Oligoclonal Expansion of CD8+CD57+ T Cells With Restricted T-Cell Receptor β Chain Variability After Bone Marrow Transplantation

By Guy Gorochov, Patrice Debre, Véronique Leblond, Béhazine Sadat-Sowti, François Sigaux, and Brigitte Autran

A major expansion of CD8+57+ lymphocytes expressing an αβ T-cell receptor (TCR) is frequent after bone marrow transplantation (BMT). We examined the clonality of the TCR β gene repertoire in these expanded CD8+57+ cells after allogeneic or autologous BMT. We performed a polymerase chain reaction (PCR) analysis of the Vβ chain usage in CD8+57+ cells purified from nine BMT recipients with a series of oligonucleotides specific for 20 Vβ gene families. PCR products from selected TCR β gene rearrangements were sequenced. The CD8+57+ cells from eight of nine patients used a restricted set of Vβ families, with a marked predominance of two to three Vβ gene families per patient, whereas the control autologous CD57− subset expressed the whole 20 Vβ family. A direct sequencing analysis confirmed the V616 and V617 clonality in six patients, showing a striking homology in the CDR3 sequences of the V916 products. The CD8+57+ cells, but not the CD57− cells, displayed an oligoclonal pattern of TCR gene rearrangements as shown by PCR analysis of TCRγ gene rearrangements. Such an oligoclonal expansion of CD8+57+ cells, using a restricted set of the Vβ gene families, may result from a specific TCR stimulation of a limited number of T-cell clones in BMT recipients.

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Table 1. Patient Characteristics

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<th>Patient No.</th>
<th>HLA</th>
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<th>Conditioning Regimen*</th>
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<th>CMV Status</th>
<th>Additional Experiments</th>
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Abbreviations: NT, not tested; SAA, severe aplastic anemia.
* Conditioning regimen before BMT: TBI (total body irradiation); cyclophosphamide (Cy); TAM, = TBI + aracynite (A) + melphalan (M).
† Both acute and chronic GVHD expressed by using the standard clinical staging; acute GVHD, stages I to IV; chronic GVHD, limited or extensive, positive or negative.
‡ CMV status assessed on seropositivity of BMT donor or recipient; active CMV infection assessed on the presence of CMV in the peripheral blood or in bronchoalveolar lavage.
§ Time interval in months between CD8*57* analysis and BMT infusion.
| Vβ segments were considered as predominant when over 18% of all Vβ segments expressed.
disease (GVHD) was observed in five of the seven recipients of alloBMT. An active CMV infection was defined by isolation of CMV in the blood, or in the lungs, followed by active specific therapy and was detectable in three of nine patients. The donor origin of the PBLs was assessed upon karyotypic examination of the six cases of sex-mismatch allografted patients. No evidence of relapse of the original disease was observed in the patients analyzed.

**Immunofluorescence analysis.** A standard cytofluorometric analysis of peripheral blood T cells was performed using a two-color whole blood staining method with the following monoclonal antibodies (MoAbs): anti-CD4-fluoro-isothiocyanate (FITC), anti-CD8-phycocerythrin (PE), and anti-CD57-FITC (Immunotech, Marseille, France) as previously described.4 Ten thousand lymphocytes were analyzed for fluorescence on a Facsscan cytofluorometer (Becton Dickinson, San Jose, CA). T cells doubly positive for both the CD8 bright and CD57 markers were defined as CD8+57+ cells. We excluded the low CD8+, CD57+ cells that are not positive for the CD3/TCR α-β complex (data not shown).

**Preparation of purified T lymphocytes.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Fresh CD8+57+ cells were sorted using an immunomagnetic procedure as previously described.4,8 Briefly, avidin-conjugated magnetic beads (Immunotech) coupled with anti-CD57-biotin MoAb (Immunotech) were incubated for 1 hour with fresh PBLs. Rosetted CD57+ cells were then sorted. The purity of the cells was assessed by immunofluorescence (IF) analysis performed immediately after sorting. The positive fraction contained more than 95% CD57+ cells (85% CD8+57+ cells and 10% to 15% CD8−3−57− cells). In contrast, the CD57− cell fraction contained less than 5% residual CD57+ cells. Between 4 × 10⁶ and 8 × 10⁶ cells were obtained for each sample and stored as dry pellets at −80°C until use.

**cDNA synthesis and PCR analysis.** Total cytoplasmic RNA was prepared from fractionated CD8+57+ and CD8−57− PBLs according to the method of Chomczynski and Sacchi15; 15 to 20 µg of RNA was first incubated at 70°C with random hexamers (Pharmacia) and after rapid cooling on ice, M-MLV Reverse Transcriptase (Bethesda Research Laboratories [BRL], Gaithersburg, MD) was added for synthesis of the first-strand cDNA (2.000 IU/125 µl) in the presence of RNAsin (Boeringher-Ingelheim, Grenoble, France). The cDNA-RNA duplex was then treated with 2.5 U of ribonuclease H (Boehringer-Ingelheim). The single-stranded cDNA was precipitated and resuspended in water. Reverse transcriptase (RT)-PCR amplification was performed according to standard methods.16 Vβ members of the 20 Vβ families first described were used and are shown in Table 2. Their locations on the Vβ segment were chosen and was sub-

**Southern blot analysis.** To confirm the specificity of the products obtained and quantify data on the relative proportions of the different Vβ PCR products, DNA was transferred from the gels to nylon membranes (Hybond N; Amersham, Arlington Heights, IL), which were then exposed for 10 minutes to UV light and prehybridized for 2 hours at 55°C in a solution containing 6X saline solution (SSC), 1X Denhardt’s solution, 5% dextran, 1% sodium dodecyl sulfate (SDS), and 50 µg/mL denatured salmon sperm DNA. A [32P] adenosine triphosphate (ATP) end-labeled oligonucleotide internal probe (5' GTTCCACCGGAGTGCCGTTG 3') with a sequence common to CB1 and CB2 constant regions of the TCR β locus, was then added to the bags and hybridization was performed for 6 hours at 55°C in the same solution. The filters were then washed in 2X SSC, 0.2% SDS, twice at room temperature, once at 55°C for 15 minutes, and then once at 2°C below the TM of the oligonucleotide for 5 minutes. The blots were exposed to x-ray film with intensifying screens at −70°C for 8 to 36 hours. The resulting autoradiographs were then analyzed by computerized quantitative densitometry (Pharmacia LKB, St Quentin en Yvelines, France) and the results expressed as a percent-

### Table 2. Oligonucleotide Primers Used for Specific PCR Amplification of TCR Vβ and Co CDNA

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age of the sum of the signals obtained with all the Vβ-specific
primers.

PCR amplifications of TCRγ gene rearrangements. One micro-
gram of high molecular weight DNA prepared from mononuclear
cells according to standard methods was used as a template for PCR
with a mixture of oligonucleotides complementary to all Vγ and Jγ
segments. Oligonucleotides were used as previously described.21,22
Ten percent of the reaction was electrophoresed through an 8%
polyacrylamide gel and visualized under UV light. The expected
size of the TCR-amplified products ranged from 180 to 250 bp.
Bands corresponding to higher molecular weight DNA were not
taken into account. Such products presumably correspond to het-
eroduplexes of two nonhomologous DNA strands, which under
nondenaturating conditions run slower than homoduplexes.22,23

Direct sequencing of PCR products. After an initial step of puri-
fication by phenol/chloroform extraction, primers were removed
and DNA purified from 80 μL of a PCR mix with a Qiagen Tip
column (Qiagen Inc, Studio City, CA) following the manufacturer’s
protocol. The purified product was then used as a template for lin-
ear amplification DNA sequencing by using a dsDNA cycle se-
quencing kit according to the manufacturer’s instructions (GIBCO,
Cergy-Pontoise, France). The oligonucleotide used (5’ TGCTTC-
TGATGGCTCAAACAC 3’) is internal to the C8 primer that was
used for PCR amplification of the cDNA (Table 2). Reactions were
overlaid with 10 μL of mineral oil and incubations performed in a
thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 20 cycles of
1 minute at 95°C, 1 minute at 45°C, 1 minute at 70°C, followed by
10 cycles of 1 minute at 95°C and 1 minute at 70°C. Each reaction
was terminated by the addition of 5 μL of formamide dye solution,
the sample heated for 5 minutes at 95°C, and 3 μL of the aqueous
phase was immediately loaded on a sequencing gel for electropho-
resis. Gels were fixed with 10% acetic acid and 10% methanol and
dried before overnight autoradiography at room temperature. Se-
quences derived from products obtained in at least two separate
experiments were systematically compared.

RESULTS

Utilization of the Vβ repertoire by CD8+57+ cells in BMT
recipients. In a previous study we reported an expansion of the
CD8+CD57+ T-cell subset in 13 of 25 (52%) recipients

![Image](attachment:image.png)

**Fig 1.** Vβ repertoire expressed by CD57- cells (upper panel) and by CD8+CD57+ cells (lower panel) from patient no. 9. PCR products are ana-
yzed on an agarose gel containing Ethidium Bromide. C, expected size for Cα bands; V, expected
size for Vβ bands. No apparent restriction of Vβ usage in the CD57+ fraction.

![Image](attachment:image.png)

**Fig 2.** Southern blot analy-
sis of the PCR products ob-
tained from patient no. 6.
CD57- cells (A) and CD8+57+ cells (B), using a [P32]-labeled
C8 internal probe. Specific Vβ
products are ranging in size
from 250 to 350 bp. Predomi-
nance of the Vβ16 and Vβ2 spe-
cific products in the CD57+ fraction.
OLIGOCLONAL EXPANSION OF CD8\(^+\) CD57\(^+\) T CELLS

usage or whether they shared some important structural characteristics. Patients were selected via the criterion of a peripheral blood CD8\(^+\)CD57\(^+\) T-cell expansion to over 15% of total PBLs. Clinical characteristics of these patients and their CD8\(^+\)CD57\(^+\) PBL percentages, ranging from 15% to 60%, are described in Table 1. As shown (Fig 1), the RNA purified from the CD57\(^-\) cells of patient no. 9 contained transcripts from most of the 20 \(V\beta\) families tested. These transcripts were amplified and visualized as a band of a size appropriate to the \(V\beta\) specific oligonucleotide used (Fig 1, upper panel). In contrast, in all cases but one (no. 9, Fig 1) we observed a restricted usage of \(V\beta\) families in the CD8\(^+\)CD57\(^+\) cells, as shown for patients 6 and 8 (Figs 2 and 3). Some \(V\beta\)-specific products appeared to be clearly predominant in the CD8\(^+\)CD57\(^+\) fractions and, among these the \(V\beta17\)- and the \(V\beta16\)-specific products were overrepresented in six of the nine patients tested.

We measured the relative importance of each PCR product by optic densitometry and, as shown in Fig 4, we compared CD8\(^+\)CD57\(^+\) and CD57\(^-\) cells in terms of \(V\beta\) segment usage. A limited number of \(V\beta\) families were clearly overrepresented in the CD8\(^+\)CD57\(^+\) subset, whereas others were not detected at all.

Predominant \(V\beta\) usage is summarized in Table 1 for all patients studied. A shared pattern of \(V\beta\) expression was found in several cases. For instance, four of the nine patients studied presented a predominant or codominant expression of \(V\beta16\) (a one-member gene segment family); in two other patients, \(V\beta17\) (another one-member family) was overexpressed in the CD8\(^+\)CD57\(^+\) fraction. Patients no. 2 and 7 overexpressed distinct \(V\beta\) families. In contrast, a lack of restriction in the usage of the \(V\beta\) repertoire could be detected in the CD8\(^+\)CD57\(^+\) T cells from only one patient (no. 9).

Together, these data show that in BMT recipients, the CD8\(^+\)CD57\(^+\) T-cell expansion frequently corresponds to T cells that use a restricted \(V\beta\) repertoire.

Characterization of oligoclonal TCR rearrangements among the CD8\(^+\)CD57\(^+\) cells. The restriction of the \(V\beta\) seg-

Fig 3. Southern blot analysis of the PCR products obtained from patient no. 8. (A) CD57\(^-\) cells; (B) CD8\(^+\)CD57\(^+\) cells. Predominance of the \(V\beta17\) specific product.

Fig 4. Histogram representation of the optical densitometry analysis of the autoradiographs. A PCR ratio was calculated as follows: The signal detected for each \(V\beta\) specific primer was divided by the sum of the signals obtained with all \(V\beta\) primers. The results are expressed as a percentage of \(V\beta\) specific amplification within the CD8\(^+\)CD57\(^+\) subset (■) or within the CD57\(^-\) subset (□) for each patient.
Overall, these data indicate the oligoclonal nature of the expansion of peripheral CD8^57^ T cells that are using a restricted set of Vβ genes in recipients of both allogeneic and autologous BMT.

Nucleotide sequences of the Vβ16 and Vβ17 rearrangements in CD8^57^ T cells. This oligoclonal pattern of TCRγ rearrangements, together with the marked restriction of the Vβ segments usage observed in CD8^57^ T cells, prompted us to study further the predominant Vβ chains shared between these patients. This analysis allowed us to investigate whether these β chains were clonally expressed and to compare their junctional sequences.

Therefore, we purified the Vβ16-Cβ and the Vβ17-Cβ products, obtained from the six patients mentioned above, and performed a direct sequencing analysis. One predominant in-frame sequence was effectively obtained in all cases, confirming that the PCR products studied were highly homogeneous. Moreover, these results indicated that CD8^57^ T cells expressing the particular Vβ16 and Vβ17 segments were predominantly clonal (Fig 6).

Interestingly, the four β chains using the Vβ16 segment presented significant similarities in the region corresponding to the third complementary determining region (CDR3 region) (Fig 7). Indeed, in these four segments, the same number of residues were encoded partially or totally by the junctional region. It should be stated that, because no genomic sequence of Vβ16 is available, the first two bases of what was defined by us as being the N region by comparison with published cDNA sequences might possibly correspond to the 3' end of the Vβ16 segment. That uncertainty does not diminish the finding that the four sequences shown share important features. Of note, two residues encoded by the CDR3 region were charged amino acids (aspartic acid and arginine) located at similar positions in the Vβ16 chain. It

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**Fig 5.** Oligoclonality of the CD8^57^ subset. PCR analysis on genomic DNA, using a mixture of Vγ sense and Jγ antisense specific primers. CD8^57^ T cells from patients 1 and 6 (lanes 1-+ and 6-), CD57^- T cells from the same subjects (lanes 1- and 6-), and from total unfractonated PBL of patients 2 and 7 (lanes 2 and 7). Bands corresponding to individual clonal rearrangements (arrows) are visible in the CD57^- samples. For comparison, results obtained in the same experiment with lymphomatous peripheral T cells (lane L) and normal control PBL (lane C) are presented. Lane φ, molecular size markers.

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**Fig 6.** Direct sequencing of TCR Vγ16 junctional region expressed by CD8^57^ T cells from patient no. 3. Note the relatively low level of background contributed by polyclonal rearrangements indicating the predominance of a single clone in the sample. Results from two separate experiments are compared. The sequence can be read from top to bottom and is reported in Fig 5.
OLIGOCLONAL EXPANSION OF CD8+ CD57+ T CELLS

A direct sequencing was performed, confirming that the predominant Vβ16 and Vβ17 products obtained reflected the expansion of single T-cell clones. Other family-specific products, obtained from control individuals, (Vβ8, Vβ12, Vβ13, Vβ18 families) were also sequenced in a similar PCR study (G.G., manuscript submitted) and no primer infidelity or PCR crossovers were detected.

A similar marked restriction of the Vβ or Vε repertoire has already been reported in several diseases,37 suggesting a role for superantigens in the expansion of T cells expressing the corresponding Vβ-responsive elements.27,30 In our study, the Vβ16 and Vβ17 segments were predominantly used in four (patients no. 1, 3, 5, and 6), and two (patients no. 4 and 8) different patients, respectively. The Vβ3 and Vβ17 segments, which share a common stretch of 15 amino acid residues in their CDR4, were overexpressed in the same patient (no. 8). Moreover, Vβ7 and Vβ14 transcripts, which also share 50% homology with Vβ17, were predominant in patients no. 2 and 7, respectively. Such CDR4 similarities could suggest a role for a superantigen in the expansion of CD8+57+ T cells. However, the fact that these Vβ gene segments (Vβ3, Vβ7, Vβ14, Vβ17) were not simultaneously found to be overexpressed by the CD8+57+ T cells from the same individual does not strongly support the superantigen hypothesis.

Interestingly, instead we found indications for antigen selection events because the junctional regions of the Vβ16 transcripts studied encoded two similar charged residues that were located exactly at the same position in the CDR3. These aspartic acid and arginine amino acids are very likely to markedly influence the specificity of the TCR, because the CDR3 region of the TCR plays a key role in the interaction with the major histocompatibility complex (MHC)-bound antigen.33 The short sequence homology observed in the CDR3 region of Vβ16 transcripts suggests that the corresponding T-cell clones might interact with a classical MHC-bound peptide rather than with a superantigen. Adams et al34 recently reported similar sequence homologies in the VDJ junctions of TCR β-chain genes used by T-cell clones from mice with lupus nephritis, raising the hypothesis that some cationic autoantigenic peptide could be responsible for the selection of TCRs sharing anionic residues in their CDR3 regions. Of note, we did not observe any sequence homology between the Vβ17 segments, which in addition belong to a different subgroup of TCR than the Vβ16 chain.35 The Vβ2 segment was also found to be overexpressed together with Vβ16 in two cases, but these two gene segments do not present any particular sequence homology.

Therefore, although the contribution of a superantigen in such a restricted selection of the Vβ T-cell repertoire may not be excluded a priori, our data suggest that a common ligand, or a family of ligands, could interact with Vβ16+ T cells and promote the growth of specific clones in vivo.

We failed to isolate a possible candidate for TCR-specific binding. A correlation between CMV serologic status and CD8+57+ expansion in BMT recipients has been previously shown14 and all recipients of allo-BMT tested here were seropositive for CMV. Moreover, oligoclonal TCR gene rearrangements have already been reported in the CD8+ PBLs

| Patient | Vβ | JUNCTION | Jβ | Vβ2/CD4/ββ
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Fig 7. Nucleotide and deduced amino acid sequences of the TCR Vβ16 and Vβ17 V-D-J junctional regions predominantly amplified from CD8+ CD57+ T cells. Equal amount of cDNA was amplified from each source using Vβ and Cβ primers. Amino acids are indicated in single-letter code. Charged residues are in bold type. The remnants of germline Dβ sequences are underlined. For clarity, only J region sequences are aligned in this figure (according to Toyonaga et al29). Should also be noted that, although the protein sequences may be partially homologous, the nucleotide sequences were dissimilar in the junctional region, excluding the possibility that cross-contaminations could have occurred during the amplification process. In contrast, transcripts using the Vβ17 segment, which we amplified from two other patients, were very dissimilar in their junctional portions (Fig 7).

DISCUSSION

We found that CD8+57+ T cells from BMT recipients can be expanded in vivo from a limited number of T-cell clones that use a restricted fraction of the Vβ gene families. Several families of Vβ segments were predominantly used, such as the Vβ16 and Vβ17 segments that were found to be overexpressed in the CD8+57+ cells in six of nine patients. This CD8+57+ subpopulation proved to be mainly of oligoclonal origin, compared with the autologous CD57+ cells from the same patients that, in contrast, appeared to be polyclonal.

It should be pointed out that the PCR-based analysis of the TCRβ repertoire presented here cannot be considered as being truly quantitative. Different pairs of specific primers can have very different priming efficiencies. Thus, it is only possible to compare signals obtained with the same pair of primers, but on different templates. That is why valuable information was only obtained by comparing CD57+ and CD57− cells from the same patient, simply to detect clear qualitative differences between the two subsets.

Moreover, primers that we used might not cover all the Vβ repertoire, because four new Vβ families of single genes have recently been described.29 Our primers do not cover these families. Nevertheless, it seems that the early Vβ families (Vβ1 to Vβ13) are indeed the most commonly expressed in humans.36
from patients with acute (Epstein-Barr virus-induced) infectious mononucleosis. However, we could not correlate any Vβ predominance with an active CMV infection, although two of the four patients with a predominant Vβ16 expression had a clinically apparent concomitant CMV infection. Similarly, we did not observe any link between a particular Vβ usage and any other viral infection, MHC haplotype, or GVHD in this small group of patients. Nevertheless, it is possible that one or several minor histocompatibility antigens might be implicated, but it is unlikely to be the general case as a restricted CD8+CD57+ expansion was also documented in one of our two auto-BMT patients.

An alternative hypothesis would be that the physiologic bias of Vβ usage observed in normal individuals could be emphasized after BMT. However, the Vβ amplified in the CD8+57+ cells do not belong to families described as being preferentially expressed in the CD8+ cell compartment and are not even predominant in the whole Vβ repertoire, therefore excluding the hypothesis that such a skewed T-cell repertoire could simply reflect a phenotypic change. It might also be argued that the repertoire bias observed in CD8+CD57+ cells from BMT patients could be found as well in the corresponding cells from normal donors. Such controls were not performed because the incidence of these cells is relatively low in normal subjects, but, interestingly, HIV patients also frequently present a similar expansion of CD8+57+ T cells. However, preliminary results of a parallel PCR analysis on CD8+57+ T cells, purified from six HIV-infected patients, suggest that these cells are polyclonal and that their Vβ repertoire appears to be much less restricted compared with our observations in BMT recipients.

Finally, the question is raised of reasons underlying such a skewed oligoclonal T-cell expansion within the CD8+57+ subset. Previous studies, as well as a detailed phenotypic analysis of some of the patients studied here (not shown), showed correlations between the CD8+57+ T-lymphocyte expansion and the concomitant expression of activation markers on CD8+ cells, such as the CD29, CD45RO, CD38, and HLA-DR antigens, and B.S.-S., manuscript in preparation). These data strongly suggest that the CD57 cell surface expression might reflect an activated status of CD8+ T cells. A similar expansion of activated oligoclonal T cells combined with a marked restriction of the Vβ T-cell repertoire has been observed in human intraepithelial lymphocytes, where they might reflect a selective activation of the T-cell repertoire.

In conclusion, we propose that these oligoclonal CD8+ T cells, which are using a limited set of Vβ segments, could correspond to a reduced number of potentially reactive T-cell clones that are selectively activated in vivo and express the CD57 marker. Further studies are needed to determine the long-term significance of this CD8+CD57+ T-cell oligoclonal expansion.

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Oligoclonal expansion of CD8+ CD57+ T cells with restricted T-cell receptor beta chain variability after bone marrow transplantation

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