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# T Cell Repertoire Development in Humans with SCID After Nonablative Allogeneic Marrow Transplantation<sup>1</sup>

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Transplantation of HLA-identical or haploidentical T cell-depleted allogeneic bone marrow (BM) into SCID infants results in thymus-dependent T cell development in the recipients. Immunoscope analysis of the TCR V $\beta$  repertoire was performed on 15 SCID patients given BM transplants. Before and within the first 100 days after bone marrow transplantation (BMT), patients' PBMC displayed an oligoclonal or skewed T cell repertoire, low TCR excision circles (TREC) values, and a predominance of CD45RO<sup>+</sup> T cells. In contrast, the presence of high numbers of CD45RA<sup>+</sup> cells in the circulation of SCID patients >100 days post-BMT correlated with active T cell output by the thymus as revealed by high TREC values and a polyclonal T cell repertoire demonstrated by a Gaussian distribution of V $\beta$ -specific peaks. Ten years after BMT, we observed a decrease of the normal polyclonal T cell repertoire and an increase of a more skewed T cell repertoire. A decline of TREC levels and a decrease in the number of CD45RA<sup>+</sup> cells beyond 10 years after BMT was concomitant with the detection of oligoclonal CD3<sup>+</sup>CD8<sup>+</sup>CD45RO<sup>+</sup> cells. The switch from a polyclonal to a more skewed repertoire, observed in the CD3<sup>+</sup>CD8<sup>+</sup>CD45RO<sup>+</sup> T cell subset, is a phenomenon that occurs normally with decreased thymic output during aging, but not as rapidly as in this patient population. We conclude that a normal T cell repertoire develops in SCID patients as a result of thymic output and the repertoire remains highly diverse for the first 10 years after BMT. The TCR diversity positively correlates in these patients with TREC levels. *The Journal of Immunology*, 2003, 170: 2711–2718.

Human SCID is caused by several genetic defects (1–7) and it is characterized by profound deficiencies in T, B, and, in some types, NK cell function (8–10). SCID infants are lymphopenic, their small thymus lacks thymocytes, their spleen is deficient in T cell areas, and tonsils and lymph nodes are not formed (8). SCID infants rarely survive beyond 1 year of age without therapeutic intervention, such as bone marrow transplantation (BMT)<sup>3</sup> or gene therapy (9, 11, 12). Transplantation of HLA-identical or haploidentical allogeneic-related, T cell-depleted bone marrow (BM) into SCID infants, in the absence of pretransplant chemotherapy or posttransplant prophylaxis, results in close to an 80% survival rate for >20 years posttransplantation (13). After transplantation, genetically donor T cells and T cell functions gradually increase to normal in the SCID recipients.

Studies by this group have demonstrated that T cell reconstitution in SCID recipients of rigorously T cell-depleted allogeneic-related BM cells is due to the development and maturation of donor T cell precursors in the infant's vestigial thymus (10). Within the thymus, T cell precursors undergo TCR gene rearrangements

by the junction of V(D)J gene segments and by the addition of N nucleotides. The process of TCR rearrangement generates extrachromosomal DNA episomes or TCR excision circles (TRECs), which can be detected only in newly thymus-derived T cells, but not in T cells that develop extrathymically (14). In SCID patients, very small numbers of mature CD45RO<sup>+</sup> T cells (10, 15) predominate for the first 100 days, while thymus-derived naive CD45RA<sup>+</sup> T cells carrying TRECs become the predominant T cells in the circulation from 160 to 350 days after BMT (10). The number of CD45RA<sup>+</sup> cells declines thereafter, but CD45RA<sup>+</sup> T cells predominate over CD45RO<sup>+</sup> T cells until 10–12 years. Fourteen years after BMT, TREC values may decline to very low levels (10).

The rearranged TCR  $\alpha$ - and  $\beta$ -chains pair soon after their biosynthesis to yield the TCR heterodimer, which determines the specificity for Ags of the newly arising T cells. Much of the variation of each TCR chain resides in the complementarity-determining region 3 (CDR3). Each T cell has a unique TCR with a characteristic CDR3 region. In the TCR  $\beta$ -chain, the CDR3 region of any V $\beta$ -J $\beta$  combination may vary in length by as many as six to eight amino acids (16, 17). The analysis of CDR3 in a T cell population provides a measure of the diversity of the T cell repertoire and can be performed using the immunoscope technique (16). The immunoscope analysis allows the discrimination of different TCR rearrangements (according to their size) using PCR amplification of the C $\beta$  gene and of individual V $\beta$  family genes across the CDR3 (16). T lymphocytes circulating in the blood of normal subjects express a widely diverse polyclonal TCR repertoire, which can be graphically represented by a normal (Gaussian) distribution of CDR3 size fragments. In some physiological or pathological conditions, the TCR repertoire can be skewed, resulting in the expression of a single or a limited number of TCR rearrangements (16, 18).

In this study, we hypothesized that maturation and selection of donor-derived precursor T cells from transplanted BM into SCID

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<sup>3</sup> Abbreviations used in this paper: BMT, bone marrow transplantation; CDR3, complementarity-determining region 3; Jak3, Janus kinase 3; TREC, TCR excision circles; GVHD, graft-vs-host disease; BM, bone marrow.

recipients results in the development of a diverse T cell repertoire, which correlates with the rise in number of recent thymic emigrant CD45RA<sup>+</sup> T cells and the increase in TRECs. We also hypothesized that the T cell repertoire may not remain diverse in these patients at later times after transplantation, when the thymus output declines.

To our knowledge, correlation of TREC levels with TCR repertoire diversity in long-term SCID survivors who received BMT without chemoablation has not been previously reported.

## Materials and Methods

### Blood samples

We studied PBMC from 15 SCID patients who were given unfractionated HLA-identical BM transplants (1 patient), T cell-depleted HLA-identical BM transplants (1 patient), or T cell-depleted HLA-haploidentical parental BM transplants (13 patients) (Table I). These patients, who were previously reported (10), were selected from a total of 127 SCID patients, based on availability of cryopreserved PBMC. None of the patients received chemotherapy before transplantation or prophylactic drugs to prevent GVHD after transplantation. Seven of these patients had SCID due to a mutation of the gene encoding the common  $\gamma$ -chain expressed by six IL receptors (an X-linked disorder) (2, 3, 19); three had a mutation of the gene encoding Janus kinase 3 (Jak3) (4); four had a defect in the IL-7R  $\alpha$ -chain (20); and one had autosomal recessive SCID of unknown molecular type. Donor BM was depleted of T cells by agglutination with soybean lectin and two cycles of rosetting with sheep erythrocytes that had been treated with aminoethylisothiocyanate bromide as described elsewhere (21). In 13 of 15 patients, the mean number of CD3<sup>+</sup> T cells/kg administered in the graft was  $1.61 \times 10^6$ , while in 2 of 15, who received HLA-identical unfractionated marrow, the mean number of CD3<sup>+</sup> cells/kg in the graft was  $5.31 \times 10^8$ . The mean ( $\pm$ SEM) age at transplantation was 248.5 ( $\pm$ 43.6) days. Blood samples were obtained from the patients before transplantation and at varying intervals thereafter. T cell phenotypes were determined by flow cytometry on freshly isolated PBMC as previously described (10, 21). Excess cells were cryopreserved in RPMI 1640 medium containing DMSO. Blood samples were also obtained from four normal control subjects. The blood specimens were obtained with the approval of the Duke University Committee on Human Investigations and the written informed consent of the patients or their parents.

### Cell sorting

PBMC were prepared by Ficoll gradient separation and incubated for 30 min with fluorochrome-labeled Abs specific for CD3, CD4, CD8, and CD45 (Beckman Coulter, Miami, FL). Fluorochromes used were FITC, PE, and PE-cyanin 5.1 in different combinations. After washing, cells were sorted using a FACS (BD Pharmingen, San Diego, CA) and collected in TRIzol (Life Technologies, Gaithersburg, MD) for RNA analysis.

### Quantitative competitive PCR assay for TRECs

PCR analysis of TCR episomes was performed as described elsewhere (10, 14). Briefly, DNA from 2 to  $10 \times 10^6$  PBMC was isolated with the use of TRIzol. DNA (1  $\mu$ g) was amplified in a reaction mixture containing standard PCR reagents and 5000, 1000, 500, or 100 molecules of an internal competitor standard TREC molecule (60 bp shorter than the target TREC sequence). PCR products were separated by PAGE and quantified with an imaging device (PhosphorImager; Molecular Dynamics, Sunnyvale, CA.). For consistency with previously published data from our group (10), results are reported as TREC per microgram of PBMC DNA. The lower limit of confidence was 100 TREC per microgram of PBMC DNA.

### Immunoscope analysis

Immunoscope analysis of the TCR  $\beta$ -chain used in these studies was performed as published (16). Briefly, RNA was extracted from fresh or frozen PBMC samples ( $2\text{--}10 \times 10^6$  cells/sample) or from sorted T cell populations ( $0.2\text{--}2 \times 10^6$  cells/sample) using TRIzol and reverse transcribed to single-stranded cDNA with reverse transcriptase from avian myeloblastosis virus (Roche, Indianapolis, IN) using an oligo(dT) primer according to the manufacturer's protocol (Promega, Madison, WI). The newly synthesized cDNA was then used as a template for 23 PCR with 23 different unlabeled TCR V $\beta$ - and one C $\beta$ -specific primers. PCR amplifications were conducted to saturation (40 cycles). The products were visualized by an elongation reaction using a nested fluorescent C $\beta$  primer. The elongation products, which contained the CDR3, were separated on a sequencing gel. The results were analyzed using Gene Scan software (Applied Biosystems,

Foster City, CA) and are shown as graphic distribution of size peaks centered on a peak corresponding to a CDR3 of 10 aa. The peaks are spaced by three nucleotides. Normal distributions of size peaks are obtained in our laboratory using RNA extracted from as few as  $5 \times 10^4$  normal donor PBMC. Comparison with size standards shows that the peaks correspond to in-frame transcripts. Normal profiles have an average of 6–10 peaks/V $\beta$  family (polyclonal Gaussian profiles; see an example in Fig. 2A, V $\beta$ 7). Alterations of the normal profile, resulting in a shift of the center of CDR3 distribution or in a disruption of the normal peak distribution due to clonal T cell expansions result in polyclonal skewed profiles (see an example in Fig. 2B, V $\beta$ 3). Finally, oligoclonal T cell expansion results in profiles of  $\leq 4$  peaks/V $\beta$  family (see an example in Fig. 2B, V $\beta$ 5). Based on these criteria, data are expressed as numbers of oligoclonal, polyclonal skewed, or polyclonal Gaussian V $\beta$  families per total number of V $\beta$  families amplified from each sample  $\times 100$  (percent of total V $\beta$  families).

### Statistical analysis

Statistical analysis of TCR diversity with TREC values was performed using Pearson's correlation test; it was corroborated using Spearman's correlation test and it was implemented in S-Plus 6.0 software (Insightful, Hampshire, U.K.).

## Results

### Phenotype and function of PBMC from SCID patients before and after BMT

Before BMT, PBMC from the 15 SCID patients were analyzed by flow cytometry to quantify the numbers of CD20<sup>+</sup> B cells, CD3<sup>+</sup> T cells, or CD16<sup>+</sup> NK cells (Fig. 1A). All but one of the patients had a severe deficiency of T cells and when T cells were present (J-1), they were of maternal origin (9). The numbers of B cells were normal or increased above normal values, while the NK cell numbers were normal. At the most recent evaluation following BMT (Fig. 1B), T cells reached normal numbers in 12 of 14 patients (patient 7-2 died of varicella zoster virus), and the number of B and NK cells remained stable.

The in vitro response of the patients' PBMC to mitogens (PHA, Con A, and PWM) was very low or undetectable before BMT (Fig. 1C), but reached normal levels in 12 of 14 patients who had normal T cell numbers following BMT (Fig. 1D).

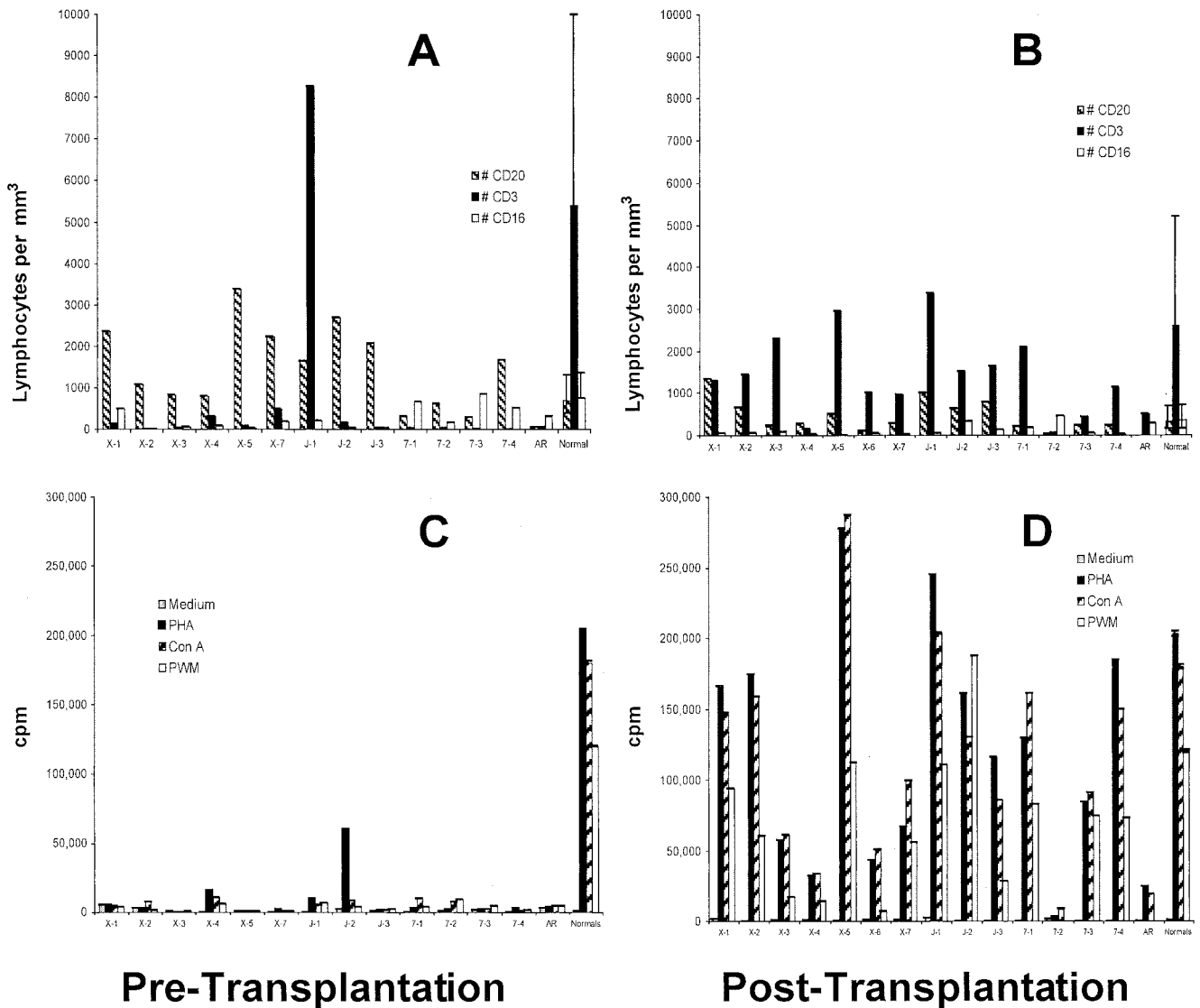
Genetic analysis of blood lymphocytes performed at the most recent evaluation showed that in 12 of 15 patients the T cells were of donor origin, while the B cells in most cases were derived from the recipients (Table I) (9), resulting in split chimerism.

### CDR3 length distribution in normal control and SCID samples taken before transplantation

Immunoscope analysis of the TCR V $\beta$  repertoire was performed on PBMC from the 15 SCID patients given BM transplants. The immunoscope reaction was first established on cDNA samples from normal PBMC. The results of the analysis of a cDNA sample from a normal control are shown in Fig. 2A. Each panel represents the immunoscope results of the PCR-amplified products from individual TCR V $\beta$  families (V $\beta$  1–23). The last panel is a positive control used in each reaction, showing the V $\beta$ 8 PCR-amplified product of cDNA from the Jurkat cell line (22).

The distribution of peaks seen in most of the normal controls' V $\beta$  panels (36% polyclonal skewed, 64% polyclonal Gaussian) indicates normal polyclonal expansion of T cells sharing the same V $\beta$  gene rearranged with different D $\beta$  and J $\beta$  genes, which results in CDR3 fragments of different lengths. This result is expected from normal control PBMC. Two discrete peaks demonstrate the clonal origin of the Jurkat cell line. The V $\beta$ 13A family is poorly expressed in some individuals.

In contrast, immunoscope analysis of pretransplantation PBMC (TREC negative) from a Jak3-deficient SCID patient (J-1) with known transplacental transfer of a large number of maternal T cells revealed a highly skewed TCR repertoire (Fig. 2B), with 68% of



**FIGURE 1.** Phenotype and function of patients' PBMC before and after BMT. Absolute numbers of CD20<sup>+</sup> B cells, CD3<sup>+</sup> T cells, and CD16<sup>+</sup> NK cells were obtained by flow cytometric analysis of each of the 15 studied patients' PBMC before (A) and at the most recent evaluation after (B) BMT. Values for normal controls are shown for comparison. [<sup>3</sup>H]Thymidine incorporation by proliferating lymphocytes in response to PHA, Con A, and PWM was evaluated before (C) and at the most recent evaluation after (D) BMT. Values are cpm. cpm for normal controls are shown for comparison.

the total PCR-amplified Vβ families showing oligoclonal T cell expansion, 27% showing a polyclonal skewed profile and only one family (Vβ21) with a Gaussian profile. The oligoclonality may be explained by the homeostatic expansion of transplacentally transferred maternal, mature, T cells detected before BMT (Fig. 1A, patient J-1).

*TCR oligoclonal/skewed repertoire in SCID samples taken within the first 100 days after BMT*

Soon after BMT in SCID patients, a mild expansion of TREC<sup>-</sup>CD45RO<sup>+</sup> PHA-responsive cells is often seen in the peripheral blood (10) before the appearance of TREC<sup>+</sup>CD45RA<sup>+</sup> T cells. We reasoned that the TREC<sup>-</sup>CD45RO<sup>+</sup> cells might represent an oligoclonal expansion of peripheral T cells. To test this hypothesis, we examined the TCR repertoires of PBMC from SCID patients within the first 100 days after BMT. PBMC from four such patients (X-1, X-2, J-1, 7-3), all of whom had a TREC value of ≤106 and a predominance of circulating CD45RO<sup>+</sup> T cells, were analyzed by immunoscope (Fig. 3A). TCR diversity was severely skewed in these samples, with a mean ± SD of 48 ±

17% of the total PCR-amplified Vβ families in the respective samples showing oligoclonal T cell expansion. The polyclonal skewed profiles averaged 52 ± 17%, while polyclonal Gaussian profiles were undetectable in these samples. In contrast, simultaneously evaluated normal control samples had no evidence of oligoclonality (0%), 33% polyclonal skewed Vβ profiles, and 67% polyclonal Gaussian profiles.

*Polyclonal T cell expansion following development of T cell function*

In SCID patients who developed T cell function, the mean number of CD45RA<sup>+</sup> T cells present in the peripheral blood was highest at 350 days after BMT (10). The newly arising CD45RA<sup>+</sup> cells represented recent thymic emigrant T cells populating the peripheral blood, as demonstrated by the presence of TRECs (10, 14). The mean peak value of TRECs in peripheral PBMC was reached between 1 and 2 years after BMT (10). To test whether the SCID thymus is capable of producing a fully diverse TCR repertoire, we analyzed the TCR repertoire in SCID patients at 1 year or more after BMT, when the circulating CD45RA<sup>+</sup> T cell numbers and

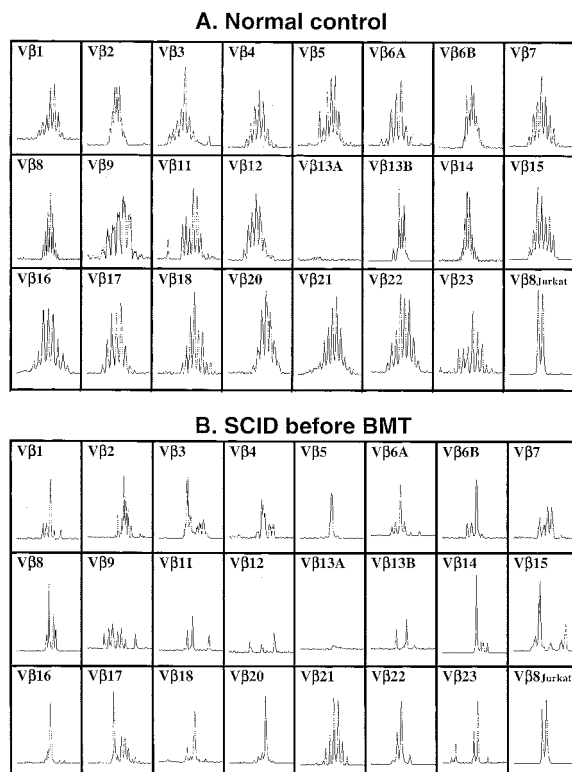
Table I. SCID: genetic types and current statuses

Patient	Type of SCID	Type of BMT	Age (days) at BMT	Years After BMT	Chimerism	IVIG	Infections
X-1	X-linked	Haploidentical	282	5.3	100% T, 0% B	Yes	No
X-2	X-linked	Haploidentical	249	3.8	100% T, 15% B	No	No
X-3	X-linked	Haploidentical	45	17.5	100% T, 1% B	Yes	No
X-4	X-linked	Haploidentical	289	9.5	No	Yes	Skin, gastrointestinal
X-5	X-linked	Haploidentical	516	14.2	100% T, 100% B	No	No
X-6	X-linked	Haploidentical	148	13.7	100% T, 0% B	Yes	Lung
X-7	X-linked	Haploidentical	113	18.1	100% T, 0% B	Yes	No
J-1	Jak3 def <sup>a</sup>	Identical	222	12.9	100% T, 6% B	Yes	No
J-2	Jak3 def	Identical	290	10.8	100% T, 7% B	No	No
J-3	Jak3 def	Haploidentical	24	6	100% T, 1% B	Yes	No
7-1	IL-7R $\alpha$	Haploidentical	325	6.4	100% T, 1% B	No	No
7-2	IL-7R $\alpha$	Haploidentical	559	2.4	No	Yes	Died of varicella zoster virus
7-3	IL-7R $\alpha$	Haploidentical	10	3.4	100% T, 100% B	Yes	No
7-4	IL-7R $\alpha$	Haploidentical	509	8.7	100% T, 0% B	No	No
AR	AutoRec <sup>b</sup>	Haploidentical	147	4.8	No	Yes	No

<sup>a</sup> def, Deficient.<sup>b</sup> AutoRec, autosomal recessive.

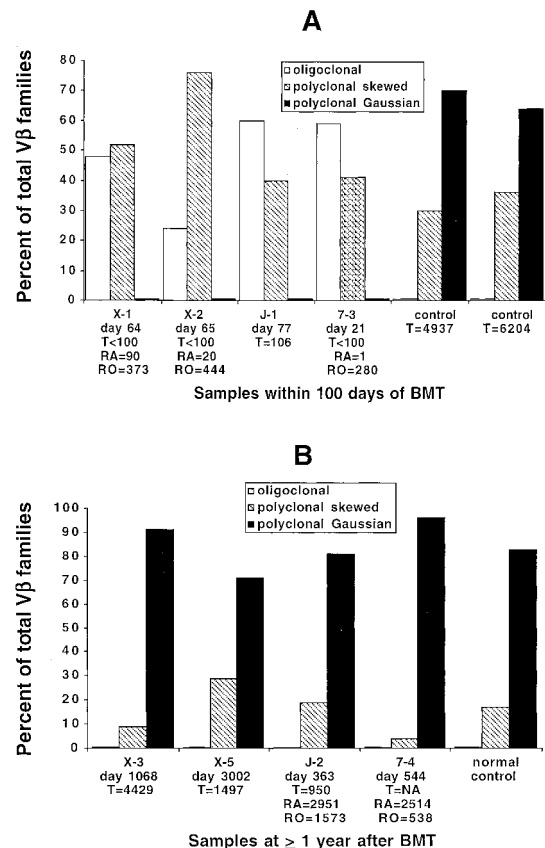
TREC values were high. Samples from four SCID patients (Fig. 3B) showed a prominent polyclonal Gaussian TCR repertoire ( $85 \pm 11\%$  polyclonal V $\beta$  families),  $15 \pm 11\%$  polyclonal skewed profiles, and no oligoclonal V $\beta$  profiles, at a time in which the TREC values ( $2292 \pm 1527$ ) and the number of circulating CD45RA<sup>+</sup> cells were high.

Two patients were then followed longitudinally to study these three functional parameters at different times after BMT. When a Jak3-deficient SCID patient (J-1) was studied for 3123 days after BMT, increasing polyclonal expansion of all of the V $\beta$  families was observed with time posttransplantation. The immunoscope

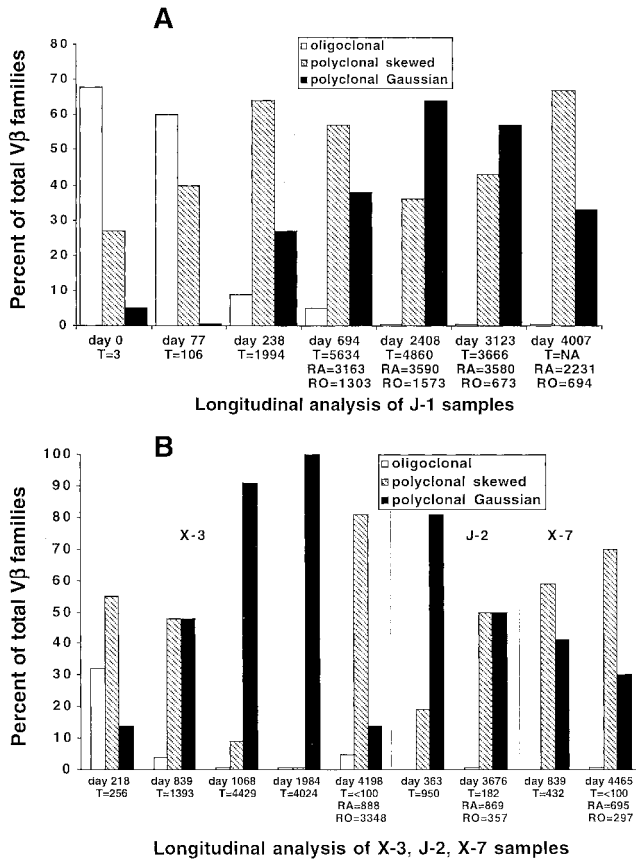


**FIGURE 2.** Immunoscope profile of TCR V $\beta$  families. Each V $\beta$  family was examined by PCR amplification and run-off reaction. Results are shown for each V $\beta$  family as a density peak histogram. CDR3 sizes are shown on the x-axis and the peak fluorescence intensity is shown on the y-axis. A, Immunoscope profile of a normal subject. B, Immunoscope profile of a Jak3-deficient SCID patient (J-1) before BMT.

results are shown in Fig. 4A. A predominant oligoclonal expansion of T cells was observed at  $\leq 77$  days after BMT. This was followed by polyclonal expansion of T cells, detectable by day 238, which



**FIGURE 3.** Immunoscope analysis of TCR V $\beta$  families in SCID patients early and late after BMT. Bar graph illustration of the immunoscope profiles analysis. Results are expressed as number of oligoclonal, polyclonal skewed, or polyclonal Gaussian V $\beta$  families per total number of V $\beta$  families amplified from each sample  $\times 100$  (percent of total V $\beta$  families) as described in *Materials and Methods*. A, Two X-linked (X-1, X-2), a Jak3-deficient (J-1), and an IL-7R $\alpha$ -deficient (7-3) SCID patients were tested at  $< 100$  days after BMT. B, Two X-linked (X-3, X-5), a Jak3-deficient (J-2) and an IL-7R $\alpha$ -deficient (7-4) SCID patients were tested at  $\geq 1$  year after BMT. T, TREC/ $\mu\text{g}$  DNA; RA, CD3<sup>+</sup>CD45RA<sup>+</sup> lymphocyte numbers per mm<sup>3</sup>; RO, CD3<sup>+</sup>CD45RO<sup>+</sup> lymphocyte numbers per mm<sup>3</sup>; day, days after BMT. For some samples (X-3, X-5, and J-1) the RA and RO values were not available.



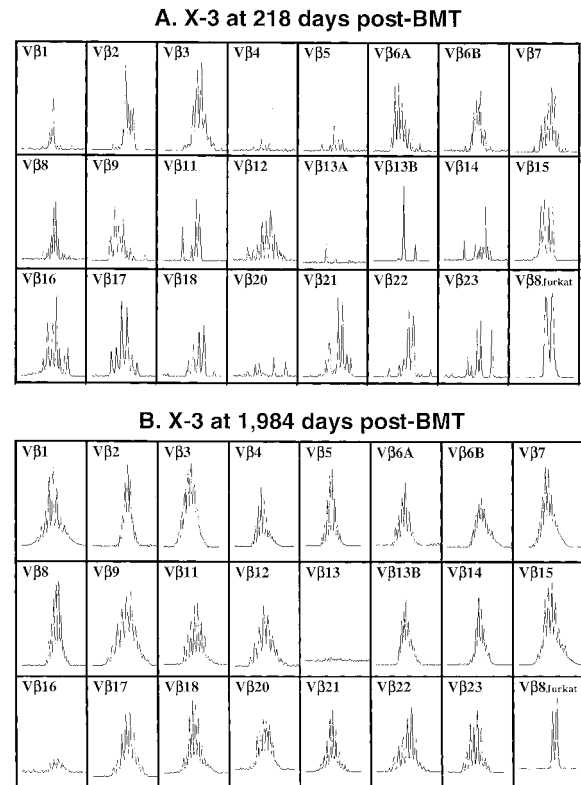
**FIGURE 4.** Immunoscope analysis of SCID patients with time after BMT. Bar graph illustration of the immunoscope profiles analysis (see Fig. 3) of PBMC samples taken from patients J-1 (A) and X-3 (B) at different times after BMT and of PBMC samples taken from patients J-2 and X-7 (B) at two time points after BMT. For some samples (X-3 and J-1), the RA and RO values were not available.

correlated with an increase of the TREC value (from 106 at 77 days to 1994 at 238 days). Polyclonal T cell expansion and high TREC values were maintained in this patient for 3123 days, when the number of circulating CD45RA<sup>+</sup> was high (3580/mm<sup>3</sup>).

A second patient (X-3) followed longitudinally had a mixed oligoclonal/polyclonal skewed expansion of T cells at 218 days after BMT (32% oligoclonal, 55% polyclonal skewed, 14% polyclonal Gaussian; TREC = 256) followed by an increase of polyclonal Gaussian profiles to 100% of total Vβ families at 1984 days (TREC = 4024; Figs. 4B and 5). Flow cytometric analysis of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> was not performed on these samples from patient X-3. These data indicate that the post-BMT TCR repertoire generated after thymus-derived T cell development in SCID is diverse.

*Skewed T cell expansion following a decrease in thymic output and a decrease of CD45RA<sup>+</sup> cells*

Our group previously showed that CD45RA<sup>+</sup> T cells predominated over CD45RO<sup>+</sup> T cells in BM-reconstituted SCID PBMC until 12 years after BMT (10). This led us to question whether the TCR repertoire remained diverse or became oligoclonal at late times after transplantation as a result of extrathymic homeostatic T cell expansion. Ten years after BMT, when the TREC levels began to decline (10), four of five patients (Fig. 4: J-1, day 4007; X-3, day 4198; J-2, day 3676; X-7, day 4465) showed a decrease in the percentage of Vβ families with a polyclonal Gaussian profile (32 ± 15%), a compensatory increase in the percentage of poly-



**FIGURE 5.** Immunoscope profile of TCR Vβ families in a SCID patient early and late after BMT. Immunoscope profile of a X-linked SCID patient (X-3) at 218 days (A) and at 1984 days (B) after BMT. Results are shown for each Vβ family as a density peak histogram. CDR3 sizes are shown on the x-axis and the peak fluorescence intensity is shown on the y-axis.

clonal skewed Vβ families (67 ± 13%), and lower numbers of circulating CD45RA<sup>+</sup> cells compared with the numbers obtained at ≥1 year after BMT (Figs. 3B and 4). One of five patients (X-5, day 3800; data not shown) maintained a diverse repertoire (86% polyclonal Gaussian profile), moderate TREC (485), and a high number of CD45RA<sup>+</sup> cells (>2500/mm<sup>3</sup>).

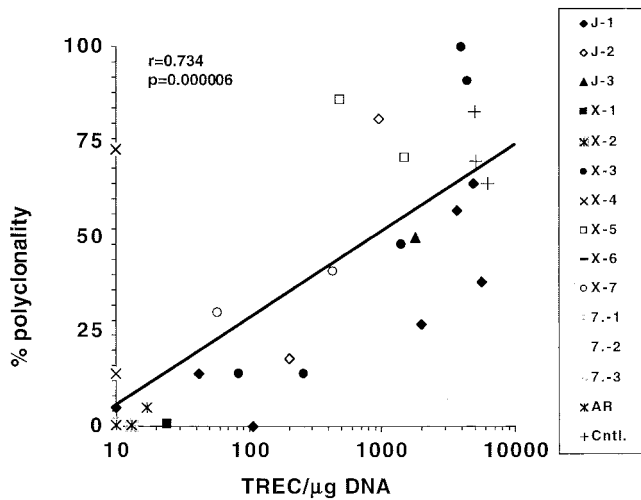
These data indicate that a more skewed T cell repertoire emerged with time after BMT (≥10 years) in 80% of the patients, when the thymic output declined.

*TCR diversity correlates with thymic output*

The above studies indicate that in SCID, TCR diversity may correlate with thymic output as determined by PBMC TREC levels in a manner that is independent of age or transplantation status. To test this hypothesis, we evaluated the correlation of TCR diversity with TREC levels in 29 samples taken from all of the studied patients (Fig. 6). TCR diversity as measured by the percentage of polyclonal Vβ families with a Gaussian distribution was highly and positively correlated with TREC levels ( $r = 0.734$ ,  $p = 0.00006$ ). Three normal controls used in these studies also demonstrated a positive correlation between percent polyclonality and TREC levels.

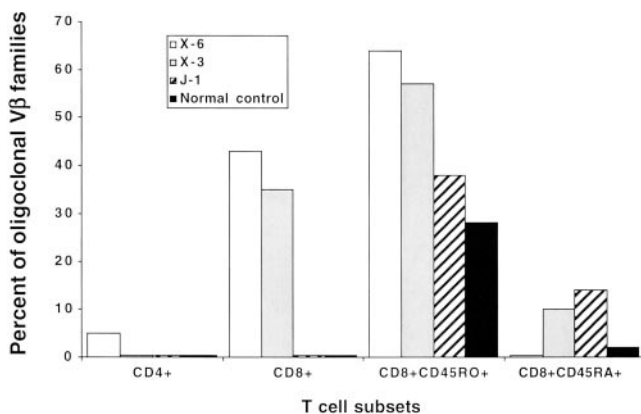
*Loss of TCR diversity is due to an expansion of the CD8<sup>+</sup> T cell compartment*

Normal individuals frequently have a marked clonal predominance of TCR transcripts within the CD8<sup>+</sup>CD45RO<sup>+</sup> population (23). This phenomenon has been associated with aging and it may be a consequence of decreased thymic output and/or function (24, 25). To determine whether the more skewed TCR repertoire observed



**FIGURE 6.** Thymic output correlates with TCR diversity. Shown are dot plots of the percent  $V\beta$  families with a polyclonal Gaussian distribution compared with TREC levels of samples taken from 14 of 15 SCID patients at multiple time points after BMT. The TREC value for patient 7-4 was not available. Data from three normal controls are also shown. Values for 7-1, 7-2, and 7-3 overlap at the origin of the axes. The correlation coefficient and  $p$  value, determined using Pearson's and Spearman's tests, are shown.

10 years after BMT (patients J-1 and X-3) or in patients that maintained a skewed repertoire beyond 1000 days of BMT (X-6: 52% oligoclonal and 48% polyclonal skewed  $V\beta$  profiles) resided in the  $CD8^+CD45RO^+$  T cell subset, we separated T cell subsets by flow cytometric sorting and tested them by TCR immunoscope (Fig. 7). The  $CD4^+$  T cell subset demonstrated minimal oligoclonal expansion ( $2 \pm 3\%$ ) in all of the samples, while a mean of 39% oligoclonal expansion was detected in the  $CD8^+$  T cell subset of only those patients that had an overall increase in  $CD8^+$  cells (X-3 and X-6:  $CD8^+$  cells  $\geq 60\%$  of  $CD3^+$  PBMC). Patient J-1 and a normal control ( $CD8^+$  cells, 38 and 30%, respectively, of  $CD3^+$  PBMC) did not have evidence of oligoclonal expansion in the  $CD8^+$  T cell subset. When the  $CD8^+$  population was further fractionated into  $CD45RO^+$  and  $CD45RA^+$  subsets, most of the oligoclonal T cell expansion ( $53 \pm 13\%$ ) was detected in the  $CD8^+CD45RO^+$  subset of patients and control samples. The



**FIGURE 7.**  $CD8^+CD45RO^+$  T cells 10 years after BMT have an increased skewed repertoire. Bar graph illustration of the immunoscope profiles analysis (see Fig. 3) of T cell subsets samples isolated by flow cytometric sorting from three patients at  $\geq 10$  years after BMT and a normal control. Results are expressed as number of oligoclonal  $V\beta$  families per total number of  $V\beta$  families amplified from each sample  $\times 100$  (percentage of oligoclonal  $V\beta$  families).

$CD8^+CD45RA^+$  subset had low oligoclonal expansion ( $12 \pm 3\%$ ) in only two of three patient samples and 2% in the control.

Thus, the majority of oligoclonal expansion was detected in the  $CD8^+CD45RO^+$  T cell population while the  $CD4^+$  and the naive  $CD8^+$  subsets remained diverse.

## Discussion

SCID patients are profoundly deficient in T cells before transplantation and, because of this, do not require pretransplant chemotherapy or posttransplant GVHD prophylaxis for successful engraftment, making it easier to study T cell reconstitution. We demonstrated that before BMT, the few T cells present in SCID patients are oligoclonal (Fig. 2B). Early after BMT, the  $CD45RO^+$  cells that expand are oligoclonal and they are not thymus derived (Figs. 3A and 4A). The initial oligoclonal expansion of T cells observed in the first 100 days after transplantation likely represents the expansion of transplantally transferred maternal T cells or residual T cells that were present in the graft, since the TREC levels were low in these patients (10, 15). Expansion of  $CD45RO^+$  cells and development of a persistent oligoclonal/skewed T cell repertoire in one of the patients studied (X-6) demonstrated that reconstitution of the peripheral T cell population by expansion of existing memory T cells does not result in a diverse repertoire.

Longitudinal studies performed in two of the patients (J-1 and X-3) demonstrated development of a diverse T cell repertoire in recent thymic emigrants of donor origin at 1–1.4 years after BMT (Fig. 4). We observed a direct correlation ( $p = 0.000006$ ; Fig. 6) between the TREC value and the T cell diversity that developed in all of the analyzed patients and in the normal controls, indicating that a fully diverse TCR repertoire develops at peak thymus output, as reported in other systems (14, 26–29). Of the 15 patients examined, 4 were tested at  $< 100$  days after BMT, too early to detect the development of a normal T cell repertoire. Three of the remaining 11 patients displayed a polyclonal skewed repertoire, while 8 (73%) developed a normal polyclonal repertoire.

A more skewed T cell repertoire emerges with time after BMT ( $\geq 10$  years; Fig. 4), when the thymic output declines in these patients (10). The reason that thymic output declines more rapidly in these immune reconstituted SCID patients than in normal controls is not known. It could be that the initially vestigial thymus is not capable of thymic output for as long a period as normal thymi. Other possibilities include: 1) that an insufficient number of normal stem cells was given, 2) that the stem cells and the thymic epithelium were not HLA identical to each other, or 3) that subclinical GVHD or infections have damaged the thymi. It has been speculated that true hemopoietic stem cells do not engraft because these SCID patients are not chemoablated (30). However, there are no comparable longitudinal studies of thymic output and T cell diversity in chemoablated BM-transplanted SCID patients that would support that hypothesis. A longitudinal TCR repertoire study in two SCID patients, who had been chemoablated before BMT, was done on patient-derived T cell lines and did not include an evaluation of TREC levels (31). That study showed that, 11 years after BMT, the TCR diversity in the SCID recipients was less marked than that of their respective donors (31), supporting our findings. Studies of the development of T cell repertoire diversity after hemopoietic cell transplantation have also been conducted in patients with various forms of malignancy (29, 32, 33). The subjects of these studies received either unfractionated (29, 33) or T cell-depleted HLA-identical BM (32), peripheral blood (after G-CSF mobilization of stem cells) (33), or unfractionated cord blood (29) transplants. As in the present study, there was abnormal T cell repertoire diversity just after transplantation. However, in contrast to the current study, the abnormal T cell diversity usually persisted

for the first year after transplantation (29). The exception was in the study of 11 adult patients with chronic myelogenous leukemia who received rigorously T cell-depleted BM from HLA-identical siblings (32). Four of these patients began to normalize their T cell repertoire by 6 mo posttransplantation. The latter study is the only one sharing similarities with the present study, i.e., T cell depletion and the fact that drugs to prevent GVHD were omitted from their treatment. None of these studies followed patients beyond 4 years posttransplantation.

The more skewed T cell repertoire may reflect the presence in these patients of T cells that have been activated by infection or immunization. Such activated or memory T cells express the CD45RO surface marker (34). Although controversy exists over the precise characterization of naive, activated, or memory cells based on the expression of the CD45 isoforms (35, 36), a strong cell staining with CD45RO-specific Abs corresponds to an activated cell (34). In our study we evaluated only the CD3<sup>+</sup> cells that showed a bright staining with anti-CD45RO Abs. Our results showed that oligoclonal V $\beta$  profiles were predominantly found in the CD8<sup>+</sup>CD45RO<sup>+</sup> T cell subset of T cells, a phenomenon occurring with aging in the normal population (24, 25). In addition, the partial loss of TCR diversity appeared to be due to CD8<sup>+</sup> T cell expansion in two patients (X-3 and X-6). In these patients, the CD4<sup>+</sup> T cells maintained their diversity, but declined in number. Although the CD4<sup>+</sup> T cell repertoire remains polyclonal (Fig. 7), it is possible that, in some of these patients, not enough naive CD4<sup>+</sup> T cells are generated to completely fill the peripheral T cell pool. Alternatively, the absence of normal-sized peripheral lymphoid tissues in SCID patients, necessary to maintain T cell homeostasis, may affect the T cell repertoire diversity. Similar oligoclonal repertoires were detected by CDR3 V $\beta$  analysis of CD8<sup>+</sup>, but not CD4<sup>+</sup> T cell populations in two nude/SCID patients tested 6 years after BMT, suggesting a differential maintenance of CD4<sup>+</sup> and CD8<sup>+</sup> TCR repertoire complexity (37). The CD8<sup>+</sup>CD45RO<sup>+</sup> cells may be Ag-specific effector T cells directed against Ags to which the individuals are exposed or they may represent regulatory T cells (25, 38). The reason why the oligoclonality developed mainly in the CD8<sup>+</sup> T cell subset is unclear at the present time, although it has been previously reported in normal subjects (23). We cannot exclude that bronchiectasis with its known chronic superinfection in patient X-6 may be related to the expansion of oligoclonal CD8<sup>+</sup> cells. However, a similar expansion occurred in patient X-3 who had no evidence of infection (Table I). Oligoclonal profiles were not the result of PCR amplification of limiting numbers of T cells obtained after flow cytometric sorting, since greater oligoclonality observed in patients X-3 and X-6 correlated with higher total numbers of CD3<sup>+</sup>CD8<sup>+</sup> PBMC compared with patient J-1 or a normal control (Fig. 7). In addition, normal distributions of size peaks were obtained in our laboratory using RNA extracted from as few as  $5 \times 10^4$  normal donor PBMC, while the oligoclonal profiles observed in Fig. 7 were obtained from the RT-PCR amplification of  $1-2 \times 10^5$  sorted T cells.

It remains to be determined whether the late-appearing oligoclonality identified in the CD3<sup>+</sup>CD45RO<sup>+</sup> T cells is an early indicator of decreased T cell functionality and/or shortened survival in these patients. However, such CD8<sup>+</sup> oligoclonal T cells are present in transplanted patients and in control subjects. Some of these patients have now survived for >18 years (Table I), have normal numbers of T, B, and NK cells, normal T and B cell function, and are healthy (Table I and Fig. 1), suggesting that these cells may not be playing a negative role.

In conclusion, these studies show that 1) allogeneic T cells that develop from donor progenitor cells in SCID recipients of rigor-

ously T cell-depleted parental BM cells are heterogeneous, as shown by the diverse TCR gene usage detected by the immunoscope analysis, and 2) the T cell repertoire remains highly diverse for the first 10 years after transplantation.

When BMT was performed in neonatal SCID recipients vs older infants (39), more rapid recovery of T cell function was found. It will be interesting to determine whether these neonatally transplanted patients also develop a normal TCR repertoire earlier in life.

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