SUPPLEMENTARY APPENDIX

Superior survival of *ex vivo* cultured human reticulocytes following transfusion into mice

Sabine Kupzig,[1] Stephen F. Parsons,[1] Elinor Curnow,[2] David J. Anstee[1] and Allison Blair[1,3]

[1]NIHR Blood and Transplant Research Unit, Bristol Institute for Transfusion Sciences, National Health Service Blood and Transplant; [2]Statistics and Clinical Studies, National Health Service Blood and Transplant, Bristol and [3]School of Cellular and Molecular Medicine, University of Bristol, UK

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Correspondence: allison.blair@bristol.ac.uk

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, an estimated starting material of 1-2x10^5 $CD34^+$ cells from adult or cord should be adequate to yield 2x10^9 leucofiltered cRBCs at the end of culture, which is sufficient to inject 10 mice. $CD34^+$ cells were seeded into tissue culture flasks at a density of 2x10^5 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.

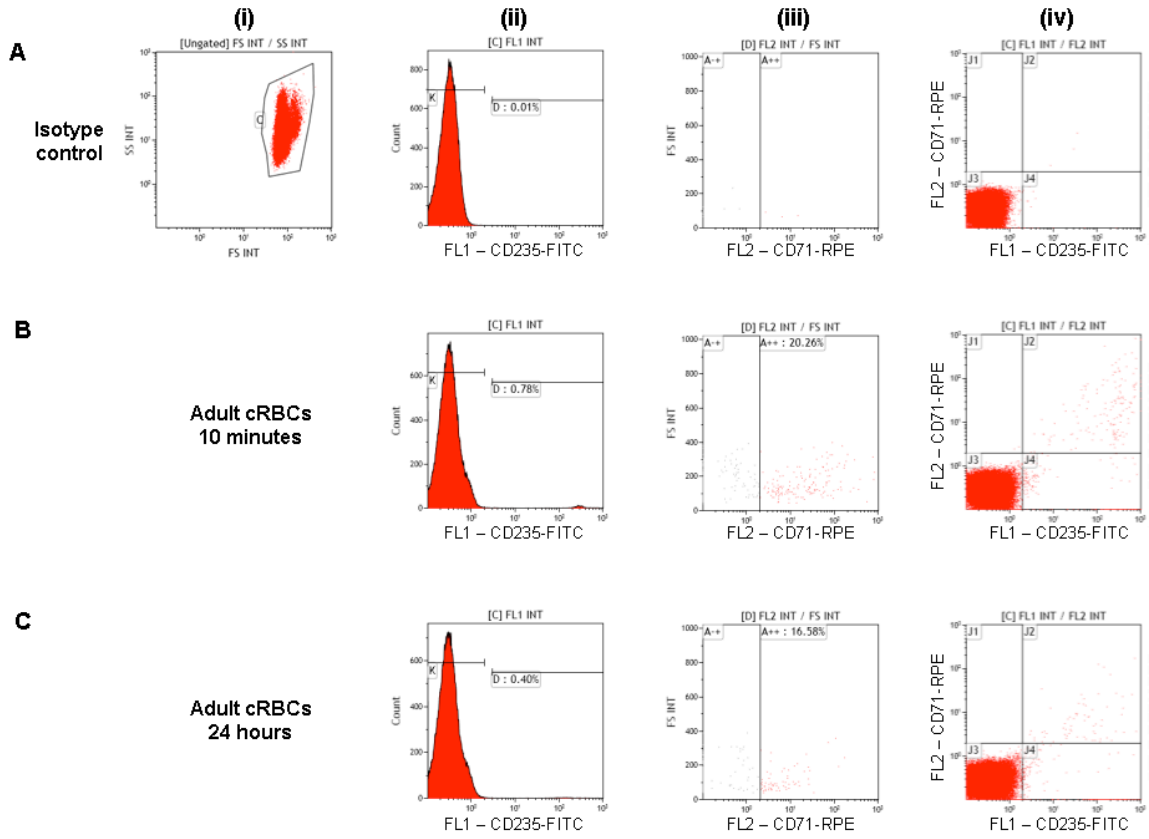
Statistical Analyses

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).

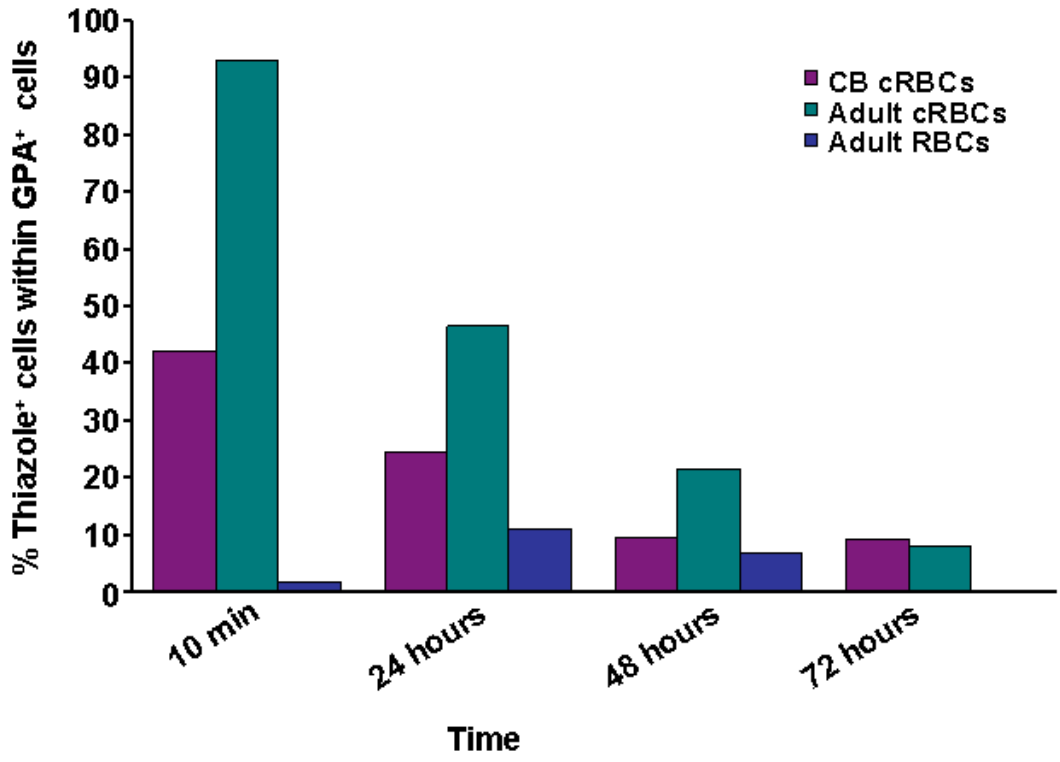
The proportion of human cells surviving at each time point in blood samples from NSG mice was calculated, taking the value 10 minutes after inoculation as 100%. Where observed cell survival reached 50%, a log-quadratic spline function was fitted to the data over time for each mouse and the human cell half-life was estimated from this. Results were categorized by source (cord cRBCs, adult cRBCs and adult RBCs) 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 cRBCs, 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

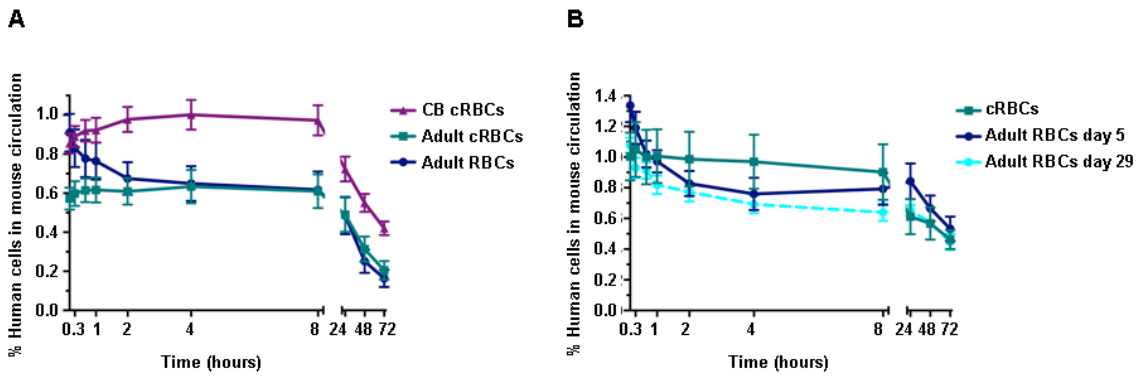


Supplemental Figure 1 Phenotypic maturation of transfused cells
Adult cRBCs were inoculated into the lateral tail vein of macrophage depleted NSG mice and blood samples were collected at 10 minutes, 24, 48 and 72 hours. Maturation of the human cells was evaluated by measuring the expression of CD235a and CD71 in murine blood by flow cytometry. (A) Isotype control of a 1:100 dilution of adult leucofiltered cRBCs 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). CD235a-FITC and CD71-RPE dual labelled cells, gated on forward and side scatter, are also shown (iv). Corresponding plots from adult cRBCs after 10 minutes (B) and after 24 hours (C) are shown. The proportion of CD71 positive cells detected decreased over time.

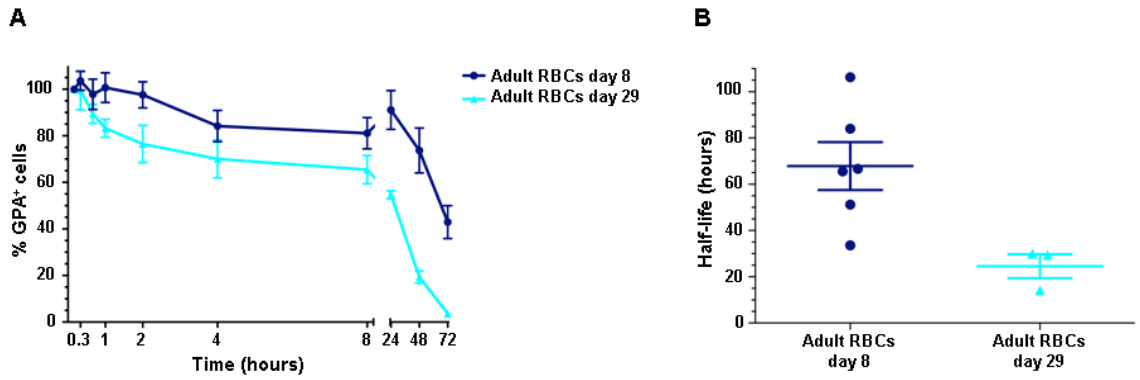


Supplemental Figure 2 ***In vivo*** **maturation of cRBCs and adult RBCs in NSG mice**

cRBCs 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 (CD235a) 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
cRBCs 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 CD235a in murine blood by flow cytometry. (A) Proportion of human cord blood cRBCs, adult cRBCs and donor RBCs surviving in the mouse circulation. Data represent mean±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 cRBCs and RBCs from the same donor surviving in the mouse circulation. The proportion of adult cRBCs and day 5 and day 26 donor cells detected in the murine circulation are shown. Data represent mean±SE.



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

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 CD235a$^+$ 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 RBCs (67.9±10 hours) than day 29 RBCs (24.6+5 hours, P=0.01). Each point represents an individual mouse, lines represent mean±SE.