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Sylvie Cointe, Loris Vallier, Pierre Esnault, Mathilde Dacos, Amandine Bonifay, Nicolas Macagno, Karim Harti Souab, Corinne Chareyre, Coralie Judicone, Diane Frankel, et al.

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1 ***Granulocyte microvesicles with a high plasmin generation capacity promote***  
2 ***clot lysis and improve outcome in septic shock***

3

4 **Running heads: Granulocyte MV plasmin generation in septic shock**

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27

1 **KEY POINTS**

- 2 • Granulocyte MV from septic shock patients display a heterogeneous profile of plasmin  
3 generation capacity driven by the uPA/uPAR system.
- 4 • High level of PGC confers to granulocyte MVs the capacity to reduce clot formation  
5 and improves survival in a mouse model of septic shock.

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1 **ABSTRACT**

2 Microvesicles (MVs) have previously been shown to exert profibrinolytic capacity, which is  
3 increased in patients with septic shock (SS) with a favorable outcome. We therefore  
4 hypothesized that the plasmin generation capacity (PGC) could confer to MVs a protective  
5 effect supported by their capacity to lyse a thrombus, and we investigated the mechanisms  
6 involved. Using a MV-PGC kinetic assay, ELISA and flow cytometry, we found that  
7 granulocyte MVs (Gran-MVs) from SS patients display a heterogeneous PGC profile driven  
8 by the uPA (urokinase)/uPAR system. *In vitro*, these MVs lyse a thrombus according to their  
9 MV-PGC levels in a uPA/uPAR-dependent manner, as shown in a fluorescent clot lysis test  
10 and a lysis front retraction assay. Fibrinolytic activators conveyed by MVs contribute to  
11 approximately 30% of the plasma plasminogenolytic capacity of SS patients. In a murine  
12 model of SS, the injection of high PGC Gran-MVs significantly improved mouse survival and  
13 reduced the number of thrombi in vital organs. This was associated with a modification of the  
14 mouse coagulation and fibrinolysis properties toward a more fibrinolytic profile. Interestingly,  
15 mouse survival was not improved when soluble uPA was injected. Finally, using a multiplex  
16 array on plasma from SS patients, we found that neutrophil elastase correlates with the effect  
17 of high-PGC-capacity plasma and modulates the Gran-MV plasmin generation capacity by  
18 cleaving uPA-PAI-1 complexes. In conclusion, we show that high PGC level displayed by  
19 Gran-MVs reduce thrombus formation and improve survival conferring to Gran-MVs a  
20 protective role in a murine model of sepsis.

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## 1 INTRODUCTION

2 Septic shock (SS) is defined as a systemic response to an infection associated with  
3 vasoactive molecule-resistant hypotension and organ failure<sup>1</sup>. SS occurs in 10 to 20% of  
4 patients in intensive care units with a high mortality rate (30-50%)<sup>2</sup>. SS is characterized by  
5 intense systemic inflammation and exaggerated coagulation activation with defective  
6 fibrinolysis, ultimately leading to disseminated intravascular coagulation (DIC). SS-induced  
7 DIC results in the widespread development of fibrin clots, microvascular occlusion and  
8 reduced oxygen delivery to tissues, leading to multiple organ failure and death<sup>3</sup>. Accordingly,  
9 plasma levels of plasminogen activator inhibitor-1 (PAI-1), prothrombin/antithrombin ratio  
10 and fibrinogen have been found to be associated with SS patient outcomes<sup>4-6</sup>.

11 Cell-derived microvesicles (MVs) are subcellular particles shed by activated or  
12 apoptotic cells that participate in the hemostatic equilibrium<sup>7,8</sup>. MVs share a procoagulant  
13 potential that relies on the exposure of anionic phospholipids, especially phosphatidylserine,  
14 and the presence of tissue factor (TF)<sup>9</sup>, according to their cellular origin. MVs also harbor  
15 molecules that regulate their procoagulant activity, such as TF pathway inhibitor<sup>10</sup>,  
16 endothelial protein C receptor<sup>11</sup> and thrombomodulin<sup>12</sup> found on circulating MVs<sup>13,14</sup>.  
17 Consistent with the cellular activation and apoptosis in SS, elevated levels of MVs have been  
18 reported in patients with sepsis<sup>15-17</sup>. These MVs play a role in tissue injury and organ  
19 dysfunction by exerting deleterious procoagulant and proinflammatory effects and inducing  
20 endothelial dysfunction<sup>18-21</sup>. Converging animal and clinical studies have emphasized this  
21 deleterious procoagulant role of MVs in sepsis and SS<sup>22-24</sup>, whereas a series of unexpected  
22 observations have suggested a potential beneficial effect of selected MV subpopulations<sup>25-29</sup>.  
23 However, the mechanisms underlying their protective effects are not fully understood.

24 Previous data have shown that the plasmin activation system is carried by MVs  
25 originating from leukocytic and endothelial cells<sup>30</sup>. MV-dependent Plasmin Generation

1 Capacity (MV-PGC) involves the binding and activation of plasminogen in plasmin by its  
2 activator urokinase (uPA) or tissue plasminogen activator (t-PA), present at the MV surface.  
3 The MV-PGC is measurable on circulating MVs in healthy donors and is altered in various  
4 pathological settings<sup>31</sup>. We recently showed that SS patients with a higher level of MV-PGC  
5 have a higher survival compared with patients with a lower level of MV-PGC<sup>32</sup>. We therefore  
6 hypothesized that an increased MV-PGC could confer to MVs a protective role *via* the  
7 capacity to lyse a thrombus and investigated the mechanisms involved.

8

## 9 **METHODS**

### 10 ***Blood collection and MV preparation and analysis***

11 Blood samples were collected from patients with a diagnosis of SS according to  
12 the guidelines of the International Sepsis Definitions Conference within the first 24 hours  
13 after admission in intensive care unit along with age- and sex-matched healthy controls<sup>33</sup>.  
14 Samples were collected and processed for MV purification and analysis according to  
15 international guidelines<sup>34,35</sup>.

16 MVs that mimic MVs from SS patients were also generated as a model by  
17 stimulating whole blood with lipopolysaccharide (LPS) and purifying them the same way as  
18 the patient-derived MVs (LPS-MVs). In certain experiments, the uPA receptor (uPAR)  
19 present on the MV surface was saturated by the addition of exogenous uPA as previously  
20 described<sup>36</sup>. Gran-MVs were also prepared from granulocytes isolated from healthy donors.  
21 MVs were characterized using immunomagnetic separation, flow cytometry, fibrin  
22 zymography, ELISA, and plasmin generation assays; the details are described in the  
23 supplemental Methods.

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25

1 ***Thrombolysis experiments***

2           The lytic effect of MVs, isolated from the blood of SS patients or LPS-stimulated  
3 normal blood, was investigated using two different *in vitro* lysis tests: 1) a fluorescent clot  
4 was incubated in MV-free platelet-free plasma (PFP) with increasing concentrations of MVs,  
5 and the released fluorescence was monitored with a plate reader, and 2) the retraction of a  
6 fluorescent lysis front was measured by fluorescence microscopy. When needed, the  
7 euglobulin fraction was prepared by the acidification of citrated plasma, as previously  
8 described<sup>37</sup>. The lytic effect of the euglobulin fraction containing or not MVs isolated from SS  
9 patients or healthy donors was evaluated by measuring the absorbance of the thrombus. The  
10 details are provided in the supplemental Methods.

11

12 ***Mouse sepsis model***

13           Pathogen-free female CD-1 mice (Envigo Society France, Gannat, France) were  
14 operated (D0) and divided into three groups receiving either Gran-MVs saturated with  
15 exogenous uPA (uPA-Gran-MVs), Gran-MVs or the wash supernatant of uPA-Gran-MVs as  
16 control (SPN uPA-Gran-MVs). Sepsis was induced by cecal ligation and puncture as  
17 previously described<sup>38</sup> with some modifications. Twenty-four hours after surgery, each animal  
18 group received a daily intravenous tail injection (D1 to D4) of uPA-Gran-MVs ( $10^7$ ), Gran-  
19 MVs ( $10^7$ ) or SPN uPA-Gran-MVs. From D0 to D5, mortality was recorded, and each living  
20 animal was monitored to establish a clinical score<sup>39,40</sup>. At D5, all animals were sacrificed. The  
21 kidney and lungs tissues were analyzed for fibrin deposits as described in the supplemental  
22 Methods.

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## 1 *Statistical analysis*

2 All statistical analyses were performed with GraphPad Prism software v.5.0 (GraphPad  
3 Software, San Diego, CA, US). The tests are detailed in the supplemental Methods.

4

## 5 **RESULTS**

### 6 **Gran-MVs from SS patients display a heterogeneous profile of PGC driven by the** 7 **uPA/uPAR system**

8           When the MV-PGC of SS patients (n=34) was compared to that of healthy donors  
9 (n=18), we found no significant difference; however, the range of values in SS patients was  
10 much wider than that in normal subjects, with 53% of the SS patients falling outside the  
11 normal range (10-90<sup>th</sup> percentile, [0.8-43] vs. [1.6-3.6]  $A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ; Figure 1A). Patients  
12 were therefore stratified according to their MV-PGC levels, as having low ( $<1.5 A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ;  
13 30<sup>th</sup> percentile; L-SS), high ( $>5 A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ; 70<sup>th</sup> percentile; H-SS) or intermediate  
14 ( $1.5 A_{405\text{ nm}} \times 10^{-3}/\text{min} < \text{MV-PGC} < 5 A_{405\text{ nm}} \times 10^{-3}$ ) capacity. These two groups of patients were  
15 not different regarding demographics, medication and source of infection. But H-MV-PGC  
16 showed as expected lower severity scores and a better outcome compared to L-MV-PGC  
17 (supplemental table 1). To investigate their cellular origin, MV subpopulations were  
18 enumerated by flow cytometry. As expected from our previous work<sup>32</sup>, Gran-MVs were  
19 significantly increased in H-SS vs. L-SS (460 [308-1336] vs. 180 [0-360] Gran-MVs/ $\mu\text{l}$ ,  
20  $p=0.01$ , Figure 1B) and moderately correlated with the MV-PGC ( $r^2=0.34$ ,  $p=0.05$ ; table 1),  
21 whereas no significant difference was observed for the other subsets. In addition to this  
22 quantitative difference in Gran-MVs, we looked for an MV qualitative difference to  
23 investigate the molecular basis of this distinct PGC profile. Using flow cytometry, we  
24 compared the expression of uPA and its receptor uPAR on Gran-MVs in L- and H-SS patients  
25 compared to platelet-derived MVs used as negative controls. Gran-MVs from the H-SS

1 patients exhibited higher surface expression of both uPA and uPAR than those from L-SS  
2 patients (Figure 1C). Moreover, ELISA measurement of lysed MVs showed higher amounts  
3 of uPAR in MVs from H-SS vs. L-SS patients ( $0.56 \pm 0.28$  vs.  $0.15 \pm 0.05$  ng/ml,  $p < 0.05$ ;  
4 Figure 1D). However, the total amount of uPA and PAI-1 levels did not differ between the  
5 two groups, ( $0.09 \pm 0.04$  vs.  $0.1 \pm 0.07$  ng/ $2 \cdot 10^6$  MVs,  $p = 0.8$  Figure 1E;  $6.3 \pm 7$  vs.  $8 \pm 6.2$  ng/ $2 \cdot 10^6$   
6 MVs,  $p = 0.6$ ; Figure 1F, respectively). It suggests that the higher MV-PGC from H-SS patients  
7 is mainly due to a higher level of uPAR which increases the capacity of the MVs to capture  
8 uPA released into the local environment.

9

#### 10 **Gran-MVs from SS patients lyse a thrombus according to their MV-PGC level in a** 11 **uPA/uPAR-dependent manner**

12 To mimics the inflammatory conditions of SS, MVs were generated from human  
13 whole blood stimulated with LPS. In a fluorescent clot lysis test, we observed a significant  
14 dose- and time-dependent increase in the fluorescence released by LPS-MVs compared to the  
15 MV SPN used as a negative control ( $3520 \pm 1540$  AU vs.  $1050 \pm 40$  AU at  $5 \cdot 10^6$  LPS-MVs/24  
16 h, respectively,  $p < 0.05$ ; Figure 2A). This lysis effect was associated with a  $44 \pm 7\%$  ( $p < 0.05$ )  
17 loss of thrombus weight (Figure 2B). These results were confirmed by those of a lysis front  
18 retraction assay (Figure 2C) showing a significant MV-dependent lysed area in the presence  
19 of LPS-MVs compared to the MV SPN ( $63 \pm 42\%$  vs.  $9 \pm 4\%$  area of lysis, at  $1 \cdot 10^7$  LPS-MV/48  
20 h,  $p < 0.05$ , Figures 2C and 2D). Interestingly, the lytic effect was amplified when uPAR on the  
21 MV surface was saturated by the addition of exogenous uPA (uPA-LPS-MV) containing MVs  
22 from SS patients with high MV-PGC (Figures 2C upper panel and 2D). Notably, the lytic  
23 response was not always complete, and a thin layer of thrombus remained undissolved, as  
24 shown by confocal microscopy (supplemental Figure 1). The clot lysis experiments were  
25 repeated using the same amount of MVs from SS patients stratified according to their MV-

1 PGC levels. As shown in Figures 2E and 2F, the retraction of the lysis front was significantly  
2 higher in H-SS patients compared to L-SS patients. These data indicate that clot lysis  
3 efficiency is driven by the MV-PGC level.

4 To confirm the molecular partners responsible for this heterogeneous thrombolytic  
5 effect, lysis front retraction experiments were conducted with specific inhibitors. As  
6 illustrated in Figures 3A and 3B, thrombolysis was significantly reduced in the presence of  
7  $\alpha$ 2-antiplasmin or an anti-uPA antibody. The involvement of uPAR in this MV thrombolytic  
8 effect was assessed using LPS-MVs treated with phosphatidylinositol-specific phospholipase  
9 C (PI-PLC). Under these conditions, the MV-PGC and lysis area were dramatically reduced  
10 (Figures 3C, 3D and 3E). Finally, the cellular origin of the subset of MV involved in the  
11 fibrinolytic effect was identified by showing that the depletion of CD15+MVs monitored by  
12 flow cytometry (Figure 4A) was associated with a decrease of more than 90% in both MV-  
13 PGC capacity and lysis front retraction (Figures 4B, 4C and 4D), whereas the depletion of  
14 erythrocyte and platelet subsets had no effect. The fibrinolytic effect of Gran-MVs was  
15 confirmed by a significantly larger lysed area compared to SPN ( $10\pm 4\%$  vs.  $4\pm 2\%$  area of  
16 lysis, at  $5.10^6$  Gran-MV/48 h,  $p < 0.05$ , Figures 4E and 4F). Additionally, the lytic effect was  
17 observed with uPA-Gran-MVs (Gran-MVs with uPAR saturated by exogenous uPA) as early  
18 as 24h and amplified at 48h (Figures 4E and 4F). The absence of residual soluble uPA was  
19 confirmed by ELISA (supplemental Figure 2). Altogether, these data identify Gran-MVs as  
20 the MV subset that mediates the thrombolytic capacity driven by the uPA/uPAR system.

21

## 22 **Fibrinolytic activators carried by MVs significantly contribute to the plasma fibrinolytic** 23 **capacity of SS patients**

24 To assess the relevance of the fibrinolytic mechanism driven by MVs, we compared  
25 the relative contributions of MV- and non-MV-derived plasma fibrinolytic capacity using a

1 euglobulin clot lysis assay<sup>41</sup> to reduce the concentration of fibrinolysis inhibitors  
2 (supplemental Figure 3A). We first found that most (90±1.9%) of the Gran-MVs previously  
3 labeled with CD15+ and Annexin V-specific antibodies coprecipitated with fibrinolysis  
4 activators (supplemental Figure 3B). Then, we used the euglobulin test with PFP and MV-free  
5 PFP from SS patients with high and low MV-PGC and healthy donors, and we tested the  
6 activator fraction for its plasmin activity or for its capacity to lyse a thrombus *in vitro*. In  
7 healthy subjects, we did not observe any significant difference in either the PGC or the time to  
8 50% lysis between the PFP and the MV-free PFP (supplemental Figures 3C and 3D). In  
9 contrast, in H-SS patients, when MVs were removed from plasma, we measured a significant  
10 decrease in the PGC (-35±37%, p=0.048, supplemental Figure 3E) and a significant increase  
11 in the time to 50% lysis (+25±15%, p=0.003, supplemental Figure 3F). Interestingly, these  
12 data are not observed in SS patients with low MV-PGC (supplemental Figures 3G and 3H).  
13 These results indicate that in SS patients with high MV-PGC, fibrinolytic activators carried by  
14 MVs contribute to approximately 30% of the plasma fibrinolytic activity.

15

### 16 **Injection of MVs with a high level of PGC improves survival and reduces clot formation** 17 **in a murine model of sepsis**

18 To investigate whether the fibrinolytic potential of MVs is protective *in vivo*, we used  
19 a murine sepsis model<sup>42</sup> with a daily intravenous caudal injection (D1 to D4) of uPA-Gran-  
20 MVs, Gran-MVs derived from granulocytes of human healthy donors or the SPN of the uPA-  
21 Gran-MVs (SPN uPA-Gran-MVs) as a negative control (Figure 5A). The MV-PGC values  
22 were 4±6 and 1±1 A<sub>405 nm</sub>×10<sup>-3</sup>/min for uPA-Gran-MVs and Gran-MVs, corresponding to the  
23 capacity of H-SS and L-SS patients, respectively. The severity of sepsis was similar between  
24 the groups before the first injection, as shown by similar temperature variations (Figure 5B)  
25 and clinical scores (Figure 5C). The plasmin generation capacity of uPA-Gran-MVs was

1 significantly higher than that of Gran-MVs ( $7.9 \pm 0.6$  vs.  $1.9 \pm 0.2$   $A_{405} \text{ nm} \times 10^{-3}/\text{min}$ ,  
2 respectively,  $p=0.0008$ ) and predominant compared to the procoagulant activity, which could  
3 be considered negligible (MV-TF=  $14.7 \pm 6.5$  fM) by reference to the procoagulant activity  
4 of Mono-MVs (MV-TF=  $353 \pm 311$  fM) (Figure 5D). Consequently, the injection of uPA-  
5 Gran-MVs compared to Gran-MVs or SPN uPA-Gran-MVs significantly improved the  
6 survival rate of SS mice at D5 (92% vs. 50% and 58%, respectively, Gehan-Breslow-  
7 Wilcoxon test  $p=0.05$  and  $p=0.02$ , respectively, Figure 5E). Moreover, fibrin clot staining  
8 revealed a significant reduction in the number of thrombi in the kidneys of mice injected with  
9 uPA-Gran-MVs compared to the two other types ( $3.7 \pm 0.9$  vs.  $7.7 \pm 1.9$  thrombi/ $2.4 \text{ mm}^2$   
10  $p=0.001$ ; and  $6.9 \pm 2.5$  thrombi/ $2.4 \text{ mm}^2$ ,  $p=0.015$ , respectively, Figures 5Fab). This  
11 observation was confirmed in lung tissues (Figures 5Fcd). Complementary experiments using  
12 fibrin/fibrinogen immunolabeling (supplemental Figure 4) confirmed that the fibrin-positive  
13 areas (supplemental Figure 4A) are superimposed with the lacquered blue stained areas with  
14 PTAH (supplemental Figure 4B) and demonstrates that the material in the vessels contains  
15 fibrins. Measurement of plasmatic parameters in surviving mice, including AST, ALT,  
16 creatinine, and LDH, did not show a significant difference between the three groups of mice.  
17 The survival benefit is maintained at day 10 with no additional death with or without MVs  
18 injection between day 5 and day 10. The injection of three different levels of uPA-Gran-MVs  
19 ( $5 - 10 - 20 \cdot 10^6$ ) for 5 days showed no significant difference between the groups. These data  
20 indicated that a daily  $5 \cdot 10^6$  dose is sufficient to observe a survival benefit after injection of  
21 fibrinolytic MVs with no additional benefit from higher doses. Taken together, these data  
22 show that the elevated PGC of Gran-MVs improves sustainably survival and reduces the  
23 number of thrombi in the vital organs in a mouse sepsis model. Interestingly, the survival  
24 benefit was not observed when an equivalent amount (25  $\mu\text{g}$ ) of soluble uPA was injected for  
25 five days compared to the injection of uPA-Gran-MVs, indicating that conveying of uPA by

1 MVs is essential for the protective effect of uPA in sepsis (Figure 5G). To better understand  
2 the protection afforded to septic mice injected with uPA-Gran-MVs, we measured the impact  
3 of the MV injection on the mouse hemostatic balance (Figure 6A). As illustrated in Figure  
4 6Bb, we observed a significant increase in PAP at D2 in the group injected with uPA-Gran-  
5 MVs compared to the other groups (124 +/- 24 ng/ml vs. 55 +/- 27 ng/ml,  $p = 0.034$  and 51.5  
6 +/- 25.8 ng/ml,  $p = 0.036$ ). This was followed by a lesser decrease in MV-PGC at D5 in this  
7 group, compared to the groups injected with Gran-MVs and SPN uPA-Gran-MVs (5.9 +/- 0.6  
8 vs. 3.2 +/- 0.5,  $p = 0.0016$  and 3.4 +/- 0.4  $A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ,  $p = 0.0016$ , respectively) (Figure  
9 6Cc). On the other hand, the injection of MVs did not significantly modify the mouse  
10 procoagulant activity. Indeed, as illustrated in Figures 6D and 6E, TAT and MV-TF levels  
11 were not different between the septic mice at D1 and D3 but were increased compared to  
12 nonseptic mice (control). Interestingly, the D-Dimers normalized at D5 only in the uPA-Gran-  
13 MV group (Figure 6F). Altogether, these data indicate that the injection of fibrinolytic MVs  
14 (uPA-Gran-MVs) modified the hemostatic balance of mice toward a more fibrinolytic profile.

15

### 16 **Soluble molecules from SS patient plasma modulate Gran-MV plasmin generation** 17 **capacity in SS**

18 To better understand how the plasma environment can modulate Gran-MV PGC  
19 capacity, we investigated the potential role of the surrounding plasma factors. To this end, we  
20 performed incubation assays using granulocytes from healthy donors and plasma (depleted or  
21 not of MVs) from L-SS and H-SS patients (Figure 7A). MVs generated by granulocytes were  
22 called “Gran-induced MVs” (Gran-iMVs). We observed that the quantities of Gran-iMVs  
23 were not significantly different between the L-SS and H-SS patients. In contrast, the Gran-  
24 iMV-PGC was significantly increased by stimulation with plasma from H-SS patients  
25 compared to plasma from L-SS patients ( $1.36 \pm 0.84$  vs.  $0.85 \pm 0.60$   $A_{405\text{ nm}} \times 10^{-3}/\text{min}$ , for  $4.10^5$

1 Gran-iMV,  $p=0.02$ ; Figure 7B). Interestingly, this result remained similar when the  
2 stimulation was performed with MV-free plasma (Figure 7C), suggesting the presence of  
3 soluble factors triggering distinct MV-PGC profiles in patients.

4 To identify these factors, SS plasma was screened by a multiplex array for 23  
5 different molecules known to modulate the uPA/uPAR system (supplemental table 2). A  
6 correlation analysis identified that among these factors, human neutrophil elastase (NE),  
7 CD40L, PDGF-AB/BB and TGF- $\alpha$  were the ones most associated with the Gran-iMV-PGC  
8 ( $r^2=0.6, 0.56, 0.48$  and  $0.46$ , respectively, table 2, supplemental figure 5). When granulocytes  
9 were directly incubated with these factors, no significant difference in the Gran-iMV-PGC  
10 was observed with CD40L, PDGF-AB/BB and TGF- $\alpha$ . In contrast, the effect of NE was  
11 demonstrated first by a significant decrease in the MV-PGC by blocking NE with the addition  
12 of a specific inhibitory antibody to the plasma (Figure 7D) and second by a significant  
13 increase in the Gran-iMV-PGC after the supplementation of L-SS plasma with  $0.3$  nM NE (no  
14 increase was found in the H-SS plasma;  $+101\%$ ,  $p=0.02$ , Figure 7E). Altogether, these data  
15 identify NE as an MV-PGC modulator present in the plasma of SS patients.

16 To further decipher the role of NE, we measured the impact of a range of NE  
17 concentrations on the PGC of Gran-MVs isolated from the plasma of LPS-treated blood by  
18 CD15-IMS. As shown in Figure 7F, NE concentrations of  $0.04$  nM and above significantly  
19 increased the MV-PGC. Because neither direct cleavage of the plasmin substrate nor direct  
20 plasminogen activation by NE were observed<sup>32</sup>, these data demonstrate that NE indirectly acts  
21 on the MV-PGC. Because plasminogen activator inhibitor (PAI-1) is a known target of  
22 NE<sup>43,44</sup>, we hypothesized that the MV-PGC might be regulated by NE in a PAI-1-dependent  
23 manner. First zymography confirmed (Figure 7G) that the uPA/PAI-1 complex was  
24 significantly reduced in the presence of NE without any impact on the uPA bands  
25 (Supplemental Figure 6). Second, if Gran-MVs incubated with PAI-1 in the absence or

1 presence of NE, we observed a significant decrease in the MV-PGC after incubation with  
2 PAI-1, which was partially restored by the presence of 0.3 nM NE (Figure 7H). Collectively,  
3 these data demonstrate that NE is one of the components of the plasma environment of SS  
4 patients which modulates the Gran-MV-PGC in a PAI-1-dependent manner.

5

## 6 **DISCUSSION**

7 In this study, we demonstrate that MVs from SS patients display a heterogeneous  
8 pattern of PGC driven by the uPA/uPAR system carried by MVs of granulocytic origin.  
9 Interestingly, Gran-MVs with a high PGC reduce clot formation *via* a mechanism involving  
10 elastase and improve survival in a mouse model of SS. These data demonstrate that high MV-  
11 PGC levels confers to MV a fibrinolytic protective response that may counterbalance the  
12 procoagulant state associated with SS.

13 As described in the literature, MV-dependent hemostatic activities (their procoagulant  
14 and fibrinolytic activities) can be modulated depending on the stimuli triggering their  
15 formation and their cellular origin<sup>45,46</sup>. Converging animal and clinical studies have  
16 emphasized the deleterious role procoagulant MVs play in sepsis and SS<sup>15,47-51</sup>, whereas a  
17 series of unexpected observations indicate a potential beneficial effect of select MV  
18 subpopulations<sup>25,27,28,52-56</sup>. Accordingly, in severe sepsis, in some studies, elevated levels of  
19 Gran-MVs were found to be associated with a more favorable outcome, as shown by reduced  
20 mortality rates and organ dysfunction<sup>27,53</sup>. Consistent with these data, in patients with acute  
21 respiratory distress syndrome, Gran-MVs were the unique subset whose level in  
22 bronchoalveolar lavages was associated with an improved prognosis<sup>25</sup>. In a mouse sepsis  
23 model, Gran-MVs enriched in  $\alpha$ 2-macroglobulin were shown to improve the resolution of  
24 infection by enhancing bacterial clearance<sup>27</sup>. Moreover, the dissemination of anti-  
25 inflammatory or antibacterial molecules such as TGF $\beta$ , annexin-1 or endothelial protein C

1 receptor by Gran-MVs has also been reported<sup>28,52,54-56</sup>. In the present work, we propose a new  
2 protective mechanism based on MV-associated high fibrinolytic capacity driven by the  
3 uPA/uPAR system through which Gran-MVs from SS patients impact survival in a mouse  
4 model of sepsis and reduce thrombus formation both *in vitro* and *in vivo*.

5 In SS, fibrinolysis is classically described as being dramatically impaired, thus leading  
6 to a procoagulant state responsible for widespread fibrin deposition<sup>3</sup>. In fact, this inhibition is  
7 heterogeneous among patients according to the levels of PAI-1<sup>57</sup> and the levels of cell- or  
8 MV-PGC<sup>32</sup>. However, whether this capacity is indirectly associated with survival or may be a  
9 significant contributor to a rescue mechanism is unknown. Here, we identified the uPA/uPAR  
10 system as the key plasminogen activator involved in this mechanism. The critical involvement  
11 of uPAR was demonstrated by the suppression of the fibrinolytic effect when uPAR was  
12 enzymatically removed from the MV surface and its enhancement when uPAR was saturated  
13 with exogenous uPA. To our knowledge, apart from matrix metalloproteinase (MMP)-17 and  
14 MMP-25, uPAR is the only GPI-anchored protein described to be involved in the fibrinolysis  
15 system<sup>58,59</sup>. Because of their substrate profiles, MMP-17 and MMP-25 are unlikely to be  
16 involved in the MV-PGC. In addition, we found that circulating Gran-MVs contribute to the  
17 plasma fibrinolysis in H-SS patients by carrying a significant proportion of the fibrinolysis  
18 activators in addition to their soluble fraction, in contrast to L-SS patients or healthy donors.  
19 Based on these results, we propose a protective mechanism that may counterbalance the  
20 procoagulant state associated with sepsis by contributing to the removal of microthromboses.  
21 This hypothesis is supported by our results in the murine SS model, where the injection of  
22 Gran-MVs enriched with uPA was associated with a more fibrinolytic profile (increase PAP  
23 and MV-PGC), without inducing a change of MV-TF that is considered as a risk factor of  
24 thrombus formation neither in TAT that reflect the activation of the coagulation cascade.  
25 More importantly, these hemostatic changes were associated with a decreased thrombus

1 formation in vital organs and an increased mouse survival. The absence of a beneficial effect  
2 from soluble uPA compared to uPA vectorized by MVs may reflect a better protection against  
3 physiological inhibitors or a better targeting of the thrombi when uPA is associated with the  
4 vesicular membrane.

5  
6 These data identify granulocytes or, more specifically, neutrophils as the key actors in  
7 this protective mechanism because they are the source of both the fibrinolytic MVs and the  
8 NE. This mechanism remains to be demonstrated *in vivo*. In sepsis, neutrophils are considered  
9 to play a paradoxical role, both protective and deleterious. By facilitating the recognition,  
10 entrapment and disposal of pathogens, neutrophils protect the host without inducing major  
11 collateral damage, as long as they function properly. However, when these reactions occur  
12 systemically and are sustained for a long period, they can lead to DIC followed by multiorgan  
13 failure and even death<sup>60,61</sup>. Through the formation of neutrophil extracellular traps and their  
14 components (DNA, histones and granules), neutrophils can propagate prothrombotic  
15 effects<sup>62,63</sup>. However, in subsequent stages, neutrophils seem to restrict thrombus growth by  
16 releasing fibrinolytic proteases such as NE<sup>64,65</sup>. The release of fibrinolytic MVs by  
17 granulocytes in SS patients may therefore represent an additional regulatory mechanism  
18 contributing to the maintenance of a vascular hemostasis equilibrium favorable to organ  
19 perfusion. A similar duality can be seen for NE. Following bacterial infection, the recruitment  
20 of vast numbers of neutrophils is associated with the extracellular release of NE, which is  
21 recognized as a key factor in the granulocyte anti-infection arsenal. Indeed, the absence of NE  
22 in mouse models of sepsis worsens the prognosis<sup>66,67</sup>. However, at the same time, NE  
23 propagates persistent neutrophilic inflammation by accelerating proinflammatory cytokine  
24 production, encouraging the development of anti-NE inhibitors as a therapeutic strategy in  
25 sepsis<sup>68-70</sup>. At the same time, NE propagates persistent neutrophil inflammation by

1 accelerating proinflammatory cytokine production, encouraging the development of anti-NE  
2 inhibitors as a therapeutic strategy in sepsis. Similarly, NE generated by activated neutrophils  
3 may contribute to thrombus formation via inactivation of TFPI<sup>62</sup>. However, this procoagulant  
4 activity is balanced by other studies showing that cleavage of active PAI-1 by NE also  
5 supports a fibrinolytic activity<sup>43</sup>, which is in agreement with the *ex vivo* observations of the  
6 present work.

7  
8 Our data illustrate that heterogeneous populations of Gran-MVs, can be generated in  
9 response to distinct inducers of vesiculation. As illustrated in the present study we identified  
10 NE as an inducer able to promote the formation of Gran-MVs with a high MV-PGC.  
11 Additionally, we showed that the fibrinolytic and procoagulant activities are modulated  
12 depending on the inducers triggering on MVs derived from a myelocyte cell line (HL-60)  
13 (supplemental Figure 7). In agreement, other studies in the literature have provided evidence  
14 that different stimuli induced granulocyte to release MVs with different biological properties.  
15 Accordingly, neutrophilic granulocytes stimulated with opsonized particles produced MVs  
16 that are able to impair bacterial growth<sup>55</sup>. More recently, using a set of 14 distinct inducers,  
17 neutrophils were found to generate different subtypes of extracellular vesicles: neutrophil-  
18 derived trails (NDTRs) generated by neutrophil migrating towards inflammatory foci,  
19 whereas neutrophil- derived macrovesicles (NDMV) were generated by neutrophils that have  
20 arrived at the inflammatory foci. NDTRs contains proinflammatory miRNAs, induce  
21 proinflammatory macrophages polarization, and showed protective effects in murine models  
22 of acute and chronic inflammation<sup>71</sup>.

23  
24 One of the key features of our data is the heterogeneous profile of MV-PGC driven by  
25 the qualitative differences in the Gran-MVs among SS patients, in line with the heterogeneous

1 profile of sepsis patients<sup>72</sup>. Indeed, sepsis patients have wide variations in their genetic  
2 background, age, gender, accompanying diseases, chronically prescribed medications, source  
3 of infection, pathogens involved and the sites of infection. This heterogeneity has been one of  
4 the major hindrances in sepsis research, particularly affecting the development of therapies.  
5 Because MVs are a miniature version of cells reflecting the fluctuating status of patients<sup>73</sup>,  
6 they form a complex signature reflecting the multifaceted responses of patients driven by their  
7 septic environment. Accordingly, as shown by the sepsis literature, MVs can have both  
8 protective and detrimental effects, either locally or at distant sites, and can take the shape of  
9 either a friend or a foe according to the cellular origin and/or the cargo they carry. Our data  
10 suggest that patients who are able to produce MVs with high PGC potential are better  
11 protected from this lethal complication. Thus, a number of promising speculations can be  
12 drawn, proposing MVs as multifaceted mediators useful in patient-tailored therapy.

13

14 In conclusion, this work demonstrates that Gran-MVs from SS patients convey an uPA-  
15 dependent MV-PGC that promotes clot lysis. This mechanism indicates that fibrinolytic MVs  
16 are key players in the thrombolytic process and an efficient protective response that may  
17 counterbalance the procoagulant state associated with sepsis. Thus, this "bloodborne  
18 fibrinolytic capacity of Gran-MVs" may be a new subcellular mechanism that could play a  
19 key role in the outcome of SS patients.

20

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25

1 **AUTHOR CONTRIBUTIONS**

2 S. C. and L. V. performed the research, analyzed and interpreted the data and write the  
3 manuscript. T. B., C. J., S. R., D. F., C. C., M. D., S. H., N. M., and A. B. performed the  
4 research, collected the data and analyzed and interpreted the data. M.C.A., P. P. and J. A.  
5 designed the research and reviewed the manuscript; F D.-G. supervised the work and helped  
6 write the manuscript, and R. L. designed the research, analyzed and interpreted the data and  
7 helped write the manuscript.

8

9 **DISCLOSURE OF CONFLICTS OF INTEREST**

10 We disclose as a conflict of interest that this work received financial support from  
11 Stago Company who has an exclusive license (patent N°07/04060). to the above-mentioned  
12 patent, and C. J. and P. P. are full-time employees of BioCytex, a Stago group company.

13

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- 26

1

	<b>r (Spearman)</b>	<b>p-value</b>
<b>AnnV+ MVs</b>	<b>0.43</b>	<b>0.01</b>
<b>Gran-MVs (AnnV/CD15)</b>	<b>0.34</b>	<b>0.05</b>
<b>P-MVs (AnnV/CD41)</b>	<b>0.16</b>	<b>0.5</b>
<b>Ery-MVs (AnnV/CD235a)</b>	<b>0.08</b>	<b>0.7</b>
<b>E-MVs (AnnV/CD146)</b>	<b>0.03</b>	<b>0.9</b>

2 *AnnV+ MVs=annexin V+ microvesicles; Gran-MVs=granulocyte-derived MVs; P-MVs=platelet-derived MVs;*  
3 *Ery-MVs=erythrocyte-derived MVs; E-MVs=endothelial-derived MVs.  $p \leq 0.05$  was considered a significant*  
4 *nonparametric correlation (Spearman's correlation).*

5

6 **Table 1. Correlations between the MV-PGC and MV subsets**

7

<b>Soluble molecule</b>	<b>r (Spearman)</b>	<b>p-value</b>
<b>Elastase</b>	<b>0.6</b>	<b>0.005</b>
<b>sCD40L</b>	<b>0.56</b>	<b>0.01</b>
<b>PDGF-AB/BB</b>	<b>0.48</b>	<b>0.03</b>
<b>TGF-<math>\alpha</math></b>	<b>0.46</b>	<b>0.04</b>
<b>TSP-1</b>	<b>0.28</b>	<b>0.23</b>
<b>FGF-2</b>	<b>0.21</b>	<b>0.37</b>
<b>IL-10</b>	<b>0.17</b>	<b>0.48</b>
<b>Resistin</b>	<b>0.15</b>	<b>0.53</b>
<b>TNF-<math>\alpha</math></b>	<b>0.1</b>	<b>0.66</b>
<b>IL-8</b>	<b>-0.04</b>	<b>0.84</b>
<b>G-CSF</b>	<b>0.03</b>	<b>0.88</b>
<b>IL-6</b>	<b>0.03</b>	<b>0.91</b>

8  *$p \leq 0.05$  was considered a significant nonparametric correlation (Spearman's correlation). Only significant*  
9 *correlations were included in the table.*

10

11 **Table 2. Correlations between the MV-PGC and SS patient plasma inflammatory**  
12 **molecule concentrations.**

13

14

15

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17

1 **FIGURE LEGENDS**

2 **Figure 1: Gran-MVs from SS patients display a heterogeneous profile of PGC driven by**  
3 **the uPA/uPAR system.** A. The microvesicle-dependent plasmin generation capacity (MV-  
4 PGC) of MVs isolated from SS patient platelet-free plasma, with a low MV-PGC (black  
5 circle), an intermediate MV-PGC (white circle) and a high MV-PGC (circle with a cross),  
6 compared to the MV-PGC of MVs isolated from healthy donor plasma (n=18, white square).  
7 Each patient and healthy control were evaluated in duplicate. B. Counts of Gran-MVs  
8 (AnnV/CD15), P-MVs (AnnV/CD41), Ery-MVs (AnnV/CD235a) and E-MVs  
9 (AnnV/CD146) by flow cytometry in PFP from SS patients with low ( $<1.5 A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ,  
10  $n=12$ , white square) or high ( $>5 A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ,  $n=10$ ; black square) MV-PGC values. Data  
11 are mean $\pm$ SEM. C. Flow cytometric detection of uPA (upper graphs) and uPAR (lower  
12 graphs) on the surface of CD15+ MVs from SS patients with low (L-SS-MV) or high (H-SS-  
13 MV) PGC values (black traces). The expression of uPA or uPAR on CD41+ platelet derived  
14 MV was used as a negative control (gray traces). D/E/F. Detection by ELISA of uPAR, uPA,  
15 and PAI-1 on purified total MVs from SS patients (SS-MVs) divided into two groups: patients  
16 with low ( $<1.5 A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ,  $n=11$ , L-SS-MV, black circle) and high ( $>5 A_{405\text{ nm}} \times 10^{-3}/\text{min}$ ,  
17  $n=11$ , H-SS-MV, circle with a cross) MV-PGC values. NS: not significant; \*  $p<0.05$ .

18

19 **Figure 2: MVs from SS patients lyse a thrombus according to their MV-PGC level.** A/B.  
20 MVs purified from blood stimulated with LPS (LPS-MVs) lyse a fluorescent human thrombus  
21 suspended in plasma in a dose-dependent manner ( $5-0.3 \times 10^6$  MVs). Thrombolysis was  
22 monitored by measuring the fluorescence released in the thrombus supernatant at 1, 2, 4 and  
23 24 h (A) and the loss of thrombus weight after 48 h (B). The results are expressed as the  
24 percentage of fluorescence increase and weight loss compared to their initial values at T0  
25 ( $n=5$ ). C/D. Thrombolysis mediated by LPS-MVs ( $10^7$  MVs) in a lysis front retraction model.

1 Lysis is amplified by the saturation of uPAR with exogenous uPA (uPA-LPS-MVs) on the  
2 MV surface. Representative fluorescence microscopy images (C) display the retraction of the  
3 lysis front (white arrows) at 0, 6, 24 and 48 h; black bar = 1000  $\mu\text{m}$ . The lysis area (D) was  
4 calculated considering the whole thrombus surface ( $4.3 \text{ mm}^2$ ) using ImageJ software. uPA-  
5 LPS-MV: black circle; LPS-MV: grey circle and SPN: white circle, E/F. Thrombolysis  
6 mediated by MVs from SS patients in a lysis front retraction model. Representative  
7 fluorescence microscopy images (E) display thrombolysis in the presence of SS-MVs ( $2.10^7$   
8 MVs) purified from a pool of SS patients with low ( $<1.5 A_{405 \text{ nm}} \times 10^{-3}/\text{min}$ , L-SS-MV) or high  
9 ( $>5 A_{405 \text{ nm}} \times 10^{-3}/\text{min}$ , H-SS-MV) MV-PGC; n=3. For each experiment, the negative control  
10 was represented by the last MV wash supernatant (SPN). black bar = 500  $\mu\text{m}$ . The lysis area  
11 (F) was calculated considering the whole thrombus surface ( $4.3 \text{ mm}^2$ ) using ImageJ software.  
12 Low-SS-PGC: black circle, high-SS-PGC: circle with a cross) and SPN: white circle.

13

14 **Figure 3: MVs lyse a thrombus in a uPA/uPAR-dependent manner.** A/B. Lysis front  
15 retraction experiments were performed with MVs purified from blood and stimulated with  
16 LPS (LPS-MVs,  $2.10^7$ ) in the presence or absence of  $\alpha 2$ -antiplasmin (A2AP) at  $0.5 \mu\text{M}$ , an  
17 inhibitory anti-uPA antibody at  $50 \mu\text{g/ml}$  and an isotype control (IgG) at  $50 \mu\text{g/ml}$ ; n=9.  
18 Representative images for each time point (0 h, 24 h and 48 h) are displayed in A; black bar =  
19  $1000 \mu\text{m}$ . The lysis area was calculated at 48 h, considering the entire thrombus surface ( $4.3$   
20  $\text{mm}^2$ ) using ImageJ software (B). The negative control was represented by the SPN. C/D/E.  
21 LPS-MVs were treated with phosphatidylinositol-specific phospholipase C (PI-PLC;  $2$   
22 IU/ml). The MV-PGC of the PI-PLC-treated LPS-MVs (PI-PLC-LPS-MVs) was compared to  
23 that of the untreated LPS-MVs ( $2.5 \times 10^6$  MVs) (C). The thrombolytic effect of both types of  
24 LPS-MVs was evaluated in the lysis front retraction model at 0, 24 and 48 h in the presence of

1  $10^7$  MVs (D). The lysis area was calculated at 48 h, considering the entire thrombus surface  
2 ( $4.3 \text{ mm}^2$ ), using ImageJ software (E); n= 6.

3

4 **Figure 4: Gran-MVs are the MV subset that mediate the thrombolytic activity.** A. Flow  
5 cytometric monitoring of MVs after selective depletion of MV subsets: platelet- derived MVs  
6 (P-MVs), erythrocyte-derived MVs (ery-MVs) and granulocyte-derived MVs (Gran-MVs).  
7 Immunomagnetic separation was performed using beads coated either with CD41 (black  
8 circle) or CD235a (black square) or CD15 (black rhombus). Negative control experiments  
9 were performed in parallel using beads coated with an irrelevant antibody (IgG, clear  
10 symbols); n=3. B. The MV-PGC test was performed on MVs after the selective depletion of  
11 CD41+, CD235a+ or CD15+ MV subpopulations or after a control IMS (IgG); n=3, CD41:  
12 black circle, CD235a: black square, CD15: black rhombus and IgG: white circle. C. The  
13 thrombolytic effect of MVs depleted of CD15-MVs or those subjected to a control IMS  
14 depletion (IgG) was evaluated in the lysis front retraction model at 0 h, 24 h and 48 h in the  
15 presence of  $10^7$  MVs. D. The lysis area was calculated at 48 h, considering the entire  
16 thrombus surface ( $4.3 \text{ mm}^2$ ), using ImageJ software. \*  $p < 0.05$ ; \*\*  $p < 0.01$ . E/F. Thrombolysis  
17 mediated by Gran-MVs ( $5 \cdot 10^6$  MVs) in a lysis front retraction model. Lysis is amplified by  
18 the saturation of uPAR with exogenous uPA (uPA-Gran-MVs) on the MV surface.  
19 Representative fluorescence microscopy images (E) display the retraction of the lysis front  
20 (white arrows) at 0, 6, 24 and 48 h; black bar =  $1000 \mu\text{m}$ . The lysis area (F) was calculated  
21 considering the whole thrombus surface ( $150 \text{ mm}^2$ ) using ImageJ software; uPA-Gran-MV:  
22 black circle; Gran-MV: grey circle; SPN: white circle. n=5

23

24 **Figure 5: Injection of MVs with a high level of PGC improves survival and reduces clot**  
25 **formation in a murine model of sepsis.** A. Cecal ligation was performed on CD-1 mice

1 (n=36 animals). Each group of animals received a daily intravenous tail injection (D1 to D4)  
2 of uPA-Gran-MVs or Gran-MVs or SPN uPA-Gran-MVs from D1 (24 h after surgery). Once  
3 sepsis was established, mice were observed daily to assess spontaneous mortality and  
4 monitored to measure their heart rate, oxygen saturation, breathing rate, and body  
5 temperature. The animals were sacrificed at D5. B/C. Temperature amplitude at D1 (°C) and  
6 the clinical score of three groups of animals injected with uPA-Gran-MVs, Gran-MVs or SPN  
7 uPA-Gran-MVs (n=12 animals/group). Data are mean+/-SEM. D. Coagulolytic balance (MV-  
8 PGC and MV-TF) of uPA-Gran-MVs ( $10^7$ ), Gran-MVs ( $10^7$ ) and supernatant (SPN-uPA-  
9 Gran-MVs) and Mono-MVs ( $10^7$ ) (n=3). E. Survival curve of animals (n=12 in each group).  
10 The results are presented as the percent survival. F. a/c Count of microthrombi in kidney and  
11 lung tissues after hematoxylin-eosin and phosphotungstic acid-hematoxylin staining (PTAH;  
12 uPA-Gran-MVs n=10; Gran-MVs n=6; SPN uPA-Gran-MVs n=7). Data are mean+/-SEM.  
13 b/d Histopathology of lung and kidney tissue samples from different groups of mice. Injected  
14 with uPA-Gran-MVs (n=11); injected with Gran-MVs (n=6); control group injected with SPN  
15 uPA-Gran-MVs (n=6). Microthrombi are indicated by black arrows; black bar=100  $\mu$ m. G.  
16 Survival curve of animals injected in uPA-Gran-MVs ( $10^7$ ), soluble uPA or SPN-uPA-Gran-  
17 MVs (n=12 in each group). The results are presented as the percent survival.

18

19 **Figure 6: Impact of the MV injection on the mouse hemostatic balance.** A. Cecal ligation  
20 was performed on CD-1 mice on three kinetic, D1: 24 h after sepsis induction; D2: 48 h after  
21 sepsis induction; D5: 5 days after sepsis induction, n=36/group). Each group of animals  
22 received a daily intravenous tail injection of uPA-Gran-MVs or Gran-MVs or SPN uPA-  
23 Gran-MVs from D1 (24h after surgery). Once sepsis was established, the mice were observed  
24 daily to assess spontaneous mortality and monitored to measure their heart rate, oxygen  
25 saturation, breathing rate, and body temperature. In according to the kinetic, the animals were

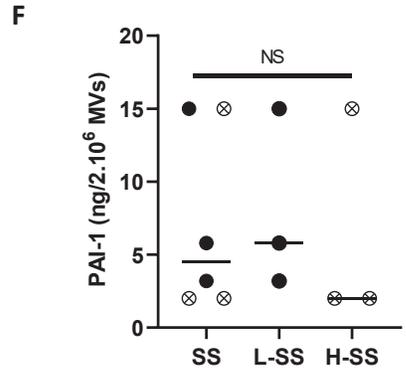
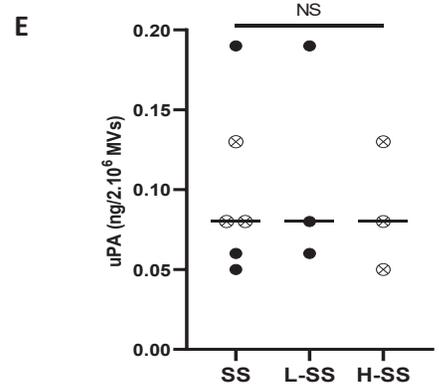
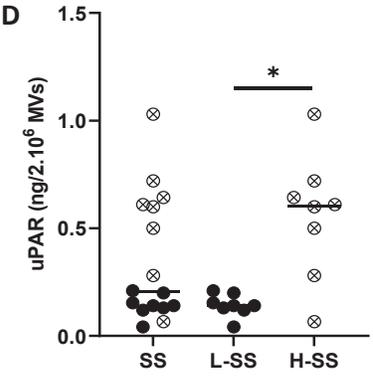
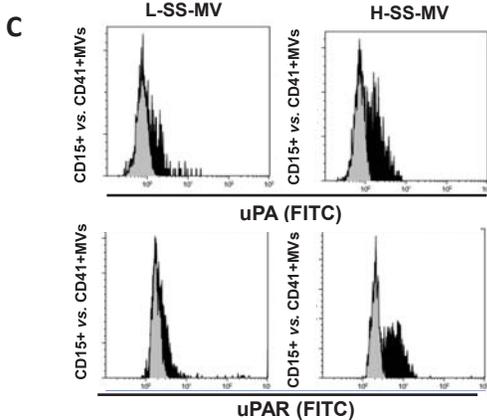
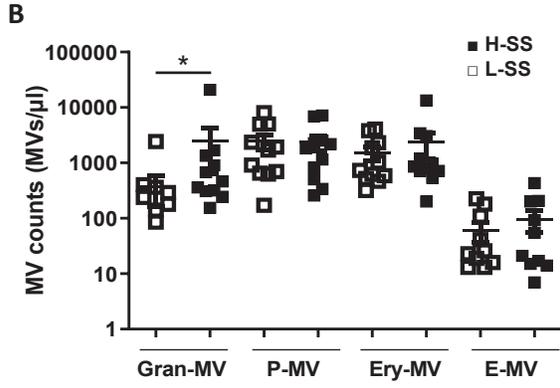
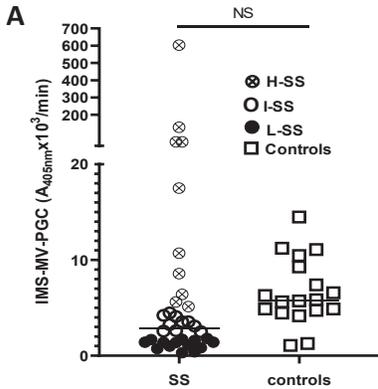
1 sacrificed at D1, D2 or D5. B/C/D/E/F. Measure of fibrinolytic and procoagulant parameters  
2 at D1, D2 and D5: plasmin-anti-plasmin complex (PAP), MV dependent plasmin generation  
3 capacity (MV-PGC), thrombin-anti-thrombin complex (TAT), MV dependent tissue factor  
4 activity (MV-TF) and D-dimer, respectively. Data are mean+/-SEM. NS: not significant; \*  
5  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.001$ .

6

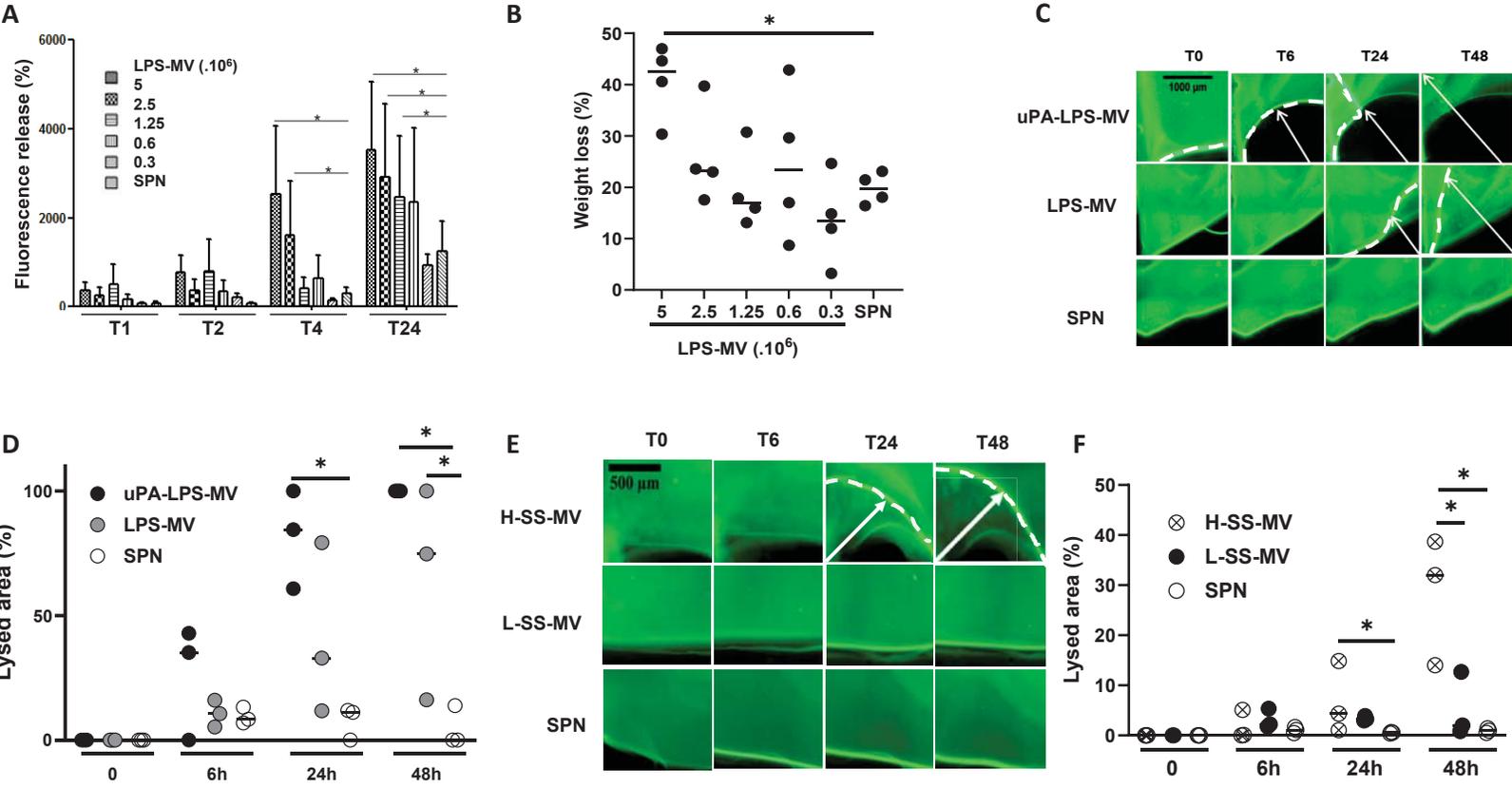
7 **Figure 7: Soluble molecules from SS patient plasma modulate Gran-MV plasmin**  
8 **generation capacity in SS.** A. Granulocytes (Gran) were purified from healthy individuals  
9 and then incubated with complete or MV-depleted plasma (PFP or MVFP) from SS patients  
10 with low (L-SS) or high (H-SS) plasmin capacity. Then, “Gran-induced MVs” (Gran-iMVs)  
11 were purified by ultracentrifugation and quantified by flow cytometry, and plasmin generation  
12 capacity was measured. B. MV-PGC of Gran-iMVs ( $4.10^5$ ) after stimulation with PFP from  
13 selected SS patients with low ( $<1.5 A_{405\text{ nm}}\times 10^{-3}/\text{min}$ ,  $n=11$ , L) or high ( $>5 A_{405\text{ nm}}\times 10^{-3}/\text{min}$ ,  
14  $n=10$ , H). C. MV-PGC of Gran-iMVs ( $4.10^5$ ) after stimulation with MVFP from selected SS  
15 patients with low ( $<1.5 A_{405\text{ nm}}\times 10^{-3}/\text{min}$ ,  $n=11$ , L) or high ( $>5 A_{405\text{ nm}}\times 10^{-3}/\text{min}$ ,  $n=10$ , H). D.  
16 MV-PGC of Gran-iMVs ( $4.10^5$ ) after stimulation with MV-depleted plasma from SS plasma  
17 treated with a human neutrophil elastase (NE) blocking antibody or isotype control ( $n=11$ ). E.  
18 MV-PGC of Gran-iMVs ( $4.10^5$ ) after stimulation with MV-depleted plasma enriched with NE  
19 (at a concentration range of 0.008-0.3 nM), from selected L-SS patient MV-PGC values  
20 ( $n=11$ ). Data are mean+/-SEM. F. PGC of MVs extract with CD15 immuno-magnetic beads  
21 (IMS-MV-PGC) from LPS-treated blood spiked with a range of NE concentrations (0.008-0.3  
22 nM;  $n=4$ ). Data are mean+/-SEM. G. Effect of a range of NE concentrations (0.008-0.3 nM)  
23 on the uPA/PAI-1 complex (95 kDa and 110 kDa) by zymography ( $n=3$ ; lytic zone in %,  
24 image digitally inverted). Data are mean+/-SEM. H. Effect of a range of NE concentrations

- 1 (0.008-0.3 nM) in the presence of PAI-1 (440 ng/ml) on the MV-PGC of purified Gran-MVs
- 2 ( $2 \cdot 10^6$ ; n=6). Data are mean $\pm$ SEM. NS: not significant; \* p<0.05; \*\* p<0.01.

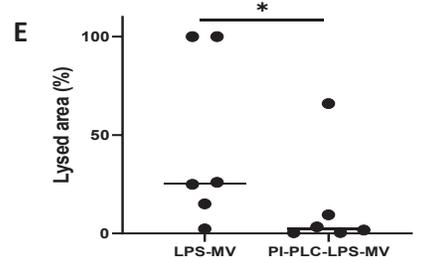
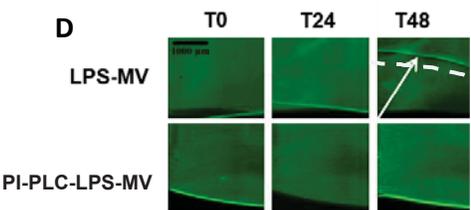
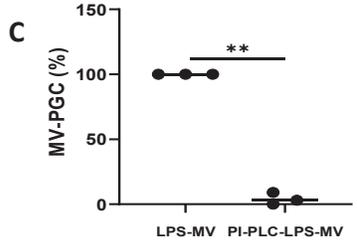
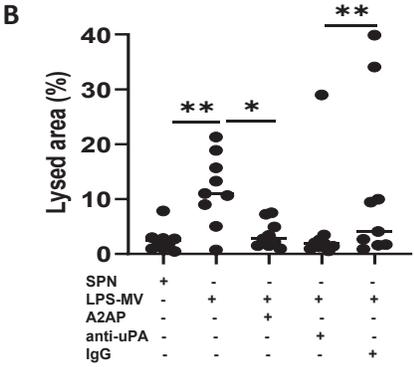
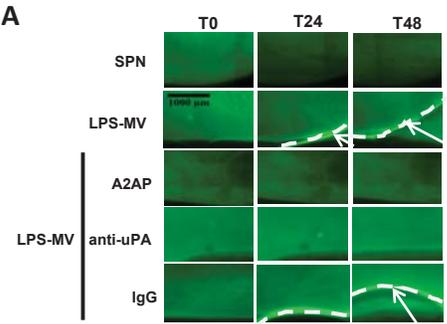
**FIGURE 1**



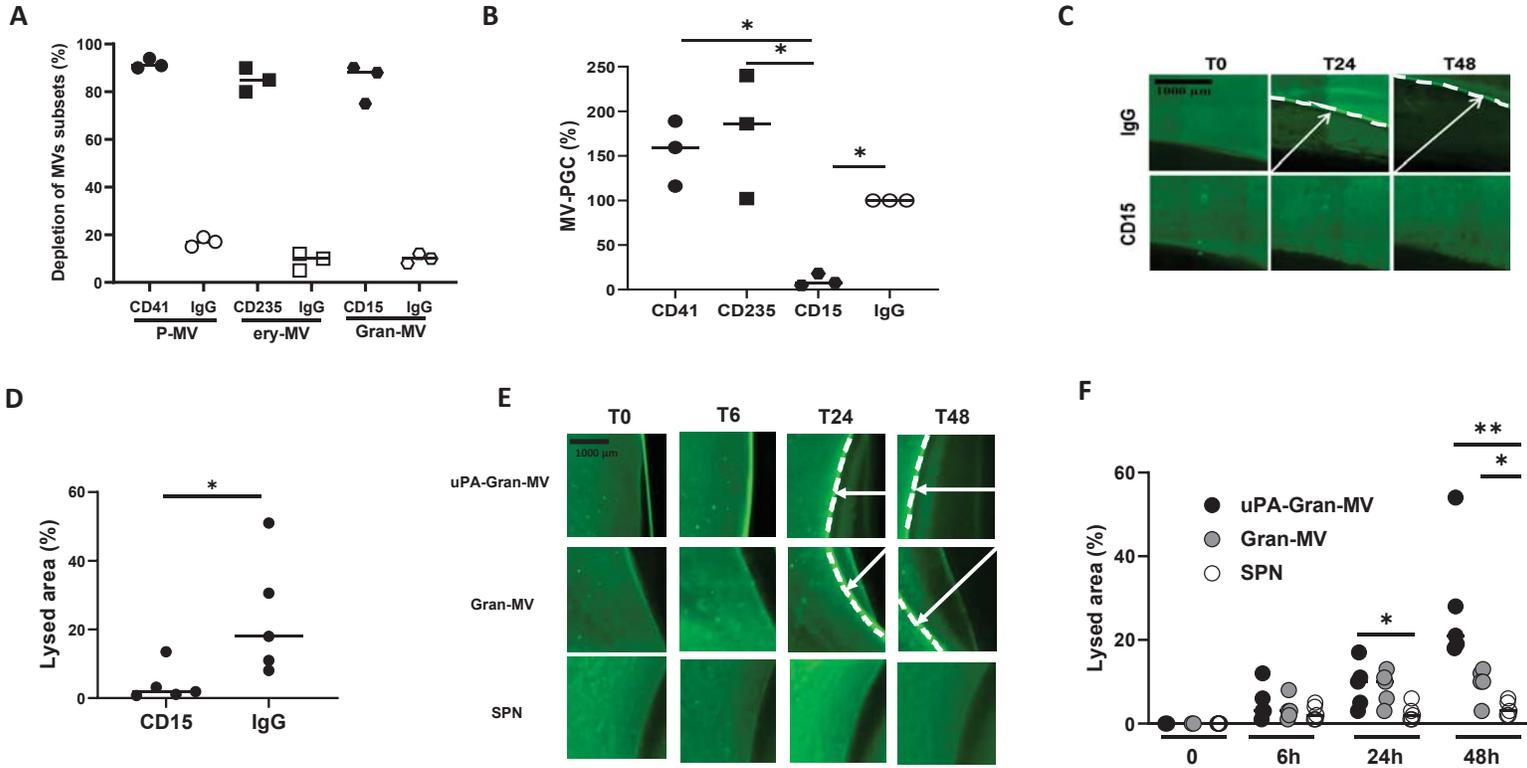
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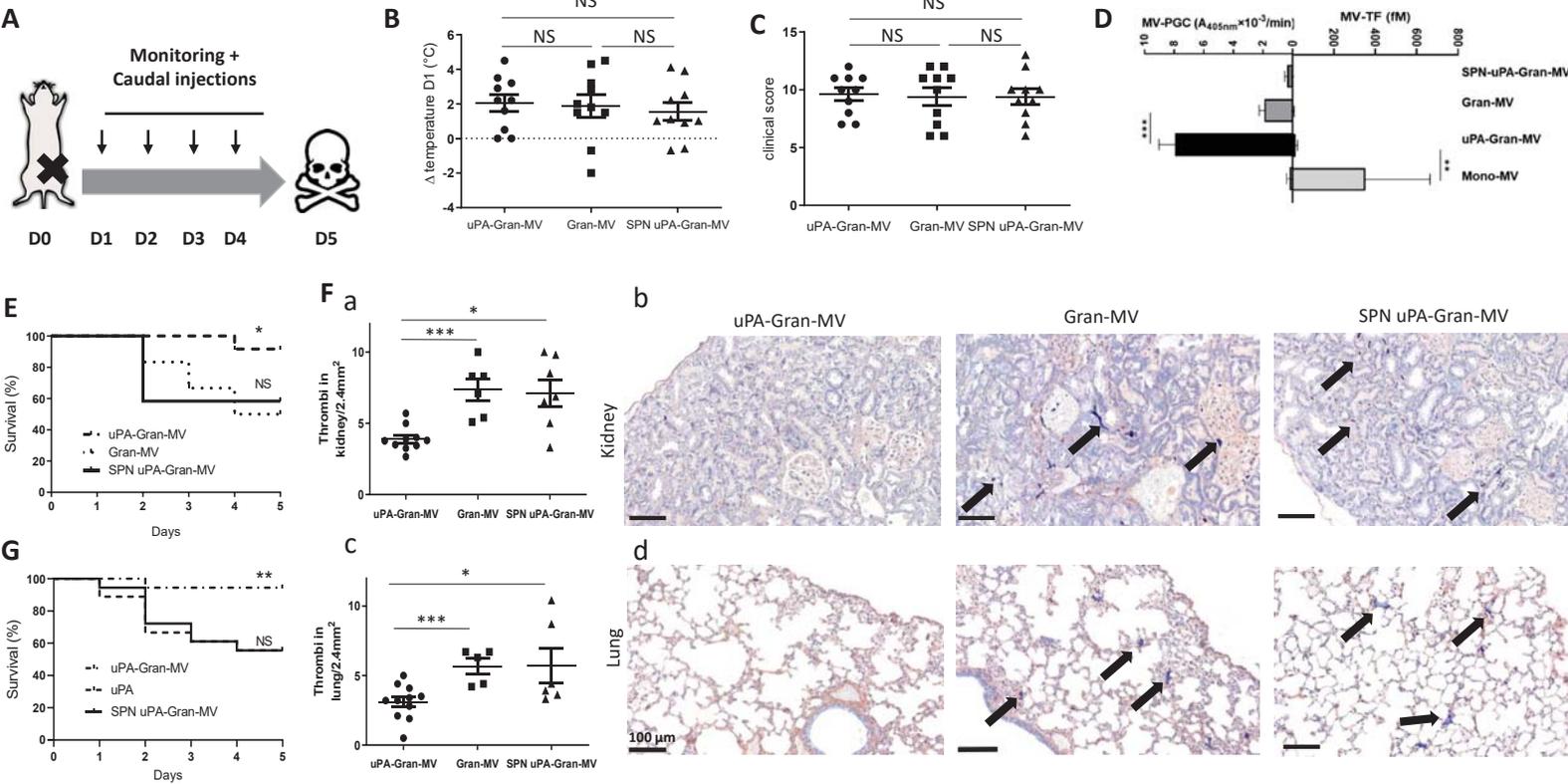
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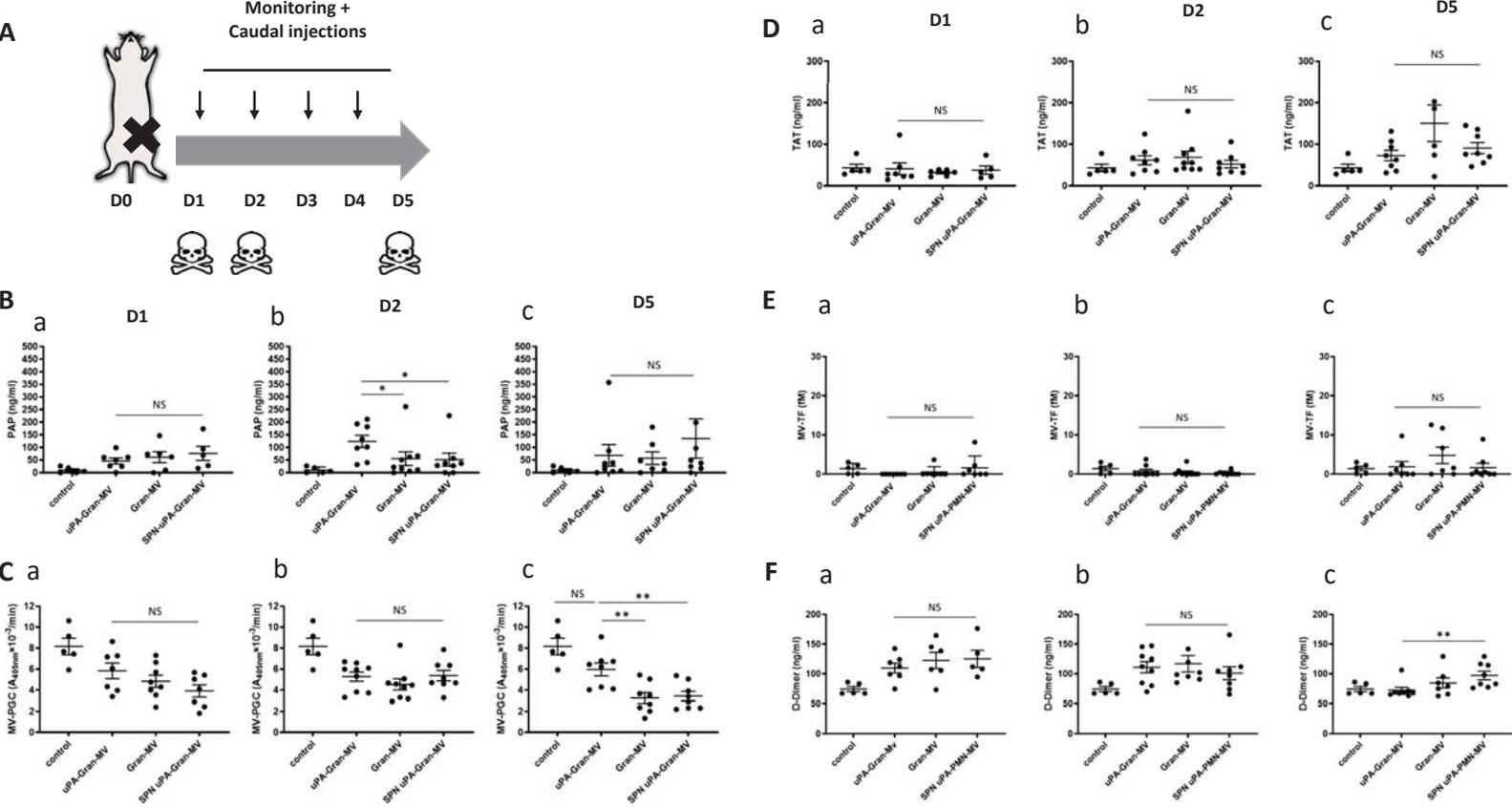
**FIGURE 4**



**FIGURE 5**

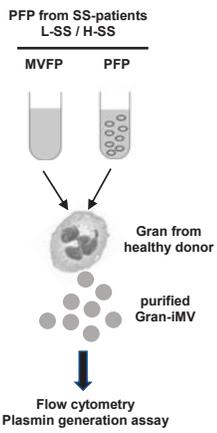


**FIGURE 6**

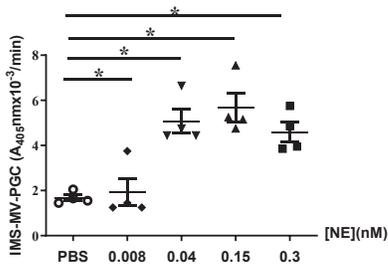


**FIGURE 7**

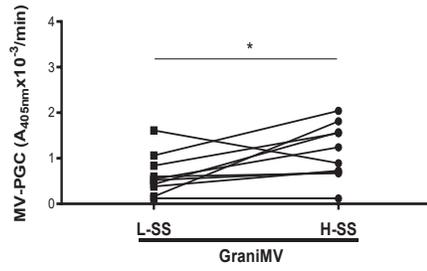
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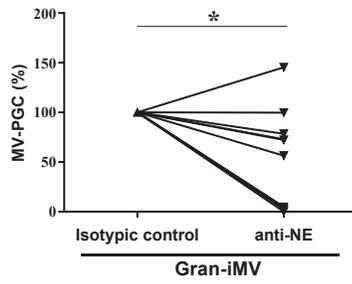
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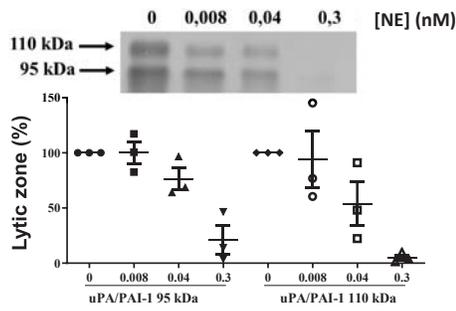
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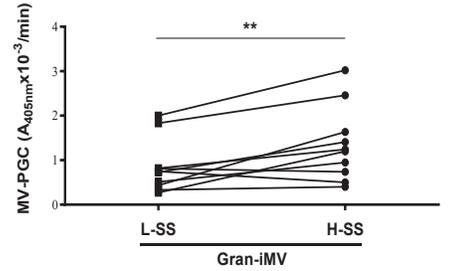
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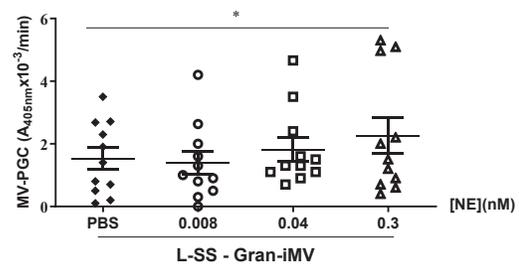
**G**



**C**



**E**



**H**

