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Analysis of immune reconstitution in children undergoing cord blood transplantation

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Objective. **The aim of this study was to investigate and compare immune reconstitution in allogeneic cord blood transplantation (CBT) and bone marrow transplantation (BMT) recipients.**

Patients and Methods. **Twenty-three children underwent CBT from either human leukocyte antigen-identical siblings (11 cases) or unrelated donors (12 cases) were enrolled in the study, together with 23 matched children receiving BMT. Patients were analyzed 2–3 and 12–15 months after transplant. Recovery of T-, B-, and NK-lymphocyte subsets, proliferative in vitro response to mitogens, as well as cytotoxic activities, were investigated.**

Results. **CBT recipients showed a marked increase in the number of B lymphocytes as compared with patients who underwent BMT ($p < 0.001$). The absolute number of $CD3^+$ and $CD8^+$ T cells, as well as the proliferative response to T-cell mitogens, recovered with time after transplantation, irrespective of the source of stem cells used. Recipients of unrelated CBT had a better recovery of $CD4^+$ T lymphocytes ($p < 0.01$). Among patients experiencing acute graft-versus-host disease (GVHD), children given CBT had a much greater production of $CD4^+$ $CD45RA^+$ T cells than BMT recipients ($p < 0.005$). Recovery of NK cell number and innate cytotoxic activities was fast, irrespective of the source of stem cells used.**

Conclusions. **Despite the much lower number of lymphocytes transferred with the graft, recovery of lymphocyte number and function toward normal in CBT recipients was rapid and comparable to that observed after transplantation of bone marrow progenitors. This prompt immune recovery possibly was favored by the reduced incidence and severity of GVHD observed in children who underwent CBT.** **© 2001 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Introduction

Over the past decade, allogeneic cord blood transplantation (CBT), especially from unrelated donors, has progressively become an extensively used treatment for patients with both malignant and nonmalignant disorders [1–5]. As compared to bone marrow transplantation (BMT), advantages of CBT include ease and safety of hematopoietic collection, low risk of viral contamination, prompt availability when an unrelated donor is used, and reduced incidence and severity of graft-versus-host disease (GVHD) [6,7]. Several immunologic properties and peculiarities of cord blood lymphocytes (CBL) may be responsible for the reduction of GVHD after CBT [8]. In fact, CBL are naive; are characterized by a lower frequency of interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α)–producing cells; have been shown to produce lower amounts of proinflammatory cytokines, such as IFN-γ and TNF-α; and display absent or markedly reduced responsiveness to allogeneic stimuli in a secondary mixed lymphocyte reaction (MLR) [9–13]. Moreover, fetal-maternal immune interaction during pregnancy favors the development of immune suppressive functions over helper ones in response to allogeneic stimuli [14–17]. Another property of CBL likely relevant to GVHD development is the preferential activation of natural killer (NK) cells, instead of T lymphocytes, in response to certain viral infections or allogeneic stimuli with noninherited maternal antigens [18,19]. Despite these advantages, the immune features of cord blood have raised concern about the potential posttransplant immune incompetence of

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patients who underwent CBT. Lack of sustained transfer of antigen-experienced T and B lymphocytes of donor origin theoretically might expose CBT recipients to an increased risk of infectious complications. Although immune reconstitution after BMT has been studied extensively [20–24], few reports, all enrolling a limited number of patients, addressed the immune recovery of CBT recipients [25–27].

The aim of this study was to compare early and late immune reconstitution in children who underwent either CBT or BMT from either compatible siblings or unrelated donors (UD). Distribution of T-, B-, and NK-lymphocyte subsets, proliferative response to T-cell mitogens, as well as NK- and lymphokine-activated killer (LAK) activity, were investigated 2–3 and 12–15 months after transplant. Data obtained from CBT patients were compared with those of BMT recipients. Our results demonstrate that, despite the low total number of nucleated cells infused with CBT and the paucity of antigen experience of CBL, early and late immune recovery of CBT recipients is comparable to or even more effective than that of BMT recipients. Patients who underwent CBT had an earlier recovery of both B lymphocytes and $CD4^+$ T cells than age-matched BMT recipients, whereas reconstitution of other lymphocyte subsets and T- and NK-mediated functions was comparable in the two groups.

Patients and methods

Patients

Twenty-three consecutive children underwent CBT from a human leukocyte antigen (HLA)-identical sibling (11 children) or an UD (12 patients), with a minimum observation time of 12 months, were enrolled into this study. All children were transplanted at either the Department of Pediatrics, University of Pavia, or the Department of Pediatrics, University of Torino, in the period between September 1996 and May 1999. Twelve patients were males and 11 females; they had a median age of 7 years (range 5 months to 19 years) and a median body weight of 22 kg (range 5–60). Results obtained in these 23 patients were compared with those of 23 age- and donor-matched BMT recipients transplanted in the same period at the two institutions participating into this study. All patients treated with BMT received an unmanipulated allograft. Details of patient and donor characteristics are listed in Table 1.

The study protocol was approved by the institutional review board of the Department of Pediatrics, IRCCS Policlinico San Matteo.

Cord blood units for unrelated transplants were provided by the cord blood banks in New York (2 cases), Milan (5 cases), and Düsseldorf (5 cases). In the majority of related CBT, cord blood was collected and cryopreserved by the Pavia cord blood bank.

HLA class I antigen serologic typing of patients, related and unrelated bone marrow donors, as well as of cord blood units, was performed by standard NIH microlymphocytotoxicity. Low-resolution generic oligotyping was used for the DRB1 antigens in all donor/recipient pairs. High-resolution molecular typing of HLA class II DRB1 was available for all children receiving the allograft from an unrelated donor. In the 12 patients who underwent unrelated CBT, the donor was HLA identical to the recipient in 3 cases,

Table 1. Patient characteristics

	CBT	BMT	*p* value
No.	23	23	
Sex			
M	12	15	NS
F	11	8	
Age at transplant (years)	7 (0.4–19)	8.5 (1–19)	NS
Donor			
Related	11	11	
Unrelated	12	12	
Diagnosis			
Malignant disorders	8	13	NS
Nonmalignant disorders	15	10	
No. of cells infused ($\times 10^7$/kg)	3.6 (1.4–18)	39 (2.5–100)	<0.0005
Conditioning regimen			
TBI-containing	8	6	NS
BU-containing	15	17	
GVHD prophylaxis			
Cs-A	13	7	<0.005
Cs-A + PDN ± ALG	10	0	
Cs-A + MTX ± ALG	0	16	
Acute GVHD			
Grade 0–I	15	7	<0.05
Grade II–IV	8	16	
Chronic GVHD			
Yes	1	9	<0.005
No	22	14	
rhG-CSF			
Yes	23	14	<0.001
No	0	9	
HCMV reactivation			
Yes	7	7	NS
No	16	16	

TBI = total body irradiation; BU = busulfan; Cs-A = cyclosporine A; PDN = prednisone; ALG = anti-lymphocyte globulin; MTX = methotrexate; rhG-CSF = recombinant human granulocyte colony-stimulating factor; HCMV = human cytomegalovirus.

whereas 5 children were transplanted from a 1-antigen disparate and 4 from a 2-antigen mismatched donor. All unrelated BMT recipients but one, who was transplanted from a DRB1 disparate donor, received the allograft from a compatible donor.

In patients who underwent CBT from a family donor, GVHD prophylaxis mainly consisted of cyclosporine A (Cs-A) alone, whereas the 12 unrelated CBT recipients received a combination of Cs-A and steroids, with or without anti-lymphocyte globulin (ALG, 10 mg/kg on days −3 and −2). In children who underwent BMT from an identical sibling, GVHD prophylaxis mainly consisted of Cs-A alone, whereas patients transplanted from an unrelated donor were given Cs-A, short-term methotrexate, and ALG. Children with malignant and nonmalignant disorders were scheduled to receive Cs-A for a total duration of 4–6 months and 8–12 months, respectively. Additional details of GVHD prophylaxis are listed in Table 1. Established GVHD was treated with steroids.

There was no substantial difference in the preparative regimen of children given CBT or marrow transplantation.

With the aim of accelerating myeloid recovery, recombinant human granulocyte colony-stimulating factor (rhG-CSF) was administered after transplantation to all patients given CBT and to 14 of the 23 BMT recipients.

To prevent endogenous reactivation of herpes simplex virus (HSV) and human cytomegalovirus (HCMV) infection, patients received prophylactic intravenous acyclovir at a dosage of 30 mg/kg/day from day −2 to day +30 after CBT. Patients with reactivation of HCMV infection were treated, according to a preemptive therapy strategy, with ganciclovir or foscarnet. Using this approach, no patient experienced HCMV disease. A commercial immunoglobulin preparation was given at a dosage of 400 mg/kg once weekly for the first 70 days after transplantation.

Complete peripheral blood count was determined daily. White blood cell (WBC) engraftment was defined as the first of 3 consecutive days when neutrophils (polymorphonuclear cells) were >0.5 $\times 10^9$/L and platelet (PLT) engraftment as the first of 3 consecutive days when the unsupported PLT count was >50 $\times 10^9$/L.

To evaluate the chimerism status after transplantation, we used a polymerase chain reaction (PCR) technique amplifying multiallele highly polymorphic loci (microsatellites or short tandem repeats [STRs]) [28]. A panel of five STRs (HumFGA, HumTH01, HumvWA, HumLPL, HumCD4) was used to identify informative polymorphisms, allowing discrimination between donor and recipient before transplantation and, subsequently, determination of the origin of hematopoietic cells after the allograft. In female donor/male recipient pairs, in combination with STRs, we used the sex-discriminating marker amelogenin and the male SRY-specific gene sequence [29]. Chimerism was detected in all donor/recipient pairs through PCR amplification of STRs and Y-chromosome markers, with sensitivity range from 0.1% to 1% (unpublished personal data). The analysis of chimerism was performed in peripheral blood leukocytes and mononuclear cells of all patients given either CBT or BMT. Chimerism was studied in both CBT and BMT recipients 2–3, 6, 9, 12, 18, and 24 months after transplantation (unless clinically indicated at other times).

Collection, processing, and thawing of CB

Cord blood units were collected and processed as previously described [30,31]. Cord blood progenitors were thawed and washed following the procedure described by Rubinstein et al. [31]. Details on the number of mononuclear cells infused in CBT and BMT recipients are listed in Table 1.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation, resuspended in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 2 mM L-glutamine and 50 μg/mL gentamicin (Gibco), 10% fetal calf serum (FCS; Euroclone-Celbio, Milano, Italy) (RPMI-FCS), and used on the same day or cryopreserved for later use.

Surface marker analysis and serum immunoglobulin determination

The monoclonal antibodies (mAb) 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-Leu-11c (CD16), anti-Leu-19 (CD56), anti-Leu-7 (CD57), anti-Leu-1 (CD5), anti-Leu-12 (CD19), anti-Leu-16 (CD20), anti-Leu-20 (CD23), anti-Hle-1 (CD45), anti-Leu-45RA (CD34RA), and anti-Leu-45RO (CD45 RO) (Becton Dickinson, Milano, Italy). Appropriate isotype-matched controls were included. Cell populations were identified on the basis of forward and side scatter analysis; CD45 mAb was used to exclude residual red blood cells. Phenotypic analysis of previously cryopreserved PBMC populations was performed by direct immunofluorescence in two-color analysis using directly labeled antibodies on a FACScan flow cytometer [32]. Data were calculated using CellQuest software (Becton Dickinson).

Serum immunoglobulin determination was measured by nephelometry.

Proliferation assays

T-cell mitogens used were phytohemagglutinin (PHA-L; Boehringer, Mannheim, Germany), concanavalin A (ConA; Pharmacia, Uppsala, Sweden), and anti-CD3 (OKT3; Ortho, Raritan, NJ, USA) mAb. T-lymphocyte activation was obtained by incubating PBMC, seeded in triplicate in flat-bottom microwells, at a concentration of 0.5 $\times 10^6$ cells/mL, with PHA (4 μg/mL), ConA (12.5 μg/mL), and OKT3 mAb (5 ng/mL), respectively, in a final volume of 0.2 mL of RPMI-FCS. After a 3-day incubation at 37°C in a humidified 5% CO_2 atmosphere, ^{3}HTdR (0.5 μCi/well, Amershampharmacia Biotec, Milano, Italy) incorporation was measured during the last 21 hours by standard procedure.

LAK activation, NK activity, and cytotoxicity assays

PBMC were cultured in 24-well plates at a concentration of 2 $\times 10^6$/mL in 2 mL of RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg/mL gentamicin, 10% pooled human sera, and 200 U/mL rIL-2 (Chiron Co., Amsterdam, Holland). After a 5-day incubation at 37°C in a humidified 5% CO_2 atmosphere, cells were recovered, and LAK activity was measured against Daudi target cells as previously described [32].

NK activity of PBMC was assessed, using K562 as target cells, according to a method previously described [32]. Results are expressed as percent of specific lysis.

Data analysis and presentation

Results of the study were analyzed as of May 31, 2000.

Normal distribution of data was evaluated using the Shapiro-Wilk's *W*-test. As all variables resulted normally distributed, data are expressed as mean ± standard deviation (SD) and parametric statistics were used for statistical analysis. Analysis of variance (ANOVA) was used to compare differences in posttransplant values of immunologic reconstitution parameters; post hoc comparisons were performed using LSD test and Scheffé test. Differences in percentages between groups were compared using χ^2 test or Fisher exact test as appropriate according to the number of subjects evaluated.

The following clinical features were considered independent variables: sex, age at transplantation, diagnosis (malignant vs nonmalignant disease), source of stem cells (cord blood vs bone marrow), donor (HLA-identical sibling vs unrelated volunteer), acute and chronic GVHD occurrence, HCMV infection reactivation, rhG-CSF administration after transplantation, and time between transplantation and immunologic evaluation (2–3 months vs 12–15 months).

As dependent variables, we considered the following immunologic parameters: absolute number of CD3$^+$, CD4$^+$ and CD8$^+$ lymphocytes; percentage and absolute number of CD4$^+$ CD45RA$^+$, CD4$^+$ CD45RO$^+$, CD8$^+$ CD45RA$^+$, and CD8$^+$ CD45RO$^+$ cells; absolute number of CD56$^+$ cells; percentage of HLA DR$^+$ cells; absolute number of CD19$^+$ lymphocytes; abso-

lute number of $CD57^+$, $CD16^+$ cells; and proliferative response to PHA, Con-A, and OKT3.

$p < 0.05$ as considered statistically significant; $p > 0.05$ were reported as not significant (NS).

The SAS package (SAS Institute, Cary, NC, USA) was used for analysis of the data.

Results

Hematopoietic recovery and engraftment

All patients engrafted. The median time needed to achieve PMN recovery was 16 days (range 12–24) and 23 days (range 17–32) for BMT and CBT recipients, respectively. Return of PLT count to $>50 \times 10^9$/L occurred at a median of 47 days (range 26–101) for children given BMT and 78 days (range 52–109) for CBT recipients. Incidence and severity of GVHD are reported in Table 1, confirming that children given CBT experience a lower incidence of this complication as compared to BMT recipients, despite the much greater HLA disparity in unrelated CBT donor/recipient pairs. Cs-A and steroid therapy were stopped at a significantly earlier time after CBT than after BMT (data not shown). In the early posttransplant period, 7 of 11 patients who underwent related CBT and all children but one receiving an unrelated CBT achieved complete chimerism. Mixed chimerism was detected in the remaining five children, and it persisted up to 6, 9, 12–15, and 24 months after CBT, respectively. All these patients had nonmalignant disorders (three thalassemia major and one each dyskeratosis congenita and severe combined immune deficiency). Three of the 11 patients who underwent an allogeneic related BMT showed transient mixed chimerism, which converted to full donor chimerism at 2, 6, and 12 months, respectively. These last three patients had nonmalignant diseases.

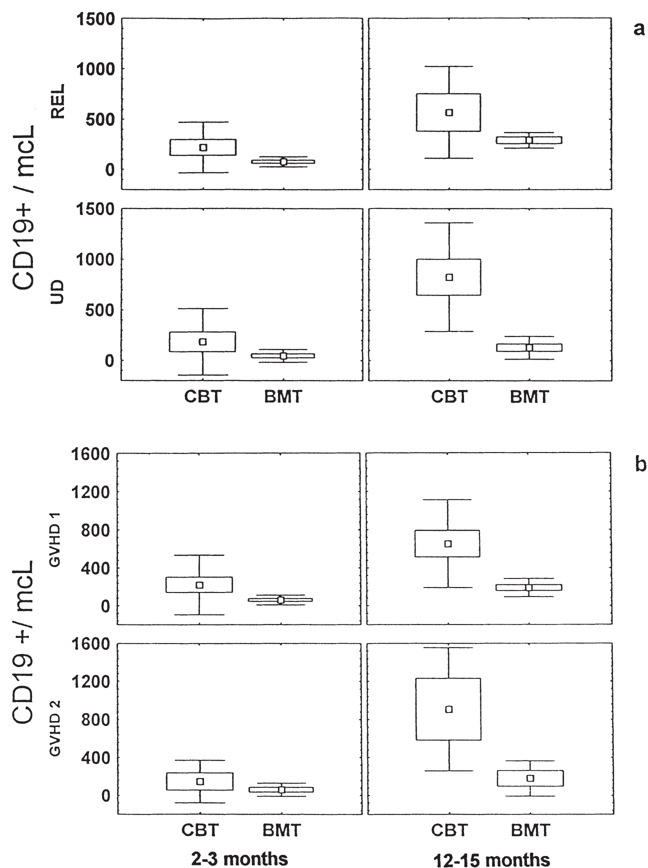

Figure 1. Number of circulating $CD19^+$ B lymphocytes/μL of patients who underwent either CBT or BMT from a related (REL) or unrelated (UD) donor, analyzed in the whole populations **(a)** or in the four cohorts subdivided according to GVHD occurrence **(b)**, where GVHD1 = grade 0–I and GVHD2 = grade II–IV. Values were obtained 2–3 months and 12–15 months after transplant. Mean (□) ± standard error (box) and ± standard deviation are reported. Range values of $CD19^+$ lymphocytes in healthy control individuals were 1.5 to 8.0×10^2/μL.

Immune reconstitution

Distribution of peripheral blood B-, T-, and NK-lymphocyte subsets, proliferative response to T-cell mitogens, as well as NK and LAK cytotoxic activities, were investigated 2–3 months (early immune reconstitution) and 12–15 months (late immune reconstitution) after transplantation. For the purpose of this analysis, children were subdivided into four groups according to the type of donor and the source of stem cell infused: related-CBT (rel-CBT), unrelated-CBT (UD-CBT), related-BMT (rel-BMT), and unrelated-BMT (UD-BMT). Despite wide variability among patients, we observed a peculiar expansion of the absolute number of $CD19^+$ B cells in most patients given CBT, at both 2–3 months (rel-CBT 218 ± 250; UD-CBT 183 ± 330; rel-BMT 75 ± 49; UD-BMT 45 ± 63) and 12–15 months (rel-CBT 566 ± 455; UD-CBT 823 ± 534; rel-BMT 291 ± 78; UD-BMT 128 ± 113) after transplant (Fig. 1a). The difference in the number of B lymphocytes between CBT and BMT recipients was statistically significant ($p < 0.001$). A percentage (between 40% and 80% in different patients) of these $CD19^+$ B cells, which also were $CD20^+$ (data not shown), coexpressed CD5 and CD23 molecules. B-cell expansion, measured as percent of PBMC, peaked at about 4–5 months after transplant (28% ± 12%; range 9–55%). The difference in B-cell recovery at 12–15 months between CBT and BMT patients also was clearly evident in the subgroup of patients who experienced GVHD (CBT 906 ± 647; BMT 179 ± 186; $p < 0.001$; Fig. 1b). The absolute number of $CD3^+$, $CD4^+$, and $CD8^+$ cells significantly increased from 2–3 to 12–15 months after transplant in all four subgroups (Fig. 2a, b, and c, respectively).

During both early and late immune reconstitution, neither the source of stem cells nor the type of donor used influenced the recovery of the number of $CD3^+$ cells (Fig. 2a).

The number of $CD4^+$ cells was comparable among the four subgroups in the early posttransplant period, whereas

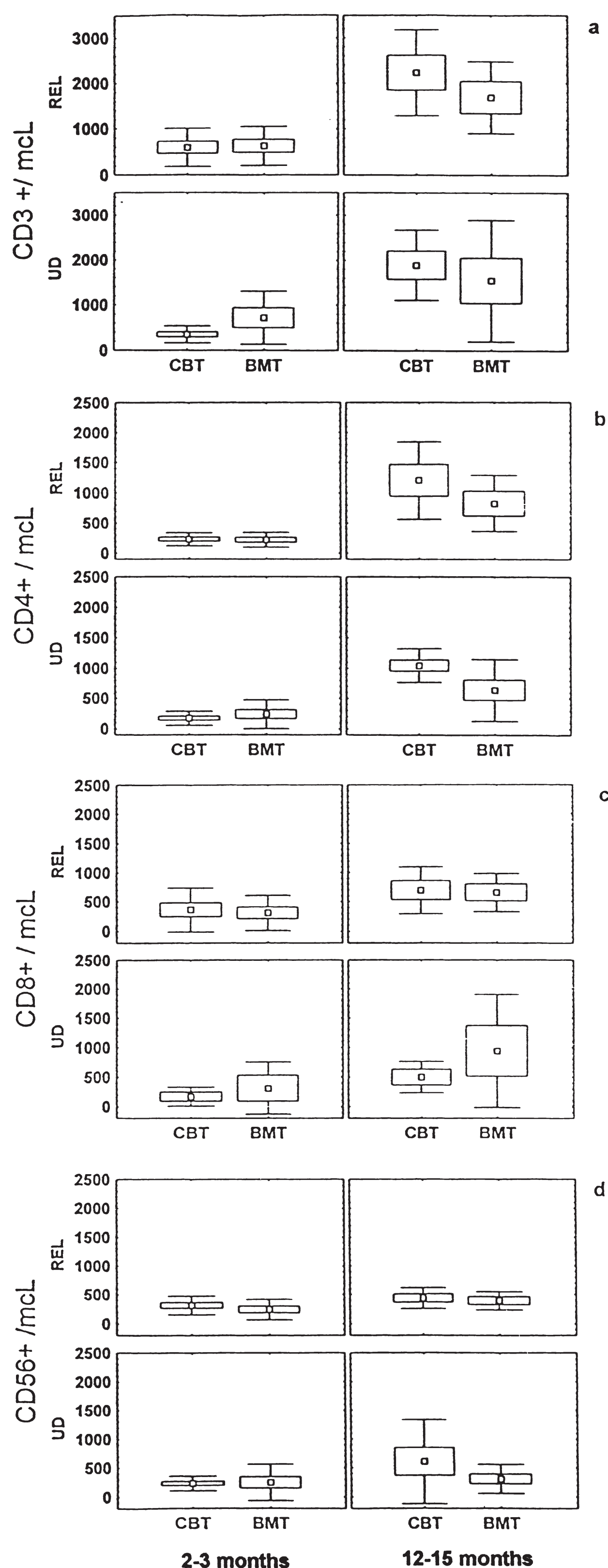

Figure 2. Number of circulating $CD3^+$ (a), $CD4^+$ (b), $CD8^+$ (c), T lymphocytes/μL, and $CD56^+$ (d) NK lymphocytes/μL of patients who underwent CBT or BMT from a related (REL) or unrelated (UD) donor. Values were obtained 2–3 months and 12–15 months after transplant. Mean (□) ± standard error (box) and ± standard deviation are reported. Range values of $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ lymphocytes in healthy control individuals were 1.1 to 6.3 × 10^3/μL, 6.7 × 10^2 to 3.6 × 10^3/μL, 3.8 × 10^2 to 2.1 × 10^3/μL, and 0.9 to 7.0 × 10^2/μL, respectively.

12–15 months after transplant, children given an UD-CBT had a significantly higher number of $CD4^+$ cells/μL (1,045 ± 280) than UD-BMT patients (637 ± 511; $p < 0.01$; Fig. 2B). At 2–3 months after transplantation, 7 of 11 patients given related and 4 of 12 patients given unrelated CBT had an absolute number of $CD4^+$ cells >200/μL, respectively. At the same time period, 6 of 11 patients given related and 5 of 12 patients given unrelated BMT recovered an absolute number of $CD4^+$ cells >200/μL, respectively.

No statistically significant difference in the number of $CD8^+$ T lymphocytes was observed among the four subgroups at both early and late evaluation of the immune recovery (Fig. 2c).

CBT and BMT recipients had a comparable recovery of $CD56^+CD3^-$ NK cells, irrespective of the type of donor used (i.e., compatible sibling or unrelated donor) (Fig. 2d). These data were confirmed by evaluation of $CD16^+CD3^-$ and $CD57^+CD3^-$ lymphocyte subsets (data not shown).

The percentage of $CD4^+CD45RA^+$ cells increased between 2–3 and 12–15 months after transplant in all four subgroups (Fig. 3a). Even though both the mean percentage and the absolute number of $CD4^+CD45RA^+$ cells were higher in CBT than in BMT recipients both at 2–3 and 12–15 months after transplant, differences among the four subgroups were not statistically significant.

Considering $CD4^+CD45RO^+$ cells, we observed a significant decline over time of the percentage of this lymphocyte subset in patients transplanted from a relative, irrespective of the stem cell source ($p < 0.01$; Fig. 3b), whereas the absolute number of $CD4^+CD45RO^+$ significantly increased over time in all four subgroups ($p < 0.0005$). Difference between CBT and BMT recipients was observed at the early evaluation only, i.e., 2–3 months after the allograft, patients given an UD-BMT had a significantly higher percentage of this subset ($p < 0.05$). Because the sum of $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ cells exceeded 100% in CBT recipients, we hypothesize coexpression of both isoforms of the CD45 molecule on circulating lymphocytes.

We did not find any significant modification over time in the percentage of $CD8^+CD45RA^+$ and $CD8^+CD45RO^+$ (data not shown). Neither stem cell source nor type of donor influenced the percentage of both naive and experienced $CD8^+$ cells (data not shown).

In the group of patients given BMT, occurrence of acute GVHD had a negative influence on recovery of $CD4^+$ $CD45RA^+$ cells. At 12–15 months after transplantation, CBT recipients experiencing acute GVHD had a much higher percentage of $CD4^+CD45RA^+$ lymphocytes (79 ±

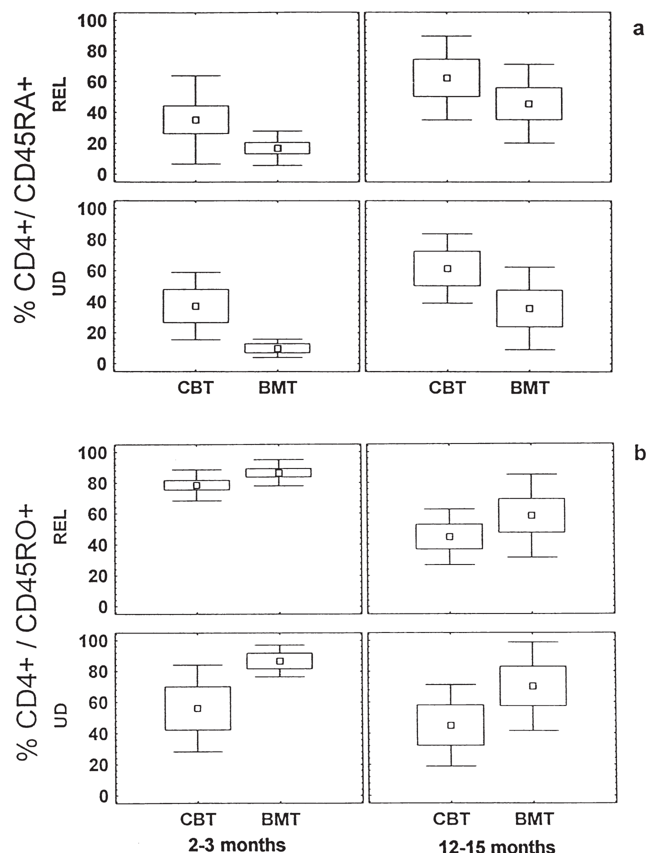

Figure 3. Percentage of circulating $CD4^+CD45RA^+$ (a) and $CD4^+CD45RO^+$ (b) T lymphocytes of patients who underwent CBT or BMT from a related (REL) or unrelated (UD) donor. Values were obtained 2–3 months and 12–15 months after transplant. Mean (□) ± standard error (box) and ± standard deviation are reported. Range values of $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ in healthy control individuals were 34–66% and 38–62%, respectively.

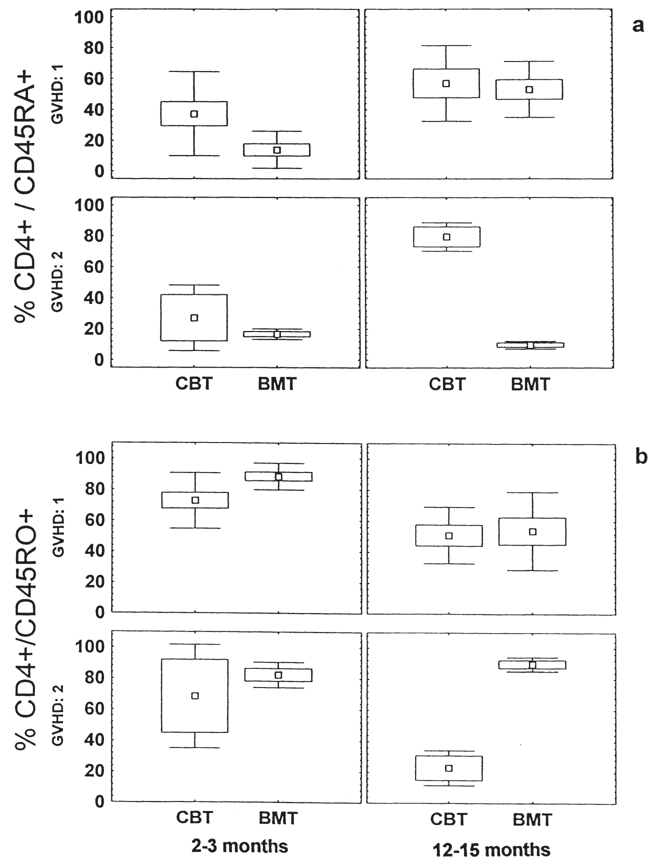

Figure 4. Percentage of circulating $CD4^+CD45RA^+$ (a) and $CD4^+CD45RO^+$ (b) T lymphocytes of patients who underwent CBT or BMT, subdivided according to GVHD occurrence, where GVHD1 = grade 0–I and GVHD2 = grade II–IV. Values were obtained 2–3 months and 12–15 months after transplant. Mean (□) ± standard error (box) and ± standard deviation are reported. Range values of $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ in healthy control individuals were 34–66% and 38–62%, respectively.

9) than children with acute GVHD after BMT (9 ± 2; $p < 0.005$; Fig. 4a). In this latter population, $CD4^+$ $CD45RO^+$ lymphocytes accounted for the vast majority of $CD4^+$ cells (Fig. 4b; $p < 0.005$).

To evaluate whether patient age had an impact on immune recovery, we subdivided patients into groups according to median age. We observed that irrespective of the source of stem cell infused, younger children preferentially reconstituted a higher percentage of both $CD4^+CD45RA^+$ ($r = -0.33$; $p < 0.01$) and $CD8^+CD45RA^+$ ($r = -0.32$; $p < 0.01$) T lymphocytes than older patients. The opposite was true for $CD4^+CD45RO^+$ ($r = 0.5$; $p < 0.05$) and $CD8^+CD45RO^+$ ($r = 0.5$; $p < 0.05$) T cells.

Proliferative response of T cells to polyclonal activators (PHA, Con-A, anti-CD3) was comparable in CBT and BMT recipients both at early and late evaluation (see Fig. 5, which shows the response to anti-CD3 mAb).

All transplanted patients recovered normal NK and LAK activities in the early posttransplant period (Fig. 6). No statistically significant difference was observed between patients given either CBT or BMT.

Other variables (including sex, HCMV reactivation, mixed or complete chimerism, use of rhG-CSF after the allograft, original disease) did not have any statistically significant influence on the immune recovery of CBT and BMT recipients.

Evaluation of serum immunoglobulin (Ig) levels showed that IgM and IgA values were comparable in CBT and BMT recipients at both early and late evaluation (data not shown). Data concerning IgG were not reliable in the first 4 months after transplantation, because patients received substitutive therapy with commercial Ig preparations. However, IgG values 12–15 months after transplantation showed no difference in the four subgroups of patients (data not shown).

Eight children given CBT were vaccinated with tetanus toxoid and inactivated poliovirus (Salk vaccine). The results in terms of immunization were comparable to those documented in 25 BMT recipients, six of whom were in the control group of this study (data not shown).

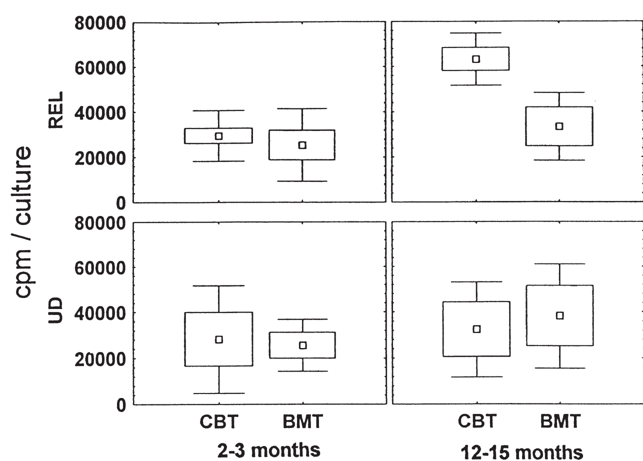

Figure 5. Anti-CD3 (OKT3) induced lymphocyte proliferation of PBMC obtained from patients given CBT or BMT from a related (REL) or unrelated (UD) donor. Values (expressed as cpm/culture) were obtained 2–3 months and 12–15 months after transplant. Mean (□) ± standard error (box) and ± standard deviation are reported. Normal values in healthy control individuals were >20,000/culture.

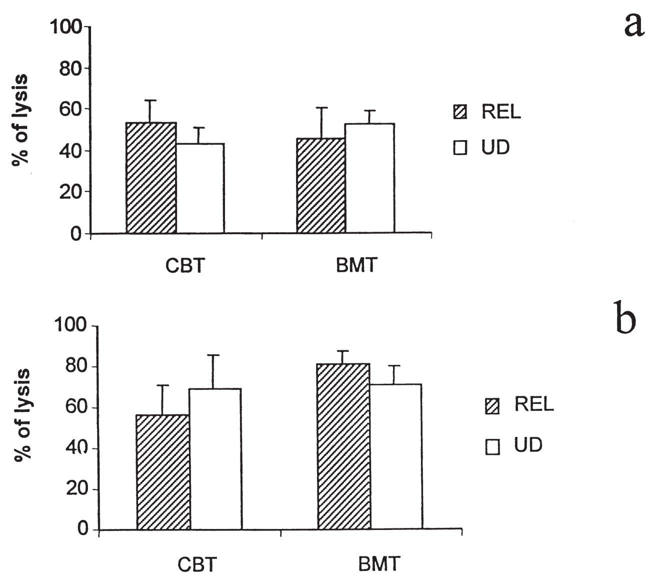

Figure 6. NK **(a)** and LAK **(b)** activity of patients given CBT or BMT from a related (REL) or unrelated (UD) donor. Values were obtained 2–3 months after transplant. Results are expressed as percent of specific lysis at the effector-to-target ratio of 100:1 and 30:1 for NK and LAK activity, respectively. Normal values of NK and LAK activity in healthy control individuals were >15% and >20% of specific lysis, respectively.

Discussion

The immune recovery after transplantation of haematopoietic stem cells has been reported to be dependent on two distinct phenomena [20–24]. In the early posttransplant period, there is an in vivo, antigen-driven expansion of mature, donor-derived lymphocytes, transferred with the graft. This early posttransplant process, considered thymus-independent [33], provides the first wave of T cells, which have a limited diversity of T-cell receptor (TCR) repertoire and persist in the periphery for up to 10–20 years after the allograft [24,34]. Following this first wave, naive lymphocytes, which derive from the differentiation of donor hematopoietic stem cells, colonize the lymphoid organs and sustains the late immune response of recipients. This second pathway involves selection of graft-derived precursor cells in the thymus and/or possibly at other peripheral selection sites [35].

The first step of immunologic recover theoretically could be expected to be much less efficient in CBT recipients than in patients given BMT, considering the lower number of lymphocytes infused and their immaturity [8–13]. Moreover, defective early immune reconstitution has been hypothesized to explain the remarkable incidence of early infectious complications observed after CBT [3–5].

To evaluate features unique to immune reconstitution in CBT recipients, lymphoid phenotype and in vitro function have been analyzed at two different time points and compared with results observed in a cohort of age- and donor-matched children who underwent BMT. The results of this study indicate that early reconstitution after CBT seems to be comparable to that observed in individuals who underwent BMT. Late evaluation of the immune system showed that, at least for some lymphocyte subsets (number of B cells and $CD4^+$ T lymphocytes), patients who underwent CBT and surviving at least 1 year after the allograft had an even more effective recovery than BMT recipients.

The advantage for late immune reconstitution documented in CBT recipients may be attributed to the lower incidence and severity of GVHD after CBT, allowing earlier discontinuation of immunosuppressive treatment. Several studies clearly documented that immune reconstitution can be hampered by the occurrence of acute and chronic GVHD [20,23,36]. These posttransplant complications are associated with lymphoid atrophy, require prolonged treatment with immunosuppressive agents, and may favor the development of severe thymus damage [21,22]. We found that GVHD had a particularly detrimental effect on immune reconstitution of $CD4CD45RA^+$ T lymphocytes (which are believed to represent recent thymic emigrants) of BMT recipients, whereas, probably due to its reduced severity and duration, it had a negligible impact on children who underwent CBT. In addition, neither B-lymphocyte recovery in CBT recipients was affected by GVHD. Because $CD4^+CD45RO^+$ lymphocytes accounted for most of the $CD4^+$ cells in the group of BMT recipients experiencing GVHD, it can be hypothesized that development of this immune complication promoted the expansion of graft-derived memory cells and/or the prompt transition of naive cells to memory lymphocytes.

We did not find a negative effect of GVHD on $CD8^+CD45RA^+$ T-cell generation of comparable magnitude. This observation can be explained by the hypothesis

that recovery of this subset of T cells may be due to selection via an alternative regeneration pathway, as recently proposed for a thymectomized pediatric patient who underwent an allogeneic BMT [37]. Future studies performed utilizing the new TREC (TCR rearrangement excision DNA circles) technology [38], which measures recent thymic emigrants, may provide further support to these speculations.

Particularly noteworthy is the comparable kinetics of recovery of $CD4^+$ T cells above the threshold of 200/μL observed in our CBT and BMT recipients. Transplanted patients with $CD4^+$ cell counts below this value have been reported to be exposed to a high incidence of potentially life-threatening opportunistic infections [23].

In our cohort of patients transplanted from an unrelated donor, the absolute number of $CD4^+$ T cells at late evaluation was higher in children receiving CBT than in those given BMT, despite the greater HLA disparity observed in the donor/recipient pairs. Because major or microvariant HLA differences have been reported to alter the capacity of the donor lymphoid progenitors to migrate to or mature within the host thymus [22,23,39], our findings argue in favor of a greater capacity of CBL to interact with host APC or thymic epithelial cells and thus to sustain redevelopment of T-cell subpopulations in the context of HLA disparity.

We found that young recipient age predicted a better production of $CD4^+CD45RA^+$ T cells. This finding is in agreement with the quick recovery of naive $CD4^+$ T lymphocytes documented in children receiving conventional chemotherapy and further points to the crucial role played by recipient thymus in supporting complete T-cell immune reconstitution [40]. One of the most peculiar characteristics of immune reconstitution after CBT is the striking expansion of B lymphocytes, which is not apparently due to any viral challenge, particularly Epstein-Barr virus infection. It is well known that, at birth, neonatal B lymphocytes are immature naive cells, only able to produce IgM antibodies [15]. In the first months of life, these cells are expected to expand vigorously in response to antigenic challenge and to differentiate into experienced B cells, capable of producing other immunoglobulin classes. It is conceivable that neonatal B lymphocytes or their precursors present in cord blood maintain an efficient self-renewal capacity even after being transplanted into an allogeneic host. This hypothesis would explain the faster recovery of the B-cell compartment after CBT as compared to BMT, and seems to be confirmed by the evidence that most B lymphocytes circulating in CBT recipients expressed a CD23CD5 activation pattern.

Our findings on effective immune recovery after CBT apparently contrast with reports of the high incidence of life-threatening infections in CBT recipients during the early posttransplant period [3–5]. This discrepancy can be explained considering that the delay in the neutrophil recovery observed in children after CBT significantly correlates with the susceptibility of these patients to infectious complications. Moreover, because the great majority of CBL are naive, CBT recipients facing a pathogen must develop a primary immune response and they cannot benefit from the contribution of antigen-experienced cells, which are the most effective population for prompt control of infection. This said, it also has been reported that the remarkable ability of CBL to mediate innate immune response [18,19,41], which is known to play a key role in the induction of adaptive immunity [42,43], might favor a prompt capacity of the majority of CBT recipients to develop a primary immune response. Support for the hypothesis pointing to the importance of aspecific cytotoxic activities in CBT recipients is provided by our observation that, in these patients, the recovery of these immune functions was comparable to those observed in children given BMT.

In conclusion, our results indicate that patients who undergo CBT do not experience a delayed recovery in the number of the different lymphocyte subsets and do not show impaired in vitro functional assays after the allograft. However, several crucial questions about the ability of CBT recipients to mount a T-cell–mediated immune response toward widespread pathogens, the contribution of either donor or recipient origin immune cells to the antigen-specific immune response, the recovery and development of antigen-presenting cells, and the regeneration of a normal TCR repertoire after CBT remain to be properly addressed.

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