T-Cell Recovery in Adults and Children Following Umbilical Cord Blood Transplantation

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ABSTRACT

T-cell reconstitution following allogeneic stem cell transplantation may involve thymic education of donor-derived precursors or peripheral expansion of mature T cells transferred in the graft. T cell–receptor excision circles (sjTRECs) are generated within the thymus and identify new thymic emigrants and those that have not divided. We measured quantitative and qualitative immunologic reconstitution and sjTREC levels in adult and pediatric recipients of umbilical cord blood transplants (UCBTs). sjTRECs were detected at normal levels in all children, starting 12 months after transplantation. sjTRECs were not detected until 18 months after transplantation in adults, and then only at a 3-fold lower level than expected for age. We used complementarity-determining region 3 (CDR3) spectratyping to measure changes in T cell–receptor diversity occurring with restoration of thymic function. T-cell repertoires were skewed in adults and children at 12 to 18 months after transplantation but recovered to near-normal diversity at 2 to 3 years post-UCBT. T-cell repertoires appeared more diverse earlier in children (at 1 to 2 years post-UCBT) than in adults (at 3 to 4 years post-UCBT). We conclude that early T-cell recovery after UCBT occurs primarily through peripheral expansion of adoptively transferred donor T cells and results in skewing of the T-cell repertoire. The reappearance of sjTREC-containing cells after UCBT is associated with increasing numbers of phenotypically naive T cells, improved mitogen and recall antigen responses, and diversification of the T-cell repertoire. The delay in central T-cell recovery in adults relative to children may be due to differences in thymic function resulting from age-related atrophy, graft-versus-host disease, or the pharmacologic effects of prophylaxis and treatment of graft-versus-host disease.

KEY WORDS

Immune reconstitution • Umbilical cord blood • Thymus • T-cell repertoire • Allogeneic stem cell transplantation

INTRODUCTION

Quantitative and qualitative immunologic reconstitution following allogeneic bone marrow transplantation (BMT) has been described extensively [1-11], yet the mechanisms by which lymphocytes recover and repopulate the immune system remain controversial. Following T-cell depletion through high-dose chemotherapy, stem cell transplantation (SCT), or human immunodeficiency virus (HIV) infection, restoration of the circulating T-cell pool may occur through central (thymus-dependent) or peripheral (thymus-independent) mechanisms, or both [12]. Bone marrow–derived precursor cells are selected in the thymus and can be released into the circulation as naive T cells.

T cells already in the circulation can expand through a process that requires antigen recognition [13]. Experiments in euthymic and thymectomized mice demonstrate T-cell recovery via both mechanisms, with a bias toward central T-cell education as long as the thymus is present [14]. Extrathymic T-cell maturation occurring in intestinal epithelium has been described [15] but does not appear to play a measurable role in recovery of the circulating pool.

T-cell reconstitution following T cell–depleted allograft is prolonged [8,10,11] and is believed to occur primarily through peripheral expansion in adults, because the natural involution of the thymus with age limits the capacity to educate new T cells [16-18]. Expansion of naive and memory

cells in the circulating pool requires recognition of specific antigen. Because the number of antigens encountered in the body after transplantation is limited, only a selected subset of available T cells will be expanded, and the resulting repertoire within the circulating pool will be skewed [19]. T cell–replete allografts do not appear to be under the same pressure to expand, and maintain a more diverse repertoire of antigen specificities [6,20]. Infusion of mature donor lymphocytes after transplantation can restore repertoire diversity [7,21]. However, repertoires may become more skewed if graft-versus-host disease (GVHD) develops as a result [22].

Umbilical cord blood (UCB) is a potential source of hematopoietic stem cells for allogeneic BMT candidates who have no related donor. Experience in the pediatric population demonstrates that UCB is more tolerant of HLA mismatch than other stem cell sources, resulting in a reduced risk of GVHD [23]. At the same time, published series of both adult and pediatric patients have shown, by qualitative and quantitative measures, a substantial delay in the reconstitution of cellular immunity and a high risk of infection following UCB transplantation (UCBT) [24,25] compared with that from other hematopoietic cell sources [3,4,8-11]. Cord blood T cells appear functionally immature: they have been shown to expand slowly in response to antigen stimulation, demonstrate a higher threshold for cytokine stimulation, and possess a lower effective cytotoxicity relative to adult-donor T-cell controls [26-28]. In adults, immune reconstitution through both central and peripheral mechanisms may be impaired following UCBT. Age-related thymic atrophy may preclude central T-cell education. At the same time, functionally immature cord blood T cells may be deficient in their ability to expand in response to antigen and may not be able to restore the circulating pool.

This study was undertaken to determine the mechanisms by which the circulating T-cell pool is reconstituted after UCBT in adults and children. Quantitative and qualitative recovery of the lymphocyte compartments as defined by surface antigen expression (CD4/8, CD45RA/RO, CD56, CD19) and proliferative responses to mitogen and specific antigen were measured prospectively. Unfortunately, CD45RA expression does not unequivocally identify naive T cells, because they can expand extrathymically without stimulation [29], and memory (CD45RO⁺) cells may revert back to the naive phenotype [30]. A recently developed polymerase chain reaction (PCR)-based assay quantifies the number of $\alpha\beta$ T cells bearing a circular DNA episome, the T cell–receptor (TCR) excision circle (sjTREC), which results from TCR gene rearrangement exclusively within the thymus [31]. As a T cell divides, it replicates the recombined TCR gene but not the episomal sjTREC. Thus, an increasing concentration of sjTREC DNA in the peripheral blood of transplant recipients indicates addition of new, thymus-educated T cells from the progenitor pool. Naive T-cell counts and proliferative responses to mitogen and specific antigen recovered earlier in children (less than 2 years post-UCBT) than in adults (at least 3 years post-UCBT). Quantitative and qualitative immunologic recovery followed restoration of sjTREC levels.

UCBT is complicated by a high rate of infection. To further define the quality of immunologic potential after transplantation, we examined TCR diversity before and after

recovery of thymic function. TCR specificity results from variability at the complementarity determining regions (CDRs), which in turn results from random recombinations of single variable (V), joining (J), and constant (C) gene segments. Further variability results from nucleotide insertions and deletions at the splice between gene segments. CDR3 spectratyping uses oligonucleotide primers specific for each of 25 V gene segments and 1 C gene segment in the TCRβ chain to amplify and detect the results of gene rearrangements that make up CDR3 [32]. The incorporation of individual Vβ, Jβ, and Cβ segments and variation in the V-J-C recombinations determine the TCR repertoire.

METHODS

Patients

A total of 39 adult patients without a suitable HLA-matched related or unrelated bone marrow donor underwent mismatched unrelated UCBT between February 1995 and August 1999 at Duke University Medical Center. Twenty-one patients survived more than 100 days, 3 of whom demonstrated autologous recovery. The remaining 18 patients were considered in this analysis. Patient characteristics are summarized in Table 1. Eight fully engrafted pediatric patients, 1 to 6 years posttransplantation, returned for follow-up between June 1999 and February 2000 and were chosen at random for analysis with the sjTREC and CDR3 spectratype assays. These patients served as the pediatric cohort for comparative purposes. All patients were treated following a single phase 1 protocol approved by the Duke University Medical Center Institutional Review Board. Informed consent was obtained from each patient or guardian.

Conditioning

The 18 adult patients with complete donor engraftment and greater than 100-day survival received chemotherapy consisting of melphalan 180 mg/m² over 3 days, melphalan 135 mg/m² over 3 days, or cyclophosphamide 120 mg/kg over 2 days. Eight of the adults received total body irradiation (TBI) given as 1350 cGy in 9 fractions of 150 cGy each over 5 days, with attenuated dose to the lungs. A further 8 of the adults were unable to receive TBI and were given busulfan 16 mg/kg over 4 days with an adjusted dose based on serum drug levels. Finally, 2 adult patients with Fanconi anemia received thoraco-abdominal irradiation (500 cGy) instead of TBI. All 18 adult patients received antithymocyte globulin (ATG) at a dose of 75 or 90 mg/m² over 3 days.

The 8 pediatric patients received conditioning chemotherapy with melphalan 180 mg/m² over 3 days, melphalan 135 mg/m² over 3 days, or cyclophosphamide 120 mg/kg over 2 days. Three of the pediatric patients received fractionated TBI regimens (1350 cGy given in 9 fractions of 150 cGy each over 5 days, with attenuated lung dose). The remaining 5 pediatric patients could not receive TBI and were given busulfan 16 mg/kg over 4 days with an adjusted dose based on serum drug levels. As in the adult cohort, all pediatric patients received ATG at a dose of 75 or 90 mg/m² over 3 days.

GVHD Prophylaxis

All patients (adult and pediatric) received cyclosporin A (CYA) for GVHD prophylaxis. Two adult patients received

Table I. *Characteristics of Adult and Pediatric Umbilical Cord Blood Transplant Recipients Who Survived More Than 100 Days After Transplantation**

| | Adult | | | |
	Total	TREC-Assayed	Pediatric	P
No. of patients	18	10	8	
Age, y	29.3 (19.9-58.1)	29.3 (19.9-54.9)	4.6 (1.3-17.2)	<.001
Weight, kg	74.7 (43.0-104.1)	70.3 (47.4-104.1)	17.1 (8.0-84.7)	.002
Diagnosis, n				
AML	5	3	1	NS†
ALL	4	2	4	
CML	3	3	0	
Other malignant	3	0	1	
Nonmalignant	3	2	2	
HLA matching (in direction of GVH), n				
5 or 6 of 6 antigens	6	4	5	NS
3 or 4 of 6 antigens	12	6	3	
TBI conditioning, n				
Yes	10	3	5	NS
No	8	5	5	
Cell dose				
Mononuclear, ×10⁷/kg	1.9 (0.65-3.7)	2.1 (0.72-2.7)	3.7 (1.0-9.3)	.002
CD34, ×10⁵/kg	2.9 (0.1-16.7)	1.4 (0.1-12.5)	4.5 (0.6-80.5)	NS
CD3, ×10⁶/kg	4.2 (1.1-8.8)	4.0 (1.1-7.9)	9.2 (0-37.9)	NS
Time to recovery, d				
ANC >500	23 (12-37)	25 (12-37)	20 (13-30)	NS
Platelet transfusion independent	59 (35-142)	56 (35-135)	60 (34-92)	NS
Acute GVHD, n				
Grade 0-1	11	8	7	NS
Grade 2-4	7	2	1	
Chronic GVHD, n				
Yes	8	6	0	0.01
No	9	4	8	

*Characteristics for both the population of all adult patients surviving at least 100 days as a whole and the sub-population of T-cell receptor excision circle (TREC)-assayed adult 100 day survivors are presented. Parameters are expressed as median (range) where applicable. There were no significant differences between the characteristics of the total adult 100-day survivor group and the TREC-assayed subpopulation, nor were there significant differences between TREC-assayed and non–TREC-assayed adult subpopulations. *P* values are for differences between the pediatric and TREC-assayed adult cohorts. NS indicates not significant; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; GVHD, graft-versus-host disease; TBI, total body irradiation; ANC, absolute neutrophil count.

†Malignant versus nonmalignant.

CYA alone, and 2 received CYA with high-dose methylprednisolone. The remaining 14 adult and all 8 pediatric patients received CYA with an intermediate, tapering dose of methylprednisolone (1 mg/kg per day on days 0 through 4, 3 mg/kg per day on days 5 through 13, 2 mg/kg per day on days 14 through 27, then a daily dose tapered by 10% each week). CYA was dose-adjusted to maintain serum levels between 200 and 400 ng/dL. Patients were assessed for GVHD according to established criteria [33]. CYA was tapered over several months, starting at day 180 in those patients without evidence of chronic GVHD. Patients failing or intolerant of CYA received tacrolimus (FK506) instead.

Immunologic Recovery

In all adult and pediatric patients, immune recovery was analyzed prospectively with quantitative and qualitative measures at least every 3 months for the first year after transplantation, then annually thereafter. Additional blood samples were drawn from adult patients at each of the follow-up times, separated by Ficoll, and frozen at –70°C following a standard protocol. Peripheral blood was ana-lyzed through the hospital clinical laboratory to measure the total white blood cell and absolute lymphocyte counts (based on an automated or manually interpreted blood smear). Donor chimerism was documented by chromosome analysis, fluorescence in-situ hybridization for sex chromosomes, or restriction fragment length polymorphism analysis as appropriate after engraftment.

Lymphocyte subsets (B, T, memory versus naive T, and natural killer [NK] cells) were enumerated using 2-color multiparameter flow cytometry. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll separation of freshly drawn, heparinized whole blood followed by staining with fluorescence-labeled monoclonal antibodies. Starting with the first patient in February 1995, all samples were stained with a standard panel of antibodies, including anti-CD2, -CD3, -CD4, -CD8, -CD16, -CD19, -CD20, -CD10, and -CD45. In subsequent years, additional antibodies were added to the standard panel, including anti-CD56, -TCRαβ, -TCRγδ, -CD45RA, and -CD45RO. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Lymphocyte subset results from 3 adult

patients could not be interpreted because the absolute lymphocyte count was not recorded.

Lymphocyte function was measured as the proliferative response of PBMCs to the plant mitogen phytohemagglutinin (PHA) and the recall antigen tetanus, following a method previously described [34]. Briefly, PBMCs were isolated from freshly drawn, heparinized whole blood by Ficoll separation and diluted to a concentration of 1×10^6/mL. One hundred microliters (1×10^5 PBMCs) was incubated with PHA at 3 different concentrations for 3 to 6 days and with tetanus toxoid at 4 different concentrations for 6 days. ^{3}H-labeled thymidine was added and further incubated for at least 6 hours. [^{3}H]thymidine uptake into proliferating PBMCs was measured by liquid scintillation counter after harvesting. The best responses to mitogen and antigen were recorded. Results were compared to unstimulated [^{3}H]thymidine-incubated patient control PBMCs (background) and normal volunteer donor PBMCs incubated simultaneously.

sjTREC Measurement

sjTREC assays were performed retrospectively on all available banked samples. The concentration of sjTREC DNA in PBMCs was measured by quantitative-competitive (QC)-PCR following a method previously described [35]. Briefly, DNA was isolated from 2 to 10 million PBMCs using the Life Technologies (Rockville, MD) Trizol reagent protocol. DNA was resuspended in 100 μL of 8 mmol NaOH; the resulting concentration of DNA was determined by spectrophotometry. DNA (1 μg) was amplified for 30 cycles using a 60°C annealing temperature and extension at 72°C for 30 seconds in a 50-μL PCR reaction mix containing 1× PCR buffer (Platinum *Taq*; Life Technologies), 1.8 mmol/L MgCl$_2$, 200 μmol/L dNTPs, sjTREC Primer A (250 nmol/L), sjTREC Primer B (250 nmol/L), 2.5 μCi [α^{32}P]dCTP, 0.5 μL Platinum *Taq* Polymerase (Life Technologies), and 5000, 1000, 500, or 100 molecules of sjTREC standard (60 base pairs shorter than the target sjTREC sequence). PCR products were separated on 6% or 8% polyacrylamide gels. The gels were dried and bands visualized on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant (Amersham Pharmacia Biotech, Piscataway, NJ) software. In this assay, the limit of detection was >100 sjTRECs/μg DNA.

CDR3 Spectratyping

The CDR3 spectratype assay was performed retrospectively on banked samples. Each spectratype analysis was performed on a peripheral blood sample that had also been assayed for sjTRECs.

CDR3 spectratyping was performed as previously described, with oligonucleotide primers specific for each of 23 Vβ gene segments and the Cβ1 segment [32]. Total RNA was extracted from PBMCs using the RNEasy Mini Kit (Qiagen, Valencia, CA) or Trizol reagent (Life Technologies) and used for first-strand complementary DNA (cDNA) synthesis using M-MLV [Moloney murine leukemia virus] Reverse Transcriptase (Life Technologies) with a random hexamer primer (Promega, Madison, WI). cDNA was amplified for 40 cycles by denaturing at 94°C for 25 seconds, annealing at 60°C for 45 seconds, and extending at 72°C for 40 seconds in a 25-μL PCR reaction

mix containing 1× PCR Buffer C (Invitrogen, Carlsbad, CA), 2.5 μmol/L MgCl$_2$, 250 μmol/L dNTPs, 2.5 μmol/L Vβ primer, 100 μmol/L Cβ1 primer, and 0.125 U Ampli-Taq DNA polymerase (Applied Biosystems, Foster City, CA). The reaction was completed with a final extension at 72°C for 5 minutes. cDNA derived from cultured Jurkat E6-1 (human T-cell leukemia) cells was amplified with the Vβ8 primer in a separate reaction mixture as a positive control.

The resulting amplified PCR products were elongated over 5 cycles in a run-off reaction with a 5'-6 carboxyfluorescein (6-FAM)-labeled nested Cβ1 primer and separated on a denaturing gel. CDR3 size and fluorescence intensity were measured using an ABI 377 XL Sequencer, and results were analyzed with ABI GeneScan software (version 3.1; Applied Biosystems). The number of peaks was counted within each Vβ family, and the total fluorescence intensity under each peak was calculated across each Vβ family.

Statistical Analysis

Where applicable, results were compared (adult versus pediatric) to determine significance using the Wilcoxon signed-rank test (for continuous data) or the Fisher's exact test (for nominal data).

RESULTS
Lymphocyte Subsets

Lymphocyte subset analyses and proliferation assays were performed on 15 of the 18 adult patients with complete donor engraftment and greater than 100-day survival. All but 1 of the 15 patients were documented to be fully donor chimeric after transplantation. One patient, who received a 6-of-6 HLA-matched and sex-matched cord blood unit, was not tested. Mean counts and standard errors from the 14 tested patients are shown in Figures 1-3. The absolute lymphocyte count remained below normal until the 2-year follow-up (Figure 1). B cells (defined as CD19$^+$ lymphocytes), on the other hand, recovered quickly after transplantation and rose to supranormal levels at 1 year. The NK cell (defined as CD56$^+$ lymphocytes) count remained relatively stable for both adult and pediatric patients through 3 years after transplantation, but made up more than half of lymphocytes 3 months after transplantation (not shown). The percentage of NK cells declined over 3 years as T-cell (defined as CD3$^+$ lymphocytes) counts recovered.

T cells remained below normal levels until the 2-year follow-up (Figure 2). Further subset analysis showed that the CD4$^+$ cells (CD4$^+$CD3$^+$ lymphocytes) remained below normal until 2 years posttransplantation, whereas CD8$^+$ cells (CD8$^+$CD3$^+$ lymphocytes) rose to the lower limit of normal by 1 year. Naive (CD3$^+$CD45RA$^+$) and memory (CD3$^+$CD45RO$^+$) T-cell populations remained flat through 2 years but increased sharply at 3 years; however, only 2 patients were evaluable at 3 years. Memory cells represented a larger percentage of the T-cell population (70% versus 27%) through the first year after transplantation, then declined to the 50% range.

Lymphocyte subset analysis was performed on all 8 pediatric patients in the comparison cohort. Normal values for children are not well established. Values based on small numbers of healthy children aged 2 years and older (median age
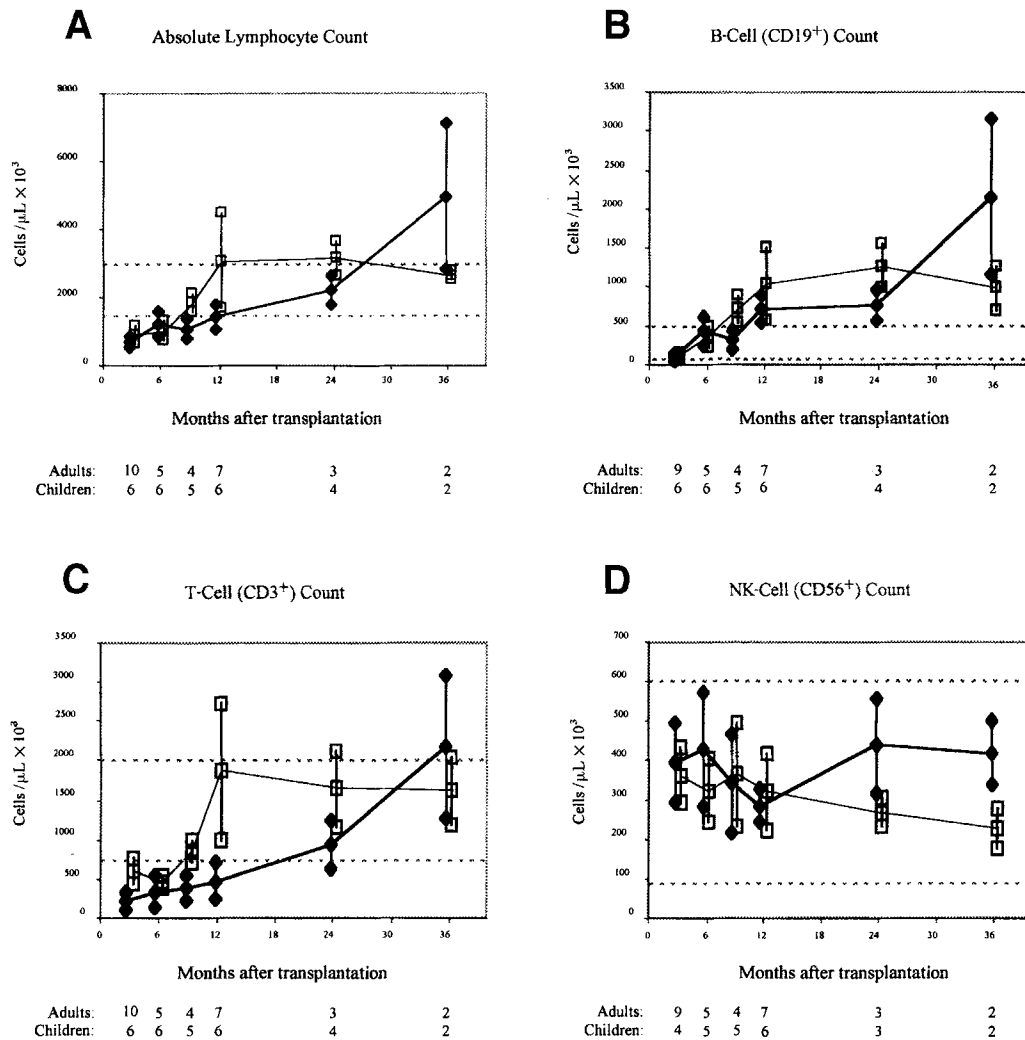
Figure 1. Median absolute lymphocyte (A), T cell (CD3⁺) (B), B cell (CD19⁺) (C), and NK-cell (CD56⁺) (D) counts versus time after umbilical cord blood transplantation for adult (◆) and pediatric (□) recipients. Vertical bars indicate the 95% confidence intervals. Horizontal dashed lines represent the institutional upper and lower limits of normal for adults. Values for the adult and pediatric cohorts have been offset from their corresponding times for clarity. There were no statistically significant differences between adults and children at the time points examined. The number of patients included at each interval appears below each figure. NK indicates natural killer.

in the pediatric cohort was 4.6 years) show great range between fifth and 95th percentiles [37]. Fifth percentile values do not differ substantially from adult lower-limit normal values and are not considered independently in this analysis. B-cell recovery paralleled that for adults, with a quick rise to supranormal levels by the first-year follow-up and remaining above normal through the 3-year mark. T-cell recovery, however, differed strikingly from adults. The absolute T-cell count recovered to normal range by 9 to 12 months and maintained a steady state. Subset analysis showed rapid and sustained recovery of both CD4⁺ and CD8⁺ T cells (Figure 2A and B). As in adults, T cells expressed predominantly the memory phenotype (CD3⁺CD45RO⁺) 3 months after transplantation, but shifted in favor of the naive phenotype (CD3⁺CD45RA⁺) over the next 9 months. This shift was marked by a 5-fold rise in naive T-cell number between 9 and 12 months after transplantation, whereas the memory T-cell number barely doubled.

Statistically significant differences in lymphocyte subset counts between adult and pediatric patients were found only in CD3⁺CD4⁺, CD3⁺CD45RO⁺, and CD3⁺CD45RA⁺ counts, all at 12 months (P values .014, .023, and .0149, respectively).

Lymphocyte Proliferation

Lymphocyte proliferative responses to PHA (Figure 3A) were generally absent at 3 months but improved thereafter in the adult cohort. The responses remained below the range of normal controls (control mean ± 95% confidence interval) until the 3-year follow-up. Proliferative responses to tetanus were very low through the first 2 years after transplantation, but improved somewhat at the 3-year follow-up (Figure 3B).

Lymphocyte proliferation assays were performed on all 8 pediatric patients in the comparison cohort. Proliferative responses to PHA essentially paralleled those measured for
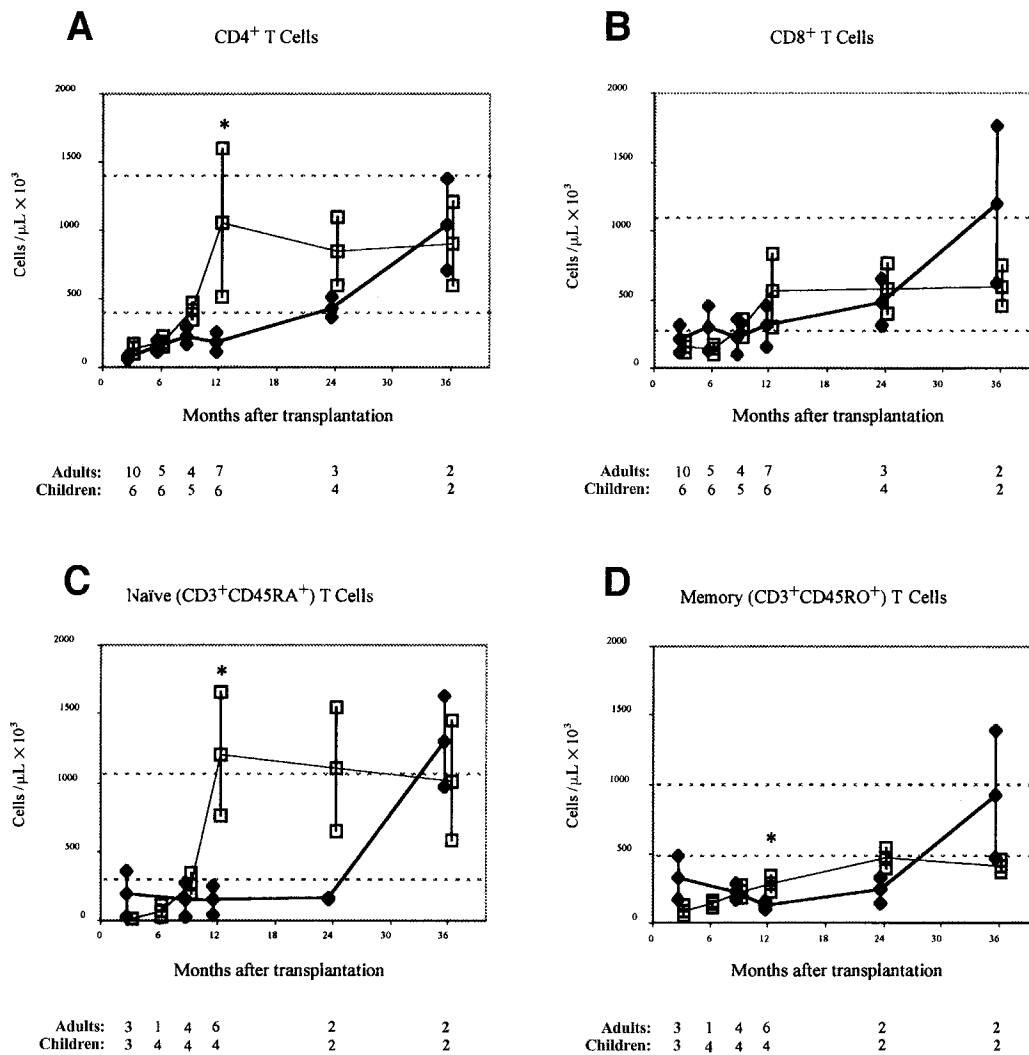
Figure 2. Median T-cell subset counts after umbilical cord blood transplantation for adult (◆) and pediatric (□) recipients: CD4⁺ (CD4⁺CD3⁺) (A), CD8⁺ (CD8⁺CD3⁺) (B), naive (CD3⁺CD45RA⁺) (C), and memory (CD3⁺CD45RO⁺) (D). Vertical bars indicate the 95% confidence intervals. Horizontal dashed lines represent the upper and lower limits of normal for adults [36]. Values for the adult and pediatric cohorts have been offset from their corresponding times for clarity. The number of patients included at each interval appears below each figure. Asterisk indicates time point at which significant differences were found between adults and children.

adults, but were higher than adults at all time points. Response to tetanus remained low through the first year, but improved in the second and third years after transplantation. No differences in mitogen or antigen responses between adults and children were statistically significant.

sjTREC Assay

QC-PCR for sjTREC DNA was performed on 27 samples of peripheral blood from 10 adult patients. Twenty-one samples were obtained 30 days or more after transplantation (median, 3.1 months; range, 1.0 to 5.9 months). Twelve assays were performed on 8 patients 100 days or more after transplantation. Seven patients had sjTRECs above the detection threshold in the peripheral blood (>100 sjTRECs/μg PBMC DNA) at a median of 36.0 months (range, 17.5 to 42 months), with a median 325 TRECs/μg PBMC DNA (range, 228 to 728). The median age of patients producing

TRECs after transplantation was 26.3 years, which would predict 1250 TRECs/μg PBMC DNA in normal individuals, following the regression analysis from Patel [35]. The sjTREC assay was performed on peripheral blood from all 8 pediatric patients in the reference cohort at a median of 30 months (range, 12 to 77 months) after transplantation. sjTRECs were assayable in 8 of 9 samples, with a median level of 6741 sjTRECs/μg PBMC DNA (range, 157 to 22,833 sjTRECs/μg PBMC DNA; $P = .028$ for sjTREC positive adults compared with children). Results are presented in Figure 4 with estimated age-adjusted normal values for the pediatric and adult cohorts.

CDR3 Spectratyping

CDR3 spectratyping was performed on 19 samples from adult and pediatric patients. Samples included aliquots from 4 UCB grafts. The remaining 15 samples were obtained
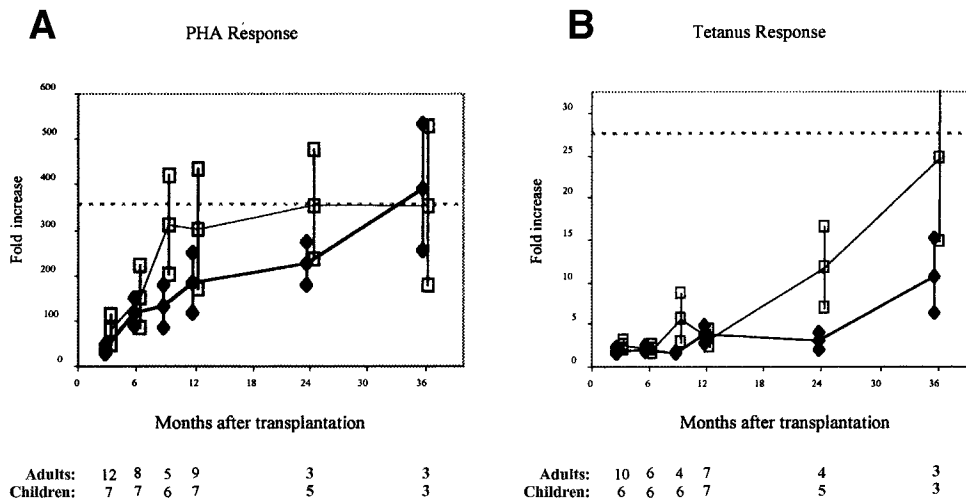
Figure 3. Lymphocyte proliferation in response to phytohemagglutinin (PHA) (A), and tetanus (B) after umbilical cord blood transplantation for adult (◆) and pediatric (□) recipients. Shown are the median fold increases in [^{3}H]thimidine uptake over unstimulated controls. Note the parallel delay in responses in the adult patients compared with the phenotype analysis in Figure 2. Vertical bars indicate the 95% confidence intervals. Horizontal dashed lines represent the lower 95% confidence limits from normal controls. Values for the adult and pediatric cohorts have been offset from their corresponding times for clarity. The number of patients included at each interval appears below each figure. There were no statistically significant differences between adults and children at the time points examined.

12 to 77 months after transplantation. The median number of peaks identified in each of the UCB grafts was 141 (range, 121 to 185), consistent with previously published data [38]. Spectratyping from 3 adult samples between 1 and 2 years after transplantation demonstrated repertoire skewing: the overall number of Vβ families represented and the number of peaks within each family were limited, particularly in 2 patients (Figure 5). T-cell repertoires appeared more diverse in 3 of 4 adult patients tested 3 years after transplantation, but remained substantially skewed in 1 patient. In contrast, spectratyping from 4 pediatric patients between 1 and 2 years after transplantation demonstrated diverse repertoires (Figure 6). Fluorescence intensity spectra for each of 23 Vβ families obtained from 1 adult and 1 pediatric patient at 2 different time points are presented in Figures 7 and 8, respectively. At 1 year, spectra for both adults and children

appear limited. The spectra at 2 years in the child appear normal, whereas the spectra at 3 years in the adult demonstrate residual skewing (in particular, Vβ13B, -20, and -21).

DISCUSSION

Reconstitution of the circulating T-cell pool after myeloablation and allogeneic SCT may occur following 2 distinct mechanisms, both of which appear to play a role after UCBT in adults. The T-cell population in the months following UCBT increased only slowly in absolute number, whereas the sjTREC concentration and T-cell proliferative responses to mitogens remained undetectable. At 1 year, T-cell repertoires were limited both in the number of Vβ families represented and in the splicing diversity within families. These findings are consistent with antigen-driven
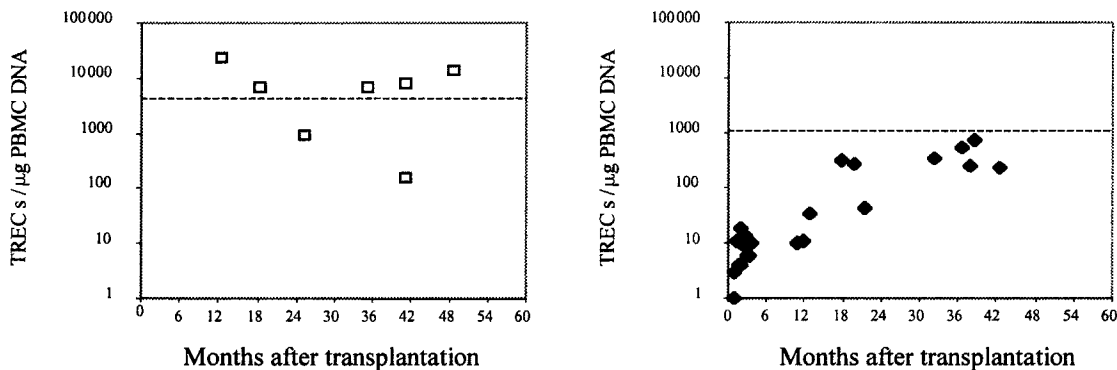

Figure 4. T-cell receptor excision circle (sjTREC) levels after umbilical cord blood transplantation for pediatric (□) (7 samples) and adult (◆) (22 samples) recipients. Pediatric sjTREC value of 4615 copies/µg of peripheral blood mononuclear cell (PBMC) DNA at the 77-month follow-up was not plotted. Horizontal dashed lines represent estimated, age-adjusted normal sjTREC levels (based on median ages of 4.6 and 29.3 years for pediatric and adult recipients, respectively) [33].
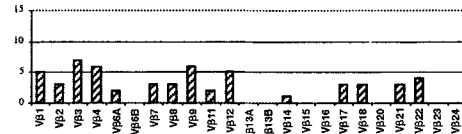
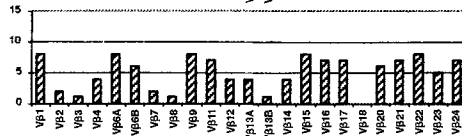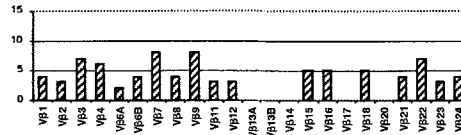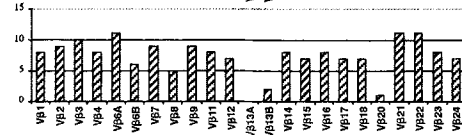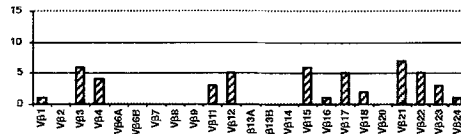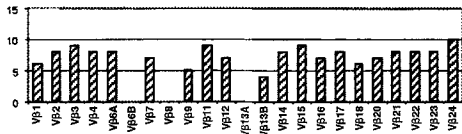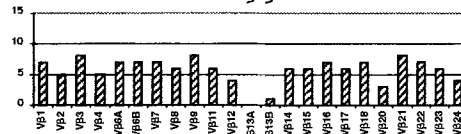**Figure 5.** Complementarity-determining region 3 (CDR3) spectratyping for six adult umbilical cord blood transplant recipients 12 to 42 months after transplantation. Results are summarized as the number of peaks within each of 24 Vβ families (▨). UPIN indicates unique patient identification number.

peripheral T-cell expansion occurring in the absence of thymic output. This analysis provides evidence that grafted UCB T-cells are able to respond to antigen stimulation and divide to begin to restore the circulating pool. The grafted T-cell pool is therefore vulnerable to repertoire skewing, presumably toward reactions against alloantigens, a factor that may exacerbate GVHD. Viral antigens may also drive expansion of the grafted T-cell reservoir [9].

Starting 18 months after UCBT, sjTRECs were detectable in substantial (although lower than expected) levels in the peripheral blood of the adult patients. The detection of sjTRECs coincided with the appearance of phenotypically naive CD4⁺ T cells and improvement in proliferative responses to mitogens and recall antigen (tetanus). T-cell repertoires 3 years after transplantation appeared substantially more diverse in 3 of 4 patients, including 1 patient who had recovered from dramatic skew-

ing. These findings support a second, thymic-dependent phase of T-cell reconstitution with attendant improvement in immunologic function and potential.

In contrast to the adults studied, T-cell recovery in children, including naive CD4⁺ lymphocytes, occurred within 6 to 12 months after UCBT. sjTRECs were detected in all pediatric patients as soon as 1 year after transplantation (the earliest time point tested). No child was tested prior to 1 year after transplantation, but other reports have documented thymic output in children at 100 days after transplantation, reaching a peak at 1 to 2 years [35]. T-cell repertoires from 2 patients were diverse at 9 and 12 months and were normal in appearance in 2 more patients at 18 and 26 months. These findings indicate that the antigen-driven, peripheral expansion phase of T-cell reconstitution is short-lived in young patients. An early dominance of the thymus-dependent phase may prevent expansion of alloreactive T-cell clones and may
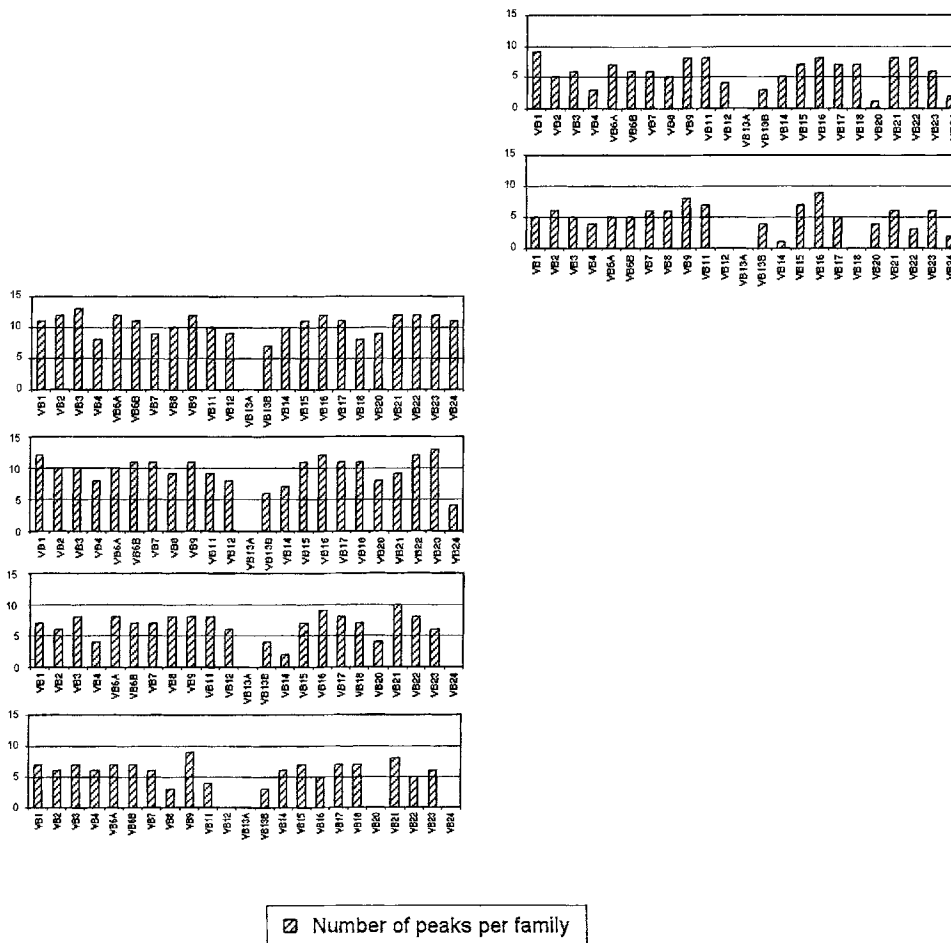
Figure 6. Complementarity-determining region 3 (CDR3) spectratyping for 6 pediatric umbilical cord blood transplant recipients 12 to 77 months after transplantation. Results are summarized as the number of peaks within each of 24 Vβ families (▨). UPIN indicates unique patient identification number.

play a role in the reduced incidence of chronic GVHD in pediatric patients. The disparity between adult and pediatric patients in this study implicates host-specific or posttransplantation differences; however, it is important to note that the small number of children in the comparison cohort necessarily limits the strength of conclusions that can be drawn from this analysis. In addition, the selection of children for the comparison group—from patients returning to clinic—may have introduced unexpected biases.

A number of factors may have influenced the mechanism of T-cell recovery. First, thymic function declines with age [16,18,39], suggesting that reconstitutive potential declines with age. The thymuses of adult recipients may have naturally atrophied and/or been exposed to more therapy (or environmental toxins, etc.) before BMT than pediatric recipients, resulting in a delayed and attenuated response. The measured sjTREC levels in adult UCB recipients were lower

than expected for normal controls based on median age. In contrast, a 3-fold higher sjTREC production has been documented in patients aged 34 to 49 years who received myeloablative chemotherapy for multiple myeloma followed by autologous stem cell rescue [40], confirming that adults can generate normal levels of sjTRECs after high-dose chemotherapy. Peak thymic function may not yet have been reached in these adult UCB recipients at the times tested.

The preparative regimen is unlikely to have played a confounding role. Adult and pediatric groups were matched in the exposure to TBI before transplantation, and both cohorts received melphalan or cyclophosphamide and ATG. In addition, posttransplantation supportive care (eg, growth factor support, antimicrobial prophylaxis) was the same for both groups.

Despite the degree of HLA mismatch, the risk of GVHD following UCBT is lower than expected for hap-
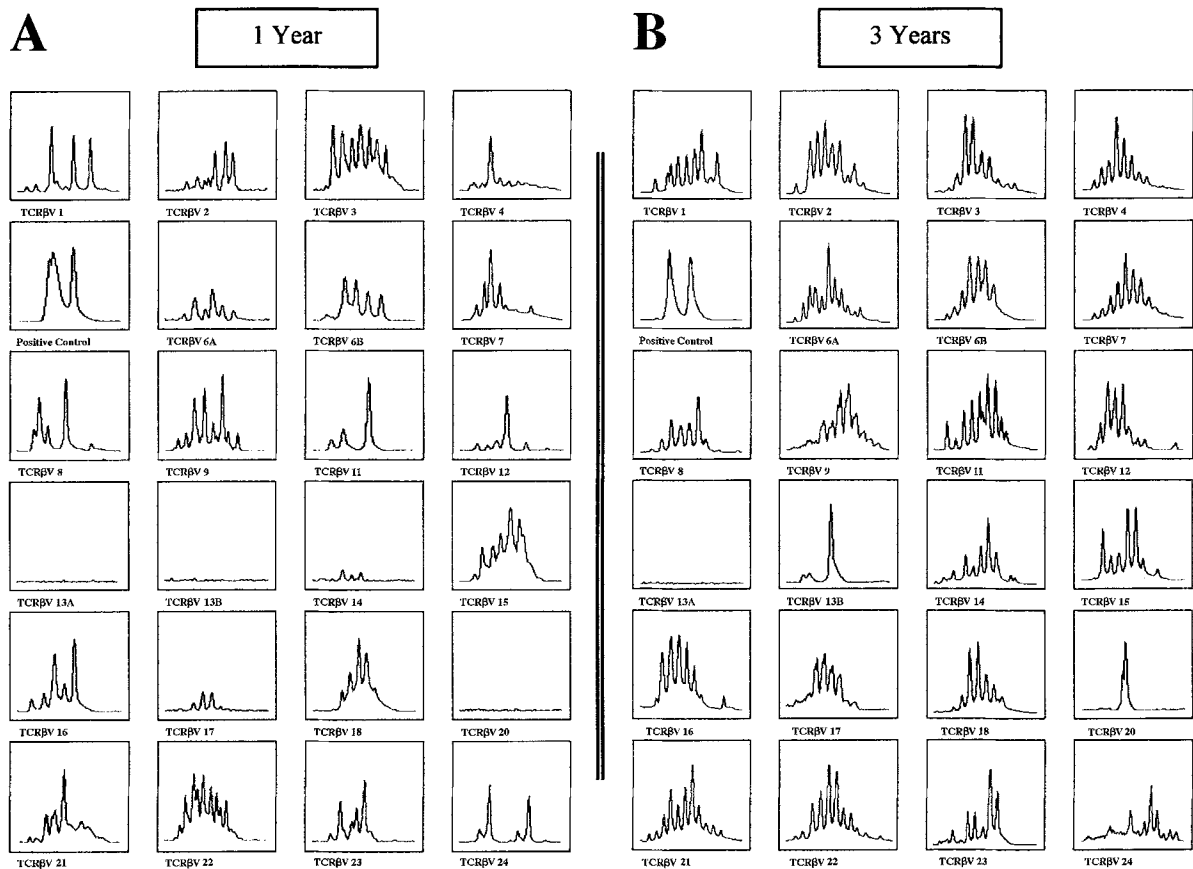
Figure 7. Fluorescence intensity spectra for each of 23 Vβ families (and 1 positive control) from a representative adult patient at 1 year (A) and 3 years (B) after transplantation. The repertoire at 1 year demonstrates skewing (a limited number of Vβ families are represented and a limited number of peaks appear within each family) but appears substantially normal at 3 years after transplantation. TCR indicates T-cell receptor.

loidentical sibling allogeneic BMT [24]. Table 1 shows that the adult and pediatric cohorts were similarly matched for HLA disparity and incidence of acute GVHD. There was, however, a significant difference in the incidence of chronic GVHD. GVHD has long been known to be associated with delayed T-cell recovery [41] and thymic tissue injury [42], which may render the thymus unable to generate new T cells. sjTREC levels are lower than expected in allogeneic BMT patients with resolved GVHD and altogether absent in patients with active (chronic) GVHD [43]. Alternatively, medical prophylaxis to treat acute or chronic GVHD (CYA, FK506, or steroids) may injure the thymus, precursor T cells, or both. The adult and pediatric patients in this study differed in the median duration of immunosuppression with CYA or FK506 (calculated for patients surviving at least 180 days: 13 versus 7 months, respectively; $P = .075$). The recovery of TRECs was delayed in each adult, although chronic GVHD was documented in only 60%. This finding could be explained by residual thymic injury from prior (resolved) GVHD or by ongoing injury from subclinical chronic GVHD in the remaining 40%.

These results may also have been observed if thymic function was be unaffected by transplantation, but the delivery of T-cell precursors from the bone marrow to the thymus was impaired. The infused mononuclear cell dose differed significantly between cohorts on a per-kilogram basis. The finite amount of fetal blood recoverable from any single postpartum placenta significantly limits the size of banked cord blood units. The weight of an adult UCBT recipient is a major limitation to the infused cell dose (number of infused cells ÷ weight of recipient) and likely accounts for the differences observed between groups. The difference in infused doses of CD34$^+$ and CD3$^+$ cells between cohorts did not reach significance, but variability in CD34$^+$ and CD3$^+$ content between cord blood units limited the power to detect a difference. The differences in cell dose may have led to differences in the production of common lymphoid progenitors from the grafts, which in turn would have led to reduced production of mature T cells. Notably, there was no significant difference in the time to absolute neutrophil count recovery or platelet transfusion independence between cohorts, suggesting that the transplanted grafts had similar capacity to differentiate at least some of the marrow precursors.

It has been argued that sjTREC concentrations may not accurately reflect thymopoiesis, because the levels depend on the number of circulating PBMCs [44]. It is possible that ongoing activation of naive thymic emigrants and turnover of the T-cell pool as a whole diluted evidence of early thymic recovery in adult patients. In addition, the sjTREC
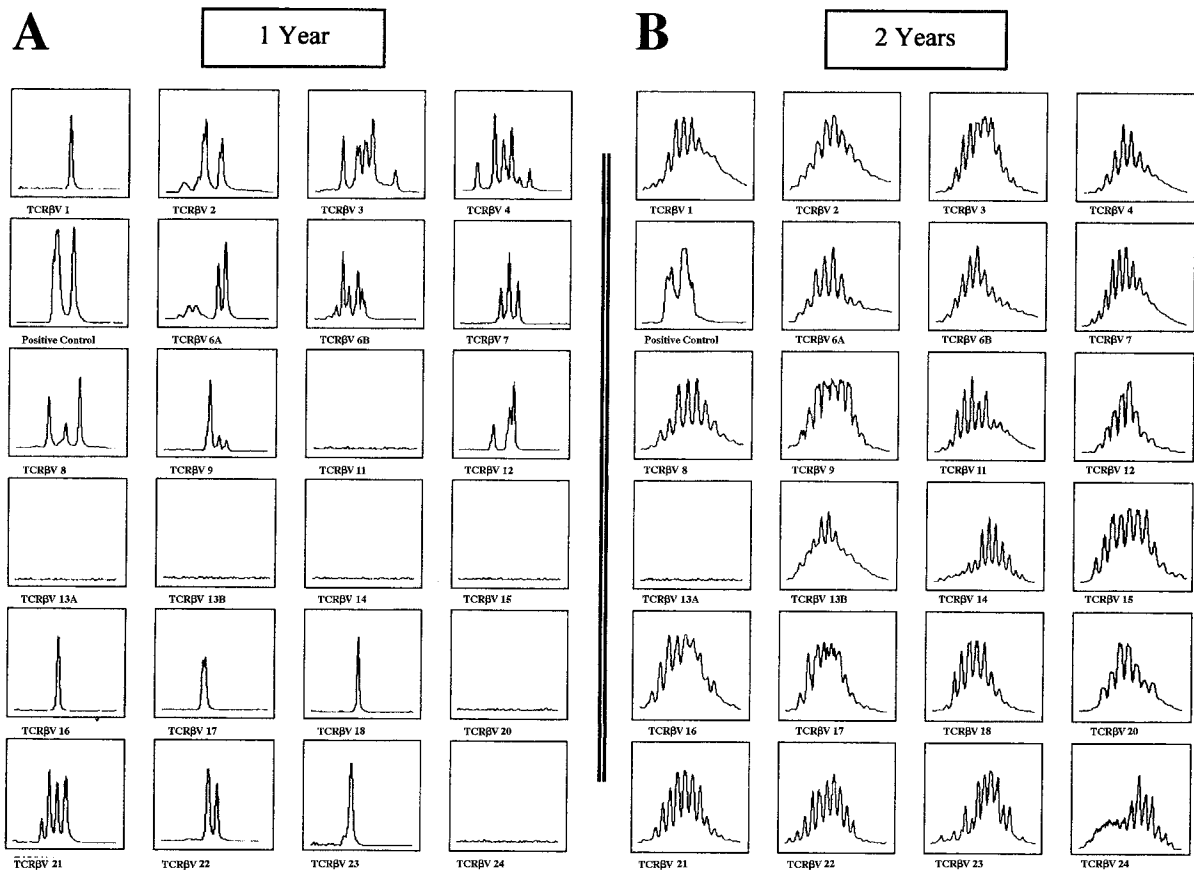
A 1 Year

B 2 Years

Figure 8. Fluorescence intensity spectra for each of 23 Vβ families (and 1 positive control) from a representative pediatric patient at 1 year (A) and 2 years (B) after transplantation. The repertoire at 1 year demonstrates skewing, but it appears essentially normal at just 2 years after transplantation. The repertoire at 2 years appears more diverse than repertoires at 3 years in adult patients (Figure 7).

and CDR3 analyses were performed on unsorted, bulk mononuclear cell fractions. Differences between CD4⁺ and CD8⁺ T-cell populations will have been missed, and improvements in sjTREC counts and T-cell repertoires cannot be attributed to one population or the other. Future studies may be improved by measuring sjTREC levels and repertoire diversities within sorted lymphocyte subsets and by including markers of cellular activation, proliferation, and apoptosis in the phenotypic analyses.

This report demonstrates the vital importance of preserving thymic function through myeloablative therapy and stem cell transplantation. UCB is prone to repertoire skewing, which may increase the risk of GVHD and may contribute to long-term immunoincompetence in the host. Infusions of mature, donor-derived T cells have been shown to improve T-cell repertoires, but they are not available to UCB recipients. Future investigations will need to focus on strategies to prevent GVHD while avoiding thymic injury. Cytokine therapy may hold promise as a promoter of central T-cell maturation [39,45].

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