# Endotoxemia-induced protein C surge protects mice against venous thrombosis based on transient lowering of natural anticoagulants

Marco Heestermans[a,b], Victorine Maillot[a], Charles-Antoine Arthaud[a], Amelie Prier[a], Marie-Ange Eyraud[a], Laurent Bertoletti[b,c], Anne-Claire Duchez[a,b], Hind Hamzeh-Cognasse[b] and Fabrice Cognasse[a,b]

**Immunothrombosis is the process by which inflammatory stimuli promote coagulation and thrombus formation. Bacterial sepsis is a well established risk factor for venous thrombosis, and numerous experimental studies have shown that sepsis indeed enhances thrombotic responses. In this study, we aimed to investigate the impact of endotoxemia – induced by either lipopolysaccharides or α-toxin – on venous thrombosis development in mice. Venous thrombosis was induced using a model based on siRNA-mediated transient inhibition of the natural anticoagulants – protein C (PC) and anti-thrombin (AT). Unexpectedly, endotoxemia attenuated rather than promoted venous thrombus formation. This counterintuitive finding appears to be explained by a transient increase in circulating protein C levels following endotoxemia. As our venous thrombosis mouse model strongly depends on the level of reduced protein C activity, this endotoxemia-induced elevation interfered with the intended prothrombotic conditions and compromised comparability between experimental groups. These results highlight the context-dependent effects of bacterial sepsis on venous thrombosis and underscore the importance of rigorous model validation in (immuno) thrombosis research. *Blood Coagul Fibrinolysis* 36:381–385 Copyright © 2025 Wolters Kluwer Health, Inc. All rights reserved.**

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[a]Etablissement Français du Sang Auvergne-Rhône-Alpes, Research Department, [b]INSERM, U 1059 SAINBIOSE, Université Jean Monnet, Mines Saint-Étienne and [c]Département of Médecine Vasculaire et Thérapeutique, CHU Saint-Étienne, Saint-Etienne, France

Correspondence to Fabrice Cognasse, Directeur de Recherche - Etablissement Français du Sang (PhD, HDR) & INSERM 1059, Directeur Scientifique - Etablissement Français du Sang Auvergne-Rhône-Alpes, Faculté de Médecine, SAINBIOSE INSERM U1059, Campus Santé Innovations, 10 rue de la Marandière, 42270 Saint-Priest-en-Jarez, France.
Tel: +33683975883; +33477421400;
e-mail: fabrice.cognasse@univ-st-etienne.fr; fabrice.cognasse@efs.sante.fr

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## Introduction

Venous thromboembolism (VTE) is a complex, multifactorial disease that affects one to three per 1000 per year, according to the WHO. Bacterial sepsis increases VTE risk 2–20 times [1]. With the growing prevalence of multidrug-resistant bacteria, bacterial sepsis represents an increasingly serious health concern [2].

Bacterial sepsis induces systemic inflammation that promotes coagulation and thrombosis, a process defined as immunothrombosis [3]. Various inflammatory mediators, including endothelial cells, platelets, neutrophils, and monocytes, are implicated in VTE pathophysiology. However, the relative contributions of these individual components *in vivo* remain incompletely understood. Bacterial components, such as lipopolysaccharide (LPS) and bacterial toxins, are commonly used to model sepsis *in vivo*, by inducing endotoxemia [4]. LPS from the outer membrane of Gram-negative bacteria and toxins, which are produced by various bacteria, are harmful agents that trigger host immune responses and can cause tissue damage. In murine models, administration of high LPS doses coincides with systemic inflammation, leading to extensive consumption of coagulation factors and death via a process resembling disseminated intravascular coagulation [5–7]. Nonetheless, data on how bacterial components directly mediate VTE remain scarce.

In line with the principles of Virchow's triad, venous thrombosis in mice can be induced by vascular injury, stasis or stenosis, or a hypercoagulable state [8]. In the present study, we employed a hypercoagulability-based venous thrombosis mouse model in which natural anticoagulants are transiently suppressed [9]. This is achieved through intravenous injection of small interfering (si)RNA-mediated, gradually lowering circulating anti-thrombin (AT) and protein C (PC). This transient decrease results in venous thrombosis, in the absence of additional pro-thrombotic stimuli or surgery. This venous thrombosis mouse model has been extensively characterized under noninfectious conditions, identifying thrombin, tissue factor, and platelets as key drivers of venous thrombosis [10]. In the current study, we aimed to investigate the contribution of endotoxemia-induced immunothrombosis to venous thrombosis development, making use of our hypercoagulability-based venous thrombosis mouse model.

## Methods

### Mouse experiments

Six-week-old female C57BL/6J mice were obtained from Charles River Laboratories (Wilmington, Massachusetts,

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USA). siRNAs targeting murine *Serpinc1* and *Proc* (#4404014, ID#S62673 and ID#S72192, respectively) and Invivofectamine 3.0 (#IVF3001) were from Thermo Scientific (Waltham, Massachusetts, USA). siRNA preparation and administration as well as venous thrombosis characterization were performed as before [9,10]. Ultrapure LPS-EB and LPS-B5 (two isoforms of LPS derived from distinct *Escherichia coli* strains) were obtained from Invivogen (San Diego, California, USA), and α-toxin (α-hemolysin, H9395) from Thermo Scientific. LPS and α-toxin (dissolved in 100 μl PBS) were administered via intraperitoneal injection, with PBS as a negative control. Blood was collected via the retro-orbital sinus under isoflurane anaesthesia. For serial collections, alternating eyes were used to minimize local trauma. Blood cell populations were determined with an MS4s cell counter (Melet Schloesing, Maria Enzersdorf, Austria). Body temperature was measured using a rectal thermoprobe (BIO-9882; Bioseb, Vitrolles, France). All animal experiments were performed according to institutionally approved protocols and national and European animal regulations (EU Directives 2010/63/EU, 86/609/EEG).

Venous thrombosis resulting from siRNA-mediated depletion of the natural anticoagulants AT and PC is characterized by the formation of large, fibrin-rich occlusive thrombi in the major veins of the masseter and mandibular regions, accompanied by secondary periocular hemorrhages [9,10]. This phenotype is associated with the consumption of circulating coagulation factors, including fibrinogen and platelets. As the thrombotic manifestations are macroscopically visible, mice can be monitored over time to determine and score the exact moment of venous thrombosis onset in individual animals. Upon detection of the characteristic macroscopic venous thrombosis-associated phenotype, which coincides with severe discomfort and is eventually deadly, mice were immediately euthanized for ethical reasons.

### Quantitative PCR and ELISA

Total RNA was extracted from homogenized, snap-frozen livers using the Monarch Miniprep Kit (#T2010, NEB, Ipswich, Massachusetts, USA). qPCR was performed with the Luna Universal One-Step RT-qPCR Kit (#E3005, NEB). Primers for *Actb*, *Serpinc1*, and *Proc*, along with the qPCR protocol, have been described previously [11,12]. Plasma PC levels were quantified using capture antibody 9-PAMPC-S (Cryopep, Montpellier, France) and detection antibody BAF4885 (R&D Systems, Minneapolis, Minnesota, USA). A standard curve was generated using C57BL/6J mouse pooled plasma (#LA-Plasma-Mouse-B6/J-C, Janvier Labs, Le Genest-Saint-Isle, France). Serum Amyloid A (SAA) plasma levels were determined by ELISA (#DY2948-05, R&D systems).

### Statistical analysis

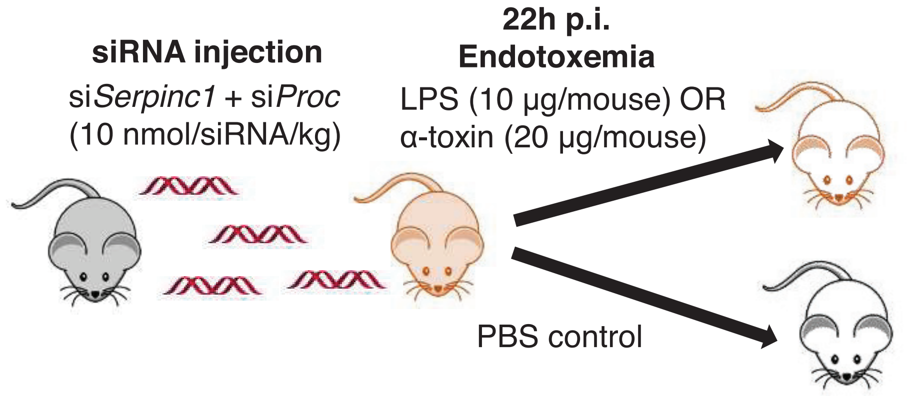
Statistical comparisons (GraphPad, La Jolla, California, USA) were performed using unpaired Student's t-tests (two groups) or ANOVA (three or more groups). Thrombosis-free survival curves were analysed using the Kaplan–Meier method, and differences between groups were assessed using the log-rank test.

## Results

We aimed to study venous thrombosis resulting from the transient inhibition of AT and PC in mice with or without endotoxemia. siRNA-induced venous thrombosis and endotoxemia are dose-dependent phenomena, and we used relatively low concentrations of these agents. In pilot experiments, mice receiving at least 15 nmol siRNA (si*Serpinc1* and si*Proc*) per kg developed venous thrombosis within 48 h, characterized by the formation of large, fibrin-rich occlusive thrombi in the major veins of the masseter and mandibular regions, accompanied by secondary periocular hemorrhages that are macroscopically visible. Only 25% of mice treated with 5 nmol siRNA/kg exhibited this phenotype within the same timeframe. Based on these observations, we selected a dose of 10 nmol siRNA/kg for subsequent experiments. For comparison, a recently published study used 75 nmol siRNA/kg to induce venous thrombosis [10]. Concentrations of LPS and α-toxin for inducing endotoxemia vary considerably across studies, likely due to differences in reagent formulation, purity, storage, and administration protocols [13]. We selected doses of 10 μg LPS/mouse and 20 μg α-toxin/mouse. These concentrations induced modest but statistically significant weight loss, alterations in peripheral blood cell profiles, and an increase of plasma SAA levels, an acute phase protein indicative for LPS-mediated inflammation (Figures S1 and S2, http://links.lww.com/BCF/A193) [14]. Phenotypical changes associated with severe endotoxemia such as alterations in body temperature and severe cytopenia were absent, and no mice died as a result of LPS or α-toxin injection.

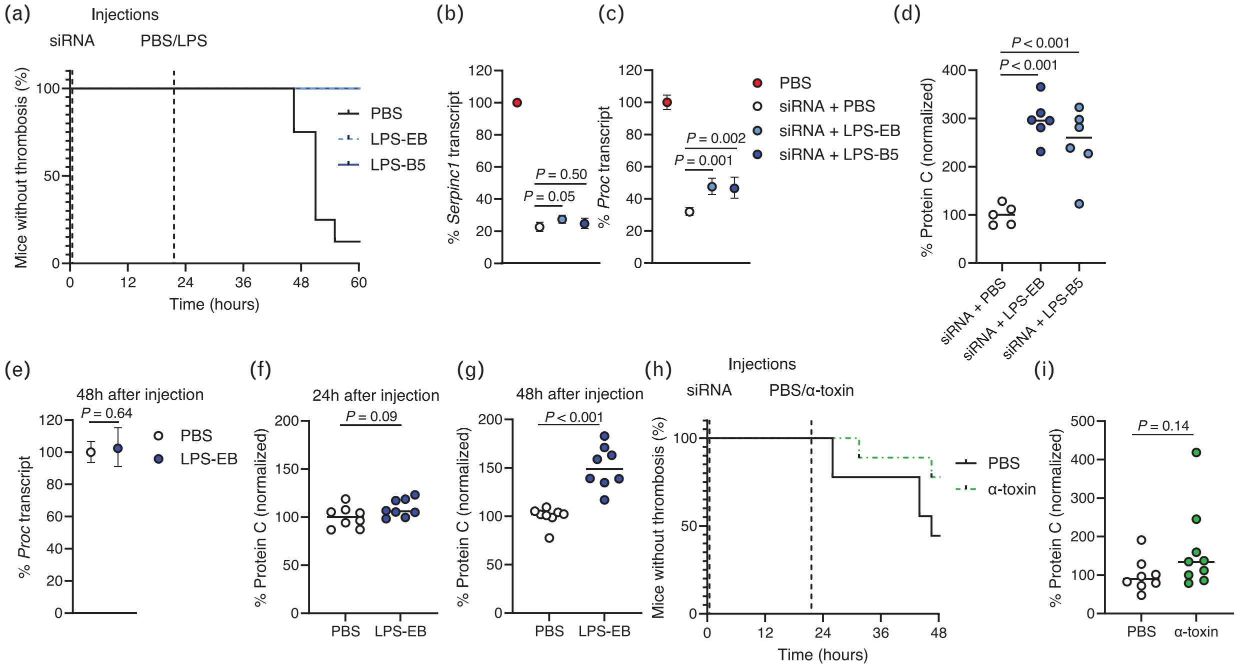
Mice were administered si*Serpinc1* and si*Proc*, and 22 h later, they received LPS or α-toxin to induce endotoxemia (Fig. 1). 22 h postsiRNA injection mice had not developed venous thrombosis yet. First, LPS (LPS-EB or LPS-B5) was used to induce endotoxemia with PBS-treated mice serving as controls. In line with our pilot experiments, 60 h after siRNA injection seven of eight PBS control mice had developed venous thrombosis (Fig. 2a). Unexpectedly, LPS-treated mice were protected from venous thrombosis, with in both LPS groups 0/8 mice affected 60 h after siRNA injection (38 h post-LPS injection, Fig. 2a, $P < 0.001$). Notably, two of eight mice from both LPS groups died during the experiment, without overt signs of venous thrombosis. Given that venous thrombosis in this model relies on siRNA-mediated downregulation of AT and PC, we quantified hepatic mRNA levels of *Serpinc1* and *Proc* in all siRNA-treated

**Fig. 1**



The experimental setup applied to test whether endotoxemia increases thrombotic coagulopathy in mice treated with siRNAs against antithrombin and protein C. Injection of siRNAs (10 nmol siRNA/kg) was performed as described before [9,10]. Twenty-two hours after siRNA injection (postinjection, p.i.), LPS (10 μg/mouse) or α-toxin (20 μg/mouse) was administered by intraperitoneal injection. The siRNA-dependent thrombotic phenotype develops with a delay, as siRNA first targets hepatocytes to reduce the expression of the target mRNA, leading to a gradual decline in circulating natural anticoagulants. Therefore, we administered the siRNA 22 h prior to the LPS/α-toxin injection, which induces an immediate inflammatory response. During the course of the experiment, mice were monitored closely for weight, body temperature, behaviour, and thrombotic coagulopathy. According to ethical guidelines, thrombotic coagulopathy-affected mice were sacrificed prematurely.

**Fig. 2**



(a) The percentage of thrombosis-free mice i.e. mice not affected by thrombotic coagulopathy characteristic for siRNA treatment was monitored in time, in mice treated with siRNAs against antithrombin and protein C, followed by administration of PBS or LPS (EB or B5) ($N=8$). The dashed line indicates the moment of PBS/LPS injection. (b and c) Upon sacrifice, mice livers were collected and homogenized to determine hepatic mRNA transcript of *Serpinc1* (panel b) and *Proc* (panel c). For reference, three livers from mice only with PBS were collected (100%, red symbols). (d) Protein C levels in plasma were determined using ELISA. Protein levels were normalized for siRNA + PBS-treated mice (100%). Of note, because some mice died because of the complications of VT (siRNA + PBS) or endotoxemia (siRNA + LPS), not from all mice plasma could be collected and included in the measurement. (e) Upon sacrifice, mice livers treated with PBS or LPS-EB were collected and homogenized to determine hepatic mRNA transcript of *Proc*. Values for PBS only-treated mice were used as reference (100%) ($N=8$). (f and g) Protein C levels in plasma were determined using ELISA, 24 h (panel f) and 48 h (panel g) after PBS/LPS injection. Protein levels were normalized for PBS-treated mice (100%) ($N=8$). (h) The percentage of thrombosis-free mice, that is, mice not affected by thrombotic coagulopathy characteristic for siRNA treatment was monitored in time, in mice treated with siRNAs against antithrombin and protein C followed by administration of α-toxin ($N=9$). The dashed line indicates the moment of PBS/α-toxin injection. (i) Protein C levels in plasma were determined using ELISA. Protein levels were normalized for siRNA + PBS-treated mice (100%).

mice. Livers from PBS-alone injected mice served as controls. *Serpinc1* levels were similar across all groups (Fig. 2b, $P \geq 0.05$), while *Proc* levels were significantly elevated in LPS-treated mice compared to controls (Fig. 2c, $P < 0.01$). This increase corresponded with a threefold elevation in plasma PC (Fig. 2d, $P < 0.01$).

To examine the time course and LPS dependence of PC elevation, mice were injected with LPS-EB or PBS alone (in the absence of siRNA). Blood was collected 24 and 48 h post-LPS injection. During the 48 h time course of the experiment, LPS-injected mice exhibited mild weight loss (Figures S1A/S1D, http://links.lww.com/BCF/A193, $P < 0.001$), but body temperature remained stable (Figures S1B/S1E, http://links.lww.com/BCF/A193, $P > 0.05$). SAA levels exhibited a marked increase at 24 h and remained significantly elevated at 48 h following LPS administration (Figures S1C/S1F, http://links.lww.com/BCF/A193, $P < 0.001$), highlighting the acute LPS-mediated inflammatory response in the mice. As expected, blood cell counts were altered in LPS mice (Figure S1G, http://links.lww.com/BCF/A193). After mice were sacrificed 48 h post-LPS injection, hepatic *Proc* levels were not significantly different between LPS and PBS groups (Fig. 2e, $P = 0.64$). However, plasma PC levels were increased over time: a nonsignificant rise of ~10% at 24 h ($P = 0.09$), and a significant increase of ~50% 48 h post-LPS injection (Fig. 2f/g, $P < 0.01$).

As an alternative method to induce endotoxemia, we used *Staphylococcus aureus*-derived α-toxin at a dose that elicited a phenotype comparable to that of LPS-treated mice (Figure S2, http://links.lww.com/BCF/A193). Using a similar experimental timeline as for the LPS experiment (Fig. 1), venous thrombosis was observed in five of nine PBS-treated mice compared to two of nine α-toxin-treated mice (Fig. 2h, $P = 0.14$). The experiment was terminated 48 h postsiRNA injection (26 h postα-toxin) to avoid undue suffering. Plasma PC levels were elevated in α-toxin-treated mice, although the difference did not reach statistical significance (Fig. 2i, $P = 0.14$).

## Discussion

Immunothrombosis as a consequence of bacterial sepsis is a widely recognized yet mechanistically underexplored concept. In the present study, we aimed to combine two well established murine models for venous thrombosis and endotoxemia, to study immunothrombosis. Unexpectedly, endotoxemia attenuated rather than promoted venous thrombosis. This appears to be caused by a transient increase in PC, following endotoxemia. As siRNA-induced venous thrombosis strongly depends on transient lowering of circulating PC, this endotoxemia-mediated elevation compromised the comparability between our experimental groups. These findings illustrate that while the siRNA-based venous thrombosis model is highly effective for studying venous thrombosis in noninfectious conditions [10,11], it is unsuitable for evaluating putative prothrombotic interventions that alter circulating PC levels.

The current study highlights the importance of validating the suitability of in-vivo models for specific pathophysiological conditions. Supporting the robustness of our findings, we observed similar effects on venous thrombosis prevention using different forms of LPS that engage distinct downstream signalling pathways [15], as well as α-toxin derived from an unrelated bacterial species (*E. coli* and *S. aureus*, respectively). The transient endotoxemia-induced increase in PC likely masked possible prothrombotic effects of endotoxemia. Previous studies monitoring PC levels following endotoxemia report conflicting results [16–18], likely due to differences in mice, endotoxemia agent, route of administration, or other methodological variations. Nevertheless, our findings are consistent with a prior report indicating that low-dose LPS increased PC levels in young mice, comparable to our experimental setup [18]. Additionally, how our endotoxemia models, as well as those reported in the literature, relate to bacterial sepsis models and the role of PC remains unknown.

We observed increased hepatic *Proc* mRNA expression in LPS-treated mice that had previously received siRNA, in contrast to mice receiving LPS alone. This differential regulation suggests that *Proc* transcript partially suppressed by siRNA is ‘responsive’ to LPS stimulation than transcript expressed at maximal level. The molecular mechanism by which LPS directly or indirectly modulates *Proc* expression and circulating PC levels remains to be elucidated. Two of eight mice treated with LPS and siRNAs died approximately 24 h after LPS injection, without developing venous thrombosis. This observation aligns with previous studies demonstrating that PC possesses cytoprotective and anti-inflammatory functions [17,19,20]. Our results suggest that transient siRNA-mediated suppression of PC impaired these protective mechanisms, potentially contributing to mortality.

Although numerous studies have reported that inflammatory conditions, such as sepsis and chronic inflammatory disorders, promote immunothrombosis and venous thrombosis [21–23], our data appear to contrast with these findings. We propose that the endotoxemia-induced transient increase in PC accounts for reduced venous thrombosis in our model. Our findings emphasize the context-dependent nature of immunothrombosis and the necessity for rigorous model validation when studying immunothrombosis.

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### Conflicts of interest

The authors declare no competing interests.

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