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Real Time Immunophenotyping of Leukocyte Subsets Early after Double Cord Blood Transplantation Predicts Graft Function

Jianqiang Li [1], Ian Nicoud [1], Joseph Blake [1], David Oliver [1], Emily Cox [1], Shelly Heimfeld [1], Filippo Milano [1,2], Suzan Imren [1], Colleen Delaney [1,3,*]

[1] *Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington*
[2] *Department of Medicine, University of Washington, Seattle, Washington*
[3] *Department of Pediatrics, University of Washington, Seattle, Washington*

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A B S T R A C T

Cord blood transplantation (CBT) recipients are at increased risk for delayed engraftment and primary graft failure, complications that are often indistinguishable early post-transplantation. Current assays fail to accurately identify recipients with slow hematopoietic recovery and distinguish them from those with pending graft failure. To address this, we prospectively examined the kinetics of immune cell subset recovery in the peripheral blood of 39 patients on days +7 and +14 after double-unit CBT (dCBT) by multiparametric flow cytometry analysis, which we term real-time immunophenotyping (RTIP). RTIP analysis at day +14 revealed distinctive patterns of reconstitution and, importantly, identified patients with slow hematopoietic recovery who went on to engraft. Strikingly, higher absolute numbers of circulating monocytes and natural killer cells at day +14 were predictive of engraftment, but only the absolute number of circulating monocytes was significantly correlated with time to engraftment. This is the first evidence that RTIP on patient peripheral blood mononuclear cells early after dCBT is technically feasible and can be used as a "signature" for predicting the kinetics of hematopoietic recovery. Furthermore, RTIP is a time- and cost-efficient methodology that has the potential to become a clinically feasible diagnostic tool to guide therapeutic interventions in high-risk patients; therefore, its utility should be evaluated in a large cohort of patients.

INTRODUCTION

Rapid and sustained donor cell engraftment following hematopoietic cell transplantation (HCT) is essential to reduce transplantation-related morbidity and mortality. Patients undergoing cord blood transplantation (CBT) are known to be at a higher risk for delayed hematopoietic recovery and graft failure [1-4] due to the low stem and progenitor cell content within individual cord blood (CB) units. Delayed hematopoietic recovery of both neutrophils and platelets in turn leads to a high risk of early transplantation-related mortality, especially compared with conventional graft donor sources [5]. To abrogate this risk, it is now common to infuse 2 individual CB units (double-unit CBT [dCBT]) at least 4/6 HLA-matched to the recipient to provide adequate numbers of hematopoietic stem and progenitor cells [6-8]. Whereas neutrophil recovery remains delayed compared with conventional donor transplantation, dCBT has resulted in an improved incidence of neutrophil and platelet recovery and reduced graft failure rates in older children and adults [9,10].

Identifying recipients with significantly delayed hematopoietic recovery but who will go on to have sustained donor engraftment has important clinical implications. However, to date it has not been possible to identify these patients, because neither routinely used daily complete blood count (CBC) analyses nor peripheral blood (PB) chimerism assays provide any useful information for predicting the kinetics of hematopoietic reconstitution early after CBT. Moreover, PB chimerism studies are quite expensive, time-consuming and technically challenging, especially in the case of lineage-specific cell-sorted PB chimerism, which often is infeasible in CBT recipients, who have very few circulating cells very early after transplantation.

In this study, we analyzed the PB of myeloablative dCBT recipients at 7 and 14 days after transplantation and found that this approach allowed for the quantitation and

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* Correspondence and reprint requests: Colleen Delaney, MD, MSc, Fred Hutchinson Cancer Research Center, Mailstop MD-B306, 1100 Fairview Ave N, PO Box 19024, Seattle, WA 98109.
E-mail address: cdelaney@fredhutch.org (C. Delaney).

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Table 1
Patient Demographics

Patient ID	Day of Engraftment	Conditioning Regimen	Sex	Age, yr	Weight, kg	Diagnosis	Day of Dominance	TNCs, $\times 10^7$/kg	CD34, $\times 10^5$/kg
1	12	Treo/FLU/2 Gy	F	26	46	ALL	11	6.9	5.2
2	13	CY/FLU/13.2 Gy	M	32	73	AML	56	7.5	3.9
3	14	Treo/FLU/2 Gy	F	41	63	MDS	21	6.7	4.9
4	15	CY/FLU/13.2 Gy	M	24	81	ALL	14	5.9	2
5	15	Treo/FLU/2 Gy	M	15	42	MDS	21	9.5	3.4
6	15	Treo/FLU/2 Gy	M	23	60	MDS	Mixed at day 28	7	5.5
7	15	CY/FLU/13.2 Gy	F	49	53	AML	55	7.5	2.7
8	16	Treo/FLU/2 Gy	F	21	45	ALL	Mixed at day 280	6.4	2.6
9	16	CY/FLU/13.2 Gy	M	45	92	ALL	14	5.4	2.4
10	16	CY/FLU/13.2 Gy	F	30	108	ALL PH$^+$	14	3.2	2.7
11	17	Treo/FLU/2 Gy	M	61	89	AML	21	7.4	4.3
12	17	Treo/FLU/2 Gy	M	46	90	AML	11	5.5	2.7
13	17	Treo/FLU/2 Gy	F	43	52	AML	14	6.3	4.1
14	17	CY/FLU/13.2 Gy	F	40	81	ALL	21	7.1	4.3
15	18	Treo/FLU/2 Gy	M	17	85	MDS	28	3.7	1.7
16	18	CY/FLU/13.2 Gy	M	13	70	ALL	15	4.5	1.5
17	18	CY/FLU/13.2 Gy	F	45	61	ALL	15	5.9	2.3
18	18	Treo/FLU/2 Gy	M	15	57	ALL	21	5.6	2.6
19	18	Treo/FLU/2 Gy	F	58	96	MDS	28	4.9	4
20	19	Treo/FLU/2 Gy	M	49	132	ALL	24	4.5	4
21	19	Treo/FLU/2 Gy	F	56	96	MDS	21	4.1	1.9
22	19	Treo/FLU/2 Gy	F	12	58	MDS	14	5.6	1.8
23	19	Treo/FLU/2 Gy	F	42	74	AML	28	4.4	4.9
24	20	CY/FLU/13.2 Gy	M	45	65	ALL	14	5.6	2.8
25	20	Treo/FLU/2 Gy	M	64	74	AML	22	6.2	3.6
26	20	Treo/FLU/2 Gy	F	49	81	AML	14	5.5	1.2
27	21	Treo/FLU/2 Gy	F	30	66	ALL	14	6.9	1.7
28	21	Treo/FLU/2 Gy	M	53	97	AML	14	3.5	1.6
29	23	Treo/FLU/2 Gy	F	38	51	ALL	Mixed @ day 424	7.2	2.2
30	25	CY/FLU/13.2 Gy	F	45	87	CML	21	5.2	2.2
31	28	Treo/FLU/2 Gy	M	24	88	MDS-RA	22	4.3	2.7
32	29	Treo/FLU/2 Gy	M	20	78	ALL	21	5.3	2.4
33	31	Treo/FLU/2 Gy	F	39	83	AML	202	6	1.7
34	32	Treo/FLU/2 Gy	F	42	62	AML	21	6.4	3
35	32	Treo/FLU/2 Gy	M	42	86	AML	180	5.1	4.4
36	36	CY/FLU/13.2 Gy	M	26	80	AML	21	4.4	1.8
37	51	CY/FLU/13.2 Gy	M	21	83	BIPHEN	31	4	1
38	PGF	Treo/FLU/2 Gy	M	55	102	MDS	Host at day 27	4.3	1.8
39	PGF	Treo/FLU/2 Gy	F	49	59	MDS/RAEB	NA*	7.5	2.5
40	PGF	CY/FLU/13.2 Gy	M	41	100	AML	Host at day 27	4.3	1.8

AML indicates acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; RA, refractory anemia; MDS, myelodysplastic syndrome; BIPHEN, biphenotypic leukemia; RAEB, refractory anemia with excess blasts; CY, cyclophosphamide; FLU, fludarabine; Treo, treosulfan; PGF, primary graft failure; NA, not available.

* Patient died of fungemia at day +28 before engraftment.

immunophenotypic characterization of circulating cells. The results of this assay then allowed for the identification of recipients with slow hematopoietic recovery based on the number of newly regenerated monocytes and natural killer (NK) cells in the PB at day +14 after transplantation. These findings provide the first evidence that a real-time immunophenotyping (RTIP) assay in PB is feasible within 2 weeks after dCBT in the majority of patients and allows tracking of the kinetics of hematopoietic recovery, and thus has the potential for use as an effective and cost-efficient routine diagnostic tool.

METHODS

Patients, Donors, and Conditioning

Forty consecutive patients who underwent myeloablative dCBT for a hematologic malignancy at the Seattle Cancer Care Alliance on investigational protocols (NCT00796068 and NCT01690520) between February 2013 and August 2016 were the subjects of this study. All patients provided written informed consent in accordance with Fred Hutchinson Cancer Research Center Institutional Review Board approval and the Declaration of Helsinki. Patient characteristics are summarized in Table 1. Typing for CB unit selection was performed at low resolution for HLA-A and -B and at high resolution for HLA-DRB1 in accordance with institutional practice. All patients received a graft matched at a minimum of 4 of 6 HLA antigens. Patients received 1 of 2 myeloablative preparative regimens: high-dose (1320 cGy) total body irradiation (TBI), fludarabine 75 mg/m^2, and cyclophosphamide 120 mg/kg, or treosulfan 42 g/m^2, fludarabine 150-200 mg/m^2, and low-dose TBI (200 cGy). All patients received cyclosporine plus mycophenolate mofetil for graft-versus-host disease (GVHD) prophylaxis. Granulocyte colony-stimulating factor was given post-transplantation until a stable absolute neutrophil count (ANC) of >2.5 $\times 10^9$/L was achieved, and then administered as needed to maintain an ANC >1.0 $\times 10^9$/L.

Real-Time Immunophenotyping

On days +7 and +14 after transplantation, 20 to 30 mL of heparinized PB was collected. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation (Histopaque; Sigma-Aldrich, St. Louis, MO), and the total number of mononuclear cells was counted using Turk's solution (0.01% crystal violet in 3% acetic acid). PBMCs (0.5 to 2 $\times 10^5$) were used to perform immunophenotyping by flow cytometry, which allowed lineage assessment of early regenerating cells using a few select markers that were sufficient to identify cell subsets and phenotypes. The following anti-human–specific monoclonal antibodies were used: CD45 PE.Cy5 (BD Biosciences Pharmingen, San Diego, CA), CD3 APC.Cy7 (BioLegend, San Diego, CA), CD14 APC (BD Biosciences, San Jose, CA), CD16 FITC (BD Biosciences), CD33 PE.Cy7 (BD Biosciences), and CD56 PE (Beckman Coulter, Brea, CA) (Supplementary Table S1). Cell events were collected with an LSR II flow cytometer (BD Biosciences), and flow data was analyzed using FlowJo software (TreeStar).

Chimerism Assessment

Analysis of donor chimerism was performed by the Seattle Cancer Care Alliance Clinical Immunogenetics Laboratory as described previously [11].