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Umbilical Cord Blood T Cells Respond against the Melan-A/MART-1 Tumor Antigen and Exhibit Reduced Alloreactivity as Compared with Adult Blood-Derived T Cells

Natacha Merindol,* Anne-Julie Grenier,* Martine Caty,* Emily Charrier,†,‡ Arnaud Duval,†§, Michel Duval,†§# Martin A. Champagne,∥‖,‡ and Hugo Soudeyns*,#,#

Umbilical cord blood (UCB) is increasingly used as a source of hematopoietic progenitor cells to treat a variety of disorders. UCB transplant is associated with comparatively reduced incidence of graft-versus-host disease, robust graft versus leukemia effect, and relatively high incidence of opportunistic infections, three processes in which donor-derived T lymphocytes are known to be predominantly involved. To examine the differential functionality of UCB T cells, CD8⁺ T cells specific for the melanoma-associated HLA-A2–restricted Melan-A26–35 A27L peptide were isolated from HLA-A2² and HLA-A2⁻ UCB samples and HLA-A2² and HLA-A2⁻ adult peripheral blood using A2/Melan-A tetramers. In UCB samples, A2/Melan-A² CD8⁺ T cells were detected at a frequency of 0.04%, were more frequent in HLA-A2² UCB, and were polyclonal and mostly naïve. Consistent with Ag-driven expansion, the frequency of A2/Melan-A² CD8⁺ T cells was increased following stimulation with cognate peptide or polyclonal activation, they acquired cell-surface markers reflective of effector/memory differentiation, their TCR repertoire became oligoclonal, and they expressed cytolytic activity and produced IFN-γ. Although functional properties of A2/Melan-A² CD8⁺ T cells derived from HLA-A2² UCB resembled those of HLA-A2² adult peripheral blood, they were more likely to reach terminal differentiation following polyclonal stimulation and produced less IFN-γ in response to cognate peptide. A2/Melan-A² CD8⁺ T cells from HLA-A2⁻ UCB were poorly cytolytic, produced little IFN-γ, and were predominantly monofunctional or nonfunctional. These properties of UCB-derived CD8⁺ T cells could contribute to the reduced incidence of graft-versus-host disease and heightened incidence of opportunistic infections observed following UCB transplant. The Journal of Immunology, 2010, 185: 856–866.

Unrelated umbilical cord blood (UCB) is increasingly being used as a source of cells for patients in need of HSC transplant. In comparison with other HSC sources such as bone marrow or peripheral blood, UCB transplant (UCBT) is characterized by a lower incidence and severity of GVHD, a robust graft versus leukemia effect, but a relatively high incidence of opportunistic infections (OIs) that represent the most important cause of mortality in graft recipients during the first 6 mo posttransplant. These differences in clinical outcome raise important questions regarding the phenotype and functional properties of UCB-derived T lymphocytes. On the one hand, as compared with T cells isolated from adults, UCB T cells have long been considered immature based on their reduced capacity for Th1/Th17 cytokine production and diminished cytolytic activity. On the other hand, neonatal T cells can mediate alloreactivity and mount efficient antiviral immune responses against CMV, influenza virus, and HIV-1, indicating that they can exhibit functional properties in some settings. Indeed, to this day, there is a paucity of data on the differential functionality of preimmune T cells in UCB as compared with adult peripheral blood.

UCB T cells that recognize the Melan-A28⁻ A27L peptide presented in the context of HLA-A2 can be isolated from peripheral blood and UCB. These cells are thought to represent the only preimmune T cell repertoire that can be studied in humans. Interestingly, A2/Melan-A² T cells can be reproducibly isolated from blood samples derived from HLA-A2⁻ subjects, providing an opportunity to compare T cell responses directed against HLA-identical and HLA-disparate targets. In this study, approaches based on peptide–MHC tetramers were used to characterize the phenotype and immunologic properties of Melan-A-specific CD8⁺ T cells derived from HLA-A2² and HLA-A2⁻ UCB to address...
lingering questions concerning the functional capabilities of UCB-derived T cells and to gain insight into the role they play in cell-mediated immune responses in the graft recipient following UCBT. Results revealed that UCB-derived A2/Melan-A+ CD8+ T cells were clonally diversified. Following polyclonal activation or stimulation with cognate peptide, these cells underwent oligoclonal expansion, exhibited cytolytic activity, produced IFN-γ, and acquired cell-surface markers reflective of effector/memory differentiation. At the functional level, A2/Melan-A+ CD8+ T cells derived from HLA-A2+ UCB exhibited properties that were similar to those observed in HLA-A2+ AB but were more likely to achieve terminal differentiation following polyclonal activation and produced significantly less IFN-γ on a per-clone basis. In contrast, A2/Melan-A+ CD8+ T cells from HLA-A2+ UCB were comparatively less cytolytic, produced less IFN-γ, and were more likely to be nonfunctional or nonfunctional.

Materials and Methods

Procurement of UCB and AB samples

This study was approved by the Institutional Review Board of Centre Hospitalier Universitaire Sainte-Justine, Montreal, Quebec, Canada. Full informed consent was obtained from all study participants. Samples of UCB mononuclear cells (UCBMCs; n = 26) were obtained from the Cord Blood Research Bank maintained by the Groupe de Recherche en Transplantation et Immunothérapie de l’Université de Montréal (Centre Hospitalier Universitaire Sainte-Justine. AB samples (n = 14) were obtained from healthy adult volunteers. UCBMCs and PBMCs were isolated on Ficoll-Hypaque (Amer sham Biosciences, Uppsala, Sweden) and cryopreserved in 90% v/v FBS (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% v/v DMSO. HLA typing was performed using the line probe assay (Inno- genetics, Gent, Belgium) (Supplemental Table 1).

Generation of T cell microcultures

Frozen UCBMCs were thawed and cultured for 24 h in RPMI 1640 supplemented with 20% v/v FBS. Cells were seeded at 1000 cells/well in 96-well round-bottom plates and were cocultured with 8 × 10^5 irradiated (300 Gy) 221.A2 cells (EBV-transformed B lymphoblastoid cell line expressing HLA-A2*0201; kindly provided by Cassian Yee, University of Washington, Seattle, WA) (16) pulsed with the HLA-A2–restricted modified Melan-A26–35 A27L peptide (ELAGIGILTV) (Peptide Synthesis Core Facility, Centre Hospitalier de l’Université Laval, Quebec City, Quebec, Canada). T cell microcultures were maintained in RPMI 1640 supplemented with 10% v/v FBS, 50 U/ml recombinant human (rh) IL-2 (Hoffmann-La Roche, Mississauga, Ontario, Canada), and 10 ng/ml rhIL-7 (R&D Systems, Minneapolis, MN). Medium was replenished biweekly. Effector cells were restimulated weekly for 3 wk with irradiated, peptide-pulsed 221.A2 cells.

Immunophenotyping, peptide–MHC tetramer staining, and cell sorting

Multiparameter fluorescence-activated cell sorting was performed on thawed UCBMCs. A minimum of 1 × 10^6 CD8+ T cells was analyzed. To detect Melan-A–specific T cells in UCBMCs, T cell microcultures, and expanded T cell clones, biotinylated HLA-A2 monomers were refolded thermodynamically with slower rates than the carrier peptide (A2/Melan-A). A2/Melan-A–specific T cells were then added. As a positive control, effector cells were incubated with 0.5 μg/ml anti-CD3 (Sanquin, Amsterdam, The Netherlands). Following 22 h of incubation, plates were washed extensively and incubated with biotinylated IFN-γ detection mAb (clone 4S.B3, BD Biosciences) and alkaline phosphatase-conjugated streptavidin (Bio-Rad, Hercules, CA). Plates were washed and incubated with BCIP/NBT-plus substrate solution (Bio-Rad). Spots were counted on an Immunospot 3A Analyzer (CTL Technology, Shaker Heights, OH).

ELISPOT

IFN-γ secretion by T cell microcultures and expanded T cells was measured using ELISPOT (19). Briefly, the wells of a polyvinylidene difluoride-backed microtiter plate (Millipore, Bedford, MA) were coated with anti-human IFN-γ capture Ab (clone NIB42, BD Biosciences) overnight at 4˚C and were then incubated for 2 h at 37˚C in RPMI 1640 medium supplemented with 10% v/v FBS. T cell microcultures or expanded T cell clones (see above) were seeded at 1 × 10^5 cells/well on Ab-coated plates and were incubated for 2 h. Irradiated 221.A2 cells (5 × 10^5 cells/well) pulsed with appropriate peptides (Melan-A26–35 A27L or NS3-1406) were then added. As a positive control, effector cells were incubated with 0.5 μg/ml anti-CD3 (Sanquin, Amsterdam, The Netherlands). Following 22 h of incubation, plates were washed extensively and incubated with biotinylated IFN-γ detection mAb (clone 4S.B3, BD Biosciences) and alkaline phosphatase-conjugated streptavidin (Bio-Rad, Hercules, CA). Plates were washed and incubated with BCIP/NBT-plus substrate solution (Bio-Rad). Spots were counted on an ImmunoSpot 3A Analyzer (CTL Technology, Shaker Heights, OH).

Amplification and sequencing of TCR β-chain transcripts

Total mRNA was extracted from pellets of tetramer-sorted cells (150–1500 cells) using the Picopure system ( Molecular Devices, Sunnyvale, CA). The TCR β-chain V region (TRBV) mRNA, including CDR3, was reverse transcribed, and corresponding cDNA was amplified by PCR (40 cycles) using universal primers, as described previously (20). PCR products were subcloned into the TOPO TA cloning vector (Invitrogen) and sequenced (Centre Hospitalier de l’Université Laval). Identification of TRBV and TCR β-chain V region (TRBV) segments and determination of CDR3 length and amino acid sequences was performed using IMGT/V-QUEST and confirmed manually (21).

Normality analysis

Standard normality of the distribution of sample data was assessed using the D’Agostino and Pearson normality omnibus test. Statistical significance in between-groups comparisons was assessed using the Mann-Whitney U test. The χ^2 test was used to examine categorical data. Spearman correlation and linear regression analysis were used to test correlations between groups. p values < 0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 4 (GraphPad, San Diego, CA).

Results

Melan-A–specific T cells are detectable in UCB

The presence of Melan-A–specific CD8+ T cells was estimated in UCB (HLA-A2+, n = 12; HLA-A2+, n = 14) and AB (HLA-A2+, n = 5; HLA-A2+, n = 9) using Melan-A26–35 A27L–HLA-A2 tetramers (A2/Melan-A). A2/Melan-A–specific T cells were detected in 19 out of 26 (73.1%) UCBMC samples tested, with a median frequency of 0.04%. Of these, 10 out of 12 (83.3%) HLA-A2+ UCBMC samples were A2/Melan-A+ and the median frequency of A2/Melan-A+ cells was 0.07% of CD8+ T cells. Nine out of 15 AB samples tested, 4 out of 5
(80.0%) HLA-A2+ AB samples were A2/Melan-A+, with a median frequency of 0.06%. In comparison, 4 out of 9 (44.4%) HLA-A2+ AB samples were A2/Melan-A+, with a median frequency of A2/Melan-A+ T cells of 0.00%. The median frequency of A2/Melan-A+ T cells was higher in HLA-A2+ UCB and HLA-A2+ AB samples than in HLA-A2+ UCB and HLA-A2+ AB samples.

FIGURE 1. A2/Melan-A+ T cells are detectable in UCB and adult blood samples. Multiparameter flow cytometric analysis was performed as described in Materials and Methods. Samples were defined as tetramer positive when: 1) A2/Melan-A+ CD8+ T cells represented >0.01% of CD8+ T cells, as this frequency corresponded to maximum background of negative controls; and 2) mean fluorescence intensity between tetramer-positive and -negative populations was >1 log10. A, Frequency of A2/Melan-A+ CD8+ T cells detected in HLA-A2+ or HLA-A2+ UCB and HLA-A2+ or HLA-A2+ AB samples using Melan-A26-35 A27L-HLA-A2 tetramers. Error bars represent the range of values. Boxes represent median and interquartile range. B, Representative dot plots of A2/Melan-A+ CD8+ T cells from UCB and AB. Percentages reflect the frequency of A2/Melan-A+ T cells in the CD8+ T cell population. C, Differentiation profiles of A2/Melan-A+ CD8+ T cells from HLA-A2+ or HLA-A2+ UCB and AB.

FIGURE 2. Repertoire of freshly isolated A2/Melan-A+ CD8+ T cells is diversified in HLA-A2+ and HLA-A2+ UCB samples. A, Clonal diversity was computed as the total number of different TCR β-chain clonotypes detected divided by the total number of sequences generated in a given UCB sample. B, The TCR β-chain was amplified from cDNA derived from A2/Melan-A+ CD8+ T cells isolated from HLA-A2+ (n = 4) and HLA-A2+ (n = 4) UCB samples as described in Materials and Methods. TRBV usage, TRBJ usage, and CDR3 length were determined using IMGT/V-QUEST (21). Gaussian distribution of CDR3 length is consistent with a preimmune T cell repertoire.
Melan-A–specific T cells from UCB express a naive phenotype

To gain insight into the differentiation pattern and proliferative history of A2/Melan-A+ CD8+ T cells from UCB, cell-surface expression of CD45RA, CCR7, and CD27 was examined, allowing the stratification of CD8+ T cells into six distinct subpopulations: 1) naive (CD45RA+CCR7+CD27+); 2) central memory (CM) (CD45RA−CCR7−CD27+); 3) effector memory (EM) (CD45RA−CCR7−), which includes EM1−2 (CD27−) and EM2−4 (CD27+); and 4) effectors (CD45RA+CCR7−), which includes effector memory RA (EMRA) (CD27+) and terminally differentiated effector memory RA (EMRA-E) (CD27−) (22). A2/Melan-A+ CD8+ T cell populations from HLA-A2+ and HLA-A2− UCB comprised a large majority of cells expressing a naive phenotype (96.6 ± 13.7% and 95.8 ± 11.7%, respectively) (Fig. 1C). Although most A2/Melan-A+ CD8+ T cells from HLA-A2+ and HLA-A2− AB were naive (61.9 ± 23.1% and 77.6 ± 18.1%, respectively), they also comprised effectors (26.8 ± 29.6% and 8.14 ± 12.2%, respectively) that could be further subdivided into EMRA (8.60 ± 8.40% and 4.54 ± 8.16%, respectively) and EMRA-E (18.2 ± 21.2% and 3.60 ± 4.04%, respectively) (Fig. 1B, 1C). Thus, freshly isolated Melan-A–specific CD8+ T cells from UCB are less differentiated than their AB counterparts.

The Melan-A–specific CD8+ T cell repertoire in UCB is polyclonal and highly diversified

To study the diversity and dynamics of the Melan-A–specific CD8+ T cell compartment, the repertoire of TCR β-chain genes expressed by sorted A2/Melan-A+ CD8+ T cells was examined using PCR amplification, cloning, and sequencing in absence of in vitro stimulation. This analysis revealed that the clonal diversity of freshly isolated A2/Melan-A+ CD8+ T cells was significantly lower in HLA-A2+ than in HLA-A2− UCB samples (p = 0.0143) (Fig. 2A). Clonal diversity was also examined in terms of TRBV gene usage, TRBJ gene usage, and CDR3 length, which represent three important structural determinants of Ag recognition by the TCR (23). Though the profiles were similar, TRBV and TRBJ gene usage was broader in A2/Melan-A+ CD8+ T cells isolated from HLA-A2− UCB samples as compared with those derived from HLA-A2+ UCB samples (Fig. 2B). In both HLA-A2+ and HLA-A2− UCB samples, the distribution of CDR3 lengths in A2/Melan-A+ CD8+ T cells was quasi-Gaussian (p = 0.5680), consistent with a preimmune repertoire (Fig. 2B) (24, 25).

Oligoclonal expansion of UCB-derived A2/Melan-A+ T cells

The capacity of A2/Melan-A+ CD8+ T cells derived from HLA-A2+ and HLA-A2− UCB to proliferate in response to stimulation with cognate peptide was then assessed. Unsorted UCBMC samples were seeded at 1000 cells/well and cultured for 2 wk with IL-2, IL-7, Melan-A26–35 A27L peptide, and irradiated 221.A2 cells. Ninety-six microwell cultures per UCB sample were pooled and characterized by flow cytometry. The proportion of A2/Melan-A+ T cells in CD8+ T cells was significantly increased in HLA-A2− and HLA-A2+ UCB samples (p < 0.0001 and p = 0.0003, respectively) following stimulation with Melan-A26–35 A27L (Fig. 3A). The overall proportion of CD8+ T cells was also significantly increased (p < 0.0001) (Fig. 3B). Similar levels of expansion were observed in AB samples (data not shown). In addition, the frequency of A2/Melan-A+ CD8+ T cells following in vitro peptide stimulation was significantly correlated with the proportion of A2/Melan-A+ CD8+ T lymphocytes in freshly isolated cell samples (p < 0.0001) (Fig. 3C).

Next, the diversity of the Melan-A–specific T cell repertoire was examined following stimulation with cognate peptide. Tetramer-sorted A2/Melan-A+ T cells derived from peptide-stimulated UCB microwell cultures showed significantly lower levels of clonal diversity as compared with freshly isolated A2/Melan-A+ T cells (p = 0.0325; HLA-A2+ and HLA-A2− samples analyzed together) (Fig. 4A). In contrast to what was observed in freshly isolated cells (Fig. 2), clonal diversity was lower in UCB-derived A2/Melan-A+ CD8+ T cells isolated from HLA-A2− donors as compared with HLA-A2+ donors, though not significantly so (p = 0.125) (Fig. 4B). TRBV3-1, TRBV5-6, and TRBV9 accounted for 81.0% of total TRBV usage in sequences derived from HLA-A2− UCB samples, whereas TRBV11-2 accounted for 64.0% of TRBV usage in sequences derived from HLA-A2+ UCB samples (Fig. 4C). TCR usage in terms of CDR3 length distribution was narrower postculture in T cell microwell cultures derived from both HLA-A2+ and HLA-A2− UCB samples (Figs. 2B, 4C). These results demonstrate that A2/Melan-A+ CD8+ T cells can undergo oligoclonal expansion following stimulation with cognate peptide, irrespective of whether they were derived from HLA-A2+ or HLA-A2− UCB.

Differentiation and Ag-specific cytotoxic activity of UCB-derived Melan-A–specific T cells

As A2/Melan-A+ CD8+ T cells underwent oligoclonal expansion following stimulation with cognate peptide, it was important to assess whether this proliferation was accompanied by phenotypic
and/or functional differentiation. To address this issue, three sets of experiments were performed. First, total UCBMCs (1000 cells per well) were plated on irradiated feeder cells and stimulated with the Melan-A26-35 A27L peptide, IL-2, and IL-7 for 2 wk. In HLA-A2+ UCB samples, a mean of 49.9 ± 14.0% of peptide-stimulated A2/Melan-A+ CD8+ T cells retained a naive phenotype. Others mainly differentiated into EMRA (37.2 ± 6.56%) and EMRA-E (6.30 ± 1.61%) (Fig. 5A). Similarly, in HLA-A2–/– UCB samples, a mean of 53.5 ± 3.35% of peptide-stimulated A2/Melan-A+ CD8+ T cells remained naive, whereas others differentiated into EMRA (37.2 ± 1.13%) and EMRA-E (6.30 ± 1.61%) (Fig. 5A).

In separate experiments, sorted A2/Melan-A+ CD8+ T cells derived from HLA-A2+ and HLA-A2– UCBMC and AB samples were expanded in the presence of IL-2, IL-7, and PHA to avoid biasing the T cell repertoire (26). After 14 d, 16.3 ± 23.5% of expanded A2/Melan-A+ cells from HLA-A2+ UCB samples retained a naive phenotype, 1.82 ± 2.48% differentiated into CM, 7.85 ± 8.53% into EM1–2, 22.4 ± 22.3% into EM3–4, 20.8 ± 25.9% into EMRA, and 30.8 ± 13.7% into EMRA-E (Fig. 5B). In contrast, 26.5 ± 28.1% of expanded A2/Melan-A+ cells from HLA-A2– AB remained naive, 1.23 ± 0.56% became CM, 17.8 ± 13.2% became EM1–2, 10.7 ± 19.5% became EM3–4, and 26.0 ± 39.7% became EMRA, but only 3.16 ± 2.73% differentiated into EMRA-E (Fig. 5C). Similarly, 28.4 ± 32.5% of expanded A2/Melan-A+ cells from HLA-A2+ UCB UC BMCs differentiated into EMRA-E, whereas only 8.80 ± 10.6% of expanded A2/Melan-A+ cells from HLA-A2– AB achieved terminal differentiation (Fig. 5B, 5C). Control stainings with CMVpp65-HLA-A2 tetramers were uniformly negative (data not shown). Taken together, these results indicate that: 1) stimulation with cognate peptide or polyclonal stimulation with PHA leads to the differentiation of Melan-A–specific T cells derived from HLA-A2+ and HLA-A2– UCB into various memory and effector subtypes; and 2) following PHA stimulation, a larger proportion of Melan-A–specific UCB-derived T cells express a terminally differentiated phenotype than AB-derived T cells.

Unsorted UCBMCs and unsorted PBMCs from AB stimulated with Melan-A26-35 A27L were then tested for cytolytic activity. Cytolytic microcultures were detected in all HLA-A2+ and HLA-A2– UCB and AB samples tested. The median frequency of T cell microcultures expressing cytolytic activity was equivalent between HLA-A2+ UCBMCs and HLA-A2– AB (p = 0.1868), but was lower in HLA-A2+ UCBMCs in comparison with HLA-A2+ AB and HLA-A2– UCBMCs (p = 0.0242 and p = 0.0058, respectively) (Fig. 6A). Furthermore, the median percent specific cytotoxicity was higher in T cell microcultures derived from HLA-A2+ UCBMCs than in corresponding microcultures derived from HLA-A2– AB (p = 0.0092) and similar between HLA-A2+ and HLA-A2– UCB-derived microcultures and between HLA-A2– UCB and AB-derived microcultures (p = 0.0923 and p = 0.3134, respectively) (Fig. 6B). Cytolytic activity was also measured in tetramer-sorted A2/Melan-A+ CD8+ T cell clones following PHA-driven expansion; again, the median frequency of cytolytic T cell clones was equivalent between HLA-A2+ UCB- and AB-derived microcultures and between HLA-A2+ and HLA-A2– UCBMCs expressing cytolytic activity was equivalent between HLA-A2+ UCB and HLA-A2– AB (p = 0.0092) and similar between HLA-A2+ and HLA-A2– UCBMCs in comparison with HLA-A2+ AB and HLA-A2– UCBMCs (p = 0.0242 and p = 0.0058, respectively) (Fig. 6A). Cytolytic activity was also measured in tetramer-sorted A2/Melan-A+ CD8+ T cell clones following PHA-driven expansion; again, the median frequency of cytolytic T cell clones was equivalent between HLA-A2+ UCB- and AB-derived microcultures and between HLA-A2+ UCB and HLA-A2– AB (p = 0.0092) and similar between HLA-A2+ and HLA-A2– UCBMCs in comparison with HLA-A2+ AB and HLA-A2– UCBMCs (p = 0.0242 and p = 0.0058, respectively) (Fig. 6A). Of note, the median frequency of cytolytic T cell clones was higher in HLA-A2+ UCB samples as compared with HLA-A2– AB samples (p = 0.0028). These results indicate that the cytolytic activity of HLA-A2+ UCBMC-derived Melan-A–specific T cells is equivalent to that of AB-derived T cells, whereas the cytolytic activity of Melan-A–specific T cells derived from HLA-A2– UCB is comparatively impaired.
HLA-A2+ UCB + Melan-A
[\(n=5\)]

HLA-A2+ UCB + Melan-A
[\(n=3\)]

A2/Melan-A
HLA-A2+ UCB + PHA
[\(n=9\)]

A2/Melan-A
HLA-A2+ UCB + PHA
[\(n=7\)]

A2/Melan-A
HLA-A2+ AB + PHA
[\(n=4\)]

A2/Melan-A
HLA-A2+ AB + PHA
[\(n=4\)]

FIGURE 5. Phenotypic analysis of A2/Melan-A+ CD8+ T cells following stimulation with cognate peptide or polyclonal activation. T cell microcultures were generated by limiting dilution of HLA-A2+ and HLA-A2+ UCB and AB-derived T cells. A. Unsorted UCBMCs were seeded (1000 cells/well) onto allogeneic feeder cells and incubated in the presence of Melan-A26-35 A27L, peptide, IL-2, and IL-7 as described in Materials and Methods. After 2 wk in culture, wells were pooled, and 1 \times 10^5 live cells were gated based on forward and side scatter. Cell-surface phenotype of A2/Melan-A+ T cells was determined on pooled microcultures using flow cytometry. Naive, CM, EM (EM1–2, EM1–4), effector (EMRA-pE1 and EMRA-pE2), and terminally differentiated effector (EMRA-E) phenotypes were defined based on cell-surface expression of CD45RA, CCR7, and CD27 as described (22). B. UCBMC sorted using Melan-A26–35 A27L-HLA-A2 tetramers were seeded (1–3 cells/well) onto allogeneic feeder cells and incubated in the presence of PHA, IL-2, and IL-7 as described in Materials and Methods. After 2 wk in culture, 1 \times 10^5 live cells were gated based on forward and side scatter for each microculture that was analyzed (23 microcultures for 11 UCB samples). Cell-surface phenotype was analyzed as above. C. AB-derived T cells sorted using Melan-A26–35 A27L-HLA-A2 tetramers were seeded (1–3 cells/well) onto allogeneic feeder cells and incubated in the presence of PHA, IL-2, and IL-7. After 2 wk in culture, 1 \times 10^5 live cells were gated based on forward and side scatter for each microculture that was analyzed. Cell surface phenotype was analyzed as above. n represents the number of UCB or AB samples that were used in the generation of T cell microcultures.

Melan-A–specific UCB-derived T cells can specifically secrete IFN-γ

To further characterize the functionality of A2/Melan-A+ T cells from UCBMCs, production of IFN-γ by expanded A2/Melan-A+ CD8+ T cell clones was examined using ELISPot. A median of 76.7%, 33.3%, 87.5%, and 55.0% of HLA-A2+ UCBMC-, HLA-A2+ UCBMC-, HLA-A2+ AB-, and HLA-A2+ AB-derived T cell clones, respectively, were able to produce above-threshold levels of IFN-γ in the presence of 221.A2 cells sensitized with Melan-A26–35 A27L as compared with unpulsed targets or targets sensitized with control peptide NS3-1406. The frequency of expanded A2/Melan-A+ CD8+ T cell clones that produced IFN-γ was equivalent between HLA-A2+ UCB and HLA-A2+ AB samples (\(p = 0.0727\)) and was significantly lower when they were derived from HLA-A2+ UCB as compared with HLA-A2+ UCB (\(p = 0.0122\)) but not to HLA-A2+ AB (\(p = 0.0741\)) (Fig. 6E). Interestingly, differences in numbers of IFN-γ-producing cells on a per-clone basis were also observed among the four groups: expanded A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ and HLA-A2– UCB comprised significantly less cells that produced IFN-γ in response to cognate peptide than HLA-A2+ and HLA-A2– AB-derived T cell clones (\(p < 0.0001\) and \(p = 0.0687\), respectively) (Fig. 6F). In summary, these data indicate that: 1) polyclonal stimulation leads to the differentiation of UCB-derived and AB-derived Melan-A–specific T cells into IFN-γ–producing cells; 2) the frequency of Melan-A–specific T cells that produce IFN-γ is higher in AB and HLA-A2– UCB than in HLA-A2– UCB; and 3) expanded A2/Melan-A+ CD8+ T cell clones derived from AB are more potent IFN-γ producers than those derived from UCB.

Expanded A2/Melan-A+ CD8+ T cells exhibit polyfunctional characteristics

Polyfunctionality of CD8+ T cells is an important characteristic of cell-mediated immune responses (23). Overall, secretion of IFN-γ by expanded A2/Melan-A+ CD8+ T cells sorted from HLA-A2+ and HLA-A2– UCBMC samples was positively correlated with their cytolytic activity (activity) (\(p < 0.0001\)) (Fig. 7A). To examine this association, expanded T cells derived from HLA-A2+ and HLA-A2– UCBMC and AB were scored according to the presence of cytolytic activity (CTL) and IFN-γ production [i.e., CTL IFN-γ (0 functions), CTL+IFN-γ/CTL+IFN-γ (monofunctional), or CTL+IFN-γ (bifunctional)]. This analysis revealed that 57.2% of expanded A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ UCB were monofunctional, 48.8% of expanded A2/Melan-A+ CD8+ T cell clones derived from HLA-A2– UCB neither produced IFN-γ nor exhibited cytolytic activity, 52.9% of expanded A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ AB were bi- or multifunctional, and 45.8% of expanded A2/Melan-A+ CD8+ T cell clones derived from HLA-A2– AB were monofunctional (Fig. 7B). Overall, the polyfunctionality profile expressed by HLA-A2– UCB T cells significantly differed from that expressed by HLA-A2– UCB T cells and HLA-A2+ AB T cells (\(p < 0.0001\) in both cases). In contrast, the polyfunctionality profiles displayed by HLA-A2+ UCB T cells did not significantly deviate from that of HLA-A2+ AB T cells (\(p = 0.8220\)). These data demonstrate that a proportion of Melan-A–specific T cells derived from HLA-A2+ UCB exhibit bifunctional properties following polyclonal expansion, though this proportion is lower than that observed in HLA-A2– AB-derived Melan-A–specific T cells. Furthermore, when evaluation was made on a per-sample basis, expanded A2/Melan-A+ CD8+ T cell clones were less likely to be nonfunctional and more likely to be monofunctional when they were derived from HLA-A2+ UCB as compared with HLA-A2– UCB (\(p = 0.0082\)) and \(p = 0.0115\), respectively). However, on a per-sample basis, the likelihood of expanded A2/Melan-A+ CD8+ T cells to be bifunctional was not significantly different whether they were derived from HLA-A2+ or HLA-A2– UCB (Fig. 7C).

Discussion

In this report, the functionality of UCB-derived Ag-specific and allogeneic T cells was studied to revisit the so-called immaturity of this T cell subset and provide a rationale for the reduced incidence of GVHD and higher incidence of OIs following UCBT. Using peptide–MHC tetramers, Melan-A–specific CD8+ T cells were detected in HLA-A2+ UCB at a frequency noninferior to that

\[\text{A}^+ \text{CD8}^+ \text{ T cell clones} \]

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Discussion

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FIGURE 6. Functional analysis of A2/Melan-A+ CD8+ T cells following stimulation with cognate peptide or polyclonal activation. Cytolytic activity and IFN-γ production were measured as described in Materials and Methods. Percent specific cytotoxicity was computed as [(sample release - spontaneous release) / (maximal release - spontaneous release)] × 100/100. Samples were considered positive when specific cytotoxicity measured in presence of cognate peptide was >10% and >2 SD above cytotoxicity measured in absence of peptide. IFN-γ production was expressed as SFUs. Cells incubated with complete medium alone were used as negative controls. Samples were considered positive when SFUs measured in presence of cognate peptide were >20 and >2 SD above SFUs measured in absence of cognate peptide. Upper cutoff was 400 SFU per well. All tests were performed in duplicate.

A, Relative frequencies of CD8+ T cell microcultures derived from HLA-A2+ UCB (n=10), HLA-A2+2 UCB (n=8), HLA-A2+ AB (n=4), and HLA-A2+2 AB (n=4) that exhibited Melan-A–specific cytolytic activity following a 2-wk stimulation with Melan-A26–35 A27L peptide in the presence of IL-2 and IL-7 as described in Materials and Methods.

B, Corresponding specific cytolytic activity exhibited by 547 T cell microcultures derived from HLA-A2+ UCB samples (n=10), 166 T cell microcultures derived from HLA-A2+2 UCB samples (n=8), 104 T cell microcultures derived from HLA-A2+ AB samples (n=4), and 185 T cell microcultures derived from HLA-A2+2 AB samples (n=4) following stimulation with Melan-A26–35 A27L peptide on a per-microculture basis.

C, Relative frequencies of expanded tetramer-sorted A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ UCB samples (n=9), HLA-A2+2 UCB samples (n=9), HLA-A2+ AB samples (n=4), and HLA-A2+2 AB samples (n=4) that exhibited Melan-A–specific cytolytic activity following a 2-wk polyclonal stimulation with PHA in the presence of IL-2 and IL-7 as described in Materials and Methods.

D, Corresponding specific cytolytic activity exhibited by 40 A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ UCB samples (n=9), 27 A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+2 UCB samples (n=9), 22 A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ AB samples (n=4), and 14 A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+2 AB samples (n=4) following polyclonal stimulation with PHA on a per-clone basis.

E, Relative frequencies of expanded tetramer-sorted A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ UCB samples (n=139), HLA-A2+2 UCB samples (n=42), HLA-A2+ AB samples (n=33), and HLA-A2+2 AB samples (n=14) that produced above-threshold levels of IFN-γ following polyclonal stimulation with PHA.

F, Corresponding frequencies of cells producing IFN-γ following polyclonal stimulation with PHA on a per-clone basis in 139 expanded A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ UCB samples (n=9), 42 A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+2 UCB samples (n=9), 33 A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+ AB samples (n=3), and 14 A2/Melan-A+ CD8+ T cell clones derived from HLA-A2+2 AB samples (n=4). Error bars represent the range of values. Boxes represent median and interquartile range. SFU, spot-forming unit.
observed in HLA-A2+ AB. Consistent with these observations, A2/Melan-A+ CD8+ T cells were also detected in HLA-A2 UCB, but their frequency was lower than that observed in HLA-A2+ UCB.

Unlike what was observed in AB, most A2/Melan-A+ CD8+ T cells from UCB expressed cell-surface markers indicative of a preimmune, naive phenotype and displayed a diversified and polyclonal TCR β-chain repertoire. Their presence at a relatively high frequency suggests that they may have already encountered cognate Ag. Hence, A2/Melan-A+ CD8+ T lymphocytes isolated from HLA-A2+ UCB may not constitute a truly preimmune repertoire but instead represent immature/transitional T cells (i.e., cells that have not been fully activated but are not Ag inexperienced)(27).

T cells from UCB were previously shown to exhibit impaired proliferative capacity (6–8, 28–30). In sharp contrast, results presented in this study demonstrate for the first time that the A2/Melan-A+ CD8+ T cells from UCB expressed cell-surface markers indicative of a preimmune, naive phenotype and displayed a diversified and polyclonal TCR β-chain repertoire. Their presence at a relatively high frequency suggests that they may have already encountered cognate Ag. Hence, A2/Melan-A+ CD8+ T lymphocytes isolated from HLA-A2+ UCB may not constitute a truly preimmune repertoire but instead represent immature/transitional T cells (i.e., cells that have not been fully activated but are not Ag inexperienced)(27).

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The relative cytolytic inefficacy of UCB T cells was associated with reduced expression of granzymes and perforin (28, 37). Costimulation mediated via NKG2D and TLRs was recently shown to be involved in the functional responsiveness of UCB-derived CD8+ T cells (38, 39). In the current study, the frequency of T cell...
microcultures derived from HLA-A2+ UCB that exhibited cytolytic activity in response to cognate peptide was similar to that observed in HLA-A2+ AB. In fact, they performed significantly better than AB on a per-microculture basis following stimulation with cognate peptide. This similarity was upheld when expanded A2/Melan-A+ CD8+ T cell clones were examined, as cytolytic activity displayed by HLA-A2+ UCB-derived T cells was noninferior to that displayed by HLA-A2+ AB-derived clones.

The frequency of expanded A2/Melan-A+ CD8+ T cells that produced above-threshold levels of IFN-γ in response to Melan-A26–35 A27L was similar in HLA-A2+ AB and HLA-A2+ UCB. In addition, there were comparatively more expanded HLA-A2+ AB-derived A2/Melan-A+ CD8+ T cells that produced IFN-γ in response to cognate peptide than HLA-A2+ UCB-derived A2/Melan-A+ CD8+ T cells on a per-clonal basis, indicating that AB-derived T cells are more robust producers of IFN-γ, a pivotal antiviral cytokine (40), than their UCB-derived counterparts. Aberrant production of the Th2 cytokine IL-13 and reduced levels of NFAT-1 in UCB T cells was shown to be associated with low levels of IFN-γ production (41, 42). Close examination of cytolytic activity and IFN-γ production revealed that HLA-A2+ UCB T cells were more likely to be nonfunctional and HLA-A2+ AB T cells more likely to be bifunctional, underscoring the notion that UCB-derived T cells exhibit attenuated, perhaps suboptimal, responses to cognate Ag. These differences could be linked to maturational stage (differential granyme and/or perforin expression) or differential sensitivity to Ag, which can vary by orders of magnitude in T cells that recognize identical peptide-MHC complexes (43, 44). In antiviral CD8+ T cell responses, functional avidity for Ag is intimately associated with polyfunctionality (44–46). As donor-derived CD8+ T cells play a key role in antiviral and antitumoral immunity in HSC transplant recipients (1, 2), reduced polyfunctionality of UCB-derived CTL might contribute to the higher incidence of OIs, in particular varicella zoster virus infection, that was reported in children following UCBT relative to recipients of bone marrow transplants (5). Functional impairment should also be taken into consideration when attempting to mobilize virus-specific CTL from UCB for use in the prevention and/or treatment of viral diseases following UCBT (47).

Even though the restricting MHC allele is not present in the donor, A2/Melan-A+ CD8+ T cells can be reproducibly isolated from HLA-A2+ blood samples, where they are thought to represent an endogenous alloreactive Melan-A–specific repertoire (15). In freshly isolated cells, clonal diversity was higher in A2/Melan-A+ CD8+ T lymphocytes from HLA-A2+ UCB as compared with HLA-A2+ UCB. As A2/Melan-A+ T cells cannot be negatively selected by Melan-A–HLA-A2 peptide–MHC class I complexes during thymopoiesis in HLA-A2+ subjects, highly reactive clones cannot be depleted, leading to a broad and diversified alloreactive T cell repertoire (48, 49). In sharp contrast, the clonal diversity of A2/Melan-A+ CD8+ T cells following peptide stimulation was lower in microcultures derived from HLA-A2+ UCB as compared with those derived from HLA-A2+ UCB. To explain this finding, we hypothesize that there were fewer individual T cell clones in HLA-A2+ UCB that were capable of proliferation in response to peptide stimulation. Alternatively, because high-affinity A2/Melan-A+ CD8+ T cells from HLA-A2+ UCB did not undergo negative selection against cognate peptide in the context of HLA-A2, these cells could have received comparatively stronger proliferative signals following stimulation, leading to reduced peptide-specific repertoire diversity.

Differentiation profiles exhibited by A2/Melan-A+ CD8+ T cells from HLA-A2+ or HLA-A2+ UCB were identical irrespective of whether stimulation with cognate peptide or PHA was used, indicating that allorecognition and cognate peptide stimulation lead to similar patterns of naïve-effector–memory differentiation. However, a lower relative proportion of UCB-derived HLA-A2− T cells exhibited cytolytic activity directed against targets sensitized with peptide. This difference held true whether T cells were stimulated with cognate peptide or PHA. Reduced frequencies were not due to nonspecific staining with the A2/Melan-A26–35 A27L tetramer, as tetramer positivity was confirmed in proliferating T cells poststimulation (data not shown). Functional A2/Melan-A+ CD8+ T cells have been previously described (15). Although differential expression of cytotoxic granule components was not examined in the current study, it is well established that differences in effector functions are reflected by the expression of distinct combinations of intracellular molecules associated with cell-mediated cytolytic activity (50–52). Indeed, the multifunctionality profile expressed by A2/Melan-A+ CD8+ T cells from HLA-A2− UCB markedly differed from that expressed by T cells from HLA-A2+ UCB: the former were more likely to be nonfunctional, whereas the latter were more likely to be mono- or bifunctional. This difference is unrelated to differentiation, because A2/Melan-A+ cells from HLA-A2+ and HLA-A2− UCB display quasi-identical naïve–effector–memory population structures. As functional avidity is closely associated with cytolytic activity, rapid proliferation of high avidity A2/Melan-A+ CD8+ T cells from HLA-A2− UCB could have led to clonal exhaustion, consistent with the reduced frequency of cytolytic T cells that was observed following peptide stimulation or polyclonal activation. Alternatively, it is possible that tetramer staining by itself led to clonal deletion of high-affinity A2/Melan-A+ CD8+ T cells from HLA-A2− UCB, as highly Ag-sensitive CTL can be killed by A2/Melan-A tetramers (53, 54). These two models are compatible with the lower levels of clonal diversity that were observed in expanded A2/Melan-A+ CD8+ T cells isolated from HLA-A2− UCB following stimulation with cognate peptide. Taken together, these results underscore fundamental differences between A2/Melan-A+ CD8+ T cells from HLA-A2+ and HLA-A2− UCB in terms of antigenic specificity, activation threshold, and function.

In summary, functional properties of UCB-derived Ag-specific CD8+ T cells that were documented for the first time in the current study (differentiation geared toward EMRA-E, reduced frequency of cytolytic and IFN-γ-producing T cell clones, and differential polyfunctionality profiles) provide new insights into UCB immunobiology and offer a rationale for the higher incidence of OIs and comparatively low rates of acute and chronic GVHD observed following UCBT, particularly in the face of HLA mismatch (55, 56). As components of posttransplant immunotherapeutic regimens, UCB-derived CD8+ T cells could potentially be used to alleviate unfavorable outcomes associated with UCBT, including failure of engraftment, leukemic relapse, GVHD, and OIs.

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Disclosures
The authors have no financial conflicts of interest.

References


