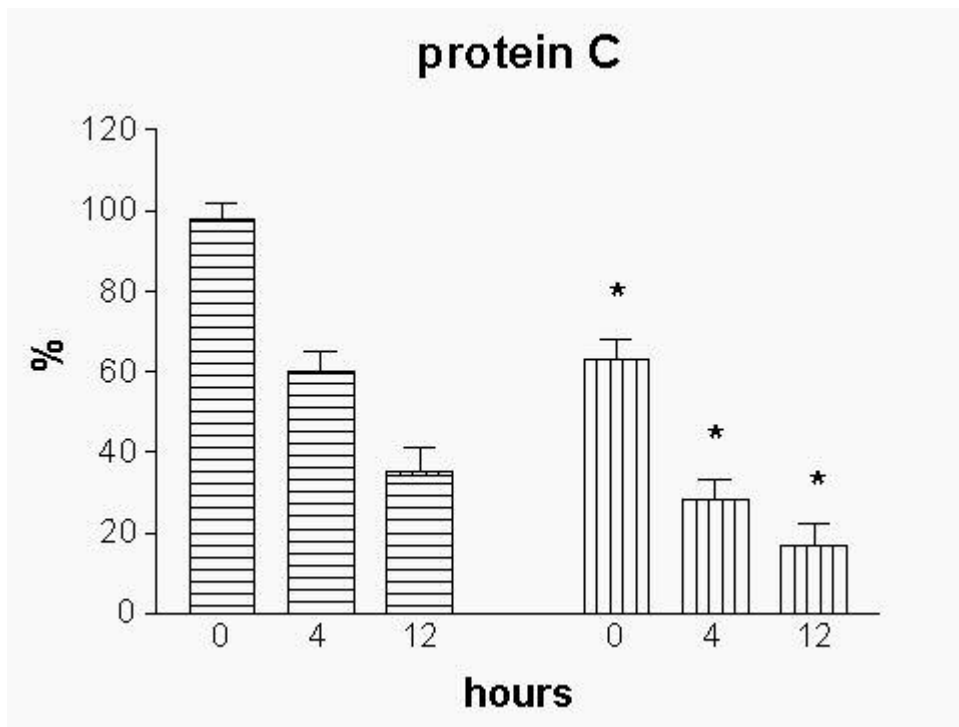


figure 1

Plasma levels of protein C activity before and at 4 and 12 hours after the injection of endotoxin in mice with a heterozygous deficiency of protein C (vertical bars) and wild-type littermates (horizontal bars). Mean values and SD are shown, statistical significance between protein C deficient mice and wild type mice at each time point is indicated (*: p=0.01).

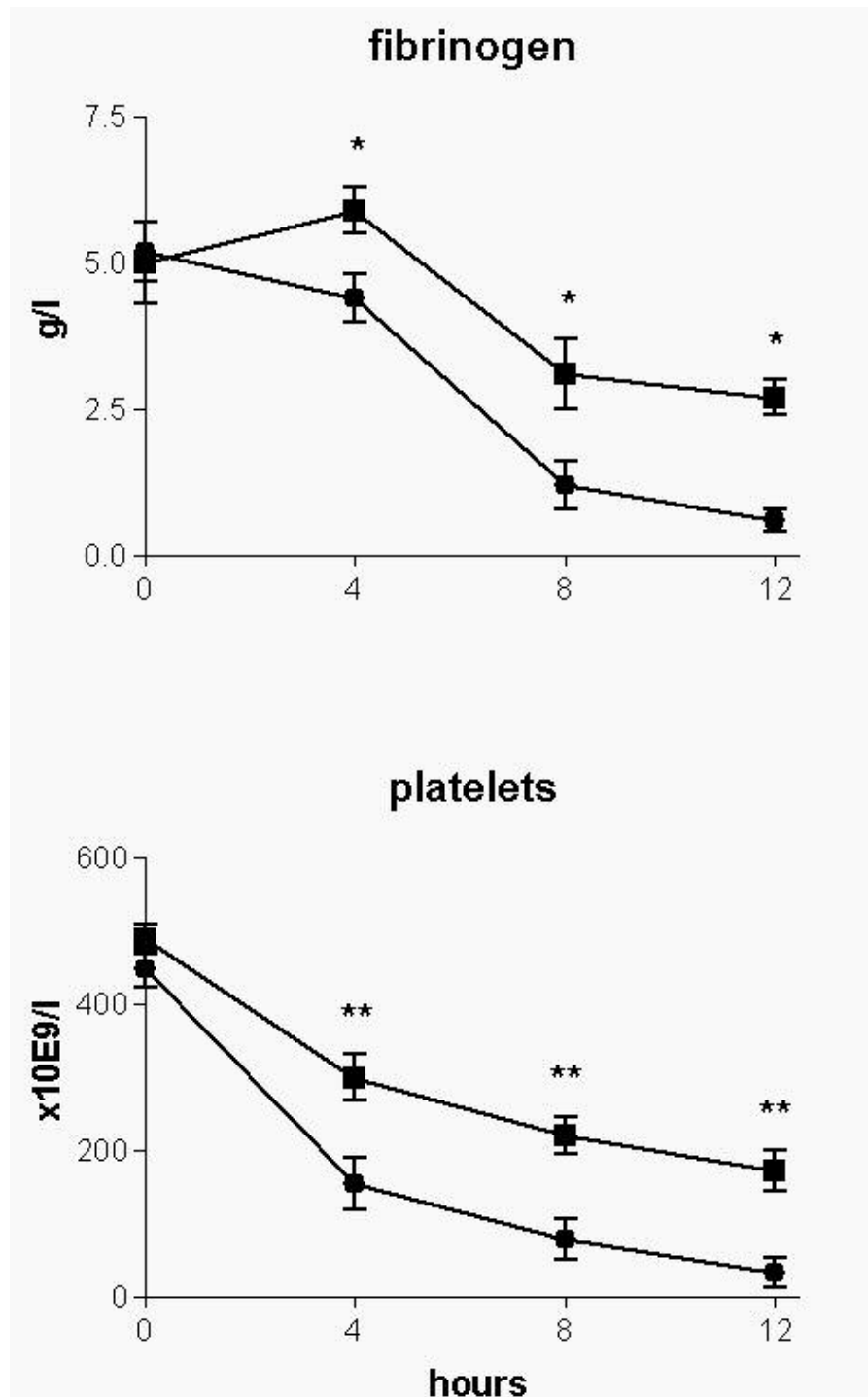


figure 2

Plasma fibrinogen concentration and platelet count after the injection of endotoxin (at t=0) in mice with a heterozygous deficiency of protein C (circles) and wild-type littermates (squares). Mean values and SD are shown, statistical significance is indicated (*: p=0.01, **:p=0.001).

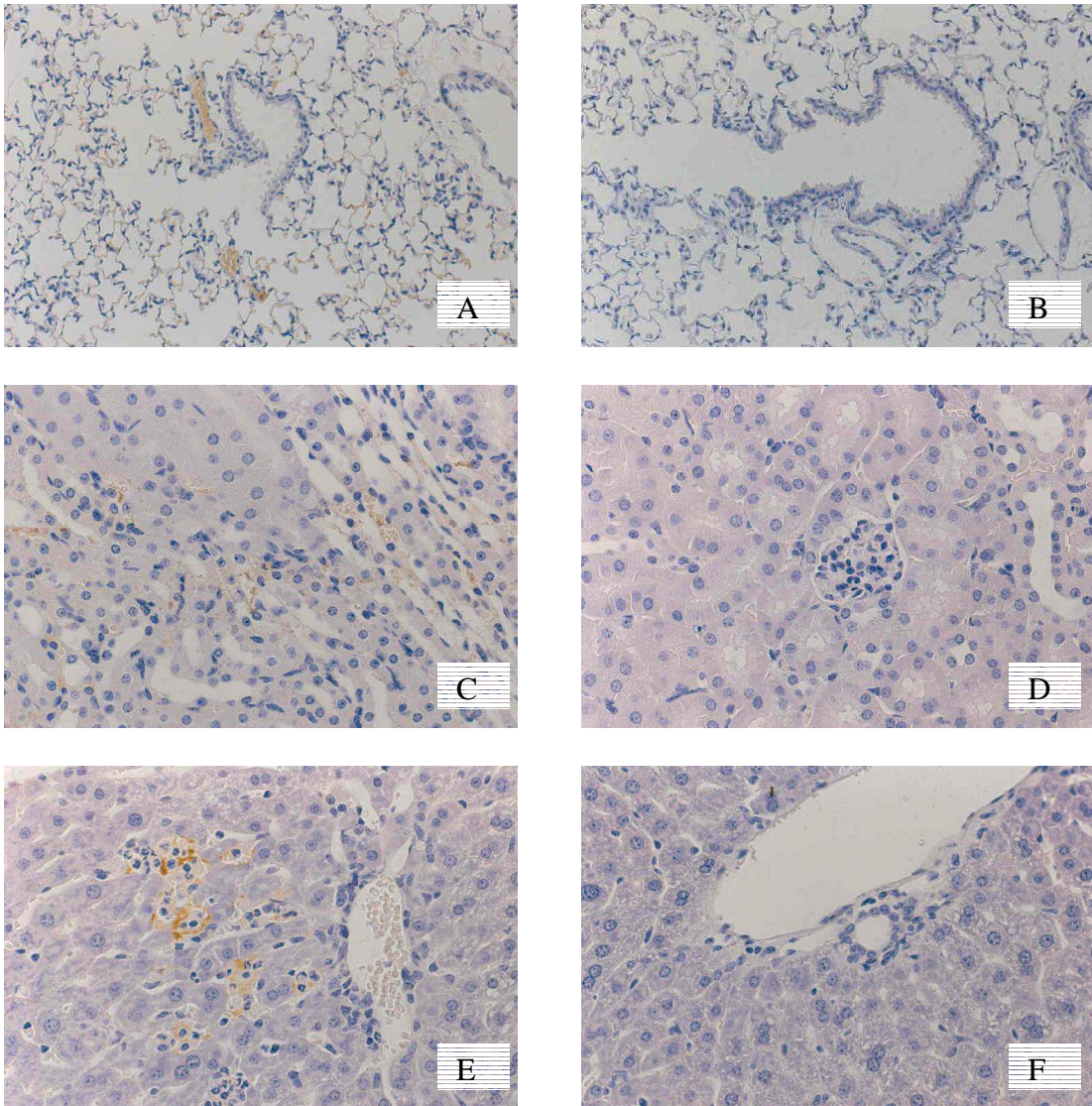


figure 3

Fibrin staining of lung (panel A and B), kidney (panel C and D), and liver (panel E and F) of mice at 12 hours after administration of endotoxin. Mice with a heterozygous deficiency of protein C (panels A, C, and E) are compared with wild type littermates (panels B, D, and F). Slides are representative for 6 mice per group.

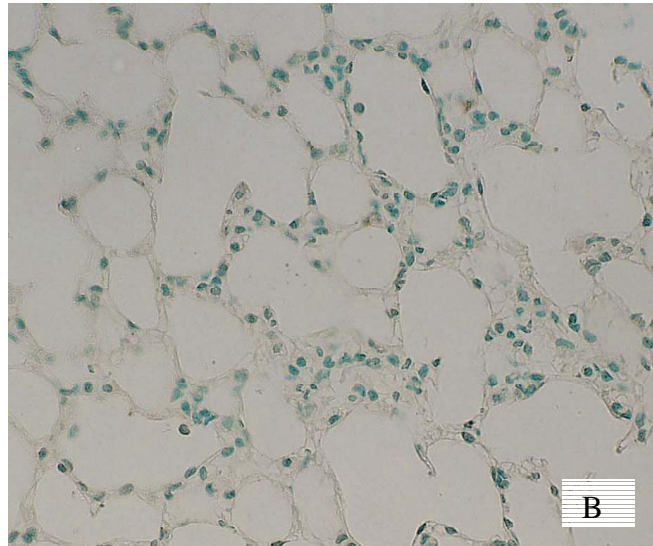
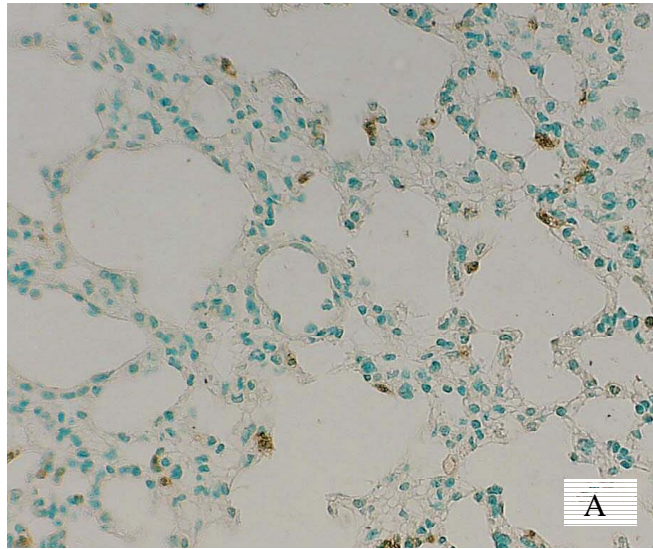


figure 4

Anti-granulocyte staining (counter staining with methylene green) of lungs of protein C +/- mice (panel A) and wild-type mice (panel B) at 12 hours after administration of endotoxin. Slides are representative for 6 mice per group.

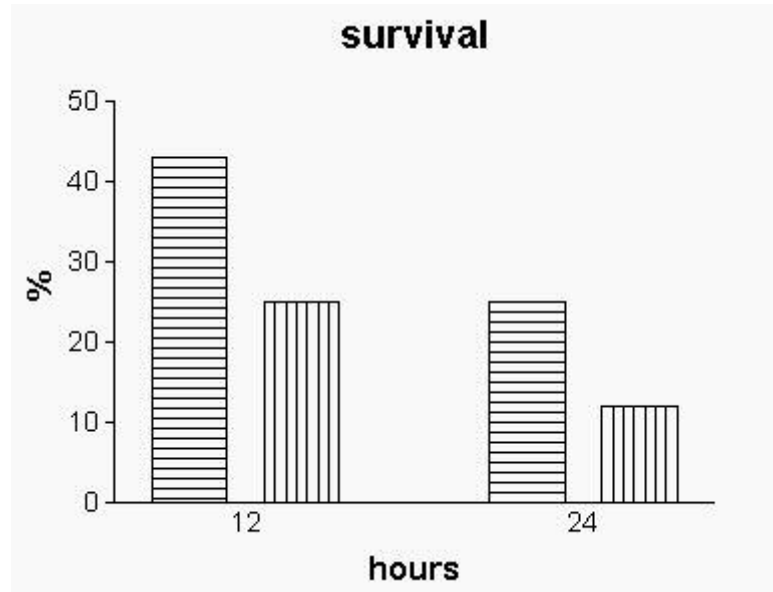


figure 5

Survival of mice (n=16) with a heterozygous deficiency of protein C (vertical stripes) and wild type littermates (horizontal stripes) at 12 and 24 hours after the administration of endotoxin. The differences are statistically significant ($p=0.02$ at 12 hours and $p=0.04$ at 24 hours).

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