T lymphocytes of recipient origin may contribute to the recovery of specific immune response toward viruses and fungi in children undergoing cord blood transplantation

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Patients undergoing allogeneic cord blood transplantation (CBT) benefit from a low risk of graft-versus-host disease (GVHD), but there are still concerns that they be able to recover an effective immune capacity early after transplantation. We investigated the ability to develop in vitro T-lymphocyte–mediated immune response toward human cytomegalovirus and *Candida albicans* antigens, early and late after transplantation, in children given cord blood transplants from either an HLA-identical sibling or an unrelated donor. Proliferative capacity and frequency of antigen-specific T cells were evaluated; antigen-specific CD4$^+$ T-cell clones were also generated and characterized for T-cell receptor repertoire diversity, cytokine phenotype, and their origin (either from donor or patient). We found that the majority of recipients can develop a specific response to viral or fungal antigens already early after transplantation. Antigen-specific T-cell clones of both donor and recipient origin contributed to the reconstitution of immune response. Antigen-specific T lymphocytes of recipient origin were detected in patients receiving a transplant from a relative, after a chemotherapy-based conditioning regimen, and who did not have GVHD. Our results document, at a clonal level, that after CBT recovery of either polyclonal or paucicional T-cell response toward widespread pathogens is prompt, with some patients benefiting from a contribution of recipient-derived cells. (Blood. 2004;103:4322-4329)

Introduction

Allogeneic cord blood transplantation (CBT), especially from unrelated donors, has progressively become an extensively used treatment for patients with both malignant and nonmalignant disorders.[1-7] As compared to bone marrow transplantation (BMT), advantages of CBT include ease and safety of cell collection, low risk of transmitting viral infections, prompt availability of stem cells, when an unrelated donor is used, as well as reduced incidence and severity of graft-versus-host disease (GVHD).[2-7] Various immunologic properties and peculiarities of cord blood (CB) may contribute to the reduction of GVHD after CBT.[8-11] In particular, it is well known that cord blood lymphocytes (CBLs) are naive cells,[8-11] with low T-cell–mediated cytotoxic capacity[12] and, in vitro, markedly reduced responsiveness to allogeneic stimuli in secondary mixed lymphocyte reaction (MLR).[13]

Several studies have demonstrated that early recovery of the immune system after hematopoietic stem cell (HSC) transplantation is predominantly sustained by peripheral expansion of mature T and B lymphocytes transferred with the graft.[14-18] Therefore, lack of sustained transfer of antigen-experienced lymphocytes of donor origin may, in theory, expose recipients of CB transplants to a slow recovery of immune response and, consequently, to an increased risk of infectious complications.

Indeed, in CB transplant recipients several studies have documented a remarkable incidence of early infectious complications, accounting for most transplant-related deaths, especially in adults.[3-5,7,19-22] Nevertheless, previously published reports have shown that, after CBT, recovery of T- and B-lymphocyte and natural killer (NK) cell numbers, as well as of the lymphocyte proliferative response to polyclonal T-cell activators and of NK activity is prompt and at least as efficient as that observed after BMT.[23-27] However, all these studies have investigated the overall immune reconstitution of recipients of CB transplants, but no study has specifically focused on the recovery of immune response toward a specific pathogen. Taking into account that CB transplant recipients cannot benefit from the contribution of the transfer of antigen-experienced cells, it is conceivable to hypothesize that their potential to effectively face infectious complications may be strictly related to the ability of the recovering immune system to promptly develop a donor-derived primary immune response. On the other hand, it has been shown previously that, in a situation where early recovery of mature donor T lymphocytes is hindered, such as that experienced by recipients of T-cell–depleted bone marrow (BM) transplants, T lymphocytes of recipient origin may provide a significant contribution to immune reconstitution.[28] In

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view of this observation, we reasoned that T lymphocytes of recipient origin, escaping the conditioning regimen, could contribute to early immune recovery also for patients undergoing CBT.

The aims of the present study were to evaluate the ability of recipients of CB transplants to develop an in vitro immune response toward 2 widespread pathogens, namely, human cytomegalovirus (HCMV) and *Candida albicans* (CA), both early (3-5 months) and late (12-15 months) after transplantation and to define the origin, either donor or patient, of T cells contributing to the immune response.

Patients and methods

Patients

Sixteen consecutive children given CB transplants from either an HLA-identical sibling (CBT-sib; 11 patients) or an unrelated donor (CBT-ud; 5 patients) were enrolled in this study. All children received the transplant at the Pediatric Hematology and Oncology Unit, IRCCS Policlinico San Matteo (Pavia, Italy) between June 1997 and January 2001. In that period, 6 more patients given either an unrelated (5 patients) or a related (1 patient) donor CB transplant were to be enrolled in this study. However, 3 of these patients died in the first 2 months and 3 in the third month after the allograft and were not, therefore, included in this analysis. The causes of death of these patients were acute GVHD (aGVHD; 3 cases), septic shock (1 case), disease recurrence (1 case), and idiopathic interstitial pneumonia (1 case). The Institutional Review Board of the Department of Pediatrics, IRCCS Policlinico San Matteo, approved the study protocol. Parents of both patients and controls gave their written informed consent to the study. For the purposes of this study, all patients were followed at least up to 12 to 15 months after the allograft. Details on patient and donor characteristics, as well as on the number of cells infused and HLA compatibility, are reported in Table 1.

HLA class I antigen serologic typing and low-resolution generic oligotyping of DRB1 antigens was performed in all donor/recipient pairs, whereas high-resolution molecular typing of HLA class II DRB1 was available for all children receiving a CB transplant from an unrelated donor. Characterization of HLA antigens or alleles was performed as previously described.[5,25,29]

In CBT-sib recipients, GVHD prophylaxis consisted of cyclosporine A alone (Cs-A, 1-3 mg/kg/d intravenously for the first 28 days and then orally at a dosage of 3-5 mg/kg/d for 4-6 further months), whereas CBT-ud recipients received a combination of Cs-A, steroids (2 mg/kg/d from day −2 to day +21 and then at progressively reduced dosage until discontinuation, in the absence of GVHD, within 6 weeks after CBT), and antithymocyte globulin (ATG; 3.5 mg/kg on days −3 and −2).

All patients with thalassemia, malignant osteopetrosis, neuroblastoma, and juvenile myelomonocytic leukemia were given a preparative regimen consisting of busulfan (16 mg/kg given from day −10 to day −7), thiotepa (10 mg/kg in 2 divided doses on day −6), and either cyclophosphamide (120 mg/kg from day −4 to day −3) or fludarabine (40 mg/m^2/d from day −6 to day −3). In children with acute lymphoblastic leukemia, the preparative regimen consisted of fractionated total-body irradiation (TBI; 12 Gy over 6 fractions from day −8 to day −6), thiotepa (10 mg/kg in 2 divided doses on day −5), and cyclophosphamide (120 mg/kg from day −4 to day −3). The child with Fanconi anemia was prepared for the allograft with a regimen including TBI (6 Gy over 3 fractions from day −8 to day −6) and low-dose cyclophosphamide (300 mg/m^2/d for 4 consecutive days from day −5 to day −2), whereas the patient with dyskeratosis congenita was prepared with a combination of low-dose cyclophosphamide (300 mg/m^2/d for 4 consecutive days from day −6 to day −3) and fludarabine (30 mg/m^2/d for 4 consecutive days from day −6 to day −3), as previously reported.[30]

With the aim of accelerating myeloid recovery, recombinant human granulocyte colony-stimulating factor (rHuG-CSF; 5-8 μg/kg/d) was administered after transplantation to all patients, starting in the first week after the allograft.

To prevent endogenous reactivation of herpes simplex virus (HSV) and human HCMV infection, patients received prophylactic intravenous acyclovir at a dose of 30 mg/kg/d from day −2 to day +30 after CBT. HCMV infection or reactivation was diagnosed on the basis of the positivity of pp65 antigenemia or quantitation of HCMV DNA performed on blood samples. Patients with reactivation of HCMV infection were treated, according to a pre-emptive therapy strategy, with either ganciclovir or foscarnet, as previously reported.[31] In detail, therapy was started in the presence of either equal to or more than 2 pp65$^+$ leukocytes/2 × 10^5 examined or a single positive leukocyte confirmed the following day or in the presence of more than 100 copies of HCMV DNA. Using this approach, no patient experienced HCMV disease. All children were given antifungal prophylaxis with fluconazole (6 mg/kg/d starting on day −2) until the neutrophil count

Table 1. Patient characteristics

Patient no.	Age at CBT	Sex	Diagnosis	No. of HLA mismatches	TBI	No. of cells infused, × 10^7/kg	Pre-CBT patient HCMV serology	aGVHD (grade)	Chimerism status	
									3-5 mo after CBT	12-15 mo after CBT
CBT-sib										
1	8 y	F	Thal	NA	No	2.7	Positive	Yes (II)	CC	CC
2	5 y	M	Thal	NA	No	4	Positive	No	CC	CC
3	3 y	F	NBL	NA	No	14	Positive	No	CC	CC
4	7 y	M	Thal	NA	No	2.1	Positive	No	MC	MC
5	10 y	F	FA	NA	No	1.4	Positive	No	CC	CC
6	4 y	M	Thal	NA	No	6	Positive	No	MC	MC
7	6 y	F	Thal	NA	No	2.5	Positive	No	MC	MC
8	13 y	F	Thal	NA	No	4	Positive	No	MC	MC
9	19 y	F	Thal	NA	No	2.6	Positive	No	MC	MC
10	10 y	M	DC	NA	No	2.2	Positive	No	MC	MC
11	4 y	F	ALL	NA	Yes	3.2	Negative	No	CC	CC
CBT-ud										
12	5 y	M	ALL	1 class II	Yes	9.7	Positive	Yes (II)	CC	CC
13	5 mo	F	Osteop	0	No	13	Negative	Yes (II)	CC	CC
14	5 y	M	ALL	0	Yes	4	Positive	Yes (I)	CC	CC
15	5 y	M	ALL	1 class I	Yes	4.7	Negative	No	CC	CC
16	2 y	M	JMML	1 class I	No	5.2	Positive	Yes (II)	CC	CC

Thal indicates thalassemia; NA, not applicable; CC, complete donor chimerism; NBL, neuroblastoma; MC, mixed donor/recipient chimerism; FA, Fanconi anemia; DC, dyskeratosis congenita; ALL, acute lymphoblastic leukemia; Osteop, osteopetrosis; and JMML, juvenile myelomonocytic leukemia.

exceeded a value of 2×10^9/L. Empirical antifungal treatment with either conventional or liposomal amphotericin B was used in all children who developed fever not responsive to broad-spectrum antibiotic therapy of at least 72 hours' duration. A commercial immunoglobulin preparation was used at a dosage of 400 mg/kg once weekly for the first 70 days after transplantation.

Acute and chronic GVHD were graded according to previously published criteria.[32,33] GVHD was usually treated with steroids as first-line therapy, whereas extracorporeal photochemotherapy was used in patients with steroid-resistant GVHD.[34]

In vitro immune response toward HCMV and CA was evaluated both early (3-5 months) and late (12-15 months) after transplantation.

Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Lymphoprep; Nycomed Pharma, Oslo, Norway) density gradient centrifugation and used on the same day of collection or cryopreserved for later use.

Surface marker analysis

The monoclonal antibodies (mAbs) used in this study included: fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)–labeled anti–Leu-4 (CD3), anti–Leu-3a (CD4), anti–Leu-2a (CD8), anti–Hle-1 (CD45) from BD Biosciences (Mountain View, CA), and anti-TCRVβ families (Valter Occhiena, Torino, Italy). Phenotypic study of cell populations was performed as previously described.[25]

Mitogen- or antigen-induced proliferation assays

The medium used was RPMI 1640 (Gibco, Life Technologies, Paisley, United Kingdom) supplemented with 2 mM L-glutamine, 50 μg/mL gentamicin, 1% nonessential amino acids (Gibco), and 5% pooled human serum (RPMI-HS). The T-cell polyclonal activator used was a purified anti-CD3 (OKT3; Ortho, Raritan, NJ) mAb. Anti-CD3–induced T-lymphocyte activation was evaluated as previously described.[25] HCMV and CA antigens were prepared by the Viral Diagnostic Service of IRCCS Policlinico San Matteo (Pavia, Italy). PBMCs (1×10^5/200 μL), resuspended in RPMI-HS were cultured, in triplicate, in round-bottom 96-wells microplates at 37°C in a 5% CO_2 humidified atmosphere. Heat-inactivated HCMV antigen was added at 1:1000 dilution; alternatively, 20 000 heat-inactivated CA organisms per well were added to the cultures. After a 7-day incubation at 37°C in a humidified 5% CO_2 atmosphere, ^{3}HTdR (0.5 μCi/well [0.185 MBq/well]; Amersham Pharmacia Biotec, Milano, Italy) incorporation was measured, during the last 21 hours, by standard procedure. Results are expressed as stimulation index (SI; cpm of stimulated cultures/cpm of unstimulated cultures). The proliferative response was considered as positive when SI was more than 2 and net cpm more than 3000.

Antigen-specific T-cell lines and T-cell clones

To obtain antigen-specific T-cell lines (TCLs) from short-term culture, patients' PBMCs were added to flat-bottom 96-well plates at a concentration of 2×10^6/mL, in a final volume of 0.2 mL RPMI-HS, in the presence of heat-inactivated HCMV or CA, at 37°C in a 5% CO_2 humidified atmosphere. Then, 20 U/mL recombinant interleukin 2 (rIL-2; Cetus, Emeryville, CA) was added after 5 to 7 days of culture. After 10 days, TCLs were cloned, by limiting dilution (0.3 cells/wells), in the presence of irradiated (30 Gy) allogeneic PBMCs, phytohemagglutinin (PHA-L 8 μg/mL; Boehringer Mannheim, Mannheim, Germany), and rIL-2 (200 U/mL), using a previously described method.[35] As a control, CA- or HCMV-specific T-cell clones (TCCs) were also prepared from PBMCs of 3 healthy immunocompetent adult volunteers (control nos. 17, 18, and 19), who had previously encountered both antigens. TCCs were screened for specificity by testing their capacity to selectively proliferate in response to antigen-pulsed autologous irradiated (30 Gy) PBMCs, used as the antigen-presenting cell (APC) in a 3-day proliferation assay. TCCs were defined as specific on the basis of their ability to proliferate in response to stimulation with CA- or HCMV-pulsed APCs, but not with unpulsed APCs.

Limiting dilution assays

To evaluate frequencies of antigen-specific proliferating T-cell precursors (PTCps), escalating numbers of responder PBMCs (from 4×10^4 down to 1.25×10^3) were cultured in RPMI-HS medium, in 96 round-bottom well microplates, with 2×10^4 irradiated (30 Gy) autologous PBMCs as feeder cells and heat-inactivated CA or HCMV antigen, in a total volume of 200 μL. Twenty-four replicates for each cell concentration were seeded. Twelve control wells, containing responder and feeder cells without antigen, were assayed for each dilution of responder cells. The cultures were incubated at 37°C in 5% CO_2 for 10 days. RPMI-HS medium (25 μL) containing a final concentration of 5 U/mL rIL-2 was added to each well on day 7. Incorporation of ^{3}HTdR was measured during the last 21 hours. Assay wells were considered positive if proliferation exceeded the average plus 3 SD of the control wells. The frequency of antigen-specific PTCps was determined by maximum likelihood estimation, using statistical software. Results were expressed as number of PTLp/10^6 input cells. Effector cells, derived from pooled positive wells of the limiting dilution assay (LDA), were also cloned, by limiting dilution (0.3 cells/well), to obtain antigen-specific TCCs.

PCR for evaluation of chimerism and identification of TCRVβ families

DNA was isolated from PBMCs of patients and donors before CBT, from PBMCs of patients at different intervals after transplantation, and from cells recovered from antigen-specific TCLs and TCCs. Chimerism was evaluated by a polymerase chain reaction (PCR)–based assay, by analyzing selected polymorphic short tandem repeat (STR) loci. To identify an informative marker for donor/patient genotype discrimination, a set of 5 STR systems, namely *HumFGA* (GenBank accession no. G33478), *HumvWA* (GenBank accession no. M25716), *HumTH01* (GenBank accession no. D00269), *HumLPL* (GenBank accession no. D83550), and *HumCD4* (GenBank accession no. M86525) was used, together with the commercial kit AmplFl Identifiler PCR amplification kit (PE Applied Biosystems, Foster City, CA). The commercial kit amplifies, in multiplex PCR, the 15 STR loci *D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA,* and the sex determination marker *Amelogenin*. DNA (4 ng) was amplified through 28 PCR cycles, according to the manufacturer's specifications and 0.2 μL of each amplified sample was analyzed by capillary electrophoresis using an ABI Prism 310 Genetic Analyser (PE Applied Biosystems); alleles were scored using the Genotyper Software version 2.1 (PE Applied Biosystems). Quantification of the degree of mixed chimerism (calculated as percent of recipient DNA) was performed by analyzing the proportion of the fluorescent peak areas corresponding to donor and patient genotypes, according to Thiede et al.[36]

Alternatively, after DNA extraction and PCR, the separation of the products was performed by polyacrylamide gel electrophoresis. PCR-amplified patterns were analyzed in visible light, after silver staining and compared with sequenced allelic ladders.[37]

PCR for identification of T-cell receptor (TCR) Vβ families was conducted as previously described.[37] Briefly, total cytoplasmic RNA was extracted from HCMV- or CA-specific TCCs, reverse transcribed into cDNA, and amplified by PCR using oligonucleotides specific for several human TCRVβ families (Vβ4, Vβ9, Vβ10, Vβ15) and for Cβ.[38]

Cytokine detection

For intracytoplasmatic cytokine detection at the single-cell level, TCCs were stimulated with 25 ng/mL phorbol-12-myristate,13-acetate (PMA; Sigma, Milano, Italy) plus 1 μg/mL ionomycin (Sigma) for 4 hours in the presence of brefeldin A (10 μg/mL; Sigma). Cells were fixed, permeabilized with Fix and Perm solution (Caltag Laboratories, Valter Occhiena), and stained with FITC–IL-2-specific, FITC-interferon γ (IFN-γ)–specific,

PE–IL-4-specific, or PE–IL-10-specific mAb (BD Biosciences), following the manufacturer's instructions.

On the basis of their capacity to produce cytokines, TCCs were classified as follows: (1) T_{H1}-dominant TCCs, including a prevalence (≥ 40%) of IFN-γ–producing cells; (2) T_{H2}-dominant TCCs, including a prevalence (≥ 40%) of IL-4–producing cells; (3) nonpolarized T_H (NP-T_H) TCCs, including a high number (≥ 75%) of IL-2–producing and less than 40% of IFN-γ– or IL-4–producing cells.

Results

Hematopoietic reconstitution and T-cell subset recovery

All patients enrolled in the study had sustained recovery of both polymorphonuclear (PMN) and platelet (PLT) counts. The median time needed to achieve PMN recovery was 23 days (range, 16-29 days). A self-sustained PLT count exceeding 50×10^9/L occurred at a median time of 67 days (range, 38-89 days) after CBT. Details on aGVHD occurrence are reported in Table 1. Only one patient (no. 14) had limited chronic GVHD. Cs-A and steroid therapy was discontinued within 6 to 12 months after transplantation, patients with nonmalignant disorders being those who received immunosuppressive therapy for a longer period (data not shown). Details on donor/recipient chimerism at early (3-5 months) and late (12-15 months) evaluation are also shown in Table 1.

At early evaluation, 3 to 5 months after CBT, the absolute number (number of cells/μL) of $CD3^+$ and of $CD4^+$ T lymphocytes in all patients receiving transplants was lower than in age-matched healthy controls (data not shown). $CD8^+$ T cells were in the normal range and lower than those of age-matched controls in 9 and 7 patients, respectively. In the late posttransplantation period, numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were within the normal range in all patients. All patients receiving transplants had already recovered the capacity to proliferate in response to anti-CD3 mAb, when tested 3 to 5 months after transplantation.

Antigen-specific proliferative T-lymphocyte response

HCMV-induced proliferation in bulk culture was assessed in the 4 patients who had experienced posttransplantation virologic evidence of HCMV reactivation within 3 months from CBT and in 8 patients who did not, whereas CA-specific response was tested in all patients receiving transplants. Between 3 and 5 months after transplantation, HCMV-specific proliferation was observed in 7 of 8 CBT-sib recipients and in 1 of 4 CBT-ud recipients (Table 2). HCMV-specific positive proliferation was observed in 3 of 4 recipients of CB transplants with evidence of virus reactivation and in 5 of 8 without evidence of HCMV reactivation within the first 3 months from CBT. CA-specific proliferation was detected in 6 of 11 CBT-sib recipients and in only 1 of 5 CBT-ud recipients. PBMCs from 9 of 12 CB transplant patients, tested 12 to 15 months after transplantation, displayed HCMV-specific lymphocyte proliferation and PBMCs from 11 of 16 children tested showed proliferation in response to CA (Table 2). The type of donor used did not influence this late proliferative response.

Frequency of antigen-specific PTLps was assessed, by LDA, both at 3 to 5 and at 12 to 15 months after CBT, in PBMCs of 9 patients. Results demonstrated a measurable frequency of PTLps, specific for either HCMV or CA, in 8 of these 9 patients, including most of those whose PBMCs were unable to display detectable proliferative activity in response to the antigen in bulk culture. As compared to 12 age-matched controls, immunocompetent toward both HCMV and CA, frequencies of antigen-specific PTLps were in the normal range, or even higher, in patients' PBMC samples displaying a positive antigen-specific proliferation in bulk culture and mostly below normal range, but still detectable, in PBMC samples with absent antigen-specific proliferation in bulk culture (Table 2).

Donor/recipient origin of antigen-specific TCLs and TCCs

Antigen-specific TCLs (6 patients) or pooled positive wells obtained from LDA (2 patients) were evaluated for chimerism,

Table 2. Antigen-specific proliferative response in bulk culture and frequency of antigen-specific PTLp in LDAs

		3-5 mo after CBT				12-15 mo after CBT			
		HCMV		CA		HCMV		CA	
Patient no.	HCMV reactive	Bulk PR	LDA F	Bulk PR	LDA F	Bulk PR	LDA F	Bulk PR	LDA F
CBT-sib									
1	Yes	+	ND	–	ND	+	ND	+	ND
2	Yes	+	ND	+	ND	+	ND	+	ND
3	No	ND	ND	–	ND	ND	ND	–	ND
4	No	ND	ND	+	ND	ND	ND	+	ND
5	No	ND	ND	+	ND	ND	ND	+	ND
6	No	+	40	+	76	+	77	+	76
7	No	+	80	–	15	+	160	+	210
8	No	+	125	+	111	+	313	+	365
9	No	+	325	–	11	+	350	+	380
10	No	+	550	–	12	+	520	+	90
11	No	–	15	+	48	–	12	–	16
CBT-ud									
12	No	–	ND	–	ND	+	ND	–	ND
13	No	ND	ND	+	ND	ND	ND	+	ND
14	Yes	+	62	–	19	+	66	+	125
15	No	–	–	–	–	–	–	–	–
16	Yes	–	12	–	43	–	12	–	6
Control ranges	NA	+	35-450	+	26-350				

Control values were obtained from 12 age-matched healthy controls. A positive (+) PR is SI > 2 and net cpm > 3000; a negative PR (–) is SI < 2 and net cpm < 3000. PR indicates proliferative response; F, frequency (expressed as PTLp/10^6 input cells); and ND, not done.

Table 3. Donor/recipient chimerism status of antigen-specific TCLs

Patient no.	3-5 mo after CBT			12-15 mo after CBT		
	PBMC	HCMV-TCL	CA-TCL	PBMC	HCMV-TCL	CA-TCL
CBT-sib						
6*	MC 15% R	MC 59% R	MC 49% R	MC 10% R	MC 46% R	MC 34% R
7†	MC 20% R	MC 28% R	ND	MC 10% R	MC 33% R	MC 19% R
8†	MC 8% R	CC	MC 10% R	MC 8% R	CC	MC 7% R
9*	MC 27% R	MC 89% R	ND	MC 18% R	MC 95% R	ND
10†	MC 25% R	MC 50% R	ND	MC 35% R	MC 70% R	ND
11†	CC	CC	CC	CC	CC	CC
CBT-ud						
14†	CC	CC	CC	CC	CC	CC
16†	CC	CC	CC	CC	CC	CC

MC indicates mixed donor/recipient (D/R) chimerism; ND, not done; and CC, complete donor chimerism.
*T-cell populations obtained from pooled positive wells of LDA.
†TCLs obtained after short-term culture.

to define the proportion of DNA of donor/recipient origin contained in cell populations that were conceivably enriched in HCMV- or CA-specific T cells. As shown in Table 3, the majority of TCLs and samples of pooled positive LDA wells, derived from PBMCs from 5 of 6 tested CBT-sib recipients and displaying mixed donor/recipient chimerism, included a proportion of DNA of recipient origin greater than that of the initial PBMC population. In particular, enrichment in recipient DNA was striking in cells obtained by pooling positive LDA wells of patient no. 9. TCLs derived from PBMCs of one CBT-sib recipient and of 2 CBT-ud recipients, displaying complete donor chimerism, were all of donor origin.

Lymphocytes recovered from 17 antigen-specific TCLs and from 4 samples of pooled positive LDA wells were cloned by limiting dilution. Antigen-specific TCCs were obtained from all samples tested (data not shown). HCMV-specific TCCs were obtained from antigen-specific TCLs, derived from short-term culture of samples from 2 CB transplant recipients who had experienced viral reactivation, and from pooled positive LDA wells of 2 children with no virologic evidence of HCMV reactivation. CA-specific TCCs were obtained from antigen-specific TCLs, derived from short-term culture of samples from 4 patients whose PBMCs displayed antigen-specific proliferative activity in bulk culture, and from TCLs of 2 children whose PBMCs were negative for CA-specific proliferation in bulk culture. All these antigen-specific TCCs were $CD3^+/CD4^+$ lymphocytes.

Table 4. Recipient/donor origin of antigen-specific TCCs derived from antigen-specific TCLs or from pools of positive wells recovered from LDAs

Patient no.	3-5 mo after CBT		12-15 mo after BMT	
	HCMV	CA	HCMV	CA
CBT-sib				
1*	0/6	0/2	0/1	0/2
2*	1/10	0/1	2/3	0/1
3*	ND	1/62	ND	2/13
4*	ND	7/4	ND	4/1
5*	ND	0/51	ND	0/47
6†	6/8	ND	4/8	ND
9†	15/0	ND	14/0	ND
CBT-ud				
12*	ND	ND	0/12	ND
13*	ND	0/32	ND	0/16

Number of TCCs of recipient/donor (R/D) origin is reported for the indicated time points. ND indicates not done.
*Cells from antigen-specific TCLs of 7 patients.
†Cells from pools of positive wells recovered from LDAs of 2 patients.

TCCs obtained from all patients were analyzed for donor/recipient origin. As shown in Table 4, TCCs of recipient origin were isolated, both at 3 to 5 and at 12 to 15 months after transplantation, from PBMCs from 5 of 7 CBT-sib recipients. In particular, the majority of TCCs obtained from patient no. 4 and all TCCs obtained from patient no. 9 were of recipient origin and 6 TCCs of recipient origin were also obtained from patients no. 2 and no. 3, whose starting PBMCs displayed complete donor chimerism. Sixty TCCs obtained from patients given an unrelated donor CB transplant were all of donor origin.

The TCRVβ usage of antigen-specific TCCs, obtained from 7 patients and 3 healthy individuals, was investigated by either cytofluorimetric analysis, using a panel of TCRVβ-specific mAbs, or by PCR analysis, using oligonucleotides specific for TCRVβ families (Table 5). TCCs obtained from the 3 healthy individuals displayed a polyclonal TCRVβ repertoire, each donor including 6 to 7 different TCRVβ families. TCCs obtained from 5 patients displayed a pauciclonal repertoire, each patient including only 2 different TCRVβ families, whereas TCCs obtained from the remaining 2 patients displayed a TCRVβ diversity comparable to that observed in healthy individuals.

Cytokine production of TCCs

Forty-seven (43 CA-specific and 4 HCMV-specific) TCCs obtained from PBMCs of CB transplant recipients were evaluated for their capacity to produce IL-2, IFN-γ, IL-4, and IL-10, in response to short-term activation with PMA and ionomycin. The same kind of evaluation was also performed on 29 (20 CA-specific and 9 HCMV-specific) TCCs derived from the 3 healthy controls. Results obtained from controls showed that stimulation with CA is able to induce in vitro the expansion of 3 different types of TCCs, which can be defined, on the basis of their cytokine phenotype, as T_{H1}-dominant, T_{H2}-dominant, or NP-T_H.[39,40] Only 9 HCMV-specific TCCs from controls were evaluated; 4 of them displayed a T_{H1}-dominant, 2 a T_{H2}-dominant, and 3 a NP-T_H phenotype. Evaluation of CA- or HCMV-specific TCCs derived from recipients of CB transplants demonstrated that, as in control individuals, stimulation of PBMCs with the relevant antigens was able to induce the expansion of all the different subsets of T_H cells (Table 6).

Discussion

The results of this study show that the majority of children given a CB transplant are able to mount a T-lymphocyte response specific for viral or fungal antigens, already at 3 to 5 months after

Table 5. TCRVβ families identified in antigen-specific TCC of recipient or donor origin or from healthy controls

Patient/control no.	No. of TCCs tested	R/D origin	No. of TCC-TCRVβ families identified
CBT-sib			
2			
3-5 mo after CBT	11	D	7-Vβ2; 4-Vβ21
3			
3-5 mo after CBT	62	D	28-Vβ2; 9-Vβ12; 13-Vβ13; 12-Vβ24
12-15 mo after CBT	13	D	2-Vβ1; 3-Vβ6; 2-Vβ8; 6-Vβ13
4			
3-5 mo after CBT	7	R	4-Vβ8; 3-Vβ13
5			
3-5 mo after BMT	51	D	42-Vβ2; 9-Vβ8
12-15 mo after CBT	47	D	37-Vβ2; 10-Vβ3
9			
3-5 mo after CBT	15	R	12-Vβ13; 3-Vβ2
12-15 mo after CBT	14	R	10-Vβ13; 4-Vβ6
CBT-ud			
12			
12-15 mo after CBT	12	D	7-Vβ1; 5-Vβ21
13			
3-5 mo after CBT	32	D	12-Vβ1; 7-Vβ13; 7-Vβ21; 6-Vβ23
12-15 mo after CBT	16	D	11-Vβ1; 2-Vβ5; 3-Vβ8
Control			
17	16	NA	4-Vβ1; 2-Vβ3; 2-Vβ5; 3-Vβ8; 3-V β13; 2-V β19
18	29	NA	6-Vβ2; 5-Vβ3; 5-Vβ5; 4-Vβ6; 5-V β13; 4-V β19
19	68	NA	10-Vβ1; 11-Vβ2; 10-Vβ8; 9-Vβ12; 9-Vβ17; 10-Vβ20; 9-Vβ22

NA indicates not applicable.

transplantation, and that HCMV- or CA-specific T lymphocytes, of both donor and recipient origin, contribute to the reconstitution of this antigen-specific immune response. HCMV and CA antigens were chosen for in vitro evaluation of immune response, reasoning that, in the first weeks after transplantation, immunocompromised recipients have a high risk of experiencing both HCMV reactivation and CA infection. Consequently, in vitro evaluation of T-cell–mediated response toward HCMV and CA should be a useful tool for investigating the capacity of CB transplant recipients to develop in vivo a donor-derived primary immune response or a recipient-derived memory response. Early reconstitution of antigen-specific T-cell–mediated immune response was particularly effective in patients given CB transplants from an HLA-identical family donor, because circulating HCMV- or CA-specific T cells were documented in all these patients, but only in 3 of 5 children given CB transplants from an unrelated donor. However, the number of patients given an unrelated donor CB transplant was too low, if compared with those receiving the transplant from an HLA-identical sibling, to draw any definitive conclusion on this point. Further studies on a larger number of unrelated donor CB transplant recipients are warranted.

Table 6. Intracytoplasmatic cytokine detection in HCMV- or CA-specific TCCs stimulated in vitro with PMA and ionomycin

Patient/control no.	Total TCCs, no.*	T_{H1}-dominant TCCs, no.	T_{H2}-dominant TCCs, no.	NP-T_H TCCs, no.
HCMV-specific TCC				
CBT-sib				
2, 12-15 mo after BMT	1	1†	0	0
CBT-ud				
12, 12-15 mo after BMT	3	1	2	0
Control				
17	2	0	1	1
18	1	0	0	1
19	6	4	1	1
CA-specific TCC				
CBT-sib				
3, 3-5 mo after BMT	15	8‡	2	5
4, 3-5 mo after BMT	7	7§	0	0
5, 12-15 mo after BMT	9	8	1	0
CBT-ud				
13, 3-5 mo after BMT	12	0	12	0
Control				
17	8	2	2	4
18	6	1	4	1
19	6	2	2	2

*Number of TCCs tested.
†TCCs of recipient origin.
‡One of 8 TCCs was of recipient origin.
§Five of 7 TCCs were of recipient origin.

Our data on the capacity of patients given CB transplants to promptly recover T cells specific for viral or fungal antigens support previously reported results, indicating a post-CBT recovery of T-lymphocyte number and a response to polyclonal activators, at least comparable to that observed in BM transplant recipients and characterized by a fully reconstituted TCR repertoire.[25,27] In particular, it is worth considering that HCMV-specific T lymphocytes were measurable even in peripheral blood of 6 recipients of CB transplants who, monitored at least weekly through evaluation of pp65 antigenemia or HCMV DNA during the first 3 months from transplantation, had not experienced virus reactivation. This last observation suggests that, as it occurs in immunocompetent individuals, the immune system of some CB transplant recipients controls HCMV reactivation before the virus reaches detectable levels of pp65 antigenemia or HCMV DNA in peripheral blood.

The presence of a donor-derived antigen-specific T-cell response early after transplantation suggests that CBT recipients can promptly develop an effective primary immune response in vivo. This may depend on the presence of a high proportion of lymphoid progenitors in CB, as well as on a low incidence and severity of

GVHD, which is known to play a detrimental role on immune reconstitution. Both these factors may compensate for the lack of adoptive transfer of antigen-experienced T lymphocytes.

HCMV- and CA-specific T cells of recipient origin were isolated, both early and late after transplantation, in 8 of 16 CB transplant recipients, who had not received TBI and did not experience GVHD. This observation prompted us to hypothesize that the persistence of residual recipient T cells after CBT is mainly favored by 3 factors: (1) the low number and, especially, the naive status of CB T lymphocytes transferred with the graft; (2) avoidance of TBI in the conditioning regimen, because radiotherapy may be more effective than chemotherapy in the eradication of lymphoid recipient cells; and (3) absent or low-grade GVHD, because alloreactive donor cells responsible for GVHD may induce lysis or apoptosis of recipient lymphocytes escaping the preparative regimen. Notably, antigen-specific T lymphocytes of recipient origin were detected only in patients given CB transplants from an HLA-identical family donor and not in those given transplants from an unrelated donor. However, among the 5 children given CB transplants from an unrelated donor, 3 had received TBI as part of the myeloablative regimen and 2 experienced grade II aGVHD. Thus, it cannot be excluded that, in some cases, T cells of recipient origin may also expand after unrelated donor CBT.

The finding that recipient T lymphocytes may contribute to immune recovery after CBT is in agreement with a previously reported study on adult patients given a T-cell–depleted BM transplant.[28] In that study, Roux and colleagues demonstrated that when a small number of donor T cells is transferred with the graft, few residual T lymphocytes of recipient origin may expand and, possibly, delay an effective recovery of thymus-dependent donor immunity. Our results indicate that residual T cells of recipient origin were immunocompetent toward widespread pathogens frequently encountered by patients early after transplantation. In 2 patients, TCCs of recipient origin represented the great majority of all isolated TCCs, whereas in the remaining 6 patients TCCs of recipient origin represented only a minority of all antigen-specific TCCs. These findings suggest that in patients given CB transplants, these recipient T cells may usefully contribute to antimicrobial immune defense in the early posttransplantation period and not necessarily hamper donor-derived thymus-dependent immune recovery.

Antigen-specific TCCs, of either donor or recipient origin, derived from peripheral blood from 5 of the 7 patients investigated, displayed a pauciclonal TCRVβ repertoire, whereas TCCs obtained from the remaining 2 patients displayed a polyclonal repertoire, comparable to that observed in healthy individuals. Recent research demonstrated that restoration of extended TCR diversity after transplantation is strictly dependent on the thymus-dependent pathway of T-cell differentiation from hematopoietic progenitors.[17] Our data indicate that antigen-specific T lymphocytes displaying a limited TCR diversity resulted from peripheral expansion of either residual recipient mature T lymphocytes or donor-derived lymphoid progenitors transferred with the graft. Because none of the CB transplant recipients included in the present study experienced HCMV- or CA-related disease and TCCs showed an effective function, it can be hypothesized that recovery of immunity derived from peripheral expansion was effective enough to protect such patients from these infectious agents.

Despite the demonstration, also at a clonal level, that prompt recovery of viral- and fungal-specific T-cell response may occur, infectious complications have been reported to unfavorably affect the outcome of CB transplant recipients, being responsible for a relevant part of transplant-related mortality and morbidity especially for adult patients, who also receive a lower number of donor T cells per kilogram of recipient body weight.[3-5,7,19-22] However, it has to be remarked that all the patients included in the present study were children, most of whom received transplants from an HLA-identical sibling, for a nonmalignant disease. These factors, together with the fact that these patients did not experience severe GVHD, may account for the prompt recovery of T-cell–mediated antigen-specific immune response, which can also take advantage of the contribution of residual recipient T lymphocytes. Altogether, the present results are in agreement with and support recently reported data, demonstrating that a CB transplant from a compatible relative can be a very attractive treatment modality for pediatric patients with nonmalignant disorders.[29]

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T lymphocytes of recipient origin may contribute to the recovery of specific immune response toward viruses and fungi in children undergoing cord blood transplantation

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