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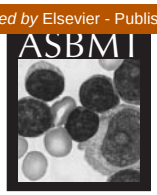

Nonmyeloablative Regimen Preserves "Niches" Allowing for Peripheral Expansion of Donor T-Cells

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ABSTRACT

T-cell recovery following myeloablative preparatory regimens and cord blood transplantation in adult patients generally occurs between 1 and 3 years following allogeneic bone marrow transplantation. T-cell reconstitution may involve thymic education of donor-derived precursors or peripheral expansion of mature T-cells transferred in the graft. We measured quantitative and qualitative immunologic reconstitution, T-cell receptor spectratyping, and T-cell receptor excision circle (TREC) levels in adult recipients of umbilical cord blood transplants following a novel nonmyeloablative regimen. These results were compared to previously published results of similar patients receiving a myeloablative regimen and cord blood stem cells. With small numbers of patients treated so far, T-cells ($CD3^+$) reached normal levels in adults 6 to 12 months following nonmyeloablative transplantation compared with 24 months in adults receiving a myeloablative regimen. At 12 months after transplantation, the numbers of phenotypically naïve ($CD45RA^+$) T-cells were higher in those receiving the nonmyeloablative regimen. The T-cell repertoire in cord blood recipients treated with a nonmyeloablative regimen was markedly more diverse and robust compared with the repertoire in those receiving the myeloablative regimen at similar time points. TRECs (which are generated within the thymus and identify new thymic emigrants and those that have not divided) were detected 12 months after transplantation in the nonmyeloablative recipients, whereas TRECs were not detected in adults until 18 to 24 months in those receiving myeloablative regimens. Thus, in adults receiving a nonmyeloablative preparatory regimen, the quantitative and qualitative recovery of T-cells occurs through rapid peripheral expansion. The ability of patients receiving a nonmyeloablative regimen to recover within a few months suggests that the peripheral niches in which T-cells can proliferate are preserved in these patients compared to those receiving ablative regimens. Moreover, the presence of TREC-positive cells within 1 year suggests that thymic recovery is likewise accelerated in nonmyeloablative compared to myeloablative regimens.

KEY WORDS

Allogeneic bone marrow transplantation • Nonmyeloablative regimens • T-cell recovery • Cord blood

INTRODUCTION

Quantitative and qualitative immunologic reconstitution following allogeneic bone marrow transplantation (BMT) has been described, but the mechanisms by which lymphocytes recover and repopulate the immune system remain unknown [1-7]. T-cells attain antigen specificity through a process of receptor gene rearrangement guided through positive and negative selection within the thymus. The circulating pool of T-cells may be maintained through addition of new/novel T-cells from the thymus or through expansion (duplication) of existing mature cells. Mackall et al. compared lymphocyte recovery in thymectomized versus thymus-bearing mice following lethal irradiation and BMT [8]. Peripheral expansion was the dominant mechanism in thymectomized animals but was suppressed and replaced by central thymic production in the control mice. Following transplantation from bone marrow or peripheral blood stem cells in adult humans, early T-cell reconstitution favors cells with the memory phenotype, then shifts to roughly equal parts memory and naïve phenotypes [6].

Until recently, no techniques were available to measure thymic production of new T-cells. Recent thymic emigrants (naïve T-cells) have been described phenotypically as expressing both CD45RA and L-selectin (CD62L). Following

Table 1. *Patient Characteristics**

	UPIN #2379			UPIN #2593			UPIN #2601			UPIN #2611			UPIN #2679		
Time	WBC	Plt	% Donor	WBC	Plt	% Donor	WBC	Plt	% Donor	WBC	Plt	% Donor	WBC	Plt	% Donor
2 wk	3.5	29†	6.5	0.2	19†	93	5.8	105	1	0.2	9†	37	7.3	387	0
6 wk	1.3	19†	18	7.7	8†	100	6.5	205	0	10.2	19†	8	5.5	135	0
8 wk	7.9	31	56	4.3	8†	100	3.5	224	0	4.3	13†	4			
3 mo	4.7	92	100	5.4	36	100				4.6	132	10			
6 mo	6.2	123	100	9.1	111	100				7.8	123	100			
12 mo	5.4	154	100	7.8	132	100									

*Plt indicates platelet count; % donor, lymphoid donor chimerism determined by microsatellite polymorphisms.

†Transfused value.

contact with its specific antigen, the T-cell is activated and may become a memory T-cell identified phenotypically by expression of CD45RO. Unfortunately, phenotypic markers are not reliable because naïve T-cells can expand extrathymically without stimulation [9], and memory cells may (spontaneously) revert back to the naïve phenotype [10].

A novel method to measure thymic production has been described [11]. T-cell precursors expressing the Vβ T-cell receptors (TCR) attain antigen specificity in the thymus. Their development requires rearrangement of the TCR genes occupying 3 loci. Rearrangement of the TCRA locus brings together a random selection of single gene segments from 2 variable segment families, V and J, and the constant segment C. Intervening segments are excised in predictable steps and form circular episomes as a by-product. Two of these recombination steps are identical in a majority of TCRA locus rearrangements and result in 2 circular episomes: the signal-joint TCR excision circle (sjTREC) and the coding-joint TREC [11]. A polymerase chain reaction (PCR)-based assay has been developed [12] to measure the amount of sjTREC DNA contained in a population of T-cells. As T-cells proliferate, they replicate the recombined TCR genes but not the episomal sjTRECs. An increasing concentration of sjTREC DNA in the peripheral blood of transplant recipients indicates maturation of new T-cells from the progenitor pool and subsequent potential for diversification of the T-cell repertoire.

Recently, several groups have measured sjTREC-containing T-cells following allogeneic transplantation [13-16]. In our previous study, immunologic reconstitution was analyzed following unrelated umbilical cord blood (UCB) transplantation in adults and children to determine the source of the regenerated T-cell pool. UCB is phenotypically naïve but T-cell replete, allowing for T-cell reconstitution through either central or peripheral mechanisms. Reconstitution of immunity following UCB transplantation occurred over a period of 2 to 3 years for both adult and pediatric recipients. Both patient groups eventually demonstrated a normal lymphocyte count with a quantitatively normal distribution of B-cells, T-cells, and natural killer (NK) cells. The sources of T-cells, however, appear strikingly different. Pediatric recipients demonstrated significant numbers of sjTREC-containing T-cells starting within 1 year after transplantation, indicating that the T-cells are recent thymic emigrants. In contrast, adult recipients failed to demonstrate any sjTREC-containing T-cells until 1½ to 2 years after transplantation, and then only at relatively low levels. These results suggest that adult recipients of cord blood had a very slow recovery of T-cell numbers and function.

Recently, less toxic nonmyeloablative regimens using peripheral blood stem cells or marrow from matched donors have allowed the expansion of allogeneic stem cell transplantation to older, more debilitated patients [17,18]. This approach is still limited by the lack of matched sibling donors, and alternative donor sources must be sought. We have been investigating the feasibility of using cord blood as the stem cell source [19]. This report describes the recovery of T-cells in 5 recipients of cord blood transplants following a nonmyeloablative regimen compared to recovery in adult recipients of cord blood following a myeloablative regimen.

METHODS

Patients

Patient characteristics are summarized in Table 1. Five adult patients without a suitable HLA-matched related or unrelated bone marrow donor underwent mismatched unrelated cord blood transplantation between May 2000 and May 2001 at Duke University Medical Center (DUMC). The diagnoses included refractory mantle cell lymphoma, refractory diffuse large cell lymphoma (after failing autologous stem cell transplantation), renal cell carcinoma, myelodysplastic syndrome, and refractory γ-δ T-cell lymphoma. None of these patients had prior radiation to a mantle field (affecting the thymus). All patients were treated following a phase I protocol approved by the DUMC Institutional Review Board. Informed consent was obtained from all patients. Adult patients receiving a myeloablative preparatory regimen and cord blood transplantation have been previously described [16]. The clinical aspects of the first 2 recipients of the nonmyeloablative regimen have also been reported [19].

Conditioning

Conditioning included the use of fludarabine 30 mg/m^2 and cyclophosphamide 500 mg/m^2 daily for 4 days (days –5 to –2) with antithymocyte globulin (ATG) 30 mg/kg per day for 3 days (days –3 to –1). Mismatched unrelated cord blood was infused on day 0. All the grafts were 4/6 HLA matches.

Graft-versus-Host Prophylaxis

All patients received cyclosporine (CYA) and methylprednisolone for graft-versus-host disease (GVHD) prophylaxis. Patients received CYA with an intermediate dose of

Table 2. *Lymphocyte Recovery*

Time	Median Lymphocyte Count (Range)	Median CD45RA (Range)	Median CD45RO (Range)
3 mo	355 (225-448)	263 (182-345)	182 (180-185)
6 mo*	2050 (87-3264)	987 (113-1860)	608 (110-1106)
1 y*	4135 (1962-6308)	2477 (980-3974)	1234 (765-1703)

*At 6 months and forward, all cells were of donor origin (total of 3 patients with engraftment).

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 day +14 through day +27, followed by a daily dose tapered by 10% each week). Methylprednisolone was changed to oral prednisone when the patient was able to tolerate oral intake. The dosages of CYA were adjusted to maintain serum levels between 200 and 400 ng/dL. Patients were scored for GVHD according to established criteria [20]. CYA and prednisone were tapered over several months starting at day +180 in those patients without evidence of chronic GVHD.

Immunologic Recovery

Immune recovery was analyzed with quantitative and qualitative measures at least every 3 months for the first year after transplantation. Peripheral blood was analyzed through the hospital clinical laboratory to measure the total white blood cell count (WBC) and absolute lymphocyte count (based on an automated or manually interpreted blood smear). Lymphocyte subsets (B, T, memory versus naïve T, and NK) were enumerated using multiparameter flow cytometry. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll separation of freshly drawn heparinized whole blood followed by staining with fluorescent-labeled monoclonal antibodies. Samples were stained with a standard panel of antibodies, including anti-CD2, CD3, CD4, CD8, CD16, CD19, CD20, CD10, and CD45, CD56, TCRαβ, TCRγδ, CD45RA, and CD45RO. FastImmune analysis (CD69, Becton Dickinson, San Jose, CA) expression was also monitored. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson).

TREC Measurement

The concentration of sjTREC DNA in PBMC was measured by quantitative competitive (QC)-PCR following a method previously described [15]. Briefly, DNA was isolated from 5×10^4 $CD3^+$ using the Life Technologies TRIZOL Reagent protocol (Gaithersburg, MD) or proteinase K digestion. DNA was resuspended in 100 µL of 8mM 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) 2.5 µL, 50-mM $MgCl_2$ 1.75 µL, 10-µM deoxynucleoside triphosphate (dNTP) 0.5 µL, 12.5-µM sjTREC Primer A 1 µL, 12.5-µM sjTREC B 1 µL, 2.5 µCi ^{32}P deoxycytidine triphosphate (dCTP), 0.125 µL Platinum Taq Polymerase (Life Technologies), and either 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 software. In this assay, the limit of detection was >100 sjTRECs/µg DNA.

CDR3 Spectratyping

CDR3 spectratyping (immunoscope) was performed every 3 months. Immunoscope analysis involves preparing cDNA and performing 23 PCR reactions using a common C-region primer and separate V-region–specific primers, followed by primer extension and PCR amplification with 1 of 12 separate J-region–specific labeled primers as previously described [21-23]. The product is then sequenced with a DNA sequencer (Applied Biosystems, Foster City, CA). Size determination of the run-off products is performed automatically. The different peaks are separated, and their CDR3 size in amino acids is calculated for each Vβ gene family. Histogram peaks were defined by the Genotyper Genescan software analysis program (Applied Biosystems). Further analysis was performed by magnetic selection of the $CD3^+$ cells into $CD4^+$ and $CD8^+$ cells and then performance of the immunoscope analysis.

RESULTS

Clinical Recovery

Four of the 5 patients had clear evidence of donor cells. One patient never had any evidence of detectable donor cells. Another patient had minimal (1%) transient detectable donor cells, but these cells did not persist. The other 3 patients had full donor chimerism established between 6 weeks and 6 months following the nonmyeloablative cord blood transplantation (Table 1).

Acute GVHD did not occur except in 1 patient (UPN 2611) who developed grade II GVHD and subsequently died of disseminated *Aspergillus flavus* infection. No other patients experienced any unusual or unexpected toxicities. As of this report, relapse of the underlying disease had not occurred in the patients with engraftment.

Lymphocyte Subsets

Lymphocyte subset analysis and proliferation assays were performed on all patients who had engraftment. We previously reported that the absolute lymphocyte count remained below normal until the 2-year follow-up [16]. In contrast, the patients who achieved engraftment had a rapid recovery to normal levels within 6 months, at which time all the cells in the peripheral blood were of donor origin according to microsatellite polymorphism analysis. Naïve ($CD45RA^+$, $CD62L^+$) and memory ($CD45RO^+$, $CD62L^-$) T-cell populations were likewise quite different (Table 2). The recovery of naïve cells for 1 patient (UPN2593) throughout the first 6 months is demonstrated in Figure 1.

Immunoscope

Five randomly chosen cord blood units that were not used clinically were tested for their T-cell repertoire. The median number of peaks identified in each of the UCB grafts was 141 (range, 121-185), consistent with previously published data [21]. Spectratyping from the 3 nonmyeloablative

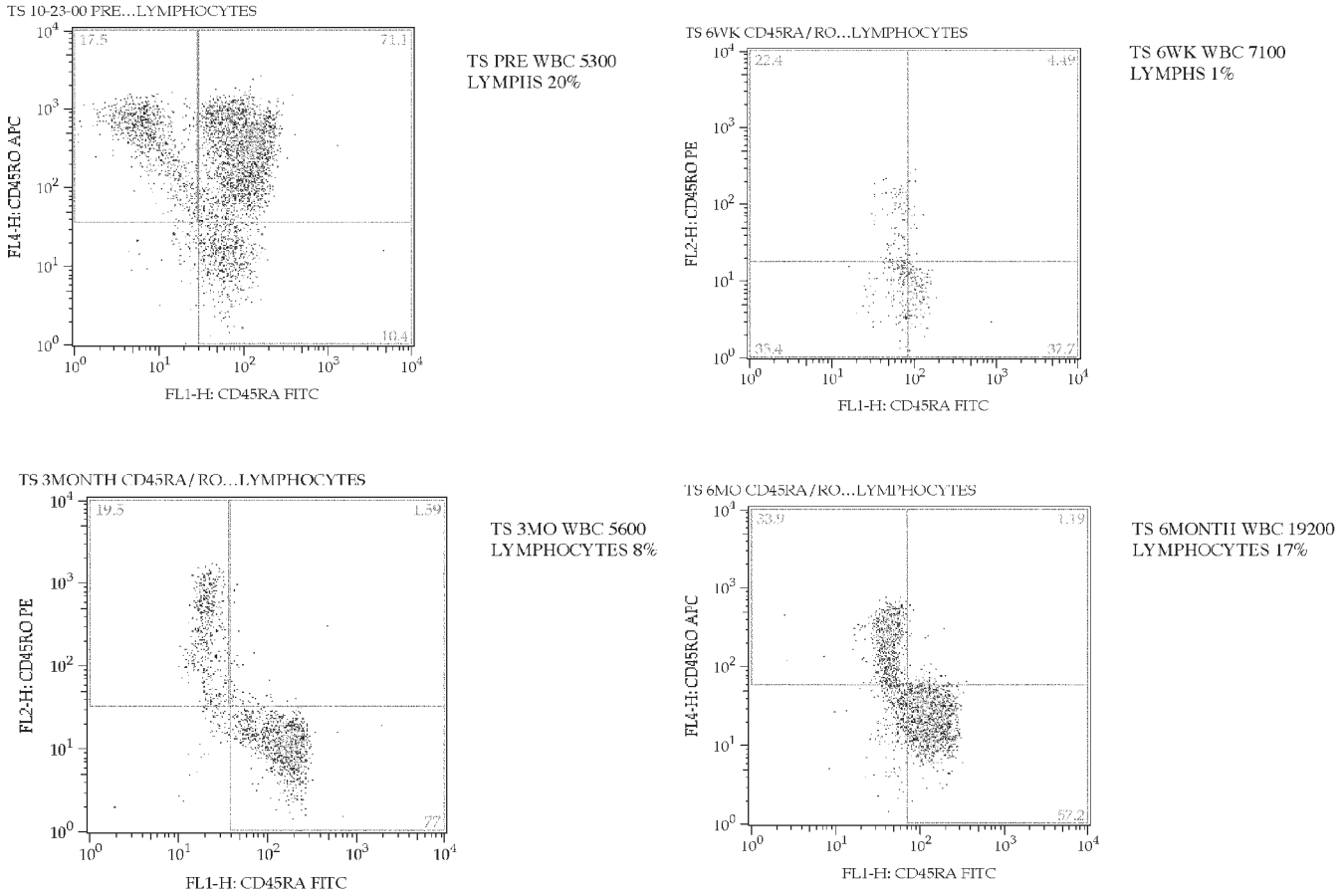

Figure 1. Fluorescence-activated cell sorting (FACS) analysis of 1 patient's recovery of naïve phenotype T-cells over the course of 6 months following a nonmyeloablative unrelated mismatched cord blood transplantation. FITC indicates fluorescein isothiocyanate; APC, allophycocyanin; PE, phycoerythrin.

recipients demonstrated a remarkable recovery of complexity as early as 3 months following the nonmyeloablative regimen. In contrast, recipients of the ablative regimen demonstrated repertoire skewing between 1 and 2 years after transplantation: the overall number of Vβ families represented and the number of peaks within each family were limited. T-cell repertoires appeared to be more diverse when tested 3 years after transplantation, but they remained substantially skewed in 1 patient. Fluorescence intensity spectra for each of 23 Vβ families obtained from 2 of the nonmyeloablative recipients are presented in Figure 2. As early as 6 months after transplantation, at a time when all the engrafted patients had full donor chimerism, the repertoire appeared complex. In contrast, Figure 3 shows results from a typical adult patient following an ablative preparatory regimen sampled 1 year after UCB transplantation compared to those from a patient receiving a nonmyeloablative regimen at 6 months. The contrast between the 2 immunoscopes is striking.

To further understand the contribution of the CD4 and CD8 cells to the complexity of the T-cell repertoire, $CD3^+$ cells were further selected into $CD4^+$ and $CD8^+$ cells using magnetic beads (Miltenyi, Auburn, CA). Figure 4 shows one representative example of a patient 1 year following a nonmyeloablative regimen. The data suggest not only that the naïve cells are recovering faster but that there is a bias toward increased complexity in the $CD4^+$ cells compared to the $CD8^+$ cells.

sjTREC Assay

QC-PCR for sjTREC DNA was performed on samples of peripheral blood. TRECs were not detectable prior to 1 year (at 3, 6, and 9 months). By the 1-year mark, both of the surviving patients had sjTRECs above the detection threshold in the peripheral blood (>100 sjTRECs/μg PBMC DNA) with values of 120 and 157 TRECs/μg PBMC DNA. Five randomly chosen cord blood units (not used in any of the patients presented) were also sampled for sjTRECs, and their median was 3913 copies/μg PBMC DNA (standard error, 2291).

DISCUSSION

This report compares quantitative and qualitative immunological recovery following an unrelated partially matched UCB transplantation in patients receiving a nonmyeloablative or a myeloablative preparatory regimen. Studies of immune recovery after matched-sibling allogeneic transplantation have demonstrated quantitative recovery of T-cells at between 6 and 12 months after transplantation. After an ablative regimen and UCB transplantation in 18 adult patients, the T-cell numbers did not reach the lower limit of

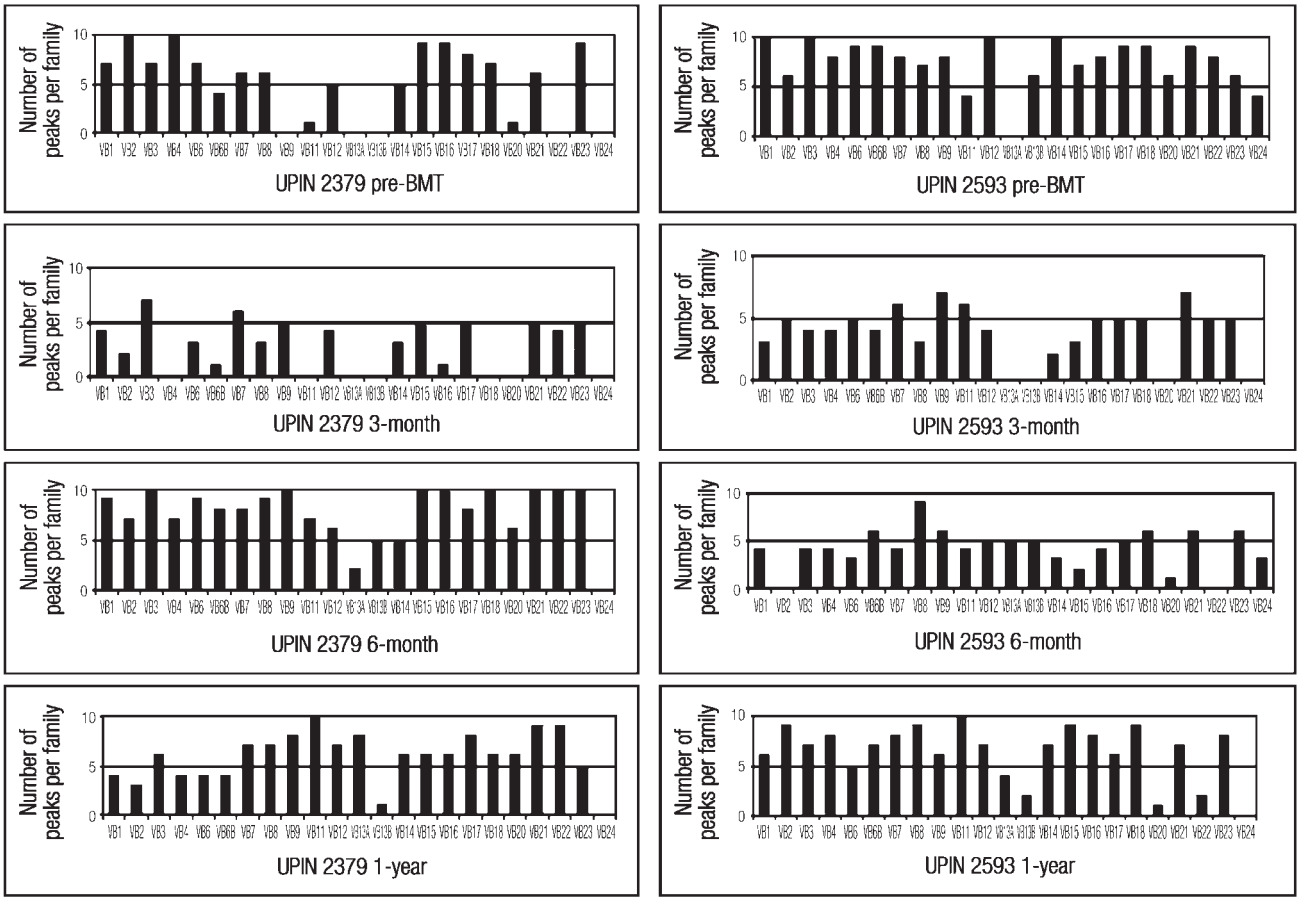

Figure 2. Composite spectratyping of 2 patients' recovery over the first year following a nonmyeloablative unrelated mismatched cord blood transplantation. Note the gradual increase in the complexity of the TCR families.

normal until the second year [16]. Memory T-cells were the dominant subpopulation for the first year after transplantation in adults. The contribution of naïve T-cells increased in the second and third years to equal that of memory cells, coinciding with the appearance of a low level of sjTREC-containing T-cells and improvement in proliferative responses. Reconstitution of T-cells, although it was marginal, occurred principally through peripheral expansion for at least the first 18 months after transplantation. Thymic production of new T-cells contributed to the T-cell pool 2 or more years after transplantation.

In contrast, the rate of T-cell recovery was markedly different for recipients of UCB transplants who were prepared with a nonmyeloablative regimen. In these patients, the preparatory regimen was the primary difference between the 2 patient populations. The GVHD prophylaxis, ATG dose, and supportive care were identical. The only other major difference was that the patient population receiving the nonmyeloablative regimen had more advanced disease or had a poorer performance status that made them ineligible for conventional ablative UCB transplantation. Although memory T-cells were the dominant subpopulation for the patients receiving the ablative regimen, a rapidly expanding naïve population outnumbered the memory cells in the recipients of the nonmyeloablative regimen. These results are similar to the rapid recovery of naïve $CD4^+$ cells seen in children following nonmyeloablative chemotherapy [24]. The naïve cells brought the total T-cell count up into the normal range by 1 year after transplantation. In contrast to those receiving the ablative regimens, 2 patients that received nonmyeloablative transplantations had detectable sjTRECs in as early as 1 year after transplantation. The third patient who also engrafted developed acute GVHD, and her recovery was not as robust. The rapid rise of naïve cells and the detectable levels of sjTRECs show that in the recipients of nonmyeloablative regimens, the thymus is functional early in the posttransplantation course, maturing bone marrow–derived precursor T-cells. Although cord blood itself does contain substantial numbers of new thymic emigrants, sjTRECs remain undetectable through the first year after transplantation. The later increase, therefore, cannot be attributed to adoptive transfer of sjTRECs in the graft but must come from new thymic function. Lastly, although the measured parameters point to a recovering immune system, functional studies are also important. Mitogen responses in the 2 patients with good engraftment have been very robust and not different from those of healthy controls (data not shown). Measurements for cytomegalovirus (CMV) responses using CMV antigen and the FASTImmune assay (CD69 measurement) at the 1-year mark have not yet demonstrated sufficient responses to convincingly demonstrate a robust CMV response. However, CMV

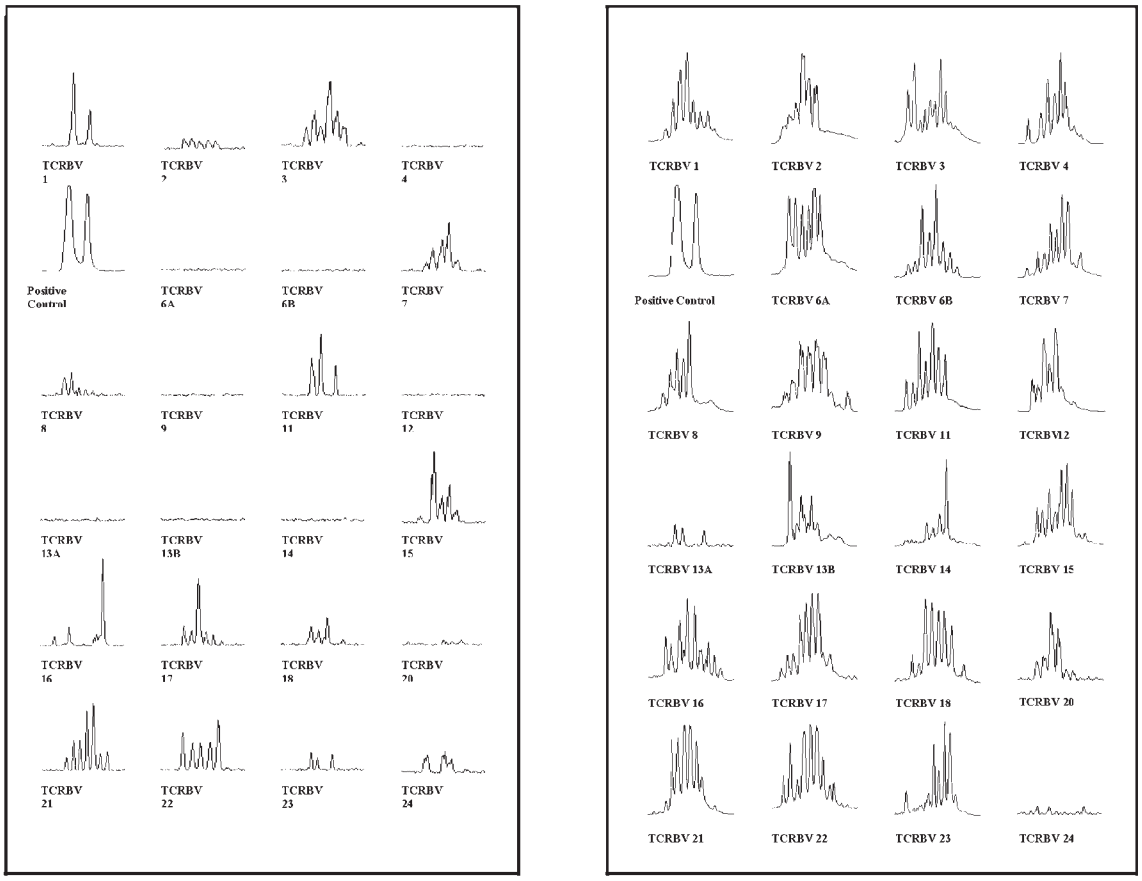

Figure 3. Fluorescence intensity spectra for each of the 23 Vβ families (and 1 positive control) from a representative recipient of an unrelated mismatched cord blood transplant following an ablative regimen (left) at 1 year compared to a recipient of a nonmyeloablative unrelated mismatched cord blood transplantation (right) at 6 months following transplantation.

reactivation has not been observed in the 3 patients that have had engraftment. Further functional studies are necessary in these patients.

UCB has proven to be a valuable source of hematopoietic progenitor cells for transplantation into pediatric and adult patients for whom a matched-related donor is not available. Advantages over HLA-matched unrelated donor sources include rapid graft availability and relative insensitivity to HLA disparity manifested as a low incidence of GVHD [1]. At the same time, adult UCB transplant recipients are at increased risk for opportunistic infections for at least 2 to 3 years after transplantation. Infection may account for half of all treatment-related deaths after UCB transplantation following an ablative regimen [2]. This risk for infection may be an intrinsic property of cord blood, because UCB T-cells have been shown to be phenotypically naïve [21] and 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 [25,26]. Moreover, thymic production of new T-cells is substantially delayed and remains limited in adult recipients of UCB following an ablative preparatory regimen.

In our previous comparison between adult and pediatric recipients of UCB transplant, the disparity between adult and pediatric patients suggested that host-specific or post-transplantation factors are at work: thymic atrophy due to age, GVHD, immunosuppressive drug effect, or other as-yet-undetermined age-related factors. Thymic function declines with age [27-29]. The thymus of adult recipients may have naturally atrophied and/or been exposed to more chemotherapy (or environmental toxin) prior to BMT than that of pediatric recipients, resulting in a delayed response. In fact, the measured TREC levels in adults were lower than expected for median age as predicted for healthy controls. However, peak thymic function may not yet have been reached in the adult UCB recipients. If the reconstituted T-cell pool after transplantation is derived solely by expansion of the adoptively transferred "mature" cord blood cells, the repertoire of antigen specificities may be severely limited.

Yet, the conclusions from our previous findings with the myeloablative preparatory regimen are challenged by the findings of immune recovery following the nonmyeloablative regimen. In a similar patient population (all adults), using identical supportive care and GVHD prophylaxis, the current study demonstrates that unrelated mismatched UCB can proliferate in the periphery quite nicely, giving rise to mature T-cells with remarkably robust complexity. These results suggest that the donor cells only need a niche in which they can proliferate and that the nonmyeloablative regimen does not destroy these niches. Moreover, although the peripheral

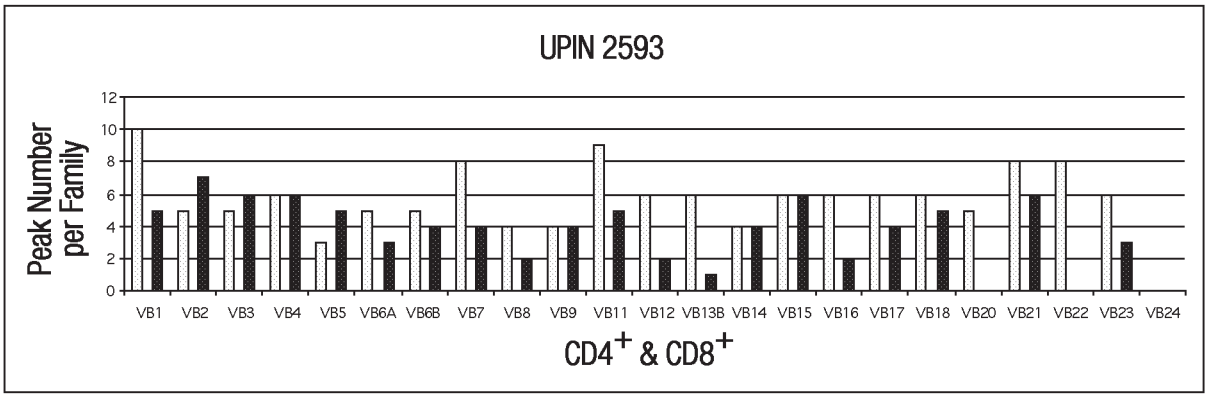

Figure 4. Composite spectratyping of 1 patient's recovery of TCR complexity in $CD4^+$ (□) and $CD8^+$ (■) T-cells 1 year following a nonmyeloablative unrelated mismatched cord blood transplantation. Note the relative higher complexity in $CD4^+$ cells.

mechanisms of T-cell expansion are preserved, there is also a suggestion that the central mechanism (thymus) is likewise preserved, in that TREC-positive cells can be detected as early as 1 year following transplantation. Similar results have been reported by Friedman et al. In that report, the authors demonstrated that the patients who received the nonmyeloablative conditioning and either bone marrow or peripheral blood matched unrelated stem cells exhibited more rapid reconstitution of T-cell repertoire complexity [30]. These authors clearly demonstrated the spectratyping differences based on the type of conditioning regimen.

Although the risk of GVHD following cord blood transplantation is lower than that following matched sibling allogeneic bone marrow or stem cell transplantation, GVHD does occur. GVHD has been shown to affect thymic tissue and may render the thymus unable to generate new T-cells. Alternatively, medical prophylaxis to prevent GVHD (cyclosporin or FK506) may injure the thymus and/or precursor T-cells. Acute GVHD did not occur in the 2 patients studied in the most detail, and the presence of sjTRECs might be a reflection of a lack of thymic injury from GVHD. This lack of injury would not be surprising given that the incidence of acute GVHD may be lower in recipients of nonmyeloablative regimens.

Although not apparent in this analysis, the potential for "holes" in the T-cell repertoire may exist in adult patients. These holes could result from transplantation of a limited number of mature donor T-cells or a subsequent toxic insult to the grafted T-cells (including GVHD prophylaxis). Such voids would be permanent without thymic maturation of new precursor T-cells with novel TCR gene rearrangements. Moreover, the delay in TREC-positive T-cells may also have important implications in the timing of immunization and posttransplantation immunotherapy [26]. The results of T-cell recovery following the nonmyeloablative regimen suggest that it may be possible to have an excellent outcome with an unrelated mismatched cord blood transplant in adult patients. Patients have a rapid recovery of myeloid cells and platelets and a rapid recovery of T-cells with a complex diversity. The primary difference between the recipients of ablative and nonablative regimens was the extent of physiologic damage caused by the preparatory regimen. When the damage is relatively mild, the donor T-cells are able to expand effectively in the periphery, and the development of new T-cells through the thymus is also accelerated compared to the rate of development in those receiving ablative regimens. Alternatively, the lower incidence of acute GVHD may also play an important role in the preservation of the peripheral and central niches for T-cell development. Future investigation will focus on increasing the chances of engraftment following this nonmyeloablative regimen and expanding these observations.

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