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Letter
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Double Umbilical Cord Blood Transplantation in High-Risk Hematological Patients: A Phase II Study Focusing on the Mechanism of Graft Predominance

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Umbilical cord blood transplantation (UCBT) is an important alternative treatment modality for patients in need of an allogeneic stem cell transplantation but lacking a matched-sibling or unrelated stem cell donor.[1] However, the availability of a sufficiently sized cord blood unit may pose a problem for adult patients.[2] Double-UCBT (dUCBT) was developed in order to increase cell dose and allow adult patients lacking a sufficiently sized single cord blood unit (CBU) to proceed for transplantation.[3] Strikingly, it appeared that hematopoietic recovery originated only from a single CBU in the majority of the cases.[3] Gutman et al showed that alloreactive interferon-γ secreting CD8+ T-cells were present early after dUCBT.[4] Subsequently, we showed that CD4+ T-cells expand early after transplantation and CD4+ T-cell chimerism predicts for graft predominance.[5,6] Given these observations we hypothesized that HLA-class II specific CD4+ T-cells from the '*winner*' cord may be responsible for rejection of the '*loser*' cord. That hypothesis was retrospectively supported by demonstrating the presence of alloreactive, HLA-class II specific effector CD4+ T-cells in a limited number of patients.[7] In addition, alloreactivity was also detected towards primary leukemic cells when the HLA-class II allele mismatches were shared with de NE-CBU. In the present study, we set out to prospectively test this hypothesis in a phase II study.[7]

A total of 70 patients aged 18 to 70 years, with high-risk hematological disease, eligible for a UCBT were included in the HOVON 115 study, a prospective, multicenter phase II trial (EudraCT 2012-001188-55). The primary endpoint was the proportion of evaluable patients with activated class-II specific T-cells. Patients received reduced intensity conditioning consisting of cyclophosphamide 60 mg/kg; fludarabine 4 × 40 mg/m^2; TBI 2 × 2 Gy. Peripheral blood (PB) from patients were collected at 1 to 3 months after dUCBT for chimerism analysis and isolating PB-mononuclear cells (PBMCs). HLA-class II negative HeLa cells were retrovirally transduced with single HLA-DRA1/B1, HLA-DQA1/B1 or HLA-DPA1/B1 molecules, based on the mismatched allele from the NE-CBU. Post-dUCBT PBMCs were cocultured with irradiated single HLA-class II molecule transduced HeLa cells and with addition of a cytokine mixture to propagate T-cells.[7] Subsequently CD4+ T-cell activation was assessed by flow cytometry using antigens and effector markers, as described.[7] Alloreactivity was measured as 'fold increase (FI) [%CD137+/CD4+]' of reactivity toward HeLa cells transduced with mismatched HLA-class II alleles relative to reactivity towards the not-transduced 'empty' HeLa cell.

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Table 1

Patients Characteristics

Number of patients	68	
Diagnosis, n (%)		
Acute myeloid leukemia	34	50
Myelodysplastic syndrome	12	18
Acute lymphoblastic leukemia	4	6
Multiple myeloma	1	1
Chronic myeloid leukemia	1	1
Chronic lymphocytic leukemia	6	9
Non-hodgkin lymphoma	7	10
Myeloprolipherative disease	2	3
Severe aplastic anemia	1	1
Gender, n (%)		
Male	35	51
Female	33	49
Age, years, median (range)	57	(20–69)
Weight, kg, median (range)	74	(41–108)
WHO performance status, n (%)		
WHO 0	33	49
WHO 1	31	46
WHO 2	2	3
Unknown	2	3
HCT-CI Score, n (%)		
0	42	62
1	19	28
2	3	4
3	3	4
4	1	1
CMV status patient, n (%)		
negative	26	38
positive	42	62

CMV = cytomegalovirus, HCT-CI = hematopoietic cell transplant comorbidity index, WHO = World Health organization.

Table 2

Graft Characteristics

Cord blood unit	Winner	Loser
Number of infused, median (range)		
Nucelated cells (10^6/kg)	26.0 (0.3–62.4)	26.3 (0.2–73.7)
Viable CD34+ cells (10^3/kg)	34.9 (0.0–140)	40.5 (0.1–430.0)
Viable CD3+ cells (10^3/kg)	1340.0 (51.7–6955.0)	1339.7 (45.6–11070.0)
HLA-class II mismatch, median (range)		
Recipient	2 (0–6)	1 (0–6)
Winner	–	2 (0–5)

CMV = cytomegalovirus, HCT-CI = hematopoietic cell transplant comorbidity index, WHO = World Health organization.

Two patients were not transplanted because of withdrawal of consent and rapid progression of the underlying disease, and were therefore excluded from all analyses. Patients' characteristics of the remaining 68 patients are described in Table 1. The majority of the patients were diagnosed with acute myeloid leukemia or myelodysplastic syndrome with a low WHO performance- and HCT-CI score. There were no significant differences in the number of infused nucleated-, viable CD34+ and CD3+ cells between winner- and loser-CBU (Table 2; P0.81, P0.19 and P0.27, respectively; Mann-Whitney test). One-year overall survival (OS) and progression free survival (PFS) from 1st UCBT were 61% (95% confidence interval (CI) 49–72%) and 60% (95% CI 47–70%) respectively (Supplementary Fig. 1, Supplemental Digital Content, http://links.lww.com/HS/A42), with median follow-up of 48.2 months (range, 3.2–65.7 months) among the 36 patients still alive. Non-relapse mortality at 12 months was 28% (standard error (SE), 5%) and the cumulative incidence of relapse (or progression) at 12 months was 12% (SE, 4%). Engraftment was observed in 62 out of 68 (91%) patients with a median time to neutrophil recovery $>0.5 \times 10^9$/L of 31 days. Primary graft failure occurred in 3 patients. Complete single donor chimerism was established in 62 out of 68 (91%) patients. HLA-class II mismatches between the 2 transplanted cords were present in 42 out of these patients. In vitro tests could be performed for 20 of the 42 patients for which an HLA-class II–transduced HeLa cell was available. T-cell numbers increased 6.7-fold (median; range, 0.2–192.4) after the HLA-class II allele-specific propagation of the post-dUCBT PBMC samples. In 18 out of the 20 (90%) patients tested, alloreactive CD4+ T-cells toward 1 or more HLA-class II mismatched alleles of the NE-CBU were detected, which is much higher than the 50% which we used for the sample size calculation. Of note, our in-vitro expansion protocols may have favored CD4+ T-cells over CD8+ T-cells as a result of the use of HLA-class II antigens for expansion. In total, CD4+ T-cell alloreactivity toward 24 of 26 (92%) mismatched alleles was detected, including 9 out of 9 (100%) for DR, 10 out of 12 (83%) for DQ and 5 out of 5 (100%) for DP alleles early (median: 1 month; range: 1–3 months) after double cord blood transplantation (Fig. 1). CD4+ T-cell alloreactivity towards DR, DQ and DP alleles were 9.3-fold (range, 3.4–44.0), 8.9-fold (range, 1.7–37.8) and 7.3-fold (5.7–31.4) increased as compared to reactivity towards the not transduced 'empty' HeLa cell, respectively. A positive CD4+ T-cell response towards '*control*' matched alleles of the PD-CBU were observed in 7 out of 26 (27%) tested alleles. The magnitude of the CD4+ T-cell response was significantly higher towards mismatched alleles as compared to matched alleles, median FI 8.9 (range, 1.7–44) vs median FI 1.7 (range, 0.2–10.1; $P < .01$; Mann–Whitney test), respectively.

Double UCBT was initially developed to allow transplantation for adult patients lacking a sufficiently sized single CBU. It resulted in less graft failure in comparison to single UCBT, but with only 1 cord surviving in most cases. We recently reported the presence of alloreactive HLA-class II specific CD4+ T-cells early after transplantation.[7] In the current study, we set out to prospectively test the hypothesis whether alloreactive HLA-class II specific CD4+ T-cells from the dominant UCB unit expand early after and transplantation and might be responsible for rapid rejection of the "loser" unit. Here we show the presence of such T-cells in 18 out of 20 patients and alloreactivity towards 24 of 26 tested alleles, further suggesting an important role for alloreactive CD4+ T-cells from the '*winner*' cord in rapid rejection of the '*loser*' cord. Rapid immunization of alloreactive CD4+ T-cells by mismatched HLA-class II antigens might occur by circulating class II expressing cells after infusion of both UCB's. Strong expression and/or a high number of class II + cells might then evoke a rapid CD4+ T-cell response. This hypothesis may, in part, be supported by findings by Purtill et al,[8] who showed that the NE-CBU total nucleated cell dose (as a surrogate of a high number of class II expressing cells) was significantly associated with neutrophil engraftment. Type and number of class II mismatches might also play a role. Avery et al[9] showed that a better HLA match between both units is associated with a mixed unit chimerism, but it is unclear how class II mismatches behave in that respect as compared to class I mismatches. Given the relatively low number of patients in our previous[7] and present study, we could not clarify the relative value of class I vs class II mismatched in this respect. However, given the matching criteria

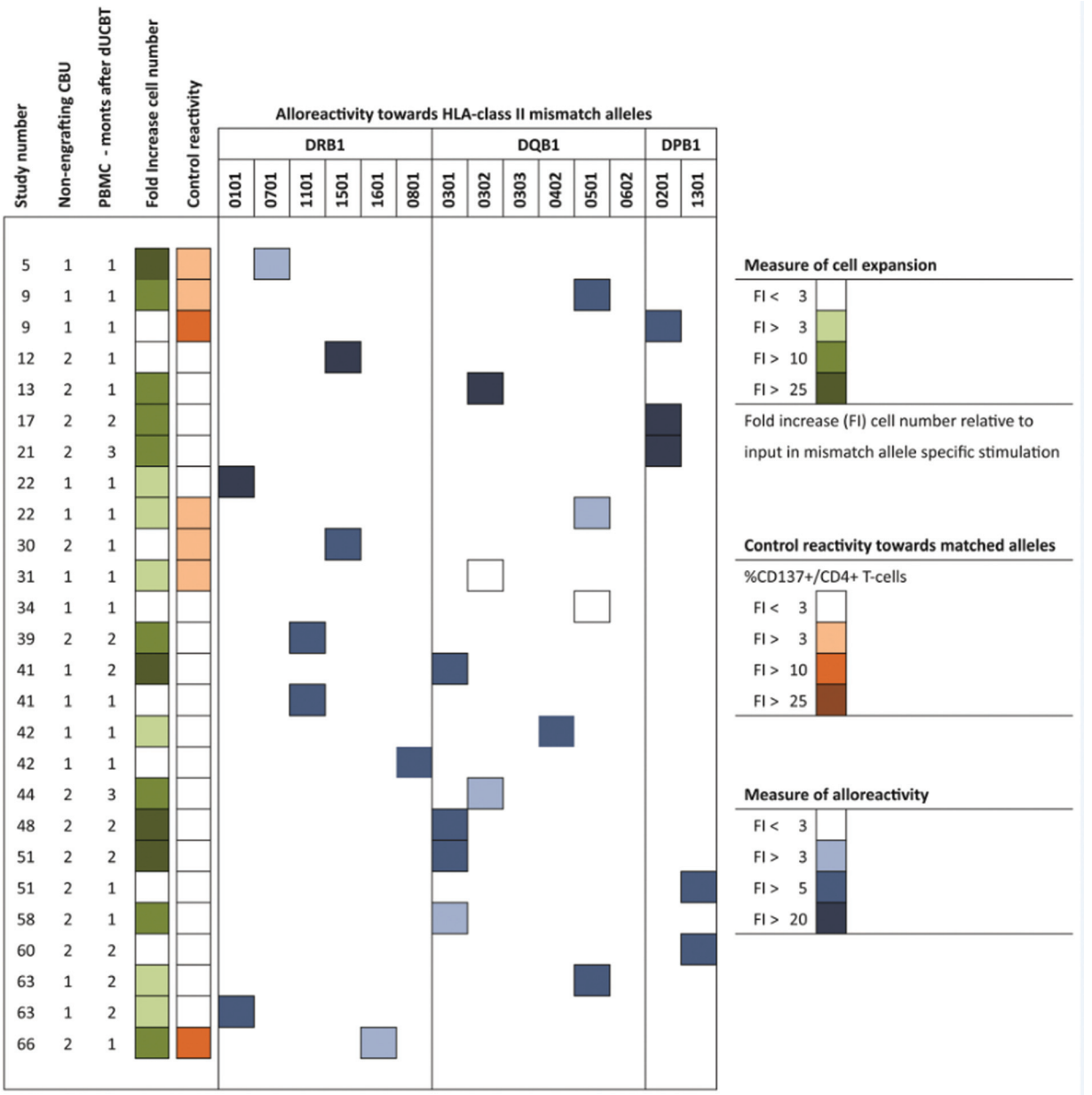

Figure 1. Twenty-six PBMC samples obtained from 20 patients at 1 to 3 months after dUCBT were propagated towards mismatched HLA-class II alleles of the non-engrafting CBU in a T cell-Hela cell coculture, using a panel of Hela cell line, each transduced with a single HLA-class II allele. T cell expansion of individual cultures at day 21 in presented as Fold increase cell number. Propagated T cells were subsequently tested for (allo)reactivity towards that same mismatched, and matched HLA-class II alleles. The response was quantified by CD137 upregulation on CD4+ T-cells [%CD137+/CD4+] and presented for the alloreactivity as 'Fold increase (FI) [%CD137+/CD4+]' of reactivity towards HeLa cells transduced with mismatched HLA-class II alleles relative to reactivity towards the not-transduced 'empty' HeLa cell. The level of T cell expansion, Control reactivity (towards matched alleles) and alloreactivity response is presented in a 4-color-grading scales.

for UCB-search, class II mismatches are more abundant than class I mismatches.

The graft-vs-graft T-cell alloreactivity towards mismatched precursor cells may result in unit predominance but also into enhanced graft vs leukemia (GvL),[10] especially if the alloreactive CD4+ T-cell response is also directed towards a mismatched allele present on recipient tissue and recipient leukemia cells.[7] The relevance of HLA-class II was indirectly supported recently by Christopher et al,[11] who showed that loss of HLA-class II expression is a mechanism by which relapsing tumors may escape immune surveillance. Immunization of CD4+ T-cells might occur by mismatched class II presenting leukemic cells and antigen presenting cells (APCs) in tissues, in which these cells are abundantly present accompanied by contributing inflammatory signals. Such tissues may include the spleen, lung, and intestine, in which large numbers of antigen presenting cells are present. Thus, a CD4+ T-cell mediated graft-vs-graft alloreactivity may involve and enhance GvL in the dUCBT setting and thereby provide a possible explanation for the relatively low relapse rate associated with dUCBT.[10] Encouragingly, the cumulative incidence of relapse in was only 12% (SE, 4%) in our cohort of high risk patients. Of note, although dUCBT may be associated with a stronger GvL in comparison to a single UCBT, dUCBT is often accompanied by a higher

incidence of acute and chronic GvHD with similar survival outcome.[12,13] However, patients with a high risk of relapse, such as those with detectable minimal residual disease, might benefit from a dUCBT.[14,15]

In conclusion, this study prospectively showed a rapid and strong alloreactive CD4+ T-cell response towards the majority of class II mismatches present and available for analysis in 24 of 26 testes alleles. These observations might explain, at least in part, the mechanism behind single unit dominance and the lower incidence of relapse after dUCBT in comparison to single UCBT. However, predicting graft predominance remains a challenge and further research into that direction is needed. In addition, the question to what extent such alloreactive T-cells are present after haplo-identical alloHSCT is of importance. While class II mismatches are abundantly present in haplo-identical alloHSCT, the stronger immunosuppression, especially anti-thymocyte globulin and/or cyclophosphamide, needed to prevent GvHD might blunt the CD4+ T-cell response with possible loss of GvL activity. Currently we are conducting a study evaluating the presence of these classes II specific T-cells after haplo-id alloHSCT.

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