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Supplemental Information

Methods

Cell Culture

Cells were cultured as described by Griffiths *et al.*¹² In brief, a >95% pure population of CD34⁺ haemopoietic stem cells (HSC) was isolated from human blood donor mononuclear cells or from thawed cryopreserved cord blood units by magnetic bead separation according to manufacturer's instructions (Miltenyi Biotec Ltd, Bisley UK). Allowing for differences in enucleation and leucofiltration rates, estimated starting material of 1-2x10⁵ CD34⁺ cells from adult or cord should be adequate to yield 2x10⁹ leucofiltered cRBC at the end of culture, sufficient to inject 10 mice. CD34⁺ cells were seeded into tissue culture flasks at a density of 2x10⁵ cells/ml using a base medium consisting of Iscove's modified Dulbecco's medium (IMDM, Source BioScience, Nottingham UK) containing 3% (v/v) AB Serum (Sigma-Aldrich, Poole UK), 2mg/ml HSA (Irvine Scientific, Newtownmountkennedy, Ireland), 10µg/ml Insulin (Sigma-Aldrich), 3U/ml heparin (Sigma-Aldrich), 500µg/ml transferrin (R&D Systems, Abingdon UK) and 3U/ml Epo (Roche, Welwyn Garden City, UK). In the first stage (days 0-10) this was supplemented with 10ng/ml stem cell factor (SCF, Medsafe, Sweden) and 1ng/ml IL-3 (R&D Systems, Abingdon UK) and in the second stage (days 11-13) with 10ng/ml SCF. In the final stage to day 21, base medium only was used.

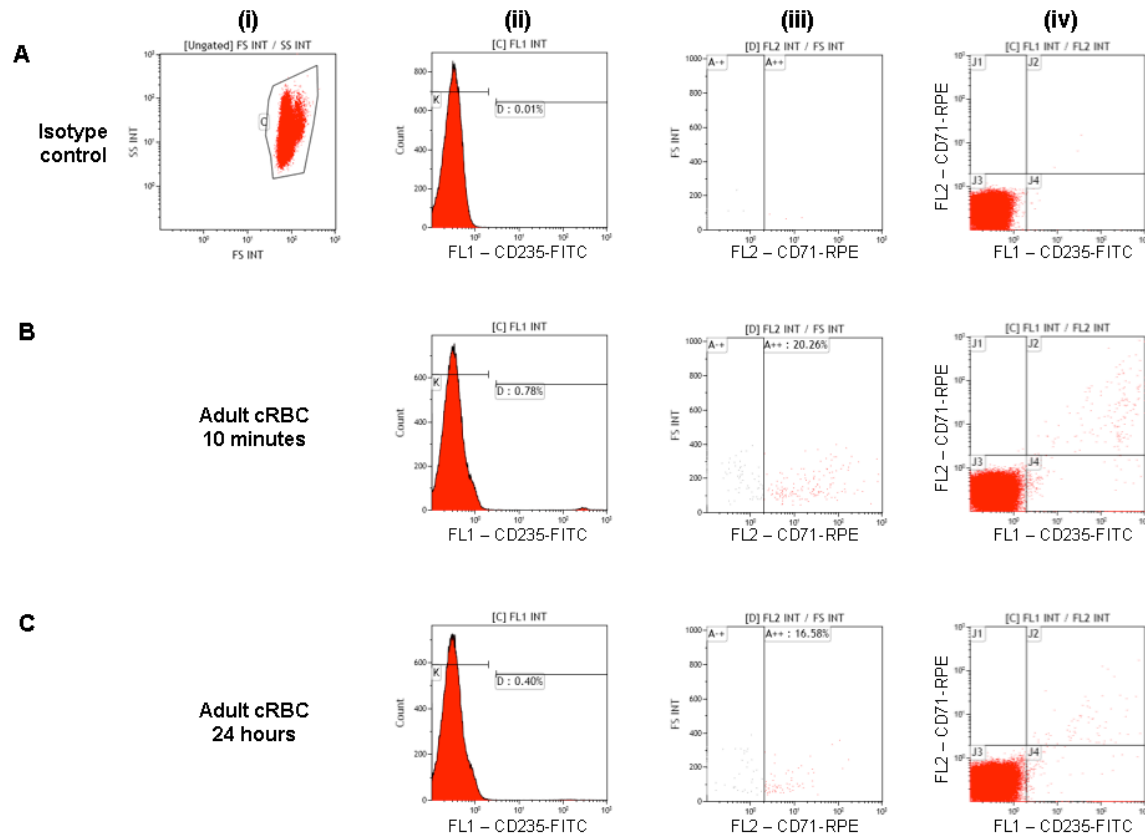
Statistical Analyses

Two-way analysis of variance (ANOVA) was used to compare results in liposome depleted and untreated mice. Post-tests were performed to compare results at each time-point. P-values for these comparisons were adjusted to take into account the multiple comparisons performed using the Holm-Sidak method. These analyses were undertaken using GraphPad Prism software, Version 6 for Windows (GraphPad Software, San Diego, CA).

The proportion of human cells surviving at each time point in blood samples from NSG mice was calculated, taking the value 10 minutes after inoculation as 100%. Where observed cell survival reached 50%, a log-quadratic spline function was fitted to the data over time for each mouse and the human cell half-life was estimated from this. Results were categorized by source (cord cRBC, adult cRBC and adult RBC) and experimental unit. All mice within an experimental unit received blood from the same donor. ANOVA was used to compare the proportion of human cells surviving at each time-point and to compare human cell half-life, by blood source. Random effects were included to allow for correlation between repeated measurements over time and between results for blood from the same donor. Separate ANOVAs were performed on experiments where adult cRBC, 5-day old and 26-day old adult red cells originated from the same donor. Blood source and age were combined into one factor and the random donor effect was omitted, since there was no variation at this level. Post-tests were performed to compare results for each blood source at each time-point. P-values for these comparisons were adjusted to take into account the multiple comparisons performed using the Tukey method. Mean values were

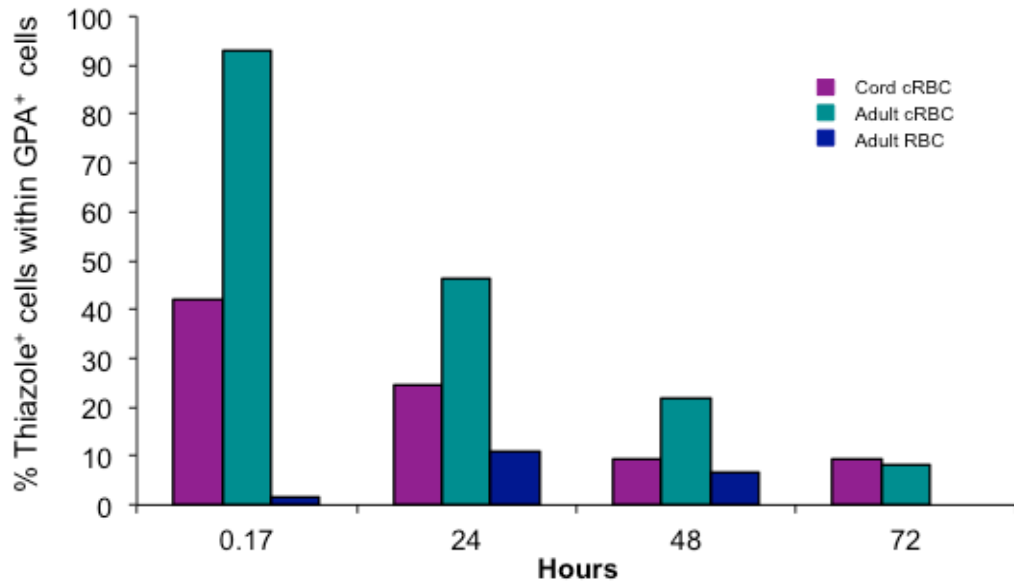
least-squares estimates, based on the analysis of variance model, as described; 95% confidence intervals (C.I.) are also stated. These analyses were undertaken using SAS/STAT software, Version 9 of the SAS System for Windows (SAS Institute Inc., Cary, NC).

Supplemental Figures



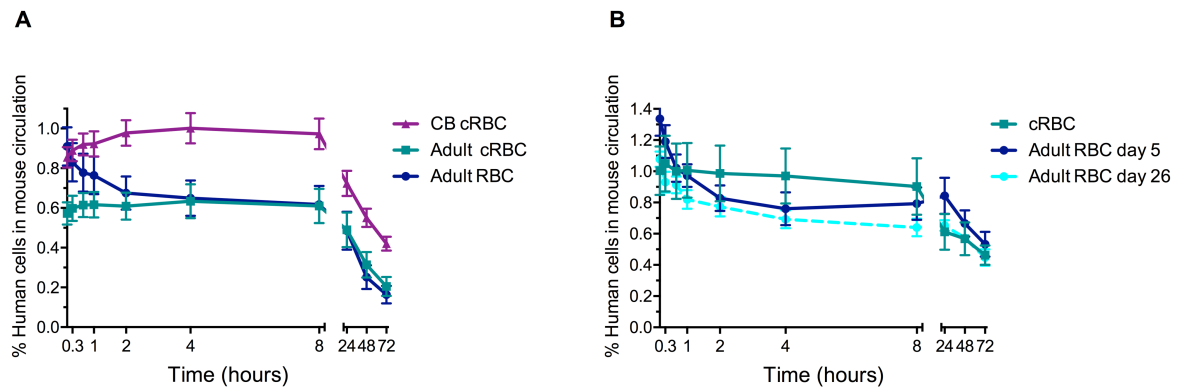
Supplemental Figure 1 Phenotypic maturation of transfused cells

Adult cRBC were inoculated into the lateral tail vein of macrophage depleted NSG mice and blood samples were collected at 10 minutes, 24, 48 and 72 hours. Maturation of the human cells was evaluated by measuring the expression of CD235 and CD71 in murine blood by flow cytometry. (A) Isotype control of a 1:100 dilution of adult leucofiltered cRBC in a murine peripheral blood aspirate. Cells were initially gated on forward and side scatter (i), Glycophorin A positive cells were gated (ii) and the proportion of CD71⁺ cells within this population is shown (iii). CD235-FITC and CD71-RPE dual labelled cells, gated on forward and side scatter, are also shown (iv). Corresponding plots from adult cRBC after 10 minutes (B) and after 24 hours (C) are shown. The proportion of CD71 positive cells detected decreased over time.



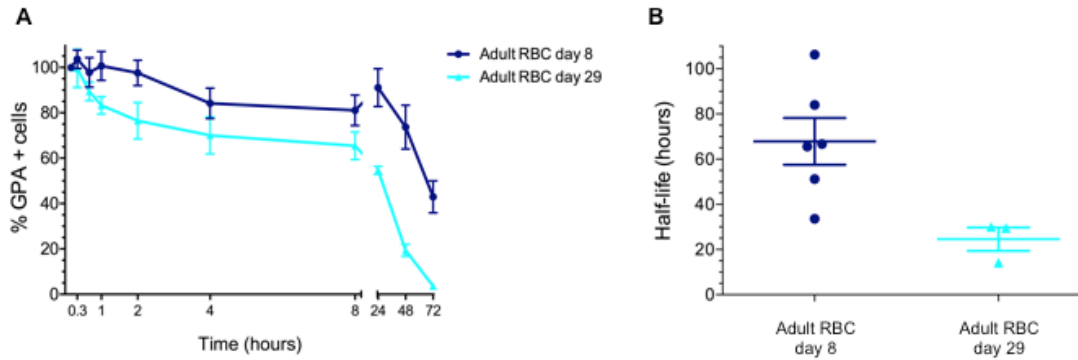
Supplemental Figure 2 In vivo maturation of cRBC and adult RBC in NSG mice

cRBC or adult blood cells were inoculated into the lateral tail vein of macrophage depleted NSG mice. Peripheral blood samples were taken from the opposite lateral tail vein at 10 minutes, 24, 48 and 72 hours. Human cells were detected by measuring expression of glycophorin A (CD235) in murine blood by flow cytometry. Cells were co-stained with thiazole orange and the amount of residual nucleic acid remaining was expressed as a percentage of the total number of GPA-positive human cells.



Supplemental Figure 3 Proportion of human cells in the mouse circulation

cRBC or adult blood cells were inoculated into the lateral tail vein of macrophage depleted NSG mice. Human cells were detected by measuring the expression of CD235 in murine blood by flow cytometry. (A) Proportion of human cord blood cRBC, adult cRBC and donor RBC surviving in the mouse circulation. Data represent mean \pm SE of 4 independent experiments for cord blood cultured reticulocytes, 8 independent experiments for the adult cultured reticulocytes and 5 independent experiments for standard donor cells. Mice in each independent experiment were injected with cells from the same donor. (B) Direct paired comparison of cRBC and RBC from the same donor surviving in the mouse circulation. The proportion of adult cRBC and day 5 and day 26 donor cells detected in the murine circulation are shown. Data represent mean \pm SE.



Supplemental Figure 4 Comparison of *in vivo* survival of fresh and stored donor RBC

Adult red blood cells from the same donor were inoculated into NSG mice at 8 days (n=6) and 29 days (n=3) following donation. Peripheral blood aspirates were taken over a 72 hour period and CD235⁺ cells were measured by flow cytometry. (A) Proportion of human cells surviving in mouse circulation. The proportion of human cells were normalized with the levels detected 10 minutes after inoculation set to 100%. The levels of human cells detected in NSG were significantly higher in recipients of day 8 cells over the entire time course of the experiment (P=0.004, T Test). Data represent mean±SE. (B) Half-life of human cells in transfused NSG mice, by source. Two-tailed T test confirmed longer half-lives of day 8 donor RBC (67.9±10 hours) than day 29 RBC (24.6±5 hours, P=0.01). Each point represents an individual mouse, lines represent mean±SE.