CD8+ T-cell reconstitution in recipients of umbilical cord blood transplant and characteristics associated with leukemic relapse

Natacha Merindol,1,3,5 Martin A. Champagne,2,3,6,8 Michel Duval,2,3,4,7 and Hugo Soudeyns1,3,5,7

1Unité d’immunopathologie virale, 2Service d’hémato-oncologie, 3Groupe de recherche en transplantation et immunologie du sang de cordon (GRETISC), and 4Laboratoire d’immunologie du sang de cordon, Centre de cancérologie Charles-Bruneau, CHU Sainte-Justine, Montreal, QC H3T 1C5, Canada; 5Departments of Microbiology & Immunology, 6Medicine, and 7Pediatrics, Faculty of Medicine, Université de Montréal, Montreal, QC H3C 3J7, Canada; 8Héma-Québec, St-Laurent, QC H4R 2W7, Canada.

Correspondence: Hugo Soudeyns, Unité d’immunopathologie virale, Centre de recherche du CHU Sainte-Justine, 3175 Côte Sainte-Catherine, Room 6735, Montreal, QC H3T 1C5, Canada; e-mail: hugo.soudeyns@recherche-ste-justine.qc.ca.

The online version of the article contains a data supplement.
ABSTRACT

Recipients of umbilical cord blood (UCB) transplantation (UCBT) face a high risk of morbidity and mortality related to opportunistic infections (OI) and leukemic relapse. To understand the molecular basis of these UCBT-related complications, the characteristics of UCB-derived antigen-specific CD8⁺ T cells were examined in a group of pediatric UCBT recipients. As compared with the UCB graft inoculum and the late post-UCBT period (12-36 months), declining clonal diversity of UCB-derived CD8⁺ T cells specific for the Melan-A26-35 A27L peptide and high frequencies of PD-1-expressing CD8⁺ T cells were observed in the first 3 months post-UCBT, a period during which OI are most frequent. The CD8⁺ T cell compartment predominantly comprised CD45RA⁺ CCR7⁻ terminally-differentiated effector-memory T cells until 6 months post-UCBT, at which time the polyfunctionality of antigen-specific CD8⁺ T cells was re-established. Finally, the frequency of PD-1⁺ CD8⁺ T cells was significantly higher in subjects who subsequently experienced leukemic relapse. This study informs the biological properties of UCB-derived CD8⁺ T cells and provides a rationale for the characteristics of UCBT in terms of immune reconstitution and OI. These results also suggest that elevated frequency of PD-1⁺ CD8⁺ T cells could be associated with leukemic relapse in pediatric UCBT recipients.
INTRODUCTION

Umbilical cord blood (UCB) transplantation (UCBT) is commonly used to treat a variety of hematologic diseases in children.\textsuperscript{1,2} Reduced incidence of graft versus host disease (GvHD) compared to BM transplantation (BMT) was documented following UCBT.\textsuperscript{3} UCBT was associated with slow engraftment, delayed CD\textsubscript{8}+ T cells recovery, high incidence of graft failure, and high incidence of opportunistic infections (OI) in the first 3 to 6 months post-transplantation.\textsuperscript{4-6} Approximately 20\% of pediatric UCBT recipients will also experience leukemic relapse, a frequency similar to that observed in BMT.\textsuperscript{7}

In the context of BMT, graft-derived CD8\textsuperscript{+} T cells are known to shield the graft recipient against OI and leukemic relapse.\textsuperscript{8,9} UCBT involves the transfer of multiple cell subtypes into the recipient, including large amounts of CD8\textsuperscript{+} T lymphocytes. While the presence of UCB-derived CD8\textsuperscript{+} T cells in the graft inoculums can provide some degree of protection from OI and leukemic relapse to UCBT recipients,\textsuperscript{10,11} recent studies suggest that these cells might only mediate minimal levels of antiviral immunity.\textsuperscript{12,13} The relative inefficacy of UCB-derived CD8\textsuperscript{+} T cells to prevent OI and their failure to prevent leukemic relapse in some patients is not well understood.

Based on cell-surface expression of CD45RA and CCR7, CD8\textsuperscript{+} T cells can be classified as naïve, central memory, effector memory, and terminally differentiated.\textsuperscript{14} Using peptide-MHC tetramers, antigen-specific CD8\textsuperscript{+} T cells isolated from UCB were shown to be mostly naïve and clonally diversified. In addition, they produced significantly less IFN-γ but were more likely to achieve terminal differentiation and less likely to exhibit
bifunctional properties (IFN-γ production and cytolytic activity) as compared with T lymphocytes derived from adult blood. In theory, these properties are compatible with low incidence of GvHD and high incidence of OI. However, little is known regarding the fate of CD8+ T cells once they are transplanted into UCBT recipients.

The objective of this study was to characterize the reconstitution of CD8+ T cells in pediatric UCBT recipients to address a series of questions: a) Do UCB-derived CD8+ T cell clones persist during immune reconstitution? b) To what extent do they participate in the composition of the reconstituted T cell repertoire? c) How does clonal diversity of antigen-specific CD8+ T cells develop? d) How do they differentiate in terms of naïve-effector-memory phenotype and expression of T cell exhaustion markers? e) How does their polyfunctionality, which reflects their efficacy to control chronic viral infections, evolve in the post-UCBT period? We examined CD8+ T cells specific for the HLA-A2-restricted Melan-A26–35 A27L peptide (A2/Melan-A) as a model for the reconstitution of an antigen-specific repertoire. A2/Melan-A-specific T cells represent one of the only preimmune T cell repertoires that can be studied in humans. Here we provide evidence that some UCB-derived CD8+ T lymphocytes persisted in paediatric recipients in the first 6 months post-UCBT. Their clonal diversity reached a nadir at 3 months post-UCBT. At this point, the CD8+ compartment was predominantly comprised of terminally-differentiated T cells and both CD4+ and CD8+ subsets were enriched in cells expressing programmed death 1 (PD-1). From 6 months post-UCBT, the CD8+ T cell repertoire was progressively restored with a naïve, diversified, and polyfunctional population. Finally, higher frequencies of PD-1+ CD8+ T cells were detected at 2 and 6
months post-UCBT in subjects who subsequently experienced leukemic relapse, suggesting that PD-1 expression on CD8+ T cells could be associated with this complication.

PATIENTS, MATERIALS, AND METHODS

Study subjects. This study was approved by the Institutional Review Board of Centre Hospitalier Universitaire (CHU) Sainte-Justine, Montreal, Quebec, Canada, and was conducted according to the standards described in the Helsinki Declaration of 1975, as revised in 2000. Full informed consent was obtained from all study participants and their parents or legal guardians. Pediatric HLA-A2+ subjects who underwent UCBT for the treatment of hematologic or neoplastic diseases were enrolled at CHU Sainte-Justine between October 2004 and December 2009 (n = 26). Median age at study entry was 5.42 years (range = 0.33-17.0 years). UCB and venous blood samples (2-10 ml) were obtained from the graft inoculums and from transplanted subjects at 1, 2, 3, 6, 12, 18, 24, and 36 months post-UCBT. UCB units used in transplant procedures were obtained from national and international UCB repositories. All UCB units used in these patients were HLA-A2+ and had a 4/6 or greater HLA-A, HLA-B, and HLA-DRB1 allele-level match with the recipient. UCB mononuclear cells (UCBMC) and PBMC were isolated on Ficoll-Hypaque gradients (Amersham Biosciences, Uppsala, Sweden) and cryopreserved in 90% v/v FBS (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% v/v DMSO. All subjects received myeloablative conditioning regimens, including total body irradiation (TBI; 1200 cGy fractionated in 8 doses) or busulfan (0.8 mg per kg, fractionated in 16 doses). GvHD prophylaxis consisted of cyclosporin A and
corticosteroids. Subjects were infused with 2 mg per kg anti-thymocyte globulin (ATG) (Thymoglobulin®; Sangstat, Mississauga, Ontario, Canada) on days -2, -1, +1, and +2 relative to the UCBT procedure. Granulocyte-colony stimulating factor (G-CSF) was also administered starting on day +1. Two subjects were lost to follow-up. Transplant procedure was detailed in Dalle et al. Clinical characteristics of study subjects are summarized in Table 1.

**Generation of T cell microcultures.** Frozen UCBMC and PBMC were thawed and cultured for 24 h in RPMI 1640 supplemented with 20% v/v FBS. Cells were seeded at 2000 cells per well in 96-well round-bottom plates and were co-cultured with 8 x 10^4 irradiated (300 Gy) 221.A2 cells (EBV-transformed B lymphoblastoid cell line expressing HLA-A2*0201 pulsed with the HLA-A2-restricted modified Melan-A_{26–35} A27L peptide (ELAGIGILTV) (New England Peptide, Gardner, MA). Alternatively, to achieve polyclonal expansion for repertoire analysis, cells were pulsed with 1 μg per ml PHA (Sigma-Aldrich, St. Louis, MO). T cell microcultures were maintained in RPMI 1640 supplemented with 10% v/v FBS, 50 UI per ml recombinant human (rh)IL-2 (Hoffmann-La Roche, Mississauga, Ontario, Canada), and 10 ng per ml rhIL-7 (R&D Systems, Minneapolis, MN). Medium was replenished twice a week.

**Isolation and immunophenotyping of Melan-A-specific T cells.** To detect Melan-A-specific CD8^+ T cells in UCBMC, PBMC and T cell microcultures, biotinylated HLA-A2 monomers were refolded around the Melan-A_{26–35} A27L peptide (CANVAC Core Facility, Montreal, Quebec, Canada) and were tetramerized using PE-conjugated
Extravidin (Sigma-Aldrich) or allophycocyanin (APC)-conjugated streptavidin (BD Biosciences, San Jose, CA). Cells were incubated for 15 min at 37°C with 0.3 µg peptide-MHC tetramers per 1 x 10^6 cells. Monoclonal antibodies (mAbs) for cell-surface staining were then added, and cells were incubated for 30 min at room temperature. Tetramer-specific CD8^+ cells were sorted at low pressure in 100% v/v FBS using a FACSVantage SE equipped with CellQuest software (BD Biosciences). Compensation and logical gates were set with single fluorochromes. Lymphocytes were gated according to forward and side scatter. Melan-A-specific T cells were gated based on expression of CD8. Samples were defined as tetramer positive when: a) A2/Melan-A^+ CD8^+ T cells represented more than 0.01% of CD8^+ T cells, as this frequency corresponded to maximum background of negative controls; and b) mean fluorescence intensity between tetramer positive and negative populations was > 1 log10 (Figure S1). Analysis of cell-surface markers was performed using FCS Express Version 3 software (De Novo Software, Los Angeles, CA). Tetramer-sorted T cells were directly seeded at 10-50 cells per well in 96-well round-bottom plates and were co-cultured with 8 x 10^4 irradiated (300 Gy) 221.A2 cells per well in RPMI 1640 supplemented with 50% v/v FBS, 50 U/l per ml rhIL-2, 10 ng per ml rhIL-7 and 1 µg per ml Melan-A_{26-35} A27L peptide. Medium was changed every 2 to 3 days, and 2 x 10^4 irradiated 221.A2 cells per well were added once a week. The functionality of Melan-A sorted T cells was tested after 2 weeks in culture (see below). Immunophenotyping was performed using the following mAbs: PE- or APC-conjugated anti-CD8 (RPA-T8), PE-Cy7-conjugated anti-CCR7 (3D12), and FITC-conjugated anti-CD45RA (HI100) (BD Biosciences). CD8^+ T cells were defined as naïve (CD45RA^+CCR7^+), central memory (CM; CD45RA^-CCR7^+), effector memory (EM;
CD45RA−CCR7), and terminally-differentiated effectors (EMRA; CD45RA+CCR7). Separately, PBMC were stained with FITC- or PE-conjugated anti-PD-1 (MIH4) and APC-conjugated anti-CD8 (RPA-T8) (BD Biosciences).

**Intracellular staining.** Tetramer-sorted microcultures (2 × 10⁵ cells) were incubated with APC-conjugated anti-CD107a mAb (H4A3) (BD Biosciences) and 221.A2 cells in the presence of 10 μg per ml Melan-A26-35 A27L peptide at 37°C in RPMI 1640 medium supplemented with 10% v/v FBS. Culture medium alone was used as negative control. Following 1 h stimulation, 10 μg per ml brefeldin A and 6 μg per ml monensin (BD Biosciences) were added. Cells were incubated for 6 h, washed with FACS buffer, stained with PerCP-Cy5.5-conjugated anti-CD8 mAb (RPA-T8) (BD Biosciences), and fixed and permeabilized using Cytofix/Cytoperm reagents (BD Biosciences). Cells were then stained with PE-conjugated anti-IFN-γ (4S.B3) and FITC-conjugated anti-IL-2 (MQ1-17H12) mAbs (BD Biosciences) for 30 min at 4°C and washed twice in Perm/Wash buffer (BD Biosciences). Viable CD8⁺ T cells were gated for analysis. Background frequencies measured in absence of cognate peptide were subtracted from each subpopulation.

**Cytotoxicity testing.** Cytolytic activity of tetramer-sorted, Melan-A-stimulated T cell microcultures was measured using a conventional 4 h [⁵¹Cr] release assay. ⁵¹Cr-labeled targets were 221.A2 cells pulsed with Melan-A26-35 A27L. Effector to target (E:T) ratio was 10:1 (2 × 10³ target cells per well). Each microculture was tested in duplicate with Melan-A26-35 A27L peptide and 221.A2 cells alone.
**Amplification and sequencing of TCR β-chain transcripts.** Total mRNA was extracted from pellets of tetramer-sorted cells (500-2000 cells) using the Picopure system (Molecular Devices, Sunnyvale, CA). TCR β-chain V region (TRBV) mRNA, including its third complementarity-determining region (CDR3), was reverse transcribed and resulting cDNA was amplified by PCR (40 cycles) using universal primers, as described previously.\(^{22}\) PCR products were subcloned into the TOPO TA cloning vector (Invitrogen) and sequenced unidirectionally using an ABI 3730xl automated sequencer (Applied Biosystems, Carlsbad, CA; Plateforme de séquençage et de génotypage des genomes, Centre de recherche du CHUL, Quebec, Quebec, Canada). Identification of TRBV and TCR β-chain J region (TRBJ) segments and determination of CDR3 length and amino acid sequences were performed using IMGT/V-QUEST and confirmed manually.\(^{23}\) Clonal diversity and dominance were estimated using Simpson’s diversity index (Ds).\(^{24}\)

**Statistical analysis.** Normality of data distribution was tested using the D’Agostino and Pearson omnibus normality test and the Kolmogorov-Smirnov test. Data were expressed as the mean and standard error or median and interquartile range (IQR). Where appropriate, statistical significance in between-groups comparisons was assessed using the Mann-Whitney \(U\) test, the Wilcoxon signed-rank test, or one way ANOVA and Bonferroni’s multiple comparison test. Relationships between variables were tested using Spearman’s rank correlation test. \(p\) values < 0.05 were considered statistically significant.
All analyses were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

**RESULTS**

*Ex vivo* detection of Melan-A-specific T cells in UCB and UCBT recipients.

Frequencies of A2/Melan-A+ CD8+ T cells were assessed in UCB grafts and in PBMC samples obtained at 1 to 36 months post-UCBT using peptide-MHC tetramers (Figure 1A). A median frequency of 0.3% of A2/Melan-A+ CD8+ T cells was detected in UCB grafts. No A2/Melan-A+ CD8+ T cells were detected at 1, 2 or 3 months post-UCBT, consistent with low numbers of CD4+ and CD8+ T cells (Figure 1A). A2/Melan-A+ CD8+ T cells were then detected from 6 to 36 months post-UCBT. Relative frequencies of A2/Melan-A+ CD8+ T cells were correlated with total CD4+ and CD8+ T cell counts in recipients over the entire post-UCBT period (p = 0.0005 and p = 0.0008, respectively). This indicates that the relative abundance of antigen-specific CD8+ T cells depends on the reconstitution of the CD4+ and CD8+ T cell compartments.

Detection of Melan-A-specific T cells post-UCBT after polyclonal stimulation. The early post-UCBT period is characterized by very low numbers of circulating CD8+ T cells.25 To circumvent this limitation, PHA stimulation was used to expand T cells independent of antigenic specificity.26 After 7-10 days of expansion, A2/Melan-A+ CD8+ T cells were detected at a frequency > 0.01% in 9 of 10 grafts and 8 of 9 study subjects at 1 month post-UCBT (Figure 1B). These cells were also detected in 2 of 6 and 1 of 5 patients at 2 months and 3 months post-UCBT, respectively, and in the majority of
subjects at 6 to 36 months post-UCBT (Figure 1B). The frequency of A2/Melan-A⁺ CD8⁺ T cells at 3 months post-UCBT was significantly lower than those observed in graft inoculums and in subjects at 1, 6, and 36 months post-UCBT (p < 0.05). Moreover, A2/Melan-A⁺ T cell frequencies in the graft inoculum and at 1 month post-UCBT were correlated (p = 0.0252). These results suggest that some of the A2/Melan-A-specific CD8⁺ T cells present in the graft inoculum persisted in the recipient during the early post-UCBT period and then progressively disappeared.

Evolution of T cell repertoire diversity following UCBT. TCR β chains expressed by A2/Melan-A⁺ CD8⁺ T cells were amplified, subcloned and sequenced. As previously reported, A2/Melan-A⁺ T cells in the graft inoculum were polyclonal (median Ds = 0.90) (Figure 2A-2B and S2). Diversity declined significantly at 2 and 3 months post-UCBT (p < 0.05) to increase again at 6 months, albeit to a level significantly lower than that observed in the graft (p < 0.05). TCR β chain diversity reached graft-comparable levels at 12 months post-UCBT (Figure 2B). Changes in diversity were also reflected at the level of CDR3 length: it was normally distributed in 6 of 6 graft inoculum but became highly biased at 1 to 3 months post-UCBT, consistent with repertoire oligoclonality (Figure S2). From then on, the distribution of CDR3 length progressively normalized, with 2 of 11 (18.2%) subjects exhibiting Gaussian profiles at 6 months, 4 of 9 (44.4%) at 12 months, 3 of 7 (42.9%) at 18 months, and 3 of 5 (60.0%) at 24 months post-UCBT (Figure S2). No common TCR β chain clonotypes were identified between graft inoculums and UCBT recipients. In contrast, two waves of persistent clonotypes were observed in the post-UCBT period. The first wave (« early clonotypes ») emerged
between 1 to 3 months and was detected until 6 months post-UCBT, whereas the second wave (« late clonotypes ») started to emerge at 6 to 12 months and persisted between 6 and 36 months post-UCBT (Figure 2A). It is unlikely that early clonotypes were of recipient origin, as 100% donor chimerism was observed at 3 and 6 months post-UCBT in most study subjects. These results are consistent with rare UCB-derived T cells transiently persisting in the recipient up to 6 months after UCBT, followed by progressive repletion of T cell repertoire diversity via thymopoïesis.

**Phenotypic reconstitution of total and A2/Melan-A^+ CD8^+ T cells following UCBT.**

Expression of CD45RA and CCR7 was monitored in total CD8^+ T cells and A2/Melan-A^+ CD8^+ T cells during the post-UCBT period (Figure 3). Total and A2/Melan-A^+ CD8^+ T cells were mostly naïve in graft inoculums (Figure 3 and S3). Low frequencies of naïve T cells (1.2%, 2.5%, and 3.0%) and high frequencies of EMRA (82.5%, 76.5%, and 70.4%) were found in total CD8^+ T cells at 1, 2 and 3 months post-UCBT (Figure 3). CD8^+ T cells were also comprised of 15.6%, 19.5%, and 22.5% of EM and 0.7%, 1.5%, and 4.1% of CM at 1, 2 and 3 months post-UCBT. At 6 months, naïve T cells emerged at a median frequency of 15.6% of total CD8^+ T cells, whereas 43.4% continued to exhibit an EMRA phenotype. At that time, A2/Melan-A^+ CD8^+ T cells were detected and a median of 54.8% of them were naïve. From 12 to 36 months post-UCBT, the vast majority of CD8^+ T cells were naïve and the second population in terms of relative abundance was EMRA. At all time points, the proportion of naïve cells was significantly higher in A2/Melan-A^+ CD8^+ T cells than in total CD8^+ T cells (p < 0.0313) (Figure 3 and data not shown). Interestingly, naïve CD8^+ T cells were observed at a frequency of
22.6% at 1 month post-UCBT in subject P11, a 5 month-old patient who had already achieved a high TCR repertoire diversity at 1 month post-UCBT (Figure 2A). These results indicate that the vast majority of CD8+ T cells display a terminally differentiated phenotype in the first 6 months post-UCBT.

**Functional reconstitution of A2/Melan-A+ CD8+ T cells following UCBT.** Polyfunctionality is the hallmark of protective T cell responses against viruses and cancer.27 PBMC from UCBT recipients were expanded *in vitro* for 7-10 days in the presence of IL-2, IL-7 and Melan-A26–35 A27L peptide, sorted with A2/Melan-A tetramers, and used in functional assays. Following stimulation with cognate peptide, no significant differences in expression of IFN-γ, IL-2, CD107a (monofunctional, bifunctional, polyfunctional) or cytolytic activity were observed in A2/Melan-A+ CD8+ T cells between graft inoculums and samples obtained at 6 to 36 months post-UCBT (p > 0.05) (Figure 4 and S4). A2/Melan-A+ CD8+ T cells were not retrieved in sufficient numbers to perform these analyses at 1 to 3 months post-UCBT. These results suggest that CD8+ T cells isolated from UCB were capable of expressing combinations of differentiated functions in response to stimulation with cognate peptide, as were those isolated from the peripheral blood of UCBT recipients at 6 to 18 months post-transplant.

**Evolution of PD-1+ T cell frequency during the post-UCBT period.** PD-1, an inhibitory receptor of the CD28 family, is highly expressed on dysfunctional CD8+ and CD4+ T cells during chronic viral infections.28 Since the first 3 months post-UCBT are associated with high risk of OI,5 with functional immunodeficiency, and with terminally-
differentiated CD8+ T cell phenotypes (Figure 3), frequencies of PD-1+ CD4+ and PD-1+ CD8+ T cells were assessed in 18 study subjects, 10 healthy children (aged 3-36 months), and 5 healthy adults (Figure 5). PD-1 was expressed at very low levels in UCB grafts (1.3% and 1.0% of CD8+ and CD4+ T cells). Major changes were observed in the mean frequency of PD-1+ T cells during the post-transplant period (p < 0.0001). These disparities were accounted for by a remarkable increase in frequency of PD-1+ CD8+ and PD-1+ CD4+ T cells at 2 and 3 months post-UCBT compared to graft inoculums and to samples obtained at 6-36 months post-UCBT (p < 0.05) (Figure 5A-5B and S5A). The frequencies of PD-1-expressing CD8+ T cells and CD4+ T cells at 3 months post-UCBT were also significantly higher than those observed in healthy adults and healthy children (p < 0.0001), consistent with recent reports.29,30 Frequencies of PD-1+ CD8+ T cells were inversely correlated with CD8+ and CD4+ T cell counts (p = 0.0077, r = -0.4124, and p = 0.0052, r = -0.4338, respectively), highlighting the association between lymphopenia and impaired CD8+ T cell function in the first months following UCBT (Figure 5C-5D). Overall, median frequencies of PD-1+ T cells were higher in the CD4+ than in the CD8+ T cell compartment (p = 0.0039) and the frequency of PD-1+ CD8+ T cells was correlated with the frequency of PD-1+ CD4+ T cells throughout the follow-up period (p < 0.0001, r = 0.7665) (Figure S5B-S5C). Finally, the normalized median fluorescence index (MFI) of PD-1 in CD8+ T cells and CD4+ T cells were not higher in the first months post-UCBT than later during follow up (p > 0.05) (Figure S5D-S5E). These results are consistent with UCB CD8+ T cells undergoing massive oligoclonal expansion while acquiring a skewed exhausted phenotype following transfer into the recipients.
**PD-1 expression is associated with leukemic relapse.** Eight study subjects experienced leukemic relapse during follow-up (« relapers »), of whom 6 relapsed > 6 months post-UCBT (Figure S3). There were no significant differences in the naïve-memory phenotype of CD8+ T cells at 2 or 3 months post-UCBT in relapsers compared to nonrelapsers (Figure S3). However, there was a trend towards higher frequencies of EM T cells ($p = 0.0823$) and significantly lower CD4+ T cells counts in relapsers at 6 months ($p = 0.0376$) (Fig. 6A, S6). There was no significant difference in total CD8+ T cell counts between relapsers and nonrelapsers (Figure 6B). Finally, the frequency of PD1+ CD8+ T cells was significantly higher in relapsers than in nonrelapsers at 2 and 6 months ($p = 0.0079$ and $p = 0.0364$, respectively) (Figure 6C-6D and S5G). The frequency of PD-1+ CD4+ T cells was also higher in relapsers at 6 months, but this difference was not statistically significant (Figure S5F and S5H). Taken together, these results suggest that a high frequency of PD-1+ CD8+ T cells in UCBT recipients during the early post-transplant period could be associated with subsequent leukemic relapse.

**DISCUSSION**

UCB is increasingly being used as an alternative source of hematopoietic stem cells for the treatment of various blood disorders in cases where a suitably-matched donor is not readily available. However, clinical outcomes associated with UCBT differ from those observed following BMT, particularly in terms of GvHD, graft failure, and OI. Leukemic relapse can occur following UCBT, particularly in patients with poor immune reconstitution. To explore the cellular and molecular basis of these outcomes, we examined the reconstitution of antigen-specific T lymphocytes in a group of pediatric
UCBT recipients. In recent years, several groups have examined the reconstitution of T cells specific for CMV,\textsuperscript{32} or for leukemia-associated antigens such as PR1\textsuperscript{33} and WT1.\textsuperscript{34} In contrast, the present study was largely focused on the repertoire of T cells that recognize the Melan-A\textsubscript{26–35} A27L peptide in the context of HLA-A2 (A2/Melan-A) because these cells represent one of the only preimmune T cell repertoires that can be studied in humans, particularly when the number of cells that can be obtained from study subjects is limited.\textsuperscript{15,17,18} Consistent with these previous reports, clonally diversified A2/Melan-A+ CD8+ T cells were reproducibly detected in UCB graft inoculums. Using peptide-MHC tetramers, these cells were not detectable \textit{ex vivo} in the peripheral blood of UCBT recipients at 1, 2 or 3 months post-UCBT, an important « window of susceptibility » to OI. When PHA stimulation was used to compensate for low numbers of total CD8+ T cells in the early post-transplant period, A2/Melan-A+ CD8+ T cells were revealed in the majority of subjects at one month post-transplant, and in one third and one fifth of subjects at 2 and 3 months, respectively. These cells exhibited reduced levels of clonal diversity and skewed distribution in CDR3 length as compared with graft inoculums and the late post-transplant period (\textit{i.e.} \textgeq 6 months post-UCBT), reflecting the oligoclonality of the antigen-specific T cell compartment. These results are consistent with previous reports on reconstitution of TCR repertoire diversity based on analysis of total CD8+ T cells.\textsuperscript{35} There were no instances of overlap between the clonotypic profiles observed in the graft inoculum and those observed post-UCBT. UCB T cells are highly polyclonal,\textsuperscript{15} possibly limiting our ability to track clonal persistence between UCB graft inoculums and UCBT recipients. ATG, which was administered to study subjects as part of the conditioning regimen, could also have contributed to the sudden drop in clonal diversity.
observed following UCBT. In contrast, the temporary persistence of T cell clonotypes was observed between 1-6 months post-UCBT. Chimerism results confirmed that these cells were of donor origin (data not shown). Taken together, these results suggest that the A2/Melan-A-specific T cell repertoire present in the graft inoculum was not preferentially mobilized following transfer into the UCBT recipient, but that some of these cells transiently persisted in UCBT recipients up to 6 months post-transplant, followed by reconstitution of the T cell repertoire via thymopoiesis. Therefore, our results are compatible with the existence of a « hole » or « through » in the T cell repertoire between 2 and 3 months post-UCBT, with little overlap between a first wave derived from homeostatic expansion of T cells contained within the UCB graft inoculum and a second wave generated by rising thymic output.

Most A2/Melan-A+ CD8+ T cells and CD8+ T cells present in graft inoculums expressed a CD45RA+ CCR7+ naïve cell surface phenotype. In contrast, the vast majority of CD8+ T cells displayed a terminally differentiated CD45RA+ CCR7− phenotype in the first 6 months post-UCBT, consistent with previous in vitro studies showing that UCB T cells differentiated mostly into EMRA following stimulation, and with observations in adult UCBT recipients and children who underwent BMT.15,36,37 The EMRA phenotype has been associated with shorter T cell t1/2 and is concordant with the short-term persistence and progressive disappearance of graft-derived CD8+ T cells during the first 3-6 months post-UCBT, followed by the emergence of clonally-diversified naïve T cells through thymopoiesis. Interestingly, naïve CD8+ T cells were observed at a frequency of 22.6% at 1 month post-UCBT in subject P11, who exhibited very high TCR β chain diversity as
early as 1 month post-UCBT and clonotypic persistence until 18 months. This suggests that naïve T cells and the persistent T cell clonotype observed in subject P11 resulted from de novo generation of thymus-derived T cells rather than homeostatic expansion of graft-derived T cells, compatible with unusually active thymopoietic processes in this 5 months-old patient. Quantification of the levels of TCR rearrangement circles (TREC) in isolated T cell subsets and other measurements which could be used to strengthen this hypothesis were not performed in the present study as a result of strict limitations in biological sample size.\textsuperscript{38,39}

There were no significant differences observed between A2/Melan-A\textsuperscript{+} CD8\textsuperscript{+} T cells isolated from the graft inoculums and from UCBT recipients at 6-18 months post-transplant in terms of IFN-\(\gamma\), IL-2, CD107a secretion and cytolytic activity following stimulation with cognate peptide. The T cell polyfunctionality profile is a key indicator of the efficacy of antiviral and antitumoral cell-mediated immune responses.\textsuperscript{27} Hence, although this profile was not determined at 1-3 months because of limitations in biological sample size, these results indicate that the functionality of antigen-specific CD8\textsuperscript{+} T cells was fully reconstituted in UCBT recipients at and beyond 6 months post-UCBT. Had larger amounts of cells (>10\textsuperscript{7}) from graft inoculums and recipients been available, it would have been of high interest to compare these results with the evolution of another biologically relevant pre-immune repertoire in the context of UCBT, such as the CD8\textsuperscript{+} T cell population specific for the A2/CMVpp65 tetramer.\textsuperscript{18}
PD-1, an inhibitory receptor of the CD28 family, is highly expressed on dysfunctional CD8$^+$ and CD4$^+$ T cells during chronic viral infections.\textsuperscript{28} PD-1 contributes to the dampening of antiviral and anti-tumoral immunity and is associated with clonal exhaustion and diminished effector functions.\textsuperscript{40-42} During the early post-transplant period, the frequency of PD-1$^+$ CD8$^+$ T cells was positively correlated with that of PD1$^+$ CD4$^+$ T cells, and inversely correlated with CD8$^+$ and CD4$^+$ T cell counts, highlighting the association between lymphopenia and impaired CD8$^+$ T cell function in the first months following UCBT. Gallez-Hawkins \textit{et al.} reported no increase in the frequency of PD-1$^+$ CD8$^+$ or CD4$^+$ T cells in the first months following BMT.\textsuperscript{43} In the present study, all UCBT recipients experienced viral infections in the first 3 months post-transplantation, making it impossible to assess the impact of OI on PD-1 expression or the impact of PD-1 expression on susceptibility to viruses. Additional studies will be needed to verify if these differences are related to a) the source of the graft inoculum (UCB versus BM); b) the use of ATG; c) the cellular dose; d) the respective ages of the transplant recipients; and/or e) the differential incidence of GvHD and OI, which could not be assessed in the context present study. PD-1 expression is preferentially observed in EM and EMRA T cells.\textsuperscript{44,45} Overall, our results are consistent with UCB CD8$^+$ T cells undergoing massive oligoclonal expansion while acquiring a skewed terminally-differentiated and exhausted phenotype following transfer into the recipients. These results provide a rationale for the increased susceptibility to OI and comparatively reduced incidence of acute GvHD observed in UCBT recipients.\textsuperscript{5} These conclusions would be strengthened by a comparison between UCBT and BMT, which should be addressed in future studies. PD-1 expression and/or the interaction between PD-1 and its ligands PD-L1 and PD-L2 could
represent attractive targets for interventions aiming to reduce the incidence of OI following UCBT.

Finally, 8 subjects experienced leukemic relapse, including 6 who relapsed later than 6 months post-UCBT, a time at which the polyfunctionality profile of antigen-specific CD8+ T cells had already completely recovered. Although the frequency of PD1+ CD8+ T cells in the first 3 months post-UCBT was high in every subject, it was significantly higher in relapsers than in nonrelapsers at 2 and 6 months. Lower CD4+ T cell counts and higher frequency of EM CD8+ T cells frequency were also observed in relapsers, potentially highlighting the importance of cooperation between CD4+ and CD8+ T cells for generation, maintenance and/or efficacy of the graft versus leukemia effect. Lower frequencies of CD28- CD8+ and EMRA CD8+ T cells following allogeneic stem cell transplantation were previously associated with a greater risk of relapse of underlying hematologic malignancies.37 To our knowledge, the present study is the first to report an association between the frequency of PD-1+ CD8+ T cells and subsequent leukemic relapse. This should be confirmed in larger series and additional studies will be required to determine whether increased frequency of PD-1+ CD8+ T cells is the underlying cause or merely a consequence of the ensuing relapse. However, these results suggest that this parameter could be used as a potential prognostic factor for leukemic relapse in pediatric UCBT recipients.
ACKNOWLEDGEMENTS

N.M. is a Ph.D. candidate at Université de Montréal and this work is submitted in partial requirement for the Ph.D. The authors wish to thank Martine Caty, Serge Sénéchal, and Samira Mezziani for expert technical assistance, and Claude Perreault and Françoise Le Deist for critical reading of the manuscript. Supported by grants from le Fonds de la recherche en santé du Québec (FRSQ), Héma-Québec, and Fondation Centre de cancérologie Charles-Bruneau (M.A.C., M.D., H.S.). N.M. was the recipient of scholarships from the Cole Foundation, la Fondation de l’Hôpital Sainte-Justine, and FRSQ. The authors declare no financial conflicts of interest.

AUTHOR CONTRIBUTIONS

N.M. performed research, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript. M.A.C. and M.D. wrote the manuscript. H.S. designed research, performed statistical analysis, and wrote the manuscript.

Correspondence: Hugo Soudeyns, Unité d’immunopathologie virale, Centre de recherche du CHU Sainte-Justine, 3175 Côte Sainte-Catherine, Room 6735, Montreal, QC H3T 1C5, Canada; e-mail: hugo.soudeyns@recherche-ste-justine.qc.ca.

REFERENCES


Table I. Clinical characteristics of transplanted patients

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%</th>
<th>Patient reference ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XX</td>
<td>11</td>
<td>42.3</td>
<td>1,5,7,8,11,12,20,21,22,23,25</td>
</tr>
<tr>
<td>XY</td>
<td>15</td>
<td>57.7</td>
<td>2,3,4,6,9,10,13,14,15,16,17,18,19,24,26</td>
</tr>
<tr>
<td>UCB graft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single unit</td>
<td>24</td>
<td>92.3</td>
<td></td>
</tr>
<tr>
<td>Double units</td>
<td>2</td>
<td>7.7</td>
<td>19, 24</td>
</tr>
<tr>
<td>Hematologic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>malignancies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>11</td>
<td>42.3</td>
<td>1,3,8,10,13,14,15,17,18,23,26</td>
</tr>
<tr>
<td>MDS</td>
<td>8</td>
<td>30.8</td>
<td>5,6,7,9,12,21,22,24</td>
</tr>
<tr>
<td>AML</td>
<td>4</td>
<td>15.4</td>
<td>2,4,20,25</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FHLH</td>
<td>1</td>
<td>3.8</td>
<td>11</td>
</tr>
<tr>
<td>Farber disease</td>
<td>1</td>
<td>3.8</td>
<td>16</td>
</tr>
<tr>
<td>Hurler syndrome</td>
<td>1</td>
<td>3.8</td>
<td>19</td>
</tr>
<tr>
<td>HLA disparity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>34.6</td>
<td>1,4,5,6,12,14,19,23,25</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>34.6</td>
<td>2,3,8,10,11,16,20,21,26</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>30.8</td>
<td>7,9,13,15,17,18,22,24</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>8</td>
<td>30.8</td>
<td>1,2,7,8,12,14,19,23</td>
</tr>
<tr>
<td>Cytomegalovirus infection</td>
<td>3</td>
<td>11.5</td>
<td>7,9,12</td>
</tr>
<tr>
<td>Herpes virus infection (other)</td>
<td>12</td>
<td>46.2</td>
<td>2,9,10,11,13,15,17,21,25</td>
</tr>
<tr>
<td>Leukemic relapse</td>
<td>8</td>
<td>34.8</td>
<td>2,8,12,14,20,22,23,26</td>
</tr>
<tr>
<td>Acute GvHD</td>
<td>4</td>
<td>15.4</td>
<td>17,18,22,24</td>
</tr>
<tr>
<td>Chronic GvHD</td>
<td>1</td>
<td>3.8</td>
<td>21</td>
</tr>
<tr>
<td>Graft failure</td>
<td>3</td>
<td>11.5</td>
<td>1,6,19</td>
</tr>
</tbody>
</table>

Conditioning regimen and GvHD prophylaxis were described under Materials and Methods. Twenty-three of 26 subjects (88.5%) were transplanted to treat persistent malignant disease. The major cause of post-transplant mortality was leukemic relapse (n = 5). The frequency of leukemic relapse was calculated on subjects who suffered from hematologic malignancies (n = 23). Subjects lost to follow-up (n = 2) were not included in the analysis. ALL: acute lymphoblastic leukemia; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; FHLH: familial hemophagocytic lymphohistiocytosis; GvHD: graft-versus-host disease.
FIGURE LEGENDS

Figure 1. Reconstitution of A2/Melan-A⁺CD8⁺ T cells following UCBT. The frequency of A2/Melan-A⁺ CD8⁺ T cells was measured *ex vivo* (A) and following PHA stimulation (B) in UCB graft inoculums and UCBT recipients. Statistical significance was tested using the Mann-Whitney *U* test. Boxes represent median and IQR. Error bars represent the range. *n* represents the number of subjects.

Figure 2. Evolution of the T cell receptor β chain repertoire in A2/Melan-A⁺ CD8⁺ T cells. A. Longitudinal analysis of the distribution of TCR β chain clonotypes amplified from graft inoculums and PBMC samples obtained from corresponding UCBT recipients (*n*=11). Each bar represents a time point (*i.e.* graft inoculum; time post-UCBT), and each box within a bar represents a particular T cell clonotype identified by a characteristic TCR β chain CDR3 sequence. Open boxes represent unique clonotypes (*i.e.* clonotypes that were not found in another time point). Colored bars represent persisting clonotypes, *i.e.* clonotypes that were observed in more than one time point. Identical colors correspond to identical clonotypes in within-patient but not between-patient analysis. Twenty to 35 independent recombinant clones were analysed per time point. P2, P8 and P20 experienced leukemic relapse. B. Simpson’s diversity index (Ds)²⁴ was used to represent clonal diversity. Statistical significance was tested using one-way ANOVA and Bonferroni’s multiple comparison test. *: *p* < 0.05. Boxes represent median and IQR. Error bars represent the range. *n* represents the number of subjects.
Figure 3. Phenotypic reconstitution of total CD8+ T cells and A2/Melan-A+ CD8+ T cells following UCBT. Representative dot plots and pie charts represent median frequencies of naïve, CM, EM, and EMRA CD8+ T cells measured ex vivo in the graft inoculum and UCBT recipients using tetramer staining. No A2/Melan-A+ T cells were detected ex vivo at 1 to 3 months post-UCBT.

Figure 4. Polyfunctionality profile and cytolytic activity of A2/Melan-A+ CD8+ T cells following UCBT. A. Staining for IFN-γ, IL-2 and CD107a was performed on T cell microcultures derived from graft inoculums and UCBT recipients following stimulation with cognate peptide. Background frequencies measured in absence of cognate peptide were subtracted. Left panel represents the frequencies of cells expressing combinations of IFN-γ, IL-2 and CD107a. Right panel depicts the frequencies of cells exhibiting 1, 2 and 3 functions. n represents the number of subjects. B. 51Cr release assays were performed using 221.A2 targets pulsed with Melan-A26-35 A27L peptide. Samples were considered positive when 51Cr release measured in presence of cognate peptide was ≥ 10% of total release and ≥ 2 standard deviations above 51Cr release measured in absence of peptide. i. Bars represent the mean and standard error of frequencies of cytolytic A2/Melan-A+ CD8+ T cell microcultures derived from the graft inoculum (G) and from samples obtained from study subjects following UCBT. n represents the number of subjects tested at each time point. ii. Box and whiskers represent % specific lysis exhibited by T cell microcultures derived from the graft inoculums or from samples obtained from UCBT recipients. % specific lysis was computed as [({51}Cr release in presence of cognate peptide - {51}Cr release in absence of peptide) x 100 / total {51}Cr release]. n represents the number of
T cell microcultures tested at each time point. iii. Squares represent the mean and SEM of lysis in presence (closed squares) or absence (open squares) of cognate peptide. n represents the number of T cell microcultures tested at each time point. There were no significant differences between time points. Statistical significance was tested using the Mann-Whitney U test and ANOVA analysis of variance. Statistical significance was tested using ANOVA or the Mann-Whitney U test.

**Figure 5. Frequencies of PD-1+ CD8+ and PD-1+ CD4+ T cells following UCBT.** A. PD-1 expression was analyzed by FACS on CD8+ T cells *ex vivo*. Gates were set on CD8^{hi} to exclude CD8+ NK cells. B. PD-1 expression was analyzed by FACS on CD4+ T cells *ex vivo*. C. Correlation between the frequency of PD-1+ CD8+ T cells and absolute CD8+ T cell counts throughout the follow-up period. D. Correlation between the frequency of PD-1+ CD8+ T cells and absolute CD4+ T cell counts throughout the follow-up period. Boxes and whiskers depict the median, IQR and range of frequencies in graft inoculums (G), UCBT recipients, and healthy adults (AB). Statistical significance was tested using Spearman’s rank correlation test or one-way ANOVA and Bonferroni’s multiple comparison test. *: p < 0.05; **: p < 0.01; ***: p < 0.001. n represents the number of subjects.

**Figure 6. Association between PD-1 expression and leukemic relapse.** Absolute CD4+ T cells counts (A) and absolute CD8+ T cell counts (B) were measured by FACS in PBMC samples obtained at 6 months post-UCBT. Frequencies of PD-1+ CD8+ T cells were measured in PBMC samples obtained at 2 months (C) and 6 months (D) post-
UCBT. Statistical significance was tested using the Mann-Whitney $U$ test. Boxes and whiskers depict the median, IQR and range. $n$ represents the number of subjects. NR: non-relapsers, R: relapsers.
Merindol et al. Figure 2

A. Frequency of T cell receptor β chain clonotypes (%)

<table>
<thead>
<tr>
<th>Patient</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>34</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>P9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>P10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>P11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
</tbody>
</table>

B. Frequency of T cell receptor β chain clonotypes (%)

| Patient | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 |
|---------|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| P2      |   |   |   |   |   | 20 | 40 | 60 | 80 | 100| 20 | 40 | 60 | 80 | 100| 20 | 40 | 60 | 80 | 100|
| P8      |   |   |   |   |   | 20 | 40 | 60 | 80 | 100| 20 | 40 | 60 | 80 | 100| 20 | 40 | 60 | 80 | 100|
| P20     |   |   |   |   |   | 20 | 40 | 60 | 80 | 100| 20 | 40 | 60 | 80 | 100| 20 | 40 | 60 | 80 | 100|

Time post-UCBT (months)

B. Simpson Index (Dw)

| Patient | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 |
|---------|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
|         | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

Time post-UCBT (months)
Merindol et al. Figure 3.
Merindol et al. Figure 4

A.

UCB graft

6 months

12 months

18 months

24 months

36 months

B.

i. Frequency of MaSC T cell microenvironment (%)

ii. Specific activity

iii. Target kill (%)
Merindol et al. Figure 6.
CD8+ T-cell reconstitution in recipients of umbilical cord blood transplant and characteristics associated with leukemic relapse

Natacha Merindol, Martin A. Champagne, Michel Duval and Hugo Soudeyns