Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron

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Abstract

Transfusions of red blood cells stored for longer durations are associated with adverse effects in hospitalized patients. We prospectively studied 14 healthy human volunteers who donated standard leukoreduced, double red blood cell units. One unit was autologously transfused “fresh” (3-7 days of storage) and the other “older” unit was transfused after 40-42 days of storage. Of the routine laboratory parameters measured at defined times surrounding transfusion, significant differences between fresh and older transfusions were only observed in iron parameters and markers of extravascular hemolysis. Compared to fresh red blood cells, mean serum unconjugated bilirubin increased by 0.55 mg/dL at 4 hours after transfusion of older red blood cells \( (P=0.0002) \), without significant changes in haptoglobin or lactate dehydrogenase. In addition, only after the older transfusion, transferrin saturation increased progressively over 4 hours to a mean of 64\%, and non-transferrin-bound iron appeared, reaching a mean of 3.2 \( \mu \text{M} \). The increased concentrations of non-transferrin-bound iron correlated with enhanced proliferation \textit{in vitro} of a pathogenic strain of \textit{E. coli} \( (r=0.94, P=0.002) \). Therefore, circulating non-transferrin-bound iron derived from rapid clearance of transfused, older stored red blood cells may enhance transfusion-related complications, such as infection. The trial was registered with clinicaltrials.gov Identifier: NCT01319552.
Introduction

The safety of transfusing red blood cells after longer durations of refrigerated storage was recently identified as “the most critical issue facing transfusion medicine.”¹ Concern was heightened when a large observational study of cardiac surgery patients found an increased risk of postoperative complications and reduced survival in those who received red blood cells stored for more than 14 days.² Although still controversial, adverse clinical consequences have since been reported in most,³–⁶ though not all,⁵,⁷ epidemiological studies of transfusions of red blood cells stored for longer durations, but still within Food and Drug Administration (FDA) guidelines. The association between the duration of red blood cell storage and increased rates of serious infections, sepsis, and mortality is particularly strong in trauma patients.⁷–¹¹ Definitive determination of the potential risks associated with transfusion of red blood cells stored for longer durations has been elusive, in part because the mechanisms responsible have not yet been identified.

Over 14 million red blood cell units are transfused in the United States each year, with a mean storage interval of 18 days before transfusion.¹² During storage, red blood cells undergo cumulative biochemical and biomechanical changes (the “storage lesion”) that reduce their survival in vivo after transfusion.¹³,¹⁴ In mouse models,¹⁵ transfusion of red blood cells stored for longer durations was followed by brisk extravascular clearance of a subpopulation of these cells, which were damaged during storage and removed by macrophages in the spleen and liver of recipient mice. The iron liberated by phagocytic digestion of these red blood cells rapidly entered the systemic circulation in amounts that exceeded the transport capacity of plasma transferrin, the physiologic iron-binding protein; in this way, circulating non-transferrin-bound iron appeared and promoted the proliferation of pathogenic bacteria both in vitro¹⁵ and in vivo.¹⁶
We hypothesized that the infectious complications observed in human patients after transfusion of red blood cells stored for longer durations were, at least in part, due to the production of circulating non-transferrin-bound iron. Therefore, we prospectively examined healthy human volunteers to determine (i) if transfusion of autologous red blood cells stored for longer durations was followed by the appearance of circulating non-transferrin-bound iron in vivo, and (ii) if this increased circulating non-transferrin-bound iron was associated with enhanced pathogenic bacterial growth in vitro.

**Materials and Methods**

**Study design**

Fourteen consecutive healthy adult volunteers were prospectively studied. Each volunteer donated, by a standard automated apheresis method (Alyx, Baxter Healthcare), a leukoreduced, double red blood cell unit, which was stored in a standard additive solution (Adsol/AS-1, Baxter) in the Columbia University Medical Center – New York Presbyterian Hospital Blood Bank in compliance with FDA standards. Each volunteer was then transfused with one autologous red blood cell unit after 3-7 days of storage (i.e., “fresh”) and subsequently with the second unit after 40-42 days of storage (i.e., “older”). Timed blood samples were obtained 90 minutes before each transfusion and at 0-, 1-, 2-, 4-, 24-, and 72-hours after transfusion. To prevent post-transfusion erythrocytosis, and to maintain a parallel study design, a single unit whole blood phlebotomy was performed 3-7 days prior to the transfusion of the older red blood cells, if the volunteer’s hemoglobin was >13.3 g/dL (Figure 1A). In addition, both fresh and older transfusions were started at approximately the same time of day (11:00 am), took place over approximately two hours at a rate of 150 mL/hour, and the same lunch was provided for both transfusion episodes at 12:00 noon. All transfusions were performed at
Columbia University Medical Center – New York Presbyterian Hospital and the double red blood cell units were collected and processed at the New York Blood Center. Study recruitment began in December, 2008 and was completed by February, 2011. The research protocol was approved by the Institutional Review Boards of both institutions, was conducted according to the principles expressed in the Declaration of Helsinki, and all participants provided written informed consent. The trial was registered with clinicaltrials.gov Identifier: NCT01319552.

**Study participants**

The inclusion criteria were: healthy adults 18-65 years of age with male body weight >59 kg (130 lbs), female body weight >70kg (155 lbs), male height >1.55 m (5’1”), female height >1.65 m (5’5”), and hemoglobin >13.3 g/dL. Exclusion criteria were: ineligibility for donation based on the New York Blood Center autologous blood donor questionnaire, systolic blood pressure >180 or <90 mm Hg, diastolic blood pressure >100 or <50 mm Hg, heart rate <50 or >100, temperature >37.5°C prior to donation, temperature >38°C or subjective feeling of illness prior to transfusion, positive results on standard blood donor infectious disease testing, and pregnancy. All screened volunteers who met the inclusion criteria, and did not meet any of the exclusion criteria, were enrolled in the study.

**Laboratory measurements**

All laboratory testing for routine clinical parameters was performed in the Columbia University Medical Center – New York Presbyterian Hospital Clinical Laboratories. Non-transferrin-bound iron was measured using an ultrafiltration assay, as described,¹⁵ and was performed in the Iron Reference Laboratory at the Columbia University Medical Center. The reference range for plasma non-transferrin-bound iron in our laboratory is
-0.71 to 0.10 μM; data are presented as a change in non-transferrin-bound iron from pre-transfusion levels. To eliminate interassay variability biasing the change in non-transferrin-bound iron levels, all the samples for a given volunteer were frozen at -80°C and were analyzed together following the final time-point of study participation. Interleukin (IL)-6 was measured with a high sensitivity ELISA kit (R&D Systems) following the manufacturer’s instructions.

**Bacterial proliferation in vitro**

Proliferation of a pathogenic strain of *E. coli*, obtained from an anonymous patient with a urinary tract infection, was measured after inoculating all serum samples obtained from study participants, both before and after transfusion, as described. Briefly, 100 μL aliquots of serum in microtiter plate wells were incubated with 1x10⁶ colony forming units of *E. coli* at 37°C with shaking. Optical density at 600 nm was measured periodically up to 5 hours after inoculation using a PowerWave XS microtiter plate reader (BioTek) and the area under the curve of the resultant growth curve was calculated using Prism 5 (GraphPad Software, Inc.). All samples were inoculated in duplicate and the mean of the two growth curves was used.

**Statistical analysis**

Differences in outcome measures after fresh or older red blood cell transfusions were compared using a paired t-test or a Wilcoxon matched pairs test, as appropriate, to compare the area under the curve of the increase in the outcome measure from 0- to 24-hours after transfusion. Normality of data was assessed using a D’Agostino and Pearson omnibus normality test. A *P* value of less than 0.05 was considered significant. Statistical
analyses were performed using Prism 5. All data are presented as mean ± SEM, unless otherwise specified.

The study was originally powered for 11 participants, a sample size appropriate for detecting 15% of the non-transferrin-bound iron difference seen in mice, assuming the same standard deviations as observed in the mouse model. Recruitment was increased to 14 volunteers to include additional female participants, who were underrepresented in the first 11 volunteers. Exclusion of these additional female volunteers from the analysis did not significantly change the results.

Results

Subject characteristics

Of 42 consecutive adults screened, 14 qualified for participation and all 14 volunteers completed the study with no dropout (study design shown in Figure 1A). There were no transfusion reactions and no significant changes in vital signs (blood pressure, heart rate, temperature) throughout the study. Two volunteers experienced lightheadedness during or immediately after donating blood and one volunteer, who suffers from chronic migraines, experienced a headache and vomited 2 hours after transfusion of fresh red blood cells; the latter event was considered to be unrelated to study participation by both the study participant and the Data Safety Monitoring Board. Because of lower baseline hemoglobin levels (Table 1), only one of the four female participants met the hemoglobin criterion (≥13.3 g/dL) for the one unit whole blood phlebotomy 3-7 days before the older red blood cell transfusion. Exclusion of the three non-phlebotomized female participants from the analyses did not significantly change the results; thus, they were included in the analysis. All male participants met the hemoglobin criterion and were phlebotomized one whole blood unit before the older red blood cell transfusion.
Effect of storage duration on complete blood counts

The mean pre-transfusion hemoglobin (i.e. after donating the red blood cell units, but 90 minutes before transfusion) was 12.7 and 13.3 g/dL, for the fresh and older red blood cell transfusions, respectively ($P = 0.07$ by paired t-test). Given the volume of blood drawn for the timed samples, the hemoglobin was expected to increase by ~0.8 g/dL following transfusion. At 4-hr post-transfusion, the mean hemoglobin increased by only 0.22 g/dL and 0.34 g/dL for the fresh and older transfusions, respectively. However, by 24-hr post-transfusion, the mean hemoglobin increased by 0.82 and 0.89 g/dL for the fresh and older transfusions, respectively ($P=0.5442$; Figure 1B-C). No significant differences between fresh and older transfusions were observed for white blood cell, absolute neutrophil, or platelet counts (supplemental Figure 1).

Effect of storage duration on basic metabolic parameters

There were no significant differences between fresh and older red blood cell transfusions in basic metabolic parameters (i.e. sodium, potassium, chloride, blood urea nitrogen, creatinine, glucose, and total calcium; Figure 2 and supplemental Figure 2). In particular, increased potassium levels were not observed following the older red blood cell transfusions (Figure 2A). However, both types of transfusions were associated with a progressive decrease in total calcium up to 4 hours after transfusion (Figure 2B), which was still evident following older red blood cell transfusions when the calcium levels were corrected for serum albumin levels (Figure 2C); ionized calcium was not measured.

Transfusion of older red blood cells results in extravascular hemolysis

As compared to transfusions of fresh red blood cells, transfusions of older red blood cells were associated with significantly increased serum unconjugated bilirubin
(\(P=0.0002\); Figure 3A), with a mean peak increase in unconjugated bilirubin of 0.55 mg/dL at 4 hours after transfusion. In addition, unconjugated bilirubin peaked above the reference range in 3 of 14 volunteers after transfusion of older red blood cells (Figure 3B). Although the bilirubin was predominantly unconjugated, there was a small, but significant, rise in serum conjugated bilirubin (Figure 3C). No statistically significant differences between fresh and older red blood cell transfusions were observed in mean serum haptoglobin and lactate dehydrogenase levels, which are indicators of intravascular hemolysis (Figure 3D). There was a significant difference between transfusions of fresh and older red blood cells in alanine aminotransferase levels (supplemental Figure 3; \(P=0.01\)) although the difference was small (a 1.7 U/L increase 4 hours following older transfusions as compared to fresh transfusions). There were no significant differences between fresh and older red blood cell transfusions in the other tested liver function parameters (i.e. aspartate aminotransferase, alkaline phosphatase, total protein, and albumin; supplemental Figure 3).

**Transfusions of older red blood cells increase iron parameters and produce circulating non-transferrin-bound iron**

Although transfusions of fresh red blood cells produced no significant change in mean serum iron or transferrin saturation, transfusions of older red blood cells led to significant increases in serum iron (\(P=0.001\)) and transferrin saturation (\(P=0.0005\)), with a mean increase of 162 \(\mu\)g/dL and 42% over baseline, respectively, at 4 hours after transfusion (Figure 4A,C). In particular, serum iron and transferrin saturation peaked above the reference range in 13 of 14 volunteers after transfusion of older red blood cells (Figure 4B,D). In addition, ferritin levels increased from the baseline pre-transfusion sample only after transfusion of older red blood cells, peaking at 15.5 ng/mL above baseline at 24 hours after transfusion (Figure 4E). Furthermore, after the fresh red blood cell
transfusions, no significant increases in circulating non-transferrin-bound iron concentration were observed. In contrast, 13 of 14 volunteers had progressively increasing circulating non-transferrin-bound iron between 1 to 4 hours after transfusion of older red blood cells reaching a mean of 3.2 µM (P=0.002) over baseline at 4 hours post-transfusion (Figure 4F).

**Effect of red blood cell storage duration on inflammation**

Prior studies in mice demonstrated increases in various markers of inflammation after transfusions of older red blood cells.15 However, in the current human study, there were no significant differences in IL-6 or C-reactive protein (CRP) levels between the groups receiving fresh and older red blood cell transfusions (Figure 5A,B). Nonetheless, one volunteer (number 14), had a CRP rise above the reference range between 4 and 72 hours after transfusion of only the fresh red blood cells and one volunteer (number 6), who had an elevated CRP level prior to the older red blood cell transfusion, manifested a progressive rise in CRP peaking 4 hours after transfusion of only the older red blood cells (Figure 5C). Interestingly, in a post-hoc analysis, this volunteer was African-American with a Duffy-negative red blood cell antigen phenotype. Because the Duffy antigen is a chemokine receptor rarely absent on red blood cells of individuals of non-African descent, none of the other volunteers’ red blood cells were tested for the Duffy phenotype.

**Transfusions of older red blood cells enhance bacterial growth in vitro**

A pathogenic strain of *E. coli* obtained from an anonymous patient with a urinary tract infection was inoculated into all serum samples from all volunteers at all time points surrounding each transfusion. At 2 to 4 hours after transfusion of older (P=0.03, Figure 6A), but not fresh, red blood cells, the growth of *E. coli* was enhanced in these serum
samples. The mean difference in the area under the growth curve between fresh and older red blood cell transfusions correlated with the mean change in non-transferrin-bound iron ($P=0.002$, Pearson $r=0.94$; Figure 6B). Prior studies confirmed the iron-dependent growth of this bacterial isolate.  

**Discussion**

These results provide evidence of physiological differences in the consequences of transfusions of red blood cells after shorter (3-7 days) or longer (40-42 days) durations of storage, despite strict adherence to current FDA standards. Transfusions of fresh red blood cells to 14 healthy volunteers produced no detected laboratory evidence of hemolysis and did not significantly alter serum iron, transferrin saturation, or circulating non-transferrin-bound iron. In contrast, despite appropriate increases in hemoglobin level, transfusions of older red blood cells led to increased mean serum unconjugated bilirubin levels with no significant changes in mean serum haptoglobin or lactate dehydrogenase levels, a pattern consistent with rapid extravascular hemolysis of a subpopulation of the transfused older red blood cells. Importantly, during the initial 4 hours after transfusion of older red blood cells, serum iron and transferrin saturation increased significantly and circulating non-transferrin-bound iron appeared. These changes returned to baseline by 24 hours after transfusion. The potential pathogenic import of these differences was shown using a bacterial growth assay with these serum samples: increased proliferation *in vitro* of a pathogenic strain of *E. coli* correlated with increased concentrations of non-transferrin-bound iron ($r=0.94$, $P=0.002$). Although no untoward clinical events occurred in these healthy volunteer recipients of older transfused red blood cells, the potential for adverse infectious outcomes is conceivable, particularly for patients after cardiac surgery or trauma, who have open entry points for bacterial invasion and may be rapidly transfused with multiple units of red blood cells of varying
storage duration. However, further studies are necessary to extend these findings in serum \textit{in vitro} to the clinical setting \textit{in vivo}.

As red blood cells age, while circulating \textit{in vivo} or stored \textit{in vitro}, they undergo changes that eventually lead to their recognition as senescent or damaged, and to their removal by macrophages in the spleen, bone marrow, and liver.\textsuperscript{15,17} In a typical healthy adult, approximately 1 mL of red blood cells reach the end of their life span and are cleared each hour, yielding about 1 mg of iron. This iron is either stored intracellularly or returned to the plasma to be bound by transferrin and transported to the erythroid marrow and other tissues for re-use. By current FDA standards, a unit of stored red blood cells is acceptable for transfusion even if 25% of the red cells are cleared within 24 hours, an amount equivalent to as much as 60 mg of iron. Because most of this clearance takes place during the first hour after transfusion,\textsuperscript{18} the rate of delivery of heme-iron to reticuloendothelial macrophages may abruptly increase by as much as 60-fold after transfusion of even a single unit of packed red blood cells. The corresponding accelerated rate of return of iron to plasma can surpass the rate of uptake by transferrin and produce circulating non-transferrin-bound iron.

In the current study, the kinetics of the appearance in the circulation of red blood cell degradation products (e.g. bilirubin) demonstrate the rapid extravascular clearance of a sub-population of the older transfused red blood cells. Thus, although the mean hemoglobin increment following transfusion did not significantly differ between fresh and older red blood cell transfusions, the serum unconjugated bilirubin and transferrin saturation levels increased rapidly and in parallel during the initial 4 hours after transfusion of older stored red blood cells (Figures 3 and 4). Despite the continued presence of unsaturated transferrin, circulating non-transferrin-bound iron appeared,
probably because the rate of iron influx into plasma overwhelmed the rate of iron acquisition by plasma transferrin.19 Because plasma non-transferrin-bound iron, a heterogeneous assortment of iron complexes,20,21 is available to pathogens reaching the blood stream and can enhance their growth,22,23 we examined proliferation in vitro of a pathogenic strain of E. coli. As shown in Figure 6A, serum samples obtained after transfusion of older red blood cells significantly enhanced bacterial growth. Furthermore, as shown in Figure 6B, increased bacterial growth in serum obtained after transfusion of older, as compared to fresher, red blood cells closely correlated with the corresponding increases in circulating non-transferrin-bound iron. Because bacteria use various mechanisms for procuring iron,24 the contribution of non-transferrin-bound iron to virulence is expected to vary among organisms. Nonetheless, withholding iron from pathogens is a central component of host defense,23 and our results in vitro illustrate the capacity of circulating non-transferrin-bound iron to enhance infection. Interestingly, patients with transfusional iron overload, as well as those with hereditary forms of hemochromatosis, may have circulating non-transferrin-bound iron levels25,26 similar to those measured in our healthy volunteers at 4 hours after transfusion with older red blood cells and are known to be at an increased risk for acute and chronic infections with specific pathogens.27-29 In addition, oral iron supplements are associated with transient increases in non-transferrin-bound iron19,30 and routine supplementation with iron and folic acid in children in a malaria-endemic region increased the risk of severe illness and death.31,32

An incidental finding from this study is the extent of variability in hemoglobin levels measured soon after transfusion (Figure 1B,C), with 6/14 volunteers exhibiting either a decrease or no increase in hemoglobin at 4-hours after transfusion of fresh autologous red blood cells. Although this finding supports classic textbook teaching33 that it requires
up to 24 hours for hemoglobin levels to equilibrate, more recent studies suggest that
hemoglobin levels quickly equilibrate after transfusion in adult\textsuperscript{34} and neonatal\textsuperscript{35} patients. Therefore, our findings suggest that hemoglobin measurements may not be ideal for
assessing the effectiveness of red blood cell transfusions until 24 hours after transfusion,
although this is assertion is limited by an absence of measurements between 4 and 24
hours after transfusion. Decreases in serum albumin and total protein levels following
transfusion of both fresh and older red blood cells (supplemental Figure 3) suggest that
there are significant volume shifts from the extravascular to the intravascular space
following transfusion, which may help explain the observed variability in hemoglobin
levels.

Contrary to the results in mouse studies\textsuperscript{15}, no difference in pro-inflammatory IL-6 levels
were observed in healthy human volunteers following transfusion of fresh or older red
blood cells. Although mice may handle transfusion-induced iron loads differently than
humans, the red blood cell dose transfused into humans may also have been too small
and may have been given too slowly to elicit a pro-inflammatory cytokine response. For
example, in the mouse studies, the rapid infusion (i.e. “IV push”) of two red blood cell
units elicited a robust pro-inflammatory cytokine response; in contrast, the human
volunteers were transfused with only one red blood cell unit over a 2 hour time period.

Several limitations of this study should be taken into consideration. For example, only
one unit was transfused over two hours per transfusion event; therefore, these results
likely underestimate the effect on markers of hemolysis following transfusion in
hospitalized patients who frequently receive multiple units at faster infusion rates. In
addition, no blood samples were drawn between 4 and 24 hours after transfusion;
therefore, the actual time intervals during which the iron parameters and markers of
hemolysis remain elevated following transfusion of older red cells are unknown. Still, these parameters predominantly return to baseline by 24 hours after transfusion, thereby indicating a relatively transient effect. Further studies are necessary to determine whether these transient effects are significant enough to affect the clinical course of transfused patients. Finally, there is an inherent time bias in the study design in that the older red blood cells were always transfused ~35 days after the fresh blood transfusions.

These studies in healthy human volunteers, and our related investigations in mice, demonstrate that increased transferrin saturation leading to production of circulating non-transferrin-bound iron after transfusion of older red blood cells is a potential mechanism for enhancing infectious complications in recipients. The concentrations of non-transferrin-bound iron observed after slow transfusion of a single unit of autologous red blood cells in healthy human volunteers may be considerably lower than those found after rapid transfusion of multiple sequential units of allogeneic red blood cells to severely ill trauma and surgical patients. Circulating non-transferrin-bound iron can also produce oxidative damage, thrombosis, cytotoxicity, and other types of injury, and may contribute to additional mechanisms of increased morbidity and mortality after transfusions of older red blood cells. Finally, other proposed mechanisms (e.g. involving nitric oxide and/or microvesicles) may contribute to the increased morbidity and mortality that may result from transfusions of older red blood cells. In conclusion, the physiological differences described here in human volunteers after transfusion of fresh or older stored red blood cells suggest that studies of novel blood storage systems, which improve post-transfusion red blood cell recovery, are warranted and that prospective clinical trials should determine whether transfusions of older red blood cells enhance infectious and other disease risks in patients.
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Authorship contributions


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References


Table 1. Baseline Characteristics of Volunteers

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* Race/ethnicity was assessed by the investigators
**Figure Legends**

**Figure 1. Study design and hemoglobin levels.** *(A)* On Day #1, each human volunteer donated two autologous red blood cell units by apheresis. One unit was transfused into the same participant after 3-7 days of storage (i.e. “fresh”); the other unit was transfused after 40 -42 days of storage (i.e. “older”). One whole blood phlebotomy was performed 3-7 days prior to the older red blood cell transfusion to prevent post-transfusion erythrocytosis and to control for effects of recent blood loss on laboratory parameters. Blood samples were collected 90 minutes pre-transfusion and at 0-, 1-, 2-, 4-, 24-, and 72-hours post-transfusion. *(B)* Mean ± SEM for hemoglobin levels from pre-transfusion to 72-hours after transfusion of either fresh or older red blood cells. The \( P \) value is as specified in the figure comparing the paired area under the curve of the mean hemoglobin levels for the N=14 volunteers from 0- to 24-hours after the fresh and older red blood cell transfusions. *(C)* The individual hemoglobin levels for each subject up to 24-hours post-transfusion. Vertical arrows denote pre-transfusion time points and horizontal dashed lines represent reference range values for men (blue) and women (pink).

**Figure 2. Potassium levels do not change and calcium levels decrease after transfusions of older red blood cells.** The mean ± SEM for serum levels of *(A)* potassium, *(B)* total calcium, and *(C)* corrected calcium calculated as \( ((0.8 \times (4.0 – \text{subject’s albumin})) + \text{serum calcium}) \). The vertical arrow denotes the pre-transfusion time point and dotted lines represent the reference ranges. The \( P \) values are as specified in the figure comparing the paired area under the curve of the mean of the outcome parameter for the N=14 volunteers from 0- to 24-hours after the fresh and older red blood cell transfusions.
Figure 3. Transfusions of older red blood cells result in laboratory values consistent with extravascular hemolysis in healthy volunteers. Mean ± SEM for serum levels of (A) total bilirubin and (B) conjugated bilirubin from pre-transfusion to 72-hours after transfusion of both fresh and older red blood cells. (C) The individual serum total bilirubin levels for all 14 volunteers from pre-transfusion to 72-hours after transfusion of both fresh and older red blood cells. (D) Mean ± SEM for lactate dehydrogenase (LDH) and haptoglobin, from pre-transfusion to 72-hours after transfusion of both fresh and older red blood cells. Although iatrogenic hemolysis was induced during a difficult blood draw for two volunteers at 1-hour after the older red blood cell transfusion, these samples were still included in the analysis; nonetheless, the analysis was not significantly altered by their exclusion. The vertical arrows in all panels denote the pre-transfusion time point and dotted lines represent the reference ranges (and in gray for LDH). The $P$ values are as specified in the figure comparing the paired area under the curve of the mean of the outcome parameter for the N=14 volunteers from 0- to 24-hours after the fresh and older transfusions.

Figure 4. Iron parameters and circulating non-transferrin-bound iron levels increase after transfusions of older red blood cells in healthy volunteers. (A) The mean ± SEM and (B) individual levels of serum iron; (C) mean ± SEM and (D) individual levels of transferrin saturation; (E) increase in ferritin as compared to baseline levels and (F) increase in plasma non-transferrin-bound iron as compared to baseline levels from pre-transfusion to 72-hours after transfusion of fresh and older red blood cells. Vertical arrows denote pre-transfusion time points and dotted lines represent the reference range (the reference range for change in ferritin and non-transferrin-bound iron is 0 by
definition). The $P$ values are as specified in the figure comparing the paired area under the curve of the mean of the outcome parameter for the N=14 volunteers from 0- to 24-hours after the fresh and older red blood cell transfusions.

**Figure 5. Serum levels of inflammatory markers do not increase after transfusions of older red blood cells as compared to fresh red blood cells in healthy volunteers.** (A) The mean ± SEM for serum interleukin (IL)-6 levels, (B) C-reactive protein (CRP) levels, and (C) individual levels of CRP from pre-transfusion to 72-hours after transfusion of fresh and older red blood cells. Vertical arrows denote pre-transfusion time points and dotted lines represent the reference range. The $P$ values are as specified in the figure comparing the paired area under the curve of the mean of the outcome parameter for the N=14 volunteers (for IL-6) and N=12 (for CRP; the first two volunteers were not tested due to inadequate sample volume) from 0- to 24-hours after the fresh and older red blood cell transfusions.

**Figure 6. Sera obtained after transfusions of older red blood cells enhance proliferation of a bacterial pathogen *in vitro*.** (A) Bacterial growth of *E. coli* in serum samples obtained following fresh or older red blood cell transfusions was determined by serial optical density measurements at 600 nm for up to 5 hours after inoculation. Each point in the graph represents the mean ± SEM of the area under the curve (AUC) of the resultant bacterial growth curve (N=14 paired values). (B) A Pearson correlation was used to determine the relationship between the mean difference in bacterial growth between fresh and older red blood cell transfusions at each time point and the corresponding differences in plasma non-transferrin-bound iron levels. The $P$ values are as specified in the figure.
Figure 1

A

2x RBC donation (apheresis)

Day #1

Blood draws

Day #0.7

Fresh transfusion

1x RBC donation

Blood draws

Day #0.42

Older transfusion

B

C

Pre-transfusion

Time post-transfusion (hr)

Hemoglobin (g/dL)

P=0.54 [ Older Fresh

Time post-transfusion (hr)

Hemoglobin (g/dL)

1

2

3

4

5

6

7

8

9

10

11

12

13

14
Figure 2

A

Potassium (mM)

Pre-transfusion
Time post-transfusion (hr)

5.5
5.0
4.5
4.0
3.5
3.0

 Older
 Fresh

P=0.91

B

Calcium (mg/dL)

Pre-transfusion
Time post-transfusion (hr)

10.5
10.0
9.5
9.0
8.5
8.0
7.5

 Older
 Fresh

P=0.65

C

Corrected Calcium (mg/dL)

Pre-transfusion
Time post-transfusion (hr)

9.0
8.5
8.0
7.5
7.0

 Older
 Fresh

P=0.28
Figure 3
Figure 4
Figure 5

A

IL-6 (pg/mL)

Pre-transfusion

Time post-transfusion (hr)

P=0.27

Older

Fresh

B

C-reactive protein (mg/L)

Pre-transfusion

Time post-transfusion (hr)

P=0.90

Older

Fresh

C

C-reactive protein (mg/L)

Time post-transfusion (hr)

31
Figure 6

A

Bacterial growth (AUC)

Pre-transfusion

Time post-transfusion (hr)

P = 0.03

Older

Fresh

B

ΔBacterial growth (Older-Fresh; AUC)

ΔNon-transferrin-bound iron (Older-Fresh; µM)

P = 0.002

r = 0.94
Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron