

7. Dreger P, Döhner H, Ritgen M, et al; German CLL Study Group. Allogeneic stem cell transplantation provides durable disease control in poor-risk chronic lymphocytic leukemia: long-term clinical and MRD results of the German CLL Study Group CLL3X trial. *Blood*. 2010;116(14):2438-2447.
8. Dreger P, Schnaiter A, Zenz T, et al. TP53, SF3B1, and NOTCH1 mutations and outcome of allotransplantation for chronic lymphocytic leukemia: six-year follow-up of the GCLLSG CLL3X trial. *Blood*. 2013;121(16):3284-3288.
9. Kovacs G, Robrecht S, Fink AM, et al. Minimal residual disease assessment improves prediction of outcome in patients with chronic lymphocytic leukemia (CLL) who achieve partial response: comprehensive analysis of two phase III studies of the German CLL Study Group. *J Clin Oncol*. 2016;34(31):3758-3765.
10. Hahn M, Böttcher S, Dietrich S, et al. Allogeneic hematopoietic stem cell transplantation for poor-risk chronic lymphocytic leukemia: dissecting immune-modulating strategies for disease eradication and treatment of relapse. *Bone Marrow Transplant*. 2015;50:1279-1285.
11. Rozovski U, Benjamini O, Jain P, et al. Outcomes of patients with chronic lymphocytic leukemia and Richter's transformation after transplantation failure. *J Clin Oncol*. 2015;33(14):1557-1563.
12. Ryan CE, Sahaf B, Logan AC, et al. Ibrutinib efficacy and tolerability in patients with relapsed chronic lymphocytic leukemia following allogeneic HCT. *Blood*. 2016;128(25):2899-2908.
13. Dreger P, Michallet M, Hoek J, et al. Ibrutinib for bridging to allogeneic hematopoietic stem cell transplantation in chronic lymphocytic leukemia and mantle cell lymphoma is safe and effective: first results of a survey by the Chronic Malignancy and the Lymphoma Working Parties of the EBMT [abstract]. *Blood*. 2016;128(22). Abstract 4657.

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To the editor:

Persistence of the losing cord blood unit following double cord blood transplantation: finding the unseen

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Double cord blood (CB) transplantation (dCBT) is an accepted treatment of patients with hematologic malignancies.^{1,2} In the vast majority of dCBT recipients, 1 unit emerges as the sole source of long-term hematopoiesis.³ As measured by standard clinical testing for chimerism (usually by short-tandem-repeat [STR] polymorphism), the “losing” unit usually becomes undetectable within the first month after transplantation.⁴⁻⁷ However, anecdotal cases in which the losing unit reemerges and contributes to hematopoiesis suggest long-term persistence of the losing unit in a quiescent state.

Although studies suggest an in vivo immune-mediated mechanism for single-donor dominance, there is no established evidence that the losing unit is definitively rejected.⁶⁻⁸ Moreover, limited data suggest that mixed-unit chimerism (the persistence of both donor CB units) may be associated with a potentially advantageous, enhanced graft-versus-tumor effect.⁹ Indeed, coexistence of semiallogeneic cells in the same individual is already a well-recognized natural phenomenon (microchimerism) resulting from bidirectional maternal-fetal exchange during pregnancy with persistence in respective individuals decades later.¹⁰ Naturally acquired microchimerism is found in healthy individuals, in organs and circulation, without apparent graft-versus-host reaction or graft rejection and has been associated with both health benefits and risks, pointing to functional capacity.^{10,11}

We hypothesized that a similar phenomenon also occurs after dCBT more commonly than would be suggested by estimates of mixed-unit chimerism by standard clinical measures, with “occult” presence of cells derived from the losing unit in the clinical setting of complete single CB unit dominance after dCBT. Using a sensitive technique developed for microchimerism analysis, we sought to determine whether very low levels of the losing unit could be identified.

Bone marrow (BM) and peripheral blood (PB) samples were collected at approximately days 28, 80, and 365 after transplant for clinical chimerism testing using a standard STR approach, and residual blood specimens were stored for research. PB mononuclear cells (PBMCs) were collected by density-based

centrifugation. Cell-lineage subsets (CD3⁺, CD33⁺, and CD56⁺) were isolated by fluorescence-activated cell sorting at the time of the blood draw.

HLA-genotyping data for subjects and CB units were reviewed to identify an HLA polymorphism unique to the losing CB unit to target using a panel of HLA-specific quantitative polymerase chain reaction (QPCR) assays. All assays were developed to detect the DNA equivalent of 1 cell in 20 000. The approach provides a standardized method for microchimerism testing that is highly sensitive and highly specific as previously described.^{12,13} DNA extracted from available BM, PBMC, and PB cell subset samples was tested with an HLA-specific QPCR assay unique to the losing unit. The total genome equivalent (GEq) tested median amounts were 9.5×10^4 (range, 1.1×10^4 to 47.0×10^4) for PBMCs, 11.6×10^4 (range, 1.5×10^4 to 16.7×10^4) for BM, and 2.1×10^3 (range, 2.5×10^2 to 3.3×10^4) for cell subsets.

Among consecutive patients undergoing a myeloablative CBT on protocols NCT00719888 and NCT00796068 between 2006 and 2014, we selected 14 patients who received a dCBT with either high-dose total-body irradiation (TBI)-based conditioning consisting of 1320 cGy TBI, fludarabine 75 mg/m², and cyclophosphamide 120 mg/kg (n = 8), or low-dose TBI-based conditioning with 200 cGy TBI, treosulfan 42 mg/m², and fludarabine 150 mg/m² (n = 6). All patients received cyclosporine plus mycophenolate mofetil for acute graft-versus-host disease (GVHD) prevention. Results were analyzed according to detection or not of the losing unit, and quantitative results summarized and expressed as DNA GEq number of cells from the losing unit per total GEq of DNA tested. All study activities were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board, and all participants provided written informed consent in accordance with the principles of the Declaration of Helsinki.

Subjects were selected according to demonstration of single CB unit dominance by clinical testing (n = 13, subjects), with 1 stable mixed unit-unit chimerism included (n = 1). The median age at transplant was 32 years (range, 10-62 years) and the median weight was 72.2 kg

	BMA			PBMC			CD3			CD33			CD56		
	D28	D80	D365	D28	D80	D365	D28	D80	D365	D28	D80	D365	D28	D80	D365
Patient 1							N	N	N	N	N	N	N		N
Patient 2				Y	Y		N	Y		N	N		N	N	
Patient 3	Y	N	N	Y	Y	N	N	N		Y	Y		Y	N	
Patient 4	N	N	N			N	N		N	N	N	N	N	N	N
Patient 5	Y	N	N				Y	Y	Y	Y	Y	N	Y	N	
Patient 6	Y	Y		Y	Y			Y	Y	Y	Y		Y	N	
Patient 7	Y	N	N				N	N	N	Y	N	N	N		N
Patient 8	N	N	N				Y	Y	Y	Y	N	Y	N	N	Y
Patient 9	N	N	N	N	N			N							
Patient 10	N			N	N	N									
Patient 11	Y	N	Y	Y	N		Y		N	Y	N		Y		
Patient 12				N	Y	N	Y	Y	N	N	Y	N	N	Y	N
Patient 13	N	N	N				N	N	N	N	N	N	N		N
Mc+	5	1	1	4	4	0	4	5	3	6	4	1	4	1	1
Mc+ (%)	50.0	11.1	12.5	57.1	57.1	0.0	40.0	50.0	33.3	54.5	36.4	14.3	36.4	14.3	16.7
Mc-	5	8	7	3	3	4	6	5	6	5	7	6	7	6	5
Non-eval	3	4	5	6	6	9	3	3	4	2	2	6	2	6	7

	Detection of the Losing Unit
	Reemergence of the Losing Unit
	No Sample or Data Available

Figure 1. Detection and reemergence of the losing unit in 13 patients with single-donor dominance in whole BM in PBMCs and in CD3, CD33, and CD56 cell subsets at days 28, 80, and 365 posttransplant. BMA, bone marrow aspiration; Mc-, negative microchimerism; Mc+, positive microchimerism; Non-eval, not evaluable.

(range, 32.5-107 kg). Pretransplant diseases included acute myeloid leukemia (n = 10), acute lymphoblastic leukemia (n = 3), and myeloproliferative disorder (n = 1). No patients experienced primary graft failure. For the overall study population (n = 14), the median time to neutrophil engraftment was 23 days (range, 14-44 days). Of the 13 subjects with single-unit dominance, absence of the losing unit by clinical STR testing was confirmed at a median time of 20 days (range, 11-28 days). The patient with clinical mixed-unit chimerism gave positive results as expected. QPCR testing results are summarized in Figure 1. Clinical characteristics of the 13 patients are shown in Table 1.

Results showed detection of the losing unit in 8 of 13 subjects (62%) for at least 1 time point and at least 1 sample type. In PBMCs, 4 of 7 (57%), 4 of 7 (57%), and 0 of 4 (0%) evaluable patients showed losing-unit detection at days 28, 80, and 365, respectively. In the CD3 subset, 4 of 10 (40%), 5 of 10 (50%), and 3 of 9 (33%) showed losing-unit detection at days 28, 80, and 365, respectively. In the CD33 subset, 6 of 11 (55%), 4 of 11 (36%), and 1 of 7 (14%) showed losing-unit detection at days 28, 80, and 365, respectively. In the CD56 subset, 4 of 11 (36%), 1 of 7 (14%), and 1 of 6 (17%) showed losing-unit detection at days 28, 80, and 365, respectively. In the BM samples, 5 of 10 (50%), 1 of 9 (11%), and 1 of 8 (13%) showed losing-unit detection at days 28,

Table 1. Patient characteristics

Patient no.	Sex	Age, y	Disease	HLA units to patient	HLA unit to unit	TNC, ×10 ⁷ /kg	CD34, ×105/kg	ANC, d	aGVHD grade	Relapse day*	Death day*
1	F	14	AML	5/6 and 5/6	4/6	7.9	4.5	14	3	1378	1471
2	F	21	ALL	4/6 and 4/6	3/6	8.1	3.0	26	2		138
3	F	50	AML	5/6 and 4/6	4/6	5.3	2.4	19	2		
4	F	24	CML	4/6 and 4/6	4/6	5.3	2.2	44	2		
5	M	39	AML	4/6 and 4/6	3/6	4.8	2.1	25	2		
6	M	62	AML	4/6 and 5/6	3/6	5.5	2.8	17	0	203	229
7	F	14	AML	5/6 and 5/6	4/6	3.8	—	19	2		
8	M	10	AML	4/6 and 4/6	4/6	14.3	—	25	3		
9	M	52	ALL	4/6 and 4/6	4/6	5.8	4.2	18	2		
10	M	25	AML	4/6 and 4/6	4/6	3.8	1.4	36	2		410
11	F	51	AML	5/6 and 5/6	4/6	4.2	1.7	20	2		
12	M	13	ALL	5/6 and 4/6	3/6	4.6	2.9	32	1		
13	M	42	AML	4/6 and 4/6	4/6	5.1	4.4	32	0		

The day of neutrophil engraftment was defined as the first of 3 consecutive days of an ANC of ≥0.5 × 10⁹/L or greater. aGVHD, acute GVHD; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ANC, absolute neutrophil count; CML, chronic myelogenous leukemia; TNC, total nucleated dose.

*Blank entries indicate that the event of interest did not happen in those patients.

80, and 365, respectively. Of samples that had losing-unit detection, the median losing-unit concentration, expressed and standardized per 10^5 total GEq, was 4.0 (range, 0.1 to 2.8×10^4) for P BMCs, 4.2 (range, 0.1 to 1.5×10^3) for BM, and 945.9 (range, 5.4 to 7.5×10^3) for cell subsets. Overall results of the current study indicate that the losing CB unit, undetectable by STR testing, was still identifiable in the majority of patients following dCBT (Figure 1).

To date, no factors reliably predict single-unit dominance or the coexistence of both units.^{5,7,8} Several reports have shown persistence for varying time periods of both CB units as stable, mixed-unit chimerism, with potential influence on clinical outcomes suggested by limited data.⁹ Dominance reversion is another uncommon situation in which cells of the predominating CB unit gradually decline giving up dominance to the other unit in the state of mixed-unit chimerism.⁹ The mechanism of dominance is multifactorial involving intrinsic features of the CB units, graft-versus-graft immune interactions mediated by T cells, as well as graft-versus-host immune interactions.^{4,6,8} In our study, the losing unit was still present even after the dominance phenomenon was well established clinically in the majority of dCBT patients. The presence of cells from the losing unit in the CD3 component suggests the possibility of dynamic regulation despite an apparent quiescent stable state.

Limited data suggest mixed-unit chimerism may confer a heightened graft-versus-leukemia benefit underscoring the need for better and more sensitive approaches to chimerism testing to further understanding of the biology and clinical implications of losing-unit persistence.⁹ Similarly, the use of this sensitive technique could be of clinical interest in other settings such as the HLA-mismatched stem cell microtransplantation¹⁴ and after haplo-cord transplantation¹⁵ where the persistence of donor cells is thought to be even more transitory.

Additional larger and prospective studies incorporating this type of approach are needed to assess losing-unit detection for correlation with clinical outcomes. Understanding the immunological interfaces for the patient, the dominant, and the “losing” unit may also yield insights into mechanisms of GVHD and graft-versus-leukemia responses.

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References

- Kurtzberg J, Prasad VK, Carter SL, et al; COBLT Steering Committee. Results of the Cord Blood Transplantation Study (COBLT): clinical outcomes of unrelated donor umbilical cord blood transplantation in pediatric patients with hematologic malignancies. *Blood*. 2008;112(10):4318-4327.
- Eapen M, Rubinstein P, Zhang MJ, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. *Lancet*. 2007;369(9577):1947-1954.
- Barker JN, Weisdorf DJ, DeFor TE, et al. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood*. 2005;105(3):1343-1347.
- Avery S, Shi W, Lubin M, et al. Influence of infused cell dose and HLA match on engraftment after double-unit cord blood allografts. *Blood*. 2011;117(12):3277-3285.
- Ramirez P, Wagner JE, DeFor TE, et al. Factors predicting single-unit predominance after double umbilical cord blood transplantation. *Bone Marrow Transplant*. 2012;47(6):799-803.
- Gutman JA, Turtle CJ, Manley TJ, et al. Single-unit dominance after double-unit umbilical cord blood transplantation coincides with a specific CD8+ T-cell response against the nonengrafted unit. *Blood*. 2010;115(4):757-765.
- Newell LF, Milano F, Nicoud IB, et al. Early CD3 peripheral blood chimerism predicts the long-term engrafting unit following myeloablative double-cord blood transplantation. *Biol Blood Marrow Transplant*. 2012;18(8):1243-1249.
- Milano F, Heimfeld S, Gooley T, Jinneman J, Nicoud I, Delaney C. Correlation of infused CD3+CD8+ cells with single-donor dominance after double-unit cord blood transplantation. *Biol Blood Marrow Transplant*. 2013;19(1):156-160.
- Hashem H, Lazarus HM. Double umbilical cord blood transplantation: relevance of persistent mixed-unit chimerism. *Biol Blood Marrow Transplant*. 2015;21(4):612-619.
- Nelson JL. The otherness of self: microchimerism in health and disease. *Trends Immunol*. 2012;33(8):421-427.
- Fugazzola L, Cirello V, Beck-Peccoz P. Fetal microchimerism as an explanation of disease. *Nat Rev Endocrinol*. 2011;7(2):89-97.
- Lambert NC, Erickson TD, Yan Z, et al. Quantification of maternal microchimerism by HLA-specific real-time polymerase chain reaction: studies of healthy women and women with scleroderma. *Arthritis Rheum*. 2004;50(3):906-914.
- Kanaan SB, Gammill HS, Harrington WE, et al. Maternal microchimerism is prevalent in cord blood in memory T cells and other cell subsets, and persists post-transplant. *Oncol Immunology*. 2017;6(5):e1311436.
- Guo M, Hu KX, Liu GX, et al. HLA-mismatched stem-cell microtransplantation as postremission therapy for acute myeloid leukemia: long-term follow-up. *J Clin Oncol*. 2012;30(33):4084-4090.
- van Besien K, Koshy N, Gergis U, et al. Cord blood chimerism and relapse after haplo-cord transplantation. *Leuk Lymphoma*. 2017;58(2):288-297.

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