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Early Recovery of CD4 T Cell Receptor Diversity after "Lymphoablative" Conditioning and Autologous CD34 Cell Transplantation

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T cell diversity posttransplantation is thought to be severely restricted, based on T cell receptor β-chain immunophenotyping or spectratyping. Using β-chain sequencing, we studied CD4 T cell diversity in 2 adult patients undergoing "lymphoablative" conditioning with cyclophosphamide (Cy), total body irradiation (TBI), and antithymocyte globulin (ATG) and autologous transplantation of hematopoietic cells depleted of T cells by enrichment for CD34 cells. The indication for the transplantation was systemic sclerosis (SSc) or multiple sclerosis (MS). Pretransplantation, the estimated number of distinct β chains (the minimum number of CD4 T cell clones) in the 2 patients was 600,000 to 700,000, similar to the number in a healthy control. This number was 200,000 to 500,000 at 1 month posttransplantation and 400,000 to 1,600,000 at 12 months posttransplantation. In conclusion, the number of T cells early after lymphoablative conditioning and autologous CD34 cell transplantation may be more diverse than previously appreciated, possibly because many T cell clones survive the conditioning or are reinfused with the graft. Thus, the therapy may not be completely T cell lymphoablative.

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The diversity of T cells posttransplantation has been studied primarily by the flow cytometric detection of T cell receptor β-chain families (with variable region-specific monoclonal antibodies [mAbs]) or by spectratyping, that is, electrophoretic detection of various lengths of the β-chain complementarity-determining region 3 (CDR3) segments of rearranged β-chain genes [1-21]. With both techniques, the repertoire of posttransplantation T cells appears to be skewed; that is, some T cell clones appear to be overrepresented, whereas others appear to be absent. This phenomenon is particularly prominent in the first several months posttransplantation and in patients who received T cell–depleted grafts (eg, in vivo with antithymocyte globulin [ATG] or ex vivo by CD34 cell graft enrichment). Normalization of the flow cytometry or spectratyping findings in adults typically takes at least 2 years [14,22-25]. This suggests that in the first several months posttransplantation, the diversity of T cells is severely restricted, and that it takes at least 2 years to restore the diversity to normal. But the skewed Vβ representation by flow cytometry or spectratyping also may reflect expansion of certain T cell clones, rather than restricted T cell diversity [26]. The expanded clones may make the detection of nonexpanded clones difficult or impossible, especially if specimens contain only a small number of T cells. In the present study, we used a sequencing-based method and specimens containing large numbers of T cells (obtained by apheresis) to estimate the number of distinct β chains (as a surrogate of T cell diversity).

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SUBJECTS AND METHODS

Three individuals were studied: a 46-year-old female with systemic sclerosis (SSc) undergoing autologous CD34 cell transplantation using conditioning with cyclophosphamide (Cy; 120 mg/kg), total body irradiation (TBI; 8 Gy), and ATG (90 mg/kg) [27]; a 28-year-old male with multiple sclerosis (MS)

Table 1. Determination of CD4 T Cell Diversity in a Healthy Control and 2 Patients

Subject (time point) [CD4 T cells per μL blood*]	Number of Vβ17⁺ CD4⁺ T cells analyzed	Rearrangement	Frequency of Vβ17 gene, %	Frequency of Jβ (1.4, 1.5, or 2.6) gene, %	Peak size, %	Number of distinct sequences within the peak(s)	Diversity (× 10^6)
Healthy control	10^5	Vβ17-Jβ1.5	1.6	13	18.5	140	0.4
	10^6	Vβ17-Jβ1.4	1.6	2.9	20.2	60	0.6
	10^5	Vβ17-Jβ1.4	1.6	2.9	20.2	60	0.6
SSc patient (pretransplantation)	10^6	Vβ17-Jβ1.4	1.0	0.6	35.1	12	0.7
	10^5	Vβ17-Jβ1.4	1.0	0.6	35.1	11	0.6
SSc patient (1 month posttransplantation)	10^5	Vβ17-Jβ1.4	0.7	6.7	100.0	12	0.3
[CD4 = 26]	10^5	Vβ17-Jβ2.6	0.7	2.0	100.0	25	0.2
SSc patient (12 months posttransplantation)	10^5	Vβ17-Jβ1.4	1.5	2.1	100.0	16	0.5
[CD4 = 347]	10^5	Vβ17-Jβ2.6	1.5	1.1	100.0	70	0.4
MS patient (1 month posttransplantation)	10^5	Vβ17-Jβ1.4	0.5	3.3	100.0	74	0.4
[CD4 = 5]	10^5	Vβ17-Jβ2.6	0.5	1.7	100.0	45	0.5
MS patient (12 month posttransplantation)	10^5	Vβ17-Jβ1.4	0.5	2.8	100.0	230	1.6
[CD4 = 186]	10^5	Vβ17-Jβ2.6	0.5	1.8	100.0	>112†	>1.2

*5th to 95th percentile reference range, 416 to 1437/μL.
†Plateau of the curve showing the number of distinct clones versus the number of clones sequenced had not been reached by the time 180 clones were sequenced.

undergoing autologous CD34 cell transplantation using the same conditioning [28]; and a healthy 44-year-old male (control). The 2 patients were selected from a cohort of 56 patients undergoing transplantation for SSc or MS based on their willingness to undergo research apheresis at 1 and 12 months posttransplantation. Because the transplantation was performed for autoimmune diseases, it was designed to be maximally lymphoablative. Thus, not only were Cy, TBI, and ATG used, but also the autologous graft was enriched immunomagnetically for CD34 cells. The typical composition of the graft using this protocol was 261 × 10^6 CD34 cells, 2 × 10^6 CD4 T cells, and 1 × 10^6 CD8 T cells [29].

Immune reconstitution of the whole cohort has been described in detail previously [29]. The immunologic studies of the 2 patients were representative of the whole cohort, in that their CD4 T cell counts were very low at 1 month posttransplantation and moderately low at 12 months posttransplantation (Table 1, first column), and their CD4 and CD8 Vβ-Jβ spectratypes exhibited a lower-than-normal median number of peaks at both 1 month and 12 months posttransplantation (data not shown). For the studies of posttransplantation T cell diversity, 8-L mononuclear cell apheresis (COBE Spectra; CaridianBCT, Lakewood, CO) was performed at 1 month and 12 months posttransplantation. For the SSc patient, pretransplantation T cell diversity also was studied, using cells obtained as the flow-through (CD34-negative) fraction during the immunomagnetic selection of CD34 cells from the autologous graft (apheresis product). The flow-through fraction of the graft was unavailable for the MS patient.

Clinically, the underlying disease was at least partially stabilized. The SSc patient's Rodnan skin score was 48 pretransplantation, 39 at 1 year posttransplantation, 32 at 2 years posttransplantation, and 42 at 4 years posttransplantation, and lung function (limited pretransplantation) was stable for 4 years posttransplantation. The MS patient's extended disability status scale was 2.0 at 1 year pretransplantation; 7.0 to 7.5 immediately pretransplantation; 8.0 at 1 year, 2 years, and 3 years posttransplantation; and 7.5 at 4 years posttransplantation. No new T2 magnetic resonance imaging (MRI) lesions were detected on any available determination (3, 6, and 12 months posttransplantation). The study design received Institutional Review Board approval.

The number of distinct β chains was estimated using the method of Arstila et al. [30], with minor modifications. The focus was on CD4 T cells, because these cells appear to play an important role in the pathogenesis of SSc and MS, and their quantitative deficiency appears to play an important role in transplant recipients' susceptibility to infection [31-34]. The frequency of cells using Vβ17 among CD4 T cells was determined by flow cytometry, using Vβ17 antibody-fluorescein conjugate (Beckman-Coulter/Immunotech, Fullerton, CA) and CD4 antibody-phycoerythrin conjugate (BD Biosciences, San Jose, CA) (Table 1, fourth column). To maximize the sensitivity and specificity of the downstream polymerase chain reaction (PCR), Vβ17⁺CD4⁺cells were sorted to > 95% purity using FACS Vantage (BD Biosciences). After centrifugation, the cells were resuspended into 100 μL of solution D, prepared by mixing 7 μL of β-mercaptoethanol (14.2 *M*) with 100 μL of lysis buffer from the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA). RNA was extracted using the same kit, and cDNA was synthesized using the Superscript II RNase H⁻ Reverse-Transcriptase Kit (Invitrogen) and Oligo(dT)$_{12-18}$ primers (Invitrogen). Each reverse transcription yielded 60 μL of cDNA. The cDNA was used for real-time PCR to determine the frequency of cells using Jβ1.4 (or Jβ1.5 or Jβ2.6) (Table 1, fifth column), for spectratyping to determine the size of the CDR3 peak(s) of interest (Table 1, sixth column),

and for gel electrophoresis for cloning and sequencing to determine the number of different sequences within the peak(s) (Table 1, seventh column). Details of the determinations of the frequency of cells using Jβ1.4 (or Jβ1.5 or Jβ2.6), the size of the CDR3 peak(s) of interest, and the number of different sequences within the peak(s), are provided below. Diversity (total number of distinct Vβ chains among CD4 T cells, ie, the minimum number of CD4 T cell clones; Table 1, last column) was calculated as

$$\frac{\text{Number of distinct sequences within the peak(s)}}{\text{Peak size }(\%)/100 \times \text{ frequency of J}\beta\text{ gene }(\%)/100 \times \text{ frequency of V}\beta 17\text{ gene }(\%)/100.}$$

Because the determination of diversity gave a similar result when 10^5 or 10^6 sorted $V\beta17^+CD4^+$ cells were used, 10^5 cells were used for most determinations (Table 1, second column). Note that in accordance with the ImMunoGeneTics nomenclature [35], Vβ17 is TRBV19, Jβ1.4 is TRBJ1-4, Jβ1.5 is TRBJ1-5, and Jβ2.6 is TRBJ2-6.

The frequency of cells using Jβ1.4 (or Jβ1.5 or Jβ2.6) was determined by real-time PCR. The forward primer was AAGGGTACAGCGTCTCTCGG (Vβ17); the reverse primer was AGACAGAGAGCTGGGTTCCA (Jβ1.4), CAGCATTTTGGTGATGGGAC (Jβ1.5), ACTTTCGGGGCCGGCAGCAG ((Jβ1.6), or GAG GTCGCTGTGTTTGAGCC (C); and the probe was FAM- TCCTTTCCTCTCACTGTGACATC GGC-TAMRA (MegaBases, Chicago, IL). Each PCR reaction contained 5 μL of cDNA as a template, 1 U of Platinum-Taq polymerase (Invitrogen), 2.5 mM $MgCl_2$, 500 μM dNTPs, 500 nM each primer, 250 nM probe, and 200 nM Blue-636 reference (MegaBases). The reactions were run at 95°C for 5 minutes, then at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 40 cycles, using an ABI Prism 7700 Sequence Detector (PE Biosystems, Norwalk, CT). Endpoint-normalized fluorescence (Rn) was detected. Vβ17-VC was considered the standard. The Jβ1.4 percentage was calculated as $Rn_{V\beta17\text{-}J\beta1.4}/Rn_{V\beta17\text{-}VC} \times 100$ (analogous for Jβ1.5 and Jb2.6) using the ABI 7700 software (PE Biosystems). Vβ17-Jβ1.4 (Vβ17-Jβ1.5, Vβ17-Jb2.6) and Vβ17-VC were assumed to have the same amplification rate. This assumption was verified by multiple 10-fold dilutions of the templates.

The sizes of the CDR3 spectratyping peaks of interest were determined as follows: Vβ17-Jβ1.4, Vβ17-Jβ1.5, or Vβ17-Jβ2.6 segments were separately amplified by PCR, using forward primer FAM-AAGGGTACAGCGTCTCTCGG (Vβ17) and the same reverse primers as in real-time PCR. Each PCR reaction contained 5 μL of cDNA, 1 U of Platinum-Taq polymerase (Invitrogen), 2.5 mM $MgCl_2$, 500 μM dNTPs, and 500 nM each primer. The reactions were run at 95°C for 9 minutes; then at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 35 cycles; and finally at 72°C for 30 minutes. The dye-labeled PCR products were detected on an ABI 3100 sequence analyzer (PE Biosystems); peaks were visualized, and their heights were quantified using GeneScan software (Applied Biosystems, Foster City, CA). If for cloning and sequencing, only a single peak was excised from the gel and analyzed, then the peak size value was < 100%. If all peaks were excised and analyzed, then the value was 100%. The decision whether to excise 1 peak or all peaks was based on whether the peak heights showed a Gaussian distribution (1 peak excised) or not (all peaks excised), because only in the case of a Gaussian distribution is it reasonable to assume that the peak height is proportional to the diversity within the peak.

The number of different (distinct) sequences within the peak(s) was determined by cloning and sequencing, as follows. Vβ17-Jβ1.4, Vβ17-Jβ1.5, or Vβ17-Jβ2.6 PCR products were separated by 2% agarose gel and 12% polyacrylamide gel and then extracted and purified using the QIAEX II Gel Extraction Kit (Qiagen, Foster City, CA) after cutting of single or multiple band(s) under UV light. Purified PCR products were inserted into vector pCR 2.1-TOPO and transformed into DH5αT1-competent *Escherichia coli* using the TOPO TA cloning Kit (Invitrogen). After culture on LB plates with 100 μg/mL of ampicillin, 25 μg/mL of kanamycin, 0.1 mM IPTG, and 60 μg/mL of X-gal for >12 hours, white or light blue colonies were chosen as positive clones. Plasmid DNA was sequenced using Bigdye dNTP (Applied Biosystems), an ABI 373 sequencer, and ABI-PRISM software. Clones were sequenced in batches of 20 until it was obvious that no new distinct sequences would be found (100 to 500 clones per each subject; time point and Vβ17-Jβ combination) (Figure 1).

RESULTS

The healthy control had 400,000 to 600,000 distinct Vβ chains (Table 1), suggesting that the minimum number of CD4 T cell clones in a normal person is approximately 500,000. This result is close to that obtained by Arstila et al. (800,000 to 1,200,000) [30]. Our estimate may be lower than that of Arstila et al., because those authors used total T cells, whereas we used CD4 T cells.

At 1 month posttransplantation, despite severe CD4 T lymphopenia and very abnormal CD4 T cell spectratypes, the calculated number of distinct Vβ chains was only 2- to 3-times lower than that detected pretransplantation or found in the healthy control in the SSc patient, and was similar to that in the healthy control in the MS patient (Table 1). This unexpected finding suggests that multiple T cell clones survived

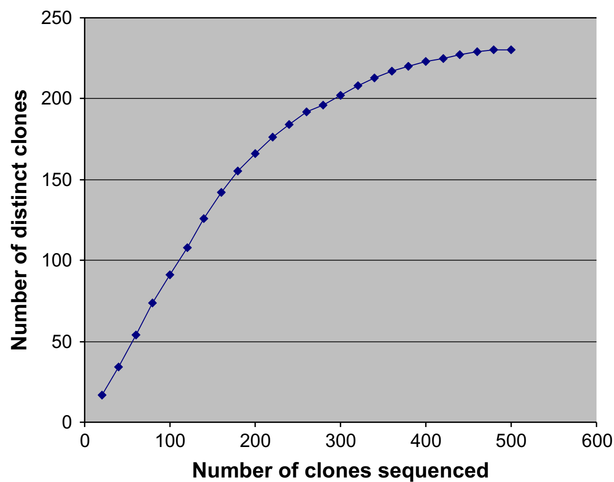

Figure 1. Example of sequencing clones in batches of 20 until it became obvious that no new distinct sequences would be found.

the conditioning or were reinfused with the graft (using this treatment protocol, ~2,000,000 CD4 T cells typically are reinfused with the graft [29]). Consistent with the finding that in the SSc patient, 2 of 2 dominant (each sequenced > 50 times) Vβ17 to -Jβ1.4 sequences in the pretransplantation sample also were detected in the 1-month posttransplantation sample. In addition, 10 nondominant (each sequenced < 10 times) Vβ17 to -Jβ1.4 sequences that were detected in the pretransplantation sample were not detected in the 1-month posttransplantation sample. It is unclear whether these clones were located in extravascular compartments at 1 month posttransplantation, were undetectable in blood from technical reasons, or were truly absent in this patient. The near-normal number of distinct Vβ chains at 1 month posttransplantation likely was not due to the generation of CD4 T cells de novo (ie, from hematopoietic stem cells) in the first month posttransplantation, because T cell receptor excision circles (TRECs) in adult autologous transplant recipients typically become detectable only at ≥ 3 months posttransplantation [24,29]. Consistent with this, in the 2 patients studied here, TRECs were undetectable in CD4 T cells at 1 month posttransplantation. The method of detection has been discussed previously [29].

By 12 months posttransplantation, the diversity of β chains in both patients became normal or near normal (Table 1). This can be attributed, at least in part, to T cell generation de novo [24,25,29]. Consistent with this finding, in the SSc patient, of the 16 distinct Vβ17 to -Jβ1.4 sequences at 1 year posttransplantation, 15 were new sequences compared with those at 1 month posttransplantation. In the MS patient, of the 230 distinct Vβ17 to -Jβ1.4 sequences at 1 year posttransplantation, 196 were new compared with those at 1 month posttransplantation. Also consistent with de novo generation is the fact that both patients exhibited a marked increase in phenotypically naïve (CD45RA^{high}) CD4 T cell counts from 1 month to 1 year posttransplantation, from 1 to 72/μL in the SSc patient and from 1 to 109/μL in the MS patient. The method of detection has been described previously [29]. Naïve T cells are more diverse than memory/effector T cells [30].

Although CD8 T cell diversity was not the focus of this study, nevertheless, we wished to determine whether, analogous to CD4 T cells, pretransplantation CD8 clones can persist after the lymphoablative conditioning with autologous CD34 cell transplantation. We explored this using cytomegalovirus (CMV)- and Epstein-Barr virus (EBV)-specific CD8 clones. As shown in Figure 2, in the MS patient, the same EBV-specific CD8 clone that was present pretransplantation also was present at 1 and 12 months posttransplantation. Similarly, in the SSc patient, the same EBV-specific and CMV-specific CD8 clones that were present pretransplantation also were present posttransplantation. TRECs were undetectable in CD8 T cells at 1 month posttransplantation; thus, all 3 CD8 clones studied likely survived the lymphoablative therapy.

DISCUSSION

The unexpectedly large number of CD4 T cell clones early after transplantation and the persistence of herpesvirus-specific CD8 T cell clones from pretransplantation to posttransplantation may explain why infections are relatively rare in autologous transplant recipients after neutrophil engraftment [29]. It also raises the question of whether clinical stabilization or improvement of patients with autoimmune disease after autologous transplantation is due to the conditioning-induced elimination of most T cell clones (including those causing the disease). On the one hand, conditioning intensity appears to be inversely related to the likelihood of autoimmune disease progression [36], suggesting that debulking of pathogenic T cell clones may be beneficial. On the other hand, Moore et al. [37] found that clinical improvement of patients with rheumatoid arthritis posttransplantation was not inversely related to the number of T cells infused with the autologous graft. Alternative explanations for clinical stabilization or improvement of autoimmune disease after autografting may include functional alteration of pathogenic T cell clones by the conditioning, faster reconstitution of regulatory T cells compared with effector T cells [38,39] and B lymphoablation [40] (B cells are undetectable on day 7 with this kind of transplant [29].)

When interpreting the results presented in Table 1, technical limitations should be kept in mind. First, the number of distinct Vβ chains is lower than the number of T cell clones, because each β chain can

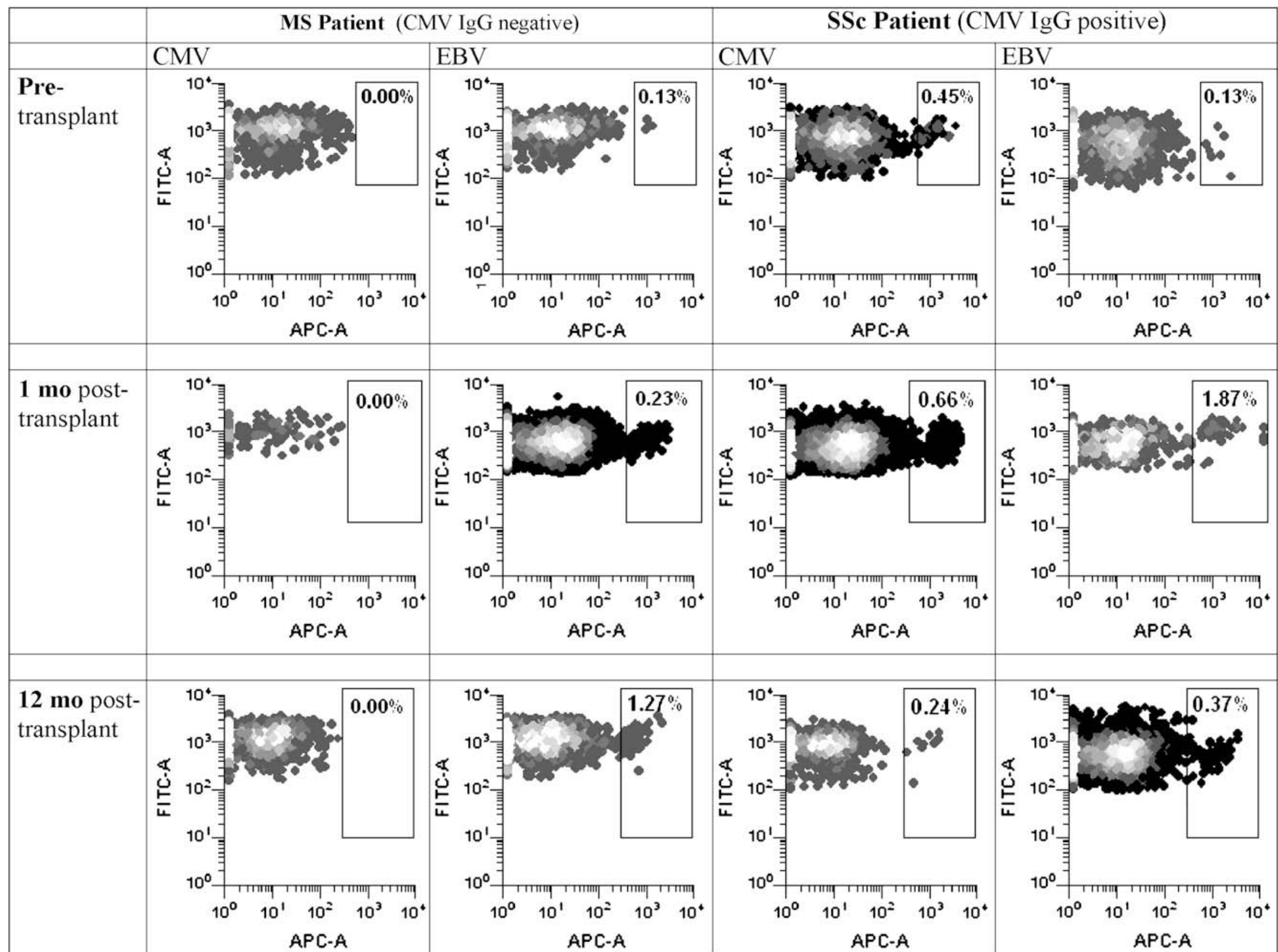

Figure 2. Persistence of CMV- and EBV-specific CD8 T cell clones, using allophycocyanin (APC)-labeled HLA-viral peptide tetramer analysis. Both patients were positive for HLA-A*0201; thus, we used NLVPMVATV (CMV peptide) conjugated to HLA-A*0201-APC tetramer and GLCTLVAML (EBV peptide) conjugated to HLA*0201-APC tetramer (both purchased from Beckman Coulter) to stain CMV- and EBV-specific clones in blood mononuclear cell specimens by flow cytometry. The cells also were stained by CD3-phycoerythrin antibody and CD8-fluorescein isothiocyanate (FITC) antibody. The density dot plots, showing tetramer-APC fluorescence on the *x*-axis and CD8-FITC fluorescence on the *y*-axis, were gated on $CD3^+CD8^+$ lymphocytes. The CMV tetramer staining of the cells from the MS patient (who was CMV-seronegative) served as a negative control for setting the border between tetramer-positive and tetramer-negative cells. The percentages of the tetramer-positive cells (of total CD8 T cells) are shown, indicating that even though the percentages varied, the same EBV-specific CD8 clones in both patients and the same CMV-specific CD8 clone in the CMV-seropositive patient were present both pretransplantation and posttransplantation.

combine with various β chains. Thus, even though the number of Vβ chains may be near normal at 1 month posttransplantation, the number of T cell clones at this time theoretically may be significantly lower than that in the same patient before transplantation or in a healthy control. Nevertheless, the lack of a marked difference in the number of distinct Vβ chains between 1 month posttransplantation and pretransplantation/healthy status suggests that the difference in the number of T cell clones is not extreme. Second, the mathematical calculation of the diversity of Vβ specificities presented here assumes that the diversity within the Vβ17-Jβ1.4 segment is similar to the diversity within other Vβ-Jβ segments. Theoretically, this may not be true; however, the fact that the calculated diversity using Vβ17-Jβ1.5 or Vβ17-Jβ2.6 was similar to that using Vβ17-Jβ1.4 (Table 1) suggests a similar diversity within various Vβ-Jβ segments in 1 individual at 1 time point . Third, given the skewed repertoire of posttransplantation T cells, underrepresented sequences can be easily missed during the sequencing of cloned CDR3 segments unless many clones are sequenced. We attempted to overcome this problem by sequencing clones until it became obvious that we would find no new distinct sequences (ie, 100 to 500 clones per each subject, time point and Vβ17-Jβ combination). Packer and Muraro [41] recently determined in autologous transplant recipients that if only 75 to 100 clones were sequenced, then the true number of distinct sequences would be underestimated, with an error of 5% to 7%. Thus, because we analyzed 100 to 500 sequences, it is likely that we underestimated the true number of distinct sequences with an error of $< 7\%$.

In conclusion, our findings suggest that the diversity of CD4 T cells may be near normal 1 month after Cy + TBI + ATG conditioning and autologous CD34 cell transplantation for autoimmune diseases. This treatment may be only partially T cell–depleting rather than completely T cell–ablative. Because the treatment is not selective for certain T cell clones, at least 1 cell or a few cells appear to be spared from most T cell clones.

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