THE EFFECT OF SICKLE HEMOGLOBIN MUTATION ON RED BLOOD CELL STORAGE INTEGRITY AND POST TRANSFUSION VIABILITY

by

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Red blood cells (RBCs) undergo progressive changes in storage, collectively referred to as the storage lesion that is associated with increases in storage and post-transfusion hemolysis. Transfusion of blood at the limits of approved storage time is associated with lower RBC post-transfusion recovery and hemolysis, which increases plasma levels of cell-free hemoglobin and iron, proposed to induce endothelial dysfunction and impair host defense, respectively. Of relevance to this study, there is noted variability among donors in the intrinsic rate of storage changes and in RBC post-transfusion recovery, suggesting that genetic determinants modulate this process. Here, I test a common genetic variable in our donor pool, sickle cell trait, present in about 8% of African Americans. Using banked human RBCs and those from a humanized transgenic sickle cell mouse, I show that sickle cell trait in both species produces storage time-dependent reductions in osmotic fragility and membrane deformability, increased storage hemolysis, and significantly reduced post-transfusion recovery in mice. Furthermore, the underlying mechanism of reduced HbAS RBC post-transfusion recovery is unrelated to macrophage uptake, reticulo-endothelial system or intravascular hemolysis, but rather by RBC intravascular sequestration in the spleen, kidney and liver. Collectively, these findings indicate that changes in HbAS RBC membrane deformability properties that are aggravated during storage lead to enhanced mechanical entrapment in tissues and rapid clearance of transfused HbAS RBCs from the circulation. These preclinical studies raise provocative questions about the use of blood from sickle cell trait individuals, particularly at the limits of approved storage.

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1 Samuel 7:12 -Thus far The Lord has helped us.

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Dedicated to King David

LIST OF ABBREVIATIONS

AAPH	2, 2'-Azobis (2-amidinopropane) dihydrochloride
ANOVA	Analysis of Variance
BFU-E	Burst-Forming Unit -Erythroid
CDC	United States Centers for Disease Control and Prevention
CPD	Citrate-Phosphate-Dextrose
CPD-A	Citrate-Phosphate-Dextrose with Adenine
DAPI	4', 6-Diamidino-2-Phenylindole Dihydrochloride
DI	Deformability Index
DiI/ DiD	(2*Z*)-2-[(*E*)-3-(3, 3-dimethyl-1-octadecylindol-1-ium-2-yl) prop-2-enylidene]-3, 3- Dimethyl-1-octadecylindole; perchlorate (DiD DiIC18 (5) and DiI (DiIC18 (3))
DMSO	Dimethyl Sulfoxide
ERD	Exercise Related Death
FDA	United States Food and Drug Administration
Fe	Iron

GFP	Green Fluorescent Protein
GMP	Granulocyte-Macrophage Progenitors
H_2O_2	Hydrogen Peroxide
Hb	Hemoglobin
HbAA	Hemoglobin A and Hemoglobin A complexes found in healthy individuals
HbAS	Hemoglobin A and Hemoglobin S complexes found in SCT individuals
LOO^-	Alkylperoxyl Radical
LPS	Lipopolysaccharide
MEP	Megakaryocyte-Erythroid Progenitors
NO	Nitric Oxide
Osm Max	Osmolality Concentration at which Red Cell exhibits maximal Deformability
PBS	Phosphate Buffered Saline
PolyI:C	Polyinosinic acid: Polycytidylic acid
PTR	Post Transfusion Recovery
PVP	Polyvinylpyrrolidone
RBC	Red Blood Cell

ROS	Reactive Oxygen Species
SCD	Sickle Cell Disease
SCT	Sickle Cell Trait
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TLR3	Toll-like Receptor 3
WB	Whole Blood
WT	Wildtype

1.0 INTRODUCTION

1.1 THE SICKLE HEMOGLOBIN MUTATION

Sickle cell trait is the carrier status of sickle cell disease (SCD), a severe hemolytic disease that is caused by a point mutation in the gene encoding beta-hemoglobin (β6Glu→Val) that increases the hydrophobicity of this protein when deoxygenated. In SCD RBCs, this mutation causes Hb polymerization under small reductions in physiologic oxygen saturation leading to cell dehydration, increased membrane rigidity and hemolysis.[1,2] These altered red cell properties promote vaso-occlusive events in the microcirculation, causing episodes of severe pain and end-organ ischemia, infarction and progressive dysfunction. Red blood cells (RBCs) from individuals with sickle cell trait (SCT) contain 25-50% HbS and polymerize only at low fractional oxygen saturations less than 50%.[2] Thus, under normal physiologic conditions, individuals with sickle cell trait are largely asymptomatic. However, under more extreme conditions of hypoxia and dehydration, vaso-occlusive events can occur.[3]

To date, the strongest evidence of SCT-related pathology is exercise related death (ERD) where military recruits and athletes suffered sudden deaths during extreme physical training.[4,5] This trend was absent in non-sickle cell trait recruits and not all sickle cell trait recruits experienced ERD suggesting additional underlying factor(s) that may be aggravated by sickle cell trait. Due to prolonged time (>10 seconds) RBCs spend in the hypoxic environment of the

vasa recta located within the renal medulla where HbS polymerize, focal lesions develop in individuals with sickle cell trait over time that can lead to loss of urine concentration ability.[6,7] Sub-clinical tissue infarction due to microvascular obstruction of non-deformable cells has also been reported in sickle cell trait individuals.[8,9] Beyond this, individuals with sickle cell trait do not present any clinically relevant symptoms and routinely donate blood for transfusion.

Historically, donor RBC genetic background is considered benign if the donor lacks clinically relevant symptoms.10 However, prolonged storage exposes RBCs to non-physiologic stress conditions and it remains possible that these conditions amplify the effects of "silent" mutations.11,12 Sickle cell trait persists at about 10%- 40% in malaria endemic regions and the estimated incidence is approximately 1.5% of all newborns screened in the U.S. in 2010 and 8% among African Americans.1,13-15 African American RBC donors are not routinely tested for this genotype and there are limited studies evaluating the properties of sickle cell trait on RBC storage or post-transfusion survival.[16,17]

1.2 TRANSFUSION OF SICKLE CELL TRAIT RED CELLS

Current transfusion practices of sickle cell trait RBCs are based on studies performed decades ago (Table 1), which found no significant differences in post-transfusion recovery of sickle trait RBCs when compared with normal RBCs. However, storage duration in these studies was relatively short (<28 days compared with present 42-day storage), and the authors utilized less sensitive methods to evaluate RBC post-transfusion survival.[10-12] Despite limited data supporting safety or efficacy, transfusion of stored HbAS RBCs remains contraindicated for neonates and SCD patients, given isolated reports of splenic infarction at high altitudes and

unexplained sudden deaths in sickle cell trait individuals under extreme physical exertion.[10,11,13,14] Routine testing for sickle cell trait is not performed in all blood banks and only discovered if blood fails to filter during leukoreduction.[15]

I therefore sought to directly examine whether sickle cell trait modulates erythrocyte susceptibility to hemolysis and post-transfusion recovery during conventional blood banking. I utilize highly sensitive methods to evaluate RBC membrane changes during storage and cell tracking techniques to quantify post-transfusion survival. I evaluated these outcome measures in human blood and blood from heterozygote humanized sickle cell transgenic mice. Our results show that sickle cell trait increases storage hemolysis and reduces post-transfusion red cell survival, which increases significantly with increasing time in storage. Interestingly, in contrast to SCD RBCs, transfused HbAS RBCs do not exhibit higher intravascular hemolysis compared to HbAA RBCs, but rather become entrapped in the systemic microcirculation. These findings raise concerns about the viability of stored sickle cell trait red blood cells after prolonged storage.

1.3 RED BLOOD CELL TRANSFUSION THERAPY

Hemorrhagic trauma is the leading cause of death in young adults in the United States[16] and to mitigate this risk, patients receive red blood cell (RBC) transfusions. RBC transfusion is also the main therapy in the management of Sickle Cell Disease and other hemolytic disorders.[17] According to Centers for Disease Control, about 15 million RBC transfusions are performed in the U.S. annually.[18] To ensure availability, RBCs are processed and stored prior to transfusion where they may undergo changes in the static and unnatural environment.[19-21] These changes are

collectively known as storage lesion and appear to occur at different rates in different RBC units; prompting the hypothesis that donor genetic background can affect RBC storage integrity.

This hypothesis has since been tested and confirmed examining the effect of gender and as glucose-6-phosphate dehydrogenase deficiency and it remains to be known whether other RBC-associated mutations severely alter RBC storage integrity.[22-24] The impact of these other mutations have not been explored because i) Low prevalence is the U.S. population and ii) Asymptomatic or sub-clinical impact of the mutation in the donor. It is estimated that 20% of the world's population has an RBC associated mutation due to the extraordinary selective pressure malaria has exerted on the human genome.[25,26] Most of these individuals have historically been concentrated in malaria endemic regions: Africa, South East Asia, Central and Southern America but due to increased immigration, Western countries are beginning to observe an increasing influx of these mutations.[27] [28] This accelerated gene flow creates a unique opportunity to evaluate these mutations on red cell storage and post transfusion survival as these individuals contribute significantly to the donor pool. (**Figure 1**).

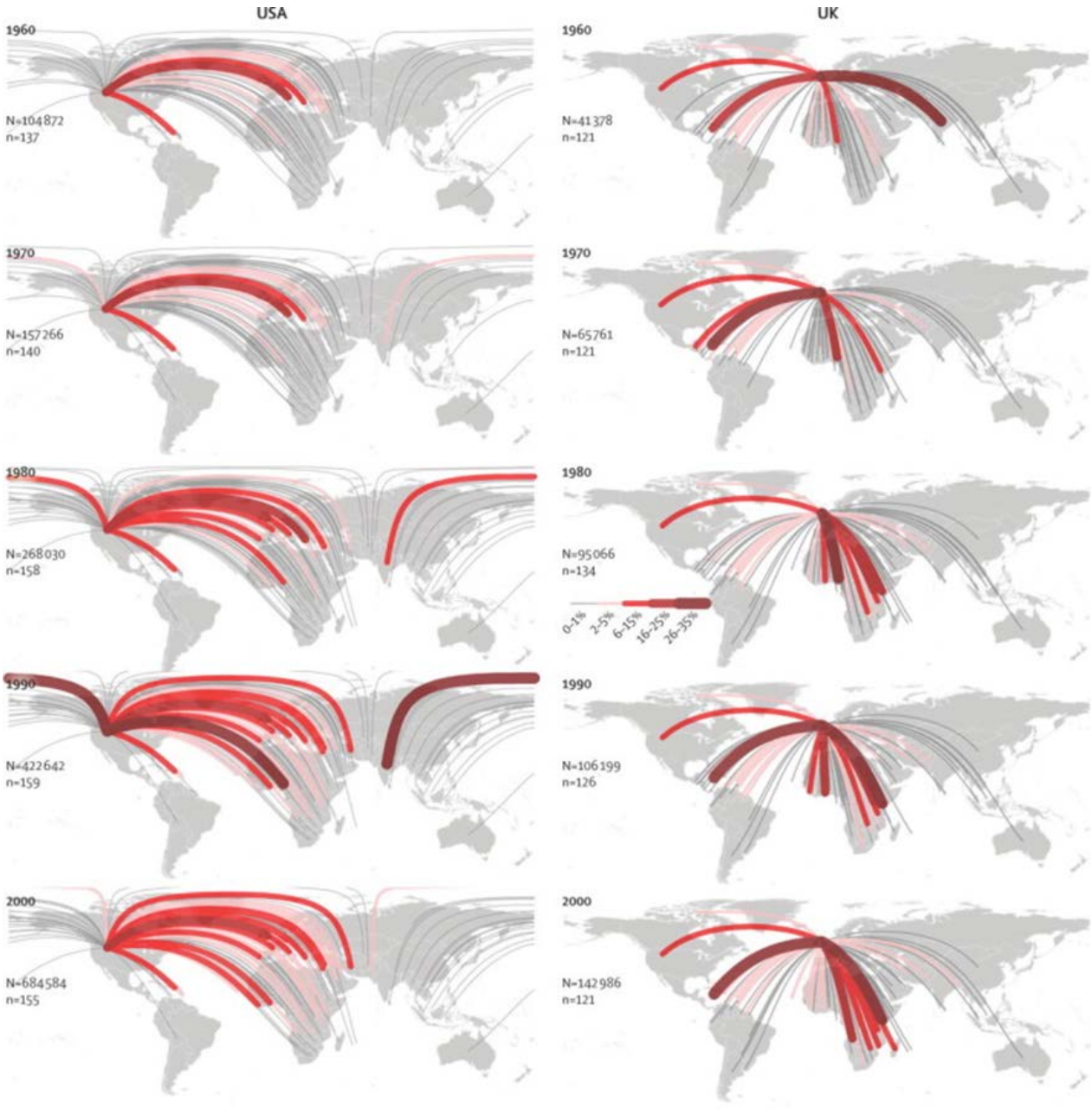

Figure 1. Migration of Individuals with HbS to the U.S. and U.K. over a 40-year period

Line thickness is proportional to number of migrant flow between the two regions shown. N= the estimated number of net migrants with HbS, n = the number of countries from which individuals migrated. HbS=sickle-cell hemoglobin (Adapted from Piel, Weatherall et. al)[29].

In this study, I focus on sickle cell trait (SCT), the carrier status of SCD and benign condition that affects 8-10% of African Americans. This sub-population routinely donates blood for transfusion and represents a higher percentage of the donor pool in urban areas. Under current blood banking practices, RBCs for transfusion are tested for SCT and excluded if the recipient is a neonate or a SCD patient. In all other cases, SCT positive units are transfused without testing. Given the high numbers of SCT RBC units currently being transfused and the known hemolytic profile of SCD RBCs[29,30] it is important to evaluate whether the presence of one sickle hemoglobin (HbS) in a donor alters RBC storage integrity and if so, whether this alteration significantly reduces post transfusion survival.

The challenge of this study and other RBC storage studies has been the clinical relevance of the measured *in vitro* changes. To circumvent this, I utilize a well-established humanized transgenic model of SCD and SCT to mimic storage and transfusion of human and murine RBC survival.[31-34] The study utilizes sensitive *in vitro* and *in vivo* methods to measure changes in the hemolytic propensity of HbS-containing RBCs. The underlying mechanisms of sickle RBC cell dehydration, membrane rigidity, sickling and hemolysis are well known and the goal of this study is to determine whether the presence of one copy of the gene (25-46% of HbS) in HbAS RBCs [35] [36] leads to a similar phenotype under storage stress to reduce storage integrity and post transfusion survival.

1.4 THE RED BLOOD CELL

1.4.1 Physiological function

Mature red blood cells are a specialized group of cells responsible for gas transport in mammals. RBCs serve a dual purpose of tissue perfusion and detoxification through the delivery of oxygen and removal of carbon dioxide to be expired in the lungs. Oxygen availability in tissues is necessary for cellular metabolism.[37] The major protein moiety in RBCs is hemoglobin, a hetero-tetrameric unit, which forms 25- 30% of the cell's intracellular content.[38] The high membrane deformability of RBCs makes it possible to passage through the microcirculation. This property is controlled by three properties namely; intracellular hemoglobin concentration, surface area to volume ratio and the elastic properties of the cell membrane. Over its 120-day lifespan, mechanical and metabolic changes lead to loss of deformability and eventually removal by splenic macrophages from circulation.[39-41] Traditionally, the role of RBCs has primarily been known to be oxygen delivery. However, it has been shown that RBCs can affect blood pressure through NO production [42] as well as platelet activation.[43]

1.4.2 Red cell Membrane deformability

Two defining and unique features of mature red blood cells are the high elasticity and ability to make large rapid shape changes in response to applied shear stress. Lacking nuclei, mature RBCs display remarkable deformability passaging through microcirculation.[44,45] This behavior is possible due to the structural organization of the membrane, which is currently believed to be composed over 50 transmembrane proteins embedded in the cholesterol: phospholipid

bilayer.[46,47] Membrane proteins have diverse functions ranging from ion and protein transport, cell adhesion and antigenicity. For example, Band 3 is an anion transporter, aquaporin transports water across the cell membrane, while Glut1 serves as a glucose and dehydro-ascorbic acid transporter. In addition to these, there exist other membrane proteins such as Kidd protein which a urea transporter and RhAG, a gas transporter.

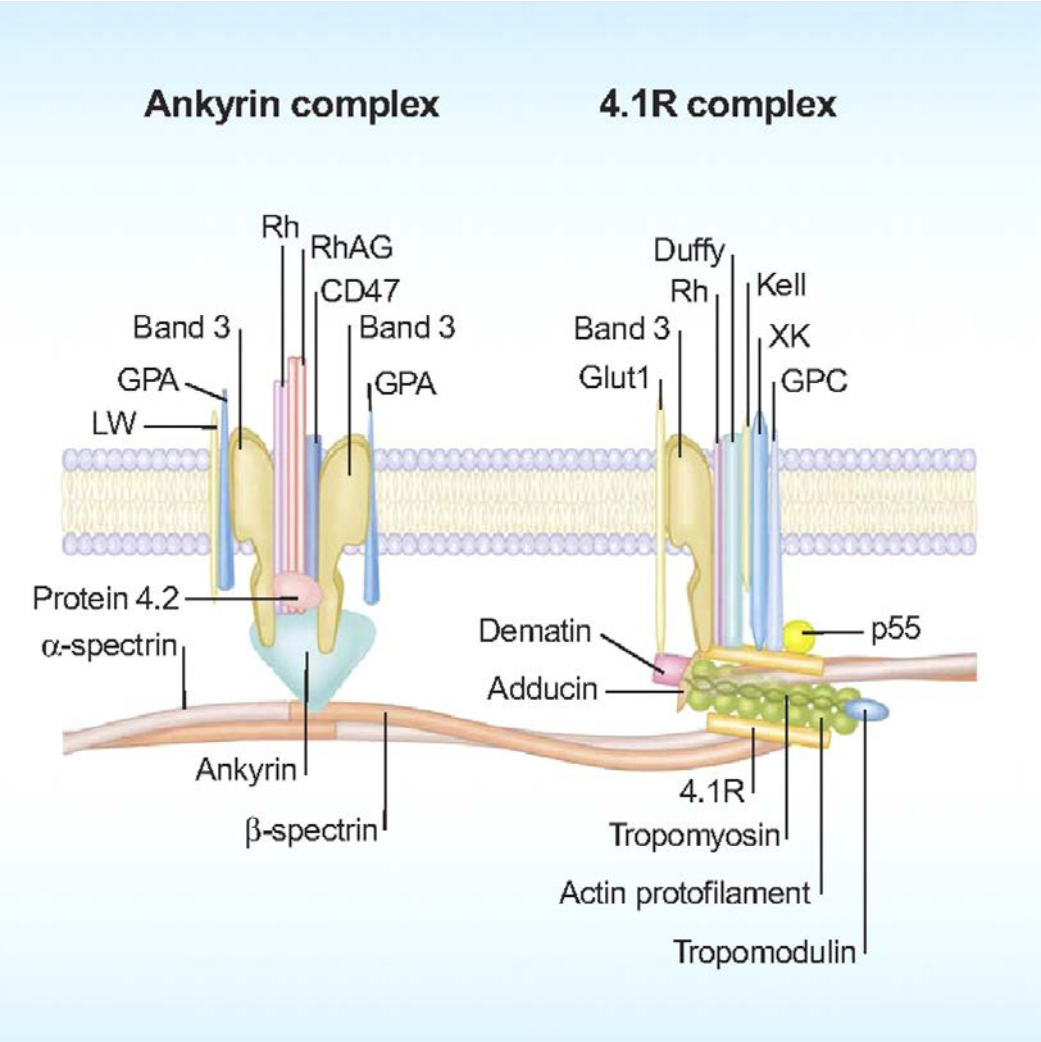

Figure 2. Schematic Model: RBC Membrane Skeletal Network.

The main components of the skeletal network are α- and β-spectrin, actin, protein 4.1R, adducin, dematin, tropomyosin, and tropomodulin. [Adapted from Mohandas and Gallagher et. al 2008.[25]

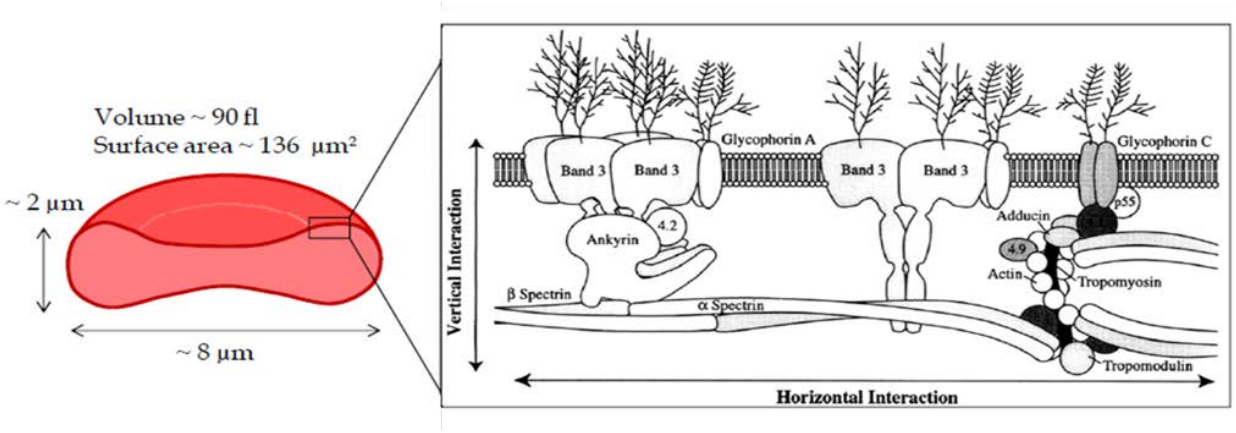

Figure 3. Schematic Figure: Red Blood Cell Membrane Structure

[Adapted from Liu, Derick et. al 1987.[62]]

The red cell membrane is further supported by a skeletal network composed of α- and β-spectrin proteins, actin, protein 4.1R, adducin, dematin, tropomyosin, and tropomodulin (**Figure 2**).[48-50] The unique tri-helical formation of α- and β-spectrin filaments provides structural and thermal stability and has been recently shown to dissociate during membrane deformation.[51-53] Junctions formed by spectrin, actin and protein 4.1R are important in providing mechanical support during high shear stress RBCs experience during circulation. The essential role of the skeletal network and its intracellular location may hold clues to how (unstable) hemoglobin-membrane protein interactions affect membrane deformability. [54,55]

1.4.3 Internal Viscosity

Another determinant of RBC Deformability is intracellular water content. A healthy mature human red blood has 7-8 micron diameter, 90-140fL volume and approximately140μm^2 surface area. Compared to a sphere with the same volume, RBCs possess an excess of 40% surface area,

a property that allows the extreme deformability RBCs exhibit. Using ektacytometry and solutions of varying osmolalities Mohandas et. al showed how internal viscosity can affect RBC deformability.[56] These findings provide the contribution of cellular dehydration to the loss of deformability of sickle RBCs. Individual mature healthy RBCs have a narrow MCHC of 27-37 g/dL and it appears that this narrow MCHC range is critical for RBC deformability. This is because the viscosity of intracellular Hb viscosity is about 5 centipoise (cp) at 27g/dL, which rises to 15cp at 40 g/dL and exponentially to 45cp at 40g/dL and even higher to 650cp 50g/Dl.[57] These exponential increases in intracellular viscosity in response to small changes in cellular dehydration demonstrate the importance of maintaining intracellular hemoglobin concentration to preserve RBC deformability. In this study, I utilize complete blood counts and ektacytometry to show that HbAS RBCs are more dehydrated compared to HbAA RBCs at the beginning and end of storage. Additionally, HbAS RBCs appear to lose membrane deformability at a faster rate during storage compared to HbAA RBCs. These observations may hold a mechanistic explanation for the reduced post transfusion survival of stored HbAS RBCs compared to HbAA RBCs and remain to be elucidated.

1.4.4 Erythropoiesis

A healthy adult human produces over 100 million red blood cells every minute in a process called erythropoiesis.[58] Erythropoiesis is a tightly regulated process through which hematopoietic stem cells (HSCs) differentiate into erythroid progenitor cells, and later into erythroid precursors, reticulocytes and finally into mature erythrocytes. During this maturation process, the cells progressively lose their proliferative capacity while becoming increasingly sensitive to erythropoietin.[59] In humans, this occurs in the erythroblastic islands of bone marrow where

terminal differentiation occurs (**Figure 3**). At maturation about 97% of the red blood cell intracellular content is hemoglobin $\alpha_2\beta_2$ tetramers. However during development, one gene cluster located on chromosome 11 encodes embryonic ε, $^{G}\gamma$, $^{A}\gamma$, adult δ and β globins and another cluster located on chromosome 16 encodes embryonic ζ, adult α1 and α2 globins.[60 61] As fetal the site of erythropoiesis shifts from the yolk sac to the liver during gestation and finally to the bone marrow as birth approaches, globin expression in the β-globin cluster transitions from the embryonic to the fetal to the adult globin genes (**Figure 4**). As a result, $\alpha_2\gamma_2$ is the main hemoglobin tetramer expression during gestation, switching to $\alpha_2\beta_2$ during infancy, thus allowing babies born with SCD to delay symptoms till about six months of age as the fetal hemoglobin transitions to adult hemoglobin.

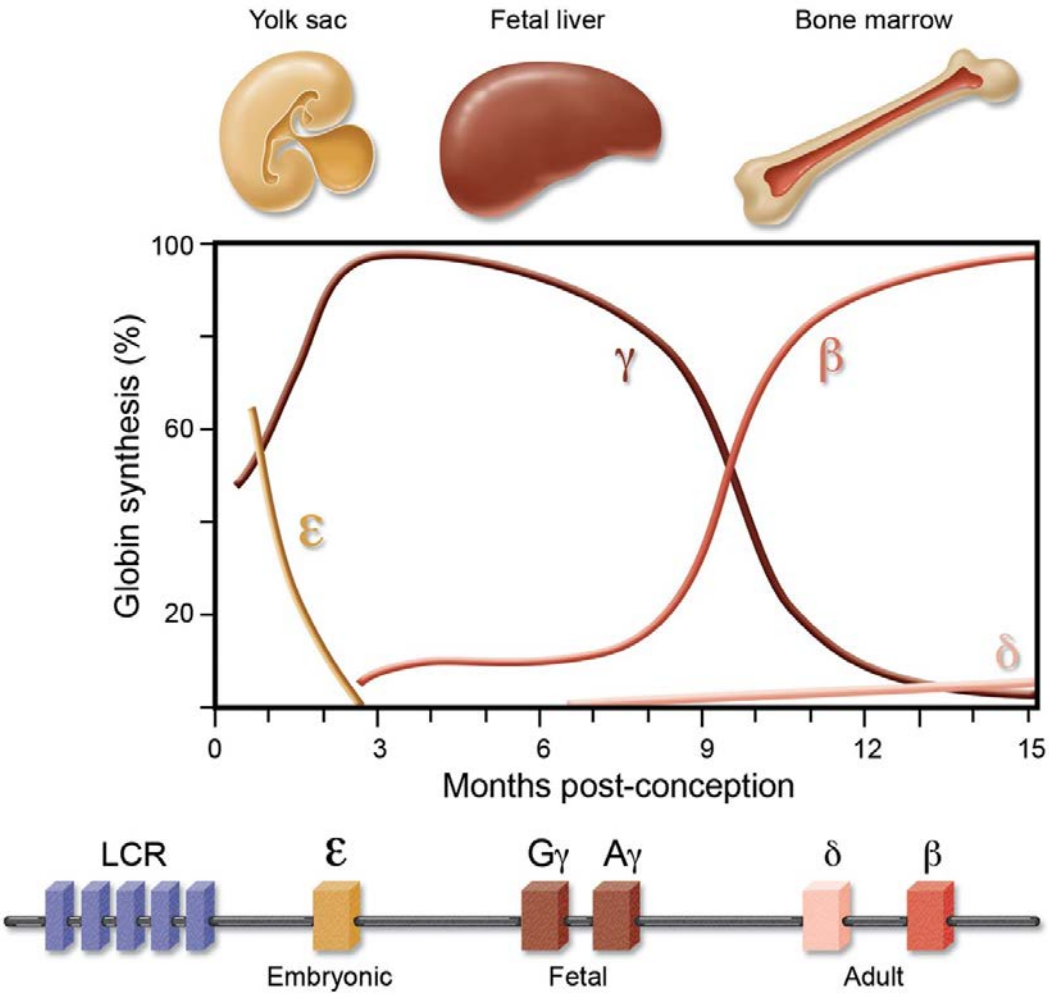

Figure 4. The regulation of β-globin during development.

β-globin genes are under differentially expressed during the embryonic and fetal stages of development under a strict control of its locus control region.[Adapted from Bauer, Kamran, Orkin., 2012 [60]

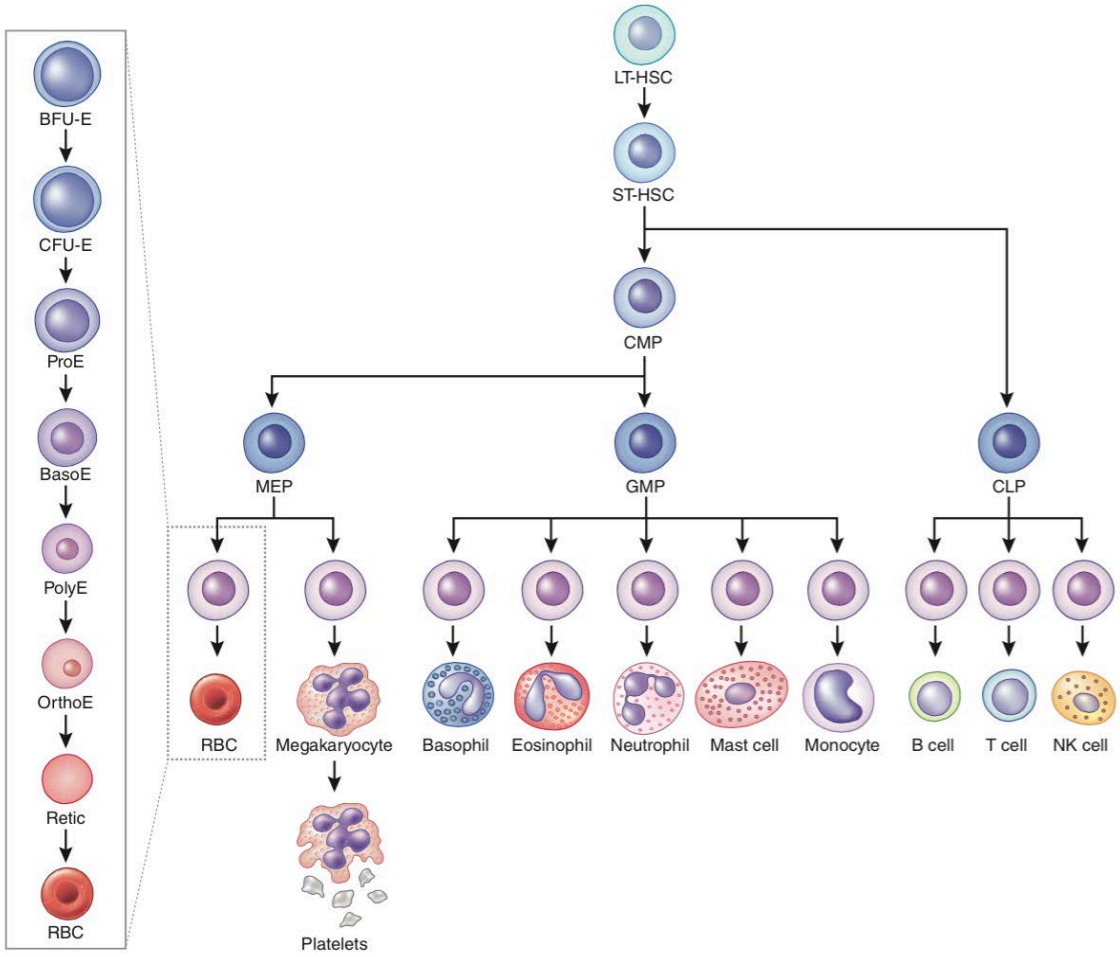

Figure 5. Erythropoiesis Model. Hematopoietic stem cells develop into multipotent common myeloid progenitor cells (CMPs).

CMPs mature into megakaryocyte-erythroid progenitor cells (MEPs) and granulocyte-macrophage progenitors (GMPs). Burst-forming unit erythroid (BFU-E) is the earliest progenitor that progressively differentiates into precursors that eventually develop into mature red blood cells (Adapted from Sankaran, V.G. and Weiss, MJ.66,69)

1.5 MALARIA

Malaria is a tropical disease characterized by episodes of massive hemolysis that causes fevers, sweating and lethargy. It is estimated that there are about 500 million new malaria infection cases worldwide annually leading to over 1 million deaths with most of the fatalities occurring in children under the age of five.[62]

1.5.1 Mechanism of Infection

Malaria infection occurs when a female Anopheles mosquito carrying *Plasmodium falciparum* sporozites punctures the skin and releases them into the bloodstream. The sporozites circulate the body and colonize the liver where they multiply into haploid forms known as merozoites. Circulating merozoites in the blood stream where they penetrate red blood cells and deplete RBC ATP levels through rapid asexual multiplication and subsequently causing hemolysis, releasing new larger numbers of merozoites about 48h post infection. This cycle is the underlying mechanism of the episodic fevers and associated hematuria every 48h during a malaria infection. A fraction of merozoites switch to sexual replication, forming male and female gametocytes. Circulating gametocytes are ingested by mosquitoes when they bite an infected individual. The male and female gametes fuse in the mosquito to form ookinetes, the diploid form. Ookinetes inhabit the midgut lining of the mosquito where they grow and develop into oocytes. Oocytes divide to produce sporozites that are subsequently released into the bloodstream of another human (**Figure 5**).

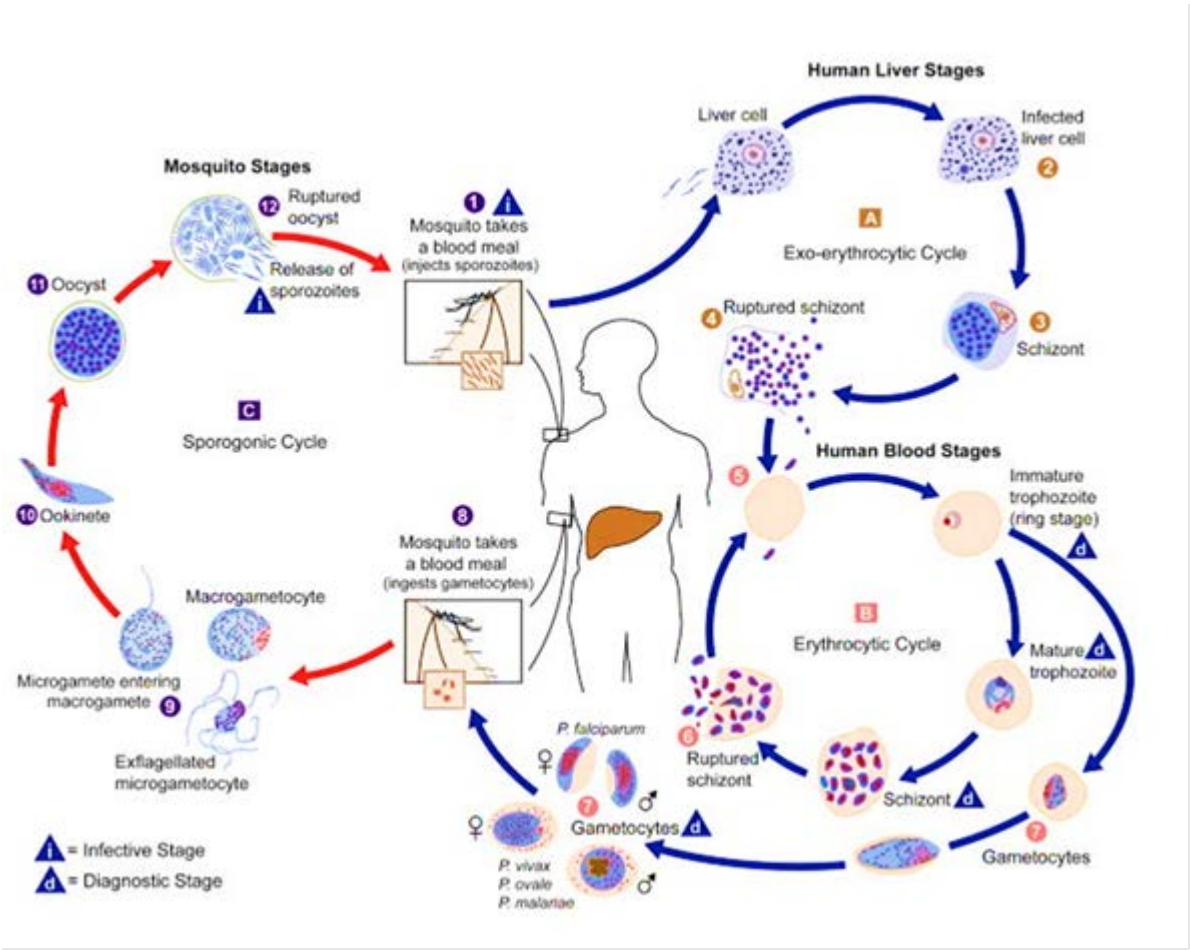

Figure 6. The Plasmodium Life Cycle

The life cycle of the plasmodium in a female *Anopheles* mosquito and a human as it cycles through the sporogonic, exoerythrocytic and erythrocytic stages (CDC Laboratory for Identification of Parasites).[63]

1.5.2 Sickle Cell Trait: An Evolutionary Response to Malarial Pressure

1.5.2.1 History

The prevalence of red blood cell mutations in malaria-endemic regions, especially the persistence of sickle cell trait provides evidence for the evolutionary pressure malaria has exerted on the human genome. In 1949, Haldane hypothesized that SCT must confer survival advantage based on the geographical similarity of malaria and SCD prevalence. He reasoned that since SCD individuals had a negligible to zero chance of reproduction, a sustained reduction in the HbS allele was expected in each subsequent generation but this was contrary to observations. The persistence of sickle cell trait in these populations therefore led Haldane to theorize that SCT must provide a survival advantage, which served as the counteracting force. Based on the geographic concentration of individuals with SCD in malaria endemic regions, Haldane hypothesized that SCT could be the force behind the persistence of HbS allele in the human population by providing protection from malaria (**Figure 5**). This hypothesis was later confirmed experimentally. [26,64-66] In evolutionary biology, the mechanism under which two alleles are maintained in a population because the heterozygous individuals display higher fitness is called balanced polymorphism and sickle cell trait remains a classic example.

1.5.2.2 Sickle cell trait Phenotype

Individuals with SCT do not display symptoms associated SCD and have normal red blood cell morphology, lifespan, and reticulocyte count as well as haptoglobin and bilirubin levels. However, under hypoxia and exertional physical activity morbidity and mortality can occur. To date, the strongest evidence of SCT-related pathology is exercise related death (ERD) where

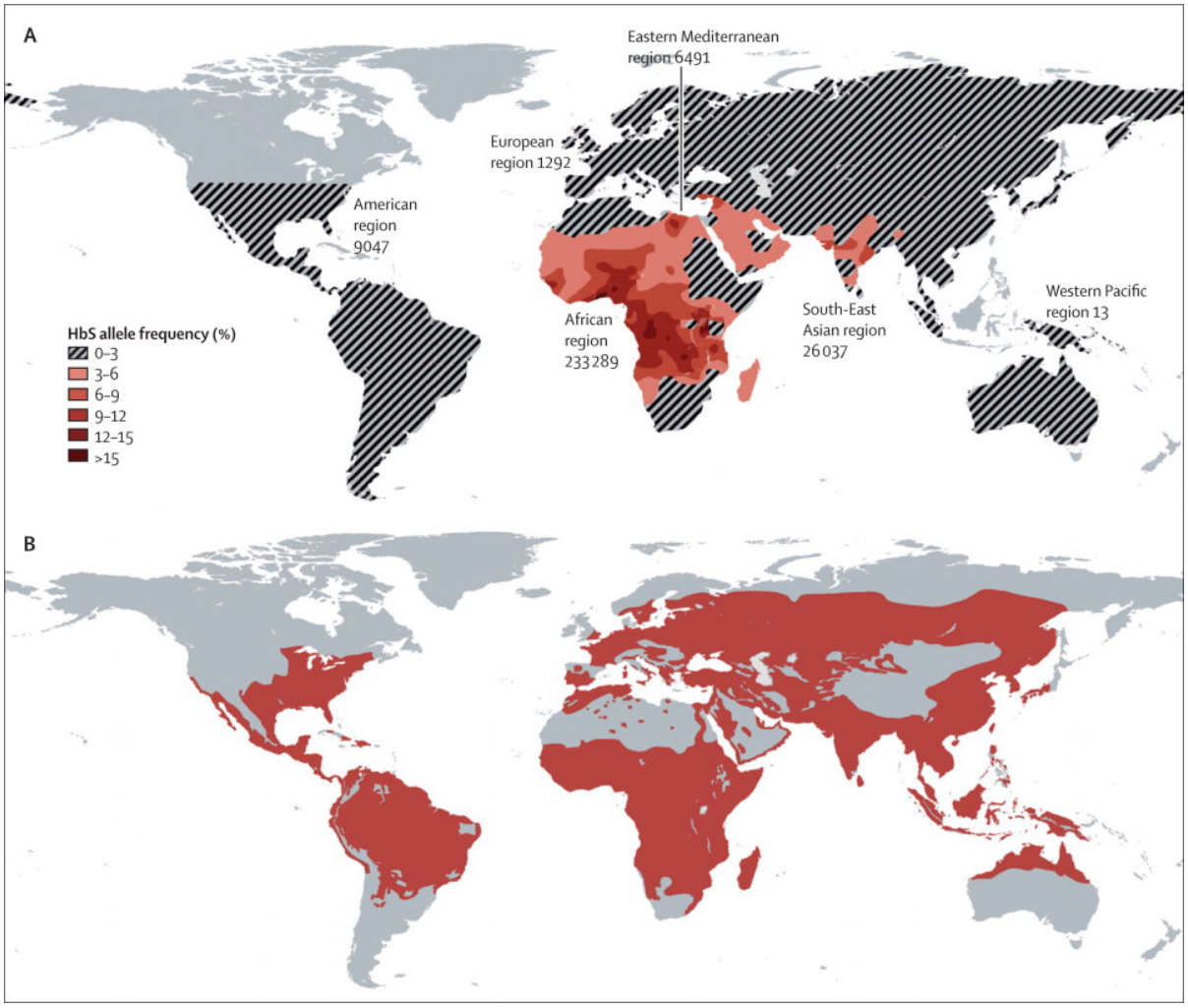

Figure 7. Global HbS Allele and Malaria Distribution.

Adapted from David C Rees, Thomas N Williams, Mark T Gladwin[1] (A) Global Hb allele distribution. Original data was obtained from Cavalli-Sforza et. al.[67] (B) Global malaria incidence distribution; originally from Lysenko et. al.[68]

military recruits and athletes have suffered sudden deaths during physical training.[4,5] While these incidents remain poorly understood, the number of deaths correlated with increasing age of SCT military recruits who also displayed increased severity of renal papillary necrosis.[5,7] This trend was absent in non-SCT recruits and not all SCT recruits experienced ERD suggesting a possible underlying factor(s) that may be aggravated by SCT. Beyond this, Individuals with SCT do not present any clinically relevant symptoms and routinely donate blood for transfusion.

In the U.S., 8% of African Americans have this condition, a statistic that tends to be higher in urban or predominantly Black inhabited regions. With the exception of neonates and SCD recipients, HbAS RBCs are transfused without restriction. Despite this routine use, there exit limited studies evaluating the suitability of these RBCs for transfusion or the effect of SCT on RBC storage and post transfusion survival. It is important to note that while SCT individuals may be asymptomatic, RBC storage is non-physiologic and presents stress conditions to the RBC. It remains unknown whether the presence of HbS allele aggravates stressors during storage and subsequently reduces the post transfusion viability of stored HbAS RBCs.

1.6 SICKLE CELL DISEASE

Sickle Cell Disease is a severe hemolytic disorder caused by a single mutation in the beta hemoglobin gene where glutamic acid is replaced with valine at the sixth amino acid position. Affected individuals inherit an HbS allele from both parents and do not have increased resistance to malaria observed in SCT individuals but rather experience pain vaso-occlusive crises, lethargy and frequent fevers. This single point mutation (transversion) increases the overall hydrophobicity of the β-hemoglobin, causing HbS protein polymer formation under low oxygen

and pH conditions. Increased auto oxidation of HbS and other associated alterations such as acidification of the cytoplasm lead to increase K^+ loss via the K-Cl co-transport system. This increased ion loss via outflow of water, causes cell dehydration and increased intracellular HbS concentration eventually leading to erythrocyte sickling caused by HbS polymerization.

Sickling of HbSS erythrocyte requires the conformational change of the RBC membrane to rigid deoxygenated HbS polymers78. There is an inverse relationship between the concentration of HbS concentration in dense RBCs and sickling. Repeated events of RBC sickling leads to irreversibly sickle RBCs that lack the deformability to traverse capillaries with diameters smaller than the RBC. Entrapment of sickled RBCs causes vaso-occlusion, a hallmark of Sickle Cell Disease. Rigid and sickled RBCs eventually rupture, releasing free hemoglobin and other intracellular contents into the intravascular space. Based on mounting scientific evidence, intravascular hemolysis appears to be the underlying event that leads to the clinical sequelae observed in SCD. Chronic hemolysis causes increased ROS, nitric oxide scavenging and a chronic inflammation (**Figure 7**). This massive RBC destruction and increased iron metabolism leads to splenomegaly and eventually auto-splenectomy in some SCD patients. To maintain adequate circulating RBCs for tissue perfusion, SCD patients typically receive RBC transfusions (3-5 units) every 3 months. Transfusions of HbAA RBCs with ~120-day life span significantly reduces splenic burden caused by the 10-day life span of SCD RBCs. However, transfusion therapy is not without risk.

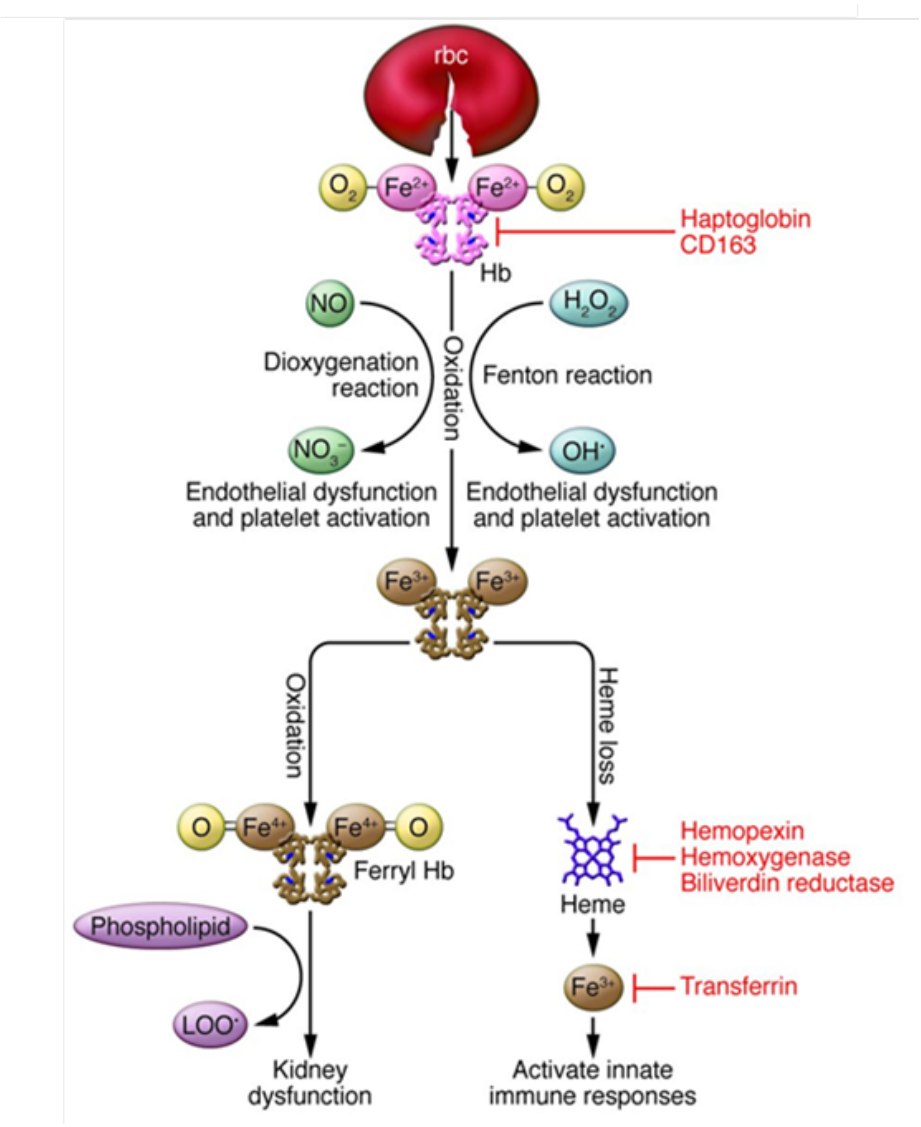

Figure 8. Schematic Model

Mechanisms by which hemolysis and cell-free hemoglobin can mediate vascular injury [Adapted from Gladwin et. al, 2012. [69]]

One major complication associated with frequent RBC transfusions is the development of anti-RBC antibodies termed allo-sensitization. Allo-sensitization occurs in a fraction of SCD patients and requires the use of extended antigen matching beyond the standard ABO matching between donor and recipients to avoid transfusion relation immune reactions 79. While most blood types fall under the ABO antigen groups, there are certain rare antigens that occur specifically in certain ethnicities, making it critical to confirm compatibility prior to transfusion. In allo-sensitized SCD patients, increasing incompatibility with RBCs by individuals from other ethnicities make them restricted to African American RBC donors. The life expectancy of SCD patients in the U.S. has significantly improved because of RBC transfusions, use of penicillin to prevent infections and the use of hydroxyurea to increase fetal Hb.

1.7 OVERVIEW AND SPECIFIC AIMS

1.7.1 Relevance and Significance

Red blood cell (RBC) transfusion is a common therapy and yet little is known regarding donor-related factors that contribute to the development of the "storage lesion", a compilation of morphologic and biochemical changes that occur in red cells during standard storage conditions that may relate to post-transfusion risk. Our group and others have shown that donor genetic background modulates storage-related "aging" of red blood cells, which is hypothesized to influence post-transfusion survival of stored RBCs.[19,29,70] Interestingly, more than 1000 genetic mutations have been identified in red blood cell proteins and enzymes, particularly in subjects of African origin related to the endemic effects of malaria infection on modern human evolution. One such common genetic variant is Sickle cell trait (SCT), the asymptomatic heterozygous condition of the hemolytic anemia sickle cell disease (SCD), which occurs in 8% of African Americans who routinely donate blood for transfusion.

Despite its prevalence, African American donors are not routinely tested for this genotype and there exist limited studies evaluating the properties of these cells in storage or effects on post-transfusion red cell survival. My preliminary data generated a very interesting paradox in which better *in vitro* cell stability was observed under stressors such as osmotic induced hemolysis during storage but yield worse *in vivo* post transfusion recovery for HbAS RBCs. These results challenge the current convention that transfusion of AS blood is as effective as healthy RBCs at the limits of storage and may potentially change our understanding of transfusion practices in patients that require this blood donor source, for example, patients with severe allo-antibody formation requiring blood. **I <u>hypothesized</u> that HbAS RBCs exhibit**

accelerated aging during storage resulting in reduced post-transfusion recovery (PTR). I further hypothesized that this accelerated "aging" during storage is a result of enhanced membrane degradation that leads to higher phosphatidylserine exposure and increased RBC clearance by the mononuclear phagocyte system. A novel approach is proposed here to use human and murine RBCs from humanized transgenic AS mice to investigate the effect of SCT on RBC storage integrity and post-transfusion recovery.

1.7.2 Specific Aim 1: To determine whether SCT (HbAS) RBCs show lower storage integrity compared to HbAA RBCs as measured by *in vitro* hemolytic assays that assess membrane resilience.

Sub Aim 1a: **To examine the *in vitro* hemolytic properties of human HbAS RBCs during storage.** *In vitro* properties that capture progressive RBC damage during storage have been traditionally used to predict post- transfusion survival although our preliminary data suggest otherwise. RBCs were collected from normal, SCT, and SCD individuals under an approved IRB protocol, processed stored under standard blood banking conditions. RBC properties that are known to change during storage such as membrane deformability, osmotic fragility, resistance to lipid peroxidation, mechanical fragility and storage hemolysis were measured at specified intervals over a 42-day period. In addition, conventionally banked HbAA and HbAS RBCs were obtained from the Central Blood Bank were subjected to similar tests at specific times during storage.

Sub Aim 1b: **To examine the *in vitro* hemolytic properties of murine HbAA and HbAS RBCs during storage.** Mice provide a mammalian system to test hypotheses and generate meaningful results that may be translatable to humans although there are limitations [31,71-73]. Utilizing Berkeley hemizygous transgenic humanized mouse model, the approximate condition for the human sickle cell trait condition, I determined whether *in vitro* membrane behavior found in human HbAS RBCs can be mimicked using Berkeley hemizygous mouse model (WT C57BL/6 used as HbAA control).

1.7.3 Specific Aim 2: To test the post transfusion survival of stored HbAA and HbAS RBCs using a humanized transgenic mouse model.

Sub Aim 2a: To determine the post transfusion survival of fresh and stored murine HbAA and HbAS RBCs in mice. To do this, I evaluated the 24 h post transfusion recovery of fresh and stored murine HbAA and HbAS RBCs in each recipient mouse using a dual cell tracking system. This was achieved by labeling RBCs with fluorescent dyes with distinct emission spectra and therefore allowed enumeration of both populations in a blood sample (obtained from transfused mouse via tail vein snip) by flow cytometry. To eliminate the effect of labeling dyes on red cell post transfusion survival, I used mice that express Green Fluorescent Protein in hematopoietic cells and therefore allowed the enumeration of transfused unlabeled RBCs.

Sub Aim 2b**: To investigate the effects of hypoxia and systemic inflammation on post transfusion red cell recovery and hemolysis in mice.** RBC transfusion is a common intervention for the treatment of severe anemia in critically ill patients who often exhibit hypoxia and the systemic inflammatory response syndrome, stress conditions that may differentially affect post-transfusion recovery. I hypothesized that the post transfusion recovery of donor

HbAS RBCs would be further reduced by recipient hypoxia by promoting HbS polymerization and intravascular hemolysis. In contrast, I hypothesized that systemic inflammation following lipopolysaccharide administration would improve RBC post transfusion recovery by impairing scavenging function of macrophages. In these experiments, recipient mice were subjected to 5.7% hypoxia immediately following transfusion for 24h, during which circulating blood volume was sampled via tail vein snip. In separate experiments, mice were treated with 1.5mg/kg lipopolysaccharide intraperitoneally 24h prior to transfusion. To further investigate the effect of factors present in the recipient on transfused RBCs, stored HbAA and HbAS RBCs were transfused in to Berkeley SCD and WT C57BL/6 to assess whether there are any differences in the post transfusion survival of the same RBCs in healthy and SCD mouse recipients.

1.7.4 Specific Aim 3: To determine whether the mechanism of reduced HbAS RBC post-transfusion recovery is due to a macrophage- mediated mechanism.

Splenic clearance is the main mechanism by which senescent RBCs are removed from circulation and since RBCs undergo storage-related aging, I hypothesized that the accelerated disappearance of stored HbAS RBCs from circulation was due to accelerated splenic clearance due to faster aging in storage compared to HbAA RBCs. Recipient mice were treated with clodronate or control (PBS) liposomes 24h prior to RBC transfusion to deplete $F4/80^{+}$ splenic macrophages and Kupffer cells. Mice were transfused with only stored HbAA or HbAS RBCs and post transfusion survival was assessed as previously described.

1.7.5 Specific Aim 5: To test whether the mechanism of reduced HbAS RBC post-transfusion recovery is due intravascular hemolysis of stored HbAS RBCs.

Chronic hemolysis is a hallmark of sickle cell disease red cells, a phenomenon driven by polymerization of sickle hemoglobin under various conditions. Here, I test whether sickle cell trait red cells hemolyze immediately following transfusion leading to the rapid clearance from circulation. WT recipient mice (n=15) per group were transfused with only stored HbAA RBCs or HbAS RBCs. Mouse blood was sampled at 5 min, 1 h and 4 h to assess cell free plasma or bladder hemoglobin content. Control groups (n=5, per group) were transfused with equal volume of PBS or equimolar concentration of cell free hemoglobin.

1.7.6 Specific Aim 6: To examine whether the increased clearance of transfused stored HbAS RBCs compared to stored HbAA RBCs is due to mechanical entrapment.

Tissue sequestration of red blood cells is a well documented phenomenon associated with Sickle Cell Disease due to mechanical entrapment of sickled or less deformable RBCs in microcirculation. Under this Specific Aim, 11-day stored cy3-labeled murine HbAA or HbAS RBCs were transfused into WT C57BL/6 recipients. Recipient spleen, kidney and liver were harvested, fixed and processed for confocal imaging to assess whether there are differences between in the entrapment of HbAA and HbAS RBCs in mouse recipient tissues. This experiment was repeated with conventionally stored human HbAA and HbAS RBCs transfused into mouse recipients. Human RBCs were unlabeled prior to transfusion and later stained with a fluorophore conjugated to glycophorin A antibody, a marker for human red blood cells during processing.

1.7.7 Specific Aim 7: To determine whether the increased clearance of transfused stored HbAS RBCs compared to stored HbAA RBCs is due to increased surface expression of heat shock proteins, CD47 or phosphatidylserine.

Heat shock proteins are stress markers that are up-regulated in cells under stress or in pathological states. SCD RBCs are known to express higher levels of HSP 90, 70 and 27 that compared to healthy RBCs. Additionally, During physiological aging, phosphatidylserine (PS) which is normally compartmentalized in the inner cell membrane becomes externalized due to accumulated injury on the membrane. Increased surface expression of phosphatidylserine is known as a marker for apoptotic cells to be phagocytosed by macrophages. Here, in vitro experiments were performed to determine the surface expression of heat shock proteins (90, 70 and 27), CD47, PS as well as the rate of microparticle formation in fresh or stored murine HbAA and HbAS RBCs.

2.0 MATERIALS AND METHODS

2.1.1 Reagents

Drabkin's reagent (D5941-6VL), Citrate-phosphate-dextrose (CPD # C7165), Citrate-Phosphate-Dextrose with Adenine, (CPD-A #C4431) were purchased from Sigma Aldrich. 2, 2'-Azobis (isobutyramidine) dihydrochloride, 2, 2'-Azobis (2-amidinopropane) dihydrochloride, (AAPH # AC401562500) was obtained from Acros ogranics. Purecell® NEO Neonatal High Efficiency Leukocyte Reduction Filter (#NEO1) was obtained from Haemonetics. Fluorescent lipophilic dyes: (2*Z*)-2-[(*E*)-3-(3, 3-dimethyl-1-octadecylindol-1-ium-2-yl) prop-2-enylidene]-3, 3-dimethyl-1-octadecylindole; perchlorate (DiD DiIC18 (5) and DiI (DiIC18 (3)) (#V-22889) were purchased from Invitrogen. Forane (Isoflurane, USP # A33J14A) was obtained from Baxter.

2.1.2 Mice

8-12 week old wildtype (HbAA) mice (C57BL/6J), Berkeley hemizygous (HbAS) (*Hba*^{tm1Paz}*Hbb*^{tm1Tow}Tg(HBA-HBBs)41Paz/J) mice and transgenic mice expressing enhanced Green Fluorescent Protein in hematopoietic cells (C57BL/6-Tg(UBC-GFP)30Scha/J) were purchased from Jackson Laboratories. Berkeley hemizygous mice express human α-globin and sickle β-globin genes in addition to one copy of the murine β-globin, making them hemizygous for Sickle Cell Disease (HbAS).

2.1.3 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.[31] These mice were subsequently bred with mice heterozygous for either mouse α-globin knockout or mouse β-globin knockout. 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 knockouts.[31,74] These mice were back crossed with C57BL/6 mice for eight generations, and one additional generation once received by Jackson laboratories.

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.[75] 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.[76] For this reason, we used C57BL/6 mouse strain as a control group in murine experiments. Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.1.4 Blood collection and storage

Leukoreduced human RBC units from sickle cell trait donors and ABO blood type matched donors were obtained from Central Blood Bank (Pittsburgh, PA) and stored at 1-6 °C until specified times for testing. Whole blood (WB) was collected from mice via the inferior vena cava immediately following euthanasia using Citrate Phosphate Dextrose solution as an anti-coagulant (Sigma St. Louis, MO). Pooled WB was leukoreduced using a Pall Purecell® NEO Neonatal High Efficiency Leukocyte Reduction Filter as previously described.[33] Leukoreduced blood was re-suspended in 14% CPDA-1 (Sigma St. Louis, MO), concentrated to a final hematocrit of 55%, and stored at 1-6 °C in glass vacutainers shielded from light for up to 11 days.[77] To replicate similar conditions for human RBCs, peripheral blood was also collected with informed consent from healthy 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.

2.1.5 In vitro hemolytic assays

Storage hemolysis and stress-induced hemolysis due to osmotic, mechanical or AAPH-induced oxidation were measured by supernatant cell-free hemoglobin using Drabkin's assay.[78] Osmotic stress was induced by incubating RBCs in hypotonic buffer containing glycerol.[79,80] Oxidative hemolysis was induced by incubating RBCs with 2, 2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH).[81] Mechanical stress was induced by shaking RBC suspensions in the presence of one metal bead (3/32") for 3 h on a plate shaker.[82] *In vitro* experiments evaluating the hemolytic propensity of human RBCs were repeated using murine RBCs 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).

2.1.6 Post-transfusion Survival Studies

Fresh or 11-day stored murine RBCs (equivalent to 39-42 day storage for stored human RBCs)[32,77] for transfusion were labeled with lipophilic dyes 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate dyes DiI (D-383) or DiD (D-307) (Invitrogen, Carlsbad CA) prior to infusion in recipient mice by retro-orbital injection. RBC 24 h post-transfusion recovery was obtained by blood sampling via tail vein snip, enumerated by flow cytometry and analyzed using FlowJo (Ashland, OR). To confirm that fluorescent labeling dyes do not alter RBC post-transfusion survival, C57BL/6 mice expressing Green Fluorescent Protein (GFP) under the direction of the human ubiquitin C promoter (C57BL/6-Tg (UBC-GFP) 30Scha/J) were transfused with unlabeled fresh or stored HbAA and HbAS RBCs and analyzed using a negative FITC gate to quantify 24 h post-transfusion recovery as previously described.[32] Methods for splenic macrophage and Kupffer cell depletion were modified from those previously published.[33,83] 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.

2.1.7 Splenectomy

To examine the role of splenic clearance in RBC post-transfusion recovery, mice were splenectomized under a standard established surgical procedure, allowed to heal and transfused 5 days later. 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.[84,85]

2.1.8 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).

2.1.9 RBC Scanning Electron Microscopy

Human and Mouse RBCs were fixed in excess 2.5% glutaraldehyde for Scanning Electron Microscopy. See Supplemental Section for details. 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.

2.1.10 Ektacytometry

A Technicon osmotic gradient ektacytometer (Technicon Instrument Corp, Tarrytown, NY) was used to measure RBC deformability and Osm Max. 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.

2.1.11 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 (GE Healthcare, Wauwatosa, WI). 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. 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.

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 (Nikon, Melville NY). Sequestration of human or murine RBCs was

assessed using segmentation analysis and object counting for FITC or cy3 positive cells in NIS Elements (Nikon Inc., Melville, NY) and expressed relative to total DAPI positive cell count.

2.1.12 Statistical Methods

To measure storage-related changes between HbAA and HbAS RBCs such as echinocyte formation, changes in hemolytic propensity and post-transfusion survival, two-way ANOVA with Bonferroni post-test for individual comparisons was used to perform statistical analysis. To determine differences in HbAA and HbAS RBC sequestration in tissues, Mann-Whitney U test was used for non-parametric analysis (GraphPad Prism 6, La Jolla, CA).

2.1.13 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.[19]

3.0 RESULTS

3.1.1 Stored HbAS RBCs exhibit accelerated 24h post transfusion clearance compared to stored HbAA RBCs.

To examine the post-transfusion recovery of HbAS and HbAA RBCs from systemic circulation, we established a murine model of allogeneic and xenogeneic transfusion as previously published (Supplemental Figure 1).[32,33,86] There was no significant difference in the post-transfusion recovery of fresh DiI-labeled human HbAA and fresh DiD-labeled HbAS RBCs, although HbAS RBCs showed a lower post-transfusion survival (storage time of RBC < 3 days) (Figure 1A). Fresh human HbAA and HbAS showed 100% and 78.9% survival in circulation 24 h post-transfusion, respectively. The >100% post transfusion survival of fresh HbAA RBCs at earlier time points is due to un-even distribution of transfused RBCs in the circulating blood volume at the time of the initial tail vein blood sampling (5 min post transfusion). As there is negligible loss of fresh HbAA transfused RBCs in circulation, the underestimation creates an artifact of >100% survival in subsequent RBC recovery estimations when normalized to the initial value. This artifact has been previously reported in studies evaluating red cell survival post transfusion.[79,87] After 39-42 day storage, the mean 24 h post-transfusion survival was 69.1% for stored human HbAA and 4.9% for stored human HbAS RBCs (Figure 1B). Consistent with the findings of human RBCs, stored murine RBCs from Berkeley hemizygous mice (herein, referred

to as HbAS) showed accelerated clearance from circulation compared with transfused, stored RBC from WT mice (herein, referred to as HbAA), whereas no differences were noted in post-transfusion recovery of fresh murine HbAS and HbAA RBCs (Figure 1C-F).

To ensure that labeling dyes did not significantly impact RBC clearance, we utilized C57BL/B6 mice expressing GFP as recipients (Supplemental Figure S2). No differences were observed in the post-transfusion survival when fresh unlabeled HbAA and fresh HbAS murine RBCs were transfused into separate groups of mice expressing GFP (Figure 1E) but transfusion of unlabeled stored HbAS murine RBCs showed accelerated clearance compared to unlabeled stored HbAA murine RBCs (Figure 1F). Experiments with stored DiD-labeled murine HbAA and HbAS RBC transfused into separate recipients showed similar findings to stored DiD-labeled HbAA and DiI-labeled HbAS RBCs transfused into the same recipients indicating that labeling dyes did not alter the accelerated clearance previously observed (Supplementary Figure S2). The post-transfusion clearance of stored RBCs shows a bi-phasic curve with rapid disappearance of both HbAA and HbAS RBCs within 2 h post-transfusion, followed by slower rate of disappearance of stored murine RBCs with HbAS exhibiting an approximately two-fold rate of disappearance (34.1%), compared with HbAA RBC (14.9%) at 2h (Figure 1F). A similar pattern was observed when stored human RBCs were transfused into WT recipient mice (rate of disappearance, HbAS: 67.6% and HbAA: 22.4%; $p<0.0001$) (Figure 1B).

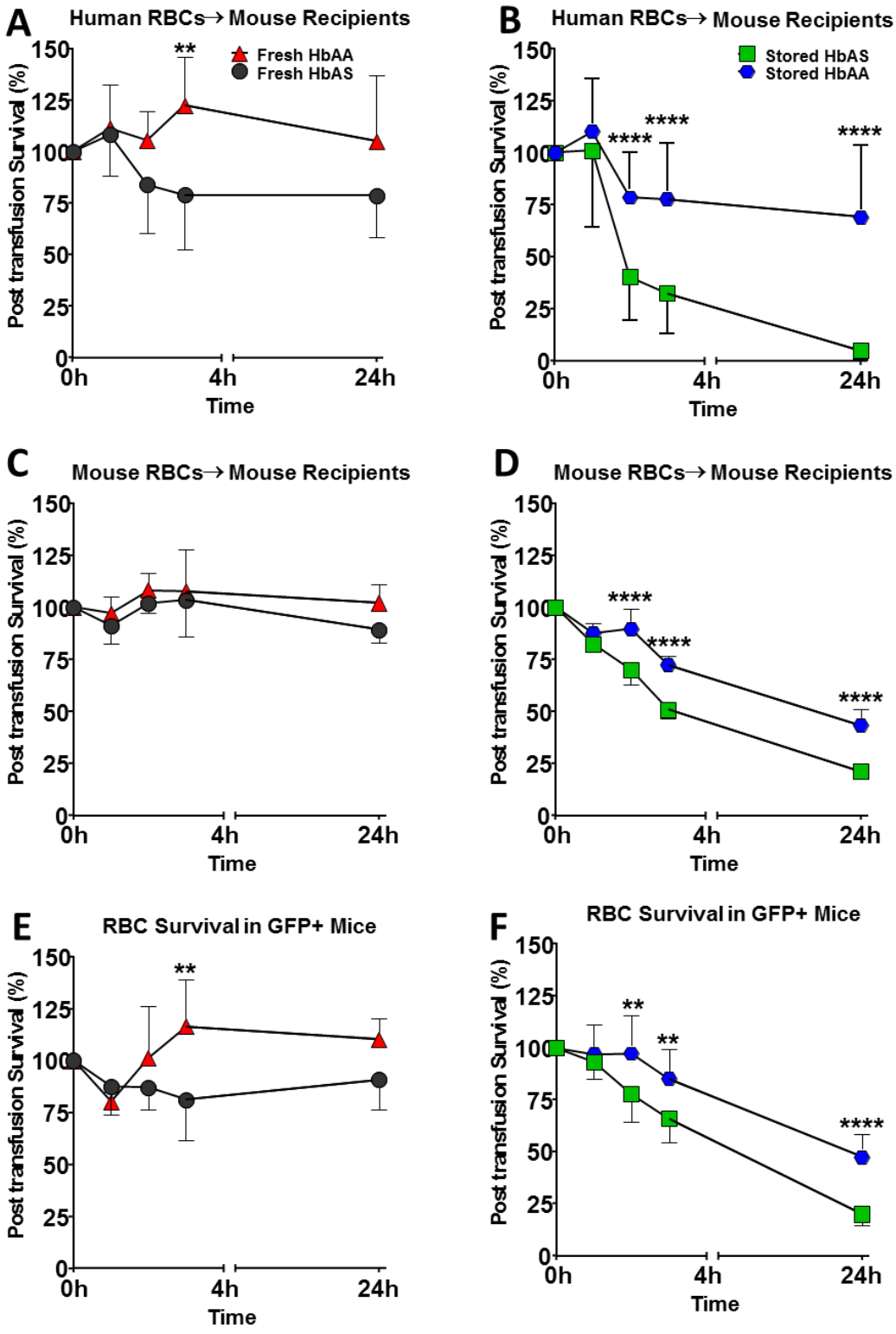

Figure 9. Stored HbAS RBCs show accelerated post transfusion clearance compared to stored HbAA RBCs.

The post transfusion recovery of RBCs from healthy and sickle cell trait individuals or from WT C57BL/6 and Berkeley hemizygous mice were evaluated at the beginning and end of storage. (A) WT C57BL/6 recipient mice (n=6) were transfused with paired samples of fresh human DiI-labeled HbAA RBCs (indicated as red triangle) and DiD-labeled HbAS RBCs (indicated as black circle) donated from one African-American healthy control and one SCT individual. (B) Pooled data from three groups of WT C57BL/6 recipients (n=5 per group, n=15 total) transfused with a 50:50 mixture of paired samples of matched 39-day stored DiI-labeled HbAA RBCs from 3 African-American healthy controls (indicated as blue circle) and stored DiD-labeled HbAS RBCs from 3 SCT individuals (indicated as green square), each recipient group receiving RBCs from one healthy and SCT donor. (C) WT C57BL/6 mice recipients were transfused with a 50:50 mixture of fresh DiD-labeled HbAA RBCs from WT mice donors (indicated as red triangle) and fresh DiI-labeled HbAS RBCs from Berkeley hemizygous mice (indicated as black circle) (n=6 recipients per group). (D) Eleven-day stored DiD-labeled HbAA RBCs (indicated as blue circle) and DiI-labeled stored HbAS (indicated as green square) RBCs (n=6 recipients per group). (E) To confirm that fluorescent labels did not interfere with RBC post transfusion survival, fresh unlabeled HbAA (indicated as red triangle) and HbAS murine RBCs (indicated as black circle) were transfused into two separate groups of GFP+ mice recipients (n=5 per group). (F) Eleven-day stored unlabeled HbAA (indicated as blue circle) and HbAS murine RBCs (indicated as green square) were transfused into two separate groups of GFP+ mice recipients (n=8 per group). All mice were 8-12 weeks of age and received a total volume of 200 µl of leukoreduced HbAA and HbAS RBCs (100 µl each) or 200 µl when HbAA or HbAS RBCs were transfused alone. Post-transfusion recovery was measured by dual-label cell tracking by flow cytometry unless

stated otherwise. The results are presented as mean ± SD.**p<0.01; ****p<0.0001 analyzed by 2-Way ANOVA, GraphPad Prism 6.0

3.1.2 HbAS red blood cells exhibit higher storage hemolysis

To assess the membrane properties of HbAA and HbAS RBCs following storage, human and murine RBCs were assayed at the beginning and end of storage. The size distribution and hemoglobin content of human HbAA and HbAS RBCs showed no differences (Supplemental Figure S3). By 39-42 days of storage, conventionally banked human HbAS RBC samples exhibited significantly higher storage hemolysis compared to human HbAA RBC samples (1.00% ± 0.57 versus 0.15% ± 0.015, *p=0.0035*, Figure 2A). Murine HbAS RBCs also exhibited higher storage hemolysis at the end of 11-day storage compared to murine HbAA RBCs (2.38% ± 0.16 versus 1.68% ± 0.06, *p=0.0022*, Figure 2B). In both human and murine HbAS RBCs, free hemoglobin concentrations were higher at the beginning of storage compared to HbAA RBCs, but did not achieve significance in the human RBC experiments (Figures 2A-B).

To determine membrane resilience to osmotic shock, packed RBCs were re-suspended in a hypotonic buffer for 3h. Similar to prior findings in HbSS RBCs,[87,88] human and murine HbAS RBCs showed striking resilience to osmotic shock compared with HbAA RBCs (human HbAS and HbAA RBCs: 9.0% ± 3.13 vs. 35.1% ± 10.28 hemolysis; p<0.0001, Figure 2C; murine HbAS and HbAA RBCs: 18.1% ± 5.40 vs. 55% ± 5.39 hemolysis; p=0.0001, Figure 2D). Human and murine HbAS RBCs exhibited reduced membrane deformability, measured by ektacytometry, compared to HbAA RBCs in both fresh and stored samples and this difference was increased by the end of storage, suggesting that reduced flexibility of the membrane is an

intrinsic property of HbAS RBCs that worsens as a result of storage-related aging (Supplemental Figure S4). Moreover, human and murine HbAS RBCs did not show increased hemolysis following AAPH-induced membrane lipid peroxidation (Figures 2E-F) or mechanical stress at the end of storage (Figures 2G-H). Taken together, the Berkeley murine HbAS RBCs appear to show similar membrane properties to that of human HbAS RBCs during storage, suggesting these mice serve as a suitable model for transfusion studies.

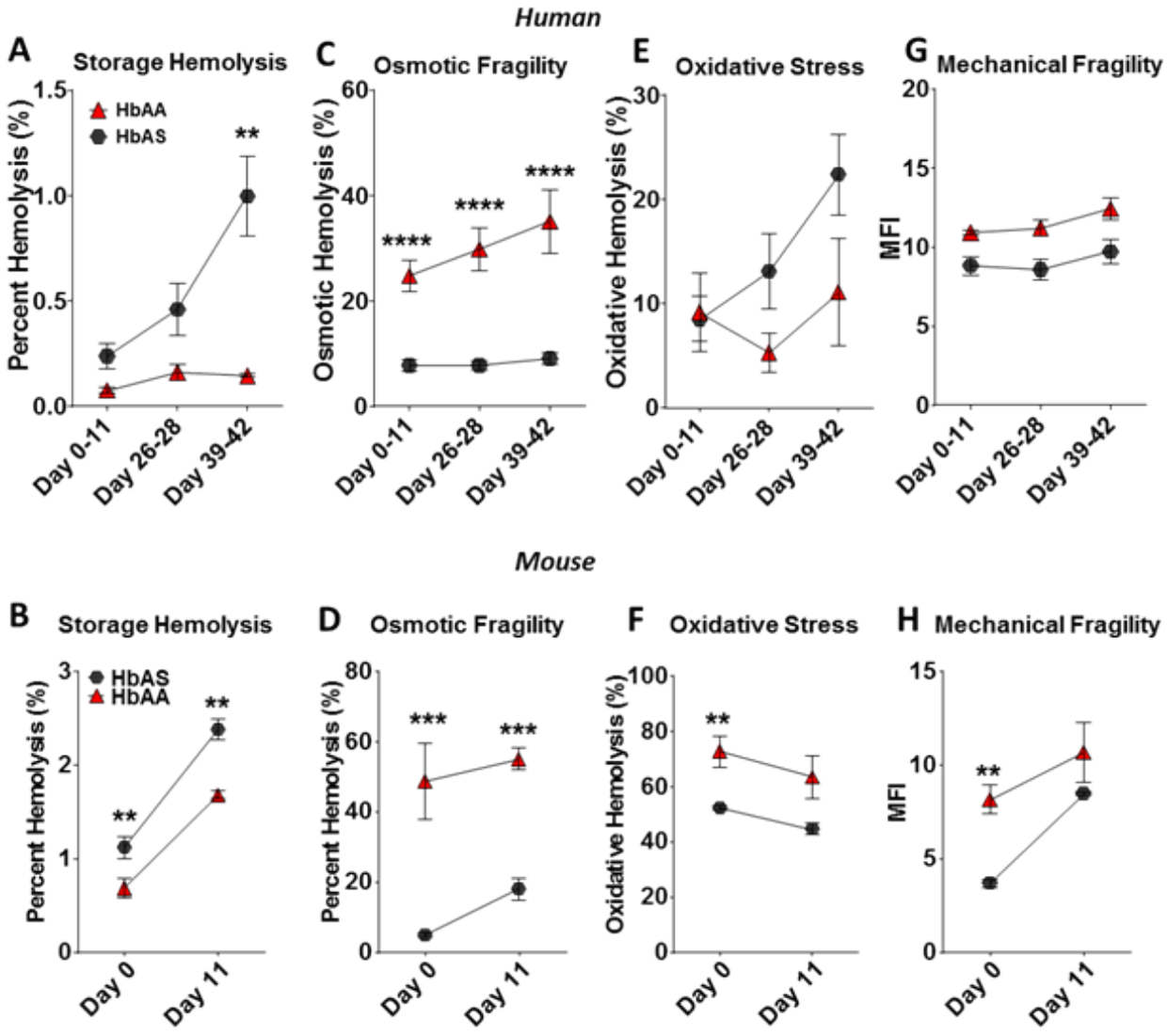

Figure 10. HbAS is associated with higher storage hemolysis in RBCs.

Fresh leukoreduced human (3 healthy donors and 9 SCT individuals) and murine RBC (representing 3 HbAA and 3 HbAS RBC pooled samples, where n=11 mice donor RBCs/pooled sample) units were stored at 4□C, sampled and tested at various times during a 42-day or 11-day storage period, respectively. (A, B) At the end of storage, there was a higher concentration of free hemoglobin in HbAS RBC samples compared to HbAA RBCs. (C, D) HbAS RBCs exhibited increased resilience under hypotonic-induced osmotic shock compared to HbAA RBCs throughout storage, as measured by the fraction of lysed RBCs in a hypotonic solution. (E, F)

There was no difference in AAPH-induced membrane lipid peroxidation, a kinetic assay used to assess the antioxidant capacity of membrane lipids. (G, H) RBCs showed no difference in mechanical fragility induced by agitation with one metal bead (3/32”) for 180 minutes in a 96-well plate. HbAA RBCs are indicated as red triangle, and HbAS RBCs are indicated as black circle. The results are presented as mean ± SEM.**p<0.01; ***p<0.01; ****p<0.0001 analyzed by 2Way ANOVA, GraphPad Prism 6.

3.1.3 Elevated echinocyte formation in HbAS RBCs compared to HbAA RBCs during storage.

Echinocyte formation occurs due to various factors such as cell dehydration and ATP depletion, and previously used as a surrogate marker for RBC storage integrity.[89-91] Human and murine RBCs were examined by scanning electron microscopy at the beginning and end of 39-42 or 11-day storage, respectively. While there were no differences in the percentage of echinocytes between human HbAA and HbAS RBCs at the beginning of storage, HbAS RBCs showed higher percentage of echinocytes at the end of storage compared to HbAA RBCs (47.6% and 24.9%; p=0.012, Figure 3A-B). A similar finding was observed in murine HbAS RBCs (68.8%) compared to HbAA RBCs (41.4%) at the end of storage (11 days; p<0.0001, Figure 3C-D). No differences in the percentage echinocytes were observed between fresh, murine HbAA and HbAS RBCs.

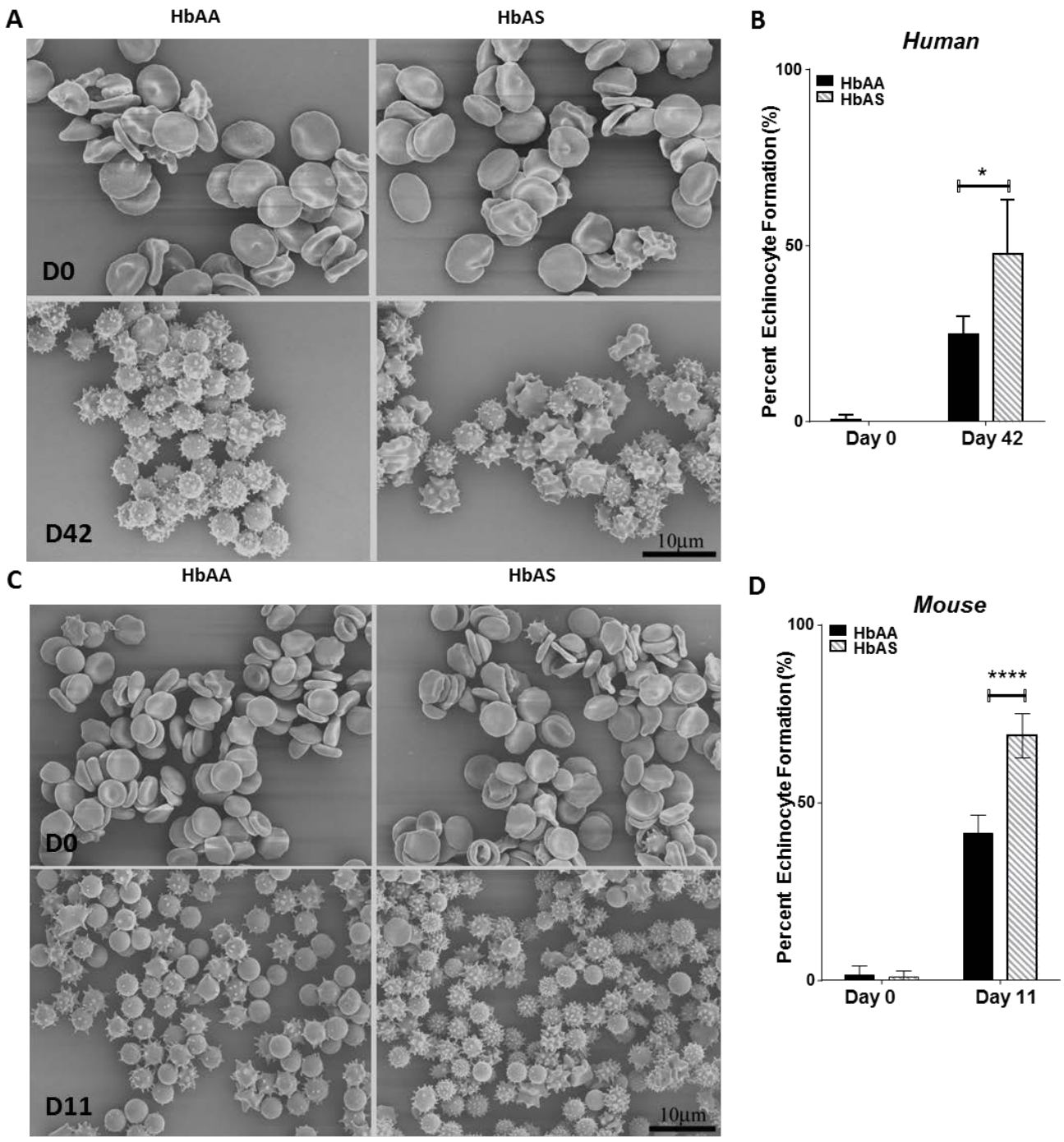

Figure 11. Echinocyte formation is increased in HbAS RBCs compared to HbAA RBCs during storage

RBC images were taken with a JEOL 6335F scanning electron microscope. (A) Upper panel shows representative fields from fresh and stored human HbAA and HbAS RBCs at the beginning and the end of 42-day storage. (B) Five fields per sample were counted analyzed from one healthy and one SCT representative donors at the beginning and end of storage. (C) Lower panel shows representative fields from fresh and 11-day stored murine RBCs obtained from WT and Berkeley hemizygous mice (one pooled sample each from n=11 mice donor RBCs). (D) Six fields were counted and analyzed for echinocyte formation from each pooled sample. The results are presented as mean ± SEM, where echinocyte formation represents % echinocyte per total number of cells in fields counted. *$p<0.05$; ****$p<0.0001$ analyzed by 2Way ANOVA, GraphPad Prism 6.0.

3.1.4 Clodronate treatment or splenectomy does not alter stored HbAS RBC 24 h post-transfusion survival

To determine whether stored HbAS RBCs were cleared by the mononuclear phagocyte system at a faster rate than HbAA RBCs, we depleted F4/80$^+$ macrophages in the liver and spleen using liposomal clodronate prior to transfusion. Intraperitoneal administration of liposomal clodronate effectively depleted F4/80$^+$ macrophages in both the liver and spleen (Figure 4A). Twenty-four hours following liposomal clodronate, >85% of splenic F4/80$^+$ macrophages were depleted as compared to spleens from mice that received liposomal PBS or no treatment (Figure 4B). Although others previously showed that macrophages were responsible for early clearance of transfused stored HbAA RBCs,[33] we did not observe any difference in the post-transfusion recovery of stored HbAS following depletion of macrophages (Figure 4C). Our *in vitro* studies showed no differences in the uptake of HbAS and HbAA RBCs by macrophages (Supplemental Figure S5), suggesting an alternative mechanism of clearance upstream of erythrophagocytosis. Furthermore, splenectomy did not alter post-transfusion recovery of stored HbAS RBCs indicating that other organs are involved in the rapid clearance in the absence of the spleen.
(Figure 4D). We further investigated whether intravascular hemolysis contributes to the reduced post-transfusion recovery of stored HbAS RBCs. Transfused mice recipients were immediately assayed for plasma and urinary free hemoglobin at various time intervals. Recipient mice transfused with HbAS RBCs did not show higher levels of plasma or urinary free hemoglobin levels at 5 min and 4 h post-transfusion compared with mouse recipients transfused with HbAA RBCs (Supplemental Figure S6). These findings indicate that intravascular hemolysis did not contribute significantly to the reduced post-transfusion recovery of HbAS RBCs.

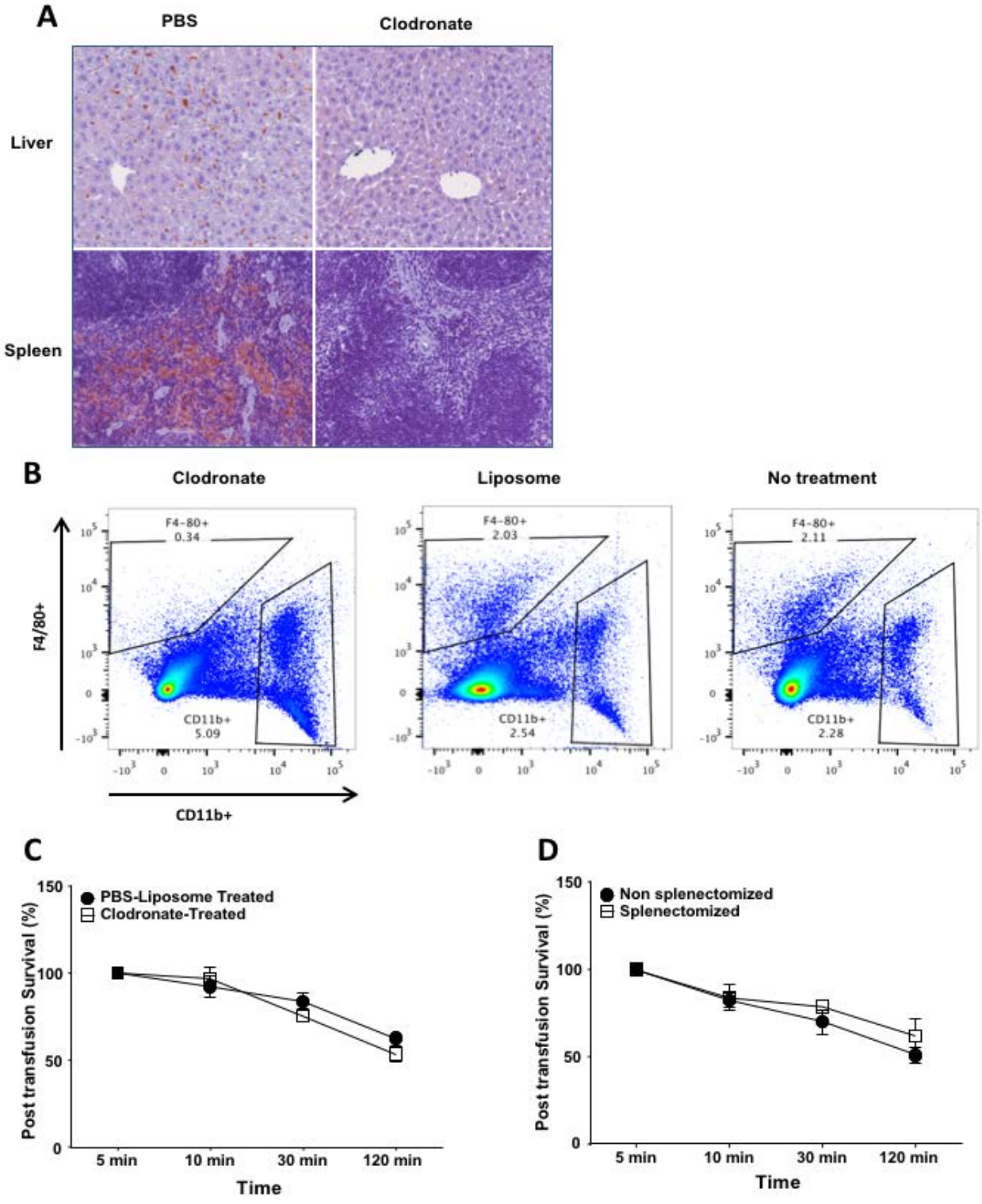

Figure 12. Clodronate treatment or splenectomy does not alter stored HbAS RBC post transfusion survival.

(A) WT mouse recipient mice were treated with clodronate or PBS liposomes 24 h prior to liver and spleen harvest for immunohistochemistry. Representative images are shown indicating staining for F4/80+ macrophages in brown. (B) WT mice were treated with clodronate or PBS liposomes and euthanized after 24 h. Spleens were homogenized and stained with F4/80 and CD11b antibodies. Samples were analyzed using flow cytometry to quantify the F4/80+ population. (C) WT recipient mice were transfused with 11-day stored HbAS murine RBCs 24 h following clodronate (n=3) or PBS liposome (n=3) injection (2 mg i.p.). (D) WT recipient mice were transfused with 11-day stored HbAS murine RBCs 5 d following splenectomy (n=4) or sham procedure (n=6). Results presented are mean ± SD**.**

3.1.5 Increased sequestration of stored murine HbAS RBCs within kidney, liver and spleen organs following transfusion.

We next examined whether tissue sequestration was the underlying mechanism for the rapid clearance of stored HbAS RBCs. WT recipient mice were transfused with cy3-labeled stored HbAA or HbAS RBCs. Kidney, liver and spleen of recipient mice were harvested at 2 h for confocal imaging and quantitative analysis (Figure 5A). There was higher sequestration of stored murine HbAS RBCs within the recipient kidney, liver and spleen when compared to recipients transfused with HbAA RBCs (Figure 5B). When stored human HbAS and HbAA RBCs were transfused into murine recipients, HbAS RBCs were entrapped in the spleen to a greater extent than HbAA RBCs (Figure 6A-B). As the spleen appeared to be the preferential site of tissue sequestration presumably due to the narrower internal diameter of splenic sinusoidal vessels,[92-94] we examined the fate of transfused stored HbAS RBC in non-splenectomized and splenectomized WT mice. There was no difference in tissue sequestration of stored HbAS RBCs

within the kidneys and livers of sham-operated and splenectomized mice (Figure 7), suggesting that the liver which is 10X the volume of the spleen in mice, are involved in the clearance of stored red cells and compensate in the absence of the spleen. Collectively, these findings indicate that enhanced mechanical entrapment in tissue accounts for the rapid clearance of transfused HbAS RBCs from circulation.

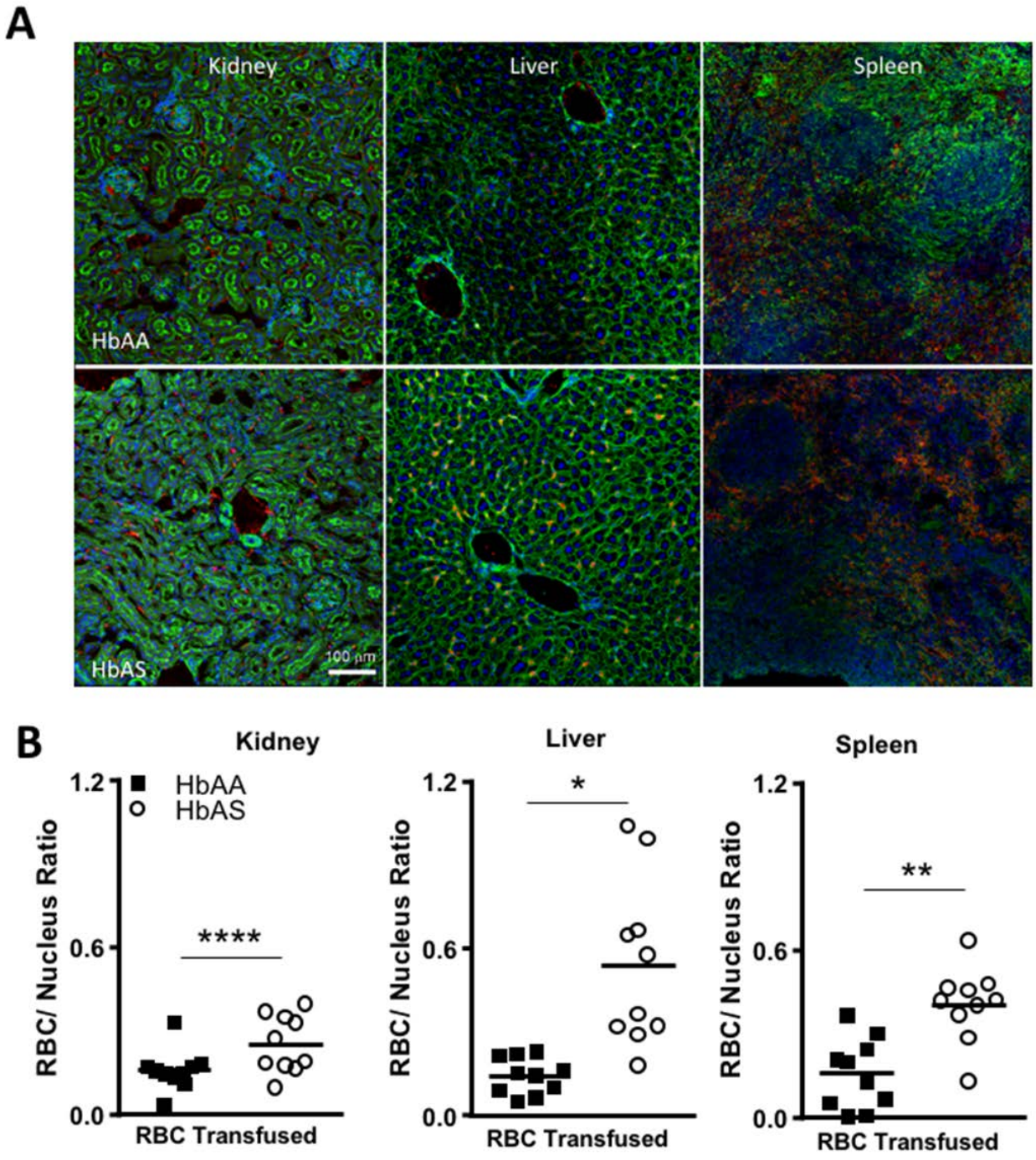

Figure 13. Increased sequestration of stored murine HbAS RBCs within kidney, liver and spleen organs following transfusion when compared with transfused stored HbAA RBCs.

WT C57BL/6 recipient mice (n=5 per group) were transfused with 11-day stored cy3-labeled HbAA or HbAS RBCs. Kidney, liver and spleen organs were harvested 2 h post transfusion for

confocal imaging. (A) Confocal images showing sequestered HbAA and HbAS in recipient mouse tissues (Magnification 20X, Green: phalloidin, Blue: DAPI, Red: cy3 RBCs). (B) Individual points represent sequestered cy3-labeled RBCs normalized to nuclei number per section quantified by a fluorescence intensity-based threshold using the average intensity of the negative staining control sample as guide, by segmentation analysis and object counting using NIS Elements. Two sections from each recipient mouse organ were analyzed (n=10 sections per group). Statistical Analysis by two-tailed Mann Whitney U, ****$p<0.0001$, **$p<0.01$, *$p<0.05$, lines indicate the median.

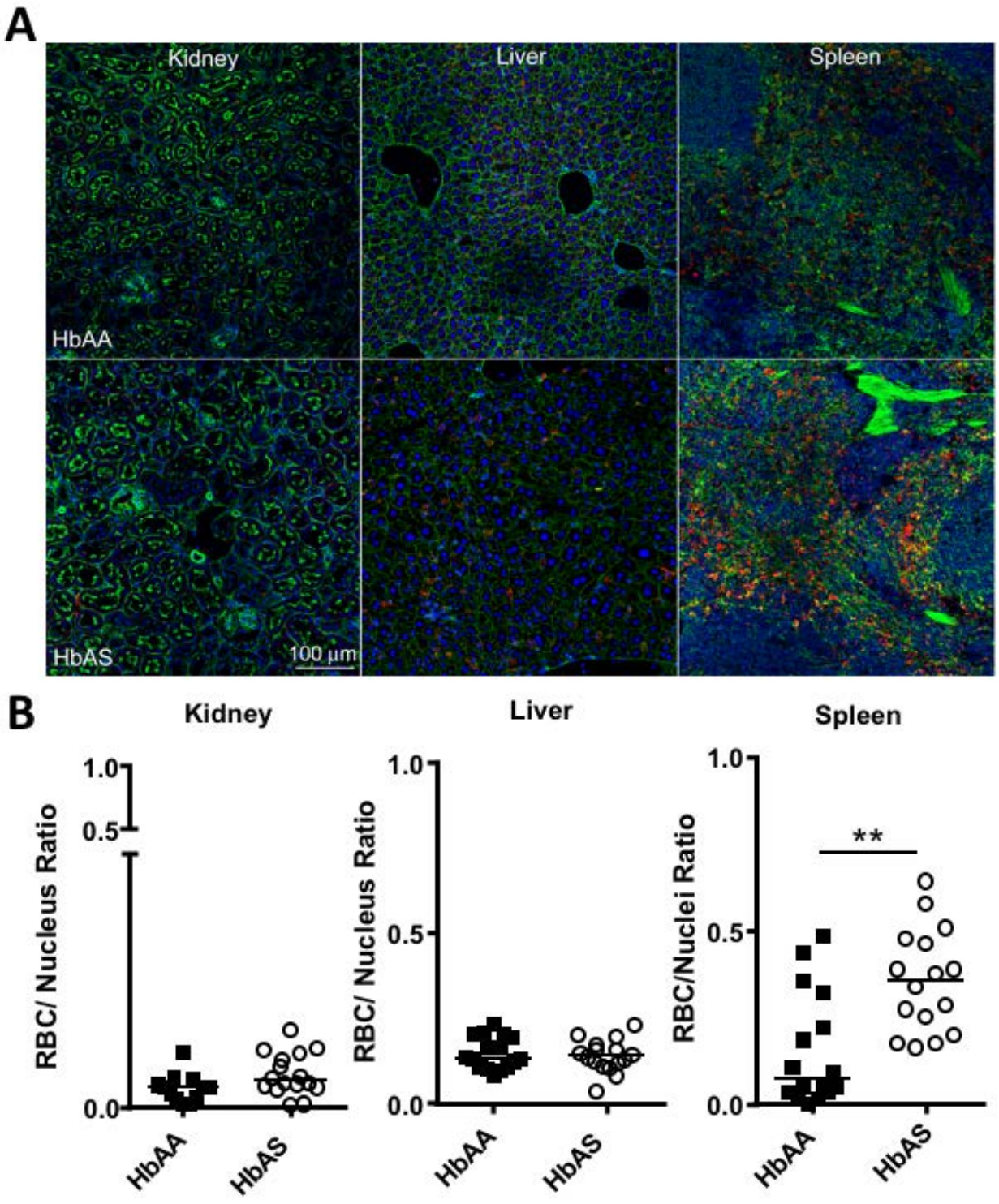

Figure 14. Increased sequestration of stored human HbAS RBCs within kidney, liver and spleen organs following transfusion when compared with transfused stored HbAA RBCs.

WT C57BL/6 recipient mice (n=8 per group) were transfused with stored human HbAA or HbAS RBCs. Kidney, liver and spleen organs were harvested 2 h post transfusion for confocal imaging. (A) Confocal images showing sequestered HbAA and HbAS RBCs in recipient mouse tissues (Magnification 20X, Green: phalloidin, Blue: DAPI, Red: Glycophorin A: FITC antibody for human RBCs). (B) Individual points represent sequestered human RBCs normalized to nuclei number per section quantified by a fluorescence intensity-based threshold using the average intensity of the negative staining control sample as guide, by segmentation analysis and object counting using NIS Elements. Two sections from each recipient mouse organ were analyzed (n=16 sections per group). Statistical Analysis by two-tailed Mann Whitney U, $**p<0.01$, lines indicate the median.

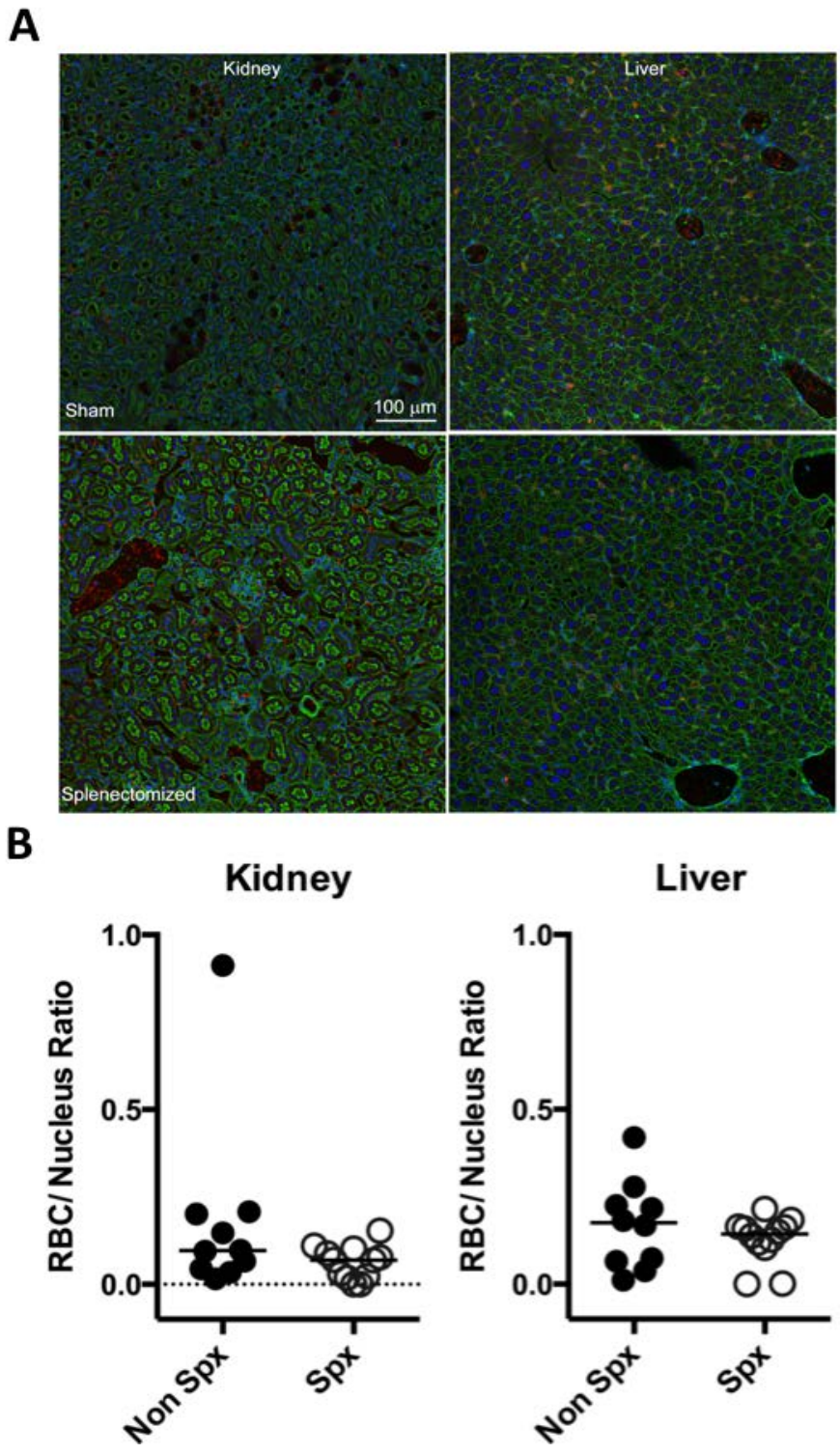

Figure 15. Splenectomy does not increase kidney and liver sequestration of stored HbAS RBCs following transfusion.

Splenectomized WT C57BL/6 recipient mice (n=7) or sham operated (n=5) were transfused with stored murine HbAS RBCs. Kidney and liver organs were harvested 2 h post transfusion, fixed in 2% PFA and processed for confocal imaging. (A) Confocal images showing sequestered HbAS RBCs in recipient mouse tissues (magnification 20X, Green: phalloidin, Blue: DAPI, Red: Cy3-labeled murine HbAS RBCs). (B) Individual points represent sequestered murine HbAS RBCs normalized to nuclei number per section quantified by a fluorescence intensity-based threshold, using the average intensity of the negative staining control sample as guide, by segmentation analysis and object counting using NIS Elements. Two sections from each recipient mouse organ were analyzed.

3.1.6 Proof of Concept: Unlabeled red cells can be tracked and enumerated following transfusion into GFP+ recipient mice.

To confirm that the fluorescent dyes used in earlier experiments to track red cells *in vivo* does not affect red cell survival, we utilized recipient mice expressing green fluorescent protein in hematopoietic cells, thus allowing us to transfuse and track unlabeled RBCs in these mice.

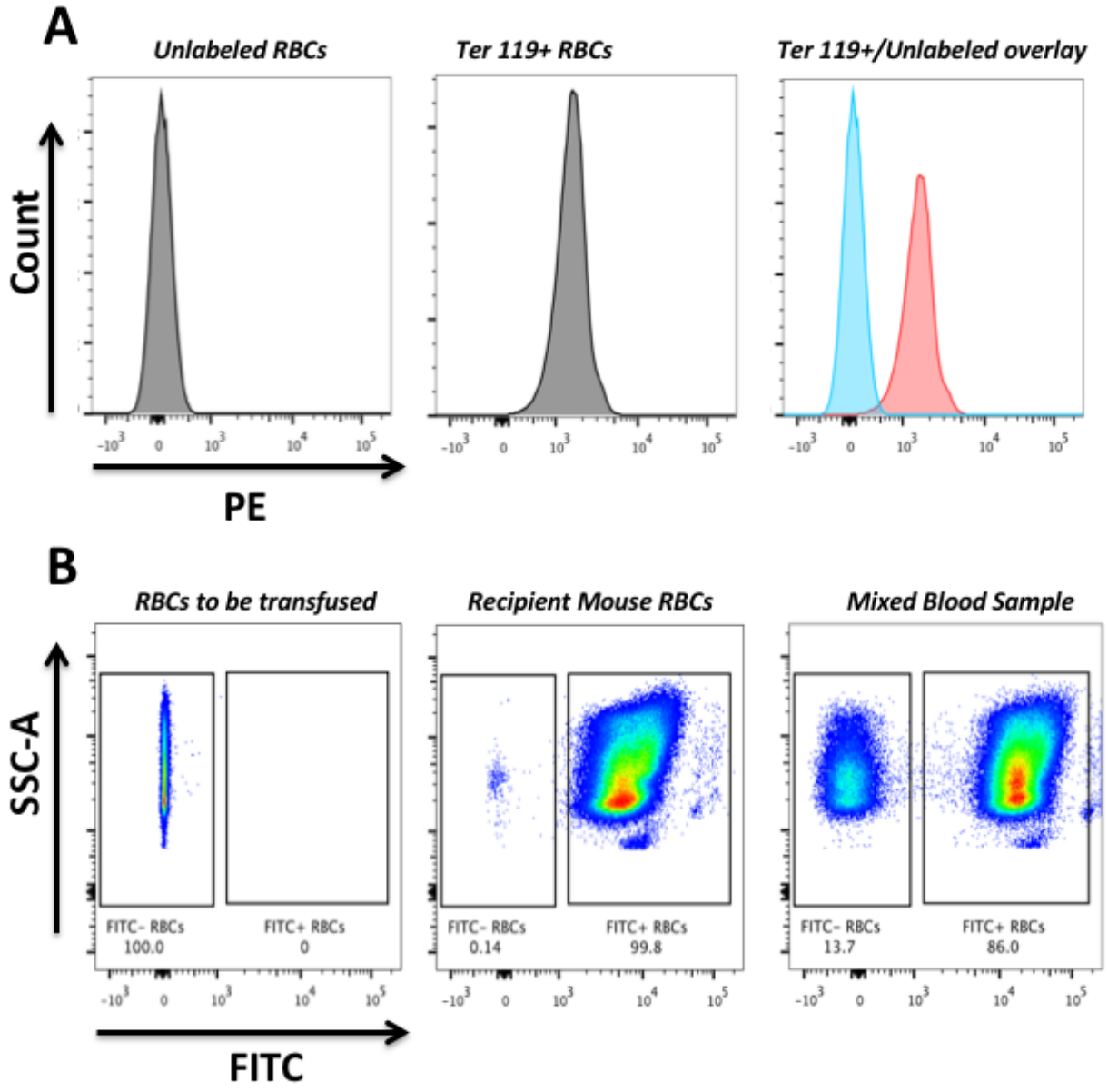

Figure 16. Assessment of Red Blood Cell Post Transfusion Survival using Flow cytometry.

(A) Charts obtained by flow cytometry to confirm that cells to be transfused are murine RBCs. This is performed using Ter-119 monoclonal antibody conjugated to PE fluorophore, an antibody that binds to murine RBC specific antigen, Ter-119. Ter-119+ events are indicated in red, unlabeled overly indicated in blue (far right panel). (B) 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 FITC fluorescence between unlabeled and GFP+ RBCs allowing quantitation enumeration of transfused unlabeled RBCs**.**

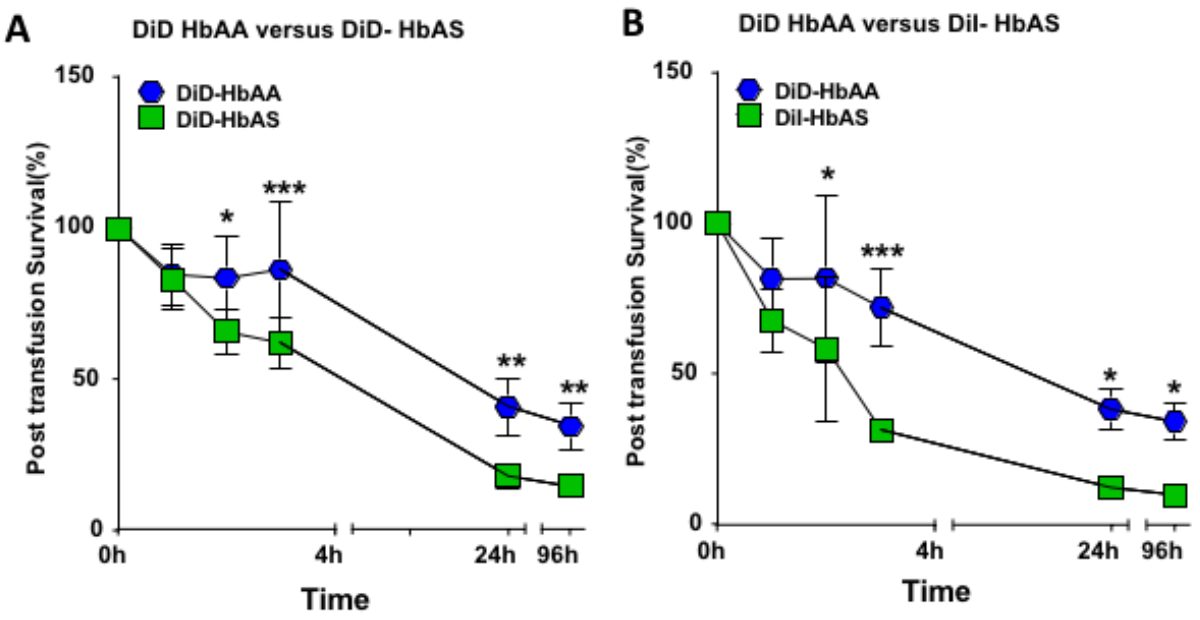

Figure 17. Labeling dye does not affect RBC post transfusion survival.

(A) To verify that fluorescent labels did not alter RBC post-transfusion survival, two separate groups of WT C57BL/6 mice recipients (n=6 per group) were transfused with either DiD-labeled 11-day stored HbAA RBCs (indicated as blue circle) or DiD-labeled 11-day stored HbAS RBCs (indicated as green square) and post-transfusion recovery analyzed. **(B)** To further confirm that fluorescent labels did not interfere with RBC post transfusion survival, fluorescence labeling was switched so that DiD-labeled stored HbAA RBCs (indicated as blue circle) and DiI labeled stored HbAS RBCs (indicated as green square) were 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

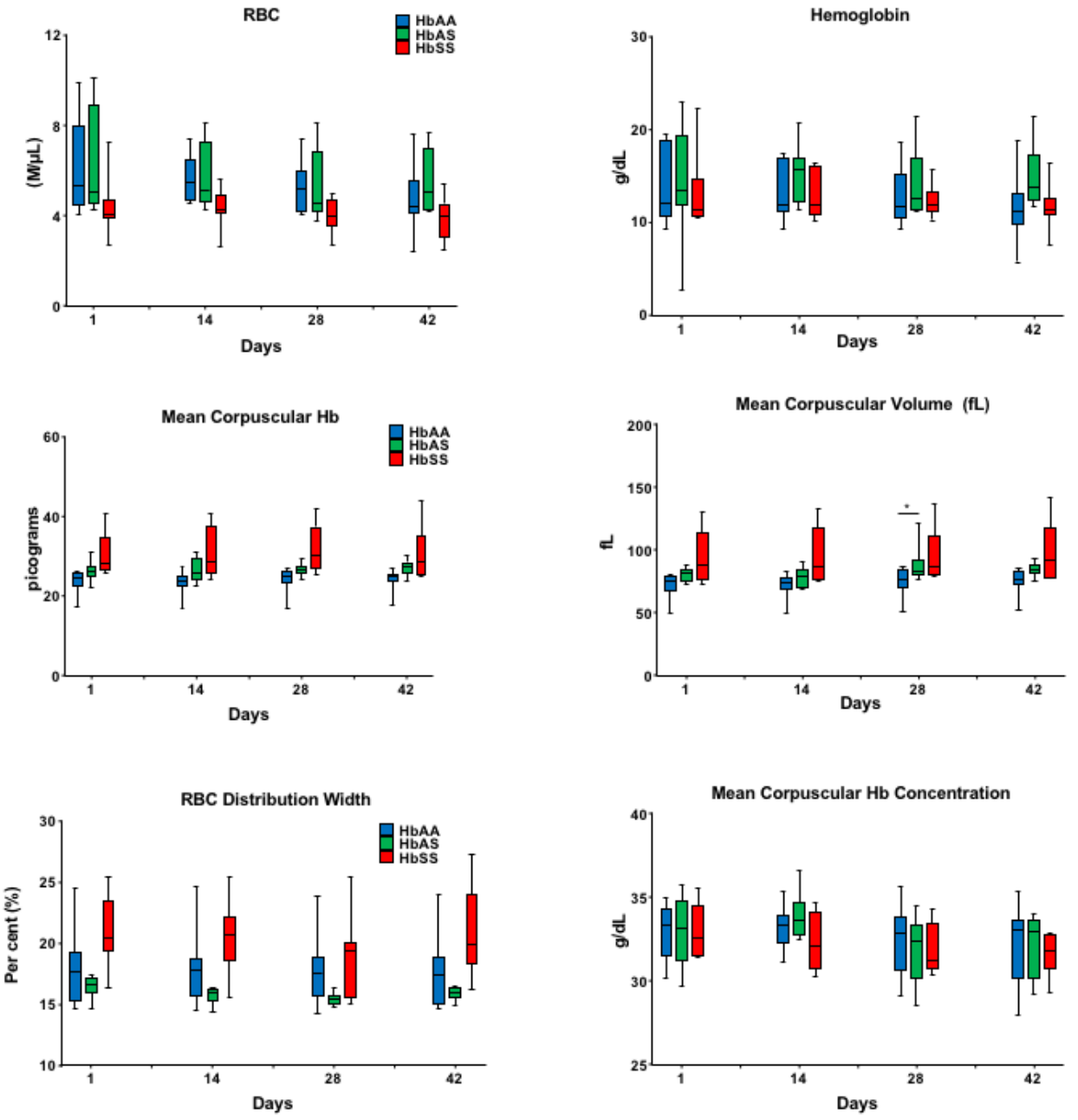

Figure 18. Complete Blood Count (CBC) measurements show no major differences in the size distribution and hemoglobin content of human HbAA and HbAS RBCs obtained from healthy and Sickle Cell Trait donors

HbAA RBCs (n=11), HbAS RBCs (n=8), HbSS RBCs (n=8). Data plotted show box and whiskers, min to max with line at median. *p<0.05 using 2way ANOVA.

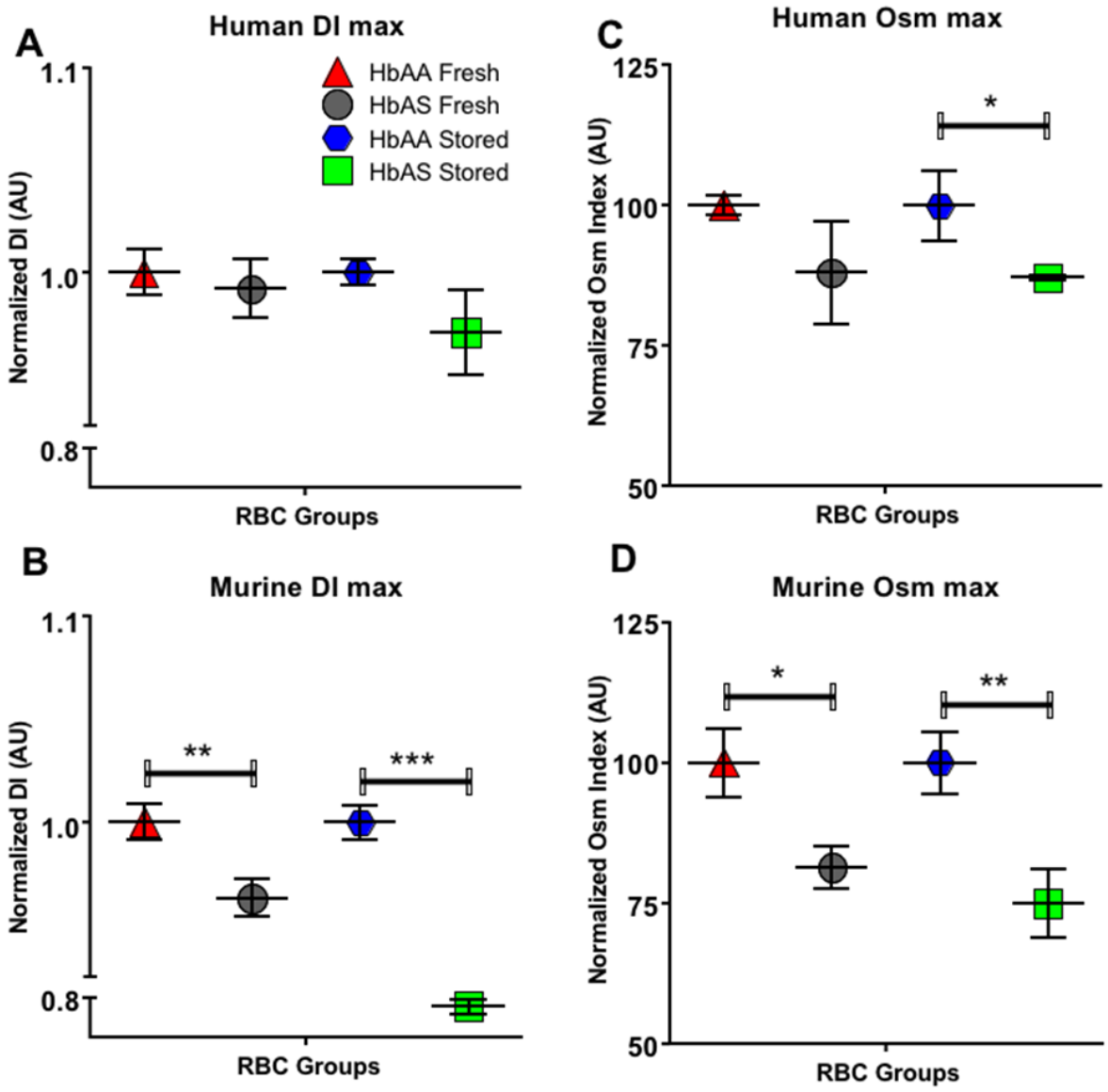

Figure 19. Membrane Deformability changes during storage.

(A) Deformability Index (DI) max of human RBCs (n=3 per group) were measured at the beginning and end of storage. (B) The maximal DI of one WT C57BL/6 and one Berkeley hemizygous pooled samples, where n=11 mice donor RBCs/pooled sample, was measured. (C, D) The osmolality at which RBCs exhibit the maximum deformability or Osm Max was measured for both murine and human HbAA and HbAS RBCs. 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.*$p<0.05$; **$p<0.01$; ***$p<0.001$ analyzed by 2Way ANOVA, GraphPad Prism 6.0

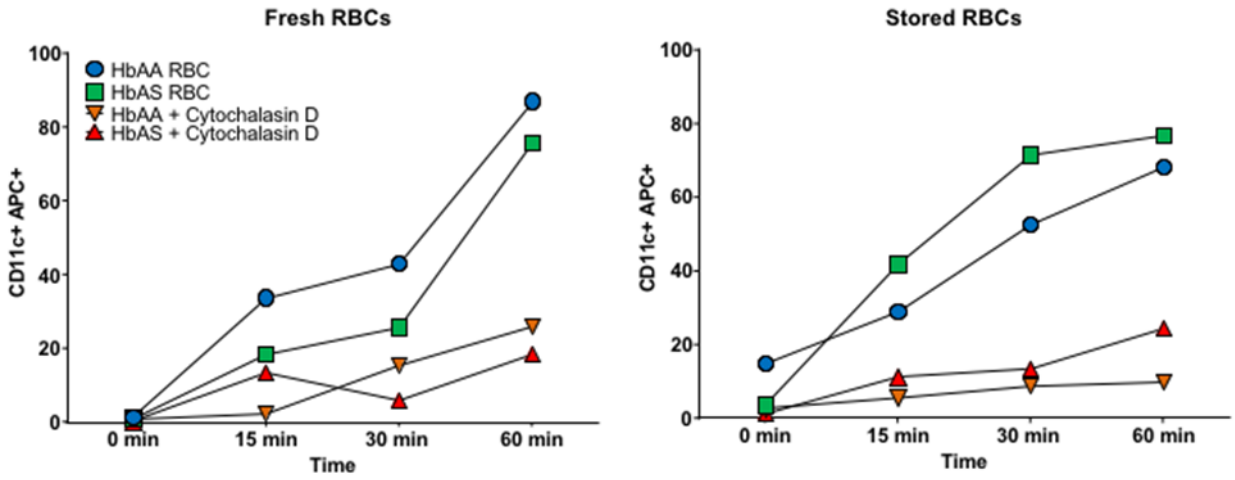

Figure 20. There is no difference in Fresh or Stored RBC uptake by CD11c+ macrophages.

Thiogylycollate-stimulated peritoneal macrophages were harvested from WT mice (n=3 donor mice). Macrophages were seeded at $1*10^6$ cells/well overnight at 37 □C in 10% FBS. 1 h prior to experiment, culture media was replaced with fresh media (1% FBS) with or without cytochalasin D (20 µM) to block phagocytosis. Di-D-labeled HbAA or HbAS RBCs were added to wells at a 1:1 ratio and incubated for up to an hour. At the end of incubation period, cell media was immediately aspirated and replaced with NH_4Cl for 30 s to lyse un-engulfed RBCs. Macrophages were scraped into 5ml-tubes and washed once at 1200 rpm for 5 min. Macrophages were re-suspended in 0.5 ml PBS and stained with anti-murine CD11c monoclonal antibody (FITC channel). Samples were washed once and analyzed by flow cytometry.

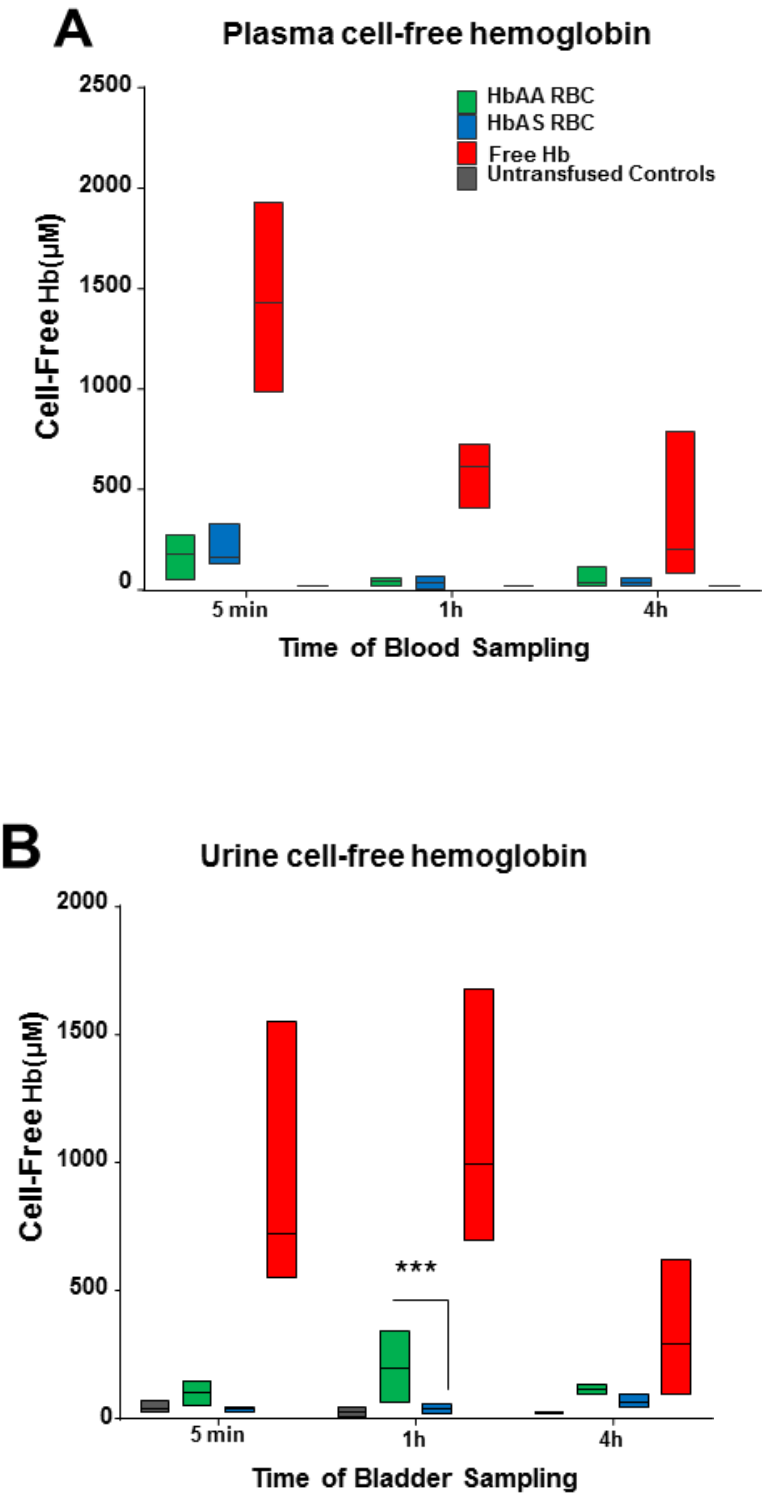

Figure 21. There is no difference between the intravascular or urinary cell- free hemoglobin following HbAA or HbAS RBC transfusion.

(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 HbAA RBCs (n=15 recipients) or HbAS RBCs (n=15 recipients), cell free hemoglobin (n=15 recipients) and compared to PBS-transfused controls (n=4). At 5 min, 1 h and 4 h following transfusion, mice in each group were bled via tail vein using a microvette to obtain 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 HbAA RBCs (n=15 recipients) or HbAS RBCs (n=15 recipients), cell free hemoglobin (n=15 recipients) and compared to PBS-transfused controls (n=4). 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 800g 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.

4.0 DISCUSSION

The main findings of this study are that both human and murine HbAS RBCs show higher storage-induced hemolysis and membrane blebbing, and increased resilience to osmotic shock compared to HbAA RBCs. Additionally, stored human and murine HbAS RBCs show reduced post-transfusion recovery from circulation compared to HbAA RBCs. These differences were less noticeable in the post-transfusion recovery of fresh HbAA and HbAS RBCs, suggesting a mechanism that is enhanced by storage-induced aging. Surprisingly, *in vivo* intravascular hemolysis was not a major contributing factor to the reduced RBC post-transfusion survival of stored HbAS RBCs. $F4/80^{+}$ macrophage depletion in the liver and spleen of recipient mice also failed to improve stored HbAS RBC 24 h post-transfusion survival thus eliminating $F4/80^{+}$ mediated erythrophagocytosis as an essential mechanism.

Our findings indicate that enhanced mechanical entrapment in organs such as the kidney, liver and spleen account for the rapid clearance of stored HbAS RBCs following transfusion. We also show that conventionally stored human HbAS RBC exhibit enhanced mechanical entrapment in the spleen following transfusion into murine recipients. Enhanced tissue sequestration may be due to altered biophysical properties of HbAS RBC membranes that accelerate storage-related aging, and increase the propensity for microvascular adhesion. It is noteworthy that splenectomy of recipient mice did not improve post-transfusion recovery of stored HbAS RBC despite increased splenic sequestration of stored HbAS RBCs in non-

splenectomized mice. This observation is likely due to the fact that the liver is significantly larger and about ten times the spleen weight in mice40 and therefore despite higher numbers of sequestered stored HbAS RBCs in the spleen compared to stored HbAA RBCs, the total number of RBCs sequestered within the spleen is relatively small compared to those sequestered in the liver. Our data supports studies performed showing no difference in the storage hemolysis of HbAA and HbAS RBCs at earlier time-points (up to 21 days).[16,17] Importantly, our data confirms that the accumulation of cell-free hemoglobin remains similar between human HbAA and HbAS RBCs during the initial four weeks of storage. However, after 28 days, this accumulation intensifies in HbAS RBC units. To our knowledge, this is the first controlled study to examine the storage integrity and post transfusion survival of HbAS RBCs at the limits of the current approved storage time of 42 days.

It is important to re-evaluate the suitability of HbAS RBCs for transfusion since prior studies only examined RBCs that were stored for shorter periods (<28 days) of time in ACD anticoagulant.[16,17] Additionally, the techniques used to evaluate RBC post-transfusion survival such as differentiation by blood group antigens may provide imprecise assessment due to formation of mixed cell populations.[95,96,97] The varying elution rates of ^{51}Cr from RBCs from donor to donor, especially since the binding site of ^{51}Cr is on the beta hemoglobin,[98,99] have also challenged the reliability of chromium labeling to assess post-transfusion survival of RBCs from patients with hemoglobinopathies. Here we utilize highly sensitive tracking methods to evaluate RBC post-transfusion survival. This study, which is performed in a well-controlled setting, provides evidence that RBC storage lesion can be intensified by silent mutations such as sickle cell trait, the asymptomatic carrier status of SCD. To prevent the negative impact of hemolysis,[19,29,69] the Food and Drug Administration set the upper threshold of hemolysis in a

RBC unit to be transfused at 1%.[100] The human HbAS RBCs purchased from a commercial blood bank for this study would not have met the regulatory requirement near the end of the FDA accepted shelf-life of 42 days, as these units exhibited a hemolysis level of greater than 1%. Occult genetic mutations such as sickle cell trait that increase storage hemolysis may explain why some RBCs age faster during storage. It is also well documented that there are leukoreduction difficulties of sickle cell trait blood from certain donors, which may increase free hemoglobin in these units at the beginning of storage.[101-103]

The finding that the reduced post-transfusion survival of stored HbAS RBCs was not due to intravascular hemolysis suggests a fundamental difference between hemolytic propensity of SCD and sickle cell trait RBCs. The increased sequestration and increased osmotic resilience indicate that HbAS RBC exhibit altered membrane properties that make them less deformable and stiffer with storage.[104,105] The effects of these membrane properties are further augmented by increased cellular dehydration in HbAS RBCs (Supplemental Figure S4). Small reductions in RBC hydration state, which is known to cause exponential changes in intracellular viscosity, may underlie the mechanical challenges of stored HbAS RBCs to passage through microcirculation leading to the observed increased entrapment.[57,106]

To date, over 1000 mutations in the human genome have been associated with malaria[66,107] with sickle cell trait representing one of the major evolutionary responses that confers protection against this endemic parasite. It still remains to be determined whether other hemoglobinopathies, such as glucose-6-phospate dehydrogenase (G6PD) deficiency and silent carrier status of alpha thalassemia, that occur with high frequency in African Americans and populations originally from malaria endemic regions, exhibit similar behavior as sickle cell trait

during storage and following transfusion. This is particularly important because these populations represent a donor pool from which SCD patients are likely to be transfused to reduce the risk of RBC allo-immunization.[23] Carriers of these mutations usually do not present with clinical symptoms, however, as shown in this study, prolonged exposure of RBCs to storage-induced stress may unmask subtle perturbations of the heterozygous state.

In conclusion, the results of this current study show that HbAS RBCs harbor subtle membrane changes that accelerate degradation during prolonged storage and prompts further investigation into whether standard blood banking procedures should include universal screening of RBCs to identify markers that accelerate storage-related aging and set shorter storage times for qualifying donor units.

4.1 PREVIOUS STUDIES EVALUATING SICKLE CELL TRAIT RED CELL STORAGE INTEGRITY AND POST TRANSFUSI3ON SURVIVAL

Authors, Journal, Year, PMID	Storage Conditions and Transfusion Methods	Main Findings/Results	Conclusions	Comments
Ray, RN et al, J Clin Pathol (1959) (PMID:14436622)	HbAS RBCs stored in ACD for 21 or up to 25 days at 5±1°C Cr^{51} labeling, autologous.	Increasing plasma free Hb in blood bag with storage time. Storage hemolysis similar to HbAA RBCs. Normal osmotic fragility. 24h survival of HbAS RBCs after 21 days of storage was 84%. Mean 24h survival of HbAS RBCs after 25 days of storage was 52% (low but suspected to be within normal range). No liver or splenic sequestration.	21-day stored HbAS RBCs exhibit similar 24h post transfusion survival compared to stored HbAA RBCs.	No stored HbAA RBC comparators were used in this study.
Levin, WC et al., J Lab Clin Med. (1960) (PMID: 14416237)	HbAA and HbAS RBCs stored in ACD for 21 days at 4°C. Cr^{51} labeling, Allogeneic HbAA recipient.	No difference in T ½ Cr^{51} after 1, 8, 15 and 21-day storage. 24h post transfusion survival of stored HbAA and HbAS RBCs were 80% after 21-day storage.	No difference in post transfusion survival of HbAA and HbAS RBCs during 21-day storage although stored HbAS appear to develop a small fragile population after 21-day storage.	Well controlled study; utilized stored HbAA RBC for storage dependent comparisons.
Callender, S.T.E et al., J Lab Clin Med. (1949) (PMID: 18122780)	The main comparison was between fresh HbAA RBCs and fresh HbAS RBCs were transfused and stored in ACD for less than 24h prior to transfusion. ABO or MN group differentiation: Allogeneic	Transfusion of HbAA and HbAS RBCs into normal or SCD recipients or HbAS RBCs into normal showed a normal lifespan of >110 days.	Fresh HbAA and HbAS RBCs have a normal life span when transfused in normal and SCD patients.	None

Authors, Journal, Year, PMID	Storage Conditions and Transfusion Methods	Main Findings/Results	Conclusions	Comments
Kaufman,M. et al, Am J Med Sci (1965) (PMID: 14254829)	Transfusion of stored RBCs. Storage conditions not described. Allogeneic	There were no reactions following transfusion of HbAS RBCs.	Transfusion of HbAS RBCs into 13 patients during this study and did not exhibit any adverse effects.	Storage conditions not described but units surveyed were chosen at random from a blood bank
Veiga S., et al, Transfusion (1963) (PMID: 14077072) Case Report	Exchange Transfusion of stored HbAS RBCs Allogeneic	Massive sequestration of deformed HbAS RBCs in spleen, liver and lungs tissues. Intravascular sickling and entrapment of HbAS RBCs observed.	Prior to exchange transfusions, units should be screened for HbS and excluded if present.	Storage duration and conditions not described. Recipient was a severely ill pre-mature neonate. Umbilical discharge tested positive for Proteus sp. and Enterocci.
Barbedo, MM. et al, Acta Haematol (1974) (PMID: 4212308)	Not stored Post transfusion survival determined using Cr^{51} and $DF^{32}P$ labeling.	Normal sickle cell trait donor hematocrit and HbAS RBC volume. The mean T ½ Cr^{51} was 30.3±1.8 days. The mean T ½ $DF^{32}P$ was 107.4±12.3 days.	No evidence to suggest significant loss of cells due to intravascular hemolysis due to normal red cell lifespan	No HbAA RBC comparators were used in this study.
Kelleher JF., et al., Transfusion (1984) (PMID: 18122780) Case Report	Frozen, deglycerolized by standard technique	Recipient experienced hemoglobinuria and hemoglobinemia following HbAS RBC transfusion. No evidence of serological mismatch. No renal dysfunction. Benign clinical discourse.	HbAS RBCs can be frozen without difficulty but aggregate during de-glycerolyzation impairing glycerol removal and leads to massive hemolysis (30-70%) of donor cells	Study does not evaluate post transfusion survival of stored HbAS RBCs but rather a processing technique that reduces quality of donor unit.

Table 1: Early Studies Performed To Evaluate The Storage Integrity and Post Transfusion Survival.

APPENDIX A

A.1 RED BLOOD CELL POST TRANSFUSION SURVIVAL CAN BE MODULATED BY FACTORS PRESENT IN MOUSE RECIPIENTS

Most of the experiments performed in this study were performed in healthy mice under normoxic conditions. However, under clinical situations, patients who receive transfusions are usually septic or hypoxic due to hemorrhage or associated pathology. To evaluate the effect of these conditions, I examined the effect of acute hypoxia, TLR3 ligands or lipopolysaccharide-induced sepsis in the mouse recipient on red blood cell post transfusion survival.

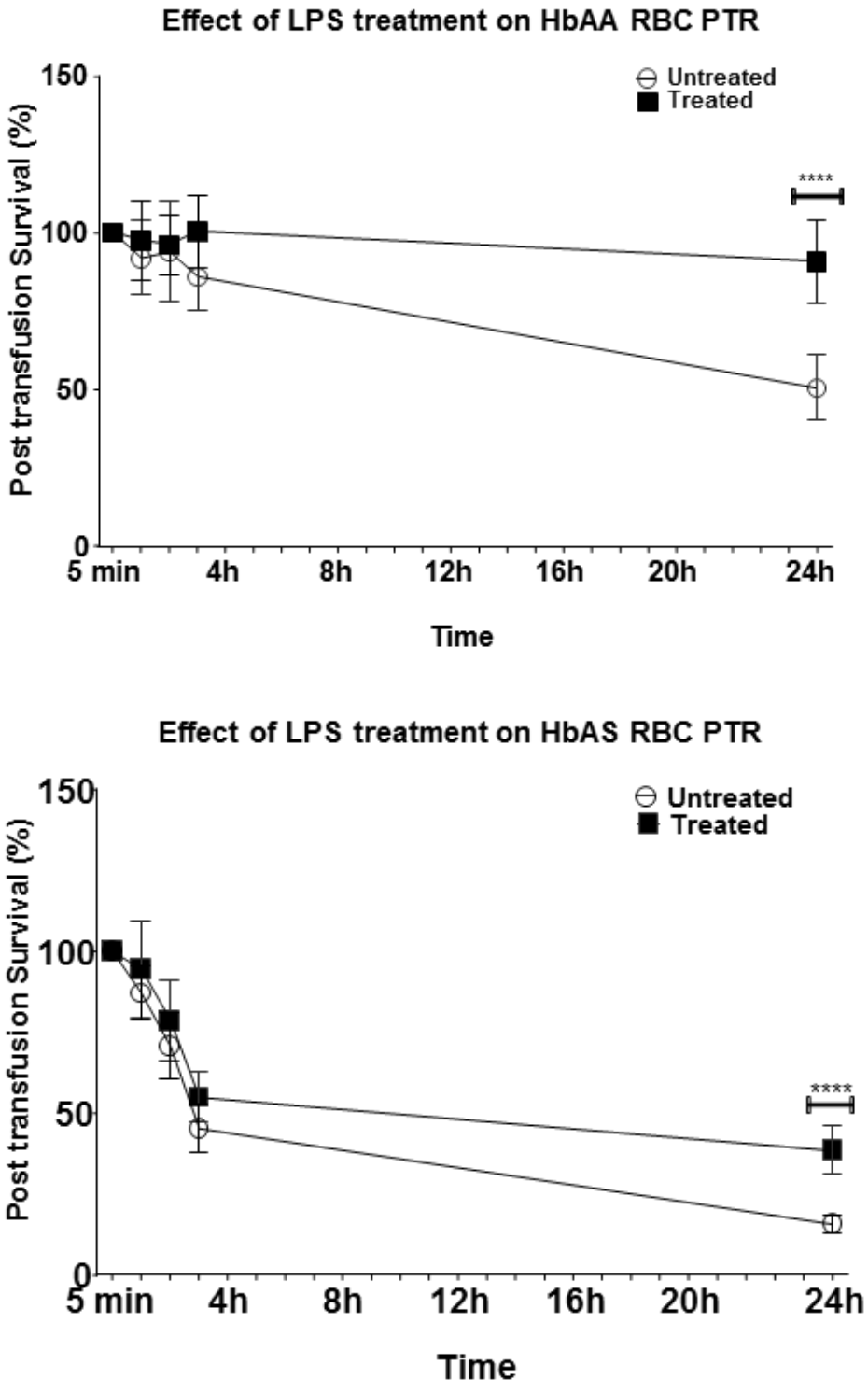

Figure 22. Lipopolysaccharide-Induced septic shock improves the post transfusion survival of stored HbAA and HbAS RBCs in mice.

WT recipient mice (n=8 per group) received intraperitoneal (i.p.) injections of LPS at a dose of 1.5mg/kg or equal volume of 1X PBS 24h prior to RBC transfusion. All mice were transfused with 100µl DiD- HbAA RBCs plus 100µl DiI- HbAS RBCs. Post-transfusion recovery was measured by dual-label cell tracking by flow cytometry. The results are presented as mean ± SD. ****$P<0.001$ analyzed by 2Way ANOVA, GraphPad Prism 6.0.

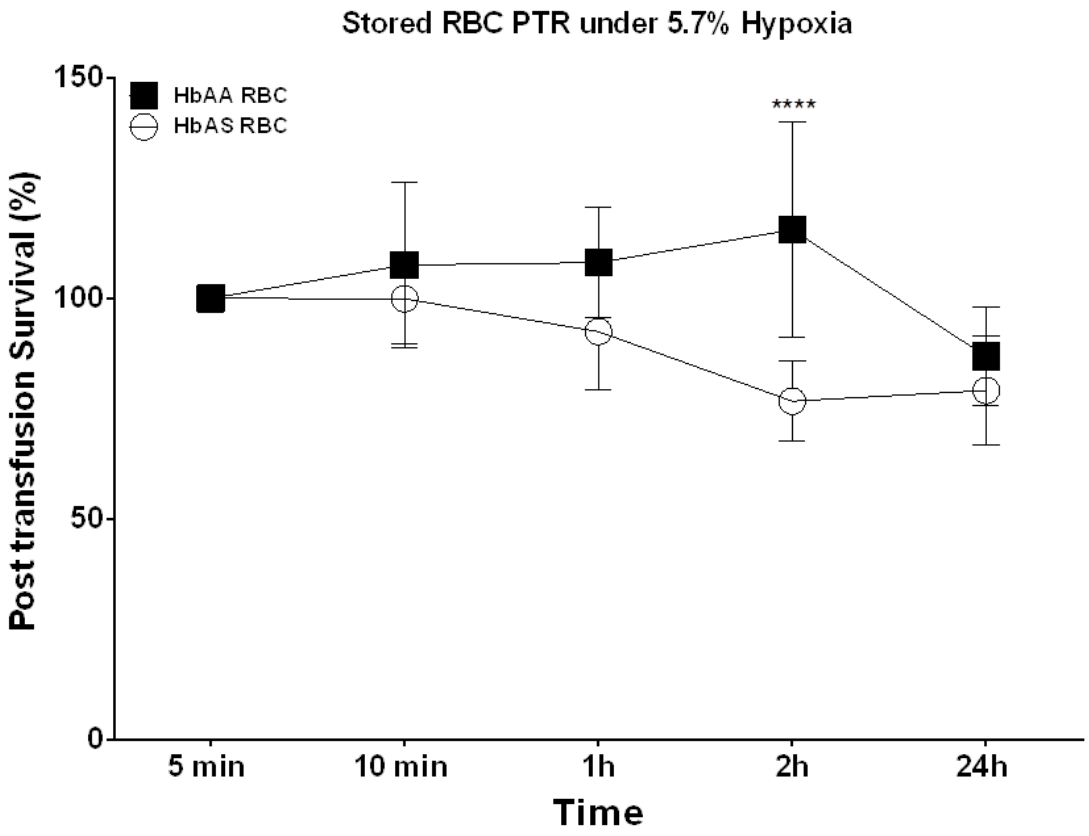

Figure 23. Acute hypoxia improves RBC post transfusion survival.

Wildtype mice (n=6 per group) were transfused with 11-day stored HbAA or HbAS RBCs. Mice were transfused with 200µl DiD- HbAA RBCs or 100µl DiD- HbAS RBCs. Post-transfusion recovery was measured by dual-label cell tracking by flow cytometry. The results are presented as mean ± SD. ****$P<0.001$ analyzed by 2Way ANOVA, GraphPad Prism 6.0.

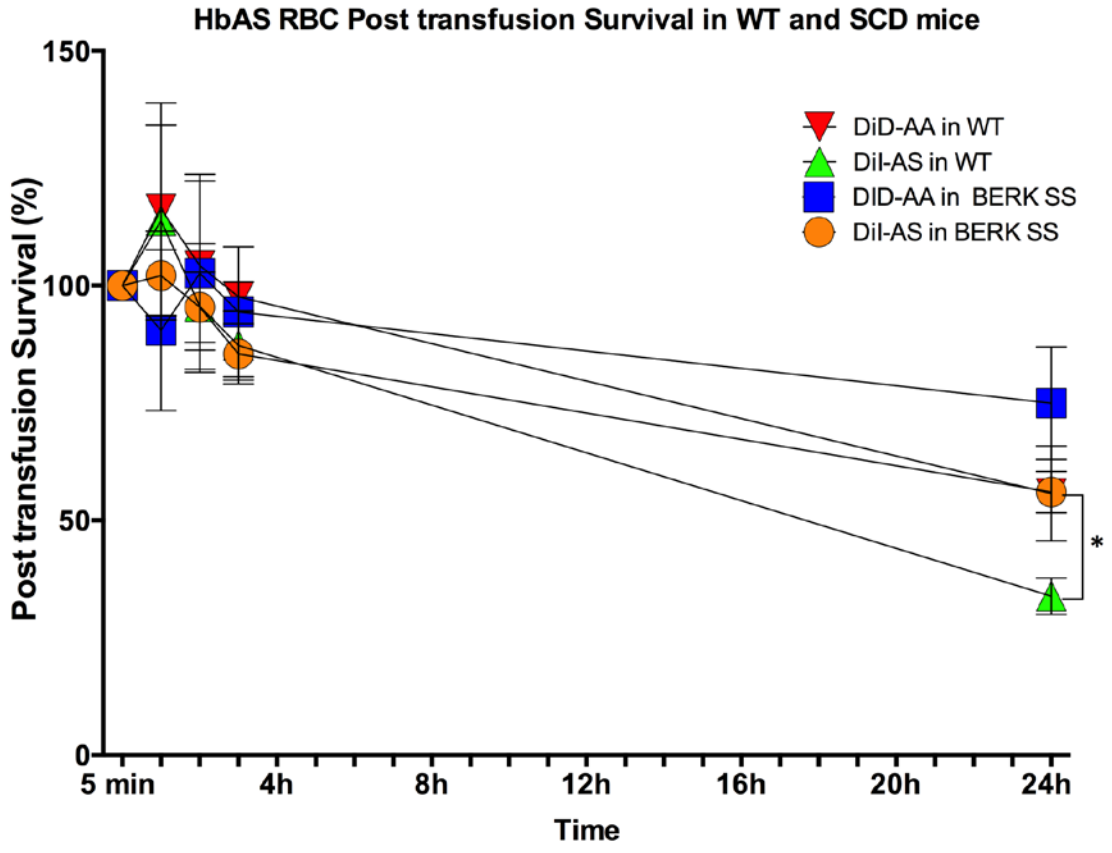

Figure 24. Stored RBCs have a higher post transfusion survival in SCD mice.

Wildtype (n=3 per group) and Berkeley SCD (n=5 per group) mice were transfused with 11-day stored HbAA and HbAS RBCs. Mice were transfused with 100µl DiD- HbAA RBCs plus 100µl DiI- HbAS RBCs. Post-transfusion recovery was measured by dual-label cell tracking by flow cytometry. The results are presented as mean ± SD. *$P<0.01$* analyzed by 2Way ANOVA, GraphPad Prism 6.0.

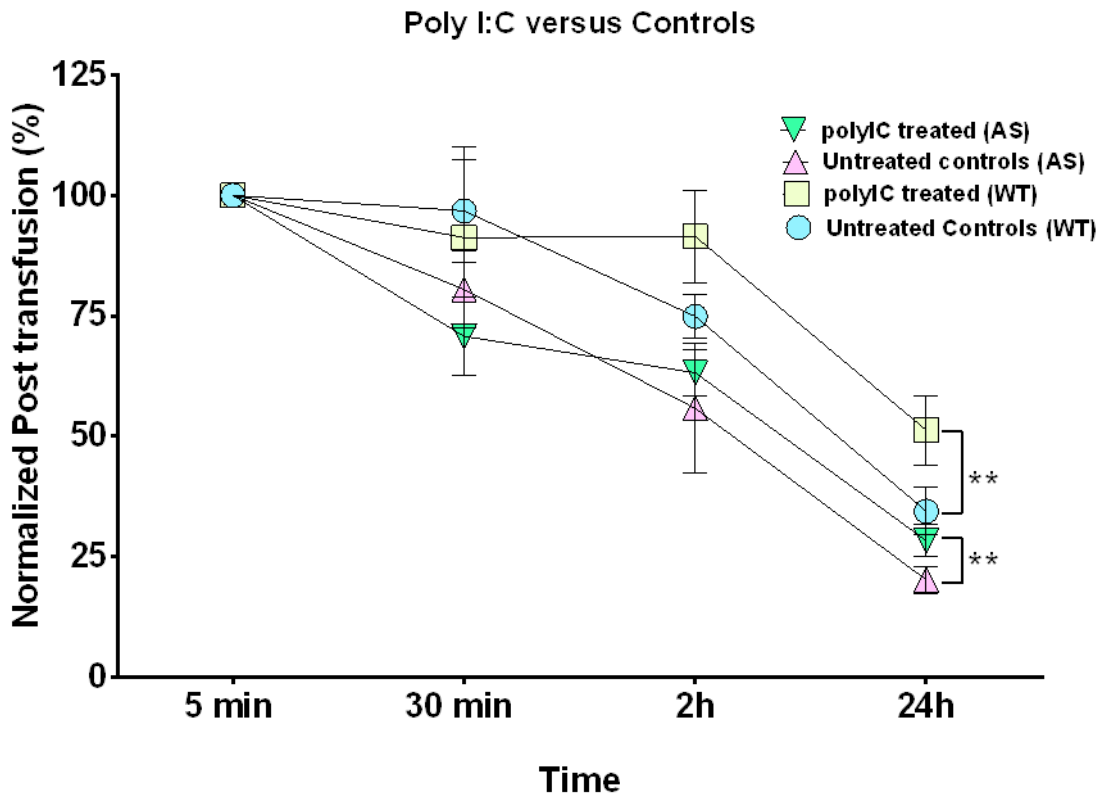

Figure 25. TLR3 ligand PolyIC improves stored RBC post transfusion survival.

PolyIC treated mice (n=4) and untreated mice (n=4) were treated with 500µg polyIC in 100µl and 100µl PBS respectively via retro orbital injection 2 minutes prior to RBC transfusion. Both groups received 200µl 11-day DiD-HbAA and DiI-HbAS RBCs. RBC post transfusion survival was enumerated by flow cytometry and analyzed with FlowJo. Statistical analysis was performed using student's t-test with GraphPad Prism 6.0 software. ***p<0.01*.

The observation that conditions such as acute hypoxia, TLR3 ligand administration or lipopolysaccharide-induced septic shock in the recipient can affect the post transfusion survival suggest that the post transfusion survival of store RBCs is modulated in part by factors extrinsic to the transfused red blood cell. This observation was further confirmed when the same stored HbAA and HbAS RBCs exhibited improved post transfusion survival in Berkeley HbSS mice; mice that are lethargic due to low circulating RBC count compared to wildtype C57BL/6 recipients (**Figure 22 and Figure 23**). This finding is counterintuitive but may hint at a regulatory system in which tissue perfusion requirements are tightly linked to RBC clearance such that when oxygen levels in tissues drop, RBC clearance is halted to commit available RBCs to tissue oxygenation.

More importantly, the ability to alter HbAS RBC post transfusion survival using factors present in the recipient suggests that the reduced post transfusion survival of stored HbAS RBCs is not due to intravascular hemolysis. This finding demonstrates a qualitative difference between HbAS and HbSS RBC sand are contradictory given that HbAS RBCs show higher hemolysis compared to HbAA RBCs at the end of storage but do not present additional challenge to recipients via intravascular hemolysis. Based on the existing data and the results from the ektacytometric analyses, it appears that cell dehydration increase the intracellular viscosity of HbAS RBCs leading to increased rigidity of the cells thus causing tissue sequestration. It would be interesting to re-hydrate cells prior to transfusion to test whether increased sequestration of stored HbAS RBCs can be reversed. These findings raise questions about the burden of tissue sequestration and extravascular hemolysis if stored HbAS RBCs are to be considered as suitable RBC transfusion products. Further investigation is needed to identify the mechanisms by which HbAS RBCs are sequestered into tissues and the ensuing process through which they are cleared.

APPENDIX B

B.1 MURINE SICKLE CELL TRAIT RED BLOOD CELLS DO NOT APPEAR TO HAVE INCREASED SURFACE MARKERS FOR MACROPHAGE CLEARANCE

To examine whether the rapid clearance of sickle cell trait red cells was due to clearance mononuclear system, I examined whether there is increased rate of microparticle formation, increased expression of heat shock proteins in HbAS RBCs as well as the increased expression of phosphatidylserine and CD47, markers known to signal macrophage engulfment of clearance apoptotic cells.

There was no increased expression of surface markers such as CD47 and phosphatidylserine in HbAS RBCs compared to HbAA RBCs. Additionally, heat shock proteins (90, 70 and 27) were not increased in HbAS RBCs compared to HbAA RBCs. These negative results further strengthened the findings that the reduced post transfusion survival of HbAS RBCs is due to mechanical difficulties experienced by the membrane traversing microcirculation due to loss of deformability.

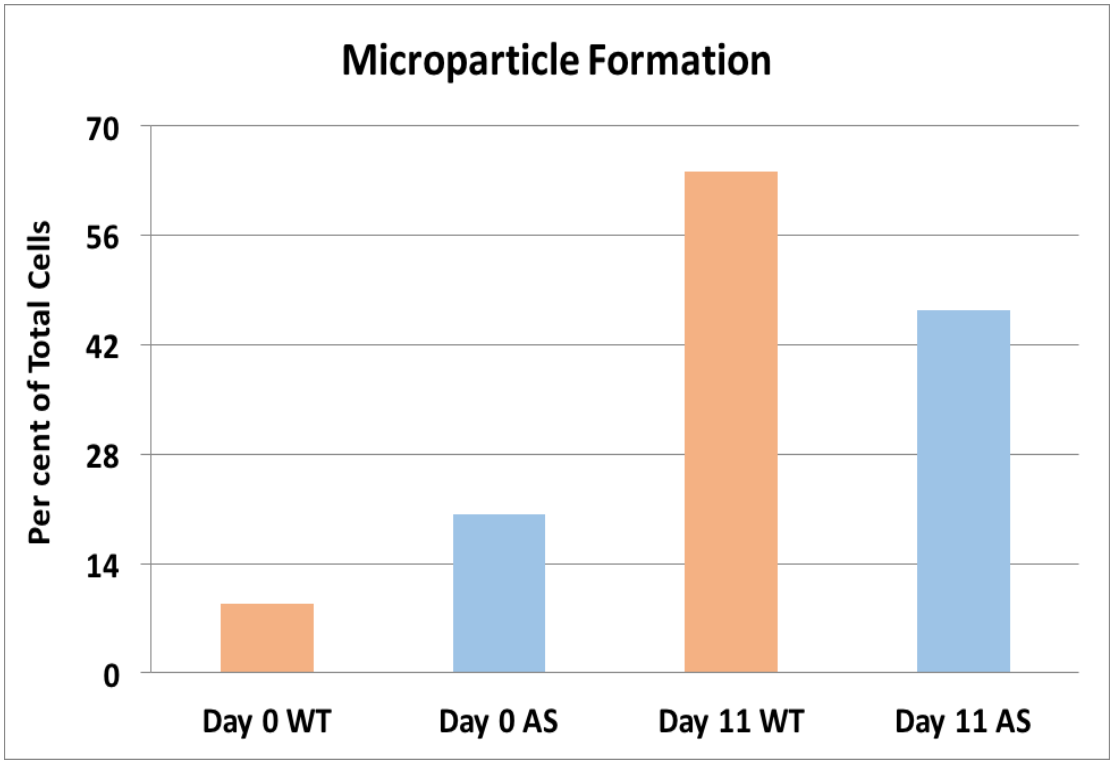

Figure 26. Sickle cell trait red blood cells do not exhibit increased microparticle formation during storage compared to healthy RBCs.

Fresh or stored HbAS RBCs were incubated with fluorescent-conjugated phosphatidylserine-antibody. Microparticle content in fresh or stored samples were determined by flow cytometry, using 2-3 micron beads to determine size and Ter 119+ and phosphatidylserine to identify RBC-derived microparticles.

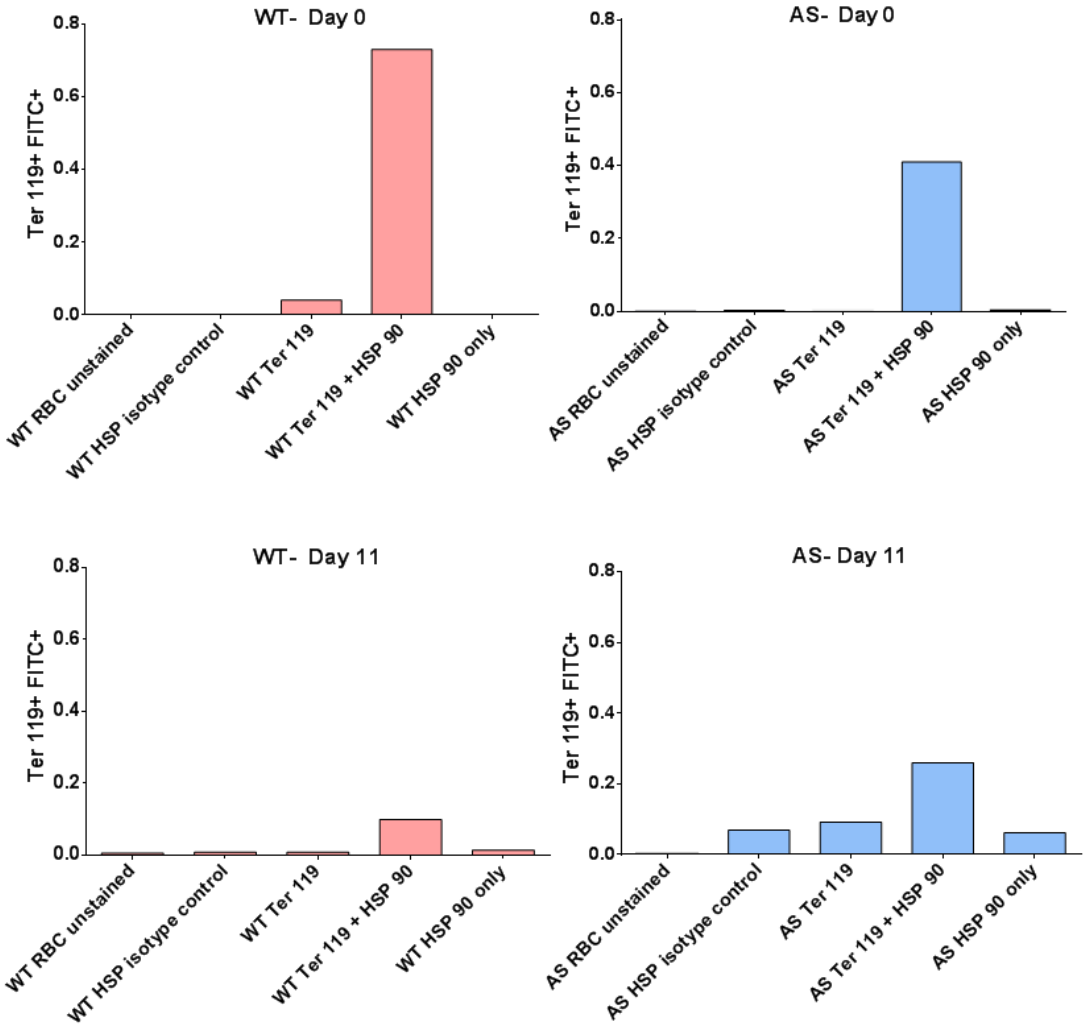

Figure 27. HSP90 Expression is decreased in HbAS RBCs compared to HbAA RBCs.

Fresh or stored HbAS RBCs were incubated with fluorescent-conjugated HSP 90-antibody. HSP expression on red blood cells in fresh or stored samples were determined by flow cytometry, using Ter 119+ and HSP90 (FITC+) events to quantify HSP90 expression on red blood cells.

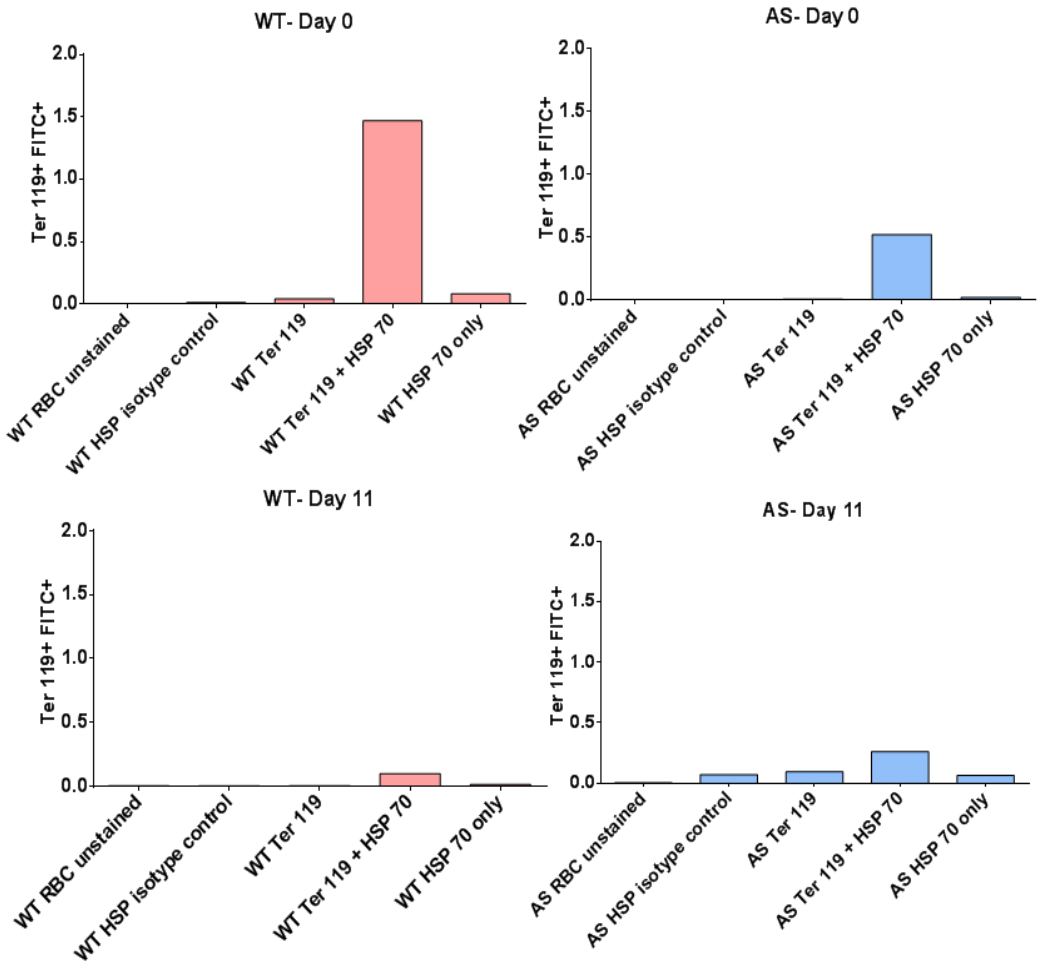

Figure 28. HSP70 Expression is decreased in HbAS RBCs compared to HbAA RBCs.

Fresh or stored HbAS RBCs were incubated with fluorescent-conjugated HSP 70-antibody. HSP expression on red blood cells in fresh or stored samples were determined by flow cytometry, using Ter 119+ and HSP70 (FITC+) events to quantify HSP70 expression on red blood cells.

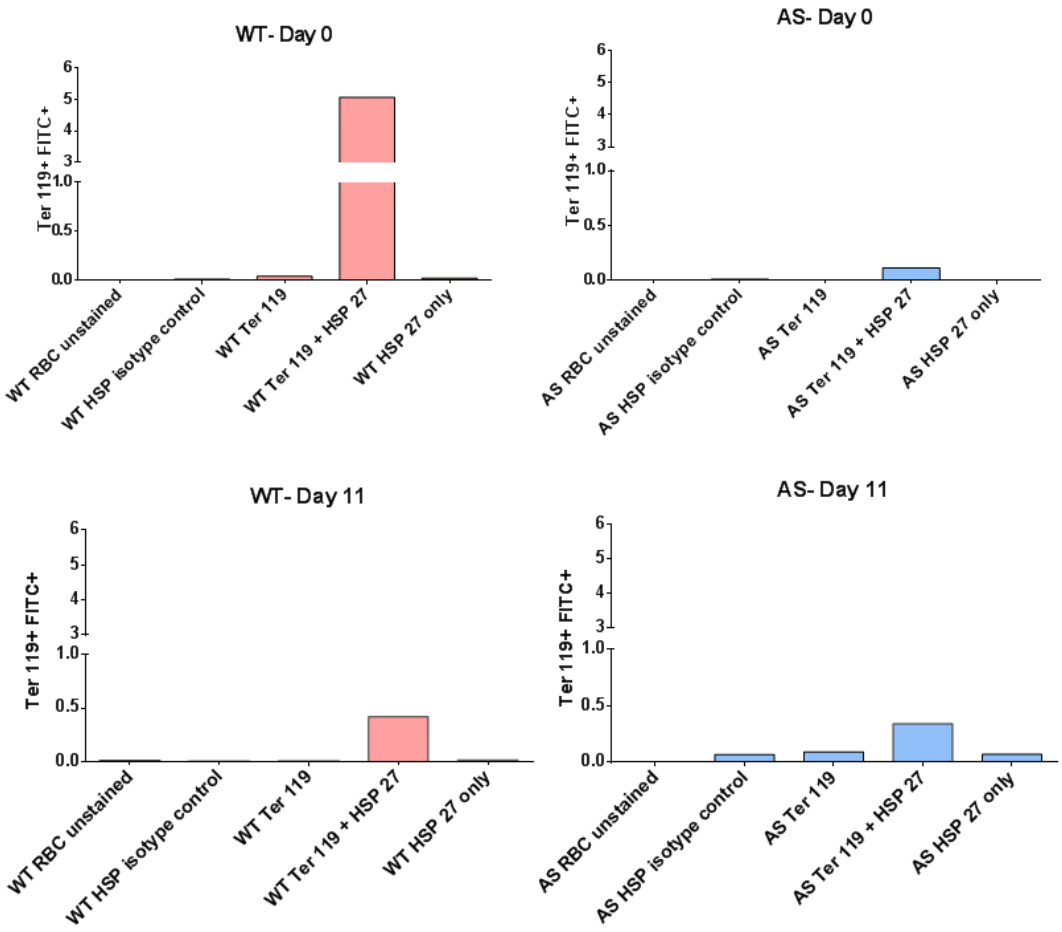

Figure 29. HSP27 Expression is decreased in HbAS RBCs compared to HbAA RBCs.

Fresh or stored HbAS RBCs were incubated with fluorescent-conjugated HSP 27-antibody. HSP expression on red blood cells in fresh or stored samples were determined by flow cytometry, using Ter 119+ and HSP 27 (FITC+) events to quantify HSP 27 expression on red blood cells.

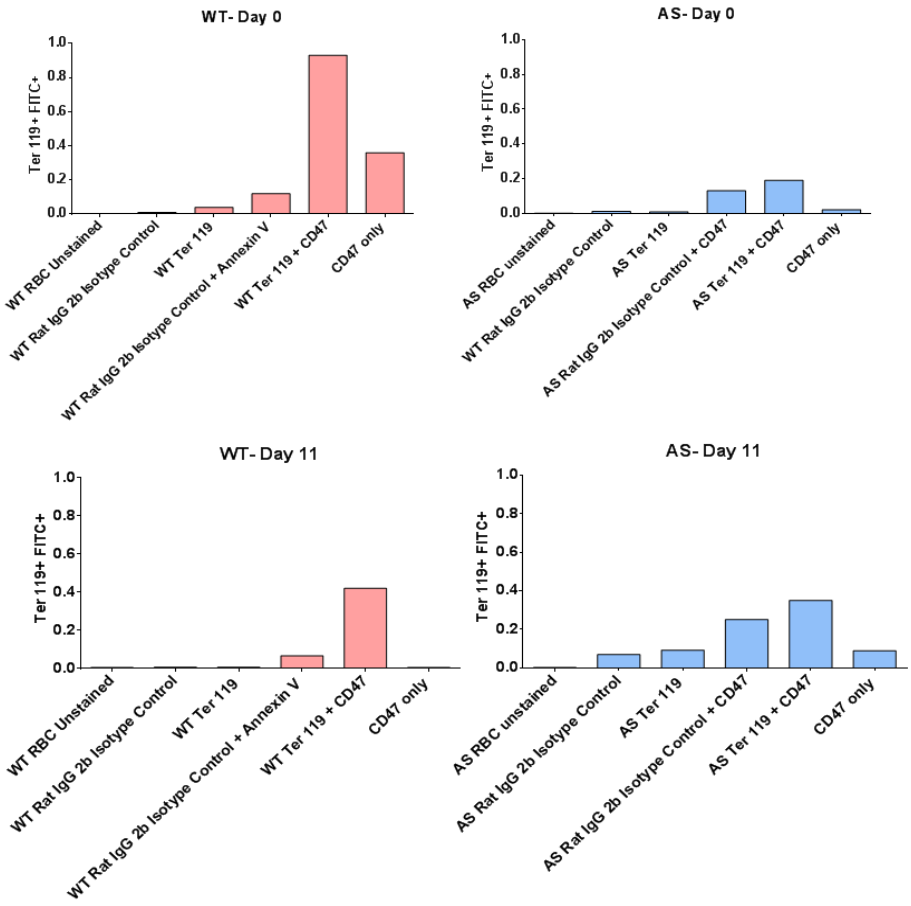

Figure 30. There is no increased CD47 expression in HbAS RBCs compared to HbAA RBCs.

Fresh or stored HbAS RBCs were incubated with fluorescent-conjugated CD47-antibody. HSP expression on red blood cells in fresh or stored samples were determined by flow cytometry, using Ter 119+ and CD47 (FITC+) events to quantify CD47 expression on red blood cells.

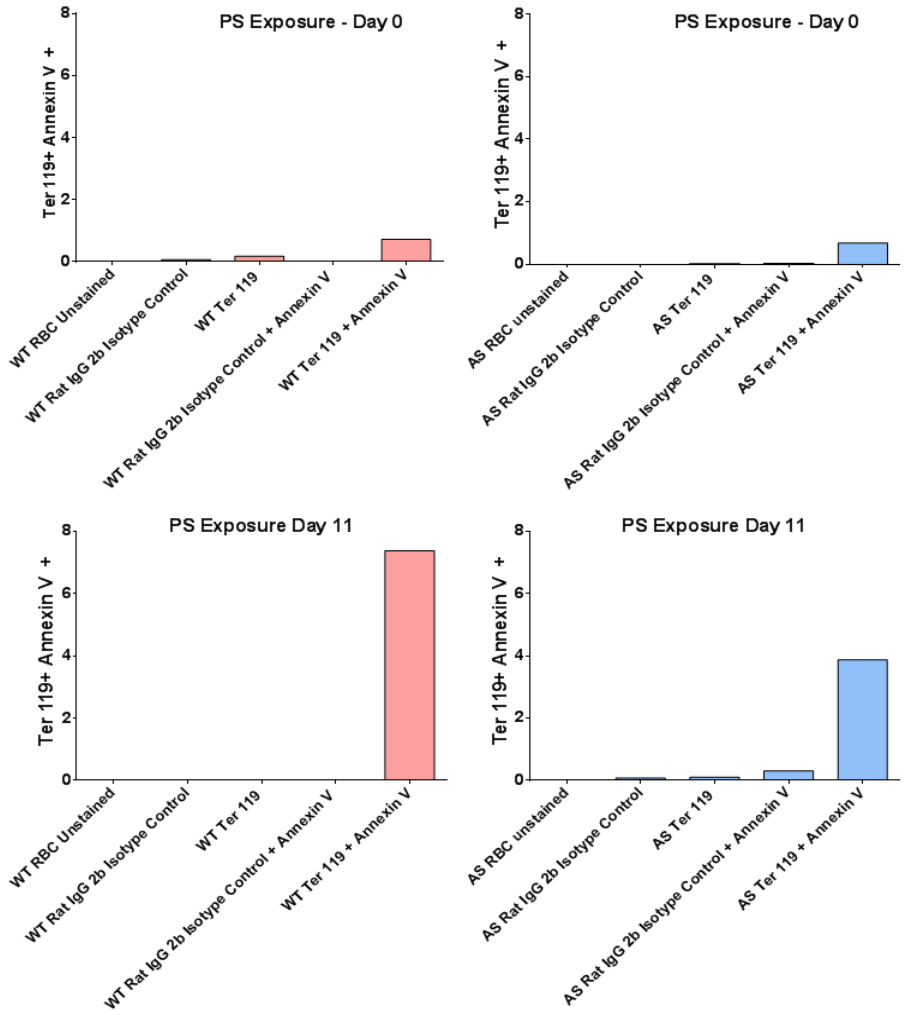

Figure 31. There is no increased phosphatidylserine expression in HbAS RBCs compared to HbAA RBCs.

Fresh or stored HbAS RBCs were incubated with fluorescent-conjugated phosphatidylserine-antibody. PS expression on red blood cells in fresh or stored samples were determined by flow cytometry, using Ter 119+ and PS (FITC+) events to quantify PS expression on red blood cells.

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