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

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ABSTRACT

The generation of cultured red blood cells from stem cell sources may fill an unmet clinical need for transfusion-dependent patients, particularly in countries that lack a sufficient and safe blood supply. Cultured red blood cells were generated from human CD34$^+$ cells from adult peripheral blood or cord blood by *ex vivo* expansion, and a comprehensive *in vivo* survival comparison with standard red cell concentrates was undertaken. Significant amplification (>10^5-fold) was achieved using CD34$^+$ cells from both cord blood and peripheral blood, generating high yields of enucleated cultured red blood cells. Following transfusion, higher levels of cultured red cells could be detected in the murine circulation compared to standard adult red cells. The proportions of cultured blood cells from cord or peripheral blood sources remained high 24 hours post-transfusion (82±5% and 78±9%, respectively), while standard adult blood cells declined rapidly to only 49±9% by this time. In addition, the survival time of cultured blood cells in mice was longer than that of standard adult red cells. A paired comparison of cultured blood cells and standard adult red blood cells from the same donor confirmed the enhanced *in vivo* survival capacity of the cultured cells. The study herein represents the first demonstration that *ex vivo* generated cultured red blood cells survive longer than donor red cells using an *in vivo* model that more closely mimics clinical transfusion. Cultured red blood cells may offer advantages for transfusion-dependent patients by reducing the number of transfusions required.

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Introduction

National Blood Services are an essential part of healthcare, playing key roles in treating patients following trauma, surgery and transplants as well as providing life saving products for patients with blood disorders. Unfortunately, in many countries there are supply shortages of red blood cell (RBC) concentrates for transfusions, and concerns about the safety of the blood supply. The majority of units transfused globally each year are used to treat individuals from developed countries that represent only around 15% of the world population.[1] Pressure on blood supply in developed countries is likely to intensify in the longer term with increasing life expectancy, concomitant with greater numbers of surgical procedures in an ageing population and notable rises in the prevalence of cancer.[2] Whilst blood transfusions are a life saving procedure for many, as evidenced by the dramatic fall (~99%) in the number of women dying in childbirth from 1920 to 1950,[3,4] they can pose significant risks. Individuals who require regular transfusions are at risk of adverse reactions following transfusion of mismatched blood. Patients with chronic transfusion-dependent anemia, such as β-thalassemia or sickle cell disease, are at particular risk of iron overload,[5] and aged stored RBCs, which contain a heterogeneous mix of cells at various ages, may have adverse clinical effects in critically ill patients.[2] A source of exclusively young RBCs, as found in cultured RBCs, could help address the above challenges for transfusion by increasing the transfusion

intervals and reducing iron overload, particularly in patients that depend on regular transfusions.[6]

Considerable effort has been made to generate cultured red blood cells (cRBCs) *ex vivo* from CD34+ hemopoietic stem cells (HSCs), human embryonic stem cells or induced pluripotent stem cells (iPSCs).[7-16] Many of the published techniques entail multi-phase culture systems, over a period of 18-38 days, and some include co-culture on stroma. The first group to produce cRBCs in the absence of stroma reported an extrapolated yield of 1.4 units of cRBCs from one cord blood (CB) unit.[8] The only clinical study to date used autologous mobilized CD34+ cells from a healthy volunteer as starting material.[11] The donor was reinfused with 2ml cRBCs,[17] and around 50% of the cultured reticulocytes could be detected 26 days after reinfusion, providing evidence for the feasibility of transfusion of *ex vivo* generated red cells. Despite these advances, problems associated with enucleation, large-scale generation and financial costs are hurdles that need to be overcome prior to clinical use.[18] We have previously described an *ex vivo* erythroid expansion method for CD34+ cells derived from adult peripheral blood (PB). Using this method it was possible to achieve significant expansion of CD34+ cells to yield 5ml (2.8x 10^{10}) of packed enucleated RBCs. This was the largest yield reported to date from PB, and represented a major advance in developing a product that is suitable for clinical use.[13,14]

However, few of the reported studies have conducted any *in vivo* evaluation of the *ex vivo* generated cells. This is an important consideration that must be addressed to prove that the *ex vivo* generated cells are suitable for transfusion. Some studies have used sublethally irradiated immune-deficient mice with intraperitoneal (IP) injection of cells following saturation with ABO type O cells, then retro-orbital sampling over a period of 5 days to detect human cells.[7,11,15,19] Hu *et al*. reported that the depletion of macrophages was essential in order to achieve human RBC chimerism in NOD/SCID mice inoculated with CD34+ fetal liver cells alone or with implanted human fetal thymic tissue.[20] Whilst these *in vivo* studies are informative, they do not accurately represent a clinical transfusion where matched red cells are administered intravenously and without additional cell products to saturate the patient.

We have developed a biologically representative *in vivo* model using NOD/LtSz-scid IL-2Rγ_c null (NSG) mice, which are more permissive hosts for the engraftment of normal and malignant human blood cells.[21,22] In the study herein, we have conducted a comprehensive *in vivo* evaluation of cRBCs generated from CB and adult PB, and demonstrate that *ex vivo* generated cRBCs are superior to donor RBCs using a model that more closely mimics clinical transfusion.

Methods

Donor samples

Blood donor mononuclear cells and CB were provided with informed consent (National Health Service National Research Ethics Committee, reference number 08/H0102/26).

Cell culture

See the *Online Supplementary Information* for full details of the three-stage *ex vivo* expansion procedure. Briefly, CD34+ HSCs isolated from human PB or thawed cryopreserved CB units were seeded into tissue culture flasks at a density of 2x10^5 cells/ml, and maintained in the range 2-5x10^5 cells/ml by division and the addition of first-stage medium until day 10. On days 11-13, cells were maintained at 5-15x10^5 cells/ml by the addition of second-stage medium. From day 14, cells were maintained in third-stage medium at 10-40x10^5 cells/ml. Once the total volume reached 200ml, cells were transferred from static flasks to 1.5 liter, stirred (15rpm), vessels. Cells were filtered using a standard leucofilter (Pall WBF, Haemonetics Ltd, Coventry, UK) prior to inoculation into NSG mice.

Microscopy

Cytospin preparations of cultured cells were stained using Leishman's Staining Solution (VWR International, Lutterworth, UK), imaged using a Leica DM750 microscope (Leica Microsystems, Milton Keynes, UK) and photographed using a Pixera Penguin 600CL camera (Digital Imaging Systems, Bourne End, UK).

For live cell confocal microscopy, cRBCs or PB aspirates from transfused mice were stained with fluorescein isothiocyanate (FITC)-conjugated BRIC 256 (mouse monoclonal anti-human CD235a; IBGRL, Bristol, UK) and imaged at 22°C using a Leica SP5 confocal imaging system.

In vivo studies

NSG mice were bred and maintained at the University of Bristol, Animal Services Unit. Adult mice were macrophage depleted by intravenous (IV) inoculation of liposome-encapsulated clodronate (dichloromethylene diphosphonate, CI2MDP, The Netherlands) on day -3 (100µl) and day -1 (50µl).

Mice were transfused with 2x10^8 cRBCs or 2x10^8 washed adult donor RBCs *via* the left lateral tail vein. PB aspirates were taken from the right lateral tail vein at 10, 20, 40, 60, 120, 240 and 480 minutes after inoculation, and once daily thereafter up to 9 days. Cells were counted and stained with anti-human CD235a (glycophorin A) antibody and analyzed by flow cytometry. Non-clodronate treated mice were also transfused and analyzed to assess the effects of murine macrophages on the inoculated cells.

For a direct paired comparison, washed RBCs from a standard red cell pack (ABO type, O RhD positive) were transfused 5 days after blood donation. CD34+ HSCs isolated from the same donation were transfused once they had been cultured for 21 days to generate reticulocytes. At the same time, a further aliquot of unmodified donor RBCs was transfused into a separate group of mice; these cells were now 26 days old.

Flow cytometry

Cells were stained with BRIC256-FITC for *in vivo* survival studies, with anti-mouse F4/80-phycoerythrin (PE) for macrophage depletion studies or with BRIC256-FITC and mouse anti-human CD71-RPE (Bio-Rad, Hemel Hempstead, UK) for maturation studies. Samples were analyzed using a Beckman Coulter FC 500 flow cytometer (Beckman Coulter, High Wycombe, UK), the gating strategy is shown in the *Online Supplementary Figure S1*.

For nucleic acid staining, 6x10^6 BRIC256-PE stained cells were washed and labelled with 0.1µg/ml thiazole orange (Sigma-Aldrich, Poole, UK).

Statistical analyses

Full details of statistical analyses are provided in the *Online Supplementary Information.*

Results

Yields and morphology of cRBCs

Using the three-stage culture technique, it was possible to achieve >10^5-fold amplification of cRBCs from 1x10^6 $CD34^+$ HSCs. On average, *ex vivo* cultured adult cells were ~60% enucleated on day 20/21 of culture (Figure 1A). In contrast, CB cells were only ~38% enucleated. The enucleated cells were separated from free nuclei, nucleated precursors and debris by leucocyte filtration, yielding >99% pure fractions (Figure 1B). Filtration yields ranged from 30.5% to 94.9% (average 60.5±7.2%) for adult cRBCs and from 40.5% to 80.5% (average 62.1±8.5%) for cord blood cRBCs. The mean corpuscular volume (MCV) of adult and cord cRBCs was 135μm^3 (range 125–142), while that of donor RBCs was 89μm^3 (range 83–96). Mouse RBCs had a MCV of 48μm^3 (range 45-52). Confocal analyses of the leucofiltered cells showed that glycophorin A (GPA) is expressed on the surface, and the morphology of the majority of cells at the end of culture was that of reticulocytes rather than mature biconcave RBCs (Figure 1C), a finding which is also supported by the cells having a larger MCV.

Macrophage depletion of NSG mice is required to allow uptake of RBCs

Prior to the commencement of clodronate treatment, macrophage levels in murine PB ranged from 23.7-26.8% (median 25.8%, Figure 2A). Macrophage levels declined significantly to 4.2±1.4% within 24 hours of the second dose of liposomes, remained low over the following 24 hour period (P<0.00003), before gradually increasing over the next 6 days (8 days after the last liposome dose) to reach similar levels to those observed in untreated controls.

To investigate the effects of murine macrophages on transfused cells, separate groups of mice were either pre-treated with clodronate liposomes or left untreated prior to inoculation of donor RBCs. Cells were inoculated by IV injection 24 hours after animals received the second dose of clodronate liposomes, when murine macrophages were at their lowest levels; control animals were injected at the same time. In the control, non-treated mice, most of the human RBCs had been removed from circulation within 10 minutes of transfusion, and the levels of human cells detected were almost 6-fold reduced compared to those in macrophage depleted mice (Figure 2B, P=0.0005). The remaining human cells were rapidly cleared and were practically undetectable after 1 hour. In contrast, clearance of human cells was significantly slower in mice that had been pre-treated with clodronate liposomes (Figure 2C, P=0.0004). Clodronate-treated animals were used for all results reported below.

In vivo *maturation of cultured reticulocytes*

Macrophage depleted NSG mice were randomized to receive cRBCs or washed donor RBCs 24 hours after receiving the second dose of clodronate liposomes. Live cell confocal imaging of PB samples from mice transfused with adult or cord cRBCs or with donor RBCs showed only human cells stained positive for GPA, and were easily distinguished from the background of mouse RBCs (Figure 3A-C). Human cells were visibly larger than the mouse RBCs, and a significant number of cRBCs appeared to have adopted the biconcave shape of mature red cells. These cells may have undergone maturation *in vivo*, most likely in the mouse spleen, when compared to the morphology of the cells that were initially transfused (Figure 1C).

Measurements of cell diameter, taken at various time points, indicated a gradual decrease in the size of human cells transfused into NSG mice (Figure 4A,B). Prior to injection (0 minutes), cord and adult cRBCs had an average diameter of 9.1±0.09μm (n=145) and 9.9±0.09μm (n=64), respectively. In comparison, standard RBCs had a mean diameter of 7.9±0.07μm (n=60) while mouse RBCs measured 5.7±0.07μm (n=60). After 48 hours in the mouse circulation, diameters had decreased to 6.4±0.11μm (n=42) and 6.1±0.19μm (n=16) for cord and adult cRBCs, respectively, indicative of a possible maturation of the cultured human reticulocytes in circulation.

Further evidence for cRBCs maturation *in vivo* was demonstrated by decreasing CD71 expression (Figure 4C). In aspirates taken 10 minutes after inoculation, 21.4±7.3% (median fluorescence intensity (MFI) 116±26) of cord cRBCs and 16.2±3.2% (MFI 159±15) of adult cRBCs expressed CD71. After 3 days this had decreased to 7.5±3.4% (MFI 93±36) and 6.4±1.5% (MFI 53±4) for cord and adult cRBCs, respectively, (P≤0.03). By comparison, CD71 expression on donor RBCs did not change significantly over the 3-day time course, ranging from 2.9±0.3% - 5.1±0.7% (MFI 61±5 – 47±5, P=0.12).

The amount of residual nucleic acid remaining in transfused human cells from the different sources was also

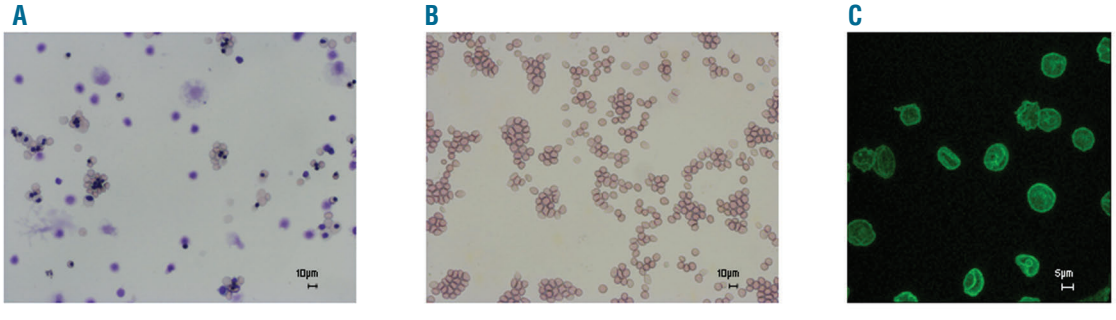

Figure 1. Cytospin and confocal analyses of day 21 cultured adult reticulocytes. Cultured reticulocytes were processed for morphological analyses by cytospin and stained with Leishman's solution before (A) and after leucofiltration (B). Leucofiltration resulted in >99% pure population of cultured reticulocytes. (C) Leucofiltered cultured reticulocytes were also processed for live cell confocal imaging using anti-CD235a-FITC antibody.

assessed by thiazole orange staining. The proportion of thiazole-positive cells decreased from 42.2% and 92.9% at 10 minutes to 9.3% and 8.16% after 3 days in cord and adult cRBCs, respectively. Such a decrease was not observed in donor RBCs (range 1.7%-11.0%, *Online Supplementary Figure S2*).

Survival of cultured reticulocytes and donor RBCs in vivo

Human cells were detectable from 10 minutes after inoculation in mice receiving adult cRBCs (median 0.51% of total circulating murine blood cells, range 0.09-1.37%, n=29), cord cRBCs (median 0.87%, range 0.38-1.57%, n=26) and adult red cells (median 0.91%, range 0.08-1.61%, n=16, *Online Supplementary Figure S3A*). Human cells could be detected in murine blood for up to 9 days. On average, the levels of human cells in animals that received cRBCs peaked 60 mins to 4 hours post-inoculation and then gradually declined. In contrast, standard RBCs peaked during the first 10 minutes followed by a sharp decline in the first 2 hours post-inoculation.

When the proportion of human cells was normalized, with the levels detected 10 minutes after inoculation set to 100%, there were significant differences in the levels of cRBCs and adult RBCs in the murine circulation over the entire 6 day evaluation period (Figure 5A, $P \leq 0.02$). Higher levels of human cells were detected in recipients of cRBCs, regardless of source, compared to recipients of adult RBCs for the duration of the experiment. The levels of adult and cord cRBCs detected in the murine circulation were not significantly different (Figure 5A, $P=0.7$). The levels of both adult and cord cRBCs were significantly higher than that of adult RBCs over the first 3 days (adult cRBCs *vs.* adult RBCs, $P=0.03$; cord cRBCs *vs.* adult RBCs, $P=0.01$), but particularly so over the initial 8 hours ($P<0.0001$). At this time point, the proportion of CB cRBCs and adult cRBCs were relatively unchanged (112±6% and 103±7%, respectively). In contrast, the proportion of human cells detected in mice inoculated with RBCs had reduced considerably to 63±7%.

The distribution of half-life, determined by experiment, is shown (Figure 5B). Data for 5 mice receiving cord cRBCs, 6 mice receiving adult cRBCs and 1 mouse receiving adult red cells were excluded from the half-life analysis, since the experiments were terminated prior to cell survival reducing to 50%. There was considerable intra-experimental variation in the half-life of transfused cells (i.e., variation between individual mice transfused with the same source of cells) and inter-experimental variation, likely due to the variation between individual donors. After taking this into account, some evidence remained of a difference in half-lives across the blood sources, although it was not significant ($P=0.1$). The mean half-life for cord cRBCs was 56±4 hours, compared with 38±5 hours and 26±8 hours for adult cRBCs and RBCs, respectively. A gradual recovery of murine macrophages was observed 24-48 hours post-transfusion (Figure 2A). Macrophages are likely to remove all human cells, indiscriminate of source, and this recovery coincides with the observed decline of human cells in the mouse circulation after 24 hours.

To directly compare the survival of cRBCs with donor RBCs *in vivo*, a matched comparison was undertaken using cells from the same donor (Figure 6A). Overall, significantly higher levels of cRBCs were detected in the murine circulation, and the extent of the difference varied over time ($P<0.0001$). The mean levels of human cells detected remained above 83% in animals transfused with adult cRBCs over the first 8 hours, whilst there was a sharp decline to <53% and <60% in the groups that received day 5 and day 26 adult cells, respectively. The levels of cRBCs detected were significantly higher than day 5 and day 26 adult RBCs ($P \leq 0.01$) over this period. There was no significant difference in levels of day 5 and day 26 adult RBCs detected in the murine circulation ($P=0.99$). From 24 hours after transfusion, similar levels of human cells were detected, regardless of cell source. The proportions of human cells detected in murine circulation are depicted in

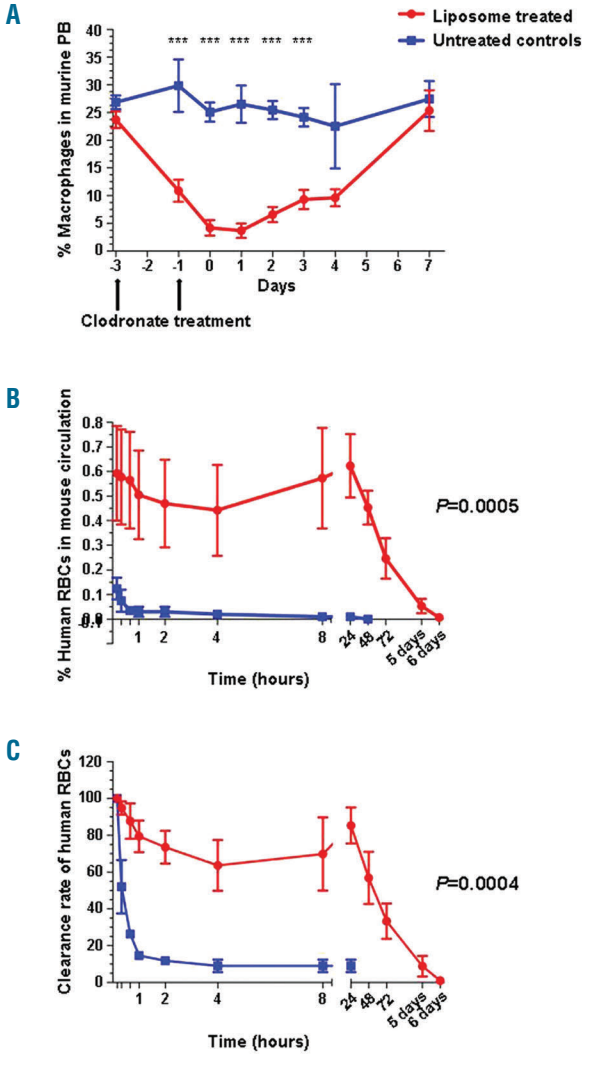

Figure 2. Validation of macrophage depletion for transfusion model. Murine peripheral blood samples were collected at designated time points and cells were labelled with PE-conjugated anti-mouse F4/80 (A) or FITC-conjugated anti-human CD235a (B & C) and analyzed by flow cytometry. (A) Circulating levels of murine macrophages measured in liposome treated (n=8) and untreated NSG mice (n=10) over a 10 day period. (B & C) NSG mice that had either been treated with clodronate liposomes to remove macrophages at day -3 and day -1 (n=3) or left untreated (n=2) were inoculated with RBCs from a single donor on day 0. (B) Percentage of human RBCs in the mouse circulation. (C) Clearance rates of human cells in untreated and macrophage depleted mice. Human cells were normalized to 100% at 10 minutes after injection. Data shown as mean±SE. ***$P<0.00003$. RBCs: red blood cells; PB: peripheral blood.

the *Online Supplementary Figure S3B*. The mean half-life for cRBCs was 47±6 hours compared with 46±16 hours and 56±4 hours for adult red cells on day 5 and day 26, respectively (P=0.74, Figure 6B). Again, the recovery of murine macrophages is likely to be responsible for the removal of human cells after 24 hours.

Discussion

There is currently a global imbalance between the supply and demand for red blood cells for transfusion. We have previously shown it is possible to generate large numbers of enucleated cRBCs from donor PB CD34$^+$ HSCs.[13,14] In the study herein, we used a good manufacturing practice (GMP) compliant procedure to achieve a >10^5-fold amplification from 10^6 CD34$^+$ HSCs, with 63% being enucleated, yielding ~10ml packed cells. To the best of our knowledge this represents the greatest yield of enucleated cRBCs reported to date. In addition, we demonstrate that the same method also permits the generation of cRBCs from CD34$^+$ HSCs isolated from cord blood samples, and have conducted a thorough *in vivo* survival assessment of these *ex vivo* generated reticulocytes.

CD34$^+$ HPCs were cultured in the presence of cytokines over a 20-21 day period in the absence of a feeder layer. During this time the cells mature to produce a mixed population of nucleated precursors, free nuclei and around 30-95% enucleated reticulocytes. Filtration, using a leucodepletion filter, removes nucleated cells, free nuclei and debris, resulting in a homogeneous suspension of reticulocytes (Figure 1B,C) for transfusion. Following enucleation, a reticulocyte needs to loose 20-30% of its plasma membrane to become a mature biconcave red cell.[23,24] We have previously shown that excess plasma membrane is internalized by maturing reticulocytes. These membrane vesicles fuse with autophagosomes, which are subsequently expelled by the cells.[13,14,25] Evidence suggests that the final maturation step occurs in the spleen, since it has long been known that splenectomised patients show an increased number of circulating reticulocytes containing autophagosomes.[26]

In order to fully evaluate the functional capacity of the cRBCs, we assessed their maturation and survival in clodronate depleted NSG mice. We initially tested the method of macrophage depletion reported by Hu *et al*. in NOD/SCID mice,[20] and found similar results with NSG mice, in that clearance of human RBCs was prevented in animals that had been pre-treated with clodronate liposomes. This model more closely mimics a clinical transfusion than those previously reported,[7,11,15,19] demonstrating non-toxic survival and maturation of *ex vivo* generated cRBCs, confirming they are suitable for transfusion *in vivo*.

Following transfusion into NSG mice, the majority of reticulocytes appear to mature into biconcave RBCs (compare Figure 1C with Figure 3A,B). We were able to measure a reduction in cell diameter over time for transfused cord and adult cRBCs in the mouse circulation. It is likely that this final maturation step takes place in the mouse spleen. Macrophages, which have largely been removed in clodronate-treated mice, do not seem to be required for the RBCs to achieve their final biconcave shape. However, they may be required to remove any membrane vesicles extruded by the maturing reticulocytes in order to reduce the amount of plasma membrane, cytoplasm and residual organelles.[13,14,25] In addition to these morphological changes, we also observed a reduction in the amount of CD71 expression on the surface of transfused human reticulocytes and in the amount of thiazole orange staining over a 3-day time course, suggesting that *in vivo* maturation of human cRBCs was occurring in the murine system. Given that human cRBCs (MCV=135μm^3) and

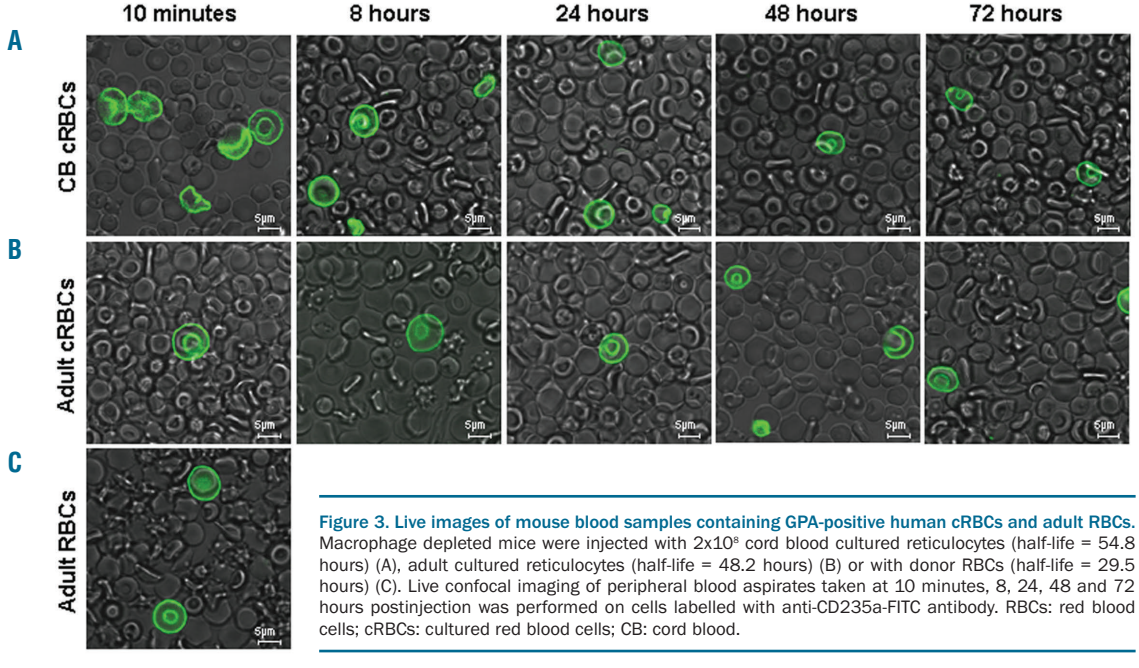

Figure 3. Live images of mouse blood samples containing GPA-positive human cRBCs and adult RBCs. Macrophage depleted mice were injected with 2x10^8 cord blood cultured reticulocytes (half-life = 54.8 hours) (A), adult cultured reticulocytes (half-life = 48.2 hours) (B) or with donor RBCs (half-life = 29.5 hours) (C). Live confocal imaging of peripheral blood aspirates taken at 10 minutes, 8, 24, 48 and 72 hours postinjection was performed on cells labelled with anti-CD235a-FITC antibody. RBCs: red blood cells; cRBCs: cultured red blood cells; CB: cord blood.

human donor RBCs (MCV =89μm^3) are significantly larger than mouse RBCs (MCV =48μm^3), it is possible that increased shear stress in the mouse capillaries contributed to the observed maturation from reticulocytes to red cells.

Survival comparisons of cRBCs and donor RBCs *in vivo* revealed that higher proportions of human cells were detected in animals transfused with cRBCs from either CB or from adult PB. In contrast, a sharp decline in the proportion of human cells was observed in animals inoculated with adult RBCs. This is most likely due to the heterogeneous cell population present in a standard adult RBC donation, while the *ex vivo* generated cRBCs comprise a much more homogeneous population of younger red cells. The dramatic decline observed following transfusion of RBCs mirrors the situation in humans where ~25% of cells are cleared within 24 hours following transfusion of packed RBCs,[27] with most cleared during the first hour.[28] Despite inoculating up to 25-fold fewer cells in the study

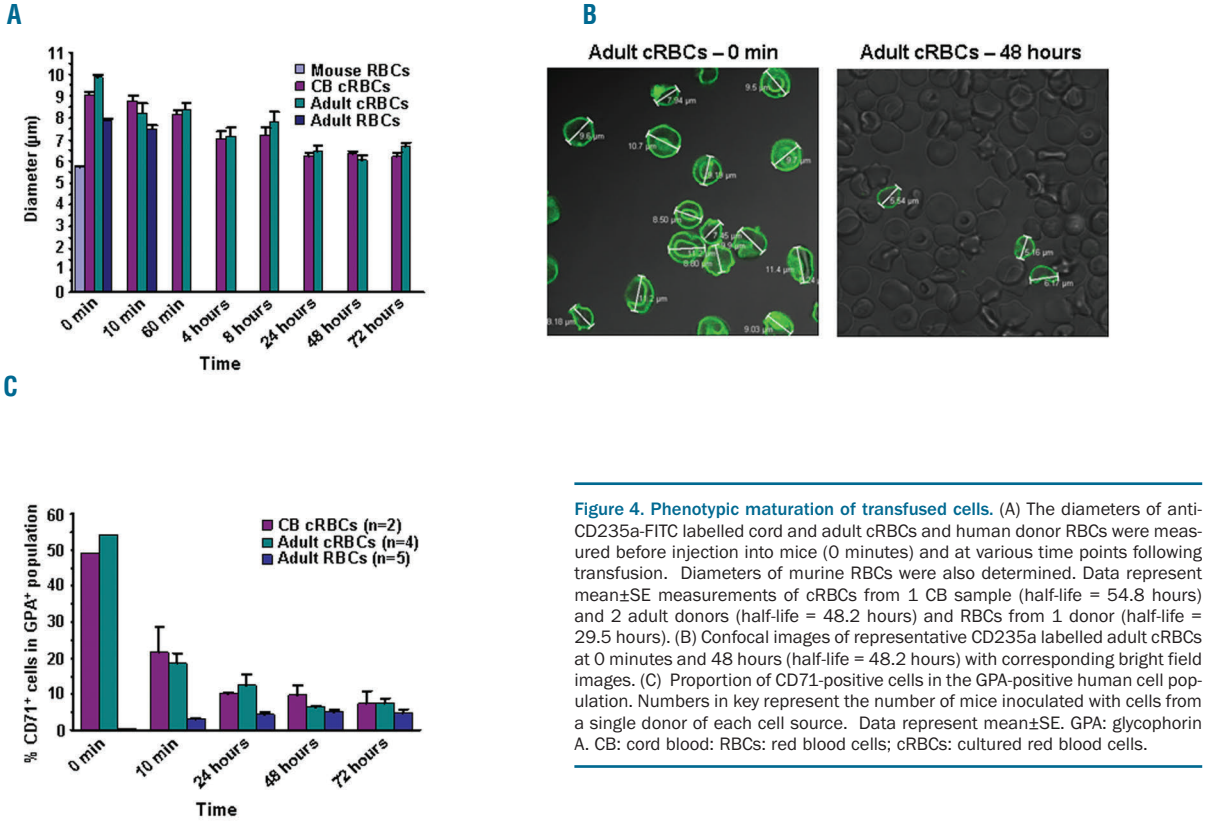

Figure 4. Phenotypic maturation of transfused cells. (A) The diameters of anti-CD235a-FITC labelled cord and adult cRBCs and human donor RBCs were measured before injection into mice (0 minutes) and at various time points following transfusion. Diameters of murine RBCs were also determined. Data represent mean±SE measurements of cRBCs from 1 CB sample (half-life = 54.8 hours) and 2 adult donors (half-life = 48.2 hours) and RBCs from 1 donor (half-life = 29.5 hours). (B) Confocal images of representative CD235a labelled adult cRBCs at 0 minutes and 48 hours (half-life = 48.2 hours) with corresponding bright field images. (C) Proportion of CD71-positive cells in the GPA-positive human cell population. Numbers in key represent the number of mice inoculated with cells from a single donor of each cell source. Data represent mean±SE. GPA: glycophorin A. CB: cord blood: RBCs: red blood cells; cRBCs: cultured red blood cells.

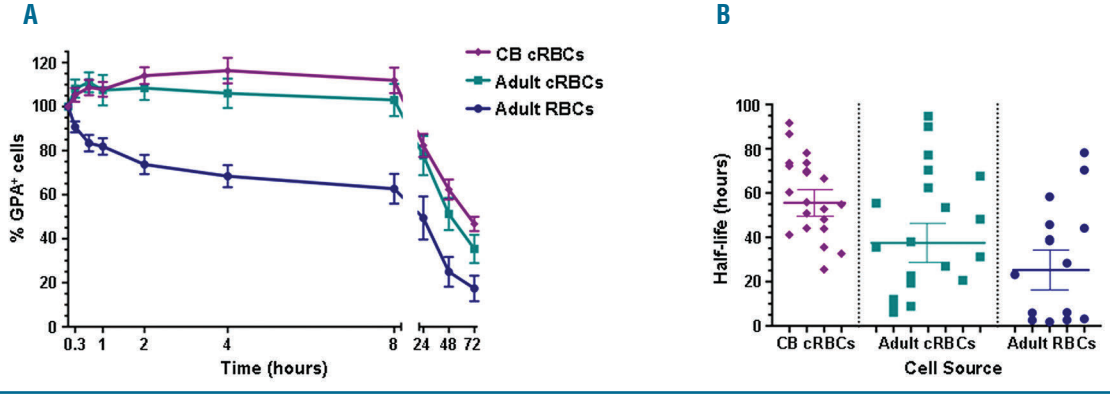

Figure 5. Detection and survival of human cRBCs and adult RBCs in transfused NSG mice. cRBCs or adult blood cells were inoculated into the lateral tail vein of macrophage depleted NSG mice. Human cells were detected by measuring expression of CD235a in murine blood by flow cytometry. (A) Proportion of human cells surviving in mouse circulation. Data represent mean±SE of 8 independent experiments for the adult cultured reticulocytes, 5 independent experiments for standard donor cells and 4 independent experiments for cord blood cultured reticulocytes. The mice in each independent experiment were injected with cells from the same donor. The proportion of human cells were normalized, with the levels detected 10 minutes after inoculation set to 100%. Survival of both adult and cord cRBCs in NSG mice was significantly greater than adult red cells at all measured time points over the entire time course of the experiment ($P \leq 0.02$). (B) Half-life of human cells in transfused NSG mice, by experiment and by source. Data from mice where the levels of human cells decreased to ≤50% by the end of the experiment were used to calculate the half-life. Each point represents an individual mouse, results from mice inoculated with cells from the same donor are stacked vertically, lines represent mean±SE. GPA: glycophorin A; CB: cord blood: RBCs: red blood cells; cRBCs: cultured red blood cells.

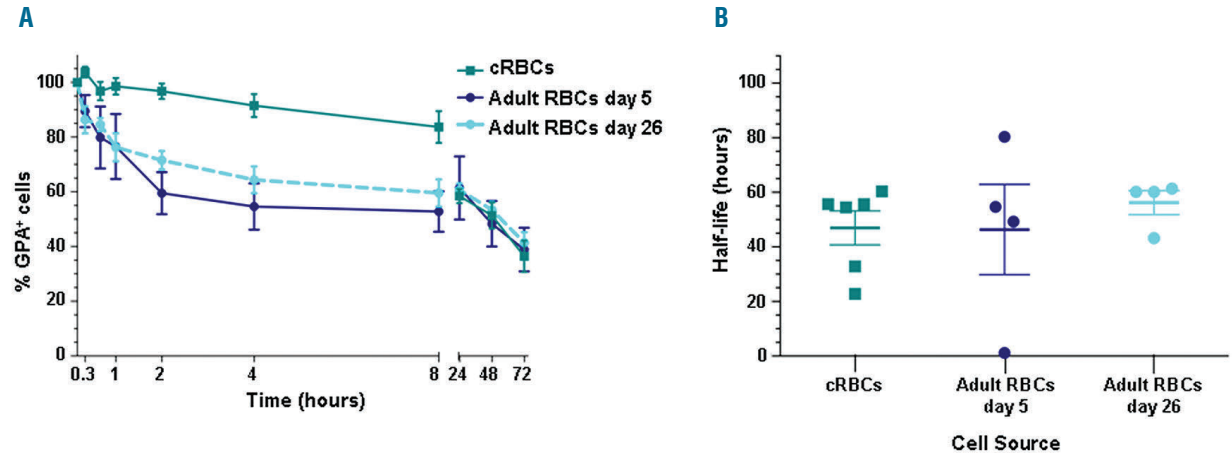

Figure 6. cRBCs demonstrate better survival than RBCs from the same donor. (A) Direct paired comparison of cRBCs and RBCs from the same donor. Five day old adult red cells were transfused into NSG mice (n=5). Twenty-one days later cRBCs generated from this sample were transfused into a separate group of mice (n=6). At the same time a third group of mice was transfused with unmodified red cells from the same donor that were now 26 days old (n=4). ANOVA showed that overall survival of cRBCs was significantly better than day 5 and day 26 adult red cells (P<0.0001). (B) Half-life of human cells in transfused NSG mice, by source. Each point represents an individual mouse, mean±SE are shown. RBCs: red blood cells; cRBCs: cultured red blood cells; GPA: glycophorin A.

herein, the levels of human red cells detected were comparable with previous reports where cRBCs derived from granulocyte-colony stimulating factor (G-CSF) primed leukapheresis or normal donor PB were inoculated into humanized NOD/SCID mice.[7,11] Moreover, we demonstrated that survival of *ex vivo* generated reticulocytes was significantly superior to that of donor RBCs. The only other published *in vivo* comparison of cRBCs and native RBCs reported survival from both sources was similar over a 3-day evaluation period, but data on half-lives were not provided.[7] Our findings also represent the first report on *in vivo* survival of cRBCs generated from CB cells. There was no difference in the levels of human cells detected or the *in vivo* survival of cRBCs from CB or PB, demonstrating that both are suitable sources for *ex vivo* reticulocyte generation. The variation observed between batches of cRBCs from CB and PB is a known issue in the field.[29] As the starting material is comprised of CD34$^+$ cells at different stages of maturation, such variation is not unexpected. Close monitoring of individual cultures, to optimize production of enucleated RBCs, will be necessary for clinical applications. In the study herein, the median half-life of donor RBCs was 30 hours while those of cRBCs from adult PB or CB were 40 and 58 hours, respectively. The larger variation in half-lives observed using adult cRBCs and RBCs can be attributed to variation in individual donor samples. cRBCs derived from CB were more uniform in this respect. Regardless of source, cRBCs remained detectable for 6-9 days while adult RBCs were largely undetectable after 72 hours. It has been previously reported that the levels of circulating human red cells in mouse models decline within a few days of the last clodronate liposome treatment, possibly due to recovery of murine macrophages.[20] We demonstrated that murine macrophages begin to recover 48-72 hours after the last clodronate treatment, 24-48 hours following transfusion, and this recovery coincides with the decline in the levels of human cells detected in murine circulation. This has a direct impact on the half-life of human cells, since all xenogeneic cells, irrespective of source, will be removed by the macrophages. Consequently, it is possible that differences in macrophage depletion may also contribute to the observed variation in half-lives.

To further evaluate the observed enhanced survival of cRBCs *in vivo*, a direct comparison of cRBCs and adult RBCs from the same donor was undertaken. Donor cells were used 5 days after leukapheresis, a typical age of RBC units issued for transfusion and, for the purposes of direct comparison with cRBCs, at day 26. Higher levels of transfused cRBCs were detected compared to both day 5 and day 26 donor red cells. In NSG mice that received cRBCs, high levels of human cells were maintained over the first 2 hours and then a gradual decline was observed. In contrast, the levels of human cells in mice that received adult RBCs declined rapidly and there was no difference in either the levels of human cells detected or the survival of day 5 and day 26 stored cells. This latter finding, showing no significant difference in survival using fresh and stored blood, was not confirmed in a subsequent experiment using day 8 and day 29 adult RBCs (*Online Supplementary Figure S4*), suggesting deterioration during storage may vary between donors.[30] Further work will be required to address this. The lack of a significant difference in the half-lives of cRBCs or day 5 and day 26 donor RBCs can be attributed to the recovery of murine macrophages. To the best of our knowledge, this is the first report of paired comparisons of cRBCs and native RBCs from the same donor.

Our rationale for generating red cells *ex vivo* is that they provide a cohort of younger cells compared to donated blood, and as such may offer clinical advantages by surviving longer and possessing superior functional characteristics. The study herein represents the first demonstration that cRBCs generated from either CB or adult PB have prolonged survival *in vivo* compared to adult RBCs. We have previously shown adult cRBCs are comparable to donor RBCs in terms of their deformability, oxygen-binding capacity and serology.[13,25] Further work is required to determine the quantity of cRBCs that constitute a therapeutic dose, and provide a cost effective manufacturing process.

A logical progression of this work will be an allogeneic

survival and recovery trial in man to compare the half-life of cRBCs with that of donor RBCs. A cRBC product with increased survival will offer several advantages over current red cell products for certain patient groups. Examples are reduction in donor exposure and iron overload in chronically transfused patients, such as those with β-thalassaemia, and difficult to transfuse patients, such as some patients with sickle cell disease. Cellular reprogramming may also be an important approach to generate stem cells for therapeutic purposes, albeit very difficult to produce on a large scale.[31] Erythroid progenitor cells that undergo enucleation and hemoglobin switching *in vivo* have been generated from human iPSCs.[19,32,33] However, such cell lines are not representative of adult hemopoiesis. We anticipate that the creation of immortalised erythroid progenitor cell lines[34] and their genetic manipulation could provide a very valuable source of cRBCs with rare blood group phenotypes that could particularly benefit immunized, difficult to transfuse sickle cell patients.

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References

1. World Health Organization Global Database on Blood Safety Summary Report. Available from: http://www.who.int/bloodsafety/global_database/GDBS_Summary_Report_2011.pdf?ua=1. 2011. Last accessed: 29th November 2017.
2. Williamson LM, Devine DV. Challenges in the management of the blood supply. Lancet. 2013;381(9880):1866-1875.
3. Chamberlain G. British maternal mortality in the 19th and early 20th centuries. J R Soc Med. 2006;99(11):559-563.
4. Goldenberg RL, McClure EM. Maternal mortality. Am J Obstet Gynecol. 2011; 205(4):293-295.
5. Anstee DJ, Gampel A, Toye AM. Ex-vivo generation of human red cells for transfusion. Curr Opin Hematol. 2012;19(3):163-169.
6. Triadou P, Girot R, Rebibo D, et al. Neocytopheresis: a new approach for the transfusion of patients with thalassaemia major. Eur J Pediatr. 1986;145(1-2):10-13.
7. Giarratana MC, Kobari L, Lapillonne H, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat Biotechnol. 2005;23(1):69-74.
8. Miharada K, Hiroyama T, Sudo K, Nagasawa T, Nakamura Y. Efficient enucleation of erythroblasts differentiated in vitro from hematopoietic stem and progenitor cells. Nat Biotechnol. 2006;24(10):1255-1256.
9. Baek EJ, Kim HS, Kim S, Jin H, Choi TY, Kim HO. In vitro clinical-grade generation of red blood cells from human umbilical cord blood CD34+ cells. Transfusion. 2008; 48(10):2235-2245.
10. Boehm D, Murphy WG, Al-Rubeai M. The potential of human peripheral blood derived CD34+ cells for ex vivo red blood cell production. J Biotechnol. 2009; 144(2):127-134.
11. Giarratana MC, Rouard H, Dumont A, et al. Proof of principle for transfusion of in vitro-generated red blood cells. Blood. 2011;118(19):5071-5079.
12. Kim HO, Baek EJ. Red blood cell engineering in stroma and serum/plasma-free conditions and long term storage. Tissue Eng. Part A. 2012;18(1-2):117-126.
13. Griffiths RE, Kupzig S, Cogan N, et al. Maturing reticulocytes internalize plasma membrane in glycophorin A-containing vesicles that fuse with autophagosomes before exocytosis. Blood. 2012; 119(26):6296-6306.
14. Griffiths RE, Kupzig S, Cogan N, et al. The ins and outs of human reticulocyte maturation: autophagy and the endosome/exosome pathway. Autophagy. 2012; 8(7):1150-1151.
15. Kobari L, Yates F, Oudrhiri N, et al. Human induced pluripotent stem cells can reach complete terminal maturation: in vivo and in vitro evidence in the erythropoietic differentiation model. Haematologica. 2012; 97(12):1795-1803.
16. Jin H, Kim HS, Kim S, Kim HO. Erythropoietic potential of CD34+ hematopoietic stem cells from human cord blood and G-CSF-mobilized peripheral blood. BioMed Res Int. 2014;2014:435215.
17. Rousseau GF, Giarratana MC, Douay L. Large-scale production of red blood cells from stem cells: what are the technical challenges ahead? Biotechnol J. 2014; 9(1):28-38.
18. Kim HO. In-vitro stem cell derived red blood cells for transfusion: are we there yet? Yonsei Med J. 2014;55(2):304-309.
19. Hirose S, Takayama N, Nakamura S, et al. Immortalization of erythroblasts by c-MYC and BCL-XL enables large-scale erythrocyte production from human pluripotent stem cells. Stem Cell Reports. 2013; 1(6):499-508.
20. Hu Z, Van Rooijen N, Yang Y-G. Macrophages prevent human red blood cell reconstitution in immunedeficient mice. Blood. 2011;118(22):9.
21. Diamanti P, Cox CV, Blair A. Comparison of childhood leukemia initiating cell populations in NOD/SCID and NSG mice. Leukemia. 2012;26(2):376-380.
22. Diamanti P, Cox CV, Moppett JP, Blair A. Parthenolide eliminates leukemia-initiating cell populations and improves survival in xenografts of childhood acute lymphoblastic leukemia. Blood. 2013;121(8):1384-1393.
23. Wiley JS, Shaller CC. Selective loss of calcium permeability on maturation of reticulocytes. J Clin Invest. 1977;59(6):1113-1119.
24. Waugh RE, McKenney JB, Bauserman RG, Brooks DM, Valeri CR, Snyder LM. Surface area and volume changes during maturation of reticulocytes in the circulation of the baboon. J Lab Clin Med. 1997;129(5):527-535.
25. Mankelow TJ, Griffiths RE, Trompeter S, et al. Autophagic vesicles on mature human reticulocytes explain phosphatidylserine-positive red cells in sickle cell disease. Blood. 2015;126(15):1831-1834.
26. Connor J, Pak CC, Schroit AJ. Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells. J Biol Chem. 1994;269(4):2399-2404.
27. Hod EA, Brittenham GM, Billote GB, et al. Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron. Blood. 2011; 118(25):6675-6682.
28. Luten M, Roerdinkholder-Stoelwinder B, Schaap NP, de Grip WJ, Bos HJ, Bosman GJ. Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods. Transfusion. 2008;48(7):1478-1485.
29. Douay L. In vitro generation of red blood cells for transfusion: a model for regenerative medicine. Regen. Med. 2014;7(1):2.
30. Hess JR, Sparrow RL, van der Meer PF, Acker JP, Cardigan RA, Devine DV. Red blood cell hemolysis during blood bank storage: using national quality management data to answer basic scientific questions. Transfusion. 2009;49(12):2599-2603.
31. Giani FC, Fiorini C, Wakabayashi A, et al. Targeted application of human genetic variation can improve red blood cell production from stem cells. Cell Stem Cell. 2016;18(1):73-78.
32. Doulatov S, Vo LT, Chou SS, et al. Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. Cell Stem Cell. 2013;13(4):459-470.
33. Huang X, Shah S, Wang J, et al. Extensive ex vivo expansion of functional human erythroid precursors established from umbilical cord blood cells by defined factors. Mol Ther. 2014;22(2):451-463.
34. Trakarnsanga K, Wilson M, Griffiths R, et al. The first human immortalised cell line generated from adult erythroid cells. Vox Sanguinis. 2015;109(Suppl. 1):197-197.