**Supplemental materials and methods**

**Porcine in vivo transfusion model** *Anaesthesia and Monitoring:* Animals were weighed and anaesthetized with Ketamine (100mg/kg, Ketaset) and Halothane (1.5-2.0%) with nitrous oxide 50% in oxygen. Buprenorphne was administered every 4 hours for anaesthesia. Animals were intubated and positive pressure ventilation commenced in a circle circuit using a Penlon Nuffield 200 (Abingdon, Oxford, UK) initially to achieve peak inspiratory pressures of 30 cmH2O in a 1:4 (inspiratory: expiratory) ratio with modifications to maintain PaCO2 between target values of 35 and 45mmHg. Venous access and measurement of central venous pressure was achieved through direct puncture of the left external jugular vein using a quad-lumen central venous catheter (MultiCath4 Expert, Vygon GmbH). Continuous measurement of arterial blood pressure was achieved using a 20G Vygon catheter placed in the left internal carotid artery. Serial measurements of arterial haematocrit, haemoglobin, lactate and central venous oxygen saturations were performed every 30min using the ABL 800 Flex blood gas analyser (Radiometer, Copenhagen, Denmark). Core body temperature was assessed using a rectal temperature probe. Central venous pressure (8–12 mmHg), hydration, and sodium load (500 mL/h, 0.9% normal saline) were strictly controlled. Post-intervention all animals were recovered, and re-anesthetized and re-evaluated after 24hrs. A schematic of experimental design is provided in Figure 1A.

Lung injury, pulmonary endothelial function, acute kidney injury and renal endothelial function were determined as described previously 1,2. For lung injury a histology scoring system was used, as described by Matute-Bello 1 by investigators blinded to intervention allocation.

**In vitro flow assay** was adapted from previously described in vitro flow assays 3,4, where whole blood is perfused over a monolayer of endothelial cells.Validation of the flow system was performed with fresh donor blood in the presence of 2 mg/mL lipopolysaccharide or 1/5 total volume of HNA-3a anti-serum (a generous gift from Dr Geoff Lucas, International Blood Group Reference Laboratory, Filton UK 5). Addition of lipopolysaccharide or HNA-3a antiserum increased numbers of CD14 positive leukocytes and platelets (CD61) attaching to a monolayer of human umbilical vein endothelial cells (data not shown) indicating that the assay can be used to monitor leukocyte and platelets activation.

Region encoding annexin V for blocking experiments was cloned into pTrcHisA bacterial expression vector (ThermoFisher). Expression was induced with 100mM IPTG overnight and proten was purified using Ni-agarose (Qiagen, .Hilden, Germany), adjusted to 1mg/mL and stored at -80˚C. For blocking equal volumes of microvesicles and annexin V were incubated for 30 min and used in the assays.

**In vivo survival of transfused red cells** was measured using a modified version of the methods of Mock et al.6. Sixty mLs of packed red cells were withdrawn from the blood storage bag and separated from supernatant by centrifugation at 600 g for 15 min at 4°C. Supernatant was stored at 4°C. The pelleted red blood cells were resuspended in phosphate buffered saline. Centrifugation was repeated, and the supernatant was discarded. Washed red blood cells were resuspended in phosphate buffered saline (60 mL) containing sulfo-N-hydroxysuccinimide-biotin (10 μg/mL; Thermo Fisher Scientific, Rockford, IL) and incubated at room temperature for 40 min. To remove unbound sulfo-N-hydroxysuccinimide-biotin, red cells were washed twice with phosphate buffered saline, as described above. After the final washing step, red cells were resuspended in the previously stored supernatant.

To assess the survival of biotinylated red cells, 4 mLs of arterial blood were drawn into heparinised tubes at 5, 10, 15, 30, 60 and 120 min and again at 24 h post-transfusion of biotinylated red cells. Samples were centrifuged at 500 × g for 5 mins at 40C, supernatant discarded and cells resuspended in 1 mL of 0.5% bovine serum albumina. Samples (50 μl) were incubated for 45 min at 2-8˚C with fluorescein isothiocyanate-tagged streptavidin (250 μl of washing solution containing 20 μg/mL streptavidin fluorescein isothiocyanate (11-4317, eBioscience, Hatfield, UK)). Fluorescein isothiocyanate-labeled red blood cells were detected in forward scatter versus the log10 of fluorescent intensity of the cells detected on a FACSort flow cytometer using; Summit V4.3.02 Build 2451, Beckman Coulter, Inc. An extrapolation of the number of biotinylated red blood cells at 0 h was performed and equated to 100%. Of 10,000 total events, the ratio of the percentage of biotinylated cells in blood samples obtained after the transfusion to the percentage of biotinylated cells calculated to be present at 0 h was calculated.

**Microscopy – fixed samples analysis** Immunohistochemistry analysis was done on snap frozen (LN2) and stored at -80˚C porcine lung and kidney tissues. The tissues were embedded in OCT, cut at 4 – 6 μm mounted on poly-L-lysine coated slides, dried at RT for 30 min and fixed by immersion into ice cold acetone for 10 min. Antibodies against CD14 (clone MIL2, IgG2b, a generous gift from Prof Mick Bailey, Grierson et al., 2007) was used at 1:20 dilution of the hybridoma supernatant. Antibodies against CD16 (clone G7, IgG1, AbD Serotec) was used at 4 μg/mL. Primary antibodies were incubated with sections at 4˚C, overnight followed by washing in phosphate buffered saline and 1 hr incubation at RT with isotype-specific secondary antibodies (Ax546-coupled anti-IgG1 and Ax488-coupled anti-IgG2a from ThermoFisher). Sections were covered with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Labelling of HMVEC and HUVEC cells was done as previously described 7. Briefly cells were washed with phosphate buffered saline and fixed with 3% formaldehyde for 25 min. Samples were then washed with phosphate buffered saline and incubated for 1 hr at RT with primary mouse monoclonal antibodies against CD14 (clone MEM-18, Exbio, Prag, Czech Republic), CD61 (clone PM6/13, AbD Serotec), E-selectin (clone BBIG-E4, R&D Systems), activated b1-integrin (HUTS-21 clone, BD Bioscience) and polyclonal rabbit antibody against CS1-fibronectin. All antibodies were used at 2 – 4 μg/mL. Samples were washed with phosphate buffered saline and incubated for 30 min at RT with fluorescently labelled (Ax488 and Ax546) antibodies against mouse or rabbit IgGs.

Tissue iron staining was performed as described before 8: cryosections were incubated with Perls staining solution (equal volumes of 4% (v/v) HCl and 4% (w/v) K-ferrocyanide) for 45 min and washed with water. Peroxidases were blocked in 0.01 M NaN3, 0.3 % H2O2 in methanol for 1 hr and washed with 0.1 M phosphate buffer pH 7.4. The reaction was enhanced with DAB solution (0.1 M phosphate buffer (pH 7.4) solution containing 0.025% (w/v) diaminobenzidine (DAB, Sigma), 0.005% (v/v) H2O2, and 0.005% (w/v) CoCl2. Reaction was stopped by rinsing in water.

Analysis of images was done either manually (positive cell counting) or using ImageJ to determine integrated density of the staining.

***In vitro* endothelial assays** To determine monolayer permeability, HUVEC cells grown on transwell PET inserts in 6 well plates were co-incubated with 2 mL of EGM™-2 BulletKit™ medium (Lonza) containing 1 mg/mL haemoglobin, 5 μg/mL lipopolysaccharide, 20 % HS Sup, or phosphate buffered saline, and incubated at 37◦C for 6 hrs. Afterwards, medium from the top chamber was transferred into a new 6-well plate, filters placed in corresponding wells and 50 μl was taken for analysis (0’ time point). 1.5 mL medium with 1 mg/mL FITC-dextran was added to the top (filter) chamber and incubated at 37˚C for 90 min. 50 μl of medium from the bottom chamber was taken for analysis at 20, 40, 60 and 90 min. Fluorescence was measured with NovoStar plate reader (BMG LabTech, Offenburg, Germany).

For endothelial activation, HUVEC cells were grown in 96 well plates until confluency and incubated for 12 hr with haemoglobin at 1 mg/mL, red cell MV, red cell HS supernatant, 5 μg/mL lipopolysaccharide and phosphate buffered saline at 20 % of total (3 wells/ treatment). The cells were then fixed and processed as described above.

**qRT-PCR** Total RNA extraction was performed using RNeasy Fibrous Kit (Qiagen) according to manufacturer’s protocol. The total RNA was quantified using a UV NanoDrop ND-1000 UV (Thermo Scientific) spectrophotometer and Agilent 2100 Bioanalyzer using the Eukaryotic RNA Assay with the RNA 6000 Nano LabChip® Kit (Agilent Technologies, Santa Clara, CA). One µg of total RNA was reverse transcribed at 42˚C using a Sensifast C-DNA synthesis kit (Bioline, London UK) according to the manufacturer’s instructions and diluted 1:10 in H2O. For each transcript a standard curve was constructed. Single reactions were prepared for each set of primers using Sensifast SYBR® Green PCR Master Mix (Bioline, London UK). Each reaction included a reverse transcription negative control to confirm the absence of genomic DNA and a non-template negative control for primer-dimer. Primers for control housekeeping genes were designed as previously reported in 9. Primers used; RPL4: CAAGAGTAACTACAACCTTC and GAACTCTACGATGAATCTTC; TBP-1: AACAGTTCAGTAGTTATGAGCCAGA and AGATGTTCTCAAACGCTTCG. Primers for porcine orthologues of HMOX-1 were designed with Primer3 10 and verified with BLASTN: HMOX1: CGCTCCCGAATGAACAC and GCTCCTGCACCTCCTC. The real time PCR was run on Rotorgene Q (Quiagen, Venlo, Limburg, Netherlands) and the cycling conditions were 1 denaturation cycle at 95˚C/10mins followed by 40 cycles of amplification (95˚C/15sec, 55˚C/15sec, 72˚C/45sec). The Rotor-Gene Q software was used to determine the Ct in each reaction and all samples were amplified in triplicates.

**Supplemental figure and movie legends**

**eFigure –Supplemental figures: A** – microvesicle size distributions were estimated with NanoSight NS500. Samples were diluted 1:50 in 0.2 µm filtered phosphate buffered saline and each sample was acquired 3 times; samples from 8 units were analysed. **B** – Schematics shows fractionation process, microvesicles were isolated at different time points from aging blood bags (RBC pack) and all resulted in similar size distribution. The histogram represent means from two isolations (day 35 and 41). Proteins from lysed cells (1% Tx-100) were separated on an 8% SDS-PAGE gel and immunoblotted with antibody against CD235a (insert). **C** – Endothelial permeability in response to red blood cells fractions and cell-free hemoglobin (see Materials and Methods, data comes from 3 independent repeats). **D,** **E** – Expression of CD62E, CS1 fibronectin and activated integrin β1 on the surface of HUVEC after 6 hr incubation with high speed supernatant (HS) sup, microvesicles (MV), free Hgb or lipopolysaccharide. Values represent mean +/-SD.

**Movie A** – Adhesion and rolling of leukocytes labelled with SYTO64 in the presence of microvesicle fraction (representative movie). Whole donor blood was depleted of CD14-positive cells using dynabeads and of platelets as described in Materials and Methods. Samples were imaged at 5 frames/sec as described, after 50 min of in vitro inflammation assay. Movies are shown at 4 times real time (20 frames/sec).Microvesicle fraction increased number of rolling leukocytes on the surface of the endothelial monolayer (left panel: +MV). This was reduced by depletion of CD14 positive cells using Dynabeads, as described in Material and Methods (middle panel: +MV CD14 depletion). Platelet depletion (right panel: +MV Plt depletion) by washing also reduced number of rolling leukocytes, however, the results were variable and data was not significant when compared with assays without depletion. The movies are quantified in Figure 4D.

**Movie B** – Adhesion and rolling of leukocytes labelled with SYTO11 in the presence of phosphate buffered saline (control), microvesicle fraction and microvesicle fraction pre-incubated with 1mg/mL annexin V (representative movies). Samples were imaged at 5 frames/sec as described in Materials and Methods after 70 min of in vitro inflammation assay. Movies are shown at 4 times real time (20 frames/sec). microvesicle fraction (middle panel: +MV) significantly increased number of rolling and adherent leukocytes on the surface of the endothelial monolayer as compared with control assays where phosphate buffered saline was used (left panel: CTRL (phosphate buffered saline)) instead of microvesicle fraction. The effect of microvesicle fraction was removed by preincubation of microvesicles with recombinant annexin V, which blocks phosphatidylserine and oxidised lipids moieties (right panel: +MV +AV). Quantification of all obtained movies is shown in Figure 4E.

**Supplemental references**

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