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

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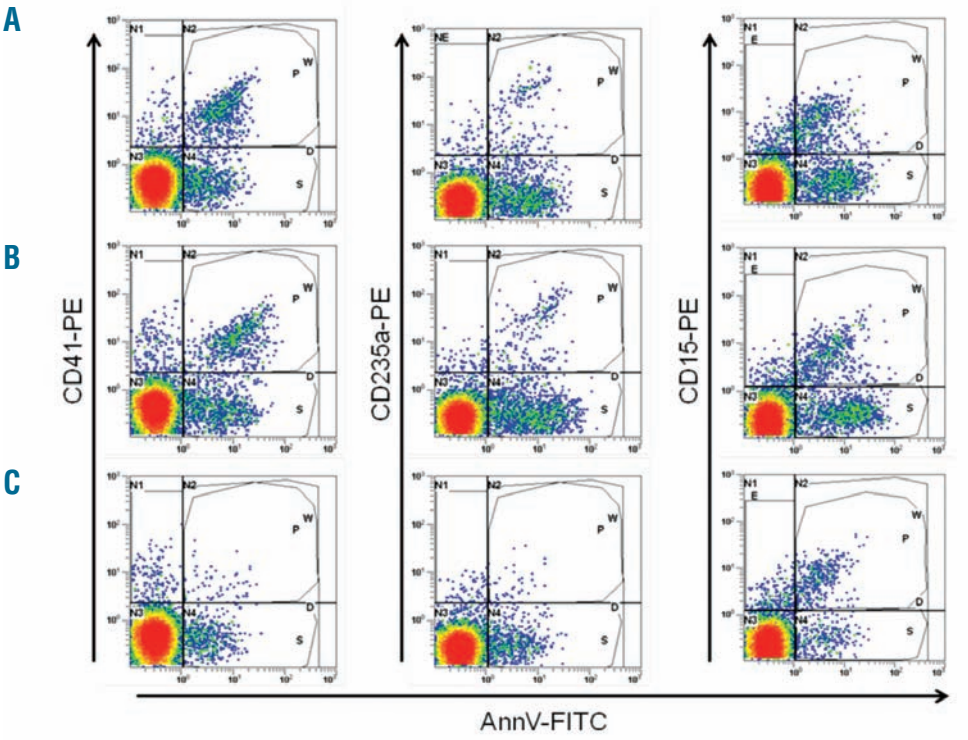
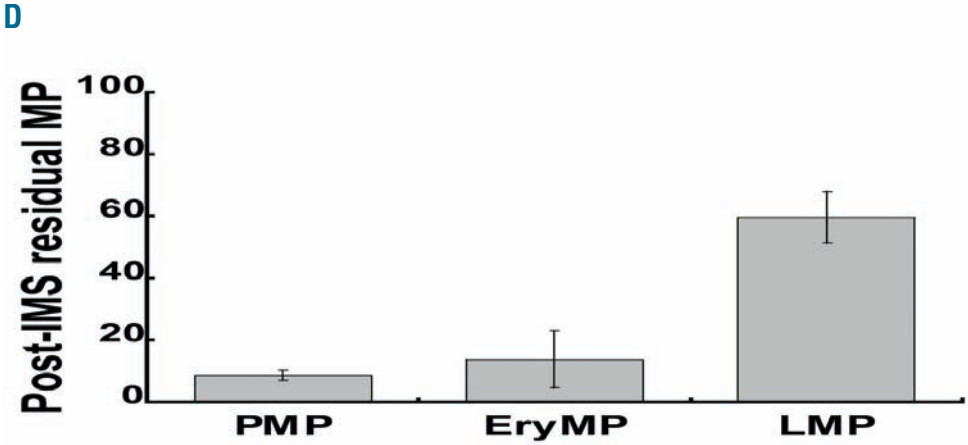
Online Supplementary data

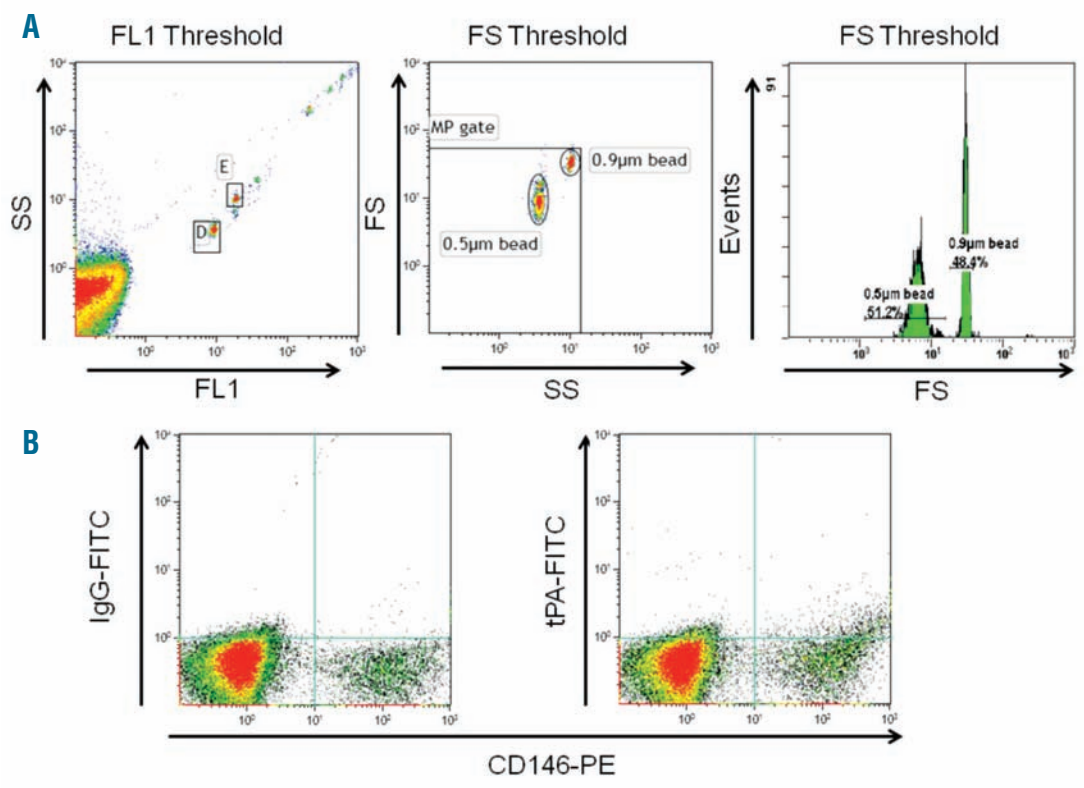
I. Immuno-phenotyping monitoring of platelet- (PMP), erythrocyte- (EryMP) and leukocyte-derived (LMP) microparticles after immuno-magnetic depletion (representative experiment).



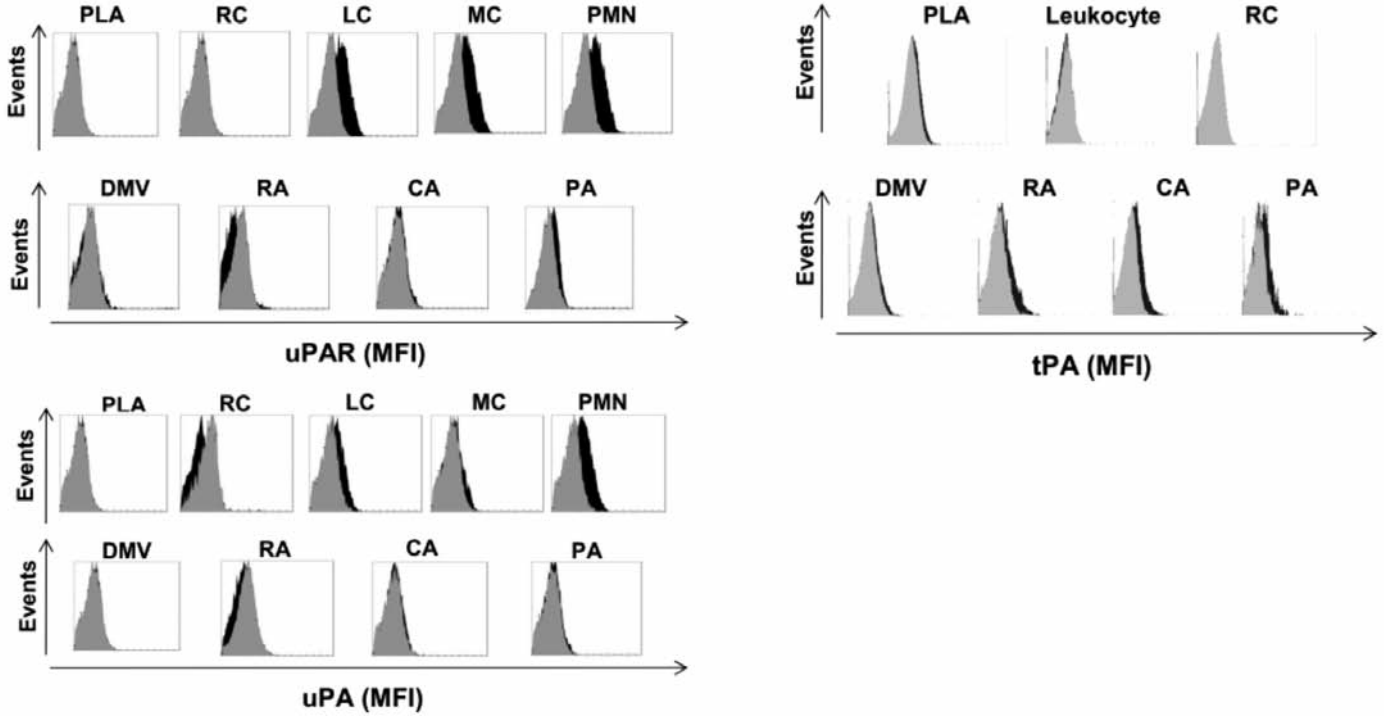


Online Supplementary Figure S1. Immuno-phenotyping monitoring of PMP and EryMP immuno-magnetic depletion. (A) Untreated samples. (B) Non-specific immuno-magnetic depletion using beads coated with irrelevant antibody. (C) PMP and EryMP immuno-magnetic depletion. Microparticle subpopulations were defined as PMP ($AnnV^+/CD41^+$) and EryMP ($AnnV^+/CD235a^+$). LMP ($AnnV^+CD15^+$) was used as a control. (D) Percentage of post immuno-magnetic separation (IMS) residual microparticles showing a selective depletion of PMP and EryMP (n=3). AnnV: annexin A5.

II. Measurement of plasminogen activators at the microparticle surface.



Online Supplementary Figure S2. Example of the flow cytometry protocol for the measurement of plasminogen activators at the microparticle (MP) 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 beads in box D, 0.9 µm beads in box E). The threshold was the switched to FS. The lower limit of the standardized MP gate was defined by settings such that the 0.5/0.9 µm bead ratio was 1 (about 50/50%) and the upper limit with a 0.9 µm bead autogate. (B) Representative density plots of t-PA detection on human coronary artery endothelial cell (HCAEC)-derived MP. HCAEC-MP were identified as $CD146^+$ events in the MP gate. Fluorescence and concentration-matched irrelevant antibody (IgG-FITC; left density plot) was used as the control of tPA-FITC antibody (right density plot).

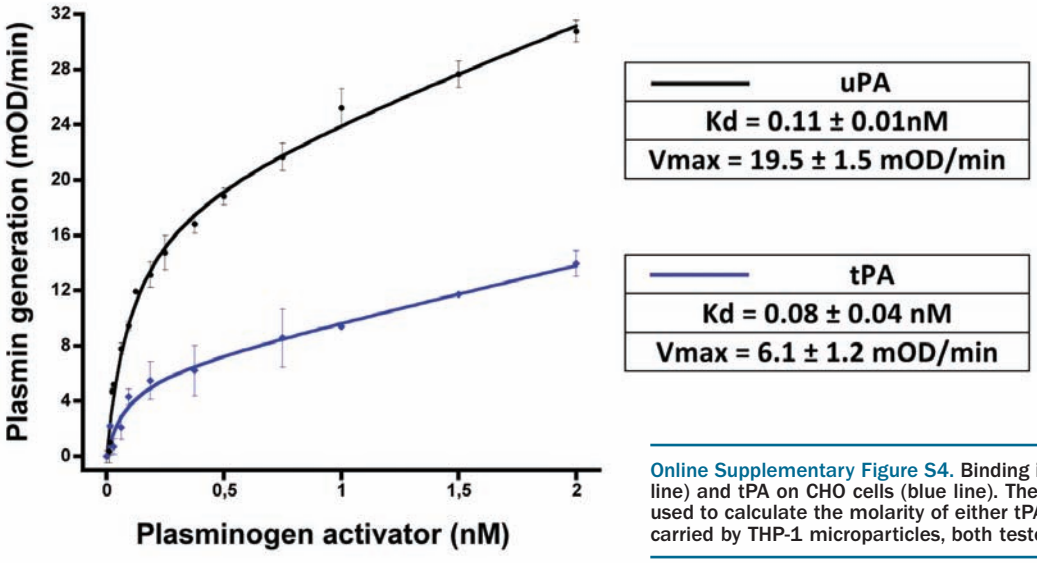


Online Supplementary Figure S3. Detection of uPAR, uPA and tPA at the surface of endothelial and circulating microparticles by flow cytometry. Graphs represent overlays of representative fluorescence histograms obtained with isotype controls (gray) and tPA, uPA or uPAR specific antibodies (black) on annexin $A5^+$ gated events. 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 microparticles were obtained from isolated polymorphonuclear cells (PMN), monocytes (MC), lymphocytes (LC), red cells (RC) and platelets (PLA). MFI: mean fluorescence intensity.

III. Preparation of reference microparticles bearing plasminogen activators

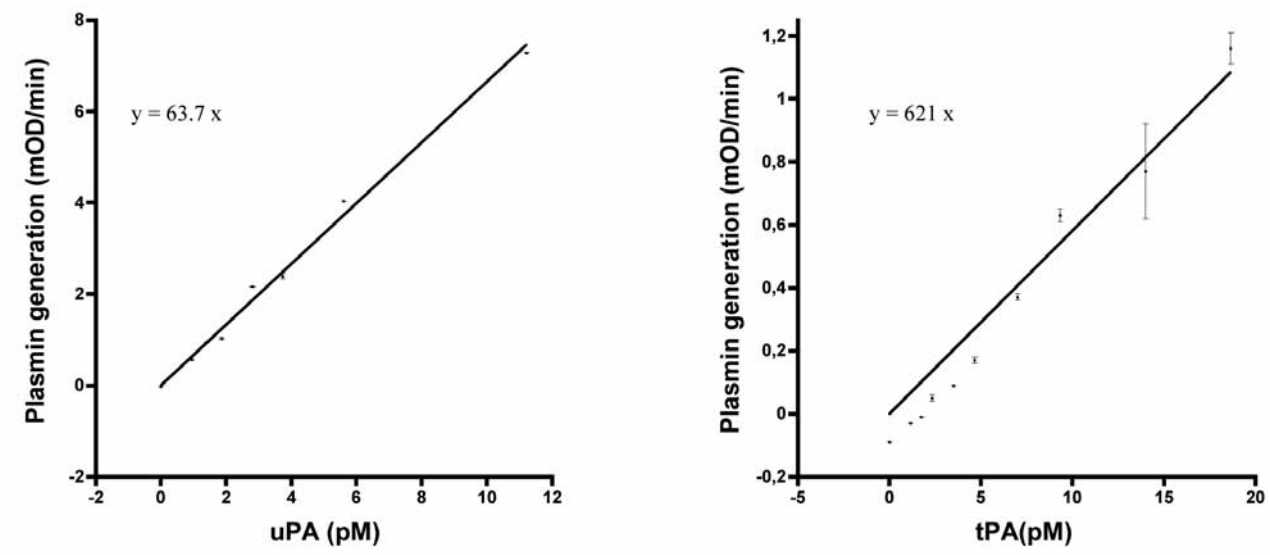
A standard curve prepared with reference microparticles (200 000 microparticles/50 μL) carrying a fixed known amount of either tPA (CHO-derived microparticles) or uPA (THP1-derived microparticles) was used to scale the degree of plasmin formation on endothelial or leukocyte microparticles, respectively.

First, reference CHO-derived microparticles or THP1-derived microparticles were prepared by incubation with a saturating amount of tPA or uPA, respectively. The molarity of activator on these microparticle suspensions was then determined by reference to a binding isotherm (*Online Supplementary Figure S4*) relating plasmin formation *versus* varying amounts of plasminogen activator bound to the corresponding cells (50 μL/well). The microparticle suspensions were tested at 200 000 microparticles per well. Parameters for each binding isotherm were calculated using the Langmuir equation (Adamson A.W. 1990 in Physical Chemistry of Surfaces, p695-606) and are indicated. The tPA molarity, 4.5 nM, of the CHO-derived microparticle reference preparation was calculated from the binding isotherm. The uPA molarity, 7.17 nM, of the THP1-derived microparticle reference preparation was also calculated from the binding isotherm.



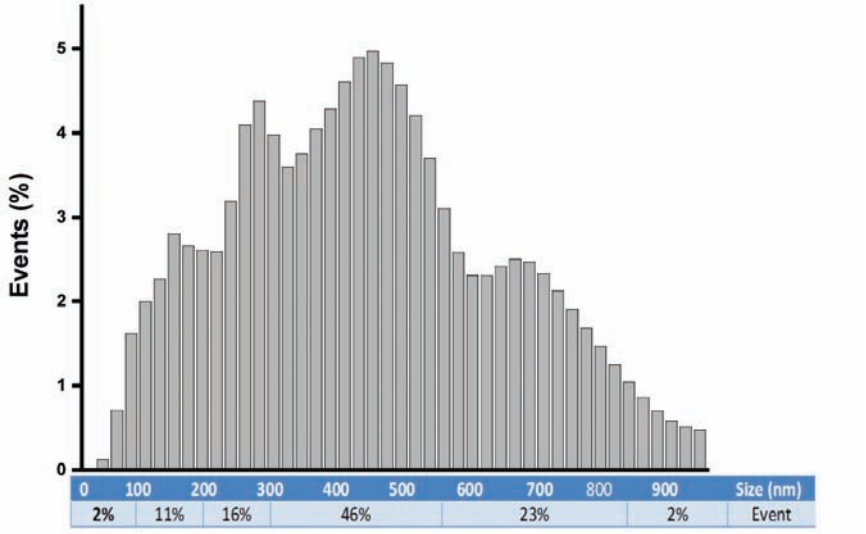
Online Supplementary Figure S4. Binding isotherms of uPA on THP-1 cells (black line) and tPA on CHO cells (blue line). The initial linear part of the isotherm was used to calculate the molarity of either tPA carried by CHO microparticles or uPA carried by THP-1 microparticles, both tested at 200 000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

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



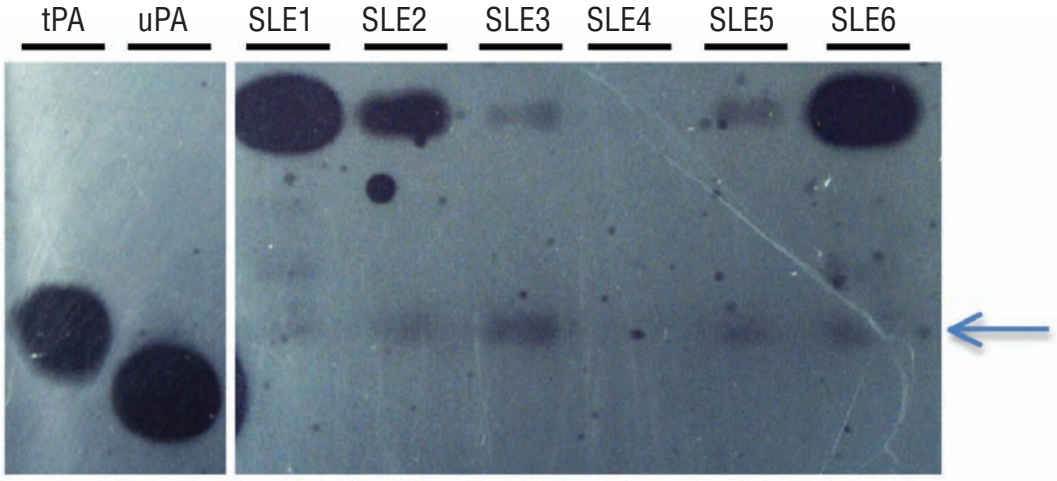
Online Supplementary Figure S5. Standard curves of plasmin generation by uPA- or tPA-bearing microparticles.

IV. Nanoparticle tracking analysis



Online Supplementary Figure S6. Nanoparticle tracking analysis (Nanosight LM10). Microparticles were isolated by centrifugation (at 20 000 *g*, 90 min) from a pool of plasmas (platelet-free plasma 1500 *g*, 15 min, then at 12 000 *g*, 2 min). The graph shows the size distribution of microparticles in the sample. Less than 2% of events were under the size of 100 nm.

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



Online Supplementary Figure S7. Fibrin-agarose zymography of microparticles isolated from patients with systemic lupus erythematosus. Proteins in microparticle 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 the *Design and Methods* section. The lysis zone in the tested samples corresponds to tPA in complex with PAI-1 (upper band) and to tPA (lower band, arrow).