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Stable Long-Term Donor Engraftment following Reduced-Intensity Hematopoietic Cell Transplantation for Sickle Cell Disease

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Reduced-intensity conditioning (RIC) regimens have the potential to decrease toxicities related to hematopoietic stem cell transplantation (HCT) in patients with sickle cell disease (SCD) and thus make HCT a more acceptable therapeutic option for this group of patients. We report the results of 7 patients enrolled on a study to evaluate safety and efficacy of HCT using bone marrow from an HLA matched sibling donor following an RIC regimen for patients with high-risk SCD. The conditioning regimen consisted of busulfan, fludarabine, equine antithymocyte globulin, and total lymphoid irradiation with shielding of the liver, lungs, heart, and gonads on day 1. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine and mycophenolate mofetil. The regimen was well tolerated, and all patients had hematopoietic recovery. Six of 7 patients are stably engrafted off immunosuppression and without sickle cell-related symptoms at 2 to 8.5 years after HCT. Consistent with the complete resolution of SCD related symptoms observed in the 6 engrafted patients, erythropoiesis of complete or predominantly donor origin was detected by red blood cell-specific chimerism assays, despite their having persistent mixed chimerism in the mononuclear and lymphoid compartments. These findings demonstrate the curative potential of allogeneic HCT after an RIC regimen in patients with SCD.

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INTRODUCTION

Despite the advances in the health care of children with sickle cell disease (SCD) over the last 30 years, this disease continues to be associated with considerable morbidity and premature mortality, with a 25- to 30-year loss of life expectancy [1,2]. Stroke, organ failure, acute chest syndrome, pulmonary hypertension, and recurrent pain crises cause significant morbidity. Transfusion therapy decreases recurrence of stroke and other complications, but at the expense of increased risk for transfusion reactions, infections, and iron overload [3]. Hydroxyurea increases the total hemoglobin concentration, reduces the vaso-occlusive complications of pain and acute chest syndrome, and attenuates mortality in adults [4]. However, <30% of eligible patients are either prescribed or take this drug; not all adults respond to this treatment, and a small and as yet unquantified risk of latent transformation to leukemia with its long-term use remains as a concern [5].

Currently, allogeneic HCT is the only curative therapy for SCD. The results of HCT after conventional myeloablative therapy in young patients (<16 years of age) with SCD are highly encouraging, with an overall event-free survival (EFS) of approximately 85% and transplant-related mortality of <10% [6-13]. Moreover, stabilization or reversal of organ damage from SCD has been documented after HCT [14]. Despite these encouraging results, the use of this treatment approach has been limited by the infrequent availability of an HLA matched sibling donor [15] and the risks of early and late regimen-related

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Table 1. Clinical Characteristics and Hematologic Recovery in Patients undergoing RIC Allogeneic HCT for Severe SCD

Pt #	Age (Years/Sex)	Recipient β - Globin Genotype and Indication for HCT	Donor β -Globin Genotype	Conditioning Regimen	Donor ABO Compatibility	Infused Marrow Product (Cell Dose/kg Body Weight) TNC ($\times 10^8$)	CD34 ($\times 10^6$)	CD3- ($\times 10^7$)	Duration of ANC<0.5$\times 10^9$/L (Days)	Number of Transfusions post HCT (Day of Last Transfusion) PRBC	Platelets
1	8/F	$\beta^s\beta^s$ Stroke, Allosensitization	$\beta\beta^s$	BU, Flu, ATG, TLI*	matched	3.49	6.25	0.5	8	3 (100)	3 (22)
2	8/M	$\beta^s\beta^s$ Repeated ACS	$\beta\beta$	BU, Flu, ATG, TLI*	matched	5	1.26	0.5	8	3 (20)	3 (28)
3	6/M	$\beta^s\beta^s$ Repeated ACS, Silent stroke	$\beta\beta^s$	BU, Flu, ATG, TLI†	matched	4.51	3.49	0.5	14	3 (35)	3 (11)
4	8/M	$\beta^s\beta^0$ Repeated ACS	$\beta\beta^0$	BU, Flu, ATG, TLI†	major mismatch	4.3	4.72	0.54	13	3 (35)	3 (18)
5	18/F	$\beta^s\beta^s$ Stroke, Thrombosis, Allosensitization	$\beta\beta^s$	BU, Flu, ATG, TLI†	matched	2.61	1.02	0.28	13	3 (39)	3 (16)
6	16/M	$\beta^s\beta^s$ Repeated ACS AVN, Recurrent pain crises	$\beta\beta^s$	BU, Flu, ATG, TLI†	matched	4.25	3.7	0.55	13	1 (35)	4 (15)
7	16/F	$\beta^s\beta^s$ Repeated ACS	$\beta\beta$	BU, Flu, ATG, TLI†	matched	3.20	1.35	0.20	17	6 (35)	14 (25)

HCT indicates hematopoietic stem cell transplantation; ANC, absolute neutrophil count; TNC, total nucleated cells; PRBC, packed red blood cell; ATG, amtithymocyte globulin; BU, busulfan; TLI, total lymphoid irridiation; FLU, fludarabine.
*Busulfan was administered orally.
†Busulfan was administered intravenously.

toxicities related to intensive myeloablative therapy, especially in patients >16 years of age. These risks include organ toxicities, acute and chronic graft-versus-host disease (aGVHD, cGVHD), and late effects, including sterility and secondary malignancies. Therefore, as a means to reduce both acute and long-term toxicities, we evaluated the safety and efficacy of a reduced-intensity conditioning (RIC) approach for SCD.

MATERIALS AND METHODS

Study Subjects, and Patient Samples

Pediatric and adult patients with SCD with severe phenotype and without end-organ failure were considered eligible for this study, using the criteria previously described in a national collaborative trial for myeloablative HCT for SCD [13]. The study protocol was approved by the institutional review boards of the University of Pittsburgh, University of Minnesota and University of Alabama, Birmingham. All subjects or their legal guardians provided signed informed consent.

Conditioning and GVHD Prophylaxis Regimens

Preparative regimen

Patients were conditioned for transplantation with a regimen consisting of busulfan (BU) 0.8 mg/kg/dose intravenously every 6 hours, or 4 mg/kg/day orally in 2 divided doses on days −8 and −7, with targeted dosing, to achieve a BU steady state of 600-900 ng/mL; fludarabine (FLU) 35 mg/m^2 i.v. daily on days −6, −5, −4, −3, and −2; and equine antithymocyte globulin (ATG) 30 mg/kg i.v. daily on days −5, −4, −3, −2, and −1. Total lymphoid irradiation (TLI) was administered on day −2 as a single fraction of 500 cGy, with shielding of the liver, lungs, heart, and gonads. As patients were accrued over a 9-year period, a number of adjustments to the conditioning regimen were implemented over time, reflecting changes in clinical practice [16-18]. As shown in Table 1, the primary change was conversion of busulfan from an oral (patients 1-2) to an intravenous (patients 3-7) formulation.

GVHD prophylaxis

Posttransplantation immunosuppression consisted of cyclosporine and mycophenolate mofetil (MMF). Cyclosponine (CSA) was initiated on day −3 at 2.5 mg/kg i.v. over 2 hours every 12 hours for adults with normal renal function. For children <40 kg, the initial dose was 2.5 mg/kg i.v. over 2 hours every 8 hours. CSA was continued until day 180, and dose adjustments were made on the basis of toxicity and to maintain CSA trough levels of 200-300 mg/L. If no GVHD was observed, the CSA dose was tapered 10% per week beginning on day 181. MMF was initiated on day 0 at 15 mg/kg intravenously or orally twice a day. Subsequently, the MMF dose was modified to 15 mg/kg/dose or 1g thrice daily (whichever was lower)

and converted to an oral dose of 600 mg/m^2/dose, in 3 doses per day once oral medications could be tolerated. MMF was tapered over 8 weeks starting days +45-220.

Supportive care

The conditioning regimen, bone marrow infusion, and initiation of the GVHD regimen were administered in the inpatient setting for all patients. Patients were discharged to the outpatient setting as soon as clinically stable. All patients received antimicrobial prophylaxis consisting of oral acyclovir, penicillin, and trimethoprim/sulfamethoxazole, and fluconazole while concurrently on immunosuppression. Blood samples were tested weekly for at least 12 weeks after transplantation for evidence of cytomegalovirus reactivation by using a nucleic acid hybridization test (Digene Corp., Gaithersburg, MD) or by testing for cytomegalovirus (CMV) antigen by shell vial assay. For patients not on a chronic transfusion protocol and having a sickle hemoglobin (Hb S) concentration >30%, a partial exchange transfusion was performed to reduce the Hb S to 30% or lower prior to initiating the conditioning regimen. Transfusions of packed red blood cells (PRBC) were administered as needed to maintain a total Hb level of 9-11 g/dL through day 100. Prophylactic platelet transfusions were given to maintain the platelet count $>50 \times 10^9$/L. To minimize the risk of central nervous system (CNS) toxicity following HCT, prophylactic phenytoin or levetiracetam was started prior to BU administration and continued for the duration of CSA therapy, while maintaining serum magnesium levels in the normal range and while keeping platelet counts $>50 \times 10^9$ cells/L [13].

Engraftment and Measurement of Overall Donor Engraftment and Chimerism

Neutrophil engraftment was defined as the presence of absolute neutrophil count $>0.5 \times 10^9$ cells/L for 3 consecutive days. Platelet engraftment was defined as platelet count $>50 \times 10^9$ cells/L maintained without platelet transfusions. For all patients, extent of overall donor engraftment in peripheral blood mononuclear cell (PBMC) and bone marrow (BM) was determined by quantitative polymerase chain reaction analysis of informative polymorphic regions of genomic DNA. Genomic DNA was extracted from 1 to 3 $\times 10^6$ PBMC or bone marrow mononuclear cells according to the manufacturer's protocol (Wizard kit, Promega, Madison, WI). For sex mismatched donor-recipient pairs, overall donor engraftment was also measured using Y-chromosome specific probes by fluorescent in situ hybridization analysis. Whole blood and marrow samples for evaluation of disease and chimerism were obtained on days 30, 60, 100, 180, and 360 after HCT and at point of last contact prior to this report.

Measurement of Lineage Specific Donor Engraftment

Mononuclear cells derived from posttransplant samples were isolated by Ficoll-Hypaque density gradient centrifugation. CD3-positive cells were isolated by flow cytometry (Hematologic, Inc., Seattle, WA) or by immunomagnetic bead selection (Miltenyi, Auburn, CA) for T cell chimerism analysis. Erythroid lineage specific chimerism was performed by β-globin RNA pyrosequencing as previously described [19,20]. Briefly, polymerase chain reaction (PCR) was performed utilizing primers specific for β-globin cDNA (reverse transcribed from RNA), such that the amplicon encompassed the sickle mutation on the β-globin locus. Quantitation of patient versus donor β-globin transcripts was performed by pyrosequencing, in which biotinylated single-strand DNA fragments were generated by mixing the PCR product with streptavidin-coated paramagnetic beads (Dynalbeads M280; Dynal, Norway) and processed according to the manufacturer's instructions on an automated pyrosequencing instrument, PSQ96 (Pyrosequencing AB, Uppsala, Sweden). Erythroid-lineage specific chimerism was quantified using Pyrosequencing software (Pyrosequencing AB).

Assessment of Organ Function and Recovery

All patients continue to be regularly followed by their primary hematologist and transplant physician with physical examination, blood counts, blood urea nitrogen, and creatinine and liver function tests. They undergo screening with transcranial Doppler ultrasound (TCD) annually. In the case of prior stroke, follow-up head magnetic resonance imaging (MRI) studies have been obtained yearly for 3 years after HCT and then as clinically indicated. Electrocardiogram, echocardiogram, and pulmonary function tests were obtained annually for 3 years post-HCT and as indicated thereafter.

RESULTS

Patient Characteristics

Seven patients with severe SCD underwent HCT. All patients or their parents were also offered myeloablative HCT, but this treatment option was refused. The early clinical results of Patient 1 has been previously reported [21]. The median age of the patients was 8 years (range: 6-18 years). Three of 7 patients underwent transplant for silent or clinical stroke, whereas 4 of 7 had a history of recurrent acute chest syndrome (Table 1). Patient 5 is a young adult whose prior clinical

Table 2. Regimen-Related Toxicity in Patients undergoing HCT

				Weaning of Immunosuppression (Days Post-HCT When Initiated)			End-organ Recovery (Pre- versus 1-Year Post-HCT)				
Pt #	Follow-up (Years)	Regimen-Related Toxicity (Days Post-HCT)	Infections (Days Post-HCT)	MMF	CSA	GVHD (Acute/Chronic)	Head MRI	Pulmonary Status*	Creatinine (mg/dL)	Splenic Fnnction†	Cardiac Status‡
1	8.5	Nil	Line-related bacteremia (d100)	45	180	None/None	Stable	Stable	0.5/0.6	No/Yes	Stable
2	8	Seizure (day +11)	Nil	45	180	Grade II skin/None	Stable	Stable	0.4/0.5	Prior splenectomy	Stable
3	5	Headache (day +1 to +4) Hemoglobinuria (day 0)	Nil	220	320	None/None	Stable	Stable	0.4/0.5	No/Yes	Stable
4	3.5	Nil	Nil	180	300	None/None	Stable	Stable	0.6/0.6	No/Yes	Stable
5	3.5	Pancreatitis (mild) (day +109)	Influenza A and herpes zoster (d106)	180	220	None/None	Stable	Stable	0.7/0.7	No/Yes	Stable
6	2	Nil	Nil	180	300	None/Limited chronic GVHD of skin	Stable	Stable	0.7/0.7	No/Yes	Stable
7	1.5	Nil	Line-related bacteremia (d244)	100	180	None/None	NA	NA	0.7/0.6	NA	NA

PFT indicates pulmonary function test; NA, not available; MRI, magnetic resonance imaging; HCT, hematopoietic stem cell transplantation.
*Pulmonary status measured by PFTs.
†Splenic recovery measured by spleen scan.
‡Cardiac status measured by EKG and Echo.

course was complicated by several severe comorbidities including sickle cell lung disease, allosensitization, autoimmune hemolytic anemia, severe transfusional iron overload of the liver and transient renal insufficiency related to iron chelation. As shown in Table 1, all patients received unmanipulated bone marrow from an HLA-matched sibling donor, with total nucleated cell number and $CD34^+$ dose ranging from 2.45-5.0 $\times$ 10^8 cells/kg and 1.2-12.0 $\times$ 10^6 cells/kg, respectively. The median follow-up was 4 years (range: 2-8.5 years).

Immediate Posttransplant Toxicity and Hematologic Recovery

The preparative regimen was very well tolerated, and immediate treatment-associated complications in all 7 patients were mild and reversible. These are summarized in Table 2. None of the patients developed mucositis. Patient 3 required analgesics for intense headaches that were attributed to MMF. Two patients required transient parenteral alimentation because of poor oral intake. Patient 2 experienced a single generalized seizure associated with subtherapeutic levels of phenytoin. Patient 3 developed hemoglobinuria immediately following stem cell infusion that recovered within 48 hours. Infectious complications consisted of coagulase-negative *Staphylococcus* bacteremia, dermatomal herpes zoster parainfluenza A infection, and line-related bacteremia with *Rhizobium radiobacter*.

The hematologic recovery of all 7 patients within the first 100 days following RIC HCT was prompt. As shown in Figure 1A, myelosuppression was limited to a median nadir absolute neutrophil count of 0.1 $\times$ 10^9/L (range: 60-130) and a median nadir platelet count of 31 $\times$ 10^9/L (range: 10-40). As shown in Table 1, median duration of neutropenia (absolute neutrophil count $<0.5 \times 10^9$/L) was 13 days (range: 8-17 days). Normal platelet and neutrophil counts were achieved by a median of 34 days (range: 27-35), and 22 days (range: 19-27), respectively. Patients received a median of 3 (range: 1-6) PRBC transfusions and 3 (range: 3-14) platelet transfusions. The median duration for requirement of transfusion of PRBC and/or platelets (Table 1) was 37 and 17 days, respectively. As shown in Figure 1B, patients achieved transfusion independence after a median of 35 days post-HCT. Six of 7 patients have normal platelet, white and red blood cell (RBC) counts at 2-8 years follow-up, whereas 1 of 7 initially engrafted, but subsequently lost the graft at day 244 with complete autologous recovery of hematopoiesis. Bone marrow examination at day 365 in all engrafted patients demonstrated normal cytogenetics.

GVHD

Patients 1 and 2 began their taper of MMF on posttransplant day 45 and of CSA doses at posttransplant day 180 (Table 2). Subsequently, early partial donor chimerism was observed in patients 3 and 4. Because mixed chimerism may be associated with graft rejection [22], in the ensuing patients, tapering of MMF and CSA was delayed until days 100-220, and 180-320 days, respectively.

As shown in Table 2, none of the patients developed greater than grade 2 aGVHD or extensive

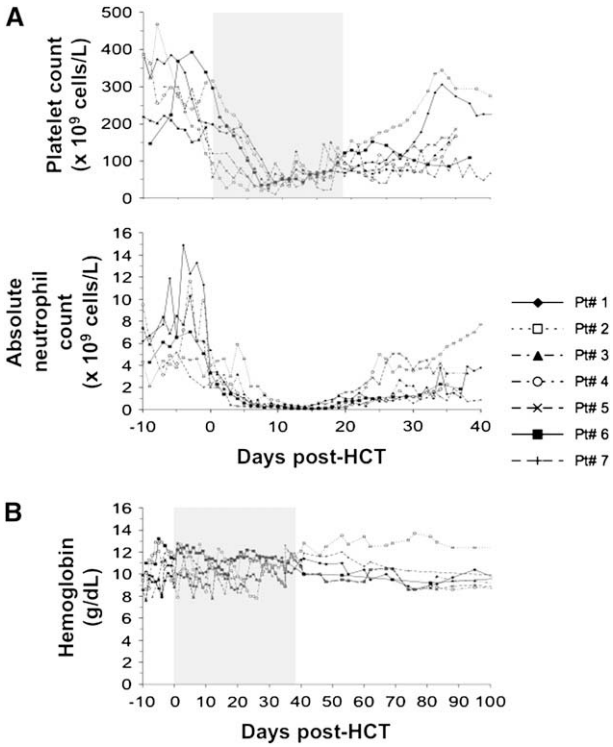

Figure 1. Recovery of (A) neutrophils and platelets, and (B) hemoglobin levels during the first 100 days following HCT in patients 1-7. Median duration of neutropenia (defined as ANC $<0.5 \times 10^9$/L) was 13 days (range: 8-17 days). Median duration thrombocytopenia requiring transfusion (platelet count $<50 \times 10^9$/L) was 17 days. Median duration of days during which platelet and PRBC transfusions were needed are indicated by the shaded gray regions.

cGVHD. Four of 7 developed neither aGVHD nor cGVHD (patients 1, 3, 4, and 7). Of the remaining 3, only 1 (patient 2) developed mild aGVHD, manifested as stage II skin GVHD, which resolved with prednisone therapy. At 6 years follow-up, this patient has not developed cGVHD. Patient 5 developed bilateral nongranulomatous uveitis 15 months after HCT with no other definite evidence of cGVHD that has been well controlled with topical steroids and weekly oral methotrexate. Patient 6 developed limited cGVHD of the skin at day 180 as CSA was being tapered. He was successfully treated with CSA and prednisone started at a dose of 2 mg/kg for 2 weeks, and then gradually weaned until all immunosuppression was removed.

Total and Lineage-Specific Chimerism following RIC HCT

We measured total PBMC chimerism on all 7 patients at 1 year following HCT, when they were no longer on immunosuppressive medications. We observed frequent mixed hematopoietic chimerism. As shown in Figure 2, 6 of 7 patients (patients 1-6) demonstrated high levels of donor engraftment, whereas patient 7 had already lost donor engraftment at this

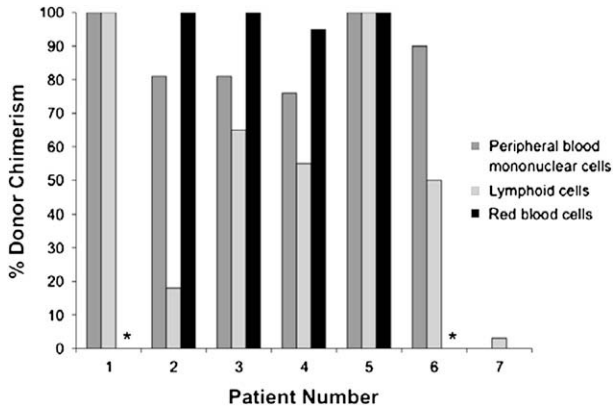

Figure 2. Lineage-specific peripheral blood donor chimerism at 1 year after HCT. Chimerism was examined in the mononuclear (dark gray bars), $CD3^+$ lymphoid chimerism (light gray), and RBC precursor (black bars) compartments. *Sample unavailable for analysis.

time point. Of the 6 engrafted patients, 2 demonstrated full donor chimerism, whereas 4 were partially engrafted, at levels ranging from 71% to 95%. The 2 patients who demonstrated full mononuclear cell chimerism (patients 1 and 5) also demonstrated full T cell donor chimerism. The 4 patients with partial donor chimerism, however, all demonstrated lower levels of T cell chimerism at 1 year following HCT, ranging from 17% to 65%.

We also sought to define the extent to which HCT corrected these patients' underlying RBC disorder. As shown in Figure 1B, patients with stable donor chimerism (patients 1-6) demonstrated a median hemoglobin level of 11.3 g/dL (range: 10.1-13.3). To determine the extent to which circulating RBCs were donor derived, we measured peripheral blood erythroid precursor chimerism by β-globin pyrosequencing [20] in 4 of the 6 patients with stable engraftment for whom samples were available. As shown in Figure 2, all 4 were measured to have >90% donor RBC chimerism. Consistent with these results, all 6 engrafted patients demonstrated the percentage Hb S in the blood expected from the donor's genotype. As shown in Figure 3, in patients transplanted from a donor with sickle cell trait, the median percentage Hb S was 33%, whereas those with nonsickle cell trait donors had undetectable Hb S levels.

To determine the long-term impact of our transplant regimen on engraftment in our patient cohort, we measured total and lineage specific chimerism in peripheral blood at the time of last contact (median of 4 years) after HCT (Figure 4). All 6 patients demonstrated a median level of donor chimerism of 78% in the mononuclear compartment, 98.5% (range: 76%-100%) in the erythroid compartment and 68.5% (range: 50%-90%) in the peripheral lymphoid compartment, 2-8.5 years post-HCT. Compared to 1 year after HCT, levels of donor lymphoid and mononuclear chimerism were stable or rose in 5 of 6

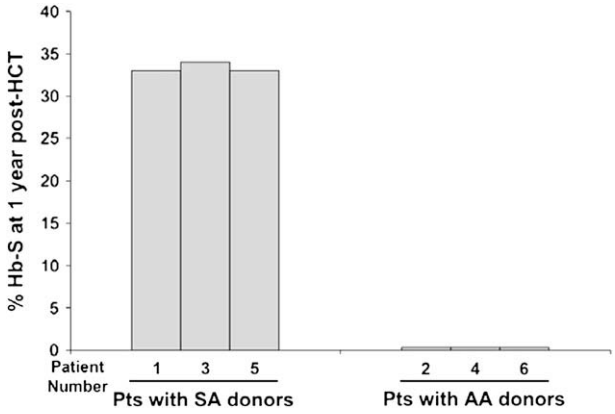

Figure 3. At 1-year post-HCT, transplanted SCD patients with stable engraftment demonstrate percentage of hemoglobin S (Hb S) in peripheral blood of a similar level to their donors. Patients 1, 3, and 5 received HCT from donors with sickle cell trait, wheres the donors of patients 2, 4, and 6 were normal nonsickle trait (AA) donors.

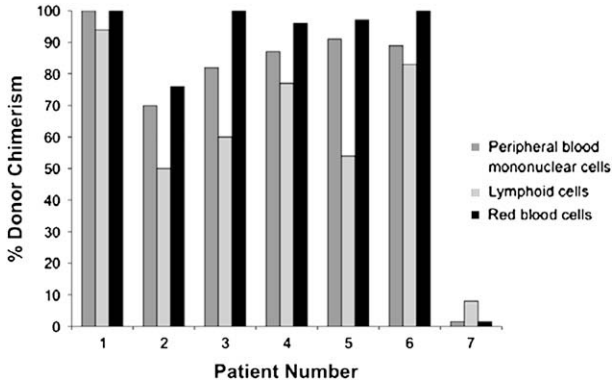

Figure 4. Lineage-specific peripheral blood donor chimerism at the time of last contact (2-8.5 years post-HCT). Chimerism was examined in the mononuclear (dark gray bars), $CD3^+$ lymphoid chimerism (light gray), and red blood cell precursor (black bars) compartments.

engrafted patients over time. Erythroid progenitor cell chimerism remained >70% donor derived in all 6 patients. These data demonstrate that long-term stable donor engraftment in the mononuclear, lymphoid, and erythroid compartments can be achieved following RIC HCT for SCD.

Noncompliance and Delayed Loss of Engraftment

Overall, 6 of 7 patients were highly compliant with the posttransplant immunosuppressive regimen. Patient 7, however, was noncompliant with cyclosporine after day 120. At day 244, following an episode of central venous line-related bacteremia, the patient became pancytopenic. During this time, peripheral blood mononuclear chimerism decreased from 90% to 3% over a period of 2 months and $CD3^+$ lymphoid chimerism decreased from 11% to 3%. Bone marrow examination revealed a very hypocellular marrow. She required platelet and RBC transfusions and intermittent administration of granulocyte-colony stimulating factor (G-CSF). Since day 330, she has demonstrated autologous recovery of her bone marrow with normal platelet counts, and white blood cell count and differential. Moreover, donor chimerism in the mononuclear, erythroid, or lymphoid subsets was not detectable, and % Hb S in peripheral blood has slowly risen from 0% without transfusion, at day 240 to 60% at day +365.

Survival and End-oOgan Effects of RIC HCT

At a follow-up of 2-8.5 years after transplantation, all 7 patients are alive. All patients are entirely off immunosuppression and 6 of 7 patients have no laboratory or clinical evidence of disease. When assessed 1 year after HCT, all 7 patients demonstrated stable head MRI findings and lung function, as measured by pulmonary function tests. None of the 7 patients had measurable pulmonary hypertension prior to transplant, and all have normal echocardiograms after transplant. In addition, normal renal function was regained following HCT in all patients. Patient 1 received HCT at age 8 and attained menarche at age 13 years. Patient 5 had primary amenorrhea at the time of HCT at 18 years and continues to be amenorrheic. Five patients with stable engraftment and no prior history of splenectomy were found to have splenic regeneration on spleen scan (data not shown).

DISCUSSION

Since we first demonstrated "proof of principle" that an RIC conditioning regimen can lead to stable donor erythropoiesis in a patient with severe SCD a number of years ago [21], a number of studies of transplantation for SCD have been published that use conditioning regimens ranging from truly nonmyeloablative, to nearly myeloablative regimens [23]. In the current study, we demonstrate stable long-term donor engraftment in 6 of 7 multiply transfused patients with SCD following HCT from a matched sibling donor using an RIC conditioning regimen. This includes a multiply transfused young adult with iron overload, ABO incompatibility and allo-sensitization—all factors associated with increased risk of engraftment failure and delayed donor erythropoiesis [24]. One of 7 patients rejected the graft because of poor compliance with immunosuppressive medications. To date, this is the single largest study of HCT following RIC conditioning for SCD where consistent stable engraftment has been demonstrated over a prolonged period of time.

Our results stand in distinct contrast to the high rate of delayed loss of donor engraftment following the withdrawal of immunosuppression, that has been

consistently observed in patients with hemoglobinopathies receiving HCT following minimal intensity (<25% of the full myeloablative regimen) conditioning regimens [25,26]. These higher rates of delayed graft loss in patients with SCD, relative to patients with hematologic malignancies, may be related to diminished marrow space because of expanded erythropoiesis, a relatively robust intact immune system unaltered by prior exposure to chemotherapy, and history of chronic transfusions leading to allo-sensitization because of chronic transfusion and exposure to alloantigens [27]. On the other hand, myeloablative regimens or RIC conditioning regimens employing near-myeloablative doses have been associated with unacceptable regimen related toxicity in adults with SCD [28]. Our regimen of BU/FLU/ATG/TLI using busulfan at approximately 50% of the myeloablative dose, together with FLU /TLI/ATG, our regimen generates a high degree of immunosuppression in conjunction with creation of marrow space, appears to be able to strike a balance between dose intensity and degree of associated toxicity. Conditioning with TLI/ATG has been reported to markedly decrease the incidence of aGVHD, possibly by enrichment of host natural killer T cells [29-31] and can promote engraftment of hematopoietic cells even in chemotherapy naïve subjects with intact hematopoietic and immune systems [29,32]. Consistent with our findings, various case reports have described successful donor engraftment in patients with hemoglobinopathies and nonmalignant disorders following HCT using alternate conditioning regimens comprised of chemotherapy at approximately 50% of the myeloablative dose, together with immunosuppressive agents [33-38].

Chimerism following a nonmyeloablative preparative regimen that is sufficient to establish stable long-term donor-derived erythropoiesis and disease amelioration in patients with SCD has long been the subject of investigation [25,26]. Stable mixed chimerism has been reported to occur in 5% to 25% of patients with SCD undergoing myeloablative HCT [24]. In the nonmyeloablative setting, because recipient hematopoiesis is not fully ablated, mixed chimerism may occur even more frequently. Full donor T cell engraftment has been reported to precede donor myeloid engraftment, aGVHD, and disease regression in patients undergoing HCT following RIC conditioning [39,40], and early achievement of 100% donor T cell chimerism by day 30 has been considered necessary for full expression of the alloimmune response [40]. However, we observed that stable donor engraftment could be achieved even in the setting of low levels of early lymphoid donor chimerism and that lymphoid donor chimerism can slowly increase over time. Further studies will be required to elucidate the mechanisms by which tolerance to donor cells is achieved in patients with SCD following RIC conditioning.

Murine studies have demonstrated that 100% erythroid chimerism is essential for organ recovery following HCT for SCD [41]. We have previously reported that donor-derived erythroid precursors are relatively enriched in the face of mixed mononuclear cell chimerism in patients with SCD because of relative ineffective erythropoiesis of sickle-derived erythroid precursors [19,42]. Consistent with our previous studies, we observed total or near-complete detection of donor-derived erythroid cells in peripheral blood in 6 of 7 patients over long-term follow-up that was associated with clinical improvement.

We observed stable neurologic, pulmonary, and cardiac status in our patient cohort following transplantation. That 1 patient achieved menarche at normal age following HCT suggests that this conditioning regimen may help preserve endocrine ovarian function. Despite the escalated degree of immunosuppression generated by our regimen, we did not observe serious morbidity or mortality related to bacterial or viral infections as has been reported in some alemtuzumab-containing regimens [43,44]. Although the use of total lymphoid irradiation is a variable risk factor for late treatment-related complications, such as malignancies [45-47], we have not observed this complication. Persistence of lymphoid cells of donor origin that have been exposed to radiation may pose an as-yet unknown increased risk of malignancies. A larger study with an even longer observation is necessary before the long-term risk of TLI associated with this preparative regimen and in this patient population can be determined.

GVHD is the other major cause of morbidity and mortality in patients with hemoglobinopathies undergoing myeloablative HCT. We observed 1 patient each with grade II aGVHD and limited cutaneous cGVHD. This is not lower than the incidence of GVHD observed in patients with SCD undergoing myeloablative or RIC conditioning [7,10,28]. Contrary to early expectations, HCT following a nonmyeloablative or RIC conditioning regimen for malignant and nonmalignant disease has been associated with a high incidence of GVHD [48]. For these reasons, it is remarkable that we did not observe any severe GVHD. Donor $CD8^+$ T cell count above the median on day +14 after HCT may be associated with increased risk of development of aGVHD grades II to IV [49]. Further studies are indicated to determine if the low incidence of severe GVHD in our patients is related to the relatively low level of early donor lymphoid mixed chimerism.

The ability to reduce the dose of myelotoxic drugs commonly used in conditioning regimen prior to HCT has the potential to reduce the risk of early and late regimen-related toxicity; decrease hospitalization

and reduce the cost of the HCT procedure. Improved tolerability of HCT may lead to this therapeutic option becoming available to older or high-risk patients, those without sibling donors, or those with significant morbidity but not meeting current criteria for HCT [23,50]. Taken together, these data demonstrate encouraging results of using an immunosuppressive RIC regimen, which create a balance between myelosuppression and immunoablation results in stable engraftment with minimal toxicity in patients with SCD.

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REFERENCES

1. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in SCD. Life expectancy and risk factors for early death. *N Engl J Med*. 1994; 330:1639-1644.
2. Powars DR, Chan LS, Hiti A, Ramicone E, Johnson C. Outcome of sickle cell anemia: a 4-decade observational study of 1056 patients. *Medicine (Baltimore)*. 2005;84:363-376.
3. Hagar RW, Vichinsky EP. Major changes in SCD. *Adv Pediatr*. 2000;47:249-272.
4. Steinberg MH. Hydroxyurea treatment for SCD. *ScientificWorldJournal*. 2002;2:1706-1728.
5. Platt OS. Hydroxyurea for the treatment of sickle cell anemia. *N Engl J Med*. 2008;358:1362-1369.
6. Abboud MR, Jackson SM, Barredo J, Beatty J, Laver J. Bone marrow transplantation for sickle cell anemia. *Am J Pediatr Hematol Oncol*. 1994;16:86-89.
7. Bernaudin F, Socie G, Kuentz M, et al. Long-term results of related, myeloablative stem cell transplantation to cure sickle cell disease. *Blood*. 2007;110. 27549–2756.
8. Ferster A, De Valck C, Azzi N, Fondu P, Toppet M, Sariban E. Bone marrow transplantation for severe sickle cell anaemia. *Br J Haematol*. 1992;80:102-105.
9. Giardini C, Galimberti M, Lucarelli G, et al. Bone marrow transplantation in sickle-cell anemia in Pesaro. *Bone Marrow Transplant*. 1993;12:122-123.
10. Panepinto JA, Walters MC, Carreras J, et al. Matched-related donor transplantation for SCD: report from the Center for International Blood and Transplant Research. *Br J Haematol*. 2007;137:479-485.
11. Walters MC. Stem cell therapy for SCD: transplantation and gene therapy. *Hematology Am Soc Hematol Educ Program*. 2005;66-73.
12. Walters MC. Cord blood transplantation for sickle cell anemia: Bust or boom? *Pediatr Transplant*. 2007;11:582-583.
13. Walters MC, Patience M, Leisenring W, et al. Bone marrow transplantation for SCD. *N Engl J Med*. 1996;335:369-376.
14. Walters MC, Storb R, Patience M, et al. Impact of bone marrow transplantation for symptomatic SCD: an interim report. Multicenter investigation of bone marrow transplantation for SCD. *Blood*. 2000;95:1918-1924.
15. Walters MC, Patience M, Leisenring W, et al. Barriers to bone marrow transplantation for sickle cell anemia. *Biol Blood Marrow Transplant*. 1996;2:100-104.
16. Andersson BS, Kashyap A, Couriel D, et al. Intravenous busulfan in pretransplant chemotherapy: bioavailability and patient benefit. *Biol Blood Marrow Transplant*. 2003;9:722-724.
17. Mielcarek M, Martin PJ, Leisenring W, et al. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood*. 2003;102:756-762.
18. Franzoni E, Sarajlija J, Garone C, Malaspina E, Marchiani V. No kinetic interaction between levetiracetam and cyclosporine: a case report. *J Child Neurol*. 2007;22:440-442.
19. Wu CJ, Krishnamurti L, Kutok JL, et al. Evidence for ineffective erythropoiesis in severe sickle cell disease. *Blood*. 2005;106: 3639-3645.
20. Wu CJ, Hochberg EP, Rogers SA, et al. Molecular assessment of erythroid lineage chimerism following nonmyeloablative allogeneic stem cell transplantation. *Exp Hematol*. 2003;31:924-933.
21. Krishnamurti L, Blazar BR, Wagner JE. Bone marrow transplantation without myeloablation for SCD. *N Engl J Med*. 2001;344:68.
22. Andreani M, Manna M, Lucarelli G, et al. Persistence of mixed chimerism in patients transplanted for the treatment of thalassemia. *Blood*. 1996;87:3494-3499.
23. Krishnamurti L. Hematopoietic cell transplantation for SCD: state of the art. *Expert Opin Biol Ther*. 2007;7:161-172.
24. Walters MC, Patience M, Leisenring W, et al. Stable mixed hematopoietic chimerism after bone marrow transplantation for sickle cell anemia. *Biol Blood Marrow Transplant*. 2001;7:665-673.
25. Horan JT, Liesveld JL, Fenton P, Blumberg N, Walters MC. Hematopoietic stem cell transplantation for multiply transfused patients with SCD and thalassemia after low-dose total body irradiation, fludarabine, and rabbit anti-thymocyte globulin. *Bone Marrow Transplant*. 2005;35:171-177.
26. Iannone R, Casella JF, Fuchs EJ, et al. Results of minimally toxic nonmyeloablative transplantation in patients with sickle cell anemia and beta-thalassemia. *Biol Blood Marrow Transplant*. 2003;9:519-528.
27. Iannone R, Ohene-Frempong K, Fuchs EJ, Casella JF, Chen AR. Bone marrow transplantation for sickle cell anemia: progress and prospects. *Pediatr Blood Cancer*. 2005;44:436-440.
28. van Besien K, Bartholomew A, Stock W, et al. Fludarabine-based conditioning for allogeneic transplantation in adults with SCD. *Bone Marrow Transplant*. 2000;26:445-449.
29. Liu YP, Li Z, Nador RG, Strober S. Simultaneous protection against allograft rejection and graft-versus-host disease after total lymphoid irradiation: role of natural killer T cells. *Transplantation*. 2008;85:607-614.
30. Lowsky R, Takahashi T, Liu YP, et al. Protective conditioning for acute graft-versus-host disease. *N Engl J Med*. 2005;353: 1321-1331.
31. Lan F, Zeng D, Higuchi M, Higgins JP, Strober S. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: the role of CD1-reactive natural killer T cells. *Biol Blood Marrow Transplant*. 2003;9: 355-363.
32. Scandling JD, Busque S, Dejbakhsh-Jones S, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med*. 2008;358:362-368.
33. Mazur M, Kurtzberg J, Halperin E, Ciocci G, Szabolcs P. Transplantation of a child with sickle cell anemia with an unrelated cord blood unit after RIC conditioning. *J Pediatr Hematol Oncol*. 2006;28:840-844.
34. Hongeng S, Pakakasama S, Chaisiripoomkere W, Ungkanont A, Jootar S. Nonmyeloablative stem cell transplantation with

a haploidentical donor in a class 3 lucarelli severe thalassemia patient. *Bone Marrow Transplant*. 2004;34:271-272.
35. Hongeng S, Pakakasama S, Chuansumrit A, et al. RIC stem cell transplantation for treatment of class 3 Lucarelli severe thalassemia patients. *Am J Hematol*. 2007;82:1095-1098.
36. Schleuning M, Stoetzer O, Waterhouse C, Schlemmer M, Ledderose G, Kolb HJ. Hematopoietic stem cell transplantation after reduced-intensity conditioning as treatment of SCD. *Exp Hematol*. 2002;30:7-10.
37. Shenoy S, Grossman WJ, DiPersio J, et al. A novel reduced-intensity stem cell transplant regimen for nonmalignant disorders. *Bone Marrow Transplant*. 2005;35:345-352.
38. Horwitz ME, Spasojevic I, Morris A, et al. Fludarabine-based nonmyeloablative stem cell transplantation for SCD with and without renal failure: clinical outcome and pharmacokinetics. *Biol Blood Marrow Transplant*. 2007;13:1422-1426.
39. Baron F, Little MT, Storb R. Kinetics of engraftment following allogeneic hematopoietic cell transplantation with reduced-intensity or nonmyeloablative conditioning. *Blood Rev*. 2005;19: 153-164.
40. Childs R, Clave E, Contentin N, et al. Engraftment kinetics after nonmyeloablative allogeneic peripheral blood stem cell transplantation: full donor T-cell chimerism precedes alloimmune responses. *Blood*. 1999;94:3234-3241.
41. Kean LS, Manci EA, Perry J, et al. Chimerism and cure: hematologic and pathologic correction of murine SCD. *Blood*. 2003; 102:4582-4593.
42. Wu CJ, Gladwin M, Tisdale J, et al. Mixed haematopoietic chimerism for SCD prevents intravascular haemolysis. *Br J Haematol*. 2007;139:504-507.
43. Myers GD, Krance RA, Weiss H, et al. Adenovirus infection rates in pediatric recipients of alternate donor allogeneic bone marrow transplants receiving either antithymocyte globulin (ATG) or alemtuzumab (Campath). *Bone Marrow Transplant*. 2005;36:1001-1008.
44. Penack O, Fischer L, Stroux A, et al. Serotherapy with thymoglobulin and alemtuzumab differentially influences frequency and function of natural killer cells after allogeneic stem cell transplantation. *Bone Marrow Transplant*. 2008;41:377-383.
45. Deeg HJ, Socie G, Schoch G, et al. Malignancies after marrow transplantation for aplastic anemia and fanconi anemia: a joint Seattle and Paris analysis of results in 700 patients. *Blood*. 1996;87:386-392.
46. Inamoto Y, Suzuki R, Kuwatsuka Y, et al. Long-term outcome after bone marrow transplantation for aplastic anemia using cyclophosphamide and total lymphoid irradiation as conditioning regimen. *Biol Blood Marrow Transplant*. 2008;14:43-49.
47. Slavin S, Naparstek E, Aker M, et al. The use of total lymphoid irradiation (TLI) for prevention of rejection of T-lymphocyte depleted bone marrow allografts in non-malignant hematological disorders. *Transplant Proc*. 1989;21:3053-3054.
48. Flowers ME, Traina F, Storer B, et al. Serious graft-versus-host disease after hematopoietic cell transplantation following nonmyeloablative conditioning. *Bone Marrow Transplant*. 2005;35: 277-282.
49. Petersen SL, Madsen HO, Ryder LP, et al. Chimerism studies in HLA-identical nonmyeloablative hematopoietic stem cell transplantation point to the donor CD8(+) T-cell count on day + 14 as a predictor of acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2004;10:337-346.
50. Storb RF, Lucarelli G, McSweeney PA, Childs RW. Hematopoietic cell transplantation for benign hematological disorders and solid tumors. *Hematology Am Soc Hematol Educ Program*. 2003;372-397.