SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Berkeley Homozygous and Berkeley-Hemizygous Genetics

As previously described by Pászty and colleagues, fragments of human DNA encoding α and β^S globin genes linked to a locus control region were injected into C57BL/6 mouse embryos to create humanized transgenic mice (Paszty et al., 1997). These mice were subsequently bred with mice heterozygous for either mouse α-globin or mouse β-globin deletion mutation. The offspring heterozygous for the human $\alpha\beta^S$ transgene and heterozygous for both knockout murine α and β globin genes were interbred to produce offspring heterozygous for the human $\alpha\beta^S$ transgene and homozygous for both murine α and β globin deletion mutations (Ryan et al., 1997, Paszty et al., 1997). These mice were back crossed with C57BL/6 mice for eight generations, and one additional generation following arrival at the Jackson laboratories (Bar Harbor, ME).

The current breeding strategy utilized by Jackson Laboratories for generation of humanized transgenic sickle mice hemizygous for the murine β globin allele is as follows: male mice homozygous for α and β globin gene deletions (mα -/- and mβ -/-) and expressing exclusively human globin ($h\beta^S$) transgenes are mated with female mice homozygous for the mouse α globin deletion and hemizygous for the mouse β globin allele (mα -/- and mβ-/+) and carrying the hβS transgene.(Noguchi et al., 2001) Several mouse strains were utilized in the generation of the transgenic mice and murine globin knockout mice. Therefore, the stock background of the Berkeley and Berkeley-hemizygous strains is a mixture of FVB/N, 129, DBA/2, Black Swiss,

and >50% C57BL/6 genomes (Szczepanek et al., 2012). For this reason, we used C57BL/6 mouse strain as a control group in murine experiments.

Blood Collection and Storage

To replicate similar conditions for human RBCs, peripheral blood was also collected with informed consent from HbAA African American and sickle cell trait donors under an approved Institutional Review Board protocol. Peripheral blood was processed and stored under similar conditions for 39-42 days prior to experiments.

In vitro Hemolytic Assays

The hypotonic solution used for the osmotic fragility assay was the Pink Test solution containing hypotonic Bis-Tris buffer containing 25 mmol/L sodium chloride, 70 mmol/L Bis–Tris buffer, and 135 mmol/L glycerol; adjusted to pH 6.6 as previously established (Vettore et al., 1984, Kanias et al., 2013). Mechanical stress was induced by shaking RBC suspensions in the presence of one metal bead (3/32") for 3 h on a plate shaker as previously established (O. K. Baskurt, 2007, Raval et al., 2010, Kanias et al., 2013). *In vitro* hemolytic hemolytic propensity of human (h-HbAA and h-HbAS) and murine RBCs (m-HbAA and m-HbAS) were evaluated to validate the suitability of Berkeley hemizygous mouse model to study human RBC transfusions. Complete blood counts were obtained by a commercial blood counter (Hemavet, Drew Scientific, Miami FL).

Splenectomy

Splenectomy was carried out under isoflurane anesthesia at a core temperature of 37°C. A 1cm incision in the peritoneal wall was made and the spleen was gently pulled onto the exterior surface of the peritoneum. The artery was tied off with a 3-0 suture by looping the suture through the mesentery making a single knot at the tip of the spleen. Mesentery and connective tissue were cut away with a cauterizer and the spleen removed. The peritoneal wall and the skin were closed with two separate sutures. Sham operation consisted of incisions and manipulation without any tissue removal (Reeves, 1991, Services, 2014). Methods for splenic macrophage and Kupffer cell depletion were modified from those previously published (Hod et al., 2010, Ramos et al., 2013). Briefly, mice were pre-treated with clodronate liposomes (Brentwood, TN) to deplete macrophages or with liposome vehicle (2 mg/kg i.p.) 24 h prior to injection.

Scanning Electron Microscopy

Human and Mouse RBCs were fixed in excess 2·5% glutaraldehyde for Scanning Electron Microscopy. RBCs were fixed in 2·5% glutaraldehyde for 1 h, washed with PBS, and post-fixed in aqueous 1% OsO4 for 1 h. Samples were washed 3X in PBS and dehydrated through a graded ethanol series (30%-100%) and washed with absolute ethanol before drying in hexamethyldisilizane solution. Samples were allowed to air dry on coverslips and affixed with double sided tape and mounted onto aluminum stubs, sputter coated with 6 nm of gold/palladium (Cressington Auto 108, Cressington, UK), and viewed (Magnification 2500X) in a JEOL JSM-6335F scanning electron microscope (Peabody, MA) at 3 kV with the SEI detector. Echinocyte formation was determined by manual counting using distinct outward projections and loss of spherical morphology as an inclusion criterion.

Histology and immunohistochemistry

Liver and spleen were harvested 24 h following clodronate or liposome vehicle treatment (2mg/kg i.p.) and fixed in 4% paraformaldehyde for 4h and paraffin embedded for sectioning. Sections were de-paraffinized and immunostained with anti-F4/80 monoclonal antibody and biotinylated anti-rat antibody. F4/80 staining was detected and developed using 3, 3'-diaminobenzidine substrate kit from Vector Labs (Burlingame, CA). Images were taken using Axiophot Microscope (Zeiss, West Germany).

In situ imaging and Quantification of Red Blood Cells Following Transfusion

Stored murine RBCs were labeled with Cy3 mono-reactive dye (GE Healthcare Life Sciences, Marlborough, MA) immediately prior to transfusion and visualized by confocal imaging. For in situ imaging of stored human RBCs transfused into murine recipients, unlabeled human RBCs were transfused and immunolabeling of human RBCs was performed using a FITC-conjugated mouse-anti-human glycophorin A (CD235a) antibody (BD Pharmingen, 561017). 2'(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2, 5'-bi-1H-benzamidazole) (Hoechst) (Sigma-Aldrich Co., B-2883) stain was used to visualize the nucleus, and rhodamine phalloidin (Invitrogen, Live Technologies, R415) used to visualize the actin-cytoskeleton.

The liver, spleen and kidney were harvested 2 h post-transfusion and fixed with 2% paraformaldehyde for 2 h and transferred into 30% sucrose solution overnight at 4 °C. Organs were frozen via submersion in liquid nitrogen cooled 2'-methylbutane and stored at -80 °C until sectioning. Organs were sectioned at 7-micron thickness. Images were taken with a Nikon A1 confocal microscope and NIS-Elements (Nikon Instruments Inc., Melville, NY). Settings for image acquisition (PMT, transmissivity and exposure time) were determined using a negative staining control and applied universally during image acquisition. The number of RBCs and

nuclei were determined by a fluorescence intensity-based threshold, using the average intensity of the negative staining control sample as guide, using NIS Elements software (Nikon, Melville NY). Sequestration of human or murine RBCs was assessed using segmentation analysis and object counting for FITC or cy3 positive cells and expressed relative to total DAPI positive cell count.

Ektacytometry

A Technicon osmotic gradient ektacytometer (Technicon Instrument Corp, Tarrytown, NY) was used to measure RBC deformability at isotonic and varying osmolality concentrations as previously described. A 20 centipoise (cP) carrier solution pH 7.4 was made from 31-g/L polyvinylpyrrolidone (PVP) (Sigma, PVP360), 0·24g/L potassium phosphate monobasic (Sigma, S0751) and 0·90g/L potassium phosphate dibasic (Fisher, BP332). Sodium chloride (Sigma, S7653) was used to make solutions of 40 mOsm (low osmolality), 290 mOsm (isotonic osmolarity) and 750 mOsm (high osmolality) relative to RBC intracellular content. RBCs (150 µl) were diluted into a 4 ml sample solution and pumped into the ektacytometer where they were exposed to an increasing osmotic gradient produced by mixtures of low and high osmolarity solution. The cells are exposed to shear of 160 dynes/cm^2 in a couette viscometer and the diffraction pattern recorded. Data was fitted using a custom MATLAB program (R2012a, Mathworks) with a Savitsky-Golay noise reduction filter of polynomial order.

Intravascular hemolysis measurements

Murine blood was sampled via tail vein at 5 min, 1 h and 4 h post-transfusion. Approximately 100 µL was collected and spun for 3 minutes at 800 g to sediment RBCs. Supernatant was

carefully removed for free hemoglobin measurements. Free Hb concentrations were determined using spectral deconvolution and least squares deconvolution.(Donadee et al., 2011)

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Complete Blood Count (CBC) measurements show no differences in the size distribution and hemoglobin content of human HbAA and HbAS RBCs from HbAA and HbAS donors. HbAA RBCs (n=11), HbAS RBCs (n=8), HbSS RBCs (n=8) obtained from volunteers. Data plotted show box and whiskers, min to max with line at median.*$P<0.05$ using two way ANOVA.

Supplementary Figure S2. HbAS RBC osmotic resilience is not a function of storage. Normalized osmolarity maximum Index (Osm Max) of human RBCs (h-RBC, n=3 per group) and murine RBCs (m-RBCs), where each sample is pooled from n=11 mice donor RBCs measured as technical replicate were measured at the beginning and end of storage. Experiments with murine RBCs were repeated once under similar conditions. The data was fit using a custom MATLAB program (R2012a, Mathworks) with a Savitsky-Golay noise reduction filter of polynomial order. The results are presented as mean ± SEM.***$p<0.01$; ****$P<0.001$; *****$P<0.0001$* analyzed by unpaired t-test, GraphPad Prism 6.0.

Supplemental Figure S3. Fluorescent labels do not interfere with RBC post transfusion survival. (a) Representative graphs showing the gating strategy and tracking of transfused unlabeled RBCs in a mouse recipient expressing enhanced Green Fluorescent Protein on hematopoietic cells. The panels show that there is no overlap in the fluorescence between unlabeled and GFP+ RBCs allowing quantitation enumeration of transfused unlabeled RBCs. **(b)** Fresh unlabeled m-HbAA and m-HbAS RBCs were transfused into two separate groups of GFP+

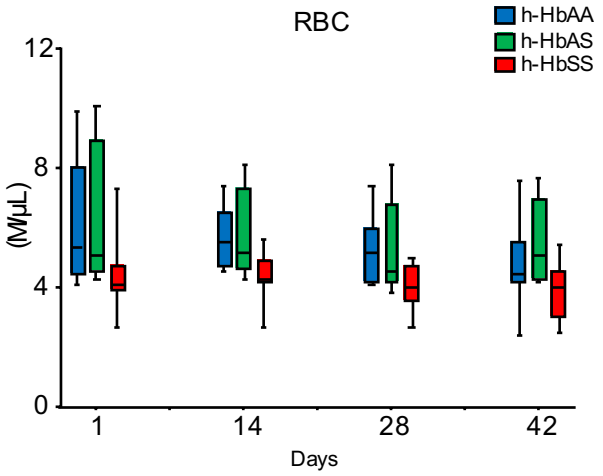
mice recipients (n=5 per group). **(c)** Stored unlabeled m-HbAA and m-HbAS RBCs were transfused into two separate groups of GFP+ mice recipients (n=8 per group). The results are presented as mean ± SD.**p<0.01; ****P<0.0001* analyzed by 2Way ANOVA, GraphPad Prism 6.0.

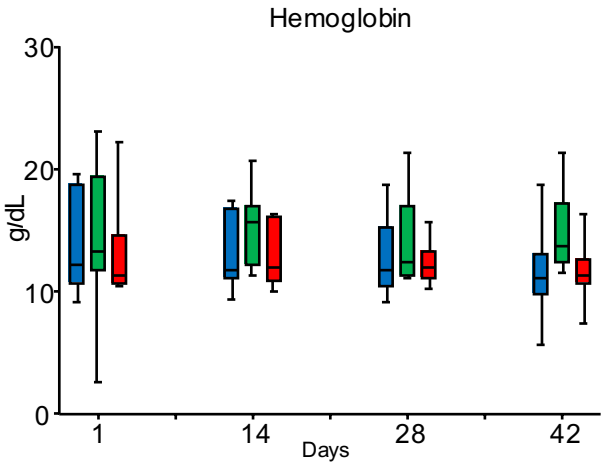
Supplementary Figure S4. Switching fluorescent labels do not alter RBC post transfusion survival patterns. **(a)** To verify that fluorescent labels did not alter RBC post-transfusion survival differentially, two separate groups of WT C57BL/6 mice recipients (n=6 per group) were transfused with either DiD-labeled stored m-HbAA RBCs or DiD-labeled stored m-HbAS RBCs and PTR analyzed. **(b)** To further confirm that fluorescent labels did not interfere with RBC post transfusion survival, fluorescence labeling was switched so that m-HbAA RBCs were labeled with DiD and m-HbAS RBCs were labeled with DiI and transfused as a 50:50 mixture into the same WT recipients (n=5). The results are presented as mean ± SD.**p<0.05; **p<0.01; ***p<0.001;* analyzed by 2Way ANOVA, GraphPad Prism 6.0.

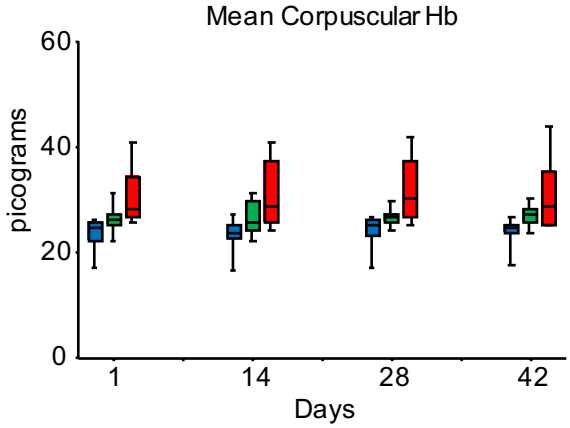
Supplementary Figure S5. **There is no difference in the post transfusion survival of conventionally stored h-HbAS RBCs following 39-42 day storage compared to glass-stored h-HbAS RBCs. (a)** WT mouse recipients, n=5 (indicated by solid triangles) were transfused with conventionally stored human HbAS RBCs following 41-day storage. WT mouse recipients, n=6 (indicated by open squares) were transfused with 39-day glass stored human HbAS RBCs.

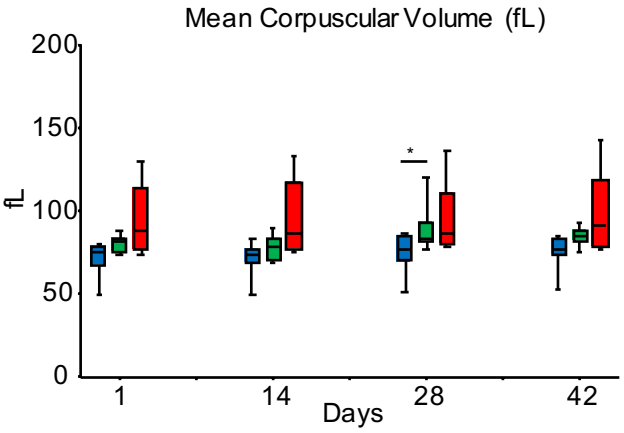
Supplementary Figure S6. Post-transfusion recovery stored m-HbAA RBCs following clodronate depletion of macrophages or splenectomy. **(a)** WT recipient mice were transfused with 11-day stored m-HbAA RBCs 24 h following clodronate (n=3) or PBS liposome (n=3) injection (2 mg i.p.). **(b)** WT recipient mice were transfused with 11-day stored m-HbAA RBCs 5 d following splenectomy (n=4) or sham procedure (n=6) Results presented are mean ± SD.***P<0.05;*****p<0.001;** analyzed by 2Way ANOVA, GraphPad Prism 6.0.

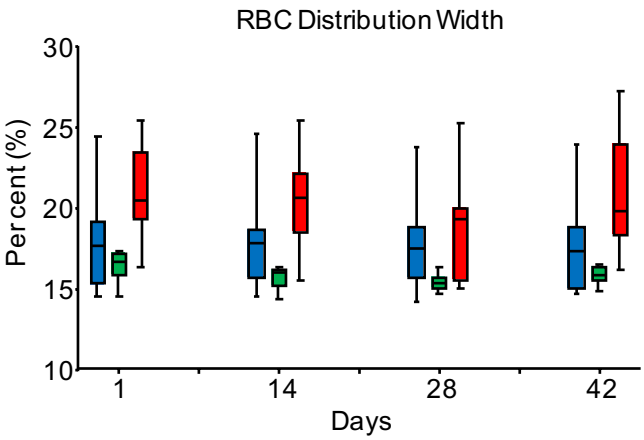
Supplementary Figure S7. **There is no difference between the plasma or urinary cell- free hemoglobin following stored m-HbAA or m-HbAS RBC transfusion. (a)** To assess the contribution of intravascular hemolysis to the observed reduced post transfusion survival of stored HbAS RBCs, WT C57BL/6 mice were transfused with equimolar concentrations of m-HbAA RBCs (n=15 recipients) or m-HbAS RBCs (n=15 recipients), cell free hemoglobin (3090 µM) (n=15 recipients) or PBS (n=4 recipients). At 5 min, 1 h and 4 h following transfusion, mice in each group were bled via tail vein using a microvette to obtain 60-100 µl of whole blood. **(b)** Accumulation of cell free hemoglobin in the bladder is rapidly cleared following transfusion. In a separate experiment, WT C57BL/6 mice were transfused with equimolar concentrations of m-HbAA RBCs (n=15 recipients) or m-HbAS RBCs (n=15 recipients), cell free hemoglobin (n=15 recipients) or PBS (n=4 recipients). At 5 min, 1 h and 4 h following transfusion, mice in each group were immediately euthanized for urine collection via bladder puncture. Whole blood or urine samples were spun for 3 minutes at 800 g to sediment RBCs. Supernatant was carefully removed for free hemoglobin measurements. Hb concentration levels were determined using spectral deconvolution and analyzed by a least squares method.

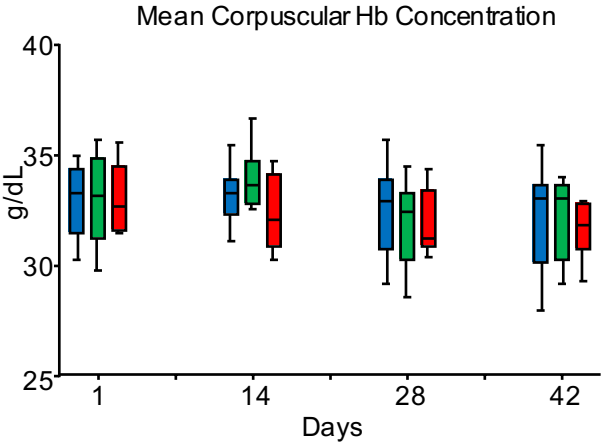

RBC
h-HbAA
h-HbAS
h-HbSS
12
8
4
0
(M/µL)
1
14
28
42
Days

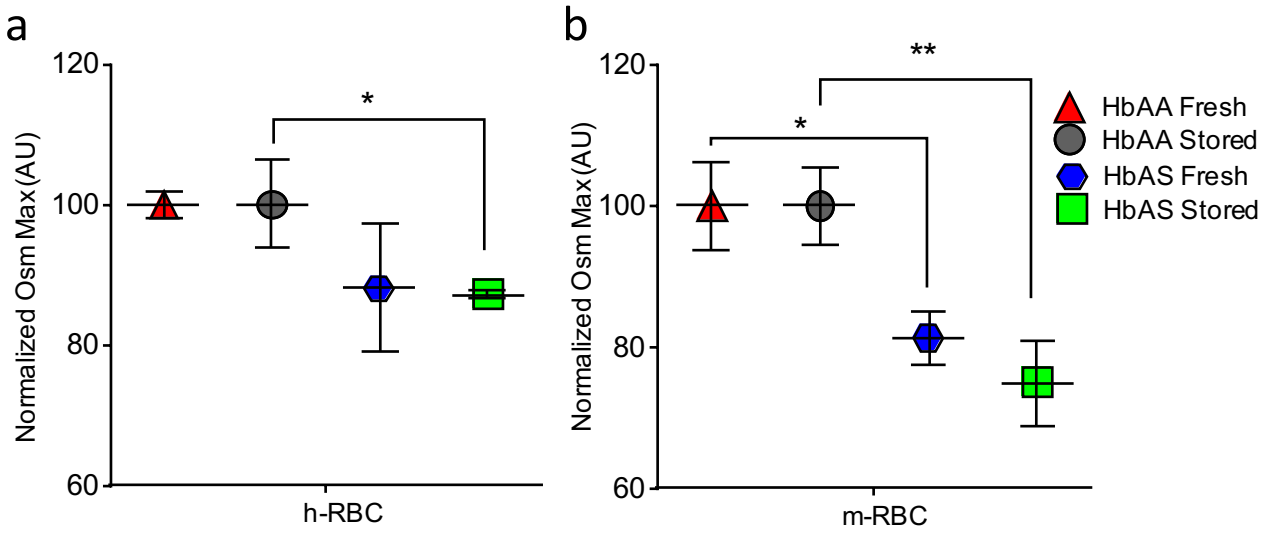

Hemoglobin
30
20
10
0
g/dL
1
14
28
42
Days

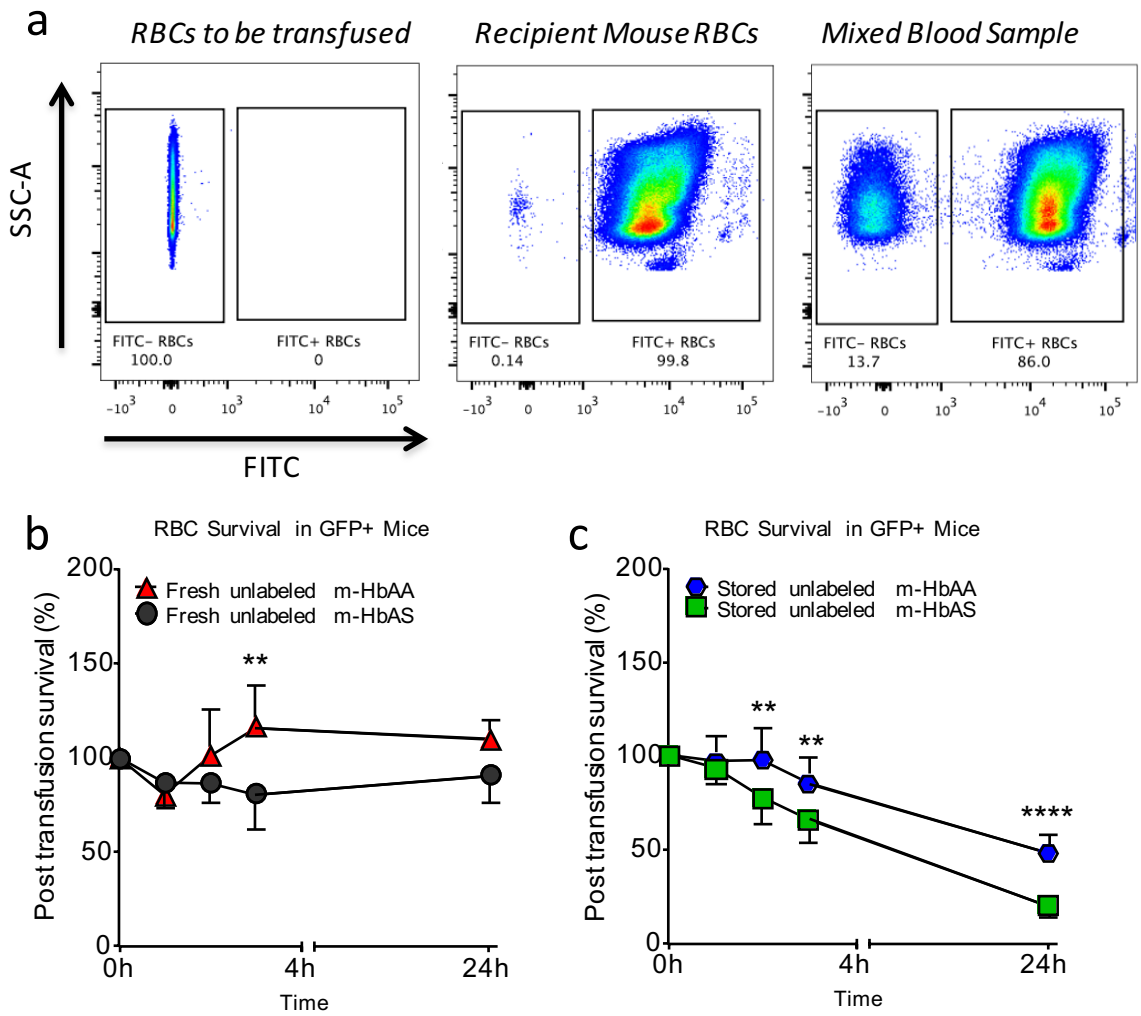

Mean Corpuscular Hb
60
40
20
0
picograms
1
14
28
42
Days

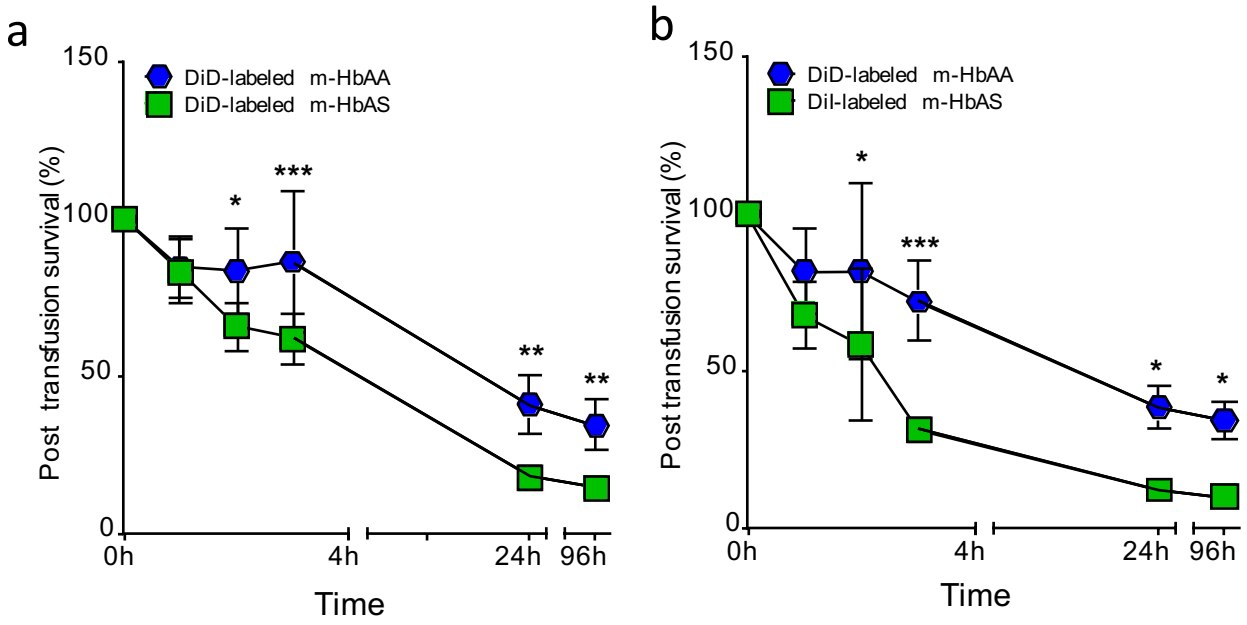

Mean Corpuscular Volume (fL)
200
150
100
50
0
fL
*
1
14
28
42
Days

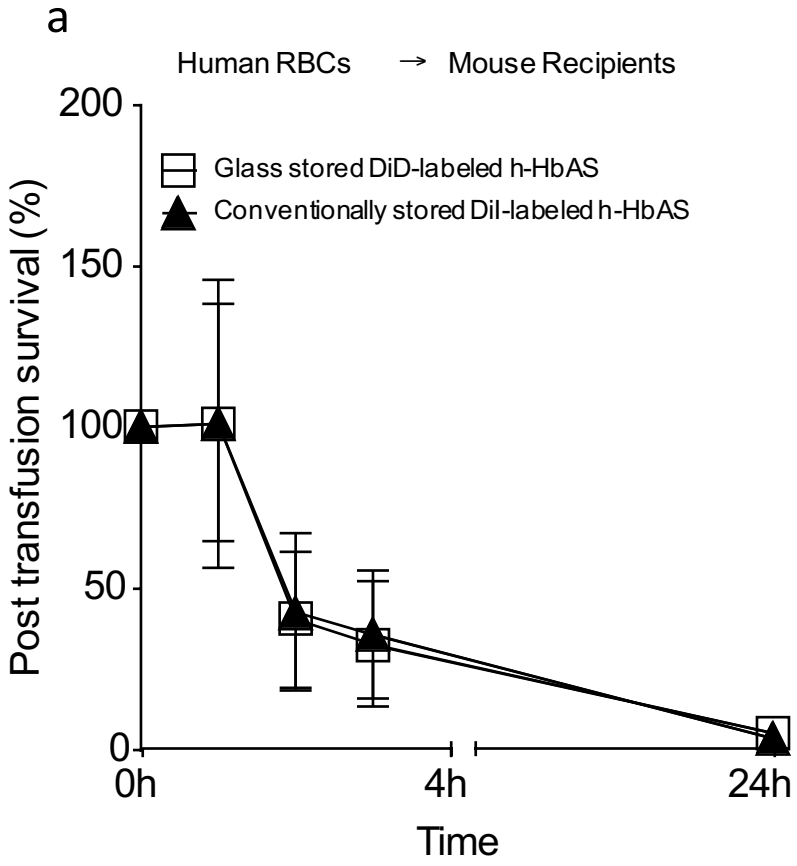

RBC Distribution Width
30
25
20
15
10
Percent (%)
1
14
28
42
Days


Mean Corpuscular Hb Concentration
40
35
30
25
g/dL
1
14
28
42
Days

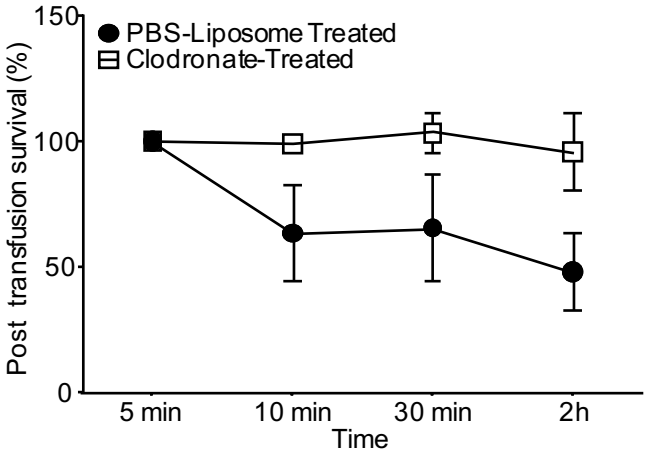

a
120
100
80
60
Normalized Osm Max (AU)
*
h-RBC
b
120
100
80
60
Normalized Osm Max (AU)
*
**
m-RBC
HbAA Fresh
HbAA Stored
HbAS Fresh
HbAS Stored


a
RBCs to be transfused
Recipient Mouse RBCs
Mixed Blood Sample
SSC-A
FITC
FITC- RBCs
100.0
FITC+ RBCs
0
FITC- RBCs
0.14
FITC+ RBCs
99.8
FITC- RBCs
13.7
FITC+ RBCs
86.0
b
RBC Survival in GFP+ Mice
Fresh unlabeled m-HbAA
Fresh unlabeled m-HbAS
Post transfusion survival (%)
Time
0h
4h
24h
**
c
RBC Survival in GFP+ Mice
Stored unlabeled m-HbAA
Stored unlabeled m-HbAS
Post transfusion survival (%)
Time
**
**
****

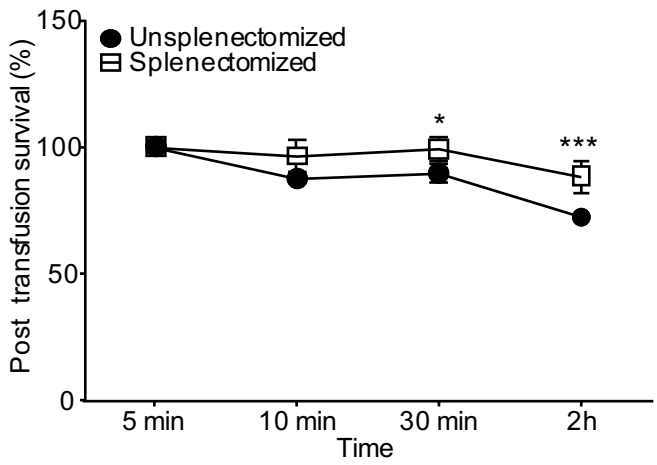

a
150
100
50
0
DiD-labeled m-HbAA
DiD-labeled m-HbAS
Post transfusion survival (%)
*
***
**
**
0h
4h
24h
96h
Time
b
150
100
50
0
DiD-labeled m-HbAA
DiI-labeled m-HbAS
Post transfusion survival (%)
*
***
*
*
0h
4h
24h
96h
Time

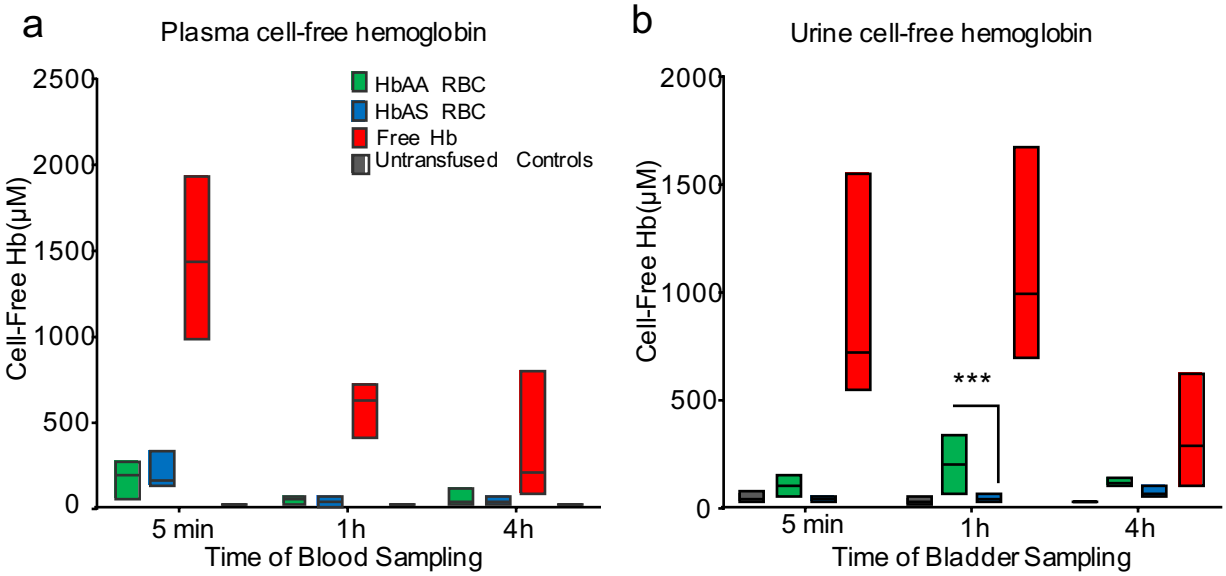

a
Human RBCs → Mouse Recipients
Glass stored DiD-labeled h-HbAS
Conventionally stored DiI-labeled h-HbAS
Post transfusion survival (%)
200
150
100
50
0
0h
4h
24h
Time

a


PBS-Liposome Treated
Clodronate-Treated
Post transfusion survival (%)
150
100
50
0
5 min
10 min
30 min
2h
Time

b


Unsplenectomized
Splenectomized
Post transfusion survival (%)
150
100
50
0
*
***
5 min
10 min
30 min
2h
Time


a
Plasma cell-free hemoglobin
HbAA RBC
HbAS RBC
Free Hb
Untransfused Controls
Cell-Free Hb(μM)
2500
2000
1500
1000
500
0
5 min
1h
4h
Time of Blood Sampling
b
Urine cell-free hemoglobin
Cell-Free Hb(μM)
2000
1500
1000
500
0
***
5 min
1h
4h
Time of Bladder Sampling