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A Novel Reduced-Intensity Conditioning Regimen for Unrelated Umbilical Cord Blood Transplantation in Children with Nonmalignant Diseases

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A B S T R A C T

Reduced-intensity conditioning (RIC) regimens have the potential to decrease transplantation-related morbidity and mortality. However, engraftment failure has been prohibitively high after RIC unrelated umbilical cord blood transplantation (UCBT) in chemotherapy-naïve children with nonmalignant diseases (NMD). Twenty-two children with a median age of 2.8 years, many with severe comorbidities and prior viral infections, were enrolled in a novel RIC protocol consisting of hydroxyurea, alemtuzumab, fludarabine, melphalan, and thiotepa followed by single UCBT. Patients underwent transplantation for inherited metabolic disorders (n = 8), primary immunodeficiencies (n = 9), hemoglobinopathies (n = 4) and Diamond Blackfan anemia (n = 1). Most umbilical cord blood (UCB) units were HLA-mismatched with median infused total nucleated cell dose of 7.9×10^7/kg. No serious organ toxicities were attributable to the regimen. The cumulative incidence of neutrophil engraftment was 86.4% (95% confidence interval [CI], 65% to 100%) in a median of 20 days, with the majority sustaining > 95% donor chimerism at 1 year. Cumulative incidence of acute graft-versus-host disease (GVHD) grades II to IV and III to IV by day 180 was 27.3% (95% CI, 8.7% to 45.9%) and 13.6% (95 CI, 0% to 27.6%), respectively. Cumulative incidence of extensive chronic GVHD was 9.1% (95% CI, 0% to 20.8%). The primary causes of death were viral infections (n = 3), acute GVHD (n = 1) and transfusion reaction (n = 1). One-year overall and event-free survivals were 77.3% (95% CI, 53.7% to 89.8%) and 68.2% (95% CI, 44.6% to 83.4%) with 31 months median follow-up. This is the first RIC protocol demonstrating durable UCB engraftment in children with NMD. Future risk-based modifications of this regimen could decrease the incidence of viral infections. (www.clinicaltrials.gov/NCT00744692).

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) can cure a variety of nonmalignant diseases (NMD) in children, either by direct replacement of abnormal bone marrow precursors, as in hemoglobinopathies and primary immunodeficiencies (PID), or indirectly, by providing cellular enzyme replacement to diseased areas, such as liver and brain, in inherited metabolic disorders (IMD) [1-3]. For patients lacking matched related donors, unrelated donor umbilical cord blood transplantation (UCBT) is increasingly being utilized because of easy availability and less restrictive human leukocyte antigen (HLA) matching requirements. Myeloablative conditioning (MAC) with UCBT has been used successfully for 2 decades to treat a variety of malignant and NMD [4-7]; however, high transplantation-related mortality (TRM) and the risks of long-term adverse effects, such as infertility and secondary malignancies, have remained a barrier for children with hopefully decades of life expectancy [8-10].

Mixed donor chimerism leads to disease resolution in patients with hemoglobinopathies and PID, which can remain stable over time [11-14]. These observations provide a rational basis to design reduced-intensity conditioning (RIC) regimens aimed at decreasing nonhematopoietic toxicity without compromising the clinical benefits of persistent donor hematopoiesis. There is a spectrum of RIC regimens, ranging from minimally intensive/nonmyeloablative to near ablative with reduced toxicity [13-18]. However, successful RIC trials were reported mainly after HSCT from living related or unrelated donors. The use of UCBT after RIC has been

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Table 1
Baseline Patient and Graft Characteristics

UPIN	Diagnosis*	Age, yr	Sex	Minority (Y/N)	Pretransplantation Comorbidities	Weight on Day 0, kg	HLA Match 6 Allele A,B, DRB1	HLA Match 8 Allele Hi res A, B, C, DRB1	CMV (Pt)	TNCC ($\times 10^7$/kg Reinfused	$CD34^+$ ($\times 10^5$/kg Reinfused)
1	Hunter	2.03	M	Y	Cardiomyopathy, obstructive sleep apnea	25.3	4/6	6/8	Neg	7.91	3.70
2	Sanfilippo B Syndrome	3.35	M	N		15.1	6/6	8/8	Neg	5.18	3.78
3	Sanfilippo B Syndrome	3.32	F	N		15.8	5/6	6/8	Neg	4.14	.87
4	Cartilage Hair Hypoplasia	1.30	M	N	CMV viremia, transfusion dependent anemia, chronic neutropenia	8.2	6/6	7/8	Pos	13.90	4.31
5	MLD	6.76	M	N		20.4	5/6	6/8	Neg	4.58	2.43
6	Zap 70 deficiency	3.32	F	Y	Resp: MAI, Pseudomonas, Parainfluenza 1, Candida, bronchiectasis; Enteroviral meningitis, hydrocephalus, VP shunt	11.2	5/6	5/8	Neg	6.60	2.6
7	HLH	.45	F	N		7.9	5/6	5/8	Neg	15.3	3.67
8	HLH	.58	F	Y	Adenoviremia, adenovirus in stool and urine	6.20	4/6	4/8	Neg	11.80	2.48
9	β thalassemia major	1.56	M	Y		9.5	5/6	6/8	Pos	8.17	5.10
10	CID	1.14	F	N	ITP, HHV-6 pneumonitis, Enterovirus encephalitis, colitis	10.1	5/6	6/8	Neg	7.80	2.73
11	Krabbe	3.09	F	Y	Blindness	12.2	4/6	5/8	Neg	14.30	4.58
12	Krabbe	3.25	F	N	Ataxia	19.1	4/6	4/8	Neg	7.36	1.55
13	DBA	2.56	M	N	Iron overload	15.9	5/6	7/8	Neg	6.22	1.50
14	Hurler	1.71	F	Y	Obstructive sleep apnea. Stool adenovirus +, h/o Parainfluenza 3 infection	11.8	4/6	4/8	Neg	10.8	4.86
15	ALD	8.02	M	Y		26.9	4/6	3/8	Neg	6.99	3.7
16	βThalassemia major	3.65	M	Y		14.2	4/6	5/8	Neg	7.07	1.63
17	B thalassemia major	5.44	F	Y	Prior myeloablative transplant; h/o severe hemorrhagic cystitis	18	5/6	6/8	Pos	11	3.5
18	Omenn syndrome	.45	M	Y		5.26	5/6	6/8	Pos	22	7.72
19	βThalassemia major	2.58	M	Y		13.2	6/6	6/8	Neg	5.04	4.58
20	CD40L deficiency	3.38	M	N		14.1	5/6	7/8	Neg	9.46	3
21	Omenn syndrome	.31	M	N	Erythroderma, FTT, CMV	6	6/6	8/8	Pos	15.8	2.4
22	PNP deficiency	4.27	M	N	FTT, food intolerance, chronic enteritis, CMV urine, adenovirus in stool	16.6	5/6	5/8	Pos	5.58	1.3

ALD indicates adrenoleukodystrophy; CID, combined immunodeficiency; CMV, cytomegalovirus; DBA, Diamond Blackfan anemia; F, female; FTT, failure to thrive; HHV-6, human herpes virus 6; Hi res, high resolution; HLH, Hemophagocytic lymphohistiocytosis; h/o, history of; ITP, immune thrombocytopenic purpura; M, male; MAI, mycobacterium avium; MLD, metachromatic leukodystrophy; Neg, negative; PNP, purine nucleoside phosphorylase; Pos, positive; Pt, patient; UPIN, patient number; VP, ventriculoperitoneal; Y/N, Yes/no; ZAP 70, zeta chain associated protein kinase 70; TNCC, total nucleated cell count.

* Inherited metabolic disorders (IMD) include Hunter, Hurler, Krabbe, Sanfilippo B, metachromatic leukodystrophy (MLD), adrenoleukodystrophy (ALD). Primary immunodeficiency diseases (PID) include Cartilage hair hypoplasia, Zap 70 deficiency, HLH, CID, Omenn syndrome, CD40 ligand deficiency, PNP deficiency.

successful in heavily pretreated adults who underwent transplantation for malignant conditions, but has been associated with high rates of primary graft failure or graft rejection and autologous reconstitution in chemotherapy-naïve children, exceeding 50% in patients undergoing single unit UCBT for hemoglobinopathies [19-22]. In these cases, graft failure might result from the fact that lower numbers of infused progenitors may have been unable to outcompete recovering host hematopoiesis and lymphopoiesis. Therefore, we developed a novel immunoablative, nevertheless RIC regimen, designed to establish durable donor cell engraftment, and we report the results of this prospective, single arm, phase I pilot study in chemotherapy-naïve children with NMD undergoing single donor UCBT.

METHODS

Patients

From December 2008 to June 2012, 22 children with NMD lacking matched related donors scheduled to undergo a single UCBT were enrolled at Duke University, Durham, NC (n = 19) and All Children's Hospital, St. Petersburg, FL (n = 3). The study was approved by the institutional review boards of both institutions. Written assent or informed consent was obtained from all parents/caretakers or, when ≥18 years of age, patients, in accordance with the Declaration of Helsinki. Eligible patients were between 2 months and 21 years of age with a diagnosis of NMD (PID, IMD, hemoglobinopathies, or other transfusion-dependent anemias). Patients with IMD exhibiting high performance status, patients with severe aplastic anemia, and those who had undergone HSCT within the previous 6 months were excluded. (www.clinicaltrials.gov NCT00744692).

Donors

Grafts were selected based on HLA Class I (A, B) intermediate-resolution, and HLA class II (DRB1) allelic level typing. The precryopreservation total nucleated cell count (TNCC) had to be $> 3 \times 10^7$/kg for 5/6 or 6/6 HLA-matched grafts and $> 5 \times 10^7$/kg for 4/6 HLA-matched grafts. Potential units were screened by international cord blood banking standards and for normal enzyme activity in IMD patients. Precryopreservation graft characteristics were obtained from the supplying cord blood banks, whereas the respective hospital laboratories that thawed, washed, and tested the units provided post-thaw data.

Conditioning Regimen, Graft-versus-Host Disease Prophylaxis, Transplantation, and Supportive Care

All patients were treated with alemtuzumab 1 mg/kg/dose I.V. on 3 successive days −21 to −19, after a test dose of .2 mg/kg on day −22; hydroxyurea 30 mg/kg/day orally from day −22 until day −10; fludarabine

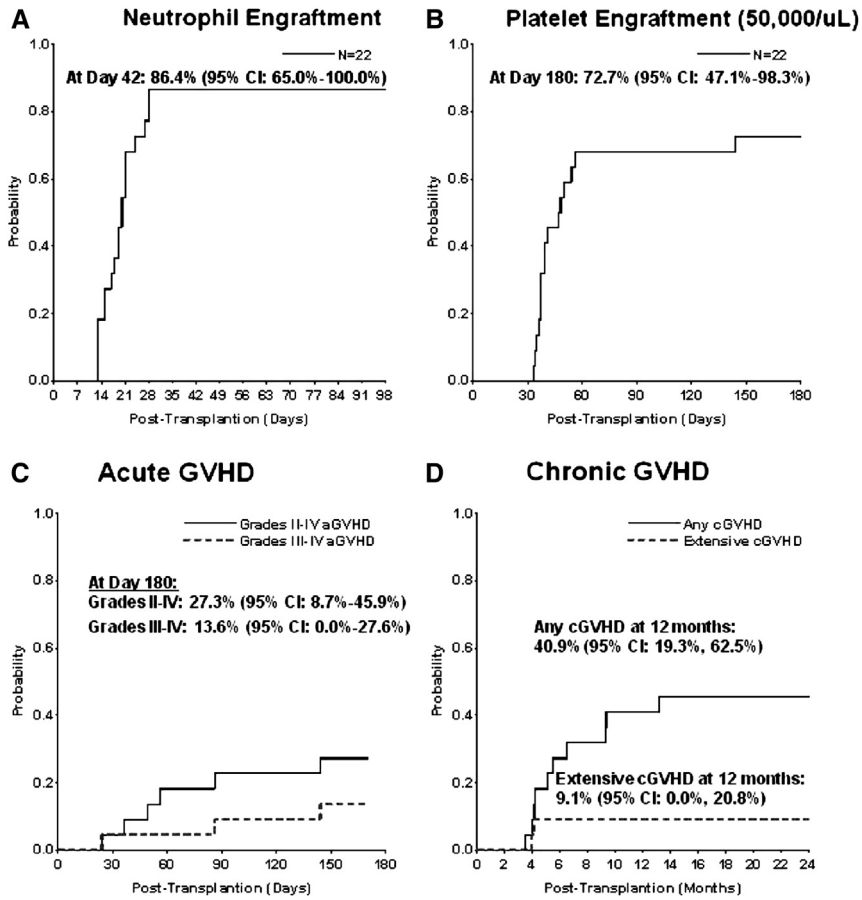

Figure 1. (A) Cumulative incidence of neutrophil engraftment > 500 cells/mm^3 with donor cells by day 42. (B) Cumulative incidence of platelet engraftment > 50,000/mm^3 by day 180. (C) Cumulative incidence of acute GVHD, grades II to IV (solid line) and III to IV (dotted line). (D) Cumulative incidence of chronic GVHD, overall (solid line) and extensive (dotted line).

(Flu) 30 mg/m^2/day I.V. from days −9 to −5; melphalan (Mel) 70 mg/m^2/day I.V. on days −4, −3; and thiotepa 200 mg/m^2 I.V. on day −2. Frequent baths (every 4 hours for 24 hours) were prescribed after thiotepa administration. The thawed and washed UCB unit was infused intravenously on day 0, as previously described [23,24].

Starting day −3, all patients received graft-versus-host disease (GVHD) prophylaxis with tacrolimus .03 mg/kg/day I.V. and mycophenolate 15 mg/kg/dose I.V. every 8 hours. Tacrolimus trough levels were maintained between 8 and 15 ng/mL. In patients without active GVHD, mycophenolate was discontinued after 45 days and tacrolimus was tapered 9 months after transplantation.

Alemtuzumab was administered in the outpatient clinic for most patients after intensive premedication with acetaminophen, diphenhydramine, ibuprofen, and corticosteroids. All patients were hospitalized in the Pediatric Blood and Marrow Transplant unit of Duke University Medical Center or All Children's Hospital before day −4 and through engraftment and stabilization post transplantation. Median length of stay for the initial hospitalization was 47 days (range, 29 to 263). Fungal and antiviral prophylaxis were voriconazole and acyclovir, respectively. Pneumocystis jiroveci pneumonia, veno-occlusive disease (VOD) prophylaxis, nutrition, and transfusion support were as previously described [24]. All patients received intravenous immunoglobulin (500 mg/kg/dose) weekly until day +100, starting day−1. Filgrastim (Amgen, Thousand Oaks, CA) was started at 5 mcg/kg/day on day +1 and weaned post engraftment. Cytomegalovirus (CMV) and adenovirus DNA were monitored weekly post alemtuzumab. One patient received irradiated, granulocyte colony-stimulating factor (G-CSF)–mobilized, parental granulocyte transfusions until engraftment for history of Mycobacterium avium, Pseudomonas, and Candida infections. Four patients subsequently enrolled on the ADV HALT study (randomized, placebo-controlled, phase 2 Study of preemptive CMX001 treatment for adenovirus disease prevention); 2 on a multicenter study of banked third-party virus-specific T cells to treat CMV, Epstein-Barr virus (EBV), or adenovirus infections after HSCT [25], and 2 on expanded access study of human mesenchymal stem cell (Prochymal) infusion for steroid-refractory acute GVHD described in more details under Results.

Post-transplantation Assessments

Engraftment studies were performed by short tandem repeat methods in whole blood, CD15$^+$, and CD3$^+$ fractions on days 30, 60, 100, 180, 270, 1 year, and annually thereafter. In parallel, immunophenotyping by 4-color FACS was performed to quantitate lymphocyte and dendritic cell subsets [26-28]. Immunoglobulin levels were tested at regular intervals post transplantation. Organ function and disease-specific evaluations were performed per institutional practices.

Assessment of Functional T Cell Responses to Microbial Antigens

Multiplex cytokine measurements using a cytokine bead array (CBA) and lymphoproliferative assays as internal controls were set up to test in parallel for the presence and magnitude of virus-specific T cell response. Mononuclear cell preps were made and 10^5 mononuclear cells were seeded per well of 96 well plates in complete medium composed of RPMI 1640, 10% PHS, 100 x antibiotic/antimycotic, L-glutamine, Hepes and β-mercaptoethanol, (Life Technologies Corp, Carlsbad, CA). Viral antigens (Meridian Life Sciences, Memphis, TN) derived from cytomegalovirus (CMV, R9A104), herpes simplex virus (HSV, R9A002), and varicella zoster virus (VZV, R02030) were diluted from frozen stocks and added for a total 200 μL. Negative control wells received no antigen, whereas positive control wells received 10^5 ClinExVivo beads (Life Technologies) as described. Duplicate wells were incubated for 5 days at 37°C, and then 75 uL medium was removed and frozen for batched CBA analysis. Secreted cytokines (IFNγ, TNFα, IL-10, granulocyte-macrophage colony stimulating factor [GM-CSF], IL-2, and IL-4) were quantitated using the BioPlex (Biorad, Hercules, CA) CBA system. The wells were pulsed with 25 μL of medium containing 1 μCi of ^{3}H-Thymidine (Perkin Elmer, Waltham, MA) and incubated for 6 to 8 hours at 37°C. Results were expressed numerically as the mean counts per minutes of duplicates as described [29,30].

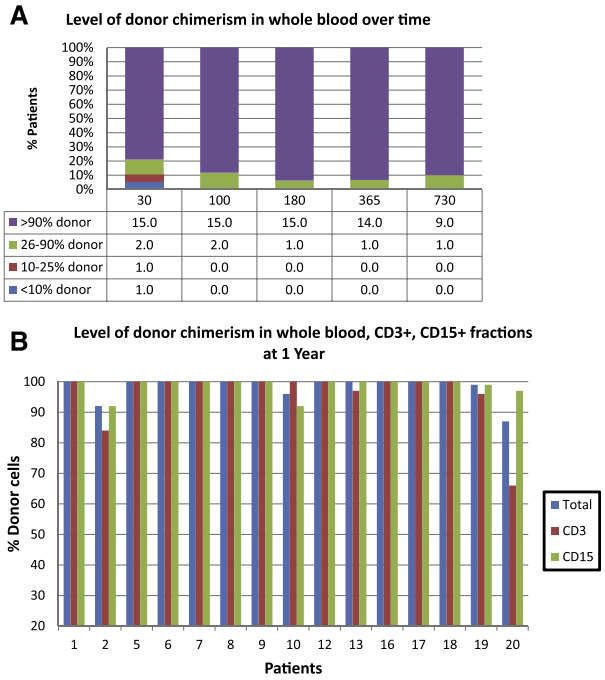

Figure 2. Donor Chimerism. (A) Level of whole blood donor chimerism over time; X-axis = Days after transplantation; Y axis = % of evaluable patients (B) Level of donor chimerism in whole blood, CD3+ & CD15+ fractions at 1 year (n = 15).

T Cell Receptor Excision Circle Measurements

Peripheral blood mononuclear cells were prospectively cryopreserved at defined intervals before and after UCBT. Thymopoiesis was assessed by amplification and quantification of delta-deletion T cell receptor excision circle (TRECs) as previously described [31]. Primers for the TREC sequence were 5′-CCC TTT CAA CCA TGC TGA CAC-3′ (forward) and 5′-GGG TGC AGG TGC CTA TGC-3′ (reverse), which produced an 80-bp fragment detected with the probe 5′-FAM-TCT GGT TTT TGT AAA GGT GCC CAC TCC TG-BHQ-1-3′. TREC quantification was normalized for cell input by parallel quantification of the human β-globin gene. The primers for human β-globin were 5′-GAA GAG CCA AGG ACA GGT ACG-3′ (forward) and 5′-CCT GGG AGT AGA TTG GCC AA-3′ (reverse), which produced an 85-bp fragment detected by the probe 5′-FAM-CTG TCA TCA CTT AGACCT CAC CCT GTG-BHQ-1-3′.

Statistical Methods

The study was designed as a prospective, single arm, safety and efficacy pilot study to evaluate the RIC regimen for single UCBT. The primary hypothesis/objective was that the RIC regimen would result in adequate engraftment defined as the presence of > 25% donor chimerism at 6-months post-UCBT. Simon's (optimum) 2-stage design was employed to test the null hypothesis that the donor engraftment rate is ≤ .30 (Ho: $P \leq .30$) versus the alternative that it is ≥ .65 (Ha: $P \geq .65$). Primary graft failure was defined as the failure to reach ANC > 500/mm^3 by day 42 or absence of donor hematopoiesis ≥ 10% in whole blood cells by day 100. The day 100 and 180 stopping rules for graft failure were not crossed. Cumulative incidence estimates of neutrophil recovery, platelet engraftment, acute GVHD grades II to IV, acute GVHD grades III to IV, and chronic GVHD were assessed using standard competing risk analyses with death as a competing risk [32]. Neutrophil recovery was defined as the first day of an absolute neutrophil count (ANC) > .5 × 10^9/L for 3 consecutive days not secondary to granulocyte infusions; and platelet engraftment as first day of platelet counts > 20 × 10^9/L or > 50 × 10^9/L for 7 consecutive days without transfusions as previously described [24]. Acute and chronic GVHD were graded per established criteria [33,34]. The cumulative incidence of infection (any, bacterial, viral, or fungal) was estimated from the start of the RIC regimen using death as a competing risk. The Kaplan-Meier method was used to describe overall survival (OS) and event-free survival (EFS) [35]. OS considered a patient death as the event and censored on the last date of contact, whereas EFS considered death, graft failure, or rejection as the events. Descriptive statistics of immune reconstitution were provided at 3, 6, 9, 12, and 24 months after UCBT. Analyses were conducted using the SAS System version 9.3 (Cary, NC).

RESULTS

Patient Characteristics

Patient demographics (N = 22) are shown in Table 1. The median age was 2.8 years (range, .3 to 8.0) and median weight was 13.7 kg (range, 5.3 to 26.9) on the day of transplantation. Most patients were males (59%) and Caucasians

Table 2
Donor Chimerism

UPIN	Day 30			Day 100			Day 180			1 yr			2 yr		
	T	L	M	T	L	M	T	L	M	T	L	M	T	L	M
1	>98	95	>98	>98	96	98	>98	98	>98	>98	>98	>98	>98	>98	>98
2	>98	>98	>98	95	78	90	92	72	89	92	84	92	88	88	86
3	10	88	10	63	91	58	Died day 170								
4	0	0	0	Autologous recovery day 30											
5	>98	>98	>98	>98	86	>98	>98	86	>98	>98	>98	>98	>98	>98	>98
6	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
7	>98	>98	>98	>98	90	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
8	>98	>98	>98	>98	83	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
9	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
10	>98	n/a	n/a	94	86	92	>98	>98	96	96	>98	92	91	>98	92
11	Engrafted day13, died day 25														
12	>98	>98	>98	>98	>98	>98	>98	98	>98	>98	>98	>98	>98	>98	>98
13	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	97	>98	>98	>98	>98
14	Graft failure, died day 24														
15	91*	8*	99*	Autologous recovery day 41											
16	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98			
17	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98			
18	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98			
19	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	96	>98			
20	87	71	41	87	55	61	88	58	96	87	66	97			
21	Engrafted day13, died day 27														
22	83	>98	68	>98	>98	>98	98	>98	97	Died day 258					

T indicates whole blood; L, CD3$^+$ fraction; M, CD15$^+$ fraction.
Data presented are %.
* = 0% on day 41, thus considered autologous recovery.

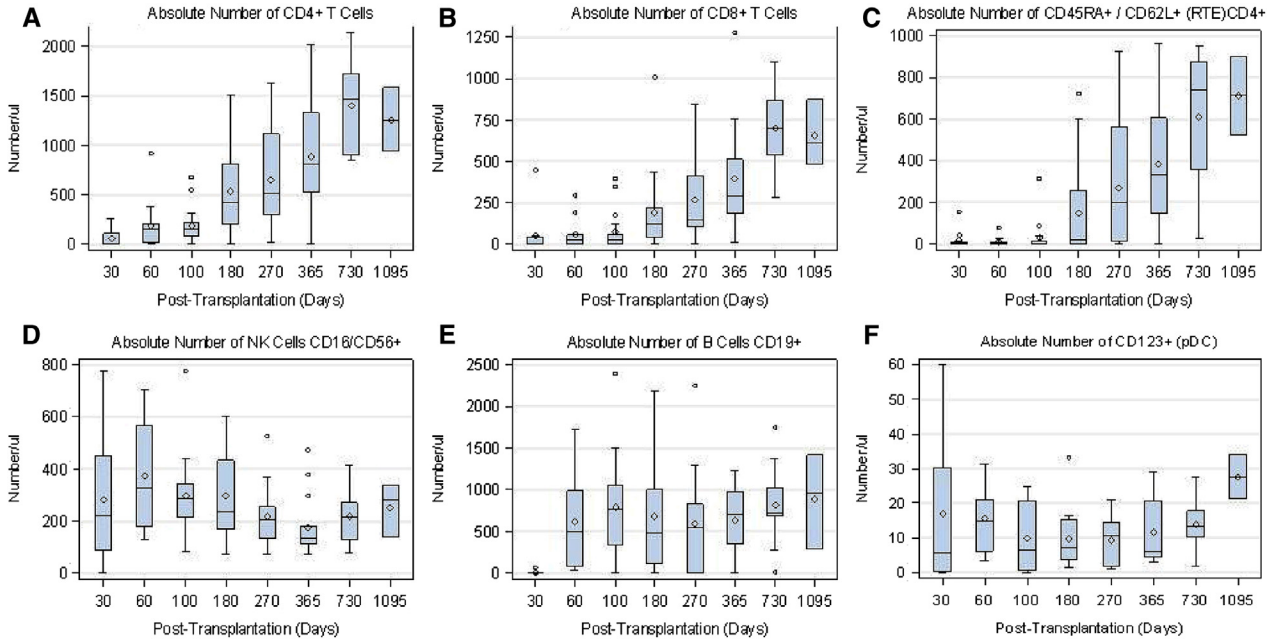

Figure 3. Immune Reconstitution. Absolute numbers of lymphocyte subsets: CD4+, CD8+, CD16,56+ (NK), CD19+, CD45RA+/62L+/CD4+ (RTE), CD123+(pDC). Box plots are shown serially over time for above immune parameters. The diamond in the box represents the mean, while the horizontal line in the box represents the median. The lower box represents the 25th percentile and the upper box represents the 75th percentile. Upper and lower whiskers represent +/− 1.5 times the interquartile range.

(68%), although 23% were African-American and 9% were Asian. Patients were diagnosed with immunodeficiency disorders (n = 9), IMD (n = 8), hemoglobinopathies (n = 4) and Diamond Blackfan anemia (n = 1). Twenty-one patients received their first HSCT, while 1 patient with thalassemia major was enrolled 15 months after rejecting a matched sibling bone marrow transplant (BMT) after MAC regimen.

Graft Characteristics

The median precryopreservation TNCC and $CD34^+$ doses were 11.1×10^7/kg (range, 5.9 to 31.3) and 4.35×10^5/kg (range, 1.71 to 8.1), respectively. Median infused TNCC and $CD34^+$ doses were 7.9×10^7/kg (range, 4.1 to 22) and 3.3×10^5/kg (range, .9 to 7.7), respectively. Using conventional HLA matching criteria to select UCB grafts, donor-recipient matching was 4/6 in 7 (31.8%), 5/6 in 11 (50%), and 6/6 in 4 (18.2%) patients. Review of HLA typing at allelic level including HLA-C revealed matches of 3/8 in 1 (4.5%), 4/8 in 3 (13.6%), 5/8 in 5 (22.7%), 6/8 in 8 (36.4%), 7/8 in 3 (13.6%), and 8/8 in 2 (9.1%). Thus, approximately 40% of recipients were mismatched for 3 or more loci at the allelic level. Donors were matched for gender in 12 (55%) and ABO blood type in 10 (45.5%) recipients. Major and/or bidirectional ABO mismatch were noted in 7 patient/donor pairs (31.8%).

Neutrophil and Platelet Engraftment

The cumulative incidence of neutrophil engraftment with donor cells by day 42 was 86.4% (95% confidence interval [CI], 65% to 100%) (Figure 1A). Nineteen patients engrafted at a median of 20 days (range, 12 to 28). Three patients experienced graft failure: primary graft failure in 1, who died on day 24 (unique patient identification number [UPIN] 14) with 100% donor cells before engrafting, and autologous reconstitution in 2. The latter 2 patients with autologous reconstitution (UPIN 15, UPIN 4) successfully underwent second transplantations 3.5 and 5 months later; with double UCBT after myeloablative busulfan (Bu)/cyclophosphamide (Cy)/antithymocyte globulin (ATG), and with matched unrelated donor BMT after thiotepa/ATG conditioning, respectively. Both are alive with donor engraftment. There have been no secondary graft failures. The cumulative incidence of platelet engraftment (50,000/uL) by day 180 was 72.7% (95% CI, 47.1% to 98.3%) occurring in a median of 48 days (range, 33 to 144) (Figure 1B).

Donor Chimerism

A primary objective of this study was to determine the proportion of patients engrafted with > 25% donor cells

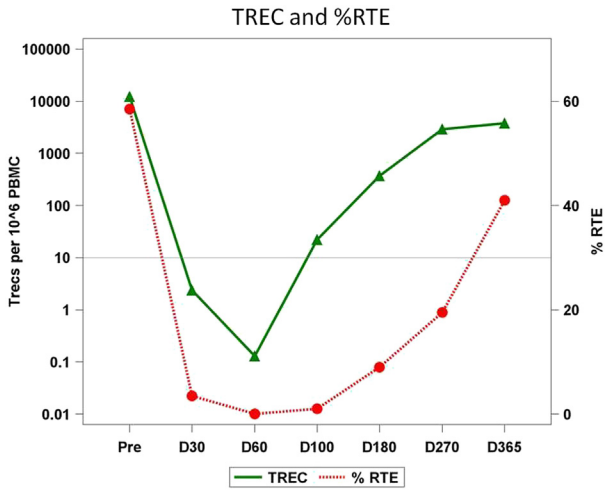

Figure 4. Thymopoiesis (TREC and RTE% reconstitution) TREC indicates T cell receptor excision circles; RTE, recent thymic emigrants (CD45RA+/CD62L+/CD4+); median TREC (filled triangle) and %RTE (filled circle) are connected serially over time.

Table 3
Details of Acute and Chronic GVHD

UPIN	Match Conventional A,B, DRB1	Match 8 Allele Hi res A, B, C, DRB1	AGVHD Max Skin Stage	AGVHD Max Gut Stage	AGVHD Max Liver Stage	Acute GVHD Grade Consensus	Chronic GVHD	cGVHD Site	AIHA	Rituximab	MSC	GVHD status at last follow-up	Immune suppression status at last follow-up
1	4/6	6/8	2	0	0	1	No		Yes	Yes (AIHA, EBV)		Resolved	Off IS
2	6/6	8/8	1	0	0	1	No					Resolved	Off IS
3	5/6	6/8	1	0	0	1	No					Resolved	Died
4	6/6	7/8	NE	NE	NE	NE	NE						
5	5/6	6/8	0	0	0	0	No						Off IS
6	5/6	5/8	3	0	0	2	Extensive	Skin	Yes	Yes (AIHA)		Ongoing	
7	5/6	5/8	0	0	0	0	Limited	Skin				Resolved	Off IS
8	4/6	4/8	0	0	0	0	Limited	Skin				Resolved	Off IS
9	5/6	6/8	1	0	0	1	Limited	Skin				Resolved	Off IS
10	5/6	6/8	0	1	0	2	Limited	Skin				Resolved	Weaning IS
11	4/6	5/8	0	0	0	0	NE						Died
12	4/6	4/8	0	0	0	0	Limited	Skin				Resolved	Off IS
13	5/6	7/8	2	0	0	1	Limited	Skin				Resolved	Off IS
14	4/6	4/8	NE	NE	NE	NE	NE						Died
15	4/6	3/8	NE	NE	NE	NE	NE						
16	4/6	5/8	1	3	0	3	No			Yes (EBV)		Resolved	Weaning IS
17	5/6	6/8	3	3	0	3	Extensive	Skin, gut			Yes	Ongoing	
18	5/6	6/8	1	0	0	1	Limited	Skin				Resolved	Off IS
19	6/6	6/8	3	0	0	2	No		Yes	Yes (AIHA)		Resolved	Weaning IS
20	5/6	7/8	0	0	0	0	Limited	Skin				Resolved	Weaning IS
21	6/6	8/8	0	0	0	0	NE						Died
22	5/6	5/8	3	4	0	3	No				Yes		Died

MSC indicates mesenchymal stem cells; NE, not evaluable; AIHA, autoimmune hemolytic anemia; IS, immune suppression.

6 months post transplantation. Eighteen patients survived > 6 months, 16 of whom achieved this endpoint. Specifically, whole blood donor chimerism > 90% was demonstrated in 15 of 16 patients at 180 days, 14 of 15 patients at 1 year, and 9 of 10 patients followed at 2 years. Similar levels of donor cell chimerism were seen in $CD15^+$ (15 of 16 at 180 days, 15 of 15 at 1 year, 9 of 10 at 2 years) and $CD3^+$ fractions (13 of 16 at 180 days, 13 of 15 at 1 year, 9 of 10 at 2 years) (Figure 2 and Table 2).

Immune Reconstitution

In engrafting patients, the median absolute lymphocyte and $CD4^+$ T cell counts at 3, 6, and 12 months were 1291 (382 to 3008) and 154 (0 to 678) (n = 17); 1405 (556 to 4470) and 418 (1 to 1507) (n = 16); and 2193 (333 to 5070) and 805 (6 to 2013) (n = 15), respectively (Figure 3). These values are similar to other pediatric reports using ATG-based rejection prophylaxis after myeloablative regimens [36-39].

At 180 days, normal, age-adjusted NK, B, and $CD4^+$ counts were found in 14 of 16 (87.5%), 11 of 16 (68.8%), and 5 of 16 (31.3%) patients respectively. At 1 year, normal, age-adjusted NK, B, and $CD4^+$ counts were seen in 13 of 15 (86.7%), 12 of 15 (80%), and 12 of 15 (80%) patients, respectively [40].

Nine of 15 engrafted and surviving patients with a minimum 1-year follow-up are off systemic immunosuppression,

Table 4
Treatment and Outcomes of EBV, CMV, Adenovirus Infections

UPIN	Infection	Treatment	Response	F/U (mo)	Outcome	Day Died	Cause of Death
1	EBV	Rituximab	Resolved	52.9			
2				45.5			
3	Adenoviremia	Cidofovir CTL	Resolved		Death	170	Hemolytic transfusion reaction
4	CMV viremia	Foscarnet, GCV	Resolved	AR			
5				40.4			
6				35.4			
7				34.8			
8	Adenoviremia	CMX001	Resolved	33.6			
9	CMV viremia	Foscarnet, GCV	Resolved	31.3			
10				30.9			
11	Adenoviremia	Cidofovir CMX001	Death	GF	Death	25	Disseminated adenoviral infection
12				26.6			
13	Adenovirus	Cidofovir	Resolved	26.4			
14	Adenovirus	Cidofovir CTL	Death		Death	24	Adenoviremia, Parainfluenza 3
15				AR			
16	EBV	Rituximab	Resolved	16.4			
17	Adenoviremia	CMX001	Ongoing treatment due to chronic GVHD	15.9			
18				15.5			
19				15.3			
20	Adenoviremia	Cidofovir		14.3			
21	CMV viremia	Foscarnet, GCV	Death		Death	27	CMV pneumonia
22	Adenoviremia CMV	Cidofovir CMX001	Death		Death	258	Acute GVHD, adenoviremia, CMV

F/U indicates follow-up; EBV, Epstein-Barr virus; CMV, cytomegalovirus; GVHD, graft-versus-host disease; CTL, trivirus (adenovirus, CMV, EBV)–specific cytotoxic T lymphocytes.

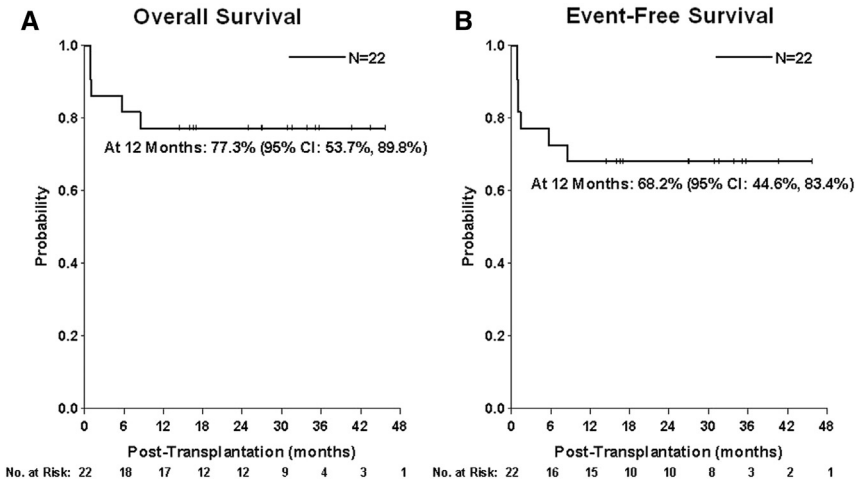

Figure 5. Kaplan-Meier estimate of (A) overall survival (B) event-free survival.

7 of whom have started immunizations. IgA levels were available for 14 of 15 patients followed at least 1 year and were normal in 11 patients, reflecting effective T cell–dependent isotype switching by B cells unaffected by possible intravenous immunoglobulin (IVIG) supplementation, and IgA levels were low in 3 patients. Two of the patients with low IgA had received rituximab, and 1 had extensive chronic GVHD.

Seven of 10 patients followed > 24 months maintain normal IgG levels without IVIG support.

Thymic Output

The appearance of recent thymic emigrants is a powerful marker of thymopoiesis, critical for acquiring a broad, naïve T-cell receptor (TCR) repertoire. It was quantitated by qPCR for TREC and independently by flow cytometry monitoring CD45RA and CD62L co-expressing CD4+ cells. There was a notable paucity of recent thymic emigrants and TREC+ cells in most patients within the first 100 days (Figure 4) followed by a steep rise at day 180, reflecting the resumption of thymopoiesis. The exceptions were UPIN 22, who died on day +258 with GVHD complications associated with profound lymphopenia and recurrence of pretransplantation CMV and adenovirus infections; UPIN 16, who had grade III acute GVHD; and UPIN 17, who had extensive chronic GVHD and had failed a prior myeloablative transplantation.

Antiviral Immunity

Sensitive BioPlex assay monitored the Duke cohort (n = 19) for virus-specific cytokines after in vitro challenge with the herpes family of viruses: CMV, HSV and VZV. The young age of this study cohort explains the relative low incidence of pretransplantation exposure: CMV (n = 5), HSV (n = 1), and VZV (n = 4). There were no VZV-specific responses detected, but only 1 seropositive patient was alive/studied beyond day 100. The 1 patient (UPIN 17) with positive pretransplantation HSV serology demonstrated specific cytokine response first at day 180 detectable by simultaneous IFNγ and GM-CSF secretion (93 pg/mL and 210 pg/mL, respectively), reflecting probable subclinical viral reactivation earlier.

There were 5 patients with pre-UCBT CMV exposure. Notably, 2 PID patients were viremic already pre-alemtuzumab: 1 died before day 30 because of CMV pneumonia (UPIN 21), and the other had autologous reconstitution (UPIN 4). Of the other 3 patients, UPIN 22 died of adenovirus and CMV with practically no circulating T cells (<10/μL) even at 6 months, whereas UPIN 9 (107 pg/mL) and UPIN 17 (77 pg/mL) both demonstrated detectable virus-specific IFNγ secretion and proliferative responses by day 100. Neither experienced recurrent CMV viremia. CMV-specific GM-CSF, IL-13, and TNFα secretion was also detected but was discordant between these 2 patients. In sum, anti-CMV and anti-HSV responses were detected only in seropositive patients and, when detected, there was no associated disease.

Graft-versus-Host Disease

The cumulative incidence of grades II to IV and III to IV acute GVHD by day 180 was 27.3% (95% CI, 8.7% to 45.9%) and 13.6% (95 CI, 0% to 27.6%), respectively (Figure 1C). The cumulative incidence of any chronic GVHD at 1 year was 40.9%

Table 5
Causes of Death

UPIN	Day Post Transplantation	Diagnosis	Cause of Death
3	170	Sanfilippo B	Hemolytic transfusion reaction
11	25	Krabbe	Disseminated adenovirus
14	24	Hurler	Adenoviremia, parainfluenza 3
21	27	Omenn syndrome	CMV pneumonia
22	258	PNP deficiency	Acute GVHD, adenoviremia, CMV

CMV indicates cytomegalovirus; GVHD, graft-versus-host disease; PNP, purine nucleoside phosphorylase; UPIN, unique patient identification number.

(95% CI, 19.3% to 62.5%). However, the cumulative incidence of extensive chronic GVHD was only 9.1% (95% CI, 0% to 20.8%) (Figure 1D). Of the 10 patients developing chronic GVHD, 8 had disease limited to skin and 2 had extensive (skin, n = 1; skin and gut, n = 1) disease. Autoimmune hemolytic anemia (AIHA) was noted in 3 patients (isolated AIHA, n = 2; AIHA with chronic GVHD, n = 1; cumulative incidence, 9.1% [95% CI, 0% to 20.8%]) and responded in all patients to steroid and rituximab therapy. Of these 12 patients (10 with classic chronic GVHD and 2 additional patients with isolated AIHA), 5 remain on chronic immunosuppression 14 to 35 months post transplantation. Two patients received mesenchymal stem cell treatment for steroid refractory GVHD. One (UPIN 17) has had a partial response, whereas the other (UPIN 22) died of acute GVHD, despite mesenchymal stem cell treatment (Table 3).

Infections

Cumulative incidence of infectious events at 1 year was high at 90.9% (95% CI, 67.5% to 100%) for any infection, 81.8% (95% CI, 57.9% to 100%) for viral, 13.6% (95% CI, 0% to 27.7%) for fungal, and 86.4% (95% CI, 63.1% to 100%) for bacterial infections. CMV, adenovirus, and EBV represented the majority of viruses encountered.

CMV

Six patients were at risk for CMV disease because of seropositivity and/or viremia (n = 2). Systemic therapy was required for 4 of them before transplantation (2 before and 2 after initiation of conditioning), 2 of whom (UPIN 21, UPIN 22), both with PID, developed CMV disease and died after transplantation.

Adenovirus

Three patients had adenovirus detected before transplantation (2 in stool alone; 1 in stool, urine, and blood), all of whom developed adenoviremia after transplantation, including 2 with adenoviral disease. Overall, adenoviremia developed in 8 of 22 patients (36%) after transplantation, all of whom received systemic antiadenoviral therapy. Two patients with inadequate response to cidofovir received banked third party virus-specific cytotoxic T lymphocytes. One of these patients cleared adenoviremia (UPIN 3), whereas the other patient did not and died on day 24 (UPIN 14). An investigational agent, CMX001, was used to treat 4 patients. One patient (UPIN 8) had clearance of adenoviremia and is off CMX001 treatment for > 2 years. Treatment is ongoing for UPIN 17 because of continued risk resulting from extensive chronic GVHD and ongoing severe lymphopenia. Two patients eventually died, 1 (UPIN 11) with fulminant adenoviral disease on day 25 and another (UPIN 22) with severe acute GVHD on day 258 with adenoviral and CMV infections. In all, 3 patients developed adenoviral disease that was associated with mortality (Table 4).

EBV

No patient had EBV infection before transplantation. After transplantation, 2 patients developed EBV infection. EBV viremia with mesenteric lymphadenopathy was noted in 1 patient (UPIN 1) on day +88. He responded to rituximab and is alive and well > 4 years after transplantation. Transient increase in EBV copy numbers in another patient (UPIN 16) responded to 2 doses of rituximab, and this patient is alive >1 year post transplantation.

AFB bacteremia

AFB bacteremia was noted in 2 patients: 1 with PID (UPIN 10) on day +123 and 1 with Krabbe disease (UPIN 12) on day +134. Both patients cleared their infections with multidrug treatment, and are well > 2 years post transplantation and off therapy.

Organ Toxicity

This regimen was well tolerated by patients, including those with significant pretransplantation comorbidities (Table 1). All patients developed some mucositis, which required parenteral nutrition and analgesia, but VOD, idiopathic pneumonitis, pericardial effusion, hemorrhagic cystitis, or neurologic events attributable to the regimen were not seen in anyone. One patient who had developed severe hemorrhagic cystitis during prior BMT needing approximately 3 months of platelet support, prostaglandin, and hyperbaric oxygen therapy was enrolled on this study for a second transplantation and tolerated the RIC regimen well without recurrence of cystitis. Several other patients had pretransplantation comorbidities, such as severe bronchiectasis, chronic pulmonary disease, enteroviral meningitis, and obstructive sleep apnea, and they tolerated the regimen well (Table 1).

Survival

Median follow-up of surviving patients is 30.89 months (range, 14.31 to 52.93). Overall and EFS at 1 year post transplantation is 77.3% (95% CI, 53.7% to 89.8%) and 68.2% (95% CI, 44.6% to 83.4%), respectively (Figure 5). Five patients died (Table 5), of whom 3 died before day +28 associated with viral infections. One immunodeficient patient died on day +258 from severe acute GVHD with concomitant adenoviral and CMV disease. Pretransplantation T cell deficiency with adenovirus and CMV detection in stool and urine, respectively, increased risk for reactivation post transplantation. One patient, a mixed chimera on day +100 (total chimerism 63%, 91% lymphoid, 58% myeloid) died on day +170 of a hemolytic transfusion reaction after empirically receiving donor type blood.

DISCUSSION

We report the outcomes of a prospective, pilot study evaluating a novel RIC regimen consisting of alemtuzumab, hydroxyurea, fludarabine, melphalan, and thiotepa in a pediatric cohort of 22 patients with NMD undergoing single unrelated donor UCBT. The majority of patients (19 of 22) were chemotherapy-naïve and 9 of 22 underwent transplantation for PID, most of whom arrived at transplantation with pre-existent viral infections (n = 7), reflecting their underlying T cell defects. The regimen was designed to decrease exposure to alkylating agents, while strengthening the immunosuppressive component (fludarabine and alemtuzumab) with the overall goal of facilitating donor engraftment while reducing nonhematopoietic organ toxicity. The cumulative incidence of neutrophil engraftment was 86%, comparable to myeloablative UCBT, and significantly higher than that reported after RIC UCBT in pediatric NMD (37.5% to 70.6%) [19,22,24,41-45]. Consistent donor engraftment was noted in patients belonging to diverse diagnostic groups, including those at a higher risk of engraftment failure.

Most patients achieved high levels of durable donor chimerism over the period of observation (median > 24 months). High-level (>90%) whole blood donor chimerism

was noted in 14 of 15 patients at 1 year and 9 of 10 patients at 2 years after transplantation. Whole blood chimerism remains > 80% and T cell chimerism remains > 65% in all engrafted surviving patients (n = 15). The durability of donor engraftment will be monitored long term. We are optimistic that donor chimerism will persist given the high (>50%) level of T cell chimerism in patients thus far. This level of chimerism is similar to our previously reported cohort of 159 patients with IMD undergoing myeloablative single UCBT [24]. All 5 patients with inherited transfusion-dependent anemias and both patients with hemophagocytic lymphohistiocytosis (HLH) maintain donor chimerism > 90% at the time of this report.

Engraftment after UCBT has been a challenge in hemoglobinopathies even with MAC regimens [19-21,46,47] because of factors such as compensatory marrow hyperactivity, alloimmunization from frequent transfusions, and the presence of an immunocompetent, chemotherapy-naïve host [48]. Retrospective registry analysis of mostly myeloablative UCBT in hemoglobinopathies showed primary graft failure as the predominant cause of failure, occurring in 27 of 51 patients with 2-year disease-free survival of 45%, despite an optimal cell dose [20]. With RIC regimens, engraftment is even more challenging. A high graft failure rate with a 2-year EFS of 50% was recently reported with Bu/Flu/alemtuzumab [21]. Similarly, in a multicenter study of sickle cell disease patients undergoing UCBT after a RIC regimen of alemtuzumab/Flu/Mel, 5 of 8 patients had autologous recovery (1-year EFS of 37.5%) [19]. In these patients, stringent criteria were used for HLA matching (≥5/6) and cell dose (median precryopreservation TNCC 6.4 × 10^7/kg; range, 3.1 to 7.6), and, thus, did not appear to affect the outcome, suggesting that perhaps the regimen needed to be intensified to ensure consistent engraftment. In the current study, the alemtuzumab/Flu/Mel backbone [18,19], which was reported with increased primary graft failure after single UCBT, was augmented with thiotepa and hydroxyurea, promoting engraftment. Thiotepa is a potent immunosuppressive and myelosuppressive drug and has been successfully used with melphalan [49-51]. It has promoted engraftment in murine transplantation models [52,53] as well as in clinical studies of T cell–depleted HSCT and UCBT [54-56]. Hydroxyurea can induce cycling of the stem cell pool, potentially sensitizing them to melphalan and thiotepa [57]. Alemtuzumab was administered distal to UCBT to maximize host immunoablation while reducing its impact on infused donor lymphocytes.

Of the 2 patients with autologous recovery, cord blood unit of 1 with cartilage hair hypoplasia lacked in vitro growth in colony forming unit assay at the time of thaw; the other with adrenoleukodystrophy received a unit with 3/8 allelic HLA matching that showed transient full donor engraftment before being replaced by host cells. Poor graft potency and immune rejection respectively may have contributed to graft failure [58,59]. Interestingly, both patients reconstituted host granulopoiesis within 4 to 6 weeks after transplantation, consistent with the notion that the preparative regimen is not fully myeloablative [60]. Both patients underwent successful second transplantations and are long-term survivors.

The 2 HLH patients are surviving with > 90% donor chimerism for > 2 years. TRM is a major barrier for success of conventional myeloablative regimens in HLH. In a CIBMTR retrospective HLH study (N = 91), mostly with matched unrelated donor BMT and Bu/Cy/VP16+/-ATG, day 100 TRM was 35% [61]. Alemtuzumab/fludarabine/melphalan has been demonstrated to decrease mortality in children with HLH receiving BMT with a 3-year survival of 92% compared with 43% for myeloablative Bu/Cy/ATG [14]. However, several patients needed donor lymphocyte infusions to sustain mixed chimerism in the RIC group. Excellent engraftment and survival was also reported for BMT from Europe [62], but data with UCBT are limited [63].

This regimen was well tolerated in terms of organ toxicity, as there was no cardiac, pulmonary, central nervous system, renal, or bladder toxicity recorded. Pericardial effusion, hemorrhagic cystitis, VOD, and multiorgan failure have been associated with Bu/Cy regimen [64]. Long-term follow-up will determine the impact of this regimen on late effects.

The major obstacle associated with this regimen is the high incidence of viral infections. Several factors are likely contributory. Clearly, patients with underlying T cell immunodeficiencies and pretransplantation viral disease were at extremely high risk for recurrent infections. Nevertheless, the alemtuzumab dose was relatively high, compared with some other pediatric studies [14,18,62], and post-UCBT exposure may have depleted most of the T cells accompanying the grafts, leading to delayed T cell reconstitution until thymic recovery [65]. Alemtuzumab dose de-escalation could be explored in the future to allow faster lymphocyte reconstitution; however, insufficient host immunoablation could jeopardize engraftment. Patients with pre-existing viral burdens should either have their transplantations delayed, when feasible, or use graft sources that would allow transfer of virus-specific memory. To further enable the use of cord blood donor grafts, patients could pre-emptively receive aggressive antiviral or adoptive immunotherapy post transplantation.

Taken together, this conditioning regimen is the first RIC regimen, to our knowledge, to result in consistent and durable donor engraftment in children undergoing UCBT for a variety of diseases, including patients with intact immunity and high risk for graft failure. It was well tolerated even in patients with significant pretransplantation comorbidities. Engraftment, survival, acute GVHD, and extensive chronic GVHD were not inferior to those previously reported after traditional myeloablative chemotherapy. Immune reconstitution and thymopoiesis were initially slow, but robust after 6 months in most patients. This regimen warrants further study and optimization for patients with nonmalignant conditions undergoing UCBT.

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