**Supplemental Information**

*Supplemental Methods*

*Materials*

Purified human FXII, FXI, FIX, FXIa, FIXa, antithrombin (AT), plasmin, anti-FIX antibody (AHIX-5041) and antibody to antithrombin (PAHAT-S) were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Purified human FXIIa, plasma prekallikrein, high molecular weight kininogen (HK) and kallikrein were purchased from Enzyme Research Laboratories (South Bend, IN).

Soybean trypsin inhibitor, calf intestinal alkaline polyphosphatase (CIP), 96-well microplates (Cat#: 16-1429-82), Streptavidin-HRP and TNB-peroxidase substrate were from Thermo Fisher Scientific (Waltham, MA). The fluorogenic substrates for thrombin generation assays in plasma (Z-Gly-Gly-Arg-AMC) and for coagulation factor activation in purified systems (Boc-Gln-Gly-Arg-AMC) were from Bachem (Bubendorf, Switzerland). Human XII-, XI-, IX- and VII-deficient plasmas were purchased from George King Biomedical Inc (Overland Park, KS). HTF1 anti-human tissue factor antibody and black 96-well CAT microplates were from Thermo Fisher Scientific (Waltham, MA). The 0.1µm filters were from Millipore (Damstadt, Germany.

*Preparation of microvesicle-free plasma*

Whole blood was collected from healthy, medication-free adult volunteers in plastic tubes containing 3.2% citrate at a ratio of 1 volume of citrate to 9 volumes of blood. Blood collection was performed under a human subjects' protocol approved by the UNC Institutional Review Board. Research was conducted in accordance with the Declaration of Helsinki. Blood cells were removed by double centrifugation at 2,500g for 15 minutes. Cell-free plasma collected from 10 individuals was pooled and stored at -80°C. After thawing and before use, cell-free plasma was filtered through a 0.1µm filter to obtain MV-free normal pooled plasma (NPP). All factor-deficient plasmas derived from patients with known severe deficiency were certified to have <1% of deficient factor activity. All deficient factor plasmas were similarly filtered to obtain MV-free plasma.

*Characterization of storage lesion-induced red blood cell microvesicles (RBC-MVs)*

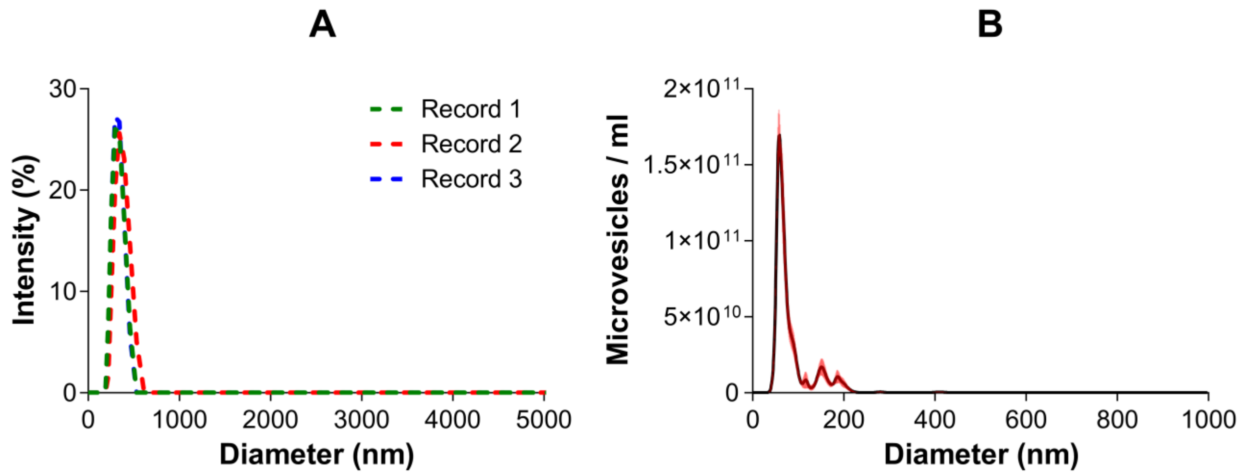
Isolated RBC-MVs were visualized after negative staining using transmission electron microscopy (JEOL JEM-1230, Peabody, MA, USA) and images were captured using a Gatan Orius SC 1000 CCD camera (Gatan Inc,

Pleasanton, CA, USA). For dynamic light scattering analysis, the Zetasizer Nano ZS (Malvern Instruments, UK) was used to confirm that isolated RBC-MVs contained only sub-micron vesicles before nanoparticle tracking analysis (NanoSight NS500 ZS, Malvern Panalytical, UK) was utilized for precise sizing and enumeration. Phosphatidylserine expression on RBC-MVs was tested using an activity-based, commercially available kit (Zymuphen MP-activity, Aniara, West Chester, OH). For subsequent functional assays, RBC-MVs were pelleted by centrifugation at 20,000g for 30 minutes, washed and re-suspended in phosphate buffered saline before use.

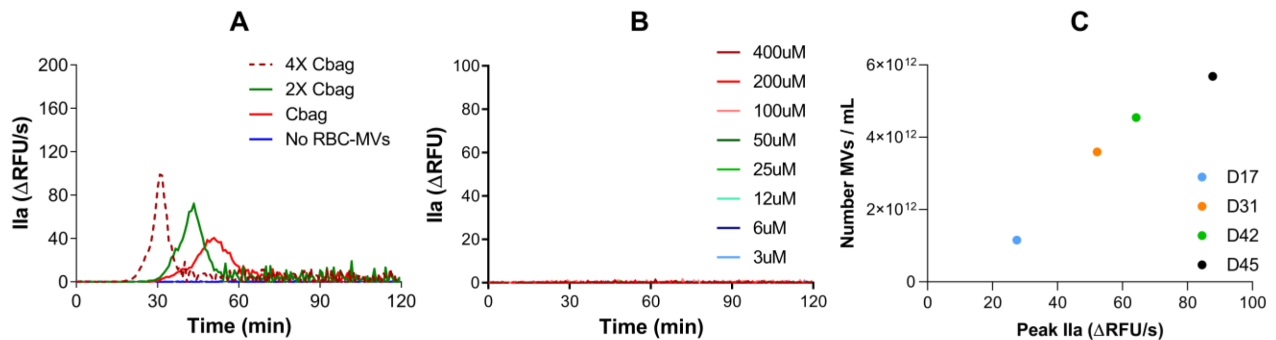
*ELISA for FIXa-Antithrombin complexes (FIXa-AT)*

FIXa-AT complexes were measured using an in-house sandwich ELISA that captures FIXa using a monoclonal anti-human FIX antibody and detects antithrombin using a biotinylated polyclonal anti-human antithrombin antibody. After washing to remove unbound antibodies, 100µl of streptavidin-HRP were added to each well and incubated for 30 minutes at 37°C. The plate was washed and 100µl of a mixture of equal volumes of the oxidizer and peroxidase substrate was added to each well. The reaction was stopped after 5-10 minutes and optical density was measured at a wavelength of 405 nm. For the ELISA standard, 100nM of human FIXa was incubated for 1 hour at 37°C with 2.5µM human antithrombin in the presence of 1U/ml unfractionated heparin. Results are expressed as concentration of FIXa-AT complexes.

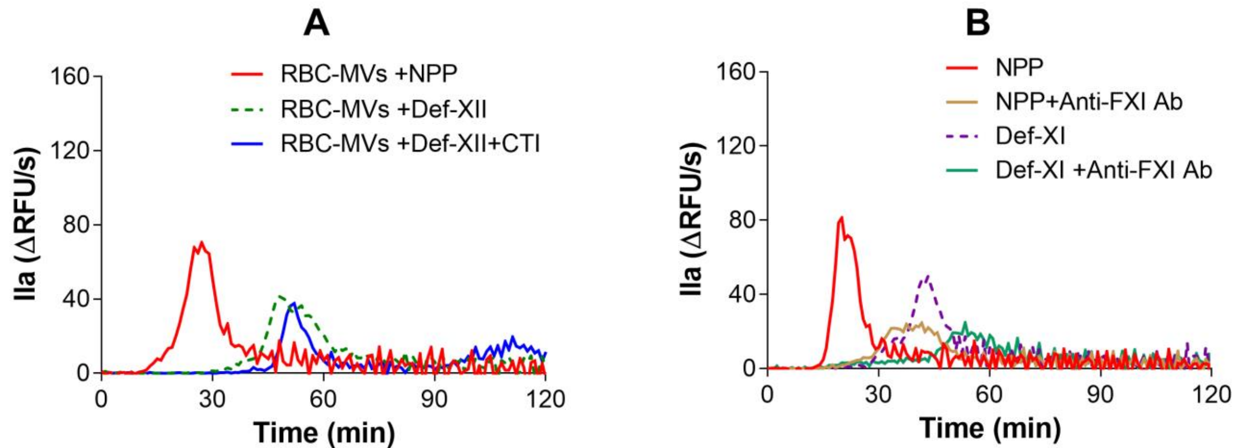
**Supplemental Data**



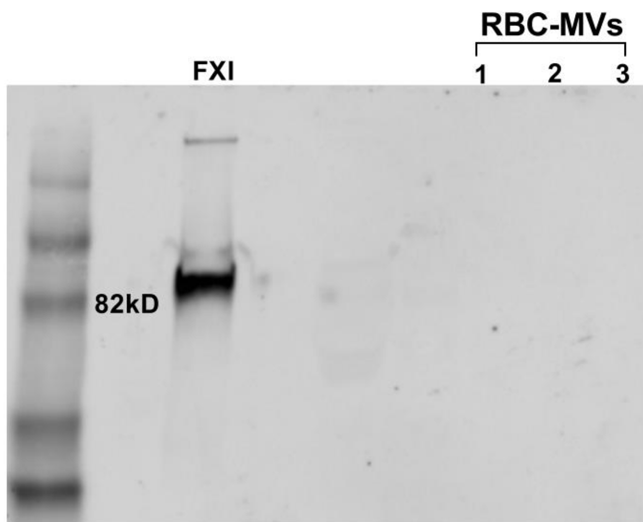

**Supplemental Figure 1**: **Size and distribution of red blood cell microvesicles (RBC-MVs) in a typical RBC concentrate unit after 43 days of storage.** A) Plot representing 3 records of dynamic light scattering analysis of a single sample of RBC-MVs. The Y axis represents the percent intensity and the X axis represents the size of the vesicles. B) Nanoparticle tracking analysis of RBC-MVs. The Y axis represents the count and the X axis represents the size. Black line represents the average of 5 independent readings, and the red denotes the standard of deviation.



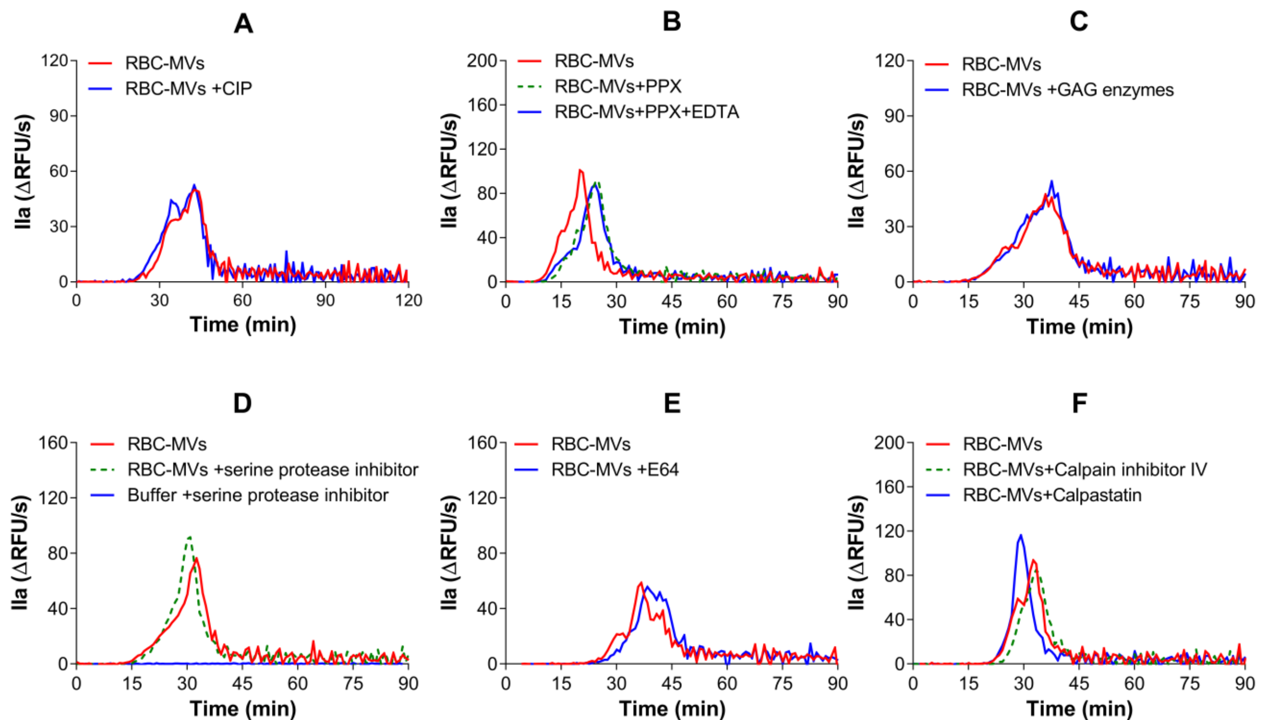

**Supplemental Figure 2**: **Thrombin generation induced by red blood cell microvesicles (RBC-MVs).** A) Three different concentrations of RBC-MVs were tested for thrombin generation capacity in normal plasma compared to buffer control (blue). B) Synthetic phospholipid vesicles added to MV-free plasma fail to induce thrombin generation in normal plasma. C) Relationship between peak thrombin generation and added number of RBC-MVs. Cbag = RBC-MV concentration in supernatant of RBC unit for 43 days.



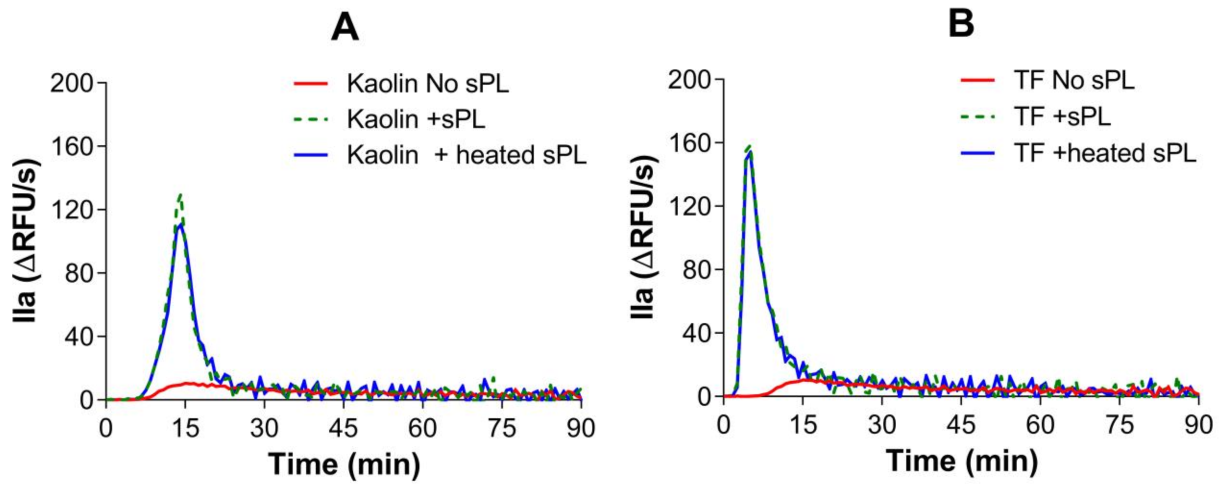

**Supplemental Figure 3**: **Red blood cell microvesicle (RBC-MV) thrombin generation is not entirely dependent on factors XI or XII.** A) Thrombin generation following addition of RBC-MVs to normal plasma (NPP) and plasma deficient (Def) in FXII, ± addition of the FXIIa inhibitor, corn trypsin inhibitor (CTI). B) Thrombin generation when RBC-MVs were added to NPP, NPP + antibody to FXI, FXI-deficient plasma, or FXI-deficient plasma + anti-FXI antibody.



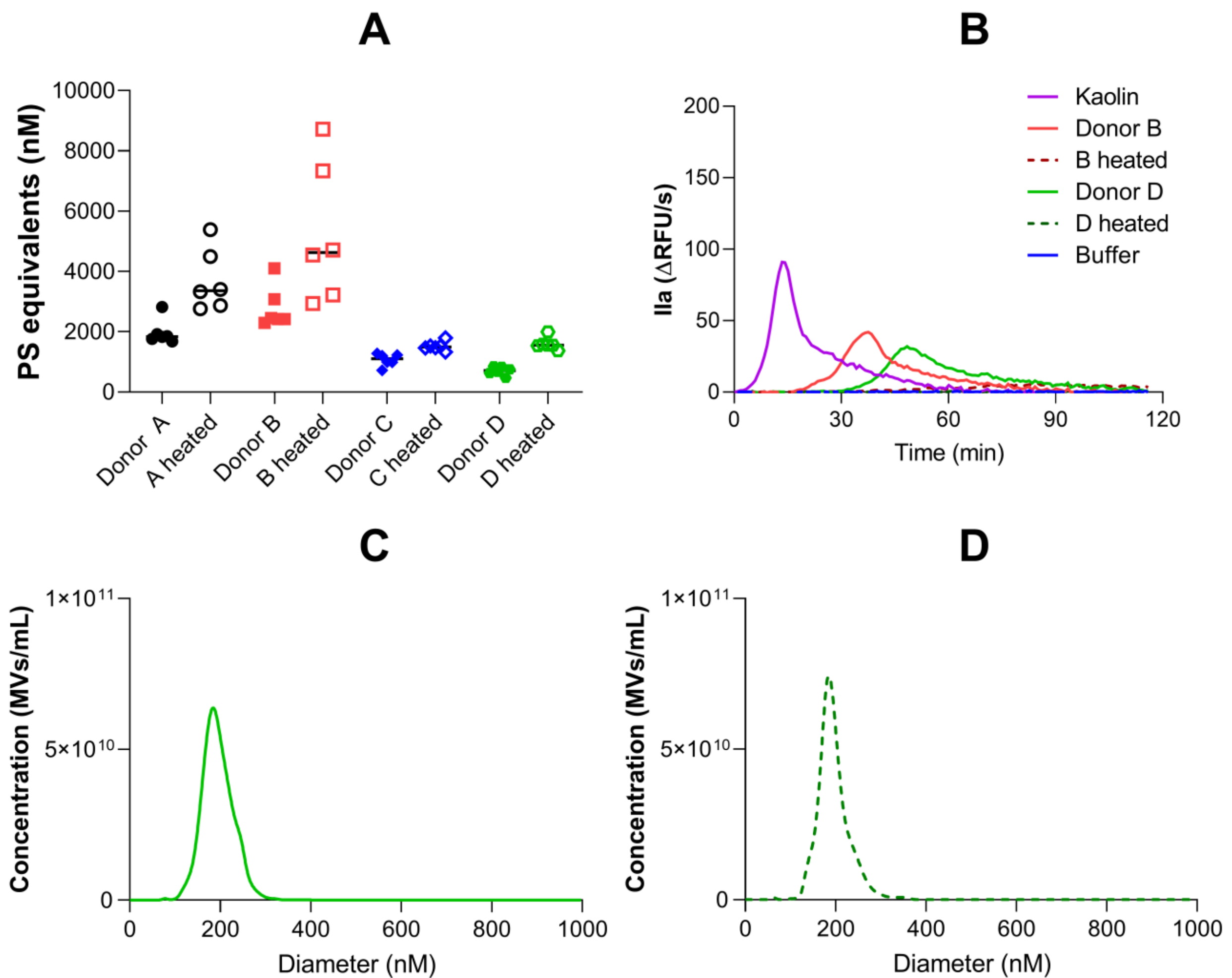

**Supplemental Figure 4**: **Absence of detectable FXI(a) on red blood cell microvesicles (RBC-MVs).** Western blot under reducing conditions depicting purified FXI control, and the absence of FXI(a) on RBC-MV preparations from 3 separate RBC units.




**Supplemental Figure 5**: **Evaluation of possible contact activators on red blood cell microvesicles (RBC-MVs).** A) RBC-MVs were added to normal plasma in the presence or absence of calf intestinal phosphatase (CIP), an inhibitor of polyphosphate. B) Similarly, thrombin generation in the presence of RBC-MVs was tested in the presence or absence of exopolyphosphatase (PPX) ± EDTA. C) RBC-MVs were incubated with glycosaminoglycans (GAG)-degrading enzymes (see text for details) prior to assay of thrombin generation in normal plasma. D) A cocktail of serine protease inhibitors (see main text) was incubated with RBC-MVs, which were then washed prior to addition to normal plasma to evaluate thrombin generation. The cocktail alone was used as control. E) The cysteine protease inhibitor E64 was incubated with RBC-MVs, which were then washed prior to addition to normal plasma to evaluate thrombin generation. F) RBC-MVs were treated with calpain inhibitor IV or calpastatin. The RBC-MVs were then washed prior to addition to normal plasma to evaluate thrombin generation.




**Supplemental Figure 6**: **Heating of RBC-MVs does not abolish phospholipid-dependent procoagulant activity.** A) Kaolin was used to activate the intrinsic pathway of coagulation in MV-free plasma which was then assayed for thrombin generation. B) use of tissue factor (TF) to initiate the extrinsic pathway. Synthetic phospholipid (sPL) vesicles, before or after exposure to 60°C for 15 minutes, were added to the samples.




**<u>Supplemental Figure 7</u>: Heating of RBC-MVs abolishes contact activation.** A) Effect of heat exposure (60°C for 15 minutes) on prothrombinase activity, expressed as phosphatidylserine (PS) equivalents, in RBC-MVs obtained from 4 different stored RBC units. B) Exposure to heat (60°C for 15 minutes) negates the ability of RBC-MVs to initiate thrombin generation in plasma. Kaolin is used as a positive control. C) Nanoparticle tracking analysis (NTA) plot of RBC-MVs prepared from a donor unit without heating. D) NTA plot of RBC-MVs from the same donor unit after exposure to 60°C for 15 minutes.