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Effects of blood storage age on immune, coagulation, and nitric oxide parameters in transfused patients undergoing cardiac surgery

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Abstract

BACKGROUND: Retrospective studies suggested that storage age of RBCs is associated with inflammation and thromboembolism. The Red Cell Storage Duration Study (RECESS) trial randomized subjects undergoing complex cardiac surgery to receive RBCs stored for shorter versus longer periods, and no difference was seen in the primary outcome of change in multiple organ dysfunction score.

STUDY DESIGN AND METHODS: In the current study, 90 subjects from the RECESS trial were studied intensively using a range of hemostasis, immunologic, and nitric oxide parameters. Samples were collected before transfusion and on Days 2, 6, 28, and 180 after transfusion.

RESULTS: Of 71 parameters tested, only 4 showed a significant difference after transfusion between study arms: CD8+ T-cell interferon-γ secretion and the concentration of extracellular vesicles bearing the B-cell marker CD19 were higher, and plasma endothelial growth factor levels were lower in recipients of fresh versus aged RBCs. Plasma interleukin-6 was higher at Day 2 and

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

lower at Days 6 and 28 in recipients of fresh versus aged RBCs. Multiple parameters showed significant modulation after surgery and transfusion. Most analytes that changed after surgery did not differ based on transfusion status. Several extracellular vesicle markers, including two associated with platelets (CD41a and CD62P), decreased in transfused patients more than in those who underwent surgery without transfusion.

CONCLUSIONS: Transfusion of fresh versus aged RBCs does not result in substantial changes in hemostasis, immune, or nitric oxide parameters. It is possible that transfusion modulates the level of platelet-derived extracellular vesicles, which will require study of patients randomly assigned to receipt of transfusion to define.

Several large observational studies have associated transfusion of older RBCs in cardiac surgical patients with adverse inflammatory and thromboembolic events.[1–3] Several possible adverse effects of transfusion of stored RBCs have been hypothesized based on in vitro studies. One proposed mechanism for adverse outcomes after receipt of stored blood is that blood becomes more procoagulant as it ages due to increasing phosphatidylserine exposure on the erythrocyte membrane,[4,5] and the production of various thrombin-generating extracellular vesicles (EVs).[6,7] While in vitro studies have shown RBC EVs to promote thrombin generation in the absence of tissue factor,[8] it is unclear what clinical effect they may have in transfused patients. One of the primary aims of the current study was to determine if patients transfused RBCs stored for 21 days or more showed differences in postoperative hemostatic parameters or systemic inflammation compared to patients transfused RBCs stored for 10 days or less.

The principal RBC function is O_2 delivery, a product of blood O_2 content and flow. Altered O_2 loading/unloading by stored RBCs is well documented;[9–11] however, less is known of how storage impacts RBC function in their role governing O_2-dependent regulation of blood flow (“hypoxic vasodilation”), in part because the role of RBCs in vascular signaling is only recently appreciated.[12–15] Specifically, it is now established that RBCs mediate nitric oxide (NO)-based hypoxic vasodilatory activity.[16,17] Recently, it has been observed that levels of the *S*-nitrosylated derivative of hemoglobin are altered in several disease states characterized by disorders of tissue oxygenation.[18–25] Where examined, RBCs from these patients exhibit impaired vasodilatory capacity.[16,20,22,24] Moreover, it has been demonstrated that, in blood that has been collected, processed, and stored, RBC *S*-nitrosylated derivative of hemoglobin levels and RBC-dependent vasodilation are profoundly depressed,[9,26,27] providing mechanistic insight to the observation of impaired vasoregulation associated with administration of banked blood.[9,28–31]

The randomized, controlled Red Cell Storage Duration Study (RECESS) trial was performed to examine the effect of RBC storage on outcome in cardiac surgery patients.[32] Patients were randomized to receive RBCs stored for 10 days or less (fresh, median of 7 days) versus 21 days or more (aged, median of 28 days). To examine if RBC storage age affects immune and coagulation function and NO flux, we performed an ancillary study of 90 subjects recruited within the RECESS trial. Our hypotheses were that patients transfused with RBCs in the aged RBC arm would have increased inflammation and a hypercoagulable profile, as well as decreased RBC NO content compared to recipients of fresh RBCs.

MATERIALS AND METHODS

Study participants

Participants in the RECESS trial were enrolled in longitudinal sampling of blood for this ancillary study from nine RECESS clinical sites (see Acknowledgments).[32] Blood was collected before surgery and before any RBC transfusion (Day 0) and on Days 2, 6, 28, and 180 after transfusion for transfused subjects and on Days 0 and 2 for nontransfused subjects. The time windows for the Day 2, Day 6, Day 28, and Day 180 samples were 0.5 to 3.5 days, 4.5 to 7.5 days, 21 to 35 days, and 166 to 194 days after the end of surgery, respectively. Informed consent was obtained for all study participants under an institutional review board–approved protocol.

Sample processing

On Days 0, 2, 6, 28 and 180, blood (7 mL) was collected in ethylenediaminetetracetic acid and shipped at ambient temperature overnight to Vitalant Research Institute for Ficoll separation and processing into peripheral blood mononuclear cells (PBMCs) and platelet-rich plasma, which were then cryopreserved in the vapor phase of liquid nitrogen or at −80°C, respectively. The PBMC samples were used for cellular function assays, and the processing protocol is consistent with prior published work demonstrating retained function of cells cryopreserved after delayed processing.[33] In addition, on Days 0, 2, 6, and 28, one 4.5-mL and one 2.7-mL citrate tube were collected and processed at each clinical site for coagulation, cytokine, and EV testing. Samples were centrifuged at 2500× g for 20 minutes at 20°C to obtain platelet-poor plasma, aliquoted into three individual cryovials, and stored at −80°C. For NO testing, blood samples were drawn on Days 0, 2, 6, and 28 into two 4-mL ethylenediaminetetracetic acid tubes and placed on ice. On-site sample processing included separation of packed RBCs from plasma and the buffy coat, aliquoting, mixing, and equilibrating packed RBCs with buffered glycerol (40% v/v), prior to freezing/storage (−80°C).

Coagulation testing and factor-level determination

Plasma samples were thawed at 37°C for 5 minutes and used immediately. The following tests and level measurements were performed at Emory: prothrombin time (PT); activated partial thromboplastin time (aPTT); prothrombin fragment 1.2 (F1.2); D-dimer; factor V, VII, VIII, and XI levels; clot-based protein C; fibrin monomer (FM); plasminogen activator inhibitor-1 (PAI-1); soluble endothelial protein C receptor (sEPCR), free tissue factor pathway inhibitor (TFPI); tissue plasminogen activator (TPA); thrombomodulin, and antithrombin (ATIII). An automated benchtop analyzer (STA Compact Max, Stago) and reagent kits (Diagnostica Stago) were used to measure PT; partial thromboplastin time (PTT); protein C; ATIII; FM; and factor V, VII, VIII, and XI levels. Factors V and VII were analyzed using one-stage clotting assay based on PT. Factors VIII and XI were similarly analyzed based on PTT. The following measurements were performed using enzyme-linked immunosorbent assays: D-dimer, TPA, PAI-1, free TFPI, and sEPCR (Diagnostica Stago), F1.2 (Siemens Healthcare Diagnostics), and soluble thrombomodulin (R&D Systems). Enzyme-linked immunosorbent assay testing was done according to the manufacturers’

directions, and readouts were performed on a microplate reader (Spectramax 340PC, Molecular Devices).

Thrombin generation testing

Calibrated automated thrombin generation was performed as described by Hemker.[34] Briefly, a 96-well microplate format and the calibrated automated thrombin generation assay (Thrombinoscope, Diagnostica Stago) were used to measure thrombin generation in plasma based on thrombin's hydrolysis of a fluorogenic substrate (Z-GLY-GLY-Arg-AMC). All samples were run in duplicate and in parallel with calibrator wells.

The following parameters were measured: lag time (LT; min) = time needed for initial thrombin generation, (i.e., 10 nM); peak thrombin generation (nM), endogenous thrombin potential (ETP; nM* min) = amount of thrombin that can be generated after in vitro activation of coagulation; and time to thrombin peak (TTP, min).

Regulatory T-cell and effector T-cell quantification

For regulatory T cells (Tregs), PBMCs were stained with cell stain (LIVE/DEAD Fixable Aqua Dead Cell Stain, Thermo-Fisher), washed, and then stained with CD4-A700 and CD25-PE-Cy7 (BD Biosciences). Cells were washed, fixed, and permeabilized using a staining kit (FoxP3 Fix/Perm Kit, eBioscience) and intracellularly stained with CD3 antibody (CD3-Pacific Blue, BD Biosciences) and an antihuman staining set (FoxP3-FITC, eBioscience). To measure production of interleukin (IL)-17A by CD4+ T cells and interferon (IFN)-γ by CD8+ T cells, 10^6 PBMCs were rested overnight and then stimulated with phorbol 12-myristate 13-acetate (PMA; 75 ng/mL) and ionomycin (1 μg/mL, Sigma-Aldrich) and incubated for 1 hour before addition of protein transport inhibitor (GolgiStop, BD Biosciences) and brefeldin A (1 μg/mL, Sigma-Aldrich) for an additional 5-hour incubation. Cells were washed and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain. Cells were then washed and stained with CD4-A700 and CD8-APC/H7 (BD Biosciences). Cells were washed, fixed, and permeabilized using a fixation/permeabilization kit (BD Cytofix/Cytoperm Kits, BD Biosciences), then stained with IFN-γ-PE-Cy7, CD3-Pacific Blue, and IL-17-A647 (eBioscience). For proliferation analysis, 10^6 PBMCs were stained with 1 μM carboxyfluorescein succinimidyl ester, then washed three times, stimulated with anti-CD3 and anti-CD28 antibodies (1 μg/mL, BD Biosciences) and incubated for 4 days. Cells were washed and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain, then washed and stained with CD3-Pacific Blue, CD4-A700, and CD8-APC/H7 antibodies. A minimum of 200,000 events were recorded for each sample on a flow cytometer (BD LSR II, BD Biosciences). Data were analyzed using computer software (FlowJo, Treestar).

Characterization of EVs and cytokines

To characterize EVs, platelet-free plasma samples were stained and acquired using 14 different fluorochrome-conjugated antibodies in three separate panels, including CD235a-FITC, CD62p-APC, CD3-PerCP/Cy5.5, CD19-A700, CD28-FITC, CD62L-APC, CD11b-PE/Cy7, CD154-APC, CD41a-PerCP/Cy5.5, CD66b-PE (Biolegend), CD15-FITC (ExAlpha), CD108a-PE, CD16-V450, and CD142-PE (BD Biosciences). Samples were washed with a 0.22-μm centrifugal filter (Millipore) at 850× *g* for 3 minutes. EVs were then

harvested from the top of the filter after washing, and data were acquired on a flow cytometer ((BD LSR II, BD Biosciences). In addition, 27 cytokines were measured using human cytokine chemokine kits (Multiplex MAG kits, Millipore): granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-10, IL-12(p70), IL-17A, IL-1β, IL-2, IL-21, IL-23, IL-6, IL-7, IL-8, CXCL11/ITAC, CCL3/MIP-1α, CCL4/MIP-1β, tumor necrosis factor-α, CXCL10/IP-10, epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), β2 microglobulin, cystatin C, myeloperoxidase, platelet-derived growth factor-AB/BB, CCL5/RANTES, soluble intercellular adhesion molecule-1, and soluble vascular cell adhesion molecule-1. A suspension array instrument (Bio-Plex 200, Bio-Rad) was used for cytokine data acquisition.

NO measurements

Hemoglobin (Hb)-adducted NO was determined in an NO analysis platform composed of a photolysis coil (TriE Medical) coupled to an NO Analyzer (TEA 810, Ellutia). All Hb-bound NO (to thiol and iron) is released upon ultraviolet light exposure, followed by chemiluminescent detection of NO after reaction with O_3 (as we have described and reviewed).[16,17,35–37] Since S-nitrosothiols are selectively eliminated by $HgCl_2$, assay ± $HgCl_2$ yields total [HbNO] or the remaining nitrosyl (Hb(Fe [II])NO) fraction. The S-nitrosothiol fraction is derived from the loss of signal following $HgCl_2$ addition. Previously frozen samples were passively thawed (room temperature) while protected from light, then freed of any remaining WBCs and platelets by passing through a column of α-cellulose and microcrystalline cellulose (1:1, w/w) that had been previously washed with 10 mM phosphate-buffered saline. Eluted RBCs were washed three times in five volumes of phosphate-buffered saline (10 mM, pH 7.4, 0.5 mM ethylenediaminetetraacetic acid), repelleted, and lysed with 0.1 mM diethylenetriaminepentaacetic acid (in deionized H_2O, final pH, 6.92) and the Hb partially purified by passing the lysate over a G-25 Sephadex column). Paired Hb samples were treated with phosphate-buffered saline ± $HgCl_2$ (0.6 mM final) and injected into the PC device. Hb-bound NO was determined by extrapolation against a standard curve generated daily by using *S*-nitrosoglutathione (8–250 nM).

Statistical analyses

Clinical characteristics were compared between subjects using Fisher's exact test for categorical variables and the Kruskal-Wallis test for continuous variables. To ascertain the effect of treatment arm (fresh vs. aged transfusion) and time after surgery and transfusion, a repeated-measures linear regression model was fit for each outcome, controlling for treatment arm, study day (as a categorical variable), the interaction between treatment arm and day (if significant), and baseline value. For terms for which interaction between treatment arm and time was significant, treatment effect was analyzed separately at each time point. The models assumed an autoregressive AR(1) variance structure of subjects over time points, as per best model fit. Outcomes were log-transformed if this produced a more normal distribution. Modeling of coagulation parameters and thrombin generation controlled for use of heparin, low-molecular-weight heparin, and apixaban for aPTT; for use of coumadin, argatroban, and apixaban for PT; and for use of coumadin for factor VII and protein C.

In addition, linear regression models were fit to assess the relationship between treatment arm and change from Day 0 to Day 2 among transfused subjects, controlling for preoperative values. Linear regression models also were fit to assess the relationship between laboratory measures at Day 2 and the clinical outcomes of 7-day ΔMODS and 28-day ΔMODS. Preliminary modeling included only one laboratory measure at a time; measures significant at level 0.05 in this preliminary modeling were included in the final multivariate models with backward selection.

To compare transfused versus nontransfused subjects, the change from the preoperative Day 0 value to the Day 2 value for each analyte was calculated for each subject. A linear regression model was fit, with this change as the dependent variable. The independent variables included the subject's preoperative value for the measure.

Statistical significance was tested at the level 0.05, and modeling was conducted using computer software (SAS version 9.4, SAS Institute Inc.). Given the testing of 71 laboratory measures, correction for multiple comparisons was conducted using the Benjamini-Hochberg method, using R software.[38]

RESULTS

Clinical cohort characteristics

The ancillary study of coagulation, immune, and NO parameters enrolled 92 subjects from the parent RECESS trial of 1481 randomized participants. Two were excluded from analyses because they did not undergo cardiac surgery. Of the remaining participants, 50 were randomly assigned to the fresh RBC arm (35 of whom were transfused RBCs), and 40 were randomly assigned to the older RBC arm (28 of whom were transfused RBCs). Those who did not receive RBCs (n = 27) were followed for only the first two visits. Follow-up was generally good; 87 of 92 eligible subjects completed the Day 0 sample, 86 of 89 Day 2, 51 of 60 Day 6, 46 of 59 Day 28, and 25 of 55 Day 180. Four subjects withdrew before Day 6, and four died between Days 28 and 180. The subjects in the two transfused arms showed no significant differences in age, sex, history of myocardial infarction, or baseline multiple organ dysfunction score (MODS) (Table 1). The patients in the fresh blood group had higher Transfusion Risk Understanding Scoring Tool (TRUST) scores,[39] received more apheresis platelets, were more likely to receive any cryoprecipitate, and had lower Hb prior to surgery compared to the aged blood group. In addition, there was a difference in the ABO types between the two study groups. Of the 28 subjects in the aged blood group, 12 received at least one RBC unit stored more than 35 days, and none in the fresh blood group received any stored more than 35 days.

Age of blood effect on coagulation factors

There were no significant differences in coagulation markers between the two treatment arms in an unadjusted analysis, though approximately half the markers changed significantly over time (Table 2). When medications known to interact with calibrated automated thrombin (principally heparin and coumadin) were added to the generalized linear model, the changes in LT and TTP were significant (p = 0.01). Several markers for coagulation

activation declined in the immediate postoperative period, then increased over subsequent days toward baseline values, including factor V, factor XI, ATIII, and sEPCR, although there was no significant difference in levels between those patients receiving aged versus fresh blood. LT, factor XI, ATIII, and TTP were significantly lower on Day 2 compared to baseline (Fig. 1A). There were no significant changes in ETP or peak height throughout the study period (Fig. S1). Factors that initially increased in the immediate postoperative period then declined over subsequent days toward baseline values were F1.2, D-dimer, factor VIII, and PAI-1, and the changes from Day 0 to Day 2 were significant for all but PAI-1 (Fig. 1B). Again, there was no significant difference between the two study arms for these parameters. Factor VII, protein C, FM, TFPI, thrombomodulin, and TPA did not have significant changes in levels throughout the study period (Fig. S1).

Age of blood effect on cellular immune function and cytokine expression

There were very few differences between treatment arms in cellular immune function or systemic cytokine levels. The ability of CD8+ T cells to secrete IFN-γ was significantly higher in recipients of aged blood, and plasma levels of EGF were higher in recipients of fresh blood (Table 3). For cytokines that showed significant interaction between study arm and time after surgery (3), significant differences between treatment arms at specific time points are noted by inverted triangles, including IL-6, FGF, VEGF, and soluble vascular cell adhesion molecule (Fig. 2). None of the differences between study arms remained significant after correction for multiple comparisons. In contrast to the limited effects of blood storage age on immune parameters, the majority of these analytes differed significantly over time. Of the 28 cytokines measured, 19 showed significant variation over time. The most common pattern showed an initial drop in systemic levels after transfusion and surgery (Fig. 2A). These included a number of innate immune mediators and chemokines (IL-1β, interferon-inducible T-cell alpha chemoattractant [ITAC], macrophage inflammatory protein [MIP]-1β, RANTES), T-cell related cytokines (IL-2, IL-7, IL-12p70, IFN-γ), cytokines in the proinflammatory IL-17 axis (IL-17A, IL-22, IL-23), and the growth factors GM-CSF, EGF, FGF, and VEGF, though the change from Day 0 to Day 2 was significant for only a few of the cytokines. A smaller subset of cytokines showed a modest increase or no distinct change immediately after transfusion and surgery (Fig. 2B). Finally, several markers showed no significant change over time (Fig. S2A), and changes in IL-22, and tumor necrosis factor -α were not significant after correction for multiple comparisons.

In addition to soluble factors, the ability of T cells to proliferate and secrete cytokines was tested, as was the frequency of Tregs (Fig. S2B). None of these factors changed significantly with time, though Treg frequencies tended to increase after baseline ($p = 0.09$). In addition, CD8+ T-cell IFN-γ secretion in response to PMA-ionomycin stimulation was significantly lower after receipt of fresh compared to aged RBCs, though not after correction for multiple comparisons. Overall, there was a strong effect of transfusion and surgery on cytokines and a more muted effect on cellular immune function, and the storage age of transfused blood had no consistent effect on these parameters.

Age of blood effect on extracellular vesicles and NO flux parameters

Of 15 EV parameters measured, only one, the B-cell marker CD19, differed significantly between arms ($p = 0.02$), and this did not remain significant after correcting for multiple comparisons. Total EV content and 50% of the EVs identified by cell of origin varied significantly over time (Table 4). EVs expressing CD62L and CD62P differed between treatment arms only at day 28 (Table 5, Fig. 2C). Total EV count and EVs derived from platelets (CD41a), T cells (CD3), and granulocytes (CD15, CD66b) all showed the same pattern of an early decrease after transfusion and surgery followed by recovery by Day 6, and the difference between Day 0 and Day 2 was significant for all but the total EV count. The change over time in CD66b + EVs was not significant after correction for multiple comparisons. EVs derived from RBCs (CD235a, CD108a), natural killer cells (CD16), B cells (CD19), endothelial cells (CD142), and those bearing co-stimulatory molecules (CD28, CD154) did not change significantly over time (Fig. S3A). Of the NO parameters measured, none differed between recipients of fresh versus aged RBCs, and none changed significantly over time after transfusion (Fig. S3B).

Posttransfusion coagulation and immune parameters correlated with organ dysfunction

A secondary aim of the RECESS ancillary study was to identify changes in coagulation or immune parameters that would predict organ dysfunction after transfusion. Given that minimal differences were noted between the two treatment arms for the parameters tested, both treatment arms were combined for analysis of whether posttransfusion fluctuation in coagulation or immune markers correlated with organ dysfunction (ΔMODS). There were several factors measured at Day 2 that were associated with ΔMODS at Day 7 or 28 (Table 6). Of these, higher total EV counts at Day 2 was associated with higher ΔMODS on both Days 7 and 28.

Comparison of transfused and nontransfused subjects

While there was very little difference in the laboratory parameters between recipients of fresh versus aged blood, many parameters showed significant modulation after surgery and transfusion. Having samples from nontransfused subjects on Days 0 and 2 allowed comparison of modulation of coagulation, immune, and NO parameters between transfused and nontransfused subjects. Because negligible differences were seen between the fresh and aged blood recipient arms for the laboratory measures, these arms were combined for this analysis. Change from baseline level to Day 2 differed for six analytes in transfused versus nontransfused subjects. One of 21 coagulation parameters, F1.2, increased at a faster rate in transfused compared to nontransfused participants (Fig. 3). Two of 29 cytokines measured, IL-23 and RANTES, decreased at a faster rate in transfused versus nontransfused subjects. Finally, 3 of 15 EV parameters measured, EVs expressing CD41a, CD62P, and CD62L, decreased in transfused participants but increased or decreased significantly more slowly in nontransfused participants. Of these analytes, only the difference in RANTES concentration remained significant after correction for multiple comparisons.

DISCUSSION

This study measured a broad array of laboratory measures to characterize coagulation, immune, and NO pathways in participants in the RECESS trial. Consistent with the finding of no clinical differences in cardiac surgery patients who received fresh versus aged blood, our study found negligible differences between the study groups across a wide range of analytes, most of which did not remain significant after correction for multiple comparisons. In spite of the lack of difference between study arms, we identified multiple coagulation, cytokine, and EV markers that changed significantly in the time after surgery and transfusion. In addition, in exploratory analyses, changes in several analytes were found to correlate with the change in MODS.

Other prospective clinical trials have investigated the impact of transfused RBC age on coagulation parameters. Wasser et al.[40] compared the transfusion of fresh whole blood collected within 12 hours to RBCs of 2to 5 days' duration in cardiac surgical patients. They reported no difference in the postoperative thrombotest (similar to a PT). More recently, Kor et al.[41] found no differences in fibrinogen levels and changes in antithrombin consumption during a randomized trial of intubated intensive care unit patients receiving RBCs that were 5 days old or less compared to standard issue RBCs with median storage duration of 26.5 days. The results of these two trials are consistent with our laboratory findings of no consistent pattern of a procoagulant state for patients transfused aged versus fresh blood. Although thrombin generation LT and TTP were slightly prolonged when adjusting for medications impacting the coagulation pathway in patients receiving aged blood, the lack of corresponding reduced ETP or peak thrombin generation suggests that this difference was due to chance rather than any important clinical effect. This is further supported by the lack of significant changes in other clotting times such as PT and PTT. Our data support the concept that the age of transfused RBCs in the range we tested has no demonstrable effect on either the up regulation of procoagulant factors or the down regulation of anticoagulant factor levels.

Although very few parameters measured differed in recipients of fresh versus stored blood, approximately one-half of the coagulation and EV parameters measured and the vast majority of cytokines showed significant changes in the time after surgery and transfusion. About one-third of the subjects enrolled in this ancillary study did not require transfusion, which gives the opportunity to see which analytes changed in postoperative patients independently of transfusion. Most analytes measured did not vary differently between transfused and nontransfused subjects, implying that much of the modulation in coagulation and immune mediators seen after cardiac surgery were due to the surgery itself rather than transfusion. This is similar to the observation made in transfused trauma patients.[42] Three of the six parameters that differed between transfused and nontransfused participants were EV related, and they included EVs expressing platelet markers (CD41a and CD62P). Both platelet-derived EV markers showed higher baseline levels in the transfused participants, then fell after transfusion (Fig. 3). It is not clear whether the differences seen in platelet-derived EV modulation is due to inherent differences in subjects who did or did not require transfusion or due to the transfusion itself, though the fact that changes in most parameters did not differ between transfused and nontransfused participants suggests that the

posttransfusion decrease in platelet-derived EVs may in fact be due to the transfusion. Of note, the same changes were seen in a companion study measuring immune modulation in transfused patients in the intensive care unit, though all subjects in that study received a transfusion (P. J. Norris, unpublished data). Definitively answering the question in humans would require comparing patients randomized to transfusion or not, such as in a transfusion trigger trial.

The limitations of our study include that there were some potentially meaningful differences in baseline characteristics between the two study groups. Higher TRUST score and transfusion requirements in the fresh RBC group indicate increased severity of illness. In addition, we were unable to account for the transfusion dose and timing of plasma, platelet, and cryoprecipitate transfusions that may have affected our hemostatic and immunologic results. It is unknown whether these differences affected our results. However, the main findings are consistent with the findings in a parallel study of RBC storage age in critically ill patients (P. J. Norris, unpublished data).

In summary, we found no major differences in potential procoagulant, immunologic, or NO effects in patients transfused with RBCs stored for 21 days or more compared to patients transfused with RBCs stored for 10 days or less. Our findings are consistent with the clinical data of the RECESS study, and offer further support of the functional equivalence of older versus newer RBCs in cardiac surgical patients. We believe this has important considerations for clinical management for clinicians, blood banks, as well as an important understanding of clinical findings in RECESS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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PJN, JHL, AD, MES and PCS designed experiments; FT, GR, and SA designed data analyses; FT and GR performed data analyses; HCI, JWH, AD, and RV performed experiments; FS and SMK analyzed data; MS and AMG coordinated studies; and PJN, RMS, and PCS wrote the manuscript.

ABBREVIATIONS:

aPTT	activated partial thromboplastin time
ATIII	antithrombin

EGF	epidermal growth factor
ETP	endogenous thrombin potential
EVs	extracellular vesicles
F1.2	prothrombin fragment 1.2
FGF	fibroblast growth factor
FM	fibrin monomer
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN	interferon
IL	interleukin
ITAC	interferon-inducible T-cell alpha chemoattractant
LT	lag time
MIP	macrophage inflammatory protein
MODS	multiple organ dysfunction score
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
PBMCs	peripheral blood mononuclear cells
PMA	phorbol 12-myristate 13-acetate
PT	prothrombin time
RECESS	Red Cell Storage Duration Study
sEPCR	soluble endothelial protein C receptor
TFPI	tissue factor pathway inhibitor
TPA	tissue plasminogen activator
Tregs	regulatory T cells
TRUST	Transfusion Risk Understanding Scoring Tool
TTP	time to thrombin peak
VEGF	vascular endothelial growth factor

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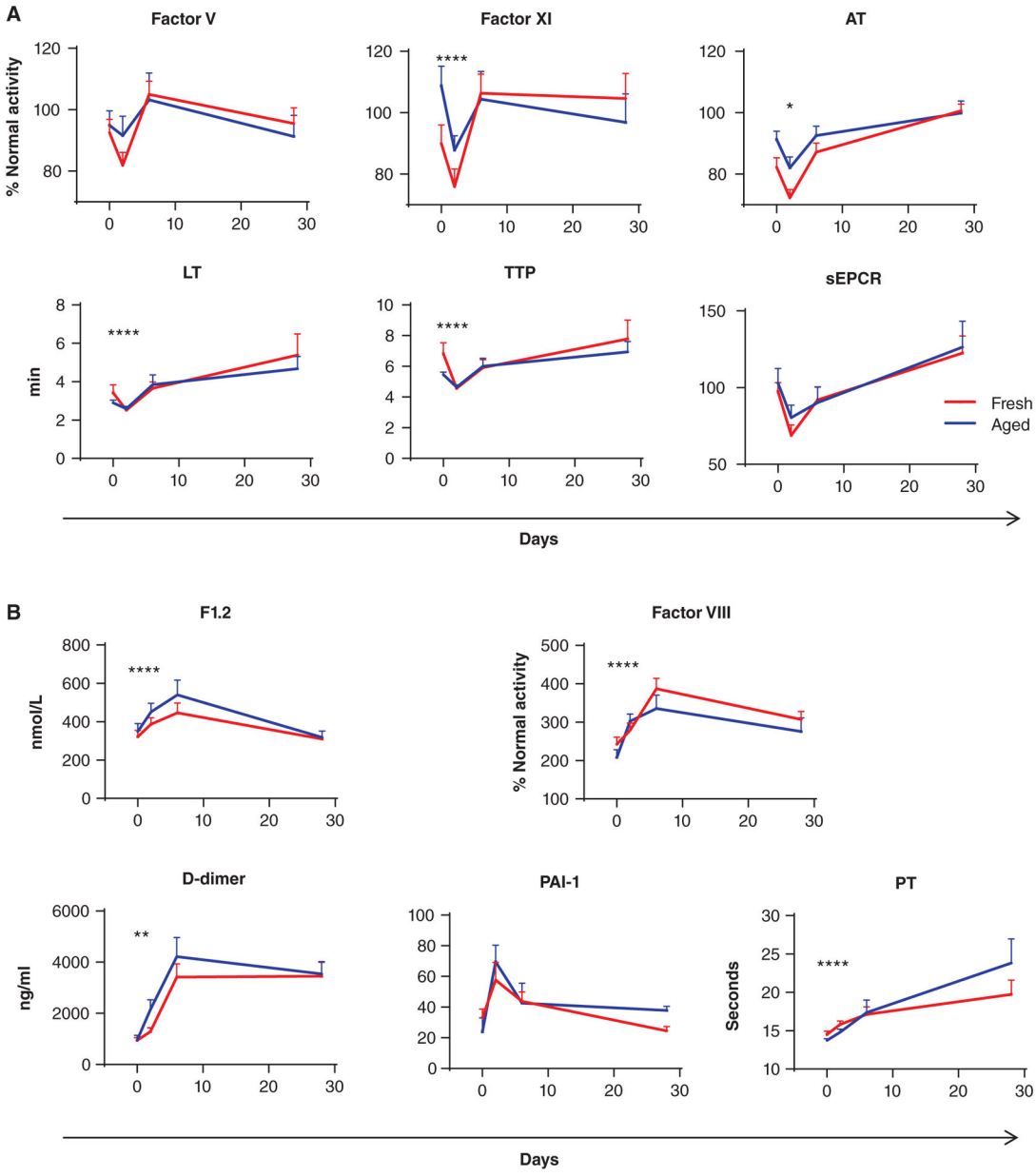
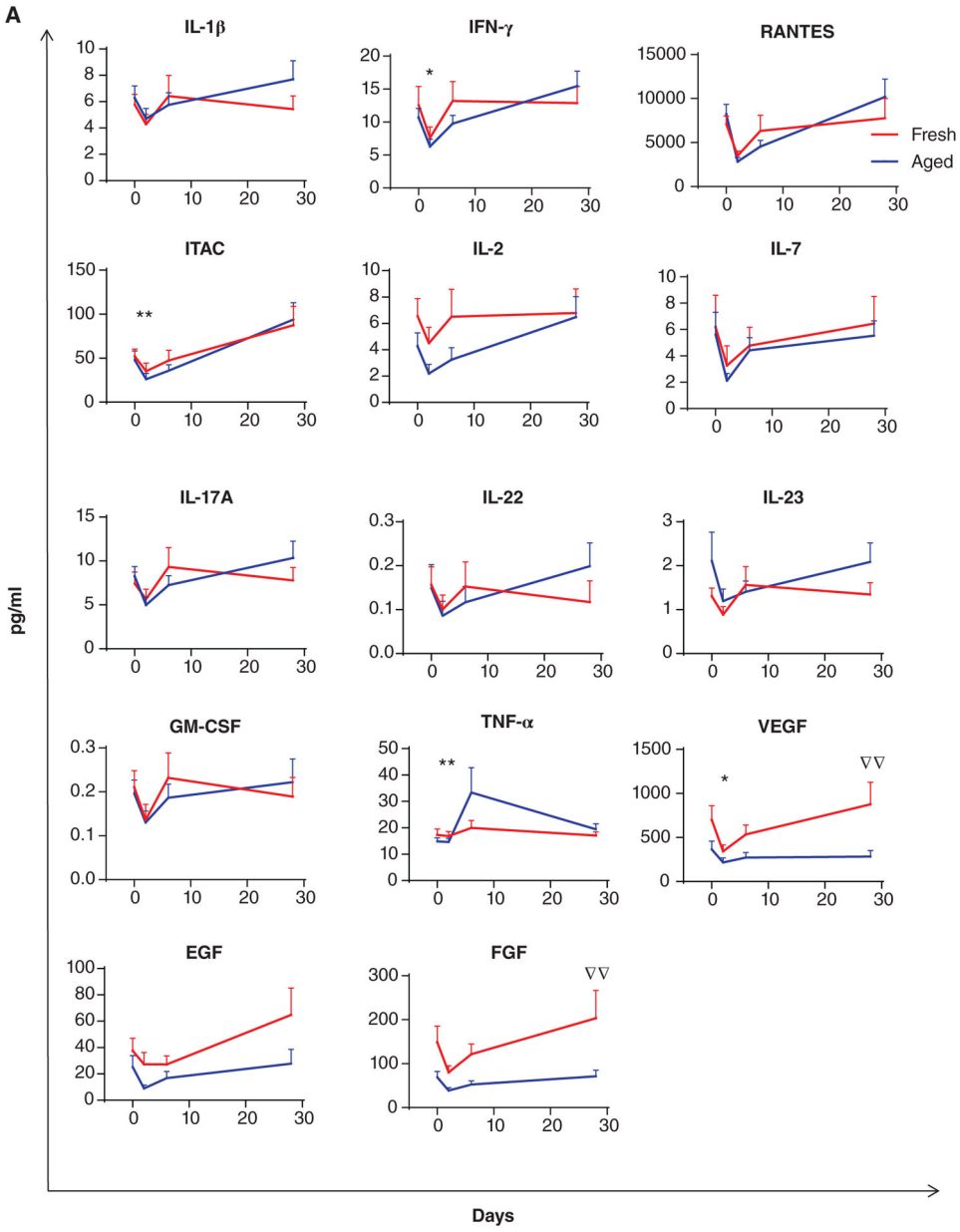


Fig. 1.

Longitudinal analysis of coagulation parameters after transfusion. Mean levels of coagulation parameters that changed significantly over time are shown on Days 0 (before transfusion) and 2, 6, and 28 after transfusion in recipients of fresh (red) and standard (blue) aged blood. (A) Values as a percentage of normal control plasma levels are shown for analytes that decreased after Day 0. (B) Analytes that increased in at least one treatment arm after Day 0 are shown. Analytes for which the change from Day 0 to Day 2 was significant are indicated by asterisks. Error bars represent standard error of the mean. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, comparing Day 0 to Day 2 values independent of treatment arm effect.



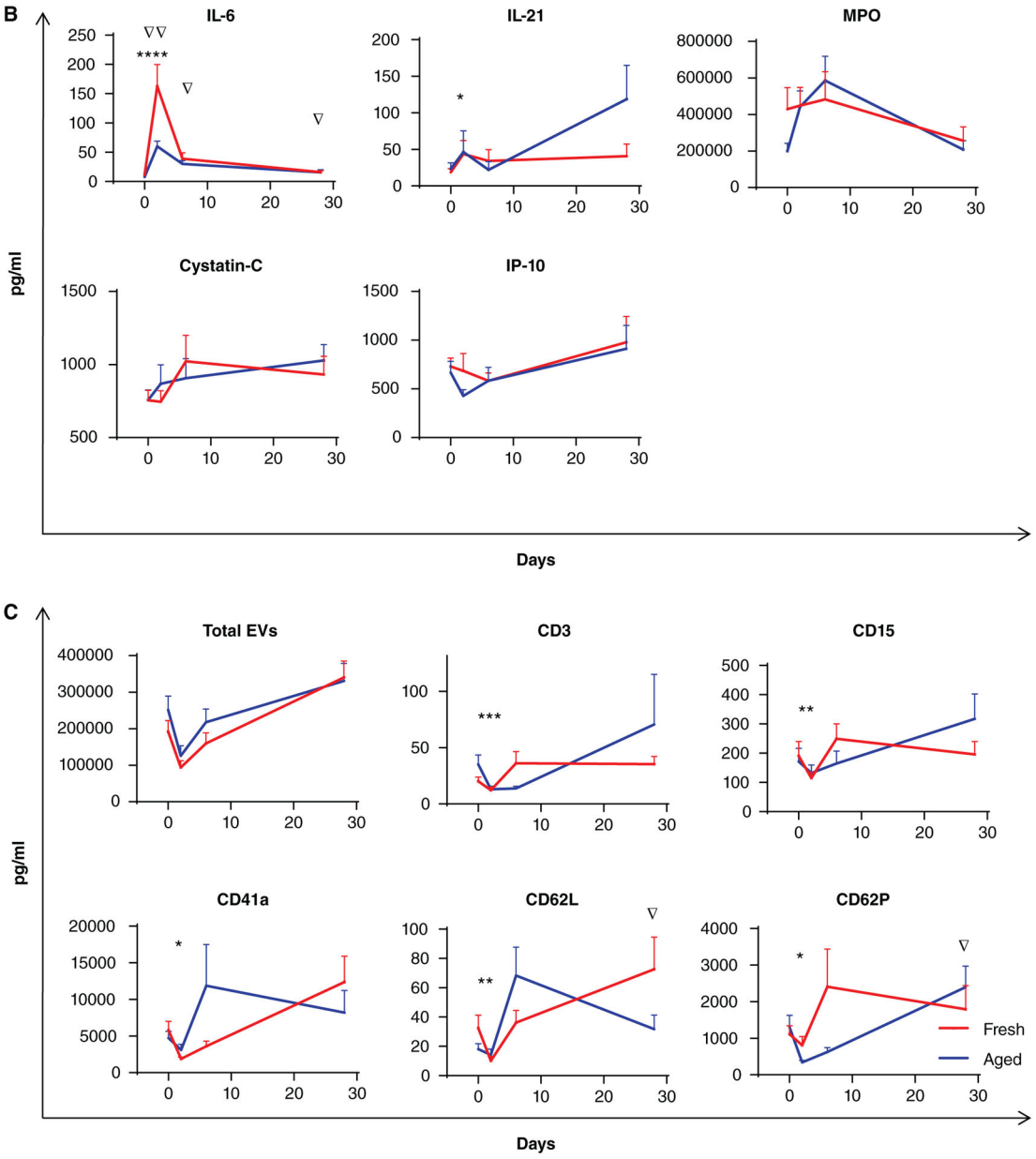


Fig. 2.

Longitudinal analysis of cytokine levels after transfusion. Mean levels of analytes are shown on Days 0 (before transfusion) and 2, 6, and 28 after transfusion in recipients of fresh (red) and standard (blue) aged blood. (A) Cytokines that decreased after Day 0 are shown. Only EGF significantly differed after baseline between the fresh and aged RBC arms. (B) Cytokines that increased after Day 0 are shown. (C) EV levels that decreased after Day 0 are shown. Analytes for which the change from Day 0 to Day 2 was significant are indicated by asterisks. Error bars represent standard error of the mean. IP-10 = CXCL10; ITAC = CXCL11; MIP-1β = CCL4; MPO = myeloperoxidase; RANTES = CCL5. *$p < 0.05$, **$p < 0.01$, ***, $p < 0.001$, ****$p < 0.0001$, comparing Day 0 to Day 2 values independent of treatment arm effect; ∇ $p < 0.05$, ∇∇ $p < 0.01$ comparing treatment arms at noted time point.

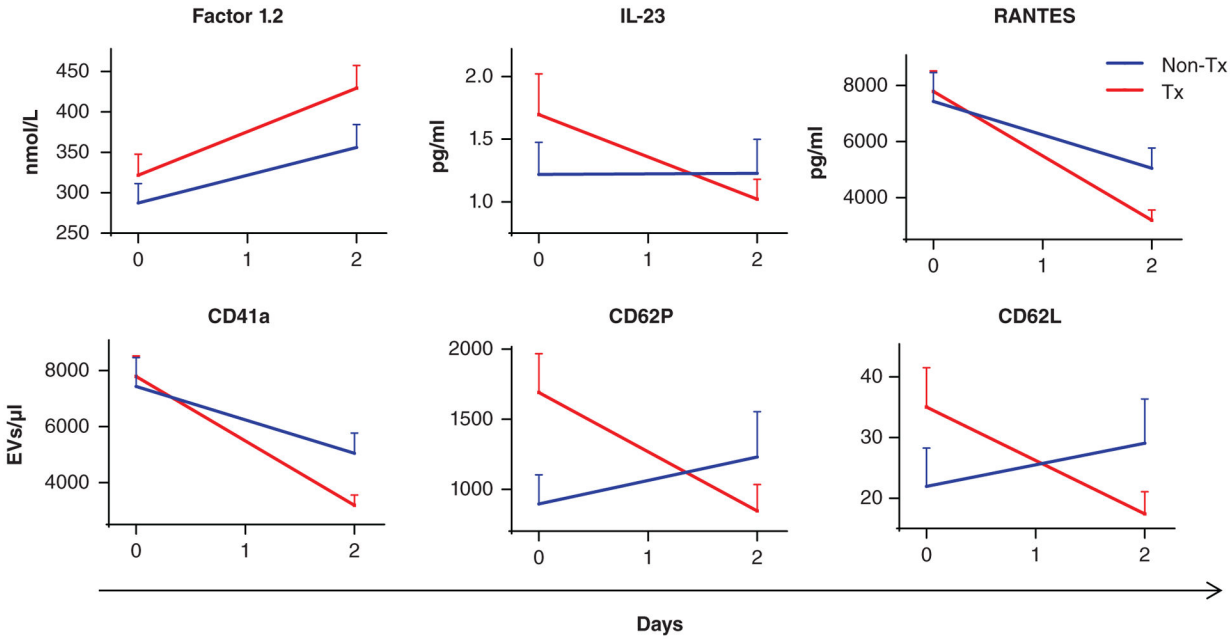


Fig. 3.

Parameters that evolved differently in transfused and nontransfused subjects. The change in levels from Day 0 to Day 2 for all parameters measured were compared between transfused (red) and nontransfused (blue) subjects. Mean levels of the six analytes for which the Day 0 to Day 2 change between the two groups differed are shown ($p < 0.05$). Error bars represent standard error of the mean.

TABLE 1.

Cohort characteristics

	Aged blood transfusion (≥21 days old) N = 28		Fresh blood transfusion (≤10 days old) N = 35		No transfusions N = 27		p values*	
	N or median	% or (Q1, Q3)	N or median	% or (Q1, Q3)	N or median	% or (Q1, Q3)	Aged versus fresh versus nontransfused	Aged versus fresh
Age (y)	74	(66, 78)	72	(68, 78)	68	(63, 75)	0.16	0.69
Male	18	64	15	43	16	59	0.24	0.13
Minimum TRUST Score	3.5	(3, 4)	4	(3, 5)	3	(3, 4)	**<0.01**	**0.04**
in ICU before surgery	3	11	6	17	9	33	0.10	0.72
Blood type							0.19	**0.05**
O	10	36	20	57	11	41		
A	13	46	10	29	12	44		
B	5	18	2	6	3	11		
AB	0	0	3	9	1	4		
Known history of myocardial infarction	2	7	9	26	8	30	0.08	0.09
Known history of congestive heart failure	9	32	13	37	7	26	0.69	0.79
Hemoglobin (g/L)	12.9	(12.0, 13.5)	11.6	(10.8, 12.6)	13.0	(12.5, 13.5)	**<0.01**	**<0.01**
Baseline MODS Score	1	(0, 1)	1	(0, 1)	0	(0, 1)	0.08	0.66
Number of RBC units transfused	3	(1.5, 5)	4	(2, 5)	-	-	-	0.49
≥1 Apheresis platelet transfusion	15	54	15	43	2	7	**<0.01**	0.40
Number of whole blood unit equivalents of apheresis platelets	6	(5, 12)	12	(10, 18)	9.5	(5, 14)	0.12	**0.05**
≥1 Whole blood-derived platelet transfusion	1	4	3	9	-	-	-	0.62
Number of pooled units of whole blood–derived platelets	12	(12, 12)	6	(6, 18)	-	-	-	1.00
≥1 Apheresis or whole blood–derived platelet transfusion	16	57	17	49	2	7	**<0.01**	0.50
≥1 Plasma transfusion	16	57	15	43	2	7	**<0.01**	0.26
Number of units of plasma	4	(2, 5)	4	(2,5)	3	(2, 4)	0.86	1.00
≥1 Cryoprecipitate transfusion	5	18	11	31	2	7	**<0.01**	**<0.01**

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	Aged blood transfusion (≥21 days old) N = 28		Fresh blood transfusion (≤10 days old) N = 35		No transfusions N = 27		p values*	
	N or median	% or (Q1, Q3)	N or median	% or (Q1, Q3)	N or median	% or (Q1, Q3)	Aged versus fresh versus nontransfused	Aged versus fresh
Number of pooled units of cryoprecipitate	4	(4, 10)	10	(6, 20)	10	(10, 10)	0.19	0.12

*Between-group differences in categorical variables were compared with the use of Fisher's exact test. Continuous variables were compared with the use of the Kruskal-Wallis test.

ICU = intensive care unit; MODS = multiple organ dysfunction score; TRUST = Transfusion Risk Understanding Scoring Tool.

TABLE 2.

Effects of RBC storage age on coagulation factors

	Treatment arm Aged versus fresh units			Time	
	Effect size	**SE**	**p value**	**p value**	**FDR corrected**
PT (sec)	0.43	1.34	0.75	**<0.0001**	**0.00059**
PTT (sec)	3.15	7.81	0.69	0.64	0.7
LT (min)	0.66	0.35	0.06 *	**<0.0001**	**0.00059**
PH (nM)	15.4	24.2	0.53	0.08	0.12
TTP (min)	0.71	0.41	0.09 *	**<0.0001**	**0.00059**
ETP (nM*min)	−49.3	99.8	0.62	**0.05**	0.074
Rate (nM/min)	2.72	14.1	0.85	**0.01**	**0.023**
PT F1.2 (nmol/L)	0.11	0.09	0.23	**<0.0001**	**0.00059**
D-dimer † (ng/mL)	4.31	3.99	0.28	**<0.0001**	**0.00059**
FM (ng/mL)	5.15	4.76	0.28	0.53	0.6
EPCR (ng/mL)	5.14	7.52	0.5	**<0.0001**	**0.00059**
Factor V (% normal)	2.76	5.55	0.62	**0.0023**	**0.0082**
Factor VII (% normal)	3.06	6.65	0.65	0.39	0.49
Factor VIII (% normal)	3.21	21.5	0.88	**0.001**	**0.0039**
Factor XI (% normal)	−3.19	6.39	0.62	**<0.0001**	**0.00059**
ATIII (% normal)	1.97	2.72	0.47	**<0.0001**	**0.00059**
Protein C (% normal)	14	8.26	0.09	0.83	0.84
TFPI (ng/ml)	2.73	5.2	0.6	0.65	0.7
TM (ng/ml)	0.05	0.06	0.39	0.38	0.48
TPA (ng/ml)	−0.12	0.1	0.21	0.12	0.17
PAI-1 (ng/ml)	6.87	10.2	0.51	**0.0006**	**0.003**

Positive effect size when value is higher in the aged RBC arm.

* p value = 0.01 after adjustment for medication use.

† Effect size per 100 ng/mL.

ATIII = antithrombin EPCR = endothelial protein C receptor; ETP = endogenous thrombin potential; F1.2 = prothrombin fragment 1.2; FDR = false discovery rate; FMfibrin = monomer; LT = lag time; PAI-1 = plasminogen activator inhibitor-1PH = peak thrombin generation; PT = prothrombin time; PTT = partial thromboplastin time; SE = standard error; TFPI = tissue factor pathway inhibitor; TM = thrombomodulin; TPA = tissue plasminogen activator; TTP = time to thrombin peak.

TABLE 3.

Effects of RBC storage age on immune parameters

	Treatment arm			Time	
	Effect size	**SE**	**p value**	**p value**	**FDR corrected**
Treg (%)	0.15	0.36	0.68	0.09	0.13
CD4 + 1 L-17+ (%)	0.17	0.16	0.28	0.19	0.25
CD8 + IFN-γ + (%)	**9.2**	**3.55**	**0.01**	**0.04**	0.064
$CFSE^{low}$ (%)	−4.73	3.95	0.23	0.83	0.84
GM-CSF (pg/mL)	−0.01	0.03	0.74	**0.01**	**0.023**
IFN-γ (pg/mL)	−1	1.98	0.61	**<0.001**	**0.0039**
IL-10 (pg/mL)	−3.69	4.21	0.38	0.19	0.25
IL-1β (pg/mL)	0.03	0.93	0.97	**0.01**	**0.023**
IL-2 (pg/mL)	−0.91	1.13	0.42	**0.001**	**0.0039**
IL-21 (pg/mL)	21.8	17.8	0.22	0.06	0.091
IL-22 (pg/mL)	−0.005	0.03	0.89	**0.04**	0.064
IL-23 (pg/mL)	−0.02	0.29	0.94	**0.003**	**0.0097**
IL-7 (pg/mL)	0.58	0.91	0.52	**<0.0001**	**0.00059**
IL-8 (pg/mL)	−0.14	0.18	0.43	**0.02**	**0.039**
ITAC (pg/mL)	0.32	10.1	0.98	**<0.0001**	**0.00059**
TNF-α (pg/mL)	5.93	3.6	0.1	**0.04**	0.064
IP10 (pg/mL)	−69.2	146	0.64	**0.02**	**0.039**
EGF (pg/mL)	**−20.2**	**8.02**	**0.01**	**0.0003**	**0.0016**
β2 microglobulin* (pg/mL)	0.09	2.09	0.97	0.16	0.22
Cystatin C (pg/mL)	53.3	98.9	0.59	**0.004**	**0.012**
MPO† (pg/mL)	0.86	0.96	0.37	**0.01**	**0.023**
PDGF (pg/mL)	−393	467	0.4	**0.02**	**0.039**
RANTES (pg/mL)	−0.05	0.16	0.77	**<0.0001**	**0.00059**
sICAM-1* (pg/mL)	−0.42	0.74	0.57	0.41	0.5

† Positive effect size when value is higher in the aged RBC arm.

* Effect size per 10,000 pg/mL.

[†]Effect size per 100,000 pg/mL.

CSFE = carboxyfluorescein succinimidyl ester; EGF = epidermal growth factor; FDR = false discovery rate; GM-CSF = granulocyte-macrophage colony-stimulating factor; IFN = interferon; IL = interleukin; IP10 = IFN-γ–induced protein; ITAC = interferon-inducible T-cell alpha chemoattractant; MPO = myeloperoxidase; PDGF = platelet-derived growth factor; SE = standard error; sICAM = soluble intercellular adhesion molecule; TNF-α = tumor necrosis factor-α; Treg = regulatory T cell.

TABLE 4.

Effects of RBC storage age on NO and EV parameters

	Treatment arm Aged versus Fresh units			Time	
	Effect size	**SE**	**p value**	**p value**	**FDR corrected**
EV concentration* (EV/μL)	0.25	0.28	0.37	**<0.001**	**0.0039**
CD3 (EV/μL)	−0.1	0.22	0.66	**0.0001**	**0.00059**
CD16 (EV/μL)	11	33.6	0.74	0.15	0.21
CD19 (EV/μL)	**−9.3**	**3.81**	**0.02**	0.7	0.74
CD28 (EV/μL)	0.1	0.3	0.75	0.27	0.35
CD142 (EV/μL)	−4.22	2.76	0.13	0.46	0.54
CD154 (EV/μL)	−13.2	13.7	0.34	0.45	0.53
CD41a† (EV/μL)	−4.73	3.87	0.22	**0.0021**	**0.0078**
CD108a (EV/μL)	0.003	0.26	0.99	0.56	0.62
CD235a (EV/μl)	0.04	0.29	0.9	0.83	0.84
CD15 (EV/μL)	13.3	53.6	0.8	**0.01**	**0.023**
CD66b (EV/μL)	77	97.2	0.43	**0.03**	0.056
RBC NO (%)	−5.2	0.15	0.44	0.53	0.6
HbNO (%)	−0.19	2.04	0.37	0.96	0.96
SNO (%)	−0.22	0.22	0.32	0.44	0.53

Positive effect size when value is higher in the aged RBC arm.

*Effect size per 100,000 EV/μl.

†Effect size per 1,000 EV/μl.

EV = extracellular vesicle; FDR = false discovery rate; NO = nitric oxide; SNO = *S*-nitrosothiol.

TABLE 5.

Cytokine and EV parameters with significant interaction between treatment group and visit

	Group by visit		Treatment arm at					
	interaction		2 days		6 days		28 days	
	p-value	FDR corrected	Effect size	p-value*	Effect size	p-value*	Effect size	p-value*
IL-12p70 (pg/mL)	**0.048**	0.074	−0.63	0.69	−1.91	0.35	2.4	0.16
IL-17A (pg/mL)	**0.01**	**0.023**	−0.34	0.85	−1.95	0.4	3.9	0.05
IL-6 (pg/mL)	**0.04**	0.064	−82	**0.001**	73	**0.04**	82	**0.02**
MIP-1α (pg/mL)	**0.03**	0.056	−5.7	0.41	19	**0.04**	−2.4	0.76
MIP-1β (pg/mL)	**0.04**	0.064	−8.8	0.46	9.4	0.58	−31	0.06
FGF (pg/mL)	**0.02**	**0.039**	13	0.67	−36	0.42	−120	**0.007**
VEGF (pg/mL)	**0.008**	**0.023**	25	0.85	−85	0.66	−540	**0.004**
sVCAM-1 (pg/mL)	**0.04**	0.064	−14	0.41	12	0.61	52	**0.02**
CD62L (#/μL)	**0.01**	**0.023**	−0.53	0.13	−0.11	0.82	1	**0.02**
CD62P (#/μL)	**0.003**	**0.01**	−0.18	0.62	−0.38	0.44	1.1	**0.01**
CD11b (#/μL)	**0.02**	**0.039**	0.17	0.58	−0.53	0.24	0.72	0.1

Positive effect size when value is higher in the aged RBC arm.

*p value represents significance of difference between treatment arms at the specified day.

FDR = false discovery rate; FGF = fibroblast growth factor; IL = interleukin; MIP = macrophage inflammatory protein; sVCAM-1 = soluble vascular cell adhesion molecule-1; VEGF = vascular endothelial growth factor.

TABLE 6.

Factors correlated with clinical outcome

	7-day ΔMODS		28-day ΔMODS	
	Slope	**p value**	**Slope**	**p value**
Day 2				
EVs	0.6	0.016	0.69	0.013
IL-2	−0.16	0.0036		
PT	0.47	0.0006		
RANTES *			−0.29	0.016
AT			−0.055	0.007
PAI-1			0.014	0.012

* Slope per 10,000 units of change: RANTES.

AT = antithrombin; EV = extracellular vesicle; IL = interleukin; PAI-1 = plasminogen activator inhibitor-1; PT = prothrombin time.