SUPPLEMENTARY APPENDIX

Extracellular mitochondria released from traumatized brains induced platelet procoagulant activity

Zilong Zhao,[1,2] Yuan Zhou,[1,2] Tristan Hilton,[1] Fanjian Li,[2] Cha Han,[1] Li Liu,[2] Hengjie Yuan,[2] Ying Li,[2] Xin Xu,[1] Xiaoping Wu,[1] Fangyi Zhang,[3] Perumal Thiagarajan,[4] Andrew Cap,[5] Fu-Dong Shi,[2,6] Jianning Zhang[2] and Jing-fei Dong[1,7]

[1]BloodWorks Research Institute, Seattle, WA, USA; [2]Tianjin Institute of Neurology, Departments of Neurosurgery and Neurology, Tianjin Medical University General Hospital, Tianjin, China; [3]Department of Neurosurgery, University of Washington School of Medicine, Seattle, WA, USA; [4]Departments of Medicine and Pathology, Michael E. DeBakey VA Medical Center and Baylor College of Medicine, Houston, TX, USA; [5]US Army Institute of Surgical Research, San Antonio, TX, USA; [6]Department of Neurology, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ, USA and [7]Division of Hematology, Department of Medicine, University of Washington, School of Medicine, Seattle, WA, USA

 doi:10.3324/haematol.2018.214932

Received: December 18, 2018.

Accepted: April 9, 2019.

Pre-published: April 11, 2019.

Correspondence: *JING-FEI DONG* - jfdong@bloodworksnw.org

JIANNING ZHANG - jianningzhang@hotmail.com

Supplemental methods and results

Mouse TBI model

Adult male C57BL/6J mice (12-16 weeks and 22-25g, Jackson Laboratory, Bar Harbor, ME) were subjected to fluid percussion injury (FPI) as previously described.[1,2] Briefly, a mouse was anesthetized by ketamine and xylazine (1 mg and 0.1 mg/10g weight, respectively) on a ventilator and constrained to a mouse surgery platform (Harvard Apparatus, Hollistone, MA). The skull was surgically exposed and a 3-mm diameter hole was drilled 2.0 mm posterior from the bregma and 2.0 mm lateral to the sagittal suture with the dura matter intact. After 60-min recovery, a female Leur-Lok was cemented to the hole on the skull and connected to an FPI device (Custom Design & Fabrication, Richmond, VA). With the head unrestrained, saline from a Plexiglas cylindrical reservoir was rapidly injected into the closed cranial cavity at a controlled pressure of 1.9 ± 0.1 atm. A sham mouse underwent the same surgery without being exposed to FPI. This mouse protocol is approved by the IACUC of the Bloodworks Research Institute.

In a subgroup experiments, exMTs were purified using Qproteome mitochondria isolation kit (Qiagen, Valencia, CA) from mice 3 hrs after TBI and used to stimulate platelets. To measure the impact of exMTs without confounding influences of injury, we infused exMTs released from mouse brains subjected to freeze-thaw injury in vitro

(supplemental method) or an equal volume of PBS into non-injured C57BL/6J male mice through the tail vein (2 x 10^8/mouse as detected in TBI mice).[2,3]

Purification of ExMTs from TBI mice and released from brains subjected to freeze-thaw injury

ExMTs were purified from plasma of TBI mice or from mouse brains subjected freeze-thaw injury using a commercial Qproteome mitochondria isolation kit (QIAgene, Valencia, CA) according to the manufacturer's instructions.[2] ExMTs thus purified were validated by an antibody against TOM22 (Translocase of Outer Membrane, Miltenyi Biotec, Auburn CA) and by the labeling of the mitochondria-specific dye MitoTracker Green using flow cytometry (Beckon Dickinson). TOM22 is a mitochondrial outer membrane protein that transports cytosolic pre-proteins into mitochondria.[4] MitoTracker Green is a membrane-potential-independent green fluorescent dye that stains live mitochondria.[2,5]

Testing exMTs from TBI mice required extensive steps to purify exMTs from heterogeneous MVs and these purification steps could damage exMT structures and metabolic activity. We would also need to sacrifice a large number of TBI mice to collect a sufficient amount of blood for purification. To address these pitfalls, we purified exMTs from mouse brains subjected to freeze-thaw injury as previously reported.[1,2] Briefly, brains were dissected aseptically from non-injured adult male C57BL/6J mice and cut into small blocks. These tissue blocks were immediately frozen in liquid

nitrogen and then rapidly thawed on ice for 20 min in 2 ml PBS containing protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL). They were then homogenized. ExMTs were purified from the brain homogenates using the Qproteome mitochondria isolation kit (QIAgene, Valencia, CA). ExMTs were freshly made before experiments. ExMTs made in vitro were similar to those found in TBI mice in their morphology (supplemental Figure S1A), surface exposure of CL,[2] and labeling with the mitochondria-specific dye MitoTracker Green (ThermoFisher Scientific, Waltham, MA, Figure S1B). ExMTs were also infused together with anti-CD36 antibody, isotype IgG, or PBS. The mice were sacrificed 30 min after infusion to analyze blood samples for platelet activation.

Platelet procoagulant activity and clot retraction

An anionic phospholipid-dependent clotting assay (Haematex Research, Homboby, Australia)[2,6] was modified to test the procoagulant activity of exMT-treated platelets. Briefly, platelets (100,000/μl final density) were treated with exMTs at platelet-to-exMT ratios of 1:1 and 1:5 for 30 min at 37°C, washed, and added to phospholipid-deficient porcine plasma together with the coagulation factor Xa (0.02 U/ml). The clotting time was then recorded on a CoaScreener coagulation analyzer (American Labor Corp., Durham, NC). Untreated platelets, platelets treated with 5 μg/ml of collagen, and 8 μg/ml of phosphatidylserine (PS, Avanti Polar Lipids, Alabaster, AB) were tested as controls.

For thrombin-induced clot retraction,[7] PRP with the platelet count normalized to 2.5 x 10^5/μl using homologous plasma was mixed with exMTs suspended in PBS to give a final platelet-to-exMT ratio of 1:1. The mixture was diluted with a Tyrode's-HEPES buffer and incubated 2 hrs at room temperature with 1U/ml of thrombin (final concentration) and 5 μl of washed erythrocytes (to visualize clot). Clot formation and retraction were recorded visually and quantified using Image-Pro Plus (Media Cybernetics, Bethesda, MD). PBS and collagen (5 μg/ml) were tested as controls.

Optical platelet aggregometry

Platelet-rich plasma (PRP) was obtained from healthy donors (n = 36, 21-56 yrs of age, 56% female) using 3.2% sodium citrate as the anticoagulant.[8] Homologous plasma was used to normalize platelet counts to 3.0 x 10^5/μl. Platelet aggregation was induced at 37°C in an optical aggregometer (Helena Laboratories, Beaumont, TX) by exMTs at 1:1 and 5:1 ratios to platelets, 5 μg/ml of fibrillar type I collagen (Helena Laboratories), or 10 μM of ADP (Helena Laboratories).[9] To increase the physiological relevance, most in vitro experiments were conducted between mouse exMTs and human platelets after the effects of mouse exMTs on mouse platelets were first identified.

Hopping Probe Ion Conductance Microscopy (HPICM)

We monitored exMT-induced morphological changes of platelets in real time using an atomic force-based HPICM imaging system (Ionscope Ltd, UK, supplemental

method).[10] Platelets were incubated for 30 min at 37°C with immobilized fibrinogen in a culture dish. After washing, adherent platelets were incubated for 30 min at room temperature with 1.5×10^4/μl of exMTs or PBS and scanned at a rate of 6-8 min per frame with a nanopipette (d = 60 nm) connected to an external Axon MultiClamp™ 700B amplifier (Molecular Devices, USA). Topographical data were continuously acquired and linearly interpolated into images with ScanIC Image Viewer V1.0 (Ionscope Ltd., UK). The supernatants were collected and examined for $CD41a^+$ platelet microvesicles using flow cytometry.

Flow cytometry

Platelet activation: we used an LSR II flow cytometry (Beckon Dickinson, San Jose, CA) to detect platelet activation in several measurements: CD62p expression, the binding of PAC-1 antibody (BD biosciences), which recognizes the active conformation of the fibrinogen receptor αIIbβ3, [11,12] annexin V binding,[2] platelet-bound fibrinogen and coagulation factor V,[13,14] and the formation platelet-leukocyte complexes (Supplemental Method). To determine whether CD62p expression was redox-dependent, exMTs were pretreated for 30 min at room temperature with 20 μM of reduced glutathione (GSH, Sigma Aldrich, St. Louis, MO), 500 μM of L-cysteine (Sigma Aldrich), or 2 mM of N-ethylmaleimide (NEM, Sigma Aldrich). To detect factor Va and fibrinogen, citrated blood was incubated with exMTs for 30 min at 37°C, followed by incubation for additional 30 min at 37°C with either a mouse anti-human factor Va antibody

(Haematologic Technologies, Essex Junction, VT) followed by an FITC-conjugated anti-mouse IgG or 5 μg/ml of Alexa 647-conjugated fibrinogen (Invitrogen). To detect platelet-leukocyte complexes, blood samples were incubated with a PE-conjugated anti-CD41a antibody (BD Biosciences, San Jose, CA) and a V450-conjugated anti-CD45 antibody (BD Biosciences) for 30 min at 37°C. Platelets treated with 5 μg/ml of collagen and 1U/ml of thrombin (0.4 mM of the peptide GPRP as anti-coagulant) or PBS served as controls. To detect whether exMTs induced platelets to express tissue factor as means to become procoagulant, platelets treated with increasing doses of exMTs (30 min at 37°C) were incubated with a PE-conjugated polyclonal antibody (Bioss, Woburn, MA) that recognizes both human and mouse tissue factor for 30 min and analyzed using flow cytometry.

Calcium influx: We used flow cytometry to measure the exMT-induced calcium influx in platelets labeled with 5 μM eFluor 514 Calcium Sensor Dye (Bioscience, San Diego, CA, 10 min at 37°C).[1]

ROS production: exMTs were suspended in the Ca^{2+}-free HEPES buffered Tyrode's solution (138 mM NaCl, 5.5 mM glucose, 10 mM HEPES, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.4 mM NaH_2PO_4, 0.4 mM $MgCl_2$, and 0.1% BSA, pH 7.2) and incubated for 30 min at 37℃ with 10 μM of the DCFH-DA dye (ThermoFisher).[12] As control, labeled exMTs were treated with either 50 μM of N-acetylcysteine (NAC, Life Technologies, Grand

Island, NY), which is a precursor of the antioxidant glutathione, or 200 μM of tert-Butyl hydroperoxide (TBHP, Life Technologies), which increases intracellular ROS production.[15] ExMTs fixed with 5% paraformaldehyde were also examined.

Image flow cytometry for exMT-platelet interaction: As we have previously described,[3] PRP was incubated for 30 min at 37°C with exMTs labeled with MitoTracker Green and an APC-conjugated CD41a antibody (BD Bioscience). The platelets were washed in PBS and fixed in 1% paraformaldehyde. Images were acquired at 60 x magnification to collect ≥ 20,000 cells from each sample using the Amnis ImageStream® X Mk II system (Amnis, Seattle, WA). To distinguish exMTs bound to platelet surface from those internalized, platelets were digested with 1% trypsin for 10 min at 37°C after incubation with exMTs. Mouse mitochondrial specific DNA was then amplified from these exMT-treated and trypsinized platelets using a protocol modified from our previous study.[2]

CD36-exMT interaction: For in vitro experiments, PRP was incubated for 30 min at 37°C with a monoclonal CD36 antibody (Abcam, ab17044, Cambridge, MA) or isotype IgG, followed by incubation with MitoTracker Green-labeled exMTs and a PE-CD41a antibody for 30 min. For in vivo experiments, blood samples from non-injured mice infused with MitoTracker Green-labeled exMTs were analyzed for exMT-bound platelets (MitoTracker Green$^+$/CD41a$^+$), platelet counts (CD41a$^+$ platelets counts in 60

sec.), platelet CD62p expression, and platelet-leukocyte complexes ($CD41a^+$/ $CD45^+$). The CD36 antibody used for this study recognizes both mouse and human CD36.[16,17]

Electron microscopy

ExMTs were incubated with washed platelets for 30 min at room temperature and fixed in 2.5% glutaraldehyde for 24 hours at 4°C. They were washed with a 0.1M cacodylate buffer and fixed again in 2% aqueous OsO4/0.2 M cacodylate for 2 hrs at 4°C before being embedded in Epon 812 for 2 hrs. Ultrathin sections were made and sequentially treated with uranyl acetate for 2 hrs and lead citrate for 5 min before being heat-dried. They were observed through transmission electron microscopy (TEM; JEM-1400 by JEOL, Tokyo, Japan).

Measurement of VWF and ATP

VWF released from platelets treated with exMTs was detected in the supernatant using a commercial ELISA kit (Ramco Laboratory Inc., Stafford, TX).[18] The result was presented as the percentage of VWF antigen found in normal reference pooled plasma (set as 100%). ATP was quantified in exMTs and platelets using a commercial bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin (Molecular Probe, Eugene, OR).[9] For both assays, platelet count was normalized to 2.5 x 10^5/μl and incubated with exMTs.

Thrombus formation under flow conditions

A microfluidic chamber system was used to study the impact of exMTs on thrombus formation on the collagen substrate.[19] Briefly, the microfluidic chambers (Cellix Mirus Nanopump™, Dublin, Ireland) were coated with 400μg/ml of type I collagen (Helena Laboratories, Beaument, TX) for 1 hour at RT, washed with PBS, and blocked with 1 % BSA for 1 hour at room temperature. Human PRP was pre-treated by mitochondria (platelet-exMT ration of 1:10) or an equal volume of the vehicle PBS for 10 minutes at RT and labeled with calcein-AM (Molecular Probes, Eugene, OR) for 5 minutes at RT. The treated PRP was mixed with red cells to reconstitute blood that reached a hematocrit of 40%. The reconstituted blood was then perfused over the collagen matrix under a shear stress of 60 dynes/cm^2 for 5 min at room temperature. The chambers were washed with PBS and platelet thrombi were recorded, and the thrombus formation was quantified as the surface area covered with platelet thrombi using the NIH ImageJ (Version 1.46r, Wayne Rasband, USA).

Supplemental Results

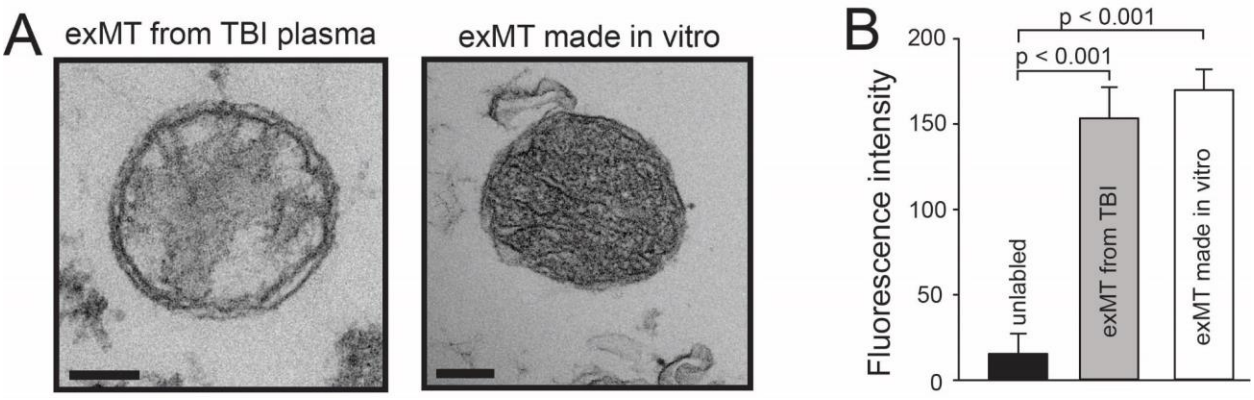

Figure S1: (A) TEM images of extracellular mitochondria found in the peripheral blood of a TBI mouse (left) and released from brains subjected to freeze-thaw injury (right, representative of 30 reviewed, bar = 50 nm) are shown. **(B)** exMTs purified from TBI mice and released from brains subjected to freeze-thaw injury were labeled with MitoTracker® Green and analyzed with flow cytometry (n = 12, paired t-test).

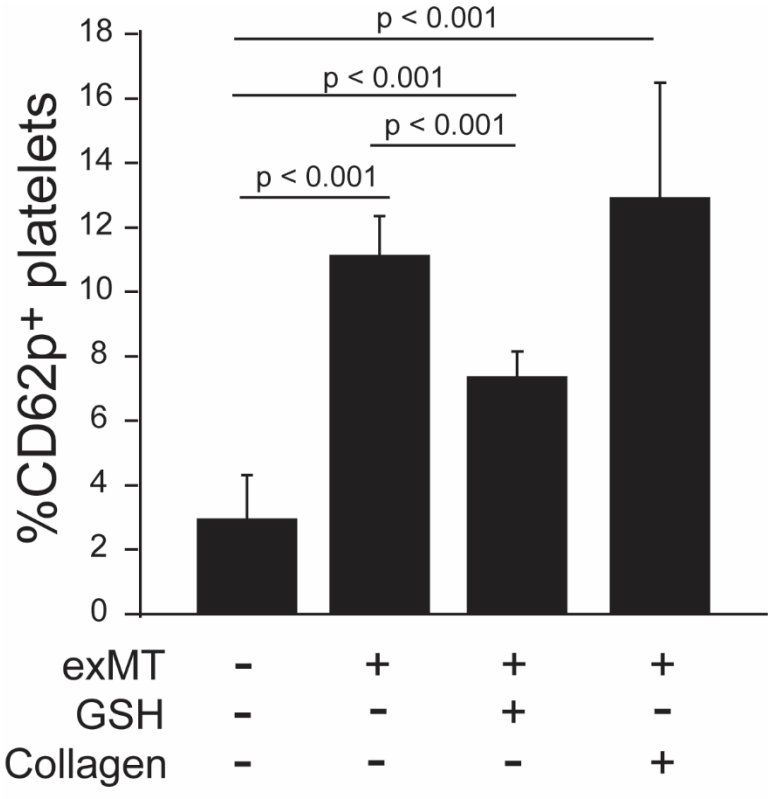

Figure S2: ExMTs purified from mouse brains activated mouse platelets defined by the expression of CD62p at a level comparable to CD62 expression induced by 5 μg/ml of collagen. This exMT-induced CD62p expression was partially blocked by the anti-oxidant GSH (n = 12, one-way ANOVA).

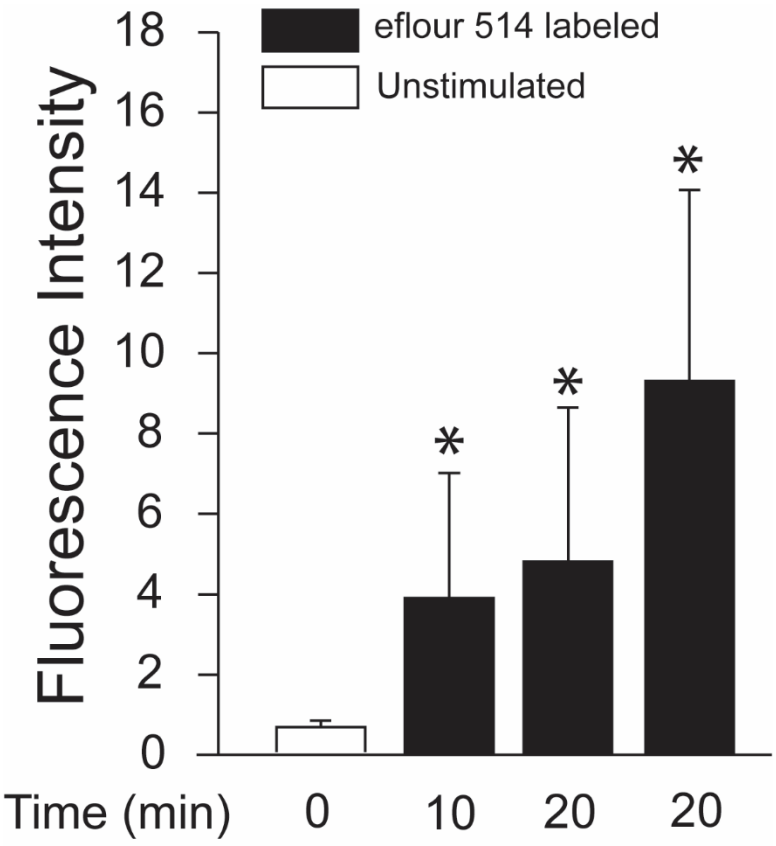

Figure S3: exMTs induced calcium influx of platelets in a time-dependent manner at a 1:5 platelet-to-exMT ratio (n = 6, one-way ANOVA, $p < 0.01$ vs. the baseline).

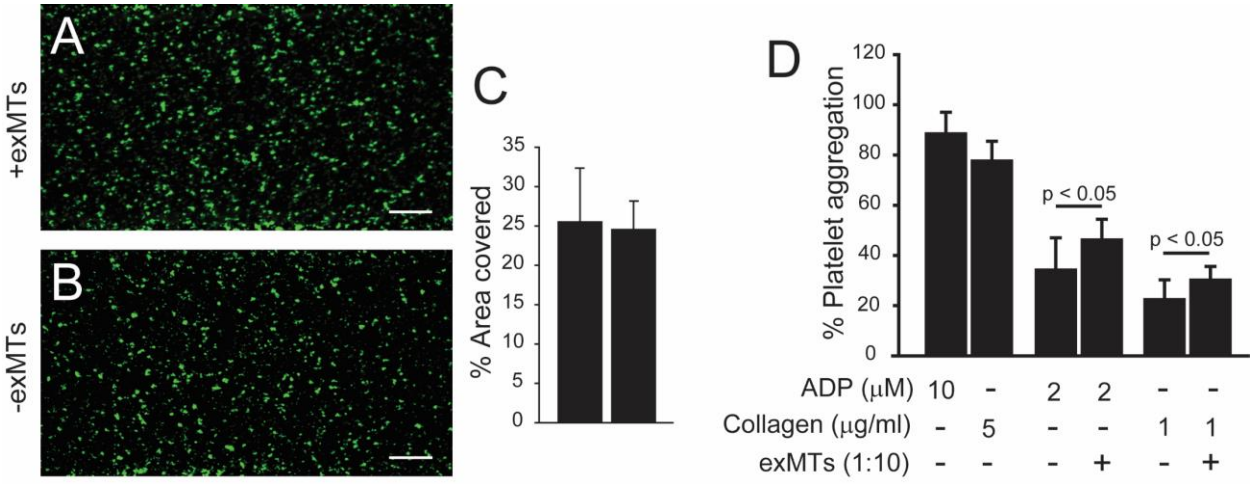

Figure S4: Reconstituted whole blood with exMT-treated platelets was perfused over the collagen matrix under 60 dynes/cm^2 of shear stress to measure the rate of platelet thrombosis defined by the areas covered by thrombi (**A** and **B**: representative images, bar = 500 μm; **C**: a summary of multiple tests, n = 6, Student's *t*-test). **(D)** Platelets that were treated exMTs for 30 min at 37^{o}C, washed with PBS, and then stimulated with full and subthreshold doses of either ADP or collagen. They were then monitored for the levels of aggregation (n = 12, one-way ANOVA).

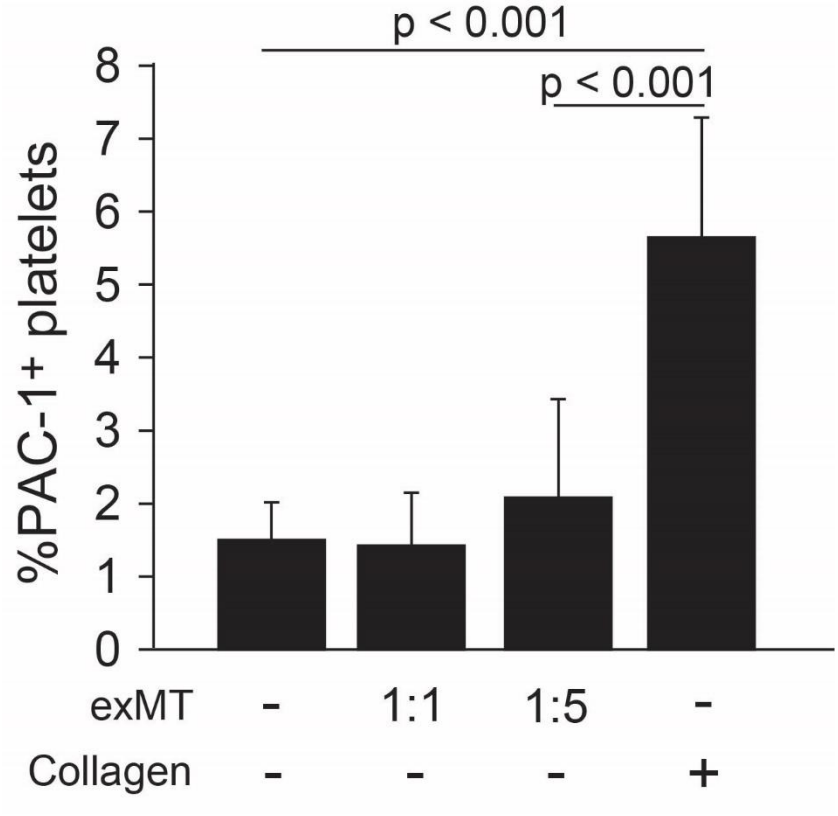

Figure S5: exMTs did not induce significant activation of the integrin αIIbβ3, as detected by the PAC-1 antibody. As control, PAC-1 binding was significantly detected on platelets treated with collagen (n = 12, one-way ANOVA).

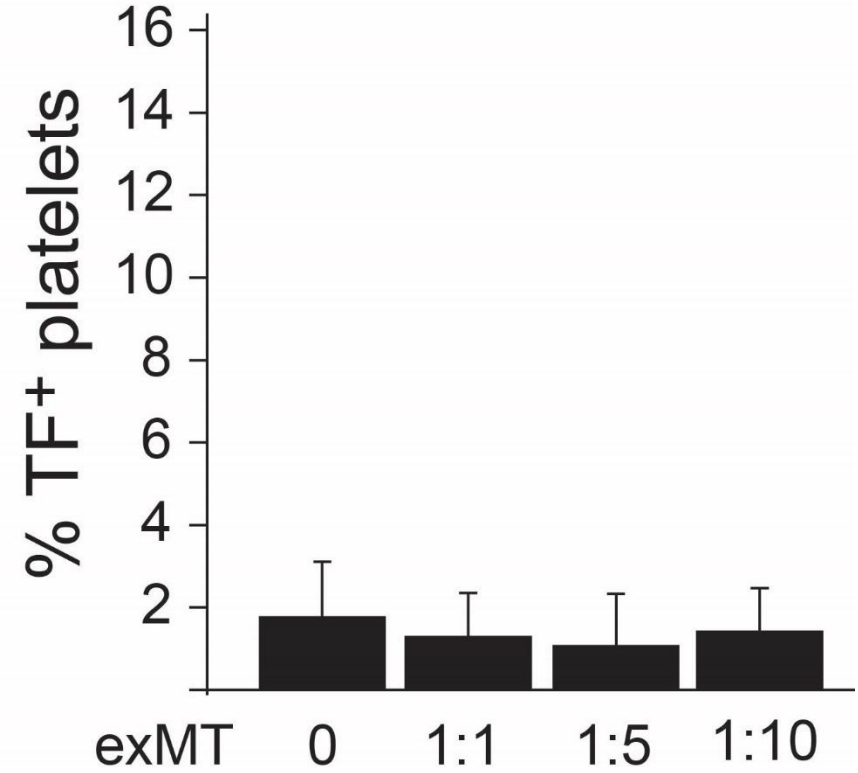

Figure S6: Human platelets were treated with exMTs for 30 min at 37°C, washed, and incubated with a polyclonal antibody that recognizes both human and mouse tissue factor. The expression of tissue factor on either exMTs or exMT-treated platelets was then detected using flow cytometry (n = 12, one-way ANOVA).

REFERENCES CITED

1. Tian Y, Salsbery B, Wang M, et al. Brain-derived microparticles induce systemic coagulation in a murine model of traumatic brain injury. *Blood*. 2015;125(13):2151-2159.
2. Zhao Z, Wang M, Tian Y, et al. Cardiolipin-mediated procoagulant activity of mitochondria contributes to traumatic brain injury-associated coagulopathy in mice. *Blood*. 2016;127(22):2763-2772.
3. Zhou Y, Cai W, Zhao Z, et al. Lactadherin promotes microvesicle clearance to prevent coagulopathy and improves survival of severe TBI mice. *Blood*. 2018;131(5):563-572.
4. Lecocq J, Ballou CE. On the Structure of Cardiolipin. *Biochemistry*. 1964;3:976-980.
5. Poot M, Zhang YZ, Kramer JA, et al. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J Histochem Cytochem*. 1996;44(12):1363-1372.
6. Exner T, Joseph J, Low J, Connor D, Ma D. A new activated factor X-based clotting method with improved specificity for procoagulant phospholipid. *Blood Coagul Fibrinolysis*. 2003;14(8):773-779.
7. Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol*. 2001;21(6):2213-2220.
8. Nascimbene A, Hilton T, Konkle BA, Moake JL, Frazier OH, Dong JF. von Willebrand factor proteolysis by ADAMTS-13 in patients on left ventricular assist device support. *J Heart Lung Transplant*. 2017;36(4):477-479.
9. Zhou Z, Gushiken FC, Bolgiano D, et al. Signal transducer and activator of transcription 3 (STAT3) regulates collagen-induced platelet aggregation independently of its transcription factor activity. *Circulation*. 2013;127(4):476-485.
10. Zhang Y, Liu X, Liu L, et al. Contact- and agonist-regulated microvesiculation of human platelets. *Thromb Haemost*. 2013;110(2):331-339.
11. Zhang JN, Bergeron AL, Yu Q, et al. Duration of exposure to high fluid shear stress is critical in shear-induced platelet activation-aggregation. *ThrombHaemost*. 2003;90(4):672-678.
12. Yuan H, Houck KL, Tian Y, et al. Piperlongumine Blocks JAK2-STAT3 to Inhibit Collagen-Induced Platelet Reactivity Independent of Reactive Oxygen Species. *PLoS One*. 2015;10(12):e0143964.
13. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood*. 2000;95(5):1694-1702.
14. Tracy PB, Mann KG. Prothrombinase complex assembly on the platelet surface is mediated through the 74,000-dalton component of factor Va. *Proc Natl Acad Sci U S A*. 1983;80(8):2380-2384.
15. Hierso R, Waltz X, Mora P, et al. Effects of oxidative stress on red blood cell rheology in sickle cell patients. *Br J Haematol*. 2014;166(4):601-606.
16. Ghosh A, Li W, Febbraio M, et al. Platelet CD36 mediates interactions with endothelial cell-derived microparticles and contributes to thrombosis in mice. *J Clin Invest*. 2008;118(5):1934-1943.
17. Pascual G, Avgustinova A, Mejetta S, et al. Targeting metastasis-initiating cells through the fatty acid receptor CD36. *Nature*. 2017;541(7635):41-45.
18. Dong JF, Moake JL, Nolasco L, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002;100(12):4033-4039.
19. Yuan H, Houck KL, Tian Y, et al. Correction: Piperlongumine Blocks JAK2-STAT3 to Inhibit Collagen-Induced Platelet Reactivity Independent of Reactive Oxygen Speciesdagger. *PLoS One*. 2016;11(1):e0146626.