Supplemental material 3:

Laboratory sample and biomarker handling.

Aim 1b: 8.5 ml of blood will be drawn from each study participant prior to transfusion, within 30 minutes following the first RBC transfusion, as well as 6 hours (± 30 minutes) and 18 hours (±30 minutes) after the end of the first RBC transfusion for all study participants. If the patient remains in the hospital, safety labs will also be drawn on study day number 5. These safety laboratory assessments (total hemoglobin, CFH, haptoglobin) will be analyzed locally at the enrolling sites using standard clinical assays.

Aim 1c: A 6 ml sample will be taken pre- and post-wash from the already anticoagulated intervention RBC units. An additional aliquot of the RBC unit will be sealed in a capillary tube, centrifuged at 2000 g, and expressed as a decimal fraction using a micro-hematocrit reader. A single 6 ml sample will also be drawn from the standard-issue RBC units prior to administration.

Aim 2: 10 ml of blood will be drawn from each study participant at baseline prior to transfusion, within 30 minutes following the first RBC transfusion, as well as 6 hours (± 30 minutes) and 18 hours (±30 minutes) after the end of the first RBC transfusion. At each time point, blood will be placed in a 10-ml EDTA tube. All samples will be centrifuged at 2500 g for 20 minutes at 20°C within 4 hours of blood draw. The platelet-poor plasma will then be stored in 1.8 ml cryotubes at -80°C. Samples will be batch shipped and analyzed in at Blood Systems Research Institute (San Francisco, CA, USA).

Biomarkers: 240 µl of thawed plasma will be diluted with assay buffers and measured on the Milliplex multi- and singleplex assay platforms (a**ims 1c/2c**: sCD40L, CCL5/RANTES; **aims 2a/2c**: IL-6, IL-8, PAI-1; Millipore, Billerica, MA). Washed, incubated, and labeled samples will be acquired on a Labscan 200 analyzer (Luminex, Austin, TX) and analyzed using Bio-Plex

manager 6.1 software (Bio-Rad). A further 150 µl of plasma will used to perform ELISA-based measurements of RAGE (a**im 2a/2c**; R&D Systems, Minneapolis, MN). NT-proBNP (a**im 2c**) will be measured using a clinical diagnostic system (Novus Biologicals, Littleton, CO). RBC-derived microparticles **(aims 1c/2c):** Thawed platelet-poor plasma will be spun at 13,000 g for 10 minutes at 20°C, then labeled in preparation for flow cytometric measurement (BD LSR II flow cytometer, San Jose, CA). Vesicles will be lysed with NP-40 detergent and samples re-run to confirm results and allow setting of gates. Free hemoglobin **(aims 1c/2c):** The Human Hemoglobin ELISA Kit will be utilized for the detection of free hemoglobin in plasma (Bethyl Laboratories, Inc., Montgomery, TX). Neutral lipids **(aims 1c/2c):** Following the addition of ice-cold methanol, proteins will be precipitated, and non-polar lipids will be extracted/analyzed using high-pressure liquid chromatography (LC) interfaced into the electrospray source of a triple quadrupole mass spectrometer (MS) (liquid chromatography coupled to electrospray ionization mass spectrometry [LC/MS/MS]). Lipid concentrations will be estimated using ratios to an internal standard ($^{2}H^{8}$-5-HETE), as previously described.[1-3]

References:

1. Gijón MA, Zarini S, Murphy RC. Biosynthesis of eicosanoids and transcellular metabolism of leukotrienes in murine bone marrow cells. Journal of lipid research 2007;48:716-25.
2. Jordan JR, Moore EE, Sarin EL, et al. Arachidonic acid in postshock mesenteric lymph induces pulmonary synthesis of leukotriene B4. Journal of applied physiology (Bethesda, Md : 1985) 2008;104:1161-6.
3. Zarini S, Gijón MA, Ransome AE, Murphy RC, Sala A. Transcellular biosynthesis of cysteinyl leukotrienes in vivo during mouse peritoneal inflammation. Proceedings of the National Academy of Sciences of the United States of America 2009;106:8296-301.