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Murine RBCs from Genetically Distinct Donors Cross-Regulate when Stored Together

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Abstract

Background: Donor variability of RBC storage has been observed in both humans and animal models. We utilized a strain of mice with RBCs known to store well (B6) and a strain known to store poorly (FVB) to test the hypothesis that RBCs affected the storage of other RBCs.

Study Design/Methods: Five strains of mice were used: 1) transgenic B6 mice expressing green fluorescent protein (GFP) in their RBCs (GFP.B6), 2) wild-type B6 mice, 3) wild-type FVB mice, 4) F1 crosses between GFP.B6 and FVB mice (GFP.F1) and 5) the analogous wild-type (B6xFVB) F1 cross. GFP.B6 or GFP.F1 RBCs were mixed with wild-type (non-GFP) RBCs from B6 or FVB strains prior to storage. 24-hour RBC recoveries were determined for stored RBCs by enumerating circulating GFP+ RBCs by flow cytometry.

Results/Findings: 24-hour recoveries of GFP.F1 RBCs was increased by co-storage with B6 RBCs but decreased by co-storage with FVB RBCs. This effect was dose dependent when tested with GFP.B6 RBCs; the more FVB blood added, the worse the 24-hour recoveries became. RBC cross-regulation did not occur when B6 and FVB RBCs were separated by a semi-permeable membrane with a 0.4µm size cut-off.

Conclusion: These findings demonstrate that RBCs affect the storage of other RBCs, in both positive and negative directions, indicating that RBC storage is not only intrinsic to the RBC but that RBC-RBC communication occurs. Additional studies will be required to determine the nature of this effect and if these findings translate into human RBC storage.

Introduction:

Red blood cells (RBCs) can be stored for up to 42 days at 4°C prior to transfusion. As per the FDA criteria for licensure of RBC storage systems, for the majority of blood donors tested, greater than 75% of their stored RBCs continue to circulate at 24 hours post

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transfusion¹. However, there can be substantial donor-to-donor variation in RBC storage. Indeed, it has been documented that RBC units from a significant percentage of donors have 24-hr recoveries of less than 75%, and for a small percentage of donors, 24-hr recoveries are very poor (in the range of 20–30%)². The basis for donor-to-donor variability is poorly understood, but there is good evidence that it has a strong genetic component, at least at the metabolic level^{3–7}. Using tractable animal models of RBC storage, we have documented that both hemolysis and decreased 24-hour recoveries vary in genetically distinct strains of inbred mice^{8,9}. These strains serve as a platform to study basic mechanisms of variant RBC storage. We have recently reported that amongst 13 different strains tested, C57BL/6 (B6) mice have the best 24-hr recoveries whereas FVB donors store poorly⁸. In the current report, we tested the hypothesis that storage biology of a given RBC is affected by the other RBCs in its storage environment. This was carried out by a series of “co-storage” experiments using the B6 and FVB strains.

Herein, we report that the storage properties of RBCs from different donor strains are altered by co-storage with RBCs from other strains. This appears to require cell-to-cell contact, or at least communication via large biological entities, as the effect is not transferred across semi-permeable membranes (0.4µm cutoff) in transwell experiments. These studies indicate that RBC storage is a property of the population of cells being stored as a cross-talking entity, not a compilation of isolated intrinsic biology of each individual RBC. These findings have mechanistic importance to understanding RBC biology, both in the context of blood storage and also normal RBC biology. In addition, these findings may have practical implications with regards to strategies of RBC storage and RBC storage studies, if the findings translate into human RBC storage biology.

Materials and Methods:

Mice: UbiC-GFP mice (on a B6 background; called GFP.B6 hereafter), C57BL/6J (B6) mice and FVB/NJ (FVB) mice were each purchased from Jackson Labs (Bar Harbor ME, USA). HOD and K1 mice were generated as previously described^{10,11}. FVB mice were crossed with GFP.B6 to generate FVBxGFP.B6 F1 mice (called GFP.F1 hereafter). Likewise, K1 mice were crossed with GFP.B6 to generate K1xGFP.B6 F1 (called GFP.K1 hereafter). GFP.B6, GFP.F1, GFP.K1, and HOD mice were bred in the BloodworksNW vivarium. Mice were maintained on standard laboratory chow and water ad libitum in a temperature- and light-controlled environment. All procedures were conducted according to approved IACUC protocols.

Collection, Storage, and testing of Blood:

For GFP co-storage experiments, RBCs were collected and stored as previously described¹²; briefly RBCs (GFP.B6, GFP.F1, FVB, B6 and/or F1) were collected via cardiac puncture into 12.3% CPDA-1 and transferred to a sterile 1.7 ml snapcap microcentrifuge tube. Hematocrits were adjusted to approximately 75% by removal of supernatant after centrifugation and RBCs were mixed at 1:1, 1:2 and/or 1:11 GFP:non-green RBC ratios (see Fig1C set-up). Samples were stored in 600 µl Eppendorf tubes for seven days at 4°C. After storage, 450 µl stored RBCs at 75% hct (600 µl total volume) were resuspended in 1850 µl

PBS and 50 μ l of fresh HOD packed RBCs were added to the suspension as an internal tracing control. The mixture of RBCs was then directly transfused into recipients by intravenous tail vein injection. Ratios of donor blood to HOD tracer RBCs was enumerated, both at baseline in the cells to be transfused (pre-transfusion mixture) and also in peripheral blood acquired from recipients 24 hours after transfusion (post-transfusion samples collected into ACD). Pre-transfusion and post-transfusion RBCs were washed 3X with PBS, and stained for 30 mins with 0.5 μ g anti-Fy3 in 50 μ l PBS (clone MIMA29; antibody was purified by BioXcell (West Lebanon, NH) with generous permission from the New York Blood Center). Stained cells were then washed 3X with PBS and incubated with 0.2 μ g APC conjugated Ggoat-anti-mouse Igs (BD Biosciences, San Jose CA, Cat. 550826) in 50 μ l PBS for 30 mins, which stains RBCs bound with MIMA-29 and thus identifies HOD tracer RBCs. Cells were then washed 3 times, re-suspended in PBS, and analyzed by flow cytometry. This approach utilizes MIMA29 to stain HOD tracer RBCs with a color that is different than the GFP RBCs, which fluoresce spontaneously. Final RBC survival was calculated by the following formula: (Circulating Stored GFP RBCs/HOD RBCs of peripheral sample) / (Stored GFP RBCs/HOD RBCs pre-transfusion sample). The use of this approach normalizes the number of RBCs injected, and avoids misinterpreting either in the tube hemolysis or decreased transfusion volume as decreased 24 hr recoveries. While HOD does represent an alloantigen, the 24 hour window for the recovery studies performed is too short for a primary humoral immune response.

For GFP.B6 transwell experiments using semipermeable membranes, RBCs were collected and stored as previously described¹² and as above. 325 μ l GFP.B6 or a mixture of GFP.B6 and B6 (or FVB) RBCs at 75% hct were added to the bottom and top wells of the plate, such that no bubbles were present that might affect free diffusion across membranes. RBCs were stored in Corning transwell polycarbonate membrane cell culture inserts (product code CLS3413), purchased from Sigma, St. Louis. In this system, the wells are separated by a membrane with average pore size of 0.4 μ m. Before storage, RBC mixture levels in bottom wells were checked to ensure that they were touching the semi-permeable membrane of the top well, allowing for diffusion across their interface. Plates were stored for 7 days at 4°C. After storage, the transwells were disassembled and RBCs from the top and bottom chambers were recovered separately, resuspended in 1003 μ l PBS, spiked with 23 μ l fresh HOD tracer RBCs and transfused into B6 recipients. Ratios of HOD RBCs to stored GFP RBCs were determined in both pre-transfusion samples and peripheral blood from recipients 24-hours after transfusion, and the same calculation was carried out as above.

For B6 and FVB transwell experiments using semipermeable membranes, RBCs were collected and stored as described above. 325 μ l B6 or FVB RBCs at 75% hct were added to the bottom and top wells of the plate and stored for 7 days at 4°C. RBCs from the top and bottom chambers were recovered separately, resuspended in 1003 μ l PBS, spiked with 23 μ l fresh HOD tracer RBCs and transfused into GFP.F1 recipients. Ratios of HOD RBCs to stored GFP RBCs were determined in both pre-transfusion samples and peripheral blood from recipients 24-hours after transfusion, and the final RBC survival was calculated by the following formula: (Circulating Stored non-GFP RBCs/HOD RBCs of peripheral sample) / (Stored non-GFP RBCs/HOD RBCs pre-transfusion sample).

Transwell plate validation:

The ability of small molecules to pass across the transwell membrane was tested by depositing anti-K1 (PUMA1)¹³ into the top well and K1 RBCs into the bottom well, followed by staining K1 RBCs recovered from bottom well with APC conjugated goat-anti-mouse Igs (BD Cat. 550826) as above.

Statistics:

Statistical analysis was performed using Graphpad PRISM 6 as follows: For GFP.B6 and GFP.F1 co-storage experiments, each replicate experiment was analyzed using means and standard deviations, and treatment conditions were compared by 2-Way ANOVA with Bonferroni post-hoc analysis (for GFP.B6) and 1-Way ANOVA with Bonferroni post-hoc analysis (for GFP.F1); reported p-values are corrected for multiple comparisons. To statistically analyze data across the three replicate experiments, fixed-effect meta-analysis was done using the web-based “Meta-light” (<http://epi.ioe.ac.uk/free-tools/meta-analysis/>). Meta data are presented as mean differences $\pm$ 95% confidence intervals.

For transwell co-storage experiments, each replicate experiment was analyzed using means and standard deviations, and then normalized to the mean value for the 1st treatment group presented (Fig 3D), or left non-normalized (Fig 4C). Values from each experiment were then averaged, and 1-Way ANOVA with Bonferroni post-hoc analysis used to identify any treatments that lead to significantly lower 24hr recoveries.

Results:**Co-storage of RBCs from different donors alters RBC storage biology**

To test the hypothesis that poorly-storing RBCs would affect RBCs that stored well, FVB RBCs were mixed with GFP.B6 RBCs prior to storage (see Figure 1A for experimental design). As GFP.B6 RBCs are on a B6 genetic background, they represent “good-storing” B6 RBCs with an easily visible internal marker. To control for any changes in volume or composition as a result of the mixing, control groups included GFP.B6 RBCs mixed with wild-type B6 RBCs. After 7 days of storage, the mixtures were spiked with a small amount of fresh HOD RBCs; HOD mice express a transgenic antigen on RBCs that can be visualized with a monoclonal antibody conjugated to a color distinct from GFP¹⁰. Thus, HOD RBCs represent a fresh RBC internal tracer population. Calculating circulation of GFP.B6 RBCs as a function of HOD RBCs controls for any differences in final volume of RBCs transfused or other systemic differences, which would affect both populations equally. Representative flow cytometry plots are shown (Figure 1B.). Wild-type FVB or B6 RBCs were co-stored with the test GFP.B6 RBCs at ratios of 1:1, 1:2 or 1:11; such that the effect of titrating out the amount of “bad-storing” RBCs added to the mixture could be assessed. In all cases, the total RBC storage volume was held constant (Figure 1C).

GFP.B6 RBCs stored with control B6 RBCs had 24-hr recoveries greater than 80%, consistent with previous reports that B6 RBCs store well (Figure 1D). In contrast, 24-hour recoveries of GFP.B6 RBCs was significantly decreased as a result of co-storage with FVB RBCs. This decrease was observed in a dose dependent fashion. Co-storage with FVB RBCs

resulted in a decrease in 24-hour recoveries with a mean difference ($\pm$ 95% confidence intervals) of 4.1 ($\pm$ 2.9)% at the 1:1 ratio, a mean difference of 25.8 ($\pm$ 2.2)% at the 1:2 ratio and a mean difference of 50.5 ($\pm$ 3.8)% at the 1:1 ratio. This was not an artifact associated with the mechanical addition of the FVB RBCs to the GFP.B6 RBCs, since wild-type B6 RBCs were added in the same fashion and at the same ratios to GFP.B6 RBCs. These data demonstrate that when co-stored, poorly-storing RBCs can transfer their storage properties to RBCs that otherwise store well.

Storage Effects Are Transferred Bi-directionally Between RBCs that Store Well and Those that Store Poorly

Because GFP transgenic mice are not available on a genetic background with RBCs known to store poorly (e.g. FVB or other strains⁸), the inverse of the experiment shown in figure 1 is not possible. However, we have observed that stored RBCs from B6xFVB F1 mice have 24-hour recoveries intermediate between B6 and FVB parental strains (data not shown). By crossing GFP.B6 mice with FVB mice, one generates mice with a B6xFVB F1 genetic background that express GFP in RBCs (GFP.F1). GFP.F1 RBCs were stored with either B6 or FVB RBCs (see Figure 2A for a schematic of experiment). As a control for manipulations and volumes, GFP.F1 RBCs were also stored with B6xFVB F1 RBCs that had no GFP. All stored RBCs were transfused into B6 recipients.

For the representative experiment shown in Figure 2B, storage of GFP.F1 by itself (along with control non-GFP F1 RBCs) had a mean 24-hour recovery of 62.4%. Storage with B6 RBCs significantly increased 24-hour recoveries of GFP.F1 RBCs to 96.7% ($p < 0.0001$). Conversely, storage with FVB RBCs significantly decreased 24-hour recoveries of GFP.F1 RBCs to 36.7% ($p < 0.0001$). Following 3 replicate experiments, the mean ($\pm$ 95% confidence intervals) decrease in 24-hour recoveries for GFP.F1 co-stored with FVB compared to syngeneic F1 RBCs was 21.4($\pm$ 3.4)%, and the mean increase ($\pm$ 95% confidence intervals) in 24-hr recoveries for GFP.F1 co-stored with B6 compared to F1 RBCs was 24.5($\pm$ 4.6)%. Together with the data presented in Figure 1, these findings indicate that different populations of RBCs can affect each other's storage, in either a positive or a negative direction.

Transfer of RBC storage biology does not occur across a semi-permeable membrane

To better characterize the nature by which RBCs affect each other during storage, transwell experiments were carried out using a semi-permeable membrane with 0.4 μ m pores, thereby preventing cell-to-cell contact of intact RBCs but allowing the free exchange of smaller microparticles and molecules (Figure 3A and B). The ability of small molecules to cross the membrane was confirmed by adding anti-K1 antibody to the top well and K1+ RBCs (K1-GFP) to the bottom well. Staining with anti-IgG demonstrated that anti-K1 IgG had passed through the membrane and bound to RBCs in the bottom chamber (Figure 3C).

Four separate transwell storage conditions were carried out (Figure 3D, bars paired by color). In each case, both the top well and the bottom well were transfused into B6 recipients, and 24-hour recoveries determined as above. Two separate conditions were set up as controls. In the first, GFP.B6 RBCs were stored in the top well and GFP.B6 + B6 RBCs

were stored in the bottom well. The second was the inverse, with GFP.B6 + B6 RBCs in the top well and GFP.B6 in the bottom well. Close to 100% 24 hour recoveries were observed after transfusion of the top well or the bottom well, for either condition. These controls demonstrate that storage does not vary significantly as a result of being in the top well or bottom well of the transwell system.

The other two conditions were such that GFP.B6 was in one well, while the other well had GFP.B6 and FVB RBCs stored together. In one case the GFP.B6 was in the top chamber with GFP.B6+FVB in the bottom chamber, the other case was the opposite. In both cases, GFP.B6 stored alone had recoveries comparable to the control GFP.B6 whereas GFP.B6 stored with FVB RBCs had poor 24-hour recoveries. Together, these findings indicate that whatever is responsible for transferring storage phenotype does not transmit across a membrane with a 0.4 μm size limit. The lack of transmission across wells was not due to the biology generated from the poorly storing RBCs not being present, as the effect of FVB RBCs upon GFP.B6 RBCs was observed in the communicating well.

One potential caveat to the interpretation of the transwell experiments is that the poor storage phenotype may have failed to transmit across the membrane because it was consumed by the B6 RBCs costored with the FVB RBCs. Likewise, the above experimental design does not address the transfer of substances improving RBC storage. To further address these issues, B6 RBCs and FVB RBCs were stored in the top or bottom wells (Figure 4A). To allow visualization of the non-GFP RBCs, GFP.F1 recipients were used for post-storage RBC recoveries (Figure 4A). An example of the flow cytometry allowing such enumeration is shown (Figure 4B). B6 or FVB RBCs were stored in top or bottom wells in the different conditions indicated (Figure 4C). There was no difference in B6 RBC 24-hr recoveries, after storage with FVB RBCs vs. B6 RBCs in the transwell system; in both cases the B6 RBCs had close to 90% 24-hr recoveries (Figure 4C). This was not because FVB RBCs didn't store poorly in the transwell system, as FVB 24-hour recoveries were approximately 35%. The same trend was observed regardless of whether B6 or FVB RBCs were stored in the top vs. the bottom well. However, storage in the bottom well was worse for either population, as demonstrated by lower 24-hour recoveries from bottom well populations when RBCs from the same donor strain were stored in both top and bottom wells.

Discussion:

The current findings presented herein indicate a communication between RBCs that are stored together in the same vessel, such that RBCs that store poorly decrease quality of RBCs that would otherwise have stored well and those that store well “rescue” the storage of those that would otherwise store poorly. This communication does not occur when particles larger than 4 μm are prevented from crossing between populations, suggesting that small molecules are not sufficient for the effect and suggesting that direct cell-cell contact may be required. However, we cannot rule out that small molecules may be responsible, but that they are too labile to survive long enough to diffuse across the membrane.

The mechanism of the observed communication is unclear. Metabolomics analysis of multiple strains (including B6 and FVB) have shown that lipid metabolism and degradation (most notably lipid oxidation) emerged as the strongest correlate with poor 24-hr recoveries^{8,14}. Strains that stored better not only had less lipid oxidation but also higher levels of antioxidants and antioxidant pathways involved in prevention and reversal of lipid oxidation (e.g. glutathione and tocopherol (vitamin E))⁸. Thus, it is possible that lipid peroxidation chain reactions transfer from oxidized RBCs to unoxidized RBCs; however, there are no data to support or refute this possibility. Moreover, lipid peroxidation would not give an obvious explanation to how B6 RBCs improve storage of FVB RBCs, unless anti-oxidant pathways somehow quenched peroxidation chain reactions. Microparticles are also known to accumulate during the storage of murine RBCs, as they do with humans, and fewer microparticles correlate with better storage¹⁵. As microparticles can have a wide range of sizes, which may or may not traverse the transwell membrane used, future studies may include such investigations. In any case, follow up studies will clearly be required to elucidate greater detail regarding the mechanism of the observed phenomenon. Nevertheless, the current findings demonstrate that stored RBCs affect the biology of other RBCs with which they are stored. To the best of our knowledge, this is a novel observation.

Although the current studies focus on RBCs stored in vitro, it raises the possibility that the same type of biology may occur in vivo, between qualitatively distinct RBC populations. For example, transfusion of damaged RBCs may have an effect upon circulating RBCs of the recipient. Additionally, RBC cross talk may affect biology outside of the context of transfusion. RBCs live approximately 120 days in humans and 55 days in mice. While the exact mechanisms by which senescent RBCs are cleared remains undetermined, numerous reports have demonstrated a progressive accumulation of damage over the life-span of RBCs. Thus, it is possible that older RBCs hasten the aging of younger RBCs, through direct interaction in circulation. This may occur in healthy humans, but also in disease states where RBC metabolism and senescence is likely altered (e.g. diabetes, sickle cell disease, glucose 6 phosphate dehydrogenase (G6PD) deficiency, etc.). G6PD deficiency is a special case. Due to X chromosome inactivation, approximately 50% of mature erythrocytes that leave the bone marrow are normal and 50% are G6PD deficient in females who are heterozygous for G6PD deficiency¹⁶. Accordingly, they will have distinct populations of RBCs circulating, which may affect each other. Paroxysmal nocturnal hemoglobinuria is a similar situation, although the defect is around GPI anchored proteins, the defect is on one population of RBCs with other RBCs being normal in the same patient¹⁷. Thus, the current studies raise the possibility that a cross-talk amongst qualitatively distinct RBCs in a given individual's circulation may affect general RBC biology.

Currently, RBCs are stored only as single donor units, and there is no mixture of RBCs from different donors during storage. However, in recognition of donor-to-donor differences in RBC storage, it has been suggested that donor RBCs might be pooled prior to storage, so as to average out the storage effects¹⁸. Similarly, research on new human RBC storage systems may employ a "pool and split" strategy, by which donors RBCs are pooled and then split into different storage conditions, so as to decrease the likelihood that one donor's particular biology will skew results. The current studies raise the possibility that these approaches may not only average out RBC units by differential storage of different RBCs in the same bag,

but may also do so through altering the biology of the whole RBC population. This distinction may be of considerable importance in considering the biology of the RBC storage. However, as the current studies are restricted to a murine model, such speculation depends upon a similar biology occurring in humans, which is as of yet untested.

In summary, the data contained herein directly challenge the notion that biology of RBC storage is exclusively intrinsic to the RBC, that the RBCs that make up a stored unit are a population of individual events simply undergoing changes in the presence of each other. In contrast, the data contained herein indicate that RBCs function as a population, with individual RBCs affecting each other. In other words, stored RBCs behave as a multicellular organism, and not as a population of isolated intrinsic events. Determination of whether these findings extend to RBC storage biology in other species and/or to in vivo RBC biology outside of the context of RBC storage, will require ongoing study.

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Figure 1:

Effect of co-storing RBCs of different storage phenotypes. **(A)** Schematic of experimental design. **(B)** Representative flow cytometry analysis of stored RBCs. GFP+ RBCs and GFP– RBCs are visualized in the lower right and lower left quadrants, respectively; whereas HOD RBCs are visualized in the upper left quadrant (left panel). In untransfused mice, the vast majority of events are in the lower left quadrant (middle panel). In transfused mice, GFP+ and HOD RBCs are easily visualized in the lower right and upper left quadrants, respectively (right panel); stored GFP– RBCs are not quantifiable as recipient RBCs are present. **(C)** Volumes of RBCs stored in each tube. **(D)** 24-hr recoveries of GFP.B6 RBCs under each storage condition. Grey bars show GFP RBCs stored with FVB RBCs; white bars indicate GFP RBCs stored with B6 RBCs. The experiment was repeated 3 times with similar results; one representative experiment is shown (n=3 mice for each group) * indicates a statistically significant difference with a p-value < 0.001; ** indicates a difference with a p-value < 0.0001. See text for meta-analysis of all 3 replicates.

Figure 2:

Co-storage of RBCs transfers both good and bad storage properties. **(A)** Schematic of experimental design. **(B)** 24-hr recoveries of GFP.F1 RBCs under each storage condition. Blue circles show GFP.F1 RBCs stored with B6 RBCs; green squares indicate GFP.F1 RBCs stored with F1 RBCs; and orange triangles indicate GFP.F1 RBCs stored with FVB RBCs. The experiment was repeated 3 times with similar results; one representative experiment is shown (n=4 mice for each group) ** indicates a statistically significant difference with a p-value < 0.0001. See text for meta-analysis of all 3 replicate experiments.

Poorly storing RBCs do not affect RBCs that otherwise store well, when separated by a semi-permeable membrane. **(A)** Schematic of experimental design **(B)** Cartoon and picture of the transwell system showing separation of the top and bottom wells by a membrane with 0.4μm pores. **(C)** Representative flow cytometry analysis demonstrating that small molecules can pass through the semi-permeable membrane in the transwell system. Results are shown for unstained B6 and K1 RBCs (left panel); RBCs stained with anti-K1 (middle panel) and RBCs stained with anti-K1 using a transwell to separate RBCs from antibody (right panel) **(D)** 24-hr recoveries of GFP.B6 under each storage condition. Paired colored bars represent cells from the same transwell, with green shading to indicate which well (top or bottom) was transfused. These data represent means $\pm$ stdev from 3 experiments. * indicates a significant difference from other conditions with a p-value < 0.05 .

Figure 4:

Neither good nor poor storage properties are transmitted when RBCs are costored separated by a 0.4um cutoff membrane. **(A)** Schematic of experimental design **(B)** Representative flow cytometry analysis of stored RBCs. GFP+ RBCs and GFP- RBCs are visualized in the lower right and lower left quadrants, respectively; whereas HOD RBCs are visualized in the upper left quadrant. In untransfused mice (middle panel) the vast majority of events are in the lower right quadrant. In transfused mice (right panel), GFP- and HOD RBCs are easily visualized in the lower left and upper left quadrants respectively. **(C)** 24-hr recoveries of non-green RBCs under each storage condition. Paired colored bars represent cells from the same transwell, with orange shading to indicate which well (top or bottom) was transfused. These data represent means $\pm$ stdev from 3 experiments. * indicates a significant difference from other conditions with a p-value < 0.05. These data represent means $\pm$ stdev from 3 experiments.