Methods

Protocol Design

Baseline BP and heart rate were measured on the morning prior to PBSC infusion and the morning of saline infusion. BP was measured from the right arm if no contraindication with the head of the bed angled at 45º, and each value was repeated two minutes later. BP and heart rate were monitored every 15 minutes for the first hour of the infusion and then once hourly for a total of four hours in the control subjects and for a total of eight hours or until the infusion was complete, whichever longer, in the transplanted subjects. Transthoracic echocardiography was performed as previously described[1] before, during or just after, and the morning after PBSC or saline infusion in all subjects. Transplant recipients were excluded from the study if they received nitrate antihypertensive medications or if they were clinically unstable, and healthy volunteers were excluded if they had evidence of renal or hepatic dysfunction, history of clinically significant cardiac or pulmonary disease, or a TRV of $\geq$ 2.5 meters per second.

In order to measure vasodilatory response before, during, and after infusion, subjects underwent peripheral arterial tonometry (EndoPAT 2000, Itamar Medical, Caesarea, Israel), a noninvasive technology that captures a beat-to-beat plethysmographic recording of the finger arterial pulse wave activity and measures vasodilatory response. A pulse wave amplitude was measured at baseline in both upper extremities, and after a 5 minute period of occlusion in the study arm. An RHI was then calculated, which is a ratio of the pulse amplitude post-occlusion to the pulse amplitude at baseline[2-5]. A value less than 1.67 was used to indicate poor endothelial function based on the manufacturer's normative testing. Transplanted subjects also underwent continuous 24-hour cardiac monitoring (eVolution, eCardio, Houston, TX or Aria, Space Labs Health Care, Issaquah, WA) on the day prior to and on the day of PBSC infusion. Control subjects wore a 24-hour cardiac monitor starting on the morning of infusion.

Transplant Procedure

Transplanted subjects received granulocyte colony-stimulating factor- (filgrastim, Amgen, Thousand Oaks, CA) mobilized PBSCs. DMSO products contained 5% DMSO and 5% hydroxyethyl starch (Hespan, Braun Medical, Irvine, CA). Transplant recipients were premedicated with acetaminophen (Tylenol, Johnson and Johnson, New Brunswick, NJ) and diphenhydramine (Benadryl, Pfizer, New York, NY). Transplant recipients received conditioning chemotherapy and medications for prophylaxis against graft-versus-host disease according to their primary protocol (supplemental Table 2).

Statistical Analysis

Descriptive statistics were performed for outcome measures. The data from the control group were used to determine baseline variability in BP. To assess whether lysed RBCs or DMSO leads to systemic toxicity and to assess safety in subjects with SCD, we compared subjects with SCD that received frozen unmanipulated products (Group 1a) to

subjects without SCD that received the same products (Group 1b, supplemental Figure 1). We also compared Group 1b to Group 2 and to Group 3 subjects. A two-sided significance level of 0.017 (Bonferroni rule, 0.05/3) was selected to control for these multiple comparisons. The Tukey-Kramer HSD and Mann-Whitney U test (where appropriate) were performed for pairwise comparison. A paired t-test was used to evaluate changes in BP from baseline to the maximum value. For skewed variables, both mean and median values are reported and p-values are based on nonparametric comparisons.

Results

PBSC graft contents among groups

The volume of PBSCs and/or normal saline infused is listed in supplemental Table 4. Group 1 subjects received a mean volume of 1,067mL of PBSCs and normal saline (used to flush the bags of PBSCs). Group 2 subjects received a similar volume with a mean of 947mL, and group 3 subjects received a mean of only 422mL. Healthy volunteers received the same volume of fluid as normal saline as an age- and sex-matched subject in group 1. The cell-free Hb concentrations measured from the PBSC grafts of subjects that received RBCs (groups 1 and 2) are listed in supplemental Table 4 and ranged from a mean level of 34.5μM (n=24) in subjects that received lysed RBCs to a mean level of 39.5μM (n=10) in group 2 subjects that received fresh RBCs. The total cell-free Hb concentrations were not significantly different between these two groups (p=0.04). These measurements, however, are unreliable as a result of technical challenges.

Changes in vasodilatory response

The mean RHI in Group 1 subjects did not significantly change during the infusion (mean 2.04 to 2.09, p=0.81). Mean RHI changed from 2.56 to 2.14 in Group 2 subjects (p=0.53), from 2.05 to 1.61 in Group 3 subjects (p=0.22), and from 1.75 to 2.04 in healthy volunteers (p=0.13). The change in mean RHI during or post-infusion as compared to baseline among groups was not statistically different.

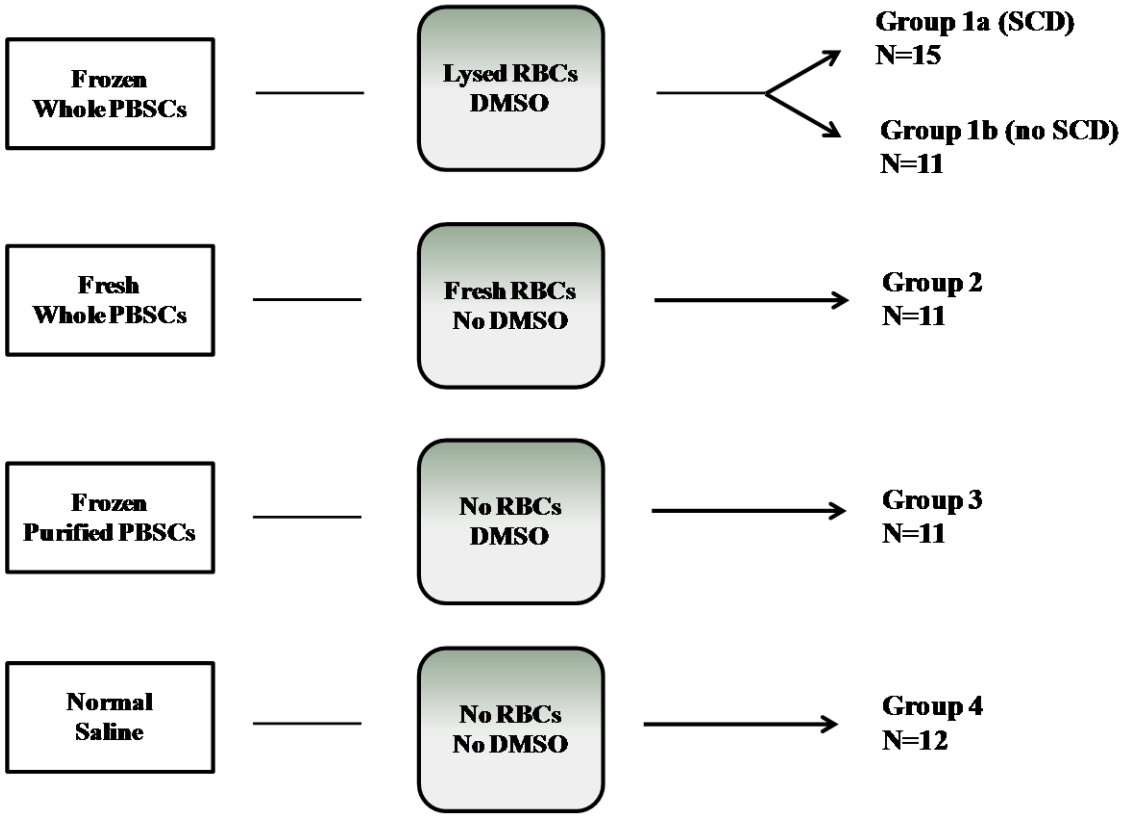
None of the subjects experienced major systemic infusion-associated toxicities

One subject from Group 2 experienced a 13 beat run of an unspecified supraventricular tachycardia approximately 3 and a half hours after the infusion was complete as determined by Holter monitoring. The subject was asymptomatic during the episode. However, none of the subjects experienced a malignant cardiac arrhythmia or severe toxicity requiring immediate medical intervention.

References:

1. Gladwin MT, Sachdev V, Jison ML, et al. Pulmonary hypertension as a risk factor for death in patients with sickle cell disease. *The New England journal of medicine.* Feb 26 2004;350(9):886-895.
2. Nohria A, Gerhard-Herman M, Creager MA, Hurley S, Mitra D, Ganz P. Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. *J Appl Physiol.* Aug 2006;101(2):545-548.
3. Bonetti PO, Pumper GM, Higano ST, Holmes DR, Jr., Kuvin JT, Lerman A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. *J. Am. Coll. Cardiol.* Dec 7 2004;44(11):2137-2141.
4. Kuvin JT, Patel AR, Sliney KA, et al. Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude. *Am. Heart J.* Jul 2003;146(1):168-174.
5. Mahmud FH, Earing MG, Lee RA, Lteif AN, Driscoll DJ, Lerman A. Altered endothelial function in asymptomatic male adolescents with type 1 diabetes. *Congenital heart disease.* May 2006;1(3):98-103.

Figure S1: Protocol study groups. Transplanted subjects were divided into groups based on whether their PBSC grafts contained RBCs and/or DMSO. The first group was subdivided into groups depending on whether or not they had SCD. The last group consisted of healthy volunteers that received an equal fluid volume of normal saline as an age- and sex-matched subject in group 1.



SCD: sickle cell disease; PBSCs: peripheral blood stem cells; RBCs: red blood cells; DMSO: dimethyl sulfoxide

Table S1. Preliminary statistics used to generate sample size

	DMSO RBCs Group 1 (N=7)		DMSO No RBCs Group 3 (N=5)	
	Mean	SD	Mean	SD
Baseline systolic pressure (mmHg)	114.8	13.1	111.5	13.6
Maximum systolic pressure (mmHg)	146.0	25.9	129.4	10.3
Change in systolic pressure (mmHg)	31.2	16.8	17.9	10.2

DMSO: dimethyl sulfoxide; RBCs: red blood cells; SD: standard deviation

Table S2. Baseline characteristics of enrolled subjects

Mean Age (range)	42.0 years (11-68 years)
Males (%) Females (%)	36 (60%) 24 (40%)
Hematologic Diagnosis (N)	Sickle Cell Disease (15) HbSS (14) HbSC (1) Healthy Volunteer (12) Non-Hodgkin's Lymphoma (7) Acute Myelogenous Leukemia (6) Chronic Myelogenous Leukemia (6) Myelodysplastic Syndrome (3) Severe Aplastic Anemia (3) Multiple Myeloma (2) Hodgkin's Lymphoma (2) Other (4)
Conditioning Therapy (N)	Fludarabine (32) Cyclophosphamide (30) Total Body Irradiation (28) Alemtuzumab (18) Horse Anti-Thymocyte Globulin (3) Busulfan (3)
Graft-Versus-Host Disease Prophylaxis (N)	Cyclosporine (30) Sirolimus (24) Methotrexate (10) Tacrolimus (2) Prednisone (1)

Other: Chronic Lymphocytic Leukemia (1), Acute Lymphocytic Leukemia (1), Disseminated Nontuberculous Mycobacterial Infection (1), Chronic Granulomatous Disease (1)

Table S3. Mean maximal blood pressure change in each group of subjects

	Baseline SBP (mmHg) Mean (SD)	Maximum SBP (mmHg) Mean (SD)	Change (mmHg) Mean (SD)	95% Confidence Interval of Change	p-value*
Group 1 (n=26)	123.1 (17.7)	138.6 (15.8)	15.5 (9.7)	(11.6, 19.4)	<0.0001
Group 2 (n=11)	125.4 (13.9)	140.6 (12.1)	15.1 (11.6)	(7.4, 22.9)	0.002
Group 3 (n=11)	121.6 (18.1)	133.0 (25.1)	11.4 (15.3)	(1.1, 21.7)	0.034
Group 4 (n=12)	120.3 (11.8)	129.3 (14.3)	9.0 (7.9)	(3.9, 14.0)	0.002
	Baseline DBP (mmHg) Mean (SD)	Maximum DBP (mmHg) Mean (SD)	Change (mmHg) Mean (SD)	95% Confidence Interval of Change	p-value*
Group 1 (n=26)	72.4 (11.6)	83.4 (9.6)	11.0 (9.2)	(7.3, 14.7)	<0.0001
Group 2 (n=11)	78.1(9.7)	85.3 (10.1)	7.2 (4.1)	(4.5, 9.9)	<0.0001
Group 3 (n=11)	71.8 (10.1)	79.8 (11.2)	8.0 (8.4)	(2.3, 13.6)	0.011
Group 4 (n=12)	72.4 (9.6)	79.4 (13.1)	7.0 (6.9)	(2.6, 11.4)	0.005

*Paired t-test, comparing maximum to baseline values within each group.
SBP: systolic blood pressure; DBP: diastolic blood pressure; SD: standard deviation

Table S4. Infused volume and measured cell-free Hb concentration in the infusate. Cell-free Hb concentrations were measured in 24 subjects from Group 1 and 10 subjects from Group 2.

	Lysed RBCs DMSO	Fresh RBCs No DMSO	No RBCs DMSO	No RBCs No DMSO
Group	1	2	3	4
Infused Volume (mL) Mean (Range)	1067 (310-2570)	947 (312-2432)	422 (215-746)	1085 (310-2560)
Cell-free Hb (μM) Mean (SD)	34.5 (6.9)	39.5 (3.8)	N/A	N/A

RBCs: red blood cells; DMSO: dimethyl sulfoxide; SD: standard deviation; N/A: not applicable

Figure S2. Cell-free hemoglobin concentrations measured in subjects post-infusion. Cell-free hemoglobin (Hb) concentrations were measured via central venous catheter in transplanted subjects and via peripheral venous catheter in healthy volunteers after the infusion was complete. Concentrations are subdivided according to study group. The median change in cell-free Hb concentrations was not significantly different among groups. The boxes represent the 25th and 75th percentiles and border the median horizontal lines. The upper and lower horizontal lines represent the maximum and minimum values, excluding outliers.

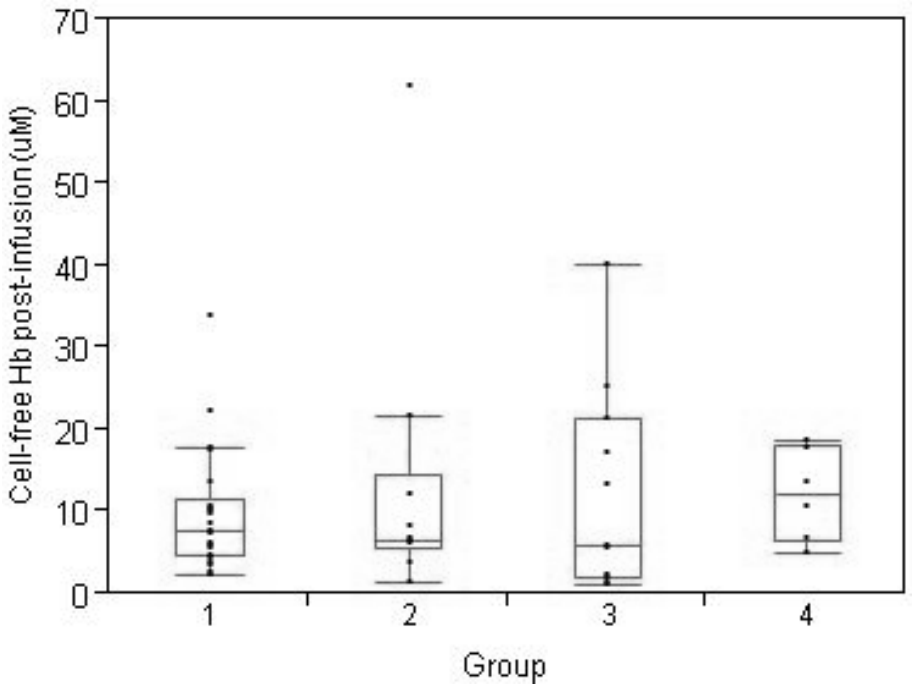


Table S5. Change in mean or median measured parameters as compared to baseline in subjects with and without SCD that received lysed RBCs

	Lysed RBCs, DMSO, SCD				Lysed RBCs, DMSO, no SCD				p-value*
	Group 1a (n=15)				Group 1b (n=11)				
Measured Parameter	Baseline (SD)	During or Post Infusion (SD)	Change (SD)	95 % CI of Change	Baseline (SD)	During or Post Infusion (SD)	Change (SD)	95 % CI of Change	
SBP (mmHg)	119 (13)	134 (12)	15 (11)	(9, 21)	129 (22)	145 (18)	16 (9)	(-2, 34)	0.82
DBP (mmHg)	68 (12)	81 (11)	13 (8)	(8, 17)	78 (8)	87 (6)	9 (10)	(3, 15)	0.24
heart rate (beats/min)	74 (17)	97 (16)	23 (20)	(12, 35)	79 (16)	89 (17)	11 (9)	(5, 17)	0.07
LDH (unit/L)	245 (108)	873 (366)	628 (360)	(418, 833)	152 (40)	604 (195)	452 (174)	(330, 579)	0.18
haptoglobin (mg/dL)	76 (59)	24 (27)	-48 (46)	(-74, -21)	166 (78)	97 (79)	-71 (33)	(-93, -53)	0.13
TRV (m/s)	2.2 (1.1)	2.3 (1.1)	0.1 (0.2)	(0.0, -0.2)	1.5 (1.2)	1.8 (1.1)	0.3 (0.7)	(-0.1, 0.7)	0.29
NT-proBNP (pg/mL)	1824 (6206)	1360 (4315)	-464 (1852)	(-1808, 625)	1412 (2405)	1960 (3960)	548 (1612)	(-5350, 1632)	0.15
Cr (mg/dL)	1.4 (1.9)	1.4 (1.9)	0 (0.1)	(-0.1, 0.1)	1.0 (0.5)	1.0 (0.5)	0 (0.1)	(0.0, 0.1)	0.17
RHI	2.0 (0.6)	2.1 (0.8)	0.1 (1.0)	(-0.5, 0.7)	2.0 (0.6)	2.1 (0.5)	0.1 (0.9)	(-1.3, 1.1)	0.68

*p-values are from t-tests or Mann-Whitney U tests (LDH, haptoglobin, and RHI)
RBCs: red blood cells; DMSO: dimethyl sulfoxide; SCD: sickle cell disease; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDH: lactate dehydrogenase; TRV: tricuspid regurgitant velocity; NT-proBNP: brain natriuretic peptide; RHI: reactive hyperemia index