Material and Methods

Cell Culture: All animal procedures were approved by the IACUC of the Scripps Research Institute. Bone marrow-derived macrophages (BMDM) were generated from total bone marrow cells of C57BL/6J, human TF knock-in (TFKI) mice[1] or Itga4 S998A (α4SA) mice[2] as described previously [3], and primed overnight with 100 ng/ml IFNγ (Peprotech, Rocky Hill, NJ) followed by 4 hours with 1 µg/ml LPS (S. abortus equi, Enzo Life Sciences, Farmingdale, NY). Primary smooth muscle cells were isolated from mouse lungs [4] and breast cancer populations were obtained by outgrowth as previously described [5]. Tumor cells were isolated from tumor bearing wt or P2xr7-deficient mice carrying the polyoma middle T oncogene expressed from the MMTV promoter.

MP release reaction: BMDM were stimulated with 5 mM ATP (Roche Applied Science, Indianapolis, IN), 80 µM Dynasore (Sigma-Aldrich, St. Louis, MO), or 25 µM mastoparan (Sigma-Aldrich) for 30 minutes or as indicated. Cellular debris was cleared (10', 1,000 g at 4ºC) and MP collected (60', 16,000 g at 4ºC) by centrifugation for functional assays or Western-blot. Where indicated, antibodies or 25 µM PACMA31 (Tocris Bio-Techne, Minneapolis) were added during the isolation of MP. MP counts in cell-free supernatants were determined by flow cytometry. In order to achieve equal MP counts for functional assays, MP were resuspended in defined volumes based on flow cytometry quantification.

MP functional assays: TF activity was determined by adding the indicated concentrations of mouse or human recombinant FVIIa (kindly provided by L. Petersen, Novo Nordisk) and 50 nM FX (Haematologic Technologies, Essex Junction, VT) to adherent cells or to resuspended MP in HBS (10 mM Hepes, pH 7.4, 137 mM NaCl, 5.3 mM KCl, 1.5 mM $CaCl_2$). FXa was measured using chromogenic substrate Spectrozyme FXa (Sekisui Diagnostics, Stamford, CT). MP prothrombinase activity was measured in HBS with 10 nM FVa, 5 nM FXa and 500 nM prothrombin (Haematologic Technologies) and thrombin was quantified with chromogenic substrate Spectrozyme TH (Sekisui Diagnostics).

FACS analysis: MP in cleared supernatants were stained with 5 µg/ml anti-TF 9C3-Alexa 647 conjugate, 1 µg/ml Lactadherin-FITC (Haematologic Technologies), and/or 66 nM Alexa633-conjugated phalloidin (Life Technologies) and analyzed on a LSR-II flow cytometer (BD Biosciences). Duramycin (1 µM, Sigma-Aldrich) or bovine lactadherin-FITC (50 nM, Haematologic Technologies) was added as indicated. For cell surface staining, macrophages were stained in PBS (2 mM EDTA, 1% FCS) for TF (5 µg/ml Alexa488-conjugated 21E10 [4]), integrin α4 (CD49d-FITC, eBioscience, San Diego, CA), or integrin α5 (CD49e-FITC, eBioscience) followed by fixation (PBS, 2 mM EDTA, 1% FCS, 1% formaldehyde). FACS data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

Microscopy: Cell surface PS exposure was quantified with FITC-labeled annexin 5 (BD Biosciences) in the provided staining buffer. Images were taken at 10x magnification, and analyzed with ImageJ (http://rsweb.nih.gov/ij). For confocal microscopy, cells were stained as described previously [3] using a cocktail of Alexa 647-conjugated anti-TF antibodies 10H10, 5G9, and 9C3 (each at 5 µg/ml) [6-8] for TFKI macrophages, or affinity-purified anti-mouse TF antibody [3] for murine TF-expressing cells. After fixation cells were counterstained with phalloidin-Alexa488/647 (Life Technologies) and 1 µg/ml Hoechst (Life Technologies). Images were taken with a 63x oil emersion objective on a Zeiss 710 LSM (Carl Zeiss, Oberkochen, Germany) and processed with Image Browser (Zeiss, Jena, Germany).

Western blotting: The following antibodies were used: polyclonal anti-mouse TF [4], polyclonal anti-human TF [9], polyclonal anti-integrin β1 [4], anti-PDI clone BD34 (BD Biosciences, San Jose, CA), anti $G\alpha_{i2}$ (EMD Millipore, San Diego, CA), anti-RGS16 (Aviva, San Diego, CA), anti 14-3-3ε and 14-3-3 α/β (Cell Signaling Technology, Danvers, MA), and from Santa Cruz Biotechnology (Santa Cruz, CA): anti-paxillin, anti-γ -actin, and anti-arf6. Cell-supernatant, MP and cells samples were prepared for Western-blotting as described previously [3].

Identification of MP proteins by mass spectroscopy: MP in cell-free supernatant were concentrated with an Amicon Ultra 3,000 MW (Millipore, Billerica, MA) and collected by centrifugation. Proteins were separated by SDS-PAGE and single protein bands based on the location of thiol-labeled bands on adjacent lanes were analyzed by nano-LC-MS/MS at The Scripps Research Institute Center for Mass Spectrometry.

Flow chamber experiments: Flow chamber experiments were performed as previously described [3, 4] with a few modifications. TF negative macrophages coated onto glass coverslips were perfused with wild-type C57BL/6J mouse blood at an initial wall shear rate of 300 s^{-1}. Blood - collected from the inferior vena cava of anesthetized mice into citrate-phosphate-dextrose buffer (12.88 mM final citrate concentration) - was mixed with anti-fibrin β-chain mouse monoclonal IgG labeled with Alexa Fluor 546 (Invitrogen, La Jolla, CA), procoagulant MP, $CaCl_2$ (1.15 mM final concentration) and immediately perfused through the chamber for 2 minutes, followed by Dulbecco's modified Eagle's Medium (Lonza, Walkersville, MD) to facilitate fibrin visualization. Stacks of confocal images at 2 μm interval through the height of fluorescent structures were collected for quantifying fibrin deposition with a Zeiss 410 LSM (Carl Zeiss) and MAX 2D projections were created using ImageJ. Background was subtracted and default signal threshold was set manually for each of the repeat experiments in the particle image analysis with ImageJ.

Thrombin generation (TG) tests with platelet-rich plasma (PRP): Procoagulant activity of MPs was determined by TG tests with PRP. PRP was prepared by centrifugation of citrate anticoagulated blood at 250 g for 10 min at 25 °C and platelets were adjusted to 180·103/μl by dilution with homologous platelet-poor plasma. PRP was mixed with re-suspended MPs in HBS (10 mM hepes, 150 mM NaCl, pH 7.4) in 96-well microtiter plates. Reactions contained 360 μM benzyloxycarbonyl-glycyl-glycyl-L-arginine coupled to fluorogenic 7-amido-4-methylcoumarin (Gly-Gly-Arg-AMC; Bachem Americas, Torrance, CA) as thrombin substrate; and were started by adding 18 mM CaCl2. Fluorescence was measured continuously at 37 °C for up to 40 min in a spectrofluorometer (355/460 nm excitation/emission). The rate of fluorescence intensity increase as a function of time (dF/dt) was calculated with Turbo Delphi 2006 (Borland Software Corporation, Austin, TX) and converted to thrombin-equivalent concentration (nM) using a calibration curve. The endogenous thrombin potential (ETP) of samples (i.e., total generated thrombin activity) was determined from the area under the TG curve.

Human whole blood MV characterization: Citrate-anticoagulated whole blood from healthy volunteers was stimulated with or without 10 μg/mL lipopolysaccharide (LPS; E. coli serotype 0111:B4, Sigma Aldrich, St. Louis, MO) for 4 hours at 37°C. Cell-free plasma was obtained by double centrifugation (2 x 10 minutes at 3,000g) and MV were isolated and washed by high-speed centrifugation in a microcentrifuge. TF activity was measured in a FXa generation assay with 150 nM FX and converted to arbitrary units (AU) based on a calibration curve with dilutions of lipidated recombinant human TF (Innovin®; Siemens Healthcare, Erlangen, Germany) obtained at a saturating

concentration of 5 nM FVIIa. Recombinant mouse FVIIa was kindly provided by Dr. L.C. Petersen (Novo Nordisk)[10]. In order to measure TF affinity of isolated MV with prebound human FVIIa, FVIIa was dissociated with a combination of FVIIa antibodies F5-13B12, F4-2.1b and F1-3G12 (50 ug/ml each) and mouse FVIIa was added at the indicated concentrations in the FXa generation assay.

Statistical analysis: Data are presented as mean ± SD, unless otherwise stated. Data were analyzed with GraphPad Prism. Parametric comparison used t-Test or ANOVA with the indicated post-tests for multiple comparison.

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