***Materials and Methods***

***Drugs, reagents and antibodies***

Human Plg and VPLCK (D-Val-Phe-Lys chloromethyl ketone) were from Calbiochem (EMD Chemicals, San Diego, CA, USA) human Pla and TXA (tranexamic acid) were from Sigma-Aldrich (St. Louis, MO, USA). LPS from *Escherichia coli* (serotype O:111:B4) was from Sigma-Aldrich. Fluorescent monoclonal anti-mouse antibodies for flow cytometry were F4/80 (PE-Cy7, Invitrogen, Carlsbad, CA, USA), GR1 (APC, eBioscience, Thermo Fisher Scientific, MA, USA), CD11b (V500, BD Biosciences, Franklin Lakes, NJ, USA).

***Treatments***

Mice received an intraperitoneal (i.p.) injection of either 10 μg of human plasmin, 10 μg of human plasminogen or vehicle. To analyse inflammatory parameters, mice received a single treatment 3 hours after sepsis induction. To analyse survival rates, mice were treated at 3 and 12 hours after sepsis induction. A group of mice also received a broad-spectrum antibiotic, imipenem (30 mg/kg i.p.) alone, or in combination with plasminogen (10 μg/mice). The doses of plasminogen or plasmin used here (10 μg/mice or 100 nM) were chosen after titration in our CLP model and are very similar to that we have used in our previous studies (2-4ug/cavity or 2-4 ug/ml or 20-40nM) (1-3) and from other studies (10-120nM) (4, 5). TXA (100mg/kg, i.p.) (6) was given 30 min prior to Pla.

***Bacterial counts***

Measurement of bacteria in blood and peritoneal lavage fluid was performed 12 hours after the induction of sepsis. Mice were euthanized and blood was collected by cardiac puncture using sodium citrate (3.2%). Peritoneal lavage was performed with 4mL of sterile PBS/EDTA (1mM). Bacterial loads were determined by plating blood and the

25 peritoneal lavage in Brain Heart Infusion (BHI) plates (37°C incubation, overnight). The
26 results were expressed as log of CFU per mL.

27 ***Phagocytosis***

28 Phagocytosis was evaluated as previously (7). Briefly, $2x10^5$ murine bone marrow-
29 derived macrophages (BMDMs) isolated from WT C57BL/6J mice, were plated, and
30 incubated with *E. coli* (MOI 1:10) for 3 hours to allow phagocytosis (1 h of adhesion at
31 4°C followed by 2 h at 37°C). Noninternalized bacteria were washed out with gentamycin
32 (5 µg/ml in PBS, 30min.). To assess phagocytosed bacteria, macrophages were lysed as
33 described (7, 8), and viable internalized bacteria were counted in LB agar plates after
34 incubation (37°C, overnight).

35 ***Neutrophil preparation***

36 Mouse bone marrow neutrophils were isolated using Percoll density gradients (1119 and
37 1077) and resuspended in RPMI 1640 supplemented with 0.5% BSA. Neutrophil purity
38 was determined by cytospin slides (>95%).

39 ***NET quantification***

40 Myeloperoxidase (MPO)/DNA assay was performed as previously described (9-11).
41 Briefly, an antibody bound to a 96-well plate captured anti-myeloperoxidase (MPO - 5
42 µg/ml; Abcam). The amount of DNA bound to the enzyme was quantified using the
43 Quant-iT™PicoGreen® kit (Invitrogen, MA, USA) according to the manufacturer's
44 instructions. The fluorescence intensity (excitation at 488 nm and emission at 525 nm)
45 was quantified using a FlexStation 3 Microplate Reader (Molecular Devices, CA, USA).
46 Measurement of citrullinated histone H3 (H3cit) was performed according to the
47 manufacturer's instructions (Cayman Chemical, MI, USA).

48

49 ***Immunofluorescence staining and confocal microscopy***

50 The procedure was performed as described (11). Initially, neutrophils were attached to
51 slides coated with a poly-L-lysine (0.1%) solution (Sigma-Aldrich, MO, USA) for 4 h
52 and then fixed with paraformaldehyde (2%). The samples were then washed with wash
53 buffer and blocked with 1% BSA and glycine (22.52 mg/mL) in PBST (PBS + 0.1%
54 Tween 20). The cells were stained with the following antibodies: rabbit anti-histone H3
55 (H3Cit; #ab5103 - Abcam; 1:500); mouse anti-MPO; 2C7; ab25989 - Abcam; 1:500).
56 Next, the samples were incubated with the secondary antibodies anti-mouse IgG Alexa
57 Fluor 647 (Abcam; 1:800); anti-rabbit IgG Alexa Fluor 594 (Abcam; 1:800). The nuclei
58 were stained with DAPI; D1306 - Life Technologies; 1:1.000). Images were acquired
59 using Axio combined with an LSM 780 confocal microscope system at 630×
60 magnification (Carl Zeiss, Germany). All acquired images were analyzed using Fiji by
61 ImageJ.

62 ***Patient samples***

63 Peripheral blood samples from 12 septic and 10 septic shock patients were obtained in
64 this study. The definitions proposed by the Third International Consensus Definitions for
65 Sepsis and Septic Shock were applied (12). Patients admitted with confirmed or suspected
66 severe sepsis or septic shock diagnosed at the time of the admission in the ICU or
67 immediately after the diagnosis of sepsis, in case they were already at the ICU, were
68 considered for inclusion. The inclusion criteria were: 1) age over or equal to 18 years old;
69 2) patients being hospitalized in one of the units participating in the study, with a
70 perspective of staying hospitalized longer than 24 hours; 3) authorization to participate in
71 the study through the signature of the informed consent (which could be done by a
72 responsible person, if the patient was not aware or with adequate conditions of
73 discernment); 4) patient's with clinical suspicion or microbiological confirmation of

74 sepsis or septic shock (12). Sepsis was defined by the presence of at least one new organ
75 dysfunction associated with infection, characterized by a variation of at least two points
76 in the Sepsis Organ Failure Assessment (SOFA) score (13). Septic shock was defined as
77 persistent hypotension requiring vasopressors to maintain a mean arterial pressure (MAP)
78 greater than 65 mmHg associated with a lactate serum level greater than 2 mmol/L despite
79 adequate fluid resuscitation (14). Microbiological confirmation of the infectious
80 condition was defined by the isolation of at least one microorganism considered
81 pathogenic, no more than 48 hours before inclusion in the study, and for which physicians
82 directed antimicrobial therapy. The clinical data were collected prospectively, and the
83 following variables were recorded: age; sex; SOFA severity score at admission; total
84 leukocytes, neutrophils and platelets counts; arterial lactate; C-reactive protein (CRP)
85 levels; and hemoglobin levels.

86 The exclusion criteria were: 1) use of antibiotic therapy against the current
87 infectious process for longer than 48 hours; 2) severely immunosuppressed patients (HIV
88 infection with CD4+ lymphocyte levels < $200/mm^3$, severe neutropenia < 500/mL, post-
89 solid organ or bone marrow transplant patients, patients using chemotherapeutic agents
90 in the last 28 days, patients with inflammatory or autoimmune diseases in chronic use of
91 immunosuppressants or in pulse therapy; 3) patients undergoing palliative care only; 4)
92 patients expected to be deceased in the next 24 hours; 5) Patients diagnosed with
93 infections known to require prolonged antibiotic therapy; 6) polytraumatized patients or
94 patients submitted to major surgeries in the last five days (except surgery for control of
95 the infectious focus).

96 Blood samples were obtained at time of inclusion (day 1) and at the third day of
97 follow-up. The serum samples were obtained from blood collected for routine ICU
98 analyses. The serum was stored in a -80ºC freezer until analysis.

99

100 ***ELISA***

101 The levels of murine IL-6, TNF-α, IL-10 and CXCL-1 were measured in the supernatants
102 of peritoneal lavage fluids and plasma of mice by ELISA, according to the manufacturer's
103 instructions (R&D Systems, MN, EUA). Human IL-6 measurement was performed in
104 plasma of samples using Peprotech (New Jersey, USA) ELISA kit, according to the
105 manufacturers' instructions. Plg measurement was performed on plasma of mice and from
106 human cohorts by using ELISA kits from Abcam (UK). PAI activity assay was
107 determined in serum of patients by ELISA (Molecular Innovations, MI, USA), according
108 to the manufacturer's instructions.

109 ***ALT, creatinine and fibrinogen measurements***

110 ALT (Gold Analisa Diagnóstica, MG, Brazil), fibrinogen (Bioclin, MG, Brazil), and
111 creatinine (Labtest, MG, Brazil) levels were determined in plasma of mice according to
112 the manufacturer's instructions. The results were expressed as indicated in graphs.

113 ***Western blotting***

114 Liver tissue from mice were processed and lysed in Tissue Extraction Reagent (Invitrogen
115 #FNN0071) containing Protease Inhibitor cocktail (Thermo Fisher Scientific). Lysates
116 were electrophoresed on Bolt 4-12% Bis-Tris Plus polyacrylamide-SDS gels under
117 reducing conditions and electrotransferred to nitrocellulose membranes. Membranes were
118 incubated with primary antibodies (anti-Fibrinogen - Abcam ab92572; anti-β-actin - LI-
119 COR, Lincoln, NE, USA), washed with PBS-Tween-20 0.1% and incubated with species
120 specific IRDye®680RD/800CW or peroxidase-conjugate antibodies. Immunoreactive
121 bands were visualized using the Odyssey Imaging System (LI-COR), or ECL detection
122 system (GE Healthcare). For densitometric analysis, membranes were scanned and
123 quantified using the software Image Studio™ Lite Software 5.2 (LI-COR).

124 ***MPO and NAG quantification***

125 Livers were processed with different buffers specific for measurement of
126 myeloperoxidase (MPO) or N-acetyl-β-D-glucosaminidase (NAG) activities used as
127 neutrophil and macrophage accumulation indexes respectively, as described previously
128 (8, 15).

129 ***Statistical analysis***

130 All statistical analyses were performed using Prism 6.0 software. The results were
131 presented as mean ± Standard Error of the Mean (SEM) per group of 3 to 8 animals.
132 Results were analysed using the parametric t-test or non-parametric Mann Whitney test
133 (two groups) or one-way analysis of variance (one-way ANOVA), followed by multiple
134 comparisons of Newman-Keuls (three or more groups). Survival curves were expressed
135 as percentage of live mice observed at 12-hour intervals for six days. The logrank test
136 was used for the statistical analysis of the survival curves. $P < 0.05$ was considered as
137 statistically significant. Outliers were excluded from the analyses.

138 Patient data analysis was performed using the SPSS Statistics software version
139 17.0 (SPSS Inc., Chicago, Ill., USA). We assumed all data as parametric, and the
140 comparisons of the continuous variables between the two groups of patients were done
141 using the Student's t-test (unpaired or paired analysis where appropriate). Continuous
142 data are expressed as mean ± standard deviation (SD). The categorical variables were
143 analysed using the chi-square test and expressed as number (percentage). Correlations
144 between the circulating levels of Plg or PAI-1 and IL-6 were calculated by Pearson's
145 coefficients (R). We also performed a binary logistic regression analysis to predict the
146 relationship between Plg or PAI-1 and IL-6 levels and calculated the $R^2$ value. $P < 0.05$
147 was considered statistically significant. Outliers were excluded from the analyses.

148

149 **References**


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203

# Supplementary Figure 1

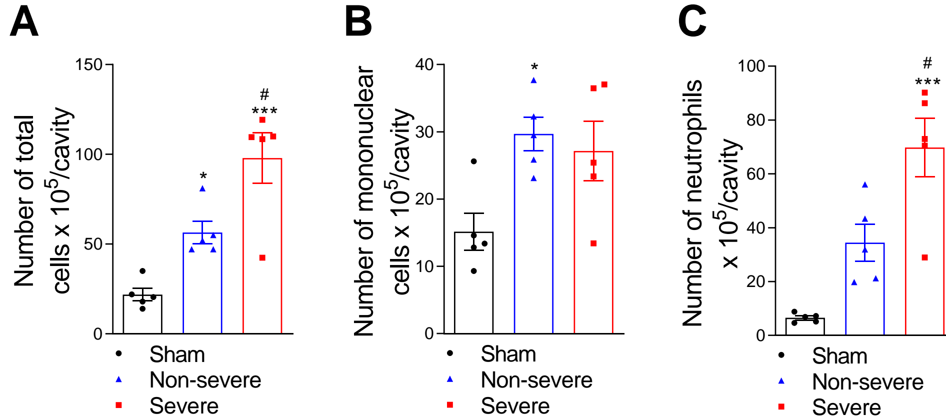


**Figure S1: Leukocyte recruitment to the peritoneal cavity in severe and non-severe sepsis**. C57BL/6J mice (n=5) were subjected to severe (18G needle) and non-severe (30G needle) CLP. Cells present in the peritoneal cavity were harvested 12 hours after CLP. The number of total cells (A), mononuclear cells (B) and neutrophils (C) were evaluated by counting cytospin slides stained with May-Grunwald-Giemsa. Results are shown as the mean ± SEM of at least five mice per group. *P < 0.05, ***P < 0.001 when comparing sham with CLP group. #P < 0.05 when comparing severe and non-severe sepsis.

# Supplementary Figure 2

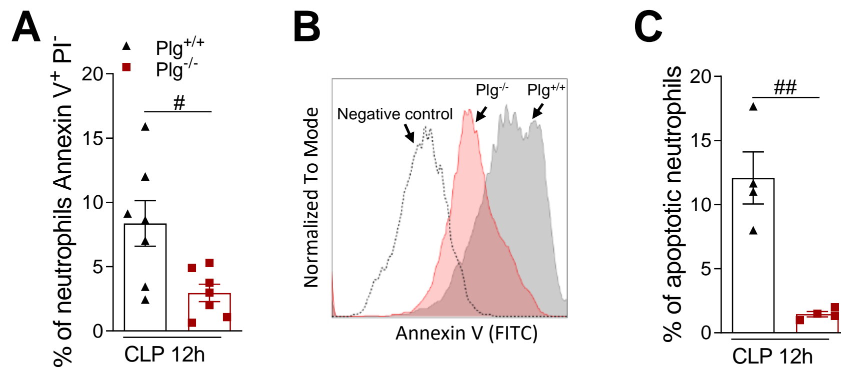


**Figure S2: Assessment of apoptosis in $Plg^{-/-}$ mice and their wild type littermates during non-severe sepsis.** $Plg^{+/+}$ and $Plg^{-/-}$ mice were subjected to non-severe (30G needle) CLP. Cells present in the peritoneal cavity were harvested 12 hours after CLP. The frequency of annexin $V^{+}$ neutrophils were determined by flow cytometry (A). Representative histograms are shown (B). The percentage of apoptotic neutrophils (C) was evaluated by counting cytospin slides stained with May-Grunwald-Giemsa. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ when comparing $Plg^{+/+}$ and $Plg^{-/-}$ mice.

## Supplementary Figure 3

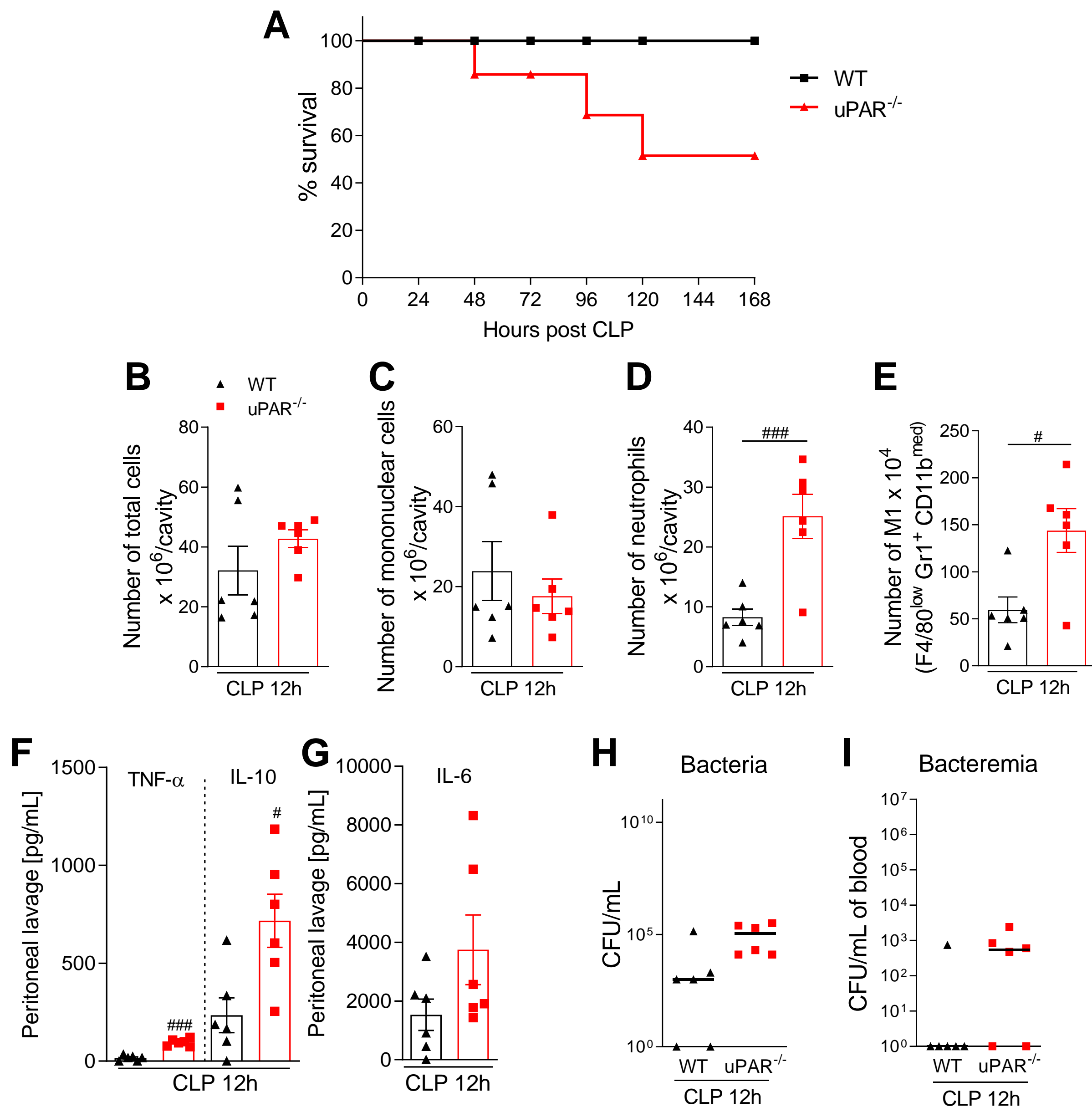


**Figure S3: Assessment of survival rates and inflammatory parameters in uPAR$^{-/-}$ mice and their wild type littermates during non-severe sepsis.** uPAR$^{+/+}$ and uPAR$^{-/-}$ mice were subjected to non-severe (30G needle) CLP. The survival rates were monitored for 7 days (A). Cells present in the peritoneal cavity were harvested 12 hours after CLP. The number of total cells (B), mononuclear cells (C) and neutrophils (D) were evaluated by counting cytospin slides stained with May-Grunwald-Giemsa. The number of M1 [F4/80$^{low}$ GR1$^{+}$ CD11b$^{med}$] macrophages were determined by flow cytometry (E). The levels of TNF, IL-10 and IL-6 were quantified in cell-free peritoneal lavages by ELISA (F, G). The peritoneal lavage (H) and blood (I) were plated in BHI media for the analysis of bacterial load. Results are shown as the mean ± SEM of at least five mice per group. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ when comparing WT and uPAR$^{-/-}$ mice. CFU, colony-forming unit.

## Supplementary Figure 4

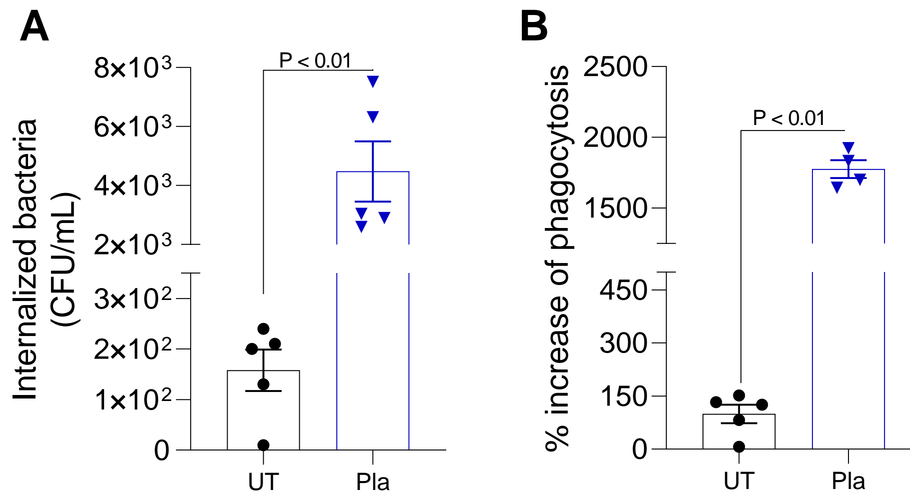


**Figure S4: Plasmin increases in vitro macrophage phagocytosis of *Escherichia coli*.** Bone marrow derived macrophages ($2 \times 10^5$) were pretreated with Plasmin (4 µg/mL) for 20 hours. Then, cells were incubated with E. coli (MOI 10) for 3 hours to allow adhesion (1h at 4 $^o$C) followed by phagocytosis (2h at 37 $^o$C). Noninternalized bacteria were excluded by the incubation of the cells with gentamicin, followed by the lysis of macrophages to identify the number of viable phagocytosed bacteria. Results are expressed as CFU/mL of internalized bacteria (A) or % increase of phagocytosis (B) (CFU counts on LB agar plates).

# Supplementary Figure 5

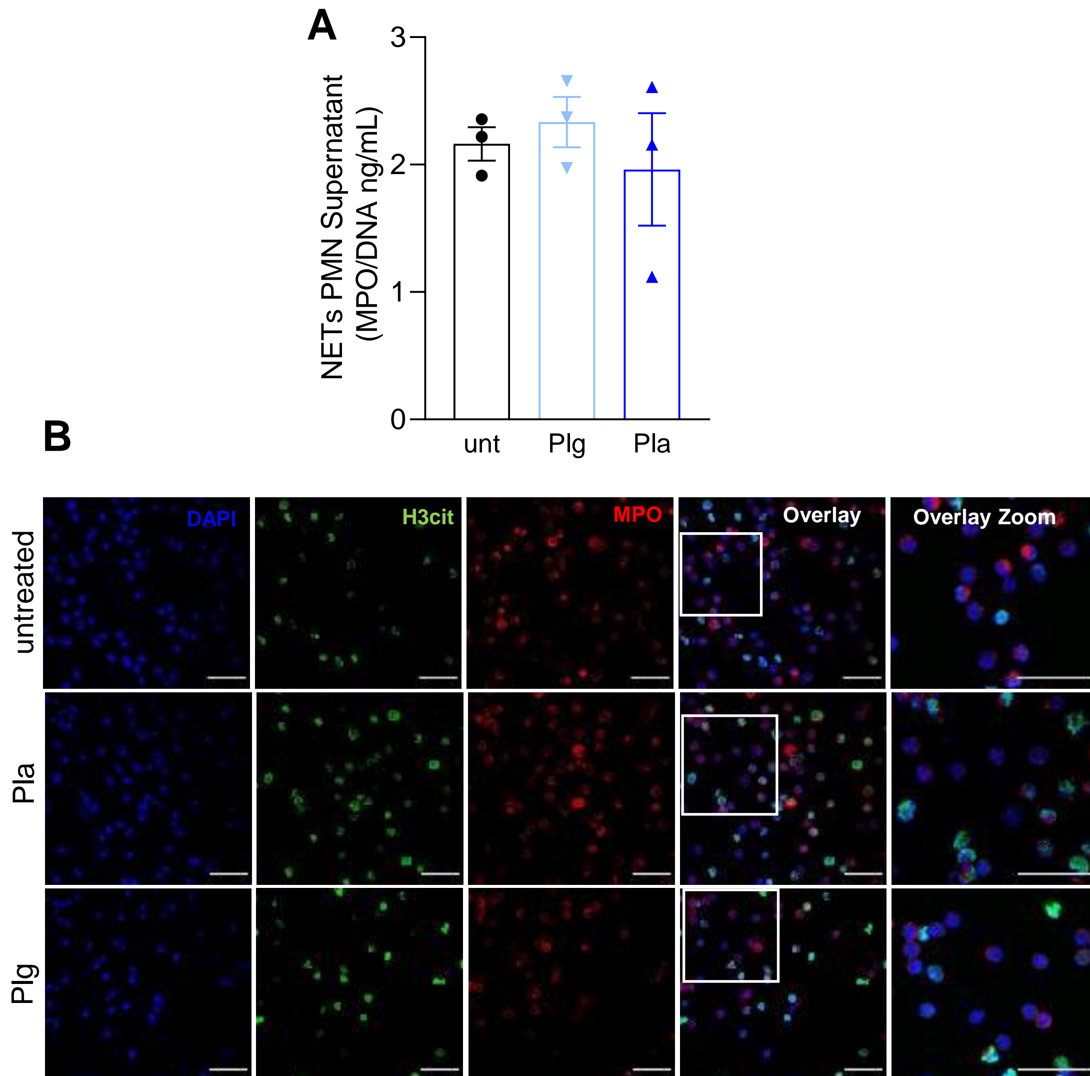


**Figure S5: Effects of plasminogen and plasmin on NETs release in vitro.** Bone marrow neutrophils obtained from C57BL/6J mice were treated with Plg (4 µg/mL) and Pla (4 µg/mL) for 5h. Quantification of NETs release (MPO/DNA) in supernatant (A). Representative fluorescence images of NETs stained for DNA (DAPI, blue), citrullinated histone H3 (H3cit, green) and myeloperoxidase (MPO, red), are shown (B). The scale bar indicates 50 µm at 630x magnification.

# Supplementary Table 1

**Table 1. Demographic and clinical parameters of patients with sepsis and septic shock.**

| | | Sepsis (n=14) | Septic shock (n=10) | *P* value |
|---|---|---|---|---|
| **Age (years-old)** | | 63 ± 15 | 61 ± 12 | 0.666[a] |
| **Sex** | **Female (n/%)** | 10 (71%) | 3 (30%) | 0.045*[b] |
| | **Male (n/%)** | 4 (29%) | 7 (70%) | |
| **SOFA at admission (points)** | | 5.0 ± 2.5 | 9.2 ± 4.4 | 0.006**[a] |
| **Total leukocytes (Ref: 3.6-11.2 K/µL)** | | 11.45 ± 5.08 | 17.75 ± 8.39 | 0.0318*[a] |
| **Neutrophils (Ref: 1.8-7.8 K/µL)** | | 9.14 ± 3.21 | 14.14 ± 6.60 | 0.0218*[a] |
| **Lactate levels (Ref: 0.1-1.3 mg/dL)** | | 1.97 ± 0.716 | 2.82 ± 0.967 | 0.0252*[a] |
| **C reactive protein (Ref: ≤ 10 mg/L)** | | 159.8 ± 130.4 | 313.9 ± 117.3 | 0.007**[a] |
| **Hemoglobin (Ref: 12.5-16.3 mg/dL)** | | 10.76 ± 2.29 | 9.84 ± 2.27 | 0.3391[a] |
| **Platelets (Ref: 159-386 K/µL)** | | 237 ± 119 | 222 ± 142 | 0.7756[a] |

Ref, reference range.; SOFA, Sequential Organ Failure Assessment. *$P < 0.05$, **$P < 0.01$ ([a] Student's t-test or [b] chi-square test). Continuous data are expressed as mean ± standard deviation and categorical variables as number (percentage).

# Supplementary Table 2

**Table 2. Significant correlations between plasminogen levels and laboratory parameters in patients with sepsis and/or septic shock according to hospitalization day.**

| | Day 1 | | Day 3 | |
|---|---|---|---|---|
| **Sepsis + septic shock** | R | *P* | R | *P* |
| Plg *vs.* platelets | 0,635 | 0,002 | NS | NS |
| Plg *vs.* C reactive protein | NS | NS | -0,525 | 0,012 |
| **Sepsis** | | | | |
| Plg *vs.* platelets | 0,615 | 0,033 | NS | NS |
| Plg *vs.* C reactive protein | -0,574 | 0,051 | NS | NS |
| **Septic shock** | | | | |
| Plg *vs.* platelets | 0,656 | 0,040 | NS | NS |

Plg, plasminogen; R, Pearson's correlation coefficient; NS, non-significant correlation.

# Supplementary Table 3

**Table 3. $Plg^{+/+}$ and $Plg^{-/-}$ mice numbers of leukocytes in blood after CLP**

| | $Plg^{+/+}$ | $Plg^{-/-}$ | *P* value |
|---|---|---|---|
| **Total cells (K/ul)** | 3.96 ± 2.88 | 11.15 ± 9.29 | $P = 0.0236$* |
| **Lymphocytes (K/ul)** | 1.19 ± 1.30 | 2.68 ± 2.23 | $P = 0.0624$ |
| **Monocytes (K/ul)** | 1.20 ± 2.29 | 2.32 ± 3.18 | $P = 0.3557$ |
| **Neutrophils (K/ul)** | 2.31 ± 1.65 | 5.81 ± 5.08 | $P = 0.0425$* |
| **Platelets (K/ul)** | 289 ± 204 | 313 ± 145 | $P = 0.7532$ |

t test *$P < 0.05$