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Leukocyte- and endothelial-derived microparticles: a circulating source for fibrinolysis

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Abstract

Background

We recently assigned a new fibrinolytic function to cell-derived microparticles *in vitro*. The relevance of this novel property of microparticles to the *in vivo* situation was explored in these studies.

Design and Methods

Circulating microparticles were isolated from plasma of thrombotic thrombocytopenic purpura or cardiovascular disease patients and from healthy subjects. Microparticles were also obtained from purified human blood cell subpopulations. Identification of plasminogen activators on microparticles was performed by flow cytometry and ELISA, their capacity to generate plasmin quantified with a chromogenic assay and their fibrinolytic activity by zymography.

Results

Circulating microparticles isolated from patients generate a range of plasmin activity at their surface. This property was related to a variable content in uPA and/or tPA. Using distinct microparticle subpopulations, we demonstrate that plasmin is generated on endothelial and leukocyte microparticles, but is absent on microparticles from platelet or erythrocyte origin. Leukocyte-derived microparticles bear uPA and its receptor uPAR whereas endothelial microparticles carry tPA and tPA/inhibitor complexes.

Conclusions

Endothelial and leukocyte microparticles, bearing respectively tPA or uPA, support a part of the fibrinolytic activity in the circulation that is modulated in pathological settings. This blood-borne fibrinolytic activity conveyed by microparticles provides a more comprehensive view on the role of microparticles in the hemostatic equilibrium.

Key words: fibrinolytic microparticles, plasmin(ogen), uPA, tPA

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Introduction

Microparticles (MPs) are small vesicles resulting from membrane blebbing of most activated or apoptotic cells. MPs derived from blood and vascular cells are detected in the circulation and over the past decade have been accepted as diagnostic and prognostic biomarkers in cardiovascular and inflammatory diseases, and in cancer.(1) Accumulating evidence indicates that beyond their clinical relevance as a biomarker, MPs also convey various bioactive effectors from the parental cell. Previous *in vitro* and *in vivo* studies indicate that MPs propagate a spectrum of biological activities and are involved in many different processes, such as activation of coagulation, inflammation, vascular remodeling and angiogenesis.(2-6) They bear tissue factor-dependent procoagulant activity and regulate procoagulant pathways in monocytes.(7) They also carry cytokines important for inflammation(8, 9) and participate in endothelial dysfunction by decreasing the production of nitric oxide.(4) Besides, it has been shown that endothelial MPs carry on their surface matrix metalloproteinase activity, suggesting their participation in extracellular matrix degradation, vascular remodeling and angiogenesis.(3)

We previously assigned a hitherto unreported fibrinolytic function to MPs(10) and more recently, we demonstrated that they participate in a new fibrinolytic crosstalk mechanism.(11) In these *in vitro* studies performed with MPs derived from the human microvascular endothelial cell line, HMEC-1, we demonstrated that these endothelial MPs constitute a catalytic surface for efficient activation of plasminogen by the urokinase-type plasminogen activator (uPA) anchored to its receptor uPAR.(10) Interestingly, these MPs may also activate plasminogen bound to fibrin, extracellular matrix proteins or platelets.(11) The possibility that circulating MPs also serve as a template for plasmin formation and fibrinolytic activity *in vivo* and their cellular origin remains unsolved questions that are the object of the present study. We demonstrate

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here that blood-borne MPs are a source of fibrinolysis that is modulated in different pathological situations. This fibrinolytic activity is selectively and specifically generated by uPA on leukocyte MPs and by tPA on endothelial MPs. This novel mechanism of plasmin formation *in vivo* may counterbalance the effect of procoagulant MPs by contributing to lysis of fibrin clots.

Design and Methods

Isolation of microparticles from human plasma

Platelet-poor plasma was separated (1500*g*, 15 minutes) from blood collected into 0.119 M sodium citrate from healthy volunteers (n=36) and from patients with systemic lupus erythematosus (n=22) according to the American College of Rheumatology criteria(12) or with atherosclerotic complications (n=16) collected during a previous study(13). Citrated plasma was also obtained by plasmapheresis from patients suffering from acute thrombotic thrombocytopenic purpura (TTP, n=10). Informed consent was obtained from all patients according to the Declaration of Helsinki.

MPs were isolated by sequential centrifugation of platelet-poor plasma, first at 12000*g* for 2 minutes to remove residual platelets. The platelet-free plasma was then centrifuged at 20000*g* for 90 minutes at 4°C. Centrifugation at 100000*g*, reported in some studies, was avoided to prevent concomitant sedimentation of exosomes, a different type of membrane vesicles.(14, 15) Pelleted MPs were washed twice (20000*g* for 90 minutes at 4°C) and re-suspended in phosphate buffer saline. In some experiments, circulating MPs were depleted in erythrocyte and platelet microparticles by magnetic immuno-separation using beads coated with CD41 and CD235a antibodies. Depletion in MPs (superior to 90%) was checked by flow cytometry (Supplemental data I). Control experiments were performed in parallel using beads coated with irrelevant antibodies.

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Generation of microparticles from blood cells

To investigate which subtype of circulating MPs may support plasminogen activation activity, MPs were generated *in vitro* from purified blood cell types. To that aim, whole blood from healthy volunteers or patients, who had not taken anti-platelet medication for at least 2 weeks, was collected into 0.119 sodium citrated tubes.

Human platelets prepared as described(16) were incubated with 1 NIH U/mL of thrombin and/or 1 µM of ionophore A23187 (Sigma, Saint Louis, MO), and/or 10 µg/mL of collagen (Stago, Asnière, France), for 15 minutes at 37°C without stirring. Platelets were subsequently pelleted by centrifugation first at 1500*g* for 15 minutes. Residual platelets were discarded by centrifugation at 12000*g* for 2 minutes and microparticles were then isolated from the supernatant and washed as described above. Purity superior to 95 % was checked by flow cytometry.

Erythrocyte MPs were generated as previously described by *Salzer et al.* with minor modifications.(17) Erythrocytes pelleted at 200*g* for 10 minutes and subsequently washed in sodium chloride 0.9%, were suspended in 9 volumes of PBS containing 1 mM $CaCl_2$ and 5 µM of A23187 and incubated at 37°C for 30 minutes. Erythrocytes were subsequently separated by centrifugation at 12000*g* for 2 minutes, and MPs isolated and washed as described above. Purity superior to 95 % was checked by flow cytometry.

Leukocyte MPs were prepared as previously described with minor modifications.(18) Peripheral blood mononuclear cells were isolated from buffy coats by FicollTM (Lymphocytes separation medium 1077). Monocytes were isolated by CD14+ immuno-magnetic separation on MS columns (Miltenyi Biotec, Bergish

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Gladbach, Germany). Purity superior to 95 % was checked by flow cytometry. Monocytes were stimulated by LPS (1µg/ml) overnight. Vesiculation was evaluated by FCM using Annexin A5-FITC/CD11b. Neutrophils were purified from buffy coats and were stimulated by N-formylmethionyl-leucyl-phenylalanine (1 µM) during 2 hours at 37ºC. Lymphocytes were purified from blood mononuclear cells and were stimulated by platelet-activating factor 500 nM/ phorbol 12-myristate 13-acetate 50 nM during 2 hours at 37ºC. MPs were isolated and washed as described above.

Generation and harvesting of microparticles from endothelial cells

Several subtypes of human endothelial cells originating from distinct vascular beds were purchased from Clonetics (Grand Island, NY) and cultured into 0.2 % gelatin coated flasks in EGM2-MV medium: renal artery endothelial cells were used at passage 8, coronary artery endothelial cells at passage 7 and both dermal human microvascular vein endothelial cells and human pulmonary artery endothelial cells at passage 6. Endothelial MPs were purified from culture medium conditioned by sub-confluent endothelial cells stimulated or not with 10 ng/mL TNF-a (PeproTech Inc, Rocky Hill, NJ, USA) for 24 hours, as previously described with some modifications.(19) Culture supernatants were centrifuged at 300*g* for 5 min and 2500*g* for 10 min to remove detached cells and debris. MPs were isolated as described above, were washed twice and re-suspended in HEPES buffer.

Characterization of microparticles by flow cytometry and ELISA

MPs suspension aliquots of 10 µL, 1/10 diluted, were labeled using fluorescein isothiocyanate (FITC)-conjugated annexin A5 (Abcys, Paris, France) or specific monoclonal antibodies. Phycoerythrin (PE)-labeled anti-CD41, FITC anti-CD31, PE anti-CD11b and PE anti-CD235a were from Beckman Coulter (Marseille, France).

DOI: 10.3324/haematol.2012.066167

Flow cytometry analysis (Supplemental data II) was performed on Cytomics FC500® instrument (Beckman Coulter,Miami, FL, USA). MPs were analyzed as previously described. (20, 21)

The presence of uPA, uPAR and tPA was evaluated by flow cytometry using FITC-labeled antibodies on MPs gated on CD146-PE (endothelial MPs), CD59-PE (endothelial MPs, erythrocyte MPs and leukocyte MPs) or CD41-PE (platelet MPs) positivity. Matched antibodies for protein concentration and fluorescence/protein ratio were used as control.

Pelleted MPs were lysed in 100 mM Tris-HCl buffer pH 8.1, containing 0.5% Triton X-100 and supplemented with a complete protease inhibitors mixture (Roche Diagnostic GmbH, Mannheim, Germany). Concentrations of uPA and uPAR were assayed by ELISA (894 and 893 IMUBIND® ELISA kits, American Diagnostica, Greenwich, GB) according to the manufacturer's instruction. The results were expressed as ng of protein per 10^6 MPs. The tPA and plasminogen activator inhibitor type-1 (PAI-1) content were measured by ELISA (Asserachrom® tPA and Asserachrom® PAI-1, Diagnostica Stago, Asnière, France). Results were expressed as ng of protein per 10^6 MPs.

Determination of plasminogen activators, plasminogen activation and fibrinolytic activity on microparticles

The presence and identity of plasminogen activators and its inhibitors borne by MPs were analyzed by direct and reverse fibrin autography following SDS-PAGE performed as described previously.(22) Briefly, MPs were lysed in 100 mM Tris-HCl buffer, pH 8.1, containing 1% Triton X-100. MPs lysates (10 µL from 2.10^5 MPs) and reference proteins (10 µ L of tPA 5 nM, uPA 1 nM and plasmin 500 nM) were electrophoresed in a 7.5% polyacrylamide gel under non-reducing conditions. SDS

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was then exchanged with 2.5% Triton X-100. After washing-off Triton X-100 excess with distilled water, the gel was carefully overlaid on a 1% agarose gel containing 1 mg/mL of bovine fibrinogen, 100 nM plasminogen and 0.2 NIH U/mL of bovine thrombin. For reverse fibrin zymography, the fibrin gel was supplemented with 0.05 i.u./mL of urokinase. Zymograms were allowed to develop at 37°C during 24 hours and photographed at regular intervals using dark-ground illumination. Active proteins in MP lysates were identified by reference to the migration of known markers (uPA, tPA, plasmin). When required the fibrin-agarose indicator gel was supplemented with antibodies (10 µg/mL) directed against specific plasminogen activators.

The capacity of MPs to activate plasminogen was determined by incubating a fixed concentration of plasminogen (1 µM) with the MPs in the presence of a chromogenic substrate selective for plasmin (CBS 0065, 0.75 mM final concentration), as described previously.(10) Plasmin formed from plasminogen bound at the surface of MPs cleaves the chromogenic substrate and the released p-nitroaniline is detected by measuring A_{405nm} as a function of time. Results are expressed in $A_{405nm}x10^{-3}$/min. When indicated, the following inhibitors were pre-incubated with the MPs: 10 µg/mL goat anti-human tPA (Biopool, Uppsala, Sweden), 20 µg/mL mouse anti-human uPA (American Diagnostica 3940), and respective irrelevant control IgGs (Biocytex, Marseille, France); amiloride and ε-aminocaproic acid (ε-ACA; Sigma-Aldrich) were used respectively at 100 µM and 0.05 M, final concentrations.

To scale the degree of plasmin formation by MPs, a standard curve prepared with reference MPs (200 000 *per* 50 µL) bearing known molar amounts of either uPA or tPA (THP1- or CHO-derived MPs, respectively) was used (see Supplemental data III for preparation of reference MPs).

The fibrinolytic activity of MP-free plasma from patients and controls was estimated by measuring the amount of plasmin formed on a fibrin surface in contact with the

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plasma euglobulin fraction in the presence of CBS 0065 (0.75 mM final concentration).(23)

Statistical analysis

Statistical analysis was performed with KaleidaGraph software (Synergy Software, Reading, PA, USA). Significant differences were determined using non-parametric Mann-Whitney test. A *p* value less than 0.05 was considered significant.

Results

In vivo evidence of blood-borne fibrinolytic activity supported by microparticles: identification of plasminogen activators

To investigate the possibility that blood-borne MPs may be involved in plasmin generation under conditions fashioned *in vivo*, we studied MPs from healthy controls and patients with pathological states reported to be associated with increased MP concentration, namely atherosclerotic vascular disease (n =16) and systemic lupus erythematosus (n = 22). MPs were isolated by centrifugation of platelet-free plasma at 20000g for 90 min. Therefore, all experiments presented in this paper excluded the presence of exosomes and concern exclusively MPs as indicated by nanoparticle tracking analysis (Supplemental data IV, figure S6). As illustrated in figure 1, it was possible to detect varying levels of plasminogen activator activity on MPs in both controls and patients. Interestingly, the plasminogen activator activity of MPs was significantly higher in the two groups of patients compared to healthy controls (median, 25-75 percentile: 1.2, 0.6-1.93 mOD/min and 0.87, 0.61-1.59 mOD/min versus 0.5, 0.4-0.6 mOD/min, $p<0.003$ and $p<0.007$, respectively). However, around 50% of patients had a plasminogen activator activity similar to healthy controls.

DOI: 10.3324/haematol.2012.066167

The nature of plasminogen activators involved in the fibrinolytic activity was analyzed using samples obtained by plasmapheresis from patients with acute TTP, a pathological situation featuring increased vesiculation.(24, 25) Plasmapheresis has the advantage over classical blood sampling, of providing sufficient amounts of plasma for MP isolation and extensive characterization. All isolated MP samples (n=10) generate plasmin activity upon incubation with 1 µM plasminogen. Figure 2A shows plasmin generation (0.7-5.9 mOD/min) in TTP samples (2.10^5 MPs *per* well) that display a variable content in plasminogen activator activity (control in the absence of MPs: 0.015 mOD/min). The inhibition of plasminogen binding and plasmin generation by ε-ACA was consistent with a lysine-dependent mechanism for plasminogen binding and activation at the surface of MPs (Figure 2B). The plasminogen activator activity in distinct samples (Figure 2C upper panel) was identified as uPA (TTP 6) or tPA (TTP 7) in fibrin-agarose gels, as indicated by the position of their fibrinolytic bands relative to known markers. For instance, the single fibrinolytic band apparent in sample TTP 6 and the corresponding uPA standard were absent in a fibrin-agarose gel containing anti-uPA antibodies (Figure 2C, lower panel). This antibody also inhibited plasmin formation by MPs from TTP 4 in a chromogenic assay (Figure 2B). The MP sample TTP 7 in figure 2C produced fibrinolytic bands of increased intensity corresponding to the position of uPA, tPA and tPA in complex with its inhibitor. The uPA fibrinolytic band did not appear in the presence of anti-uPA antibodies whereas tPA and its complexes retained their activity (Figure 2C, lower panel). In two additional TTP samples it was possible to quantify the molar activity of plasminogen activators on 2.10^5 MPs *per* 50 µL (tPA: 1.93 and 0.84 pM; uPA 1.5 and 0.83 pM). For this purpose we used reference MPs bearing known molar concentrations of either tPA or uPA and amiloride to resolve tPA activity (Supplemental data III). Free tPA and tPA in complex with its inhibitor were also identified in SLE samples (Supplemental data V, figure S7). These results provide

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the first evidence that human circulating MPs convey plasminogen activators and activate membrane-bound plasminogen. These *ex vivo* data suggest that blood-borne MPs may generate and disseminate plasmin activity *in vivo.*

The fibrinolytic activity in circulating blood is borne by leukocyte- and endothelial-derived microparticles

To further explore the fibrinolytic activity of MPs, we determined the cellular origin of MPs bearing plasminogen activator activity. We first performed experiments using specific antibodies coated on magnetic beads, allowing to deplete samples in platelet and erythrocyte MPs, representing the major subpopulation of circulating MPs. Less than 10% residual platelet MPs and erythrocyte MPs were detected in depleted samples (Figures 3A, 3B). The predominant subpopulations of endothelial MPs and leukocyte MPs in the depleted sample generated a significant increase in plasmin formation as compared to the non-depleted sample (Figure 3C). These data suggest (1) that endothelial MPs and leukocyte MPs represent the genuine support of plasminogen activator activity and (2) that platelet or erythrocyte MPs do not contribute to the fibrinolytic activity of MPs isolated from circulating blood. Of note, platelets and platelet MPs bear active PAI-1 (Figure 3D). Thus, the unexpected increase in plasmin formation observed in the depleted samples was probably related to the absence of platelet MPs bearing active PAI-1.

To define which plasminogen activators are specifically borne by MPs of endothelial and leukocyte origin, we studied the fibrinolytic activity of isolated MPs (2.10^5 *per* well) derived from distinct purified blood cell subpopulations and from primary human endothelial cells.

Endothelial-derived microparticles. Plasmin generation was detected on endothelial MPs with variations according to their anatomical origin (Figure 4A). Extremes high

DOI: 10.3324/haematol.2012.066167

and low fibrinolytic activities were displayed by endothelial MPs of renal artery (1.2±0.2 mOD/min) and dermal microvascular origin (0.03±0.01 mOD/min), respectively. By zymography, two fibrinolytic bands resistant to anti-uPA antibodies were observed in endothelial MP lysates (Figure 4B). The lower band corresponded to the migration of purified tPA whereas the top band, of higher molecular weight, corresponded to the migration of tPA in complex with its inhibitor (Cs in Figure 4B), in agreement with a previous report.(22) Consistent with this observation, significant levels of PAI-1 antigen were detected in lysates of endothelial MPs with a specific ELISA (Figure 4C). The presence of tPA was further confirmed by ELISA (Figure 4D) and by flow cytometry (Supplemental data II, figure S3), whereas uPA activity and antigen were undetectable. The molarity of active tPA borne by endothelial MPs was determined by reference to standard MPs bearing known molar amounts of tPA (Table 1).

Leukocyte-derived microparticles. Plasminogen activator activity was also detected on leukocyte MPs of monocyte, lymphocyte and neutrophil origin (Figure 4A). The presence of such activity on leukocyte MPs was further confirmed by a lytic band on zymography corresponding to the position of uPA (Figure 4B) that was absent in the presence of antibodies to uPA (not shown). Flow cytometry (Supplemental data II, figure S3) and ELISA (Figure 4D) allowed detection of uPA and its receptor uPAR on the surface of leukocyte MPs. In a parallel experiment, tPA antigen was undetectable on leukocyte MPs (Supplemental data II, figure S3). The molarity of uPA borne by leukocyte MPs was calculated using reference standard MPs bearing known molar amounts of uPA (Table 2).

Platelet- and erythrocyte-derived microparticles. Both platelet MPs and erythrocyte MPs (up to 5.10^6 MPs *per* well) failed to generated plasmin (Figure 4A) and fibrinolytic bands (Figure 4B). Accordingly, depletion of erythrocyte and platelet MPs did not decrease the fibrinolytic activity of MPs isolated from plasma (Figure 3C). Similar

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negative results on plasminogen activator activity were obtained when platelet MPs were generated using different platelet agonist combinations (collagen, thrombin and A23187; not shown). Flow cytometry experiments failed also to detect uPA or tPA antigens on platelet or erythrocyte MPs (Supplemental data II, figure S3).

Collectively, these results indicate that active plasminogen activators expressed by endothelial cells and leukocytes, tPA and uPA respectively, are conveyed in circulating blood by endothelial and leukocyte MPs.

Discussion

Using the human microvascular endothelial cell line HMEC-1,(26) we previously reported that endothelial MPs convey a fibrinolytic activity *in vitro* and participate in a fibrinolytic crosstalk.(10, 11) However, the relevance of this model to fibrinolysis *in vivo* and whether MPs derived from all circulating cell types support plasminogen activation remained unsolved questions that were addressed in the present studies. First, we provide the proof of concept that MPs produced *in vivo* support fibrinolysis as indicated by the formation of active plasmin on MPs isolated from blood of healthy subjects and patients with vascular diseases. Second, we show that specific plasminogen activators on endothelial and leukocyte MPs generate this fibrinolytic activity, whereas erythrocyte and platelet vesicles do not have this property. Third, the plasminogen activators were identified as u-PAR-bound uPA, on leukocyte MPs, and tPA on endothelial MPs. Therefore, the present study originally contributes to identify new partners of blood-borne fibrinolytic activity.

We also provide evidence that MPs with fibrinolytic activity are found at very low concentration in healthy subjects whereas various levels of plasmin formation were found in different pathological situations (atherosclerosis, systemic lupus erythematosus and TTP). This varying level of *ex vivo* fibrinolytic activity reflects

different amounts of active plasminogen activator borne by MPs whereas the plasminogen activator activity of MP-free plasma was negligible as published previously.(23) The significance of these findings with regard to the clinical status remains to be established in future studies. Notwithstanding, we have revealed the existence of a MP-dependent profibrinolytic compensatory mechanism that may counterbalance the procoagulant phenotype and reduce the thrombotic risk in these patients.(24, 27-29) These interesting features put forward the basis for a potential new biomarker.

To identify MPs that bear these plasminogen activators, we studied MPs subpopulations released by primary endothelial cells in culture or by platelets, red cells and leukocytes isolated from circulating human blood that were properly stimulated. We provide here the first demonstration that plasminogen activators are borne in a selective manner by a minor fraction of MPs circulating in human blood: endothelial MPs carry tPA and leukocyte MPs carry uPA/uPAR. This selectivity contrast with the well-described procoagulant potential common to all MPs.(30) Interestingly, we found various levels of fibrinolytic MPs in differents pathological situations (arteriosclerosis, systemic lupus erythematosus, TTP) that have been reported to be associated with increased levels of leukocyte and endothelial MPs.(13, 19, 27, 28, 31-34) This fibrinolytic activity may reflect pathophysiological leuko-endothelial activation associated with inflammation that helps to identify patients with a higher individual vascular risk in these clinical situations. Of note, leukocyte MPs and endothelial MPs are among the least accessible circulating subsets by current methodologies, such as flow cytometry, because of their low proportion, size distribution and limited specific markers. Thus, the measurement of this circulating activity brings innovative information to these MP subsets, as compared to their counting alone.

DOI: 10.3324/haematol.2012.066167

The study of MP samples from patients suffering of TTP demonstrates that plasmin may be generated either by uPA or tPA. By combining a set of tests (chromogenic, zymography, ELISA) and flow cytometry, we demonstrate that this activity was exclusive on MP subsets derived from either leukocytes (expressing uPA) or endothelial cells (expressing tPA). These distinctive characteristics may advantageously be used to identify the origin of the plasmin generation capacity of circulating MPs in patients. The presence of uPA on MPs derived from the human microvascular endothelial cell line HMEC-1 described in our previous report(10) was due to the atypical synthesis of this plasminogen activator by the modified cell line HMEC-1, that was used as a model to generate MPs after TNFα stimulation.

In contrast to uPA-bearing MPs derived from circulating leukocytes and tPA-bearing MPs derived from human primary endothelial cells, we did not detect plasmin generation on erythrocyte and platelet MPs. Accordingly, plasminogen activators have not been described on red blood cells and their expression by human platelets is not clearly demonstrated, except for the ectopic production of uPA in the platelet Quebec syndrome.(35) It is therefore possible that platelet MPs may, in some cases, develop plasminogen activator activity. Indeed, since platelets bind plasminogen,(36-38) platelet MPs may be a source of substrate for enhanced fibrinolysis by scuPA(39) via a fibrinolytic cross-talk mechanism we demonstrated recently.(11) However, active PAI-1/vitronectin complexes present in platelets(40) and active PAI-1 detected in platelet MPs as shown in this study, may modulate this fibrinolytic activity. Given that both platelet and erythrocyte MP subpopulations account for the bulk of circulating MPs,(13) it was relevant to demonstrate the effect of their depletion from the pool of MPs isolated from plasma. The unexpected increased fibrinolytic activity generated by the depleted samples may reflect the absence of platelet MP bearing active PAI-1.

DOI: 10.3324/haematol.2012.066167

Thus, we have identified MPs of endothelial and leukocyte origin as the main sources of the plasmin generation capacity on MPs in human plasma. However, in pathological settings, it remains possible that MPs from other origins may carry proteolytic activity in plasma. For instance, tumor MPs were proved to circulate and have been associated to thrombosis due to the tissue factor they convey.(41, 42) Facing this property, MPs from tumor cell lines and from ascites of cancer origin were found to bear vesicle-associated proteolytic activity represented by matrix metalloproteinases 2 and 9 and plasminogen activators.(43-47) The discovery of fibrinolytic MPs that circulate in human plasma raises the possibility to detect such activity in some cancer patients with potential role in diagnostic relevance or prognostic value in metastatic evolution.

Beyond fibrinolysis, plasmin formation by microparticles bearing the uPA/uPAR system may participate, in concert with matrix metalloproteinases, in other proteolytic functions.(48) Thus, the proteolytic activity of uPA-bearing MPs may be of relevance in cell migration, angiogenesis and outside-in signal transduction. Indeed, we have previously shown that tube formation was stimulated at low concentrations of MPs, (10) an effect that was related to crosstalk activation of plasminogen present in the Matrigel.(49) This proangiogenic effect is consistent with plasmin associated proteolytic activity that favors cell migration via extracellular matrix processing. The role of MP-bound plasmin in pathological settings involving inflammation, atherosclerosis and angiogenesis remains to be investigated. The high concentration of MPs reported in atherosclerotic plaques suggests that plasmin generation on MP could participate in the modulation of cell apoptosis/angiogenesis balance influencing plaque vulnerability.

In summary, our study indicates (1) that circulating human MPs display a range of plasmin generation in various pathological conditions, (2) that this activity is

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specifically generated by tPA on endothelial MPs and by uPA on leukocyte MPs, and (3) that these MPs support blood fibrinolytic activity. We provide the first evidence that surface plasminogen activation is a functional feature of plasma MPs that significantly differ between healthy subjects and pathological samples. Altogether, these results suggest that the presence of profibrinolytic MPs during clot formation anticipates their potential for clot lysis.

The existence of profibrinolytic MPs in the circulation raises the question about the physiological relevance of this activity. Our data suggest that pro-fibrinolytic MPs may compensate the effect of procoagulant MPs. Further studies will be necessary to correlate procoagulation versus fibrinolytic effects of MPs with the clinical status of patients. Microparticle blood-borne fibrinolytic activity not only provides a more comprehensive view on their role in the hemostatic equilibrium but also puts forward the basis for a potential new biomarker.

Authorship and Disclosures

RL and LP, first co-authors, performed the research, collected, analyzed and interpreted data, and participated in manuscript drafting. SR, SN and SMdeL contributed to isolation and purification of microparticle subpopulations. AM, FA and LD participated in data interpretation. AL and CB contributed to atherosclerotic patient experiments and editing of the manuscript. PP contributed to thrombotic thrombocytopenic patient experiments. NJ contributed to systemic lupus patient experiments. FS participated in research design and data analysis. EAC and FDG designed the research, analyzed the data and participated in manuscript drafting.

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Table 1. tPA activity on endothelial cell-derived microparticles.

200 000 MPs *per* 50µL	tPA (pM)
Renal artery endothelial cells	7.6 ± 1.0
Coronary artery endothelial cells	3.9 ± 0.1
Human microvascular endothelial cells	1.2 ± 0.2

Table 2. uPA activity on leukocyte-derived microparticles**.**

200 000 MPs *per* 50µL	uPA (pM)
Neutrophils	0.5 ± 0.07
Lymphocytes	0.2 ± 0.06
Monocytes	0.4 ± 0.03

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Figure 1. Plasmin generated by circulating microparticles from patients with cardiovascular disease (CVD), systemic lupus erythematosus (SLE) and healthy controls (HC).

Microparticles **(**2.10^5 per well) were isolated as indicated in Methods and then incubated with plasminogen (1μM) in the presence of a plasmin-selective chromogenic substrate (CBS 0065, 0.75mM). Dots represent the amount of plasmin formed (mOD/min). Results represent the median (25-75 percentile) of a duplicate experiment. The median for each category is represented by the dotted lines. CVD 1.2 (0.6-1.93) mOD/min and SLE 0.87 (0.61-1.59) mOD/min versus ◇ HC 0.5 (0.4-0.6) mOD/min, *p<0.01.

Figure 2. Plasmin generation and fibrinolytic activity of microparticles isolated from patients with thrombotic thrombocytopenic purpura (TTP).

A. Plasmin generation by 10 MP TTP samples. Experiments were performed as indicated in figure 1. Results in mOD/min (mean±SD).

B. Effect of inhibitors (100mM ε-aminocaproic acid, ε -ACA and 20μg/mL anti-uPA antibody, a-uPA) and IgG, a control immunoglobulin, on plasmin generation by TTP 4 MPs. Experiments performed as indicated in figure 1. Results in mOD/min (mean±SD).

C. Fibrin-zymography analysis of TTP MPs. Proteins in MP lysates were separated by SDS-PAGE and their fibrinolytic activity revealed on fibrin-agarose gels. Reference standards are: purified tPA, plasmin (Pn) and uPA. The fibrinolytic activity was tested in the absence (-, upper panel) or presence (+, lower panel) of 10 μ g/mL of a

DOI: 10.3324/haematol.2012.066167

polyclonal antibody against uPA. The high molecular weight fibrinolytic band observed in the purified tPA standard lane correspond to tPA dimers.(50)

Figure 3. Platelet- and erythrocyte-derived microparticles do not exhibit fibrinolytic activity.

A. Immuno-phenotyping monitoring of platelet MPs (Annexin A5-FITC+/CD41-PE+) and erythrocyte MPs (Annexin A5-FITC+/CD235a-PE+) before and after immuno-magnetic depletion (representative experiment). Left panels show MP content after non-specific immuno-magnetic separation (IMS control). Right panels show MP content after anti-CD41/CD235a IMS.

B. Efficiency of the IMS depletion in MPs of platelet and erythrocyte origin (right panel, n=3, 11.9%+/-1.43%, $p<0.05$) expressed as a percentage of the non-depleted samples (left panel).

C. Plasmin generation by MPs in depleted and non-depleted samples. Left bar shows activity of the non-depleted sample compared to the activity, right bar, of the sample depleted of erythrocyte and platelet MPs (145%+/-11%, $p<0.05$). An equal volume of 25µL of depleted and non-depleted MP samples was tested.

D. Reverse fibrin-zymography analysis of platelet MPs. Proteins in platelet MPs lysates (20 µg) and non-activated platelets (80 µg) were separated by SDS-PAGE and their plasminogen activator inhibitory activity was revealed on a fibrin-agarose gel containing urokinase (0.025 I.U./mL). The reference standard is purified recombinant PAI-1 (80 ng). Representative samples are shown.

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Figure 4. Plasminogen activators and fibrinolytic activity of human circulating blood cell- and primary endothelial cell-derived microparticles.

MPs derived from circulating human platelets, leukocytes and erythrocytes, and from primary cultures of human endothelial cells were prepared and isolated as indicated in Methods. Endothelial MPs were obtained from primary cultures of coronary artery (CA), pulmonary artery (PA), renal artery (RA) and dermal microvascular (DMV) endothelial cells. Circulating blood cell-derived MPs were obtained from isolated polymorphonuclear cells (PMN), monocytes (MC), lymphocytes (LC), red cells (RC) and platelets (PLA).

A. Plasmin generation by MPs (2.10^5 per well) was tested as indicated in figure 1. Bars represent the amount of plasmin formed (mOD/min). Results are the mean ± SD of duplicate experiments (n=3).

B. Fibrin-zymography analysis of endothelial and circulating MPs. Zymography was performed as indicated in Methods. Reference standards are purified tPA, plasmin (Pn), uPA and tPA in complex with PAI-1 (Cs). Representative samples are shown.

C. Concentration of uPAR and tPA determined by ELISA in cell-derived MP lysates (10^6 endothelial MPs per well). Results are the mean ± SD of duplicate experiments.

D. Concentration of PAI-1 as determined by ELISA in cell-derived MP lysates (10^6 endothelial MPs per well). Results are the mean ± SD of duplicate experiments.

DOI: 10.3324/haematol.2012.066167

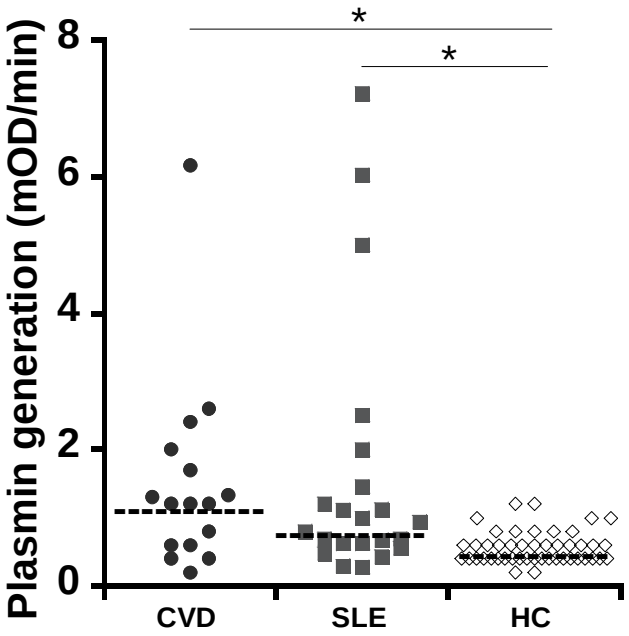

Figure 1

DOI: 10.3324/haematol.2012.066167

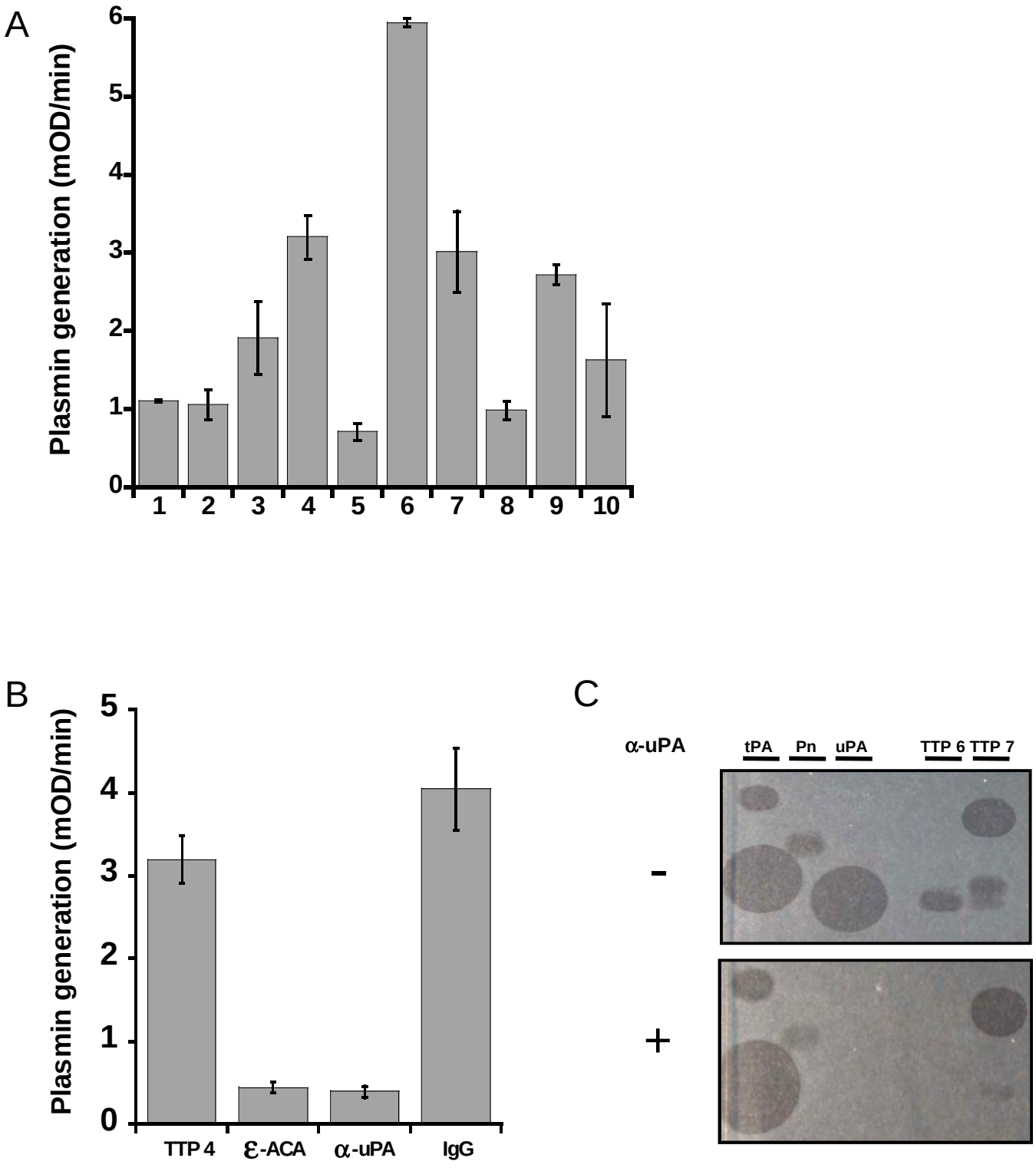

Figure 2

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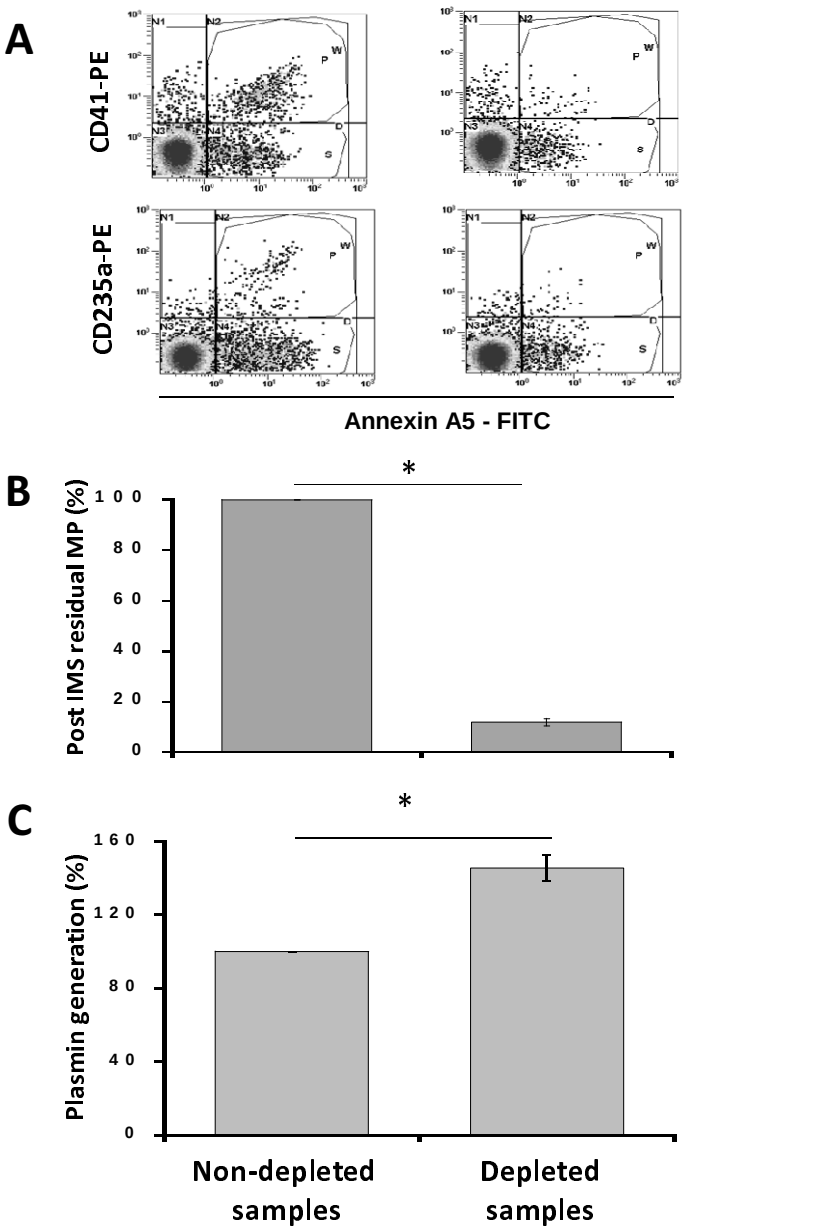

Figure 3

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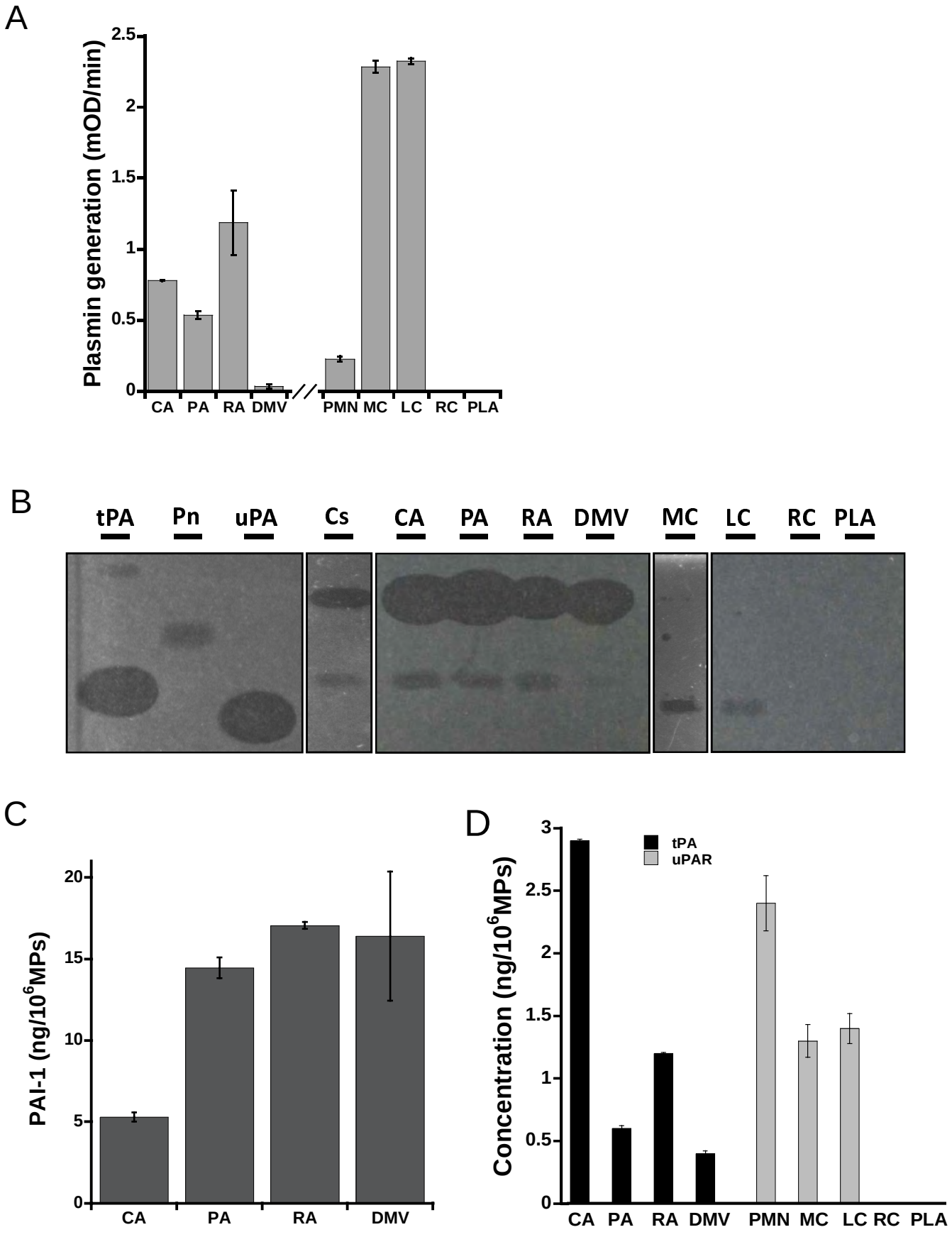

Figure 4

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Leukocyte- and endothelial-derived microparticles: a circulating source for fibrinolysis

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Online Supplementary data

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I. Immuno-phenotyping monitoring of platelet- (PMP), Erythrocyte- (EryMP) and leukocyte-derived (LMP) microparticles immuno-magnetic depletion (representative experiment).

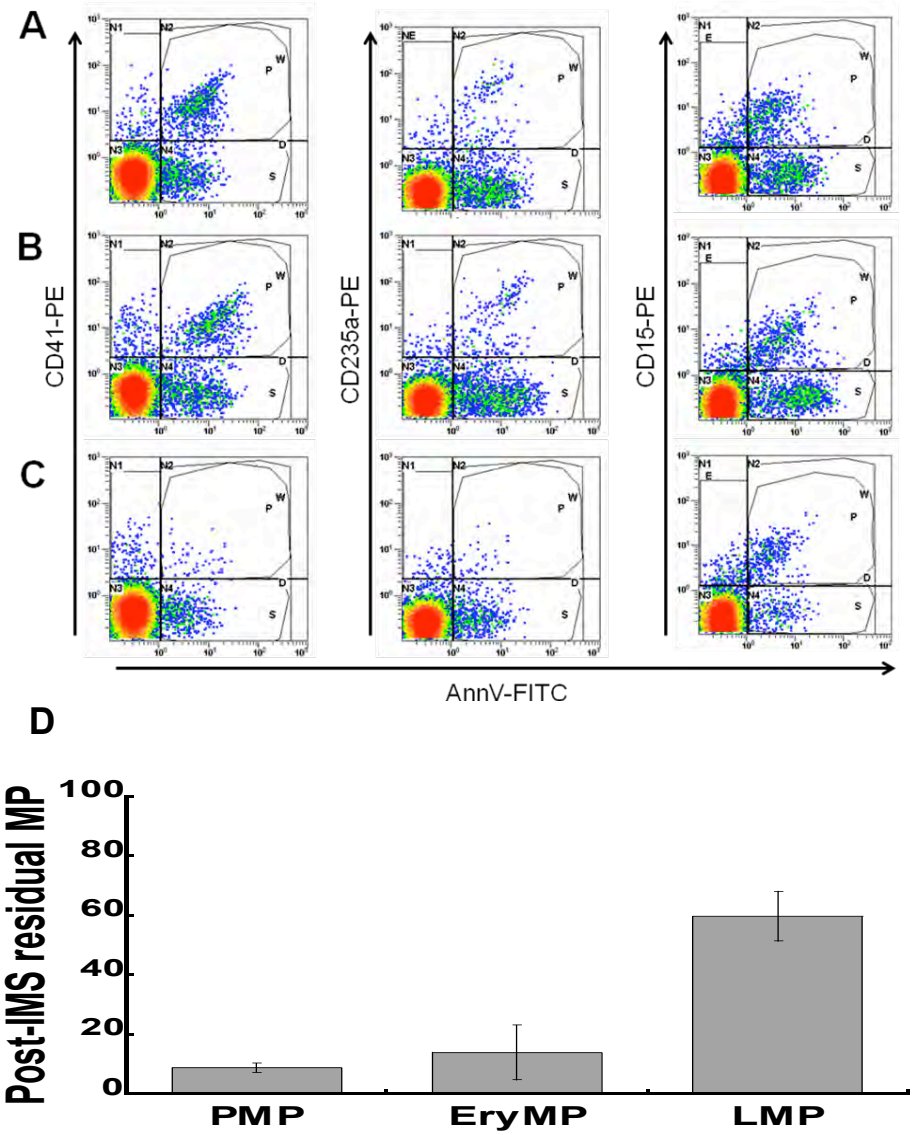

Figure S1. Immuno-phenotyping monitoring of PMP and EryMP immuno-magnetic depletion.

A. Untreated samples. **B.** Non-specific immuno-magnetic depletion using beads coated with irrelevant antibody. **C.** PMP and EryMP immuno-magnetic depletion. MP subpopulations

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were defined as PMP (AnnV+/CD41+) and EryMP (AnnV+/CD235a+). LMP (AnnV+CD15+) was used as control. **D.** Percentage of post immuno-magnetic separation (IMS) residual MP showing a selective immunomagnetic depletion of PMP and EryMP (n=3).

II. Measurement of plasminogen activators at the microparticle surface.

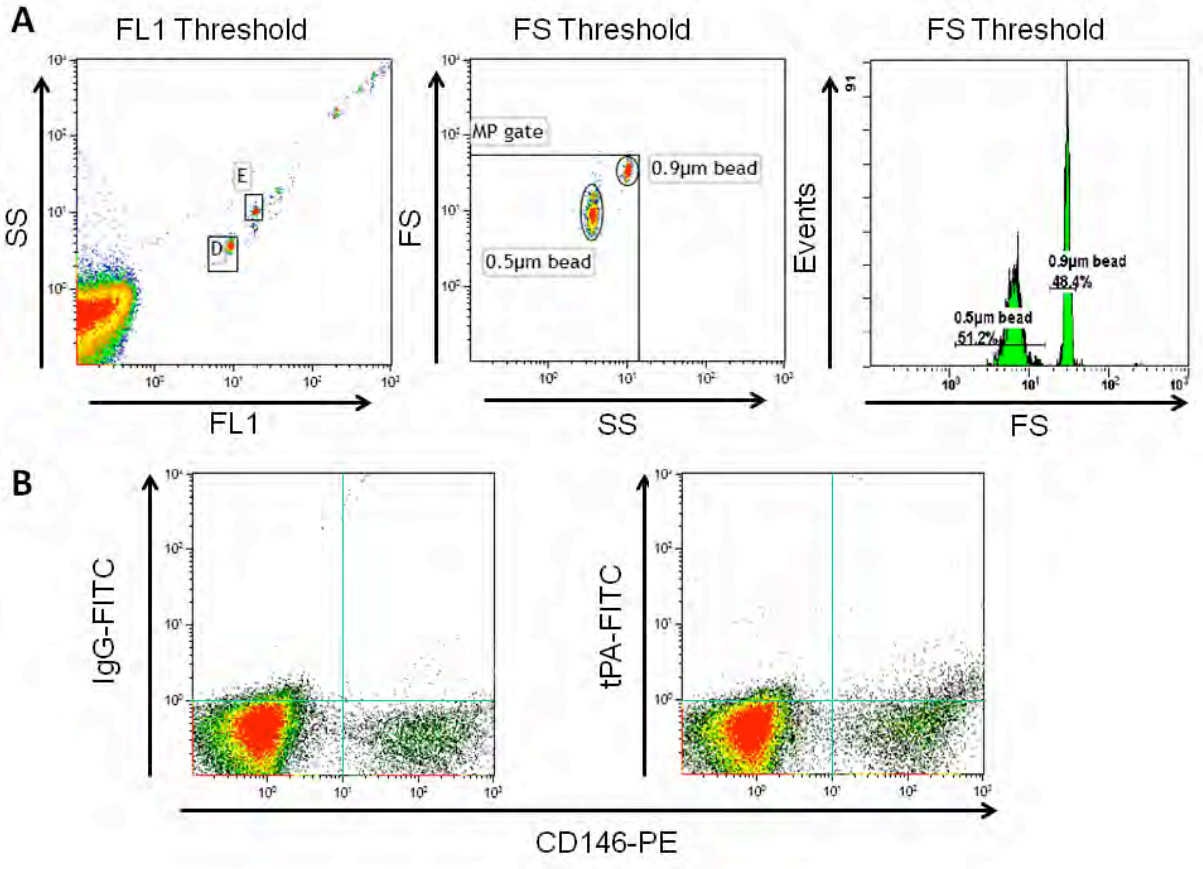

Figure S2. Example of flow cytometry protocol for the measurement of plasminogen activators at the microparticle surface.

A. Calibration protocol with Megamix beads. Megamix (BioCytex, Marseille, France) are fluorescent size-calibrated beads allowing standardization of the MP window of analysis (*Robert et al., J Thromb Haemost 2009*). Briefly, Megamix beads have a specific 2:1 ratio between 0.5 µm and 0.9 µm beads. First, beads were detected using a FL1 threshold (0.5 µm

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beads in D box, 0.9 µm beads in E box). Then, threshold was switched to FS. The lower limit of the standardized MP gate was defined by settings such as the 0.5/0.9 µm beads ratio was 1 (about 50/50%) and the upper limit with a 0.9 µm beads autogate. **B.** Representative density plots of t-PA detection on HCAEC derived-MP (Human Coronary Arterial Endothelial Cells). HCAEC-MP were identified as CD146+ events in MP gate. Fluorescence and concentration matched irrelevant antibody (IgG-FITC; left density plot) was used as control of tPA-FITC antibody (right density plot).

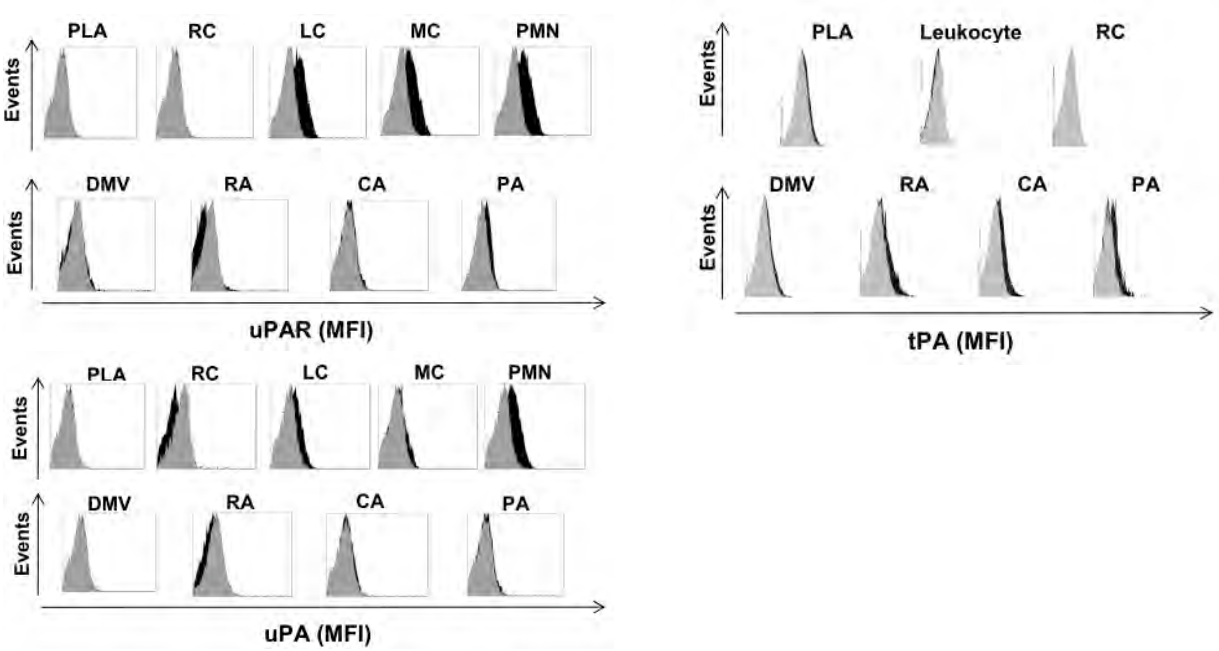

Figure S3. Detection of uPAR, uPA and tPA at the surface of endothelial and circulating MPs by flow cytometry. Graphs represent overlays of representative fluorescence histograms obtained with isotype controls (grey) and tPA, uPA or uPAR specific antibodies (black) on annexin A5+ gated events. MFI: mean fluorescence intensity. Endothelial cell-derived microparticles were obtained from primary cultures of coronary artery (CA), pulmonary artery (PA), renal artery (RA) and dermal microvascular (DMV) endothelial cells. Circulating blood cell-derived MPs were obtained from isolated polymorphonuclear cells (PMN), monocytes (MC), lymphocytes (LC), red cells (RC) and platelets (PLA). MIF: mean fluorescence intensity.

DOI: 10.3324/haematol.2012.066167

III. Preparation of reference microparticles bearing plasminogen activators

A standard curve prepared with reference microparticles (200 000 MPs per 50 µL) carrying a fixed known amount of either tPA (CHO-derived MPs) or uPA (THP1-derived MPs) was used to scale the degree of plasmin formation on respectively endothelial or leukocyte MPs.

First, reference CHO-derived MPs or THP1-derived MPs were prepared by incubation with a saturating amount of respectively tPA or uPA. The molarity of activator on these MP suspensions was then determined by reference to a binding isotherm (figure S1) relating plasmin formation versus varying amounts of plasminogen activator bound to the corresponding cells (50 µL per well). The MPs suspensions were tested at 200 000 MPs per well. Parameters for each binding isotherm were calculated using the Langmuir equation (Adamson A.W. 1990 in Physical chemistry of surfaces, pp695-606) and are indicated at the figure bottom. The tPA molarity, 4.5 nM, of the CHO-derived MPs reference preparation was calculated from the binding isotherm. The uPA molarity, 7.17 nM, of THP1-derived MPs reference preparation was calculated from the binding isotherm.

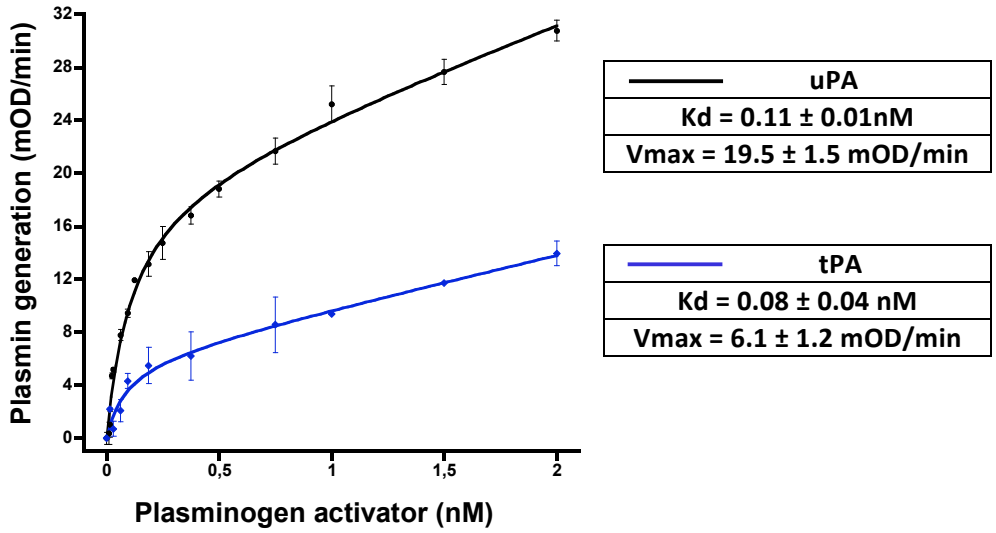

Figure S4. Binding isotherms of of uPA on THP-1 cells (black line) and tPA on CHO cells (blue line). The initial linear part of the isotherm was used to calculate the molarity of either tPA carry by CHO MPs or uPA carry by THP-1 MPs, both tested at 200 000 MPs per 50 µL.

DOI: 10.3324/haematol.2012.066167

Second, standard curves of plasmin generation by tPA-bearing or uPA-bearing MPs (50 µL) were prepared by plotting the amount of plasmin generated versus varying concentrations of the activator on MPs. The amount of tPA carry by endothelial-derived MPs and uPA carry by leukocyte-derived MPs were then determined using the equation of this curve.

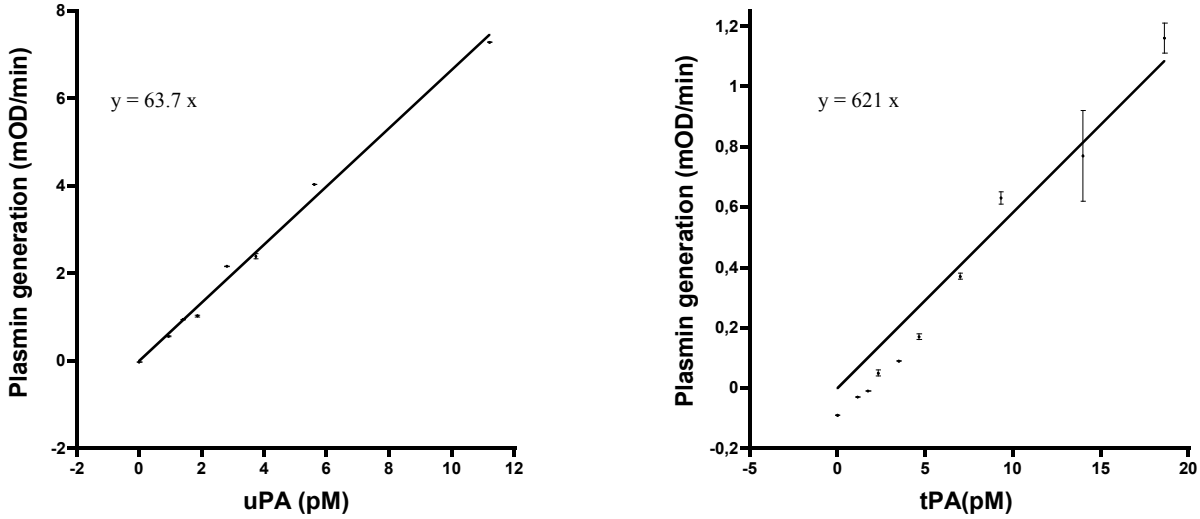

Figure S5. Standard curves of plasmin generation by uPA- or tPA-bearing MPs.

IV. Nanoparticle tracking analysis

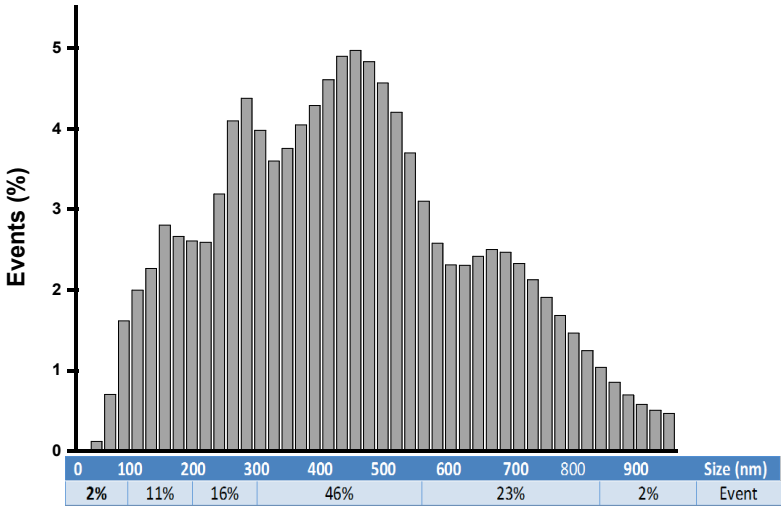

Figure S6. Nanoparticle tracking analysis (Nanosight LM10). Microparticles were isolated by centrifugation (20 000g, 90min) from a pool of plasmas (platelet-free plasma 1 500g,

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15min, then 12 000g, 2min). The graph shows the size distribution of microparticles in the sample. Less than 2% of events were under 100nm size.

V. Identification of plasminogen activators in microparticles of systemic lupus erythematosus patients.

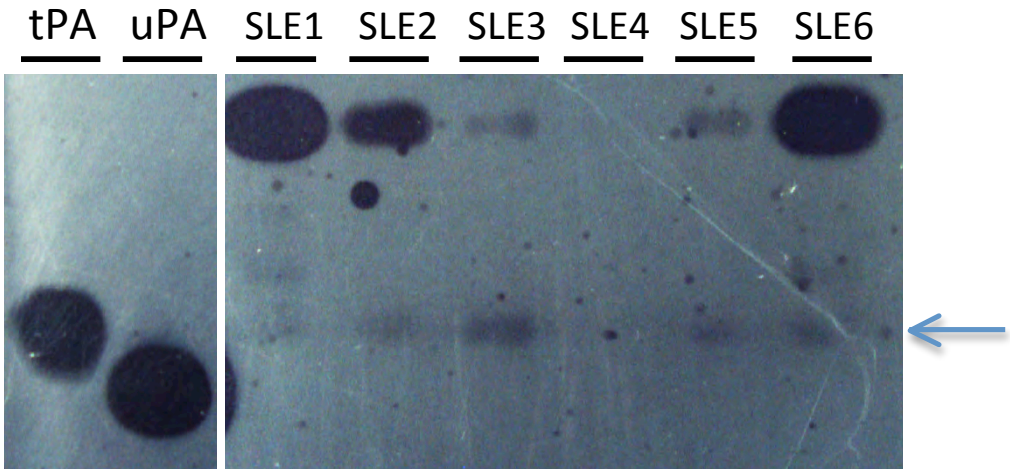

Figure S7. Fibrin-agarose zymography of microparticles isolated from patients with systemic lupus erythematosus. Fibrin-zymography analyses of TTP MPs. Proteins in MP lysates were separated by SDS-PAGE and their fibrinolytic activity revealed on fibrin-agarose gels. Reference standards are: purified tPA and uPA. Zymography was performed as indicated in Methods. The lysis zone in the tested samples corresponds to tPA in complex with PAI-1 (upper band) and to tPA (lower band, arrow).