

Persistently Biased T-Cell Receptor Repertoires in HIV-1-Infected Combination Antiretroviral Therapy–Treated Patients Despite Sustained Suppression of Viral Replication

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Abstract: In most HIV-1–infected patients, highly active antiretroviral therapy (HAART) reduces plasma viral load to <50 copies/mL and increases CD4⁺ T-cell number and function. However, it is still unclear whether alterations of T-cell receptor (TCR) β -chain variable region (BV) repertoire, tightly related to disease progression, can be fully recovered by long-term treatment with HAART. This study analyzed the evolution of both T-cell subset composition and TCRBV perturbations in chronically HIV-1–infected patients with moderate immunodeficiency during 36 months of HAART. Despite persistently suppressed HIV replication, the rate of CD4⁺ T-cell repopulation, after an initial burst, progressively declined throughout the study period, resulting in a mean CD4⁺ T-cell count at the end of follow-up that was still significantly lower in HIV patients than in HIV-seronegative controls. This was seen in association with an incomplete restitution of both CD4 and CD8 TCRBV repertoire disruptions and was also demonstrated by the appearance of new TCRBV oligoclonal expansions occurring during HAART. In conclusion, these data indicate that 3 years of fully suppressive HAART may be not adequate to normalize CD4 counts and TCRBV repertoires in patients starting HAART with moderately advanced disease.

Key Words: HIV-1, HAART, T-cell receptor, T lymphocytes

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HIV-1 infection results in dramatic changes in the distribution of T cells in both peripheral blood and lymphoid tissues. Primary HIV infection associates with the generation of large clonal and oligoclonal expansions of CD8⁺ T cells.¹ These expansions of selected T-cell receptor (TCR) β -chain variable region (BV)-expressing CD8⁺ T cells are thought to be driven by viral antigens and are at least partially constituted by HIV-specific cytotoxic T lymphocytes (CTLs).² A correlation between expanded HIV-specific CTLs and the rate of disease progression has been proposed because the appearance of CTLs during the primary infection coincides with the decline of viremia and with the control of the acute illness.^{3,4} Unfortunately the CTL responses became early inadequate to limit virus spread by the occurrence of mechanisms of viral escape^{5–8} or by clonal exhaustion of HIV-specific CD8⁺ cells.⁹ Failure of CTL responses—in the absence of any other immune mechanisms capable of effectively restricting HIV replication—results in high rates of virus replication and, consequently, in the progressive erosion of the CD4⁺ T-cell pool.

Skewing of the CD4⁺ TCR repertoire observed in advanced HIV infection would therefore be the consequence of both deletion of CD4⁺ cells, occurring in a clonewise or random fashion, and antigen-driven expansion of CD4⁺ cells bearing selected TCRBV.^{10–12}

In most patients, highly active antiretroviral therapy (HAART) reduces plasma viral load below the limit of standard quantification and improves CD4⁺ T-cell number and function.¹³ However, residual HIV replication persists despite prolonged suppression of viremia.¹⁴ Previous studies performed to assess the effects of HAART on TCRBV diversity have shown a significant but incomplete normalization of the TCRBV repertoire in CD4⁺ or in CD8⁺ T cells.^{15–23} However, many of these studies suffered from restrictions such as short follow-up,^{15,17,18,21,22} limited number of patients^{15,18} and BV families investigated,^{16,17} absence of phenotypic information on T-cell subsets,^{16,20,21,23} and noncomparable cell populations (eg, peripheral blood mononuclear cells vs. purified CD4⁺ or CD8⁺ T cells).²²

Because the characterization of events leading to immune reconstitution is essential for our understanding of HIV pathogenesis and for the development of new therapeutic strategies, we investigated the evolution of both TCRBV perturbations and T-cell subset composition in chronically HIV-1-infected patients with moderate immunodeficiency who were naive for protease inhibitors during prolonged suppression of HIV replication.

PATIENTS AND METHODS

Patients

Fifteen subjects with chronic HIV-1 infection who showed optimal virologic and immunologic responses to HAART were studied over a period of 36 months. Inclusion criteria were as follows: at baseline, CD4 cell count ranging between 100–600 cells/ μ L, viral load $> 4 \log_{10}$ copies/mL, and naive for protease inhibitors; and during follow-up, improvement of CD4 cell counts and plasma viral load persistently under the detection limit of the test ($1.69 \log_{10}$ copies/mL). Patients meeting the inclusion criteria were selected among a previously described larger patient population undergoing HAART.²⁴ At entry, CD4⁺ T-cell count ranged from 141–556 cells/ μ L (mean 274 ± 124 cells/ μ L), whereas mean viral load was $4.86 \pm 0.56 \log_{10}$ copies/mL. Detailed demographic, clinical, and laboratory characteristics of patients investigated are reported in Table 1. No concomitant active opportunistic infections were present at enrollment or during the follow-up.

Blood samples were collected at baseline (t0) and after 3, 6, 12, 24, and 36 months of therapy (t3, t6, t12, t24, and t36, respectively). All subjects enrolled in this study reached the t24 time point, whereas 9 patients gained the t36 time point. As controls, we included 15 age-matched healthy blood donors. All subjects gave informed written consent according to the Ethical Committee of the University of Rome, La Sapienza, Italy.

Flow Cytometric Analysis of CD4⁺ and CD8⁺ T-Cell Subsets and TCRBV Repertoire

Five hundred microliters of whole blood were lysed using 10 mL of Ortho lysing reagent (Ortho-Clinical Diagnostics, Raritan, NJ), washed, labeled with a cocktail of 4 monoclonal antibodies for 30 minutes at 4°C, and fixed within 1 hour from blood collection. The peripheral distribution of naive and memory T cells has been investigated by evaluating the differential expression of CD45RA and CD62L molecules on both CD4⁺ and CD8⁺ T cells. Anti-CD4 allophycocyanin, anti-CD8 peridinin chlorophyll protein, anti-CD45RA and anti-CD45R0 fluorescein isothiocyanate (FITC), anti-CD62L phycoerythrin, and anti-HLA-DR FITC were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA); FITC-labeled anti-Fas was obtained from Medical & Biologic Laboratories Co., Ltd. (Nagoya, Japan). Direct staining with anti-TCRBV antibodies (IOtest Beta Mark; Immunotech, Marseille, France) was performed according to manufacturer's instruction. After staining, cells were washed once in phosphate-buffered saline containing 2% fetal bovine serum, and

TABLE 1. Baseline Clinical and Laboratory Characteristics

Patients	Age (years)	Sex	Risk Factors	Disease Stage*	CD4 (Cells/ μ L)	Viral Load (log copies/mL)	NRTI experience (years)	CD4 Nadir (Cells/ μ L)	Antiretroviral Treatment
1	56	M	Homosexual	A2	287	4.27	—	287	AZT + 3TC + IDV
2	36	M	Intravenous drug user	B3	197	4.66	4	197	3TC + d4T + IDV
3	52	M	Homosexual	B2	228	4.70	1	228	AZT + 3TC + IDV
4	35	M	Intravenous drug user	B3	181	4.85	8	181	3TC + d4T + IDV
5	29	F	Heterosexual	A2	475	3.90	2	376	3TC + d4T + IDV
6	37	F	Intravenous drug user	B2	282	4.34	7	282	3TC + d4T + RTV
7	36	M	Heterosexual	B3	172	5.71	—	153	AZT + 3TC + IDV
8	31	M	Heterosexual	B3	141	5.05	—	141	3TC + d4T + IDV
9	30	M	Heterosexual	C3	143	5.72	—	84	3TC + d4T + IDV
10	33	F	Heterosexual	A2	334	4.04	—	266	AZT + 3TC + IDV
11	56	M	Homosexual	C2	334	4.56	4	189	AZT + 3TC + IDV
12	52	M	Homosexual	A2	241	5.00	1	241	AZT + 3TC + IDV
13	43	M	Homosexual	B1	556	5.60	—	433	AZT + 3TC + IDV
14	27	F	Heterosexual	A3	154	4.89	—	154	AZT + 3TC + NFV
15	35	M	Homosexual	B2	383	5.13	—	326	3TC + d4T + IDV

*According to the 1993 revised AIDS definition⁶¹.

AZT, zidovudine; d4T, stavudine; IDV, indinavir; NFV, nelfinavir; NRTI, nucleoside reverse transcriptase inhibitor; 3TC, lamivudine.

were analyzed on a FACSCalibur cytofluorometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) using the Cell Quest software. To determine marker expression on CD4⁺ and CD8⁺ cells, total lymphocytes were first identified and gated by forward and side scatter. The cells were then additionally gated for CD4 or CD8 expression. TCRBV gene segment denomination is in accordance with that of Wei et al.²⁵ The normal distribution of TCRBV families was obtained from 40 healthy subjects aged between 25–65 years, with no history, at the time of sampling, of chronic infection, allergy, or autoimmunity.

Determination of Viral Load

Plasma HIV RNA levels were determined by reverse transcriptase polymerase chain reaction (PCR) (Amplicor kit; Roche Molecular Systems, Branchburg, NJ) and the threshold was 1.69 log₁₀ HIV RNA copies/mL.

Molecular Studies

CD4⁺ and CD8⁺ T cells were separated by using CD4 and CD8 MicroBeads and MACS columns according to the manufacturer's protocols (Miltenyi Biotec, Bergisch Gladbach, Germany). Total mRNA was extracted directly from 1 × 10⁶ bead-coated cells using Trizol-LS Reagent (Gibco BRL, Grand Island, NY) and Micro-carrier (Molecular Research Center, Cincinnati, OH) and precipitated with isopropyl alcohol. The pelleted RNA was resuspended in diethylpyrocarbonate-treated water and the poly-(A)+ portion of total RNA was converted into cDNA using 2.5 μM oligo(dT) as primer for reverse transcription, 50 mM KCl, 10 mM TRIS-HCl, 5 mM MgCl₂, 1 mM of each dNTPs, 1 U/μL of RNase Inhibitor, and 2.5 U/μL MULV reverse transcriptase (Applied Biosystem, Foster City, CA).

To analyze the TCRBV transcript size patterns cDNA samples were amplified by using a TCRβ C1/C2-specific primer (CGG GCT GCT CCT TGA GGG GCT GCG) and a set of 24 TCRBV-specific primers (BV 1, 2, 3, 4, 5.1, 5.3, 6.1, 6.2, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 24).²⁶ Briefly, 2 μL of the reverse transcriptase product was brought to a final reaction volume of 50 μL containing 50 mM KCl, 10 mM TRIS-HCl, 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 25 pmol of each oligonucleotide, and 2 U of Taq DNA polymerase (Applied Biosystems). After an initial denaturation step of 3 minutes at 95°C, the reactions were subjected to 35 cycles of PCR (30 seconds at 94°C, 30 seconds at 60°C, 30 seconds at 72°C) followed by a final elongation step for 10 minutes at 72°C. Aliquots of the unlabeled PCR products were then labeled by 10 cycles of elongation in a 10-μL "run-off" reaction with the FAM TCRβ constant primer (CTG CAC CTC CTT CCC ATT) mixed with deionized formamide and carboxytetramethylrhodamine (TAMRA) 500 size standard (Applied Biosystems). Finally, run-off products were electrophoresed for 24 minutes on a 310 ABI PRISM automated sequencer (Applied

Biosystems, Foster City, CA) by using a 47-cm capillary and Performance Optimized Polymer-4 (POP-4) (Applied Biosystems, Foster City, CA) polymer. The third complementarity-determining region (CDR3) profile was then analyzed with the Genescan software (Applied Biosystem). Analysis of the level of perturbation of TCRBV repertoire of HIV-infected patients was performed as previously described.^{27,28} Briefly, the CDR3 length profiles (spectratypes) were first translated into probability distributions as function of the area under the profile for each CDR3 length. A control profile, representing the nonperturbed repertoire, was determined for each BV by calculating the average distribution of the corresponding CD4 and CD8 profiles from 5 healthy blood donors. The extent of perturbation for each CDR3 fragment was then calculated by the difference between the sample's distribution and the control's distribution. Finally, the TCR repertoire perturbation per BV family was defined as the sum of the absolute values of the differences between each sample's CDR3 length and the corresponding control distribution. Values of BV perturbation greater than the sum of the standard deviations calculated in normal blood donors for each CDR3 profile were considered abnormal.

For CDR3 sequencing, amplification products were inserted into a plasmid vector (pCRII-TOPO; Invitrogen, Carlsbad, CA), cloned in *Escherichia coli*, and sequenced onto an Applied Biosystems Sequencer Model 377-96. Sequence similarities were identified using the multiple sequence alignment application, Align X, of the Vector NTI Suite 6.0 (InforMax, North Bethesda, MD) based on the Clustal W algorithm.²⁹ The identification of the V(D)J junctions was performed comparing the sequences with those reported in "IMGT, the international ImMunoGeneTics database" (<http://imgt.cines.fr:8104-Initiator> and coordinator: Marie-Paule Lefranc, Montpellier, France).³⁰

Statistical Analysis

Baseline and follow-up data were compared using Wilcoxon matched pairs test, and for data of different groups Mann-Whitney *U* test was used; values of *P* < 0.05 were considered significant. With regard to TCRBV expression we define as expansion a value greater than the mean of the controls plus 3 SD.³¹ Differences in the number of expansions at each time point were evaluated by using the PROC MIXED procedure of the statistical software SAS 8.2 (SAS Institute, Cary, NC).

RESULTS

T-Cell Subsets and Plasma Viremia Before and After HAART

Changes in lymphocyte subpopulations during HAART are summarized in Figure 1 and Table 2. Before therapy, the frequency of CD4⁺ T cells was 21 ± 10% with an absolute count of 274 ± 124 cells/μL, thereby consistent with a less advanced disease. The distribution of CD4⁺ naive T cells

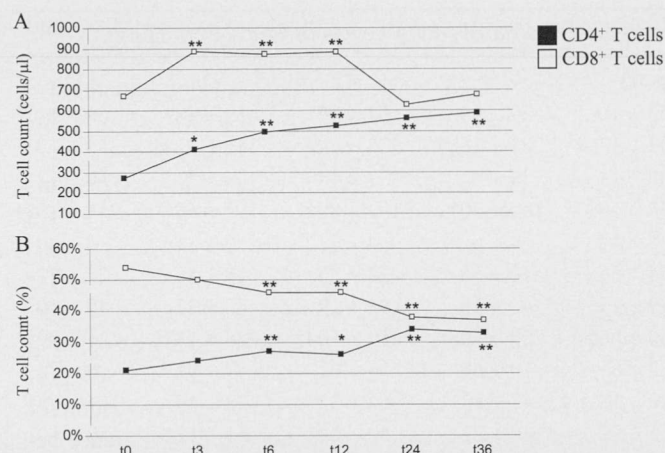


FIGURE 1. Changes in CD4⁺ and CD8⁺ lymphocyte count under HAART. Data are expressed as mean values (A, absolute count, and B, percentage) for each time point studied. Significance in comparison with baseline values: * $P < 0.05$; ** $P < 0.01$.

(CD45RA⁺CD62L⁺) as well as that of CD4⁺ memory cells (CD45RA⁺CD62L⁺ + CD45RA⁺CD62L⁺) and CD4⁺CD95⁺ cells was within normal range. As opposite, the percentage of CD4⁺ effector memory cells (CD45RA⁺CD62L⁺) and activated CD4⁺ HLA-DR⁺ cells was significantly increased in HIV-1-infected patients ($P < 0.0001$ and $P = 0.006$, respectively). However, due to the restriction of the CD4⁺ T-cell pool, the absolute numbers of all these T-cell subsets, with the exception of CD4⁺ effector memory cells, were significantly downregulated in comparison to healthy controls.

The frequency of CD8⁺ T cells, as well as that of CD8⁺ effector memory, CD8⁺CD95⁺, and CD8⁺ HLA-DR⁺ T cells, was at baseline increased in HIV-1-infected patients ($P < 0.0001$, $P = 0.003$, $P = 0.007$, and $P = 0.001$, respectively), whereas the percentage of naive cells was, conversely, decreased ($P = 0.001$). When the absolute counts of CD8⁺ T-cell subsets were considered, only naive cells were found within normal range, whereas all other subsets investigated were significantly upregulated in comparison to healthy controls ($P = 0.036$, $P = 0.009$, $P = 0.006$, $P = 0.007$, and $P = 0.002$ for CD8⁺, CD8⁺ memory, effector memory, CD95⁺, and HLA-DR⁺ cells, respectively).

After starting therapy, plasma viral load, which was $4.86 \pm 0.56 \log_{10}$ copies/mL at baseline, fell to undetectable levels in all patients throughout the study (with the only exception of patient 8 at t12, $2.59 \log_{10}$ copies/mL). This was seen in association with a significant rise in both absolute number and percentage of CD4⁺ T cells at all studied time points. Naive and memory cells contributed equally to the recovery of CD4⁺ cells according to a similar rate of growth: 1.77 versus 1.87 at t3; 2.13 versus 2.22 at t6; 2.4 versus 2.29 at t12; 2.57 versus 2.37 at t24; and 2.50 versus 2.73 at t36 for naive and memory cells (including effector memory cells), respectively.

As a result of this progressively declining rate of T-cell repopulation, the absolute number of both CD4⁺ and CD4⁺ naive cells was at t36 still significantly downregulated in comparison to age-matched controls ($P < 0.001$). No significant changes were observed in the percentage of CD4⁺ naive and memory T cells in comparison to pretherapy determinations. The proportion of HLA-DR⁺-activated cells was significantly decreased within the CD4 subset from t6 to t36, whereas the percentage of CD95⁺ cells remained unchanged.

The percentage of CD8⁺ cells decreased during HAART, while the absolute number, after an initial significant increase (from t3 to t12), returned to values overlapping those detected at baseline after 24 months of therapy. A significant increase of both proportion and absolute numbers of CD8⁺ naive cells was observed during HAART, whereas CD8⁺ memory, effector memory, and CD95⁺ cells were not significantly modified. As opposite, the percentage of CD8⁺ HLA-DR⁺ cells was only transiently reduced at t3, t6, and t12 time points.

TCRBV Repertoires

Anomalies in the relative BV usage were evaluated longitudinally in cytofluorometry using a panel of BV family-specific monoclonal antibodies covering approximately 60–70% of T cells expressing TCR $\alpha\beta$ in normal individuals. The distributions of CD4 and CD8 BV families are shown in Tables 3 and 4, respectively, and refer to the 9 patients who reached the t36 time point.

Although all patients had several underexpressed BV families, we did not detect clear-cut repertoire holes, nor a trend for the restricted BV subsets to cluster in selected families. However, in some patients, underexpression of several BV families could be ascribed to the predominance of expanded clones (eg, within CD4⁺ cells of patients 1 and 3 and within CD8⁺ cells of patients 1 and 9). In fact, expansions of BV families were detected at baseline in most patients (8/9), both in CD4⁺ and CD8⁺ T cells. Unlike underrepresentations, these expansions tended to cluster to selected BV families rather than disperse in a random manner. The BV families preferentially expanded were BV12 and BV14 for CD4⁺ and CD8⁺ T cells, respectively.

The breadth of TCRBV repertoire of CD4⁺ cells improved early after the initiation of HAART. The number of BV expansions decreased from 28, observed at t0, to 20 at t3 ($P > 0.05$), 17 at t6 ($P > 0.05$), and 11 at t12 ($P = 0.002$). However, an additional 2 years of suppressive therapy did not result in a further widening of the CD4 repertoire. In fact, the number of BV expansions reascended after 36 months of HAART to 16 ($P > 0.05$), a value closely resembling those observed at t3 and t6 (Table 3).

Although the BV expansions were relatively constant over time—particularly those that were most prominent—it should be noted that approximately half of the expansions de-

TABLE 2. Cytofluorometric Analysis of CD4⁺ and CD8⁺ T-Cell Subsets and Plasma HIV RNA Levels of Study Population

T-cell subsets*	Analysis	Controls	t0	t3	t6	t12	t24	t36
Naive	% of CD4 ⁺	49 ± 10	55 ± 14	55 ± 16	57 ± 13	61 ± 17	59 ± 18	56 ± 18
Naive	cells/μL of CD4 ⁺	516 ± 189	134 ± 89	237 ± 170†	285 ± 168†	323 ± 141†	345 ± 228†	335 ± 173†
Memory	% of CD4 ⁺	50 ± 10	44 ± 15	38 ± 11	39 ± 9	37 ± 14	36 ± 9	40 ± 12
Memory	cells/μL of CD4 ⁺	510 ± 138	94 ± 32	142 ± 68‡	189 ± 70†	194 ± 89†	195 ± 69†	234 ± 103†
Effector memory	% of CD4 ⁺	0.8 ± 1.3	4 ± 3	8 ± 14	4 ± 6	4 ± 7	5 ± 11	4 ± 8
Effector memory	cells/μL of CD4 ⁺	8 ± 12	10 ± 9	34 ± 55	20 ± 36	22 ± 35	28 ± 56	23 ± 51
CD95 ⁺	% of CD4 ⁺	48 ± 11	43 ± 13	39 ± 15	43 ± 16	42 ± 19	42 ± 22	40 ± 16
CD95 ⁺	cells/μL of CD4 ⁺	492 ± 146	96 ± 44	160 ± 66‡	209 ± 108‡	219 ± 104‡	228 ± 123‡	226 ± 99‡
HLA-DR ⁺	% of CD4 ⁺	6 ± 2	16 ± 10	12 ± 6	10 ± 6‡	11 ± 6‡	9 ± 9‡	10 ± 8‡
HLA-DR ⁺	cells/μL of CD4 ⁺	59 ± 20	40 ± 30	40 ± 18	47 ± 13	54 ± 32	48 ± 49	61 ± 55‡
Naive	% of CD8 ⁺	56 ± 13	36 ± 16	35 ± 17	46 ± 22‡	50 ± 24‡	51 ± 12‡	50 ± 19‡
Naive	cells/μL of CD8 ⁺	256 ± 125	217 ± 85	278 ± 104‡	381 ± 216‡	390 ± 204†	315 ± 123‡	338 ± 174‡
Memory	% of CD8 ⁺	29 ± 10	34 ± 12	32 ± 13	27 ± 13	27 ± 20	26 ± 8	25 ± 13
Memory	cells/μL of CD8 ⁺	129 ± 62	254 ± 199	300 ± 223	251 ± 228	247 ± 219	168 ± 100	164 ± 98
Effector memory	% of CD8 ⁺	15 ± 11	29 ± 13	33 ± 17	28 ± 15	28 ± 16	25 ± 11	24 ± 13
Effector memory	cells/μL of CD8 ⁺	76 ± 87	199 ± 150	308 ± 226	232 ± 134	285 ± 267	168 ± 113	171 ± 127
CD95 ⁺	% of CD8 ⁺	49 ± 17	65 ± 17	65 ± 25	60 ± 22	65 ± 19	58 ± 17	52 ± 17
CD95 ⁺	cells/μL of CD8 ⁺	229 ± 150	447 ± 248	612 ± 426‡	506 ± 259‡	604 ± 421‡	377 ± 201‡	352 ± 199‡
HLA-DR ⁺	% of CD8 ⁺	17 ± 9	38 ± 21	23 ± 12‡	23 ± 15‡	23 ± 15‡	28 ± 18	32 ± 20
HLA-DR ⁺	cells/μL of CD8 ⁺	78 ± 62	275 ± 264	138 ± 72	192 ± 185	203 ± 213	165 ± 101	220 ± 184
HIV1-RNA	(log ₁₀ copies/mL)		4.86 ± 0.56	<1.69	<1.69	<1.69 (8/9), 2.59 (1/9)	<1.69	<1.69

*CD45RA⁺CD62RL⁺ were defined as naive cells, CD45RA⁺CD62L⁺ and CD45RA⁺CD62L⁻ as memory cells, and CD45RA⁺CD62L⁻ as effector memory cells. Results are expressed as mean values ± SD. As controls, we included 15 age-matched healthy blood donors. HIV-1-infected patients were studied before starting therapy (t0) and after 3 (t3), 6 (t6), 12 (t12), 24 (t24), and 36 (t36) months of HAART. Data regarding t36 are referred to 9 of 15 patients. Significance in comparison with baseline values: †*P* < 0.01; ‡*P* < 0.05.

tected at t36 were seen in BV families showing normal values at previous time points.

In contrast, a substantial stability of the CD8 TCRBV repertoire was observed. The number of expanded BV families did not significantly change during 36 months of HAART, passing from 17, detected at baseline, to 19 at t3, 20 at t6, and 14 at t12 and t36 (Table 4). The slight increase of BV expansions seen at t3 and t6 was consistent with the parallel increase of CD8⁺ T-cell count observed up to 12 months from the onset of therapy probably consequent to redistribution of cells from previously inflamed tissues. Like that observed for the CD4 subset, the CD8 BV expansions were quite stable over time and clustered to limited BV families including BV14 and BV16. Statistical analysis of these data showed that the diversity of CD4, but not CD8, TCRBV repertoire, as a whole, significantly increased over time (*P* = 0.018 and *P* = 0.35 for CD4⁺ and CD8⁺ T cells, respectively). However, when the individual BV families were considered, no significant changes in the relative BV usage could be demonstrated during the 36 months of follow-up both in CD4 and in CD8 subset.

Because the evaluation of TCRBV usage with monoclonals does not allow an exclusion of reduced diversity of the

TCR repertoire even in the presence of a normal distribution of individual BV families, we looked for further restrictions of repertoires by performing the more informative CDR3 spectratyping, namely the quantitative analysis of CDR3s with different sizes generated by the random insertion/deletion of nucleotides during V(D)J rearrangement. A normal, polyclonal repertoire results in a spectratype with a gaussian-like distribution of CDR3 lengths, differently from oligoclonal repertoires showing profiles altered by ≥1 predominant fragments outside the peak of median length.

The levels of CDR3 perturbation detected at t0, t12, and t36 in 6 of 9 patients who reached the t36 time point are shown in Figures 2 and 3 for CD4⁺ and CD8⁺ cells, respectively. Mathematical analysis of the deviation of patients' histograms from the normal distribution revealed significantly altered patterns at baseline in most BV genes examined in CD4⁺ cells (56–82% of the BV families investigated). As expected, CDR3 profiles were more perturbed within the CD8⁺ subset, with oligoclonal peaks found in almost all BV families (96%–100%). In this regard it should be noted that disruptions of the gaussian-like CDR3 profile of some BV families are often observed in CD8⁺ lymphocytes of normal individuals. This abnormal

TABLE 3. Quantitative Cytofluorometric Analysis of BV Subsets among CD4⁺ T Cells of 9 HIV-1-Infected Patients Before and During HAART

Patient	Months	BV2	BV3	BV5.1	BV5.2	BV5.3	BV6.7	BV7	BV8	BV9	BV11
	0	9.6	4.4	3.7	0.5	0.2	19.7	1.2	1.9	1.3	0.9
	3	8.7	8.7	4.0	2.5	2.5	ND	9.0	1.6	3.2	1.9
1	6		9.7	6.6	4.4	0.4	0.8	16.3	1.0	2.3	3.0
	12		5.2	4.7	2.7	0.6	1.8	18.0	1.3	2.3	2.0
	36		9.0	5.0	4.1	0.7	0.9	ND	4.1	2.3	2.7
	0	9.1	5.0	7.0	0.8	2.8	ND	6.9	4.3	2.9	1.2
2	3	12.8	11.2	7.4	1.2	1.9	3.6	1.6	3.9	4.2	0.4
	6	13.2	6.2	5.2	0.6	0.6	5.0	1.9	3.8	2.7	0.7
	12	12.6	8.7	5.0	0.9	1.6	3.2	1.2	3.2	3.7	0.5
	36	12	7.4	5.7	1.3	0.8	ND	4.2	4.5	3.7	0.7
	0	16	1.4	7.0	1.0	1.0	2	5.0	3.0	4.6	0.5
3	3	4.9	1.5	5.0	0.3	0.6	1.9	0.8	5.0	3.9	1.2
	6	5.6	1.6	6.5	3.6	0.6	1.8	1.5	2.9	3.3	1.2
	12	6.3	1.2	7.5	1.3	1.7	2.7	0.9	3.8	3.7	0.9
	36	8.4	2.1	7.0	0.9	1.0	ND	2.2	3.4	3.8	1.1
	0	5.0	5.0	9.2	1.2	1.2	2.4	1.0	4.0	2.9	1.0
4	3	6.6	3.4	3.5	0.6	1.5	5.5	1.5	4.6	2.4	2.5
	6	4.0	2.3	2.2	1.6	1.7	3.0	1.2	4.9	3.5	1.0
	12	5.2	4.0	3.4	0.9	0.8	6.3	1.9	5.2	2.5	1.0
	36	5.3	2.8	4.4	0.9	1.0	ND	1.7	4.8	2.3	0.9
	0	9.9	3.0	6.0	0.6	1.9	5.8	2.0	2.8	2.6	1.0
5	3	11.0	5.0	8.0	1.5	2.0	7.0	1.2	4.0	4.0	1.4
	6	12.5	5.0	7.9	2.3	1.6	7.0	1.9	4.9	3.8	0.7
	12	11.8	3.2	7.5	1.5	1.8	6.5	3.5	3.2	3.6	0.5
	36	12	3.2	7.0	1.3	1.5	ND	4.6	3.0	4.3	0.7
	0	11.1	1.2	6.5	1.4	1.5	8.8	2.4	4.1	3.5	0.6
6	3	9.1	0.8	5.7	1.1	0.6	5.6	2.7	2.9	3.6	0.8
	6	7.0	2.0	6.8	2.3	2.1	5.7	2.1	3.1	4.1	2.8
	12	11.7	0.7	5.5	0.7	1.0	7.0	0.3	3.9	2.3	0.8
	36	10.7	0.7	7.0	0.8	1.1	ND	1.8	4.2	3.4	0.7
	0	8.6	6.0	10.4	0.2	1.0	1.3	3.0	3.0	3.6	0.6
7	3	8.9	4.0	9.0	0.2	1.2	3.3	1.2	3.9	4.6	1.7
	6	5.6	3.0	7.6	0.7	0.9	5.3	3.0	4.0	5.6	0.8
	12	1.8	4.1	6.3	6.3	1.3	3.1	2.1	3.1	4.8	0.9
	36	8.3	3.5	8.9	1.1	1.4	ND	4.0	6.0	4.8	0.8
	0	8.5	2.1	5.0	7.9	3.0	ND	1.7	5.0	4.0	1.0
8	3	8.0	1.3	6.0	0.7	0.6	4.2	0.8	4.4	3.6	1.0
	6	8.0	1.2	6.3	0.4	1.0	5.0	1.0	3.6	1.4	2.2
	12	8.4	1.5	5.8	1.1	1.2	ND	2.0	3.6	3.8	1.3
	36	8.4	1.5	6.8	0.9	1.3	ND	4.1	4.7	3.8	1.1
	0	8.7	4.0	8.6	1.0	0.6	ND	4.5	4.6	3.7	0.9
9	3	6.5	4.5	6.5	1.3	2.2	ND	5.3	4.3	3.7	1.3
	6	9.5	5.7	6.4	0.9	1.5	ND	3.6	5.0	3.5	0.1
	12	8.1	5.2	6.7	1.2	1.3	ND	3.1	3.5	3.7	0.8
	36	8.3	3.6	8.8	1.0	1.2	ND	4.2	5.7	4.7	0.7

TABLE 3. (Continued).

Patient	Months	BV12	BV13.1	BV13.6	BV14	BV16	BV17	BV18	BV20	BV21.3	BV22	BV23
1	0	4.9	2.8	8.8	1.8	1.6	4.9	1.0	1.8	2.6	1.5	1.2
	3	4.4	2.3	9.9	2.9	0.0	4.6	1.0	1.7	1.0	1.6	0.2
	6	4.0	2.8	9.6	2.2	1.0	3.0	0.6	1.0	2.5	1.5	0.3
	12	4.6	2.2	6.5	1.8	0.4	2.6	0.5	2.4	0.9	1.5	0.5
	36	4.5	2.7	7.7	2.9	3.5	4.5	0.8	2.0	1.8	2.4	1.7
2	0	4.1	5.3	3.4	3.2	0.8	3.8	1.7	1.2	4.0	3.1	0.8
	3	3.5	5.4	1.4	4.5	2.0	3.4	2.3	2.6	2.7	3.0	1.0
	6	1.6	3.9	1.4	2.8	0.8	3.5	1.0	1.0	1.2	2.7	0.3
	12	0.8	4.1	1.7	3.7	1.4	4.0	0.9	1.4	2.7	4.0	0.2
	36	2.4	3.8	1.4	3.5	2.2	4.5	1.0	2.8	3.0	3.8	0.8
3	0	1.6	10.9	1.7	1.7	1.4	5.9	1.8	4.0	3.8	2.7	0.5
	3	3.5	13.7	0.7	2.7	1.2	3.9	1.5	3.5	3.6	2.0	0.3
	6	3.0	10.0	2.0	2.1	0.7	7.0	1.4	2.7	5.0	3.4	0.2
	12	2.9	10.3	1.2	1.5	0.5	5.8	1.4	3.7	4.4	2.8	0.2
	36	2.8	7.0	2.0	2.8	1.4	5.6	0.9	3.0	3.7	4.0	0.5
4	0	1.5	5.8	1.5	5.2	4.5	6.0	2.7	1.9	2.4	3.8	1.0
	3	2.0	5.7	3.6	5.4	1.3	5.0	1.5	3.2	1.5	2.7	1.1
	6	3.0	5.4	1.9	3.5	2.4	5.5	2.5	1.8	4.8	0.6	3.2
	12	2.0	4.6	3.3	3.7	2.1	5.3	0.6	3.2	2.2	3.4	0.5
	36	1.9	3.9	2.6	2.7	1.5	5.8	0.6	2.0	2.5	3.0	0.1
5	0	2.2	3.7	2.2	1.5	1.6	3.7	1.7	1.4	3.0	3.8	0.9
	3	2.2	5.3	1.9	3.8	1.4	4.2	2.6	ND	4.4	2.6	0.9
	6	2.6	2.8	2.2	2.4	0.8	4.9	1.8	0.1	2.4	4.3	0.4
	12	1.7	4.2	1.7	1.2	1.5	4.1	2.6	0.4	3.0	3.8	0.2
	36	1.8	4.4	1.9	1.9	1.8	4.9	2.3	0.4	2.6	4.7	0.8
6	0	4.0	3.7	2.7	2.0	1.3	3.4	1.6	1.8	2.5	3.4	0.6
	3	4.1	3.6	1.1	2.4	2.3	4.1	1.6	ND	2.1	3.2	0.8
	6	4.0	8.7	2.0	2.8	1.5	1.4	2.5	3.0	2.5	4.3	2.0
	12	4.2	4.4	1.7	2.1	1.2	2.1	1.2	2.2	0.6	2.1	0.4
	36	2.1	3.4	2.4	2.7	1.0	4.5	1.5	2.4	1.8	3.9	0.2
7	0	0.9	6.3	2.4	2.4	0.3	6.7	2.5	2.1	3.7	2.8	3.7
	3	0.8	6.5	1.5	1.5	0.4	4.7	3.0	5.0	1.9	3.8	0.7
	6	1.9	4.5	2.0	2.0	1.0	5.0	1.5	3.9	0.6	4.0	0.4
	12	1.8	4.0	4.8	3.6	0.5	4.5	0.4	6.3	2.4	5.4	0.1
	36	1.4	5.0	1.8	2.8	2.4	5.5	2.3	3.7	2.7	4.7	1.0
8	0	5.0	5.0	1.6	2.0	3.9	8.5	3.0	4.0	1.5	4.0	2.5
	3	1.9	4.1	2.9	2.5	1.3	6.3	1.9	4.5	1.7	3.3	0.6
	6	1.8	3.0	3.0	1.9	0.8	9.9	1.1	4.9	1.4	3.5	0.7
	12	2.3	4.5	1.7	2.5	1.5	5.3	1.0	4.4	2.4	4.1	0.6
	36	2.1	4.5	1.8	2.2	1.9	6.6	1.2	4.6	2.5	4.5	0.6
9	0	2.4	4.2	1.5	4.5	2.4	6.4	0.9	3.4	2.4	3.9	1.3
	3	4.3	4.2	1.6	7.5	1.8	5.2	1.3	4.3	2.7	3.9	1.0
	6	1.9	3.5	1.6	5.6	4.1	5.2	1.1	5.0	2.7	4.0	1.1
	12	2.2	3.3	1.5	5.8	2.6	5.8	0.4	4.8	2.9	3.4	0.5
	36	1.4	4.8	1.8	2.4	1.6	5.5	1.9	3.7	2.2	4.6	0.5

BV, variable region of β -chain. ND, not determined.

Analyses were done with BV-specific monoclonal antibodies. Results are the percentage of TCR $\alpha\beta$ T cells expressing the indicated TCRBV gene. For each patient, before (0) and after 3, 6, 12, and 36 months of therapy. Boldface indicates overexpressed TCRBV families.

TABLE 4. Quantitative Cytofluorometric Analysis of BV Subsets among CD8⁺ T Cells of 9 HIV-1-Infected Patients Before and During HAART

Patient	Months	BV2	BV3	BV5.1	BV5.2	BV5.3	BV6.7	BV7	BV8	BV9	BV11
1	0	4.8	4.0	3.0	0.5	0.9	1.1	4.0	3.3	2.0	0.5
	3	3.7	4.5	3.4	ND	5.0	0.3	2.6	2.1	0.9	0.8
	6	4.8	3.4	3.0	0.4	0.3	0.7	2.0	2.7	1.6	0.3
	12	5.1	3.5	0.6	0.4	0.6	2.2	1.3	1.5	0.5	0.1
	36	5.1	5.2	0.7	0.6	0.8	ND	5.6	1.3	0.7	0.2
2	0	4.7	5.5	6.0	0.7	2.9	ND	4.9	3.7	2.7	3.7
	3	3.8	6.7	4.4	1.7	3.3	2.6	3.0	5.8	3.5	1.6
	6	ND	7.6	3.5	0.6	1.3	5.2	3.3	4.2	2.5	0.7
	12	5.2	8.6	3.2	0.5	1.4	4.7	4.3	4.2	2.4	0.5
	36	5.0	6.6	2.9	2.6	1.4	ND	4.1	3.2	3.0	0.7
3	0	2.4	1.7	2.9	3.0	1.3	3.4	7.0	5.0	3.2	1.4
	3	1.8	7.8	2.9	0.7	0.1	3.6	3.3	4.9	2.6	0.7
	6	2.3	7.8	4.8	2.5	2.6	4.2	3.7	5.0	2.5	3.4
	12	2.0	6.8	3.4	0.7	0.5	5.0	3.2	4.9	2.1	0.5
	36	3.0	5.7	2.8	0.8	0.5	ND	3.9	5.0	2.2	1.0
4	0	2.8	2.5	2.0	0.9	1.0	1.6	2.5	6.0	1.9	1.4
	3	6.9	3.0	3.0	1.8	5.0	1.5	0.7	2.4	0.8	0.4
	6	3.0	3.4	3.3	1.6	1.8	1.7	1.3	2.7	2.2	1.0
	12	4.8	2.3	1.4	0.7	1.3	2.0	3.3	1.4	1.3	1.0
	36	2.2	2.3	1.9	0.9	1.2	ND	2.4	2.0	1.6	1.1
5	0	7.0	3.5	3.6	1.3	2.0	6.0	2.9	1.9	1.3	1.7
	3	4.0	5.6	2.4	1.2	1.5	4.0	1.5	2.0	1.1	0.6
	6	3.4	1.9	4.9	0.0	2.9	4.6	2.6	1.8	1.9	1.2
	12	4.3	3.7	1.1	0.9	0.8	5.2	3.2	0.8	2.7	0.2
	36	5.0	4.3	1.3	0.9	1.1	ND	7.0	1.8	3.2	0.7
6	0	6.5	1.2	2.4	0.9	1.3	2.2	3.9	7.0	1.6	0.3
	3	5.3	3.8	1.8	1.2	2.2	2.7	3.8	7.4	1.4	0.7
	6	5.5	2.2	2.5	1.1	1.7	0.1	3.0	6.6	1.3	0.7
	12	5.2	1.6	2.2	0.5	1.5	1.3	3.1	7.0	0.8	0.3
	36	4.9	2.7	1.6	0.4	1.2	ND	3.6	ND	1.6	0.5
7	0	2.0	2.0	5.0	0.4	1.8	0.8	1.9	3.4	1.9	0.2
	3	2.1	2.3	5.0	0.7	0.4	0.8	1.6	2.1	2.1	0.2
	6	1.3	1.0	4.0	0.4	0.6	2.3	2.0	1.9	2.0	0.4
	12	1.5	2.6	3.0	1.7	0.4	0.9	4.9	3.0	2.6	0.4
	36	2.6	3.2	3.2	1.0	1.0	ND	5.3	3.6	2.3	0.3
8	0	7.9	2.9	2.2	2.0	1.9	ND	3.1	3.1	0.7	2.1
	3	9.0	1.4	1.4	2.8	0.9	0.8	6.0	5.3	0.8	0.5
	6	8.0	1.4	4.6	3.0	0.0	4.6	4.9	4.4	2.4	0.6
	12	10.2	2.1	2.1	1.0	0.5	3	5.4	4.2	1.3	0.9
	36	10.3	2.2	2.3	1.9	1.5	ND	6	3.6	1.5	0.8
9	0	8.5	3.2	2.0	0.4	0.1	ND	1.9	1.9	1.9	0.2
	3	8.0	7.1	2.0	0.3	0.9	ND	6.3	2.0	2.3	0.1
	6	8.0	1.4	2.2	0.9	5.5	ND	3.5	1.1	5.1	2.0
	12	10.3	1.4	1.7	0.6	1.8	ND	3.5	1.1	1.3	0.1
	36	2.5	2.7	3.1	0.8	0.8	ND	3.6	3.5	2.2	0.4

TABLE 4. (Continued).

Patient	Months	BV12	BV13.1	BV13.6	BV14	BV16	BV17	BV18	BV20	BV21.3	BV22	BV23
1	0	2.0	3.9	0.7	17.3	17.0	7.0	1.0	0.2	2.7	2.9	2.7
	3	3.4	1.6	0.6	17.0	0.5	4.0	0.4	0.4	0.7	3.6	1.0
	6	2.6	1.3	0.4	17.9	0.3	5.0	0.3	1.7	1.8	3.1	2.3
	12	2.5	1.3	0.9	21.2	1.2	4.9	0.1	1.2	0.4	3.8	1.0
	36	2.2	1.6	0.6	26.4	2.2	5.7	0.4	0.9	0.6	2.6	1.0
2	0	1.5	2.0	1.5	7.3	1.3	4.5	1.4	0.9	4.2	6.4	2.0
	3	3.3	4.2	1.0	5.3	5.7	4.2	0.9	1.4	2.4	5.8	2.2
	6	2.8	3.2	1.4	6.8	0.7	4.6	0.4	1.2	1.9	2.8	1.6
	12	3.5	3.4	1.5	5.1	0.6	4.2	0.6	1.3	2.8	4.0	3.3
	36	2.7	4.4	1.5	6.5	1.0	4.5	0.5	3.1	3.0	3.7	2.0
3	0	2.6	3.0	1.4	8.9	7.3	4.9	0.4	6.0	2.5	2.0	4.7
	3	1.2	3.2	0.7	9.4	8.0	4.4	0.1	3.0	2.5	2.0	5.0
	6	1.0	4.7	1.9	9.0	5.5	4.2	0.8	4.2	1.6	2.9	5.9
	12	1.1	2.5	0.9	7.0	6.1	3.7	0.2	4.0	3.8	2.1	6.0
	36	2.2	3.6	0.9	6.7	7.7	3.6	0.1	4.4	4.6	3.0	5.6
4	0	2.9	3.7	1.8	4.9	5.0	4.6	0.3	1.0	4.5	2.2	0.8
	3	3.0	1.9	1.0	3.0	6.0	2.3	0.5	1.0	2.6	5.6	1.6
	6	1.3	3.4	2.2	4.9	9.1	2.9	0.3	1.0	3.7	1.5	1.8
	12	2.5	2.1	1.2	4.5	13.2	2.1	0.3	0.5	3.9	1.6	0.5
	36	1.4	1.8	1.7	5.0	11.3	2.8	0.3	1.2	2.6	1.4	0.1
5	0	1.9	5.4	3.4	2.4	3.6	3.5	0.9	1.4	3.0	4.7	2.0
	3	1.6	3.6	1.8	3.2	5.7	2.4	0.7	ND	3.3	2.5	2.8
	6	1.9	3.1	2.4	4.8	3.9	2.3	1.4	0.3	2.7	3.0	2.4
	12	2.1	2.9	2.6	4.1	10.7	2.9	1.3	1.6	2.5	2.3	2.6
	36	1.5	3.0	3.1	4.3	13.0	3.2	0.4	0.9	2.2	2.0	2.3
6	0	3.9	4.4	1.7	8.2	0.8	3.6	0.8	1.0	2.1	1.3	3.1
	3	1.3	12.4	1.2	10.8	9.3	3.6	0.7	ND	1.8	1.4	2.1
	6	9.0	10.5	1.5	8.0	0.4	4.6	0.3	0.8	1.7	0.1	3.0
	12	0.3	9.0	2.2	9.5	1.3	3.5	0.2	2.2	1.8	1.0	0.6
	36	0.6	ND	ND	8.0	0.7	2.6	0.2	1.5	1.2	1.9	0.6
7	0	2.6	5.3	3.8	14.0	0.4	3.6	1.3	2.6	0.8	2.8	1.1
	3	3.6	8.4	4.5	21.0	0.8	4.7	0.4	4.3	1.4	2.1	1.5
	6	2.9	9.7	3.9	11.8	0.8	3.5	0.1	1.2	0.7	2.9	1.2
	12	1.5	8.7	3.8	15.3	1.1	4.7	0.1	3.0	1.7	3.1	1.1
	36	1.8	8.0	2.0	25.0	3.2	5.6	0.7	5.1	2.0	5.9	2.6
8	0	5.8	3.9	2.7	3.5	4.1	6.4	2.0	4.0	3.9	8.0	10.4
	3	1.4	3.2	0.4	3.5	0.6	1.8	3.1	4.3	1.0	12.0	6.9
	6	1.2	1.3	3.5	6.0	1.5	2.3	1.5	2.0	3.5	11.0	8.5
	12	1.5	4.2	0.7	6.1	1.6	2.0	2.0	2.5	1.4	10.3	5.3
	36	2.8	4.1	1.1	7.4	1.2	2.9	0.8	2.1	1.4	9.3	6.3
9	0	0.3	2.9	1.5	5.3	0.1	9.0	0.1	15.0	1.3	1.1	0.6
	3	1.2	3.4	0.8	4.8	0.5	14.0	0.1	14.0	1.2	1.1	1.0
	6	1.9	4.4	0.9	1.5	0.9	12.9	0.3	11.0	1.5	0.0	3.7
	12	1.0	3.3	0.5	5.3	0.5	20.0	0.3	7.8	0.5	1.3	0.9
	36	2.7	8.1	2.0	23.7	2.8	5.4	0.7	1.5	1.8	5.7	2.5

BV, variable region of β -chain. ND, not determined.

Analyses were done with BV-specific monoclonal antibodies. Results are the percentage of TCR $\alpha\beta$ T cells expressing the indicated TCRBV gene. For each patient, before (0) and after 3, 6, 12, and 36 months of therapy. Boldface indicates overexpressed TCRBV families.

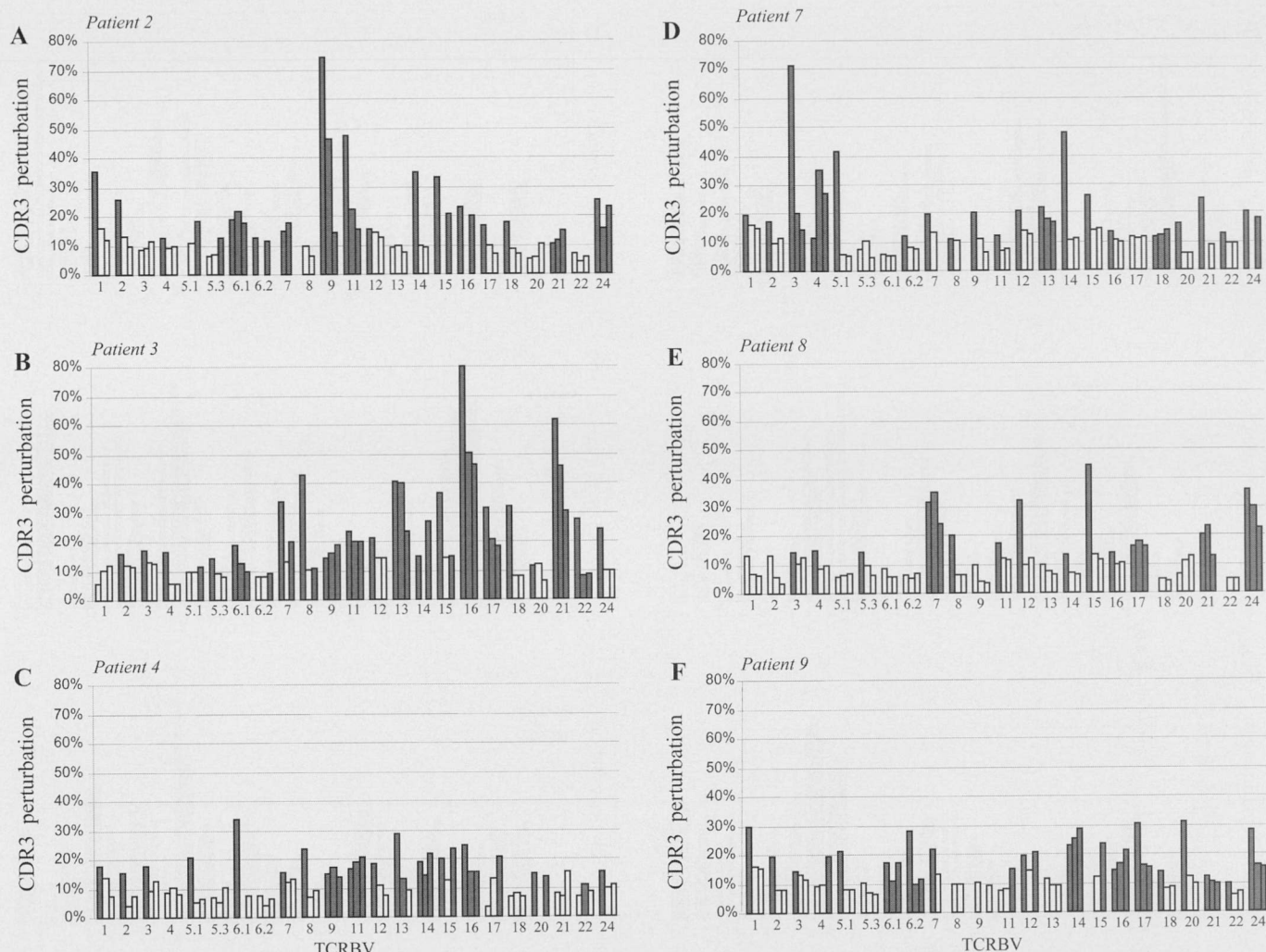


FIGURE 2. Diversity of CD4 TCRBV repertoire analyzed by CDR3 spectratyping. The extent of TCRBV repertoire perturbation is represented as the percent difference between the patient's CDR3 distribution and the corresponding control distribution. A value of perturbation greater than the sum of the standard deviations relative to each CDR3 length in normal blood was considered abnormal. A–F, TCRBV data for 6 of 9 patients who reached the t36 time point. The bars on the left of the triplets represent CD4⁺ cells at t0, the bars on the middle CD4⁺ cells at t12, and the bars on the right CD4⁺ cells at t36. Open bars, normal pattern; solid bars, abnormal pattern.

distribution of the CD8 repertoire can be observed in normal donors, tending to be relatively constant over time, and does not necessarily underlie ongoing immune responses. However, in HIV-infected patients, the perturbations of the CD8⁺ TCRBV repertoire were much more prominent and systematic than in normal controls, suggesting that they were most likely related to the ongoing viral replication.

During 36 months of suppressive therapy, the number of BV families showing gaussian-like profiles increased, passing in CD4⁺ cells from 34 at t0 to 95 at t12 and 82 at t36 and in CD8⁺ cells from 2 at t0 to 20 at t12 and 28 at t36. However, despite the prolonged suppression of viral replication, numerous BV families continued, at this time point, to show oligoclonal CDR3 profiles.

Finally, in selected patients, we sequenced the CDR3 regions of more disrupted CD4 BV families to prospectively follow the evolution of expanded clones. The redundancy of some sequences that showed identical predicted amino acid sequences confirmed the oligoclonal nature of peaks detected by CDR3 spectratyping. In patient 3 (Fig. 4), the most restricted repertoire was observed, at baseline, within the CD4 BV16 family, where the repertoire diversity, expressed as the percentage ratio between the number of different sequences obtained and the total number of sequences, was 63%, compared with 98% observed in the normal control. Also, we did not notice any preferential usage of TCRB joining genes in HIV patients. According to results obtained with CDR3 spectratyping after 36 months of suppressive HAART, we found in

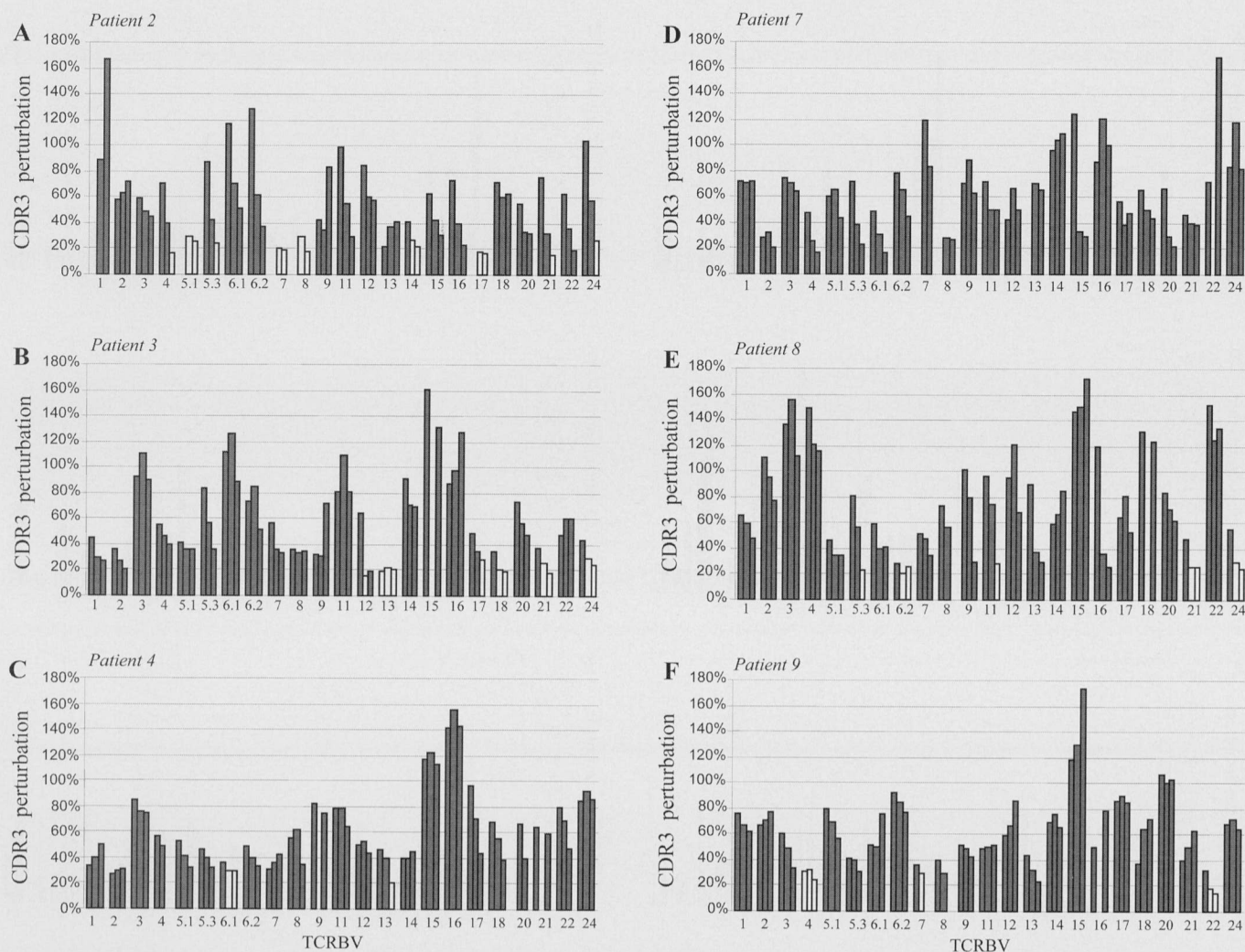


FIGURE 3. Diversity of CD8 TCRBV repertoire analyzed by CDR3 spectratyping. A–F, TCRBV data for 6 of 9 patients who reached the t36 time point. The bars on the left of the triplets represent CD8⁺ cells at t0, the bars on the middle CD8⁺ cells at t12, and the bars on the right CD8⁺ cells at t36. Open bars, normal pattern; solid bars, abnormal pattern.

the CD4 BV16 of patient 3 a general reduction of the size of previously expanded clones. However, we also could demonstrate the appearance of new TCRBV expansions at this time point as well as the further broadening of previously expanded clones.

DISCUSSION

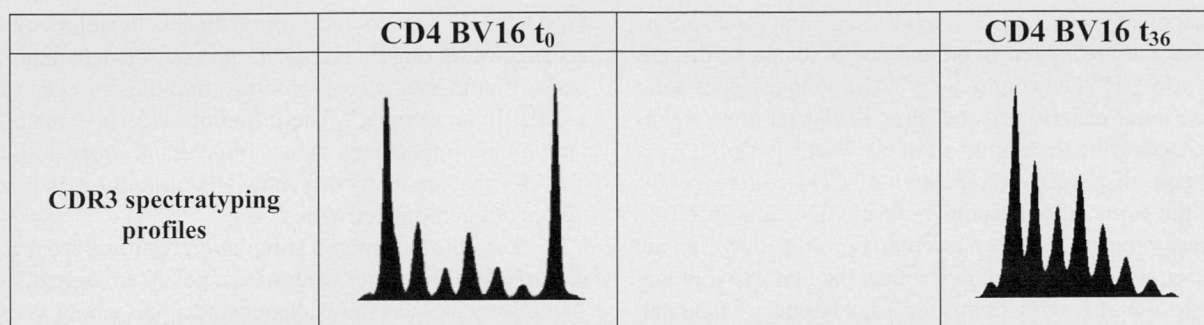
In this report, we analyzed the diversity of the TCRBV repertoire of CD4⁺ and CD8⁺ T cells in a longitudinal study of HAART-treated, HIV-1-infected patients by cytofluorometry, CDR3 spectratyping, and sequencing of the V(D)J region.

Untreated HIV-infected individuals progressively lose their CD4⁺ cells and develop a severe immunodeficiency with the appearance of life-threatening opportunistic infections and finally death. HAART can arrest this almost inexorable evolu-

tion of HIV disease allowing for a gradual immune reconstitution with replenishment of previously lost T-cell pools.³²

The main goal of our study was to characterize the evolution of HIV-induced TCRBV perturbations during HAART to define the extent of immune reconstitution achievable after long-term suppression of viral replication. In fact, it is still unclear whether alterations of TCR repertoires, tightly related to disease severity and progression, may be fully recovered by HAART.

Initial studies on TCRBV repertoires during HIV infection suggested a superantigen-like activity of HIV proteins causing expansions and deletions of CD4⁺ cells bearing selected TCRBV families.^{33–38} This early pathogenetic hypothesis on events leading to CD4⁺ T-cell depletion during HIV infection has been mitigated by subsequent studies showing



CDR3 sequences (t0)	No. clonotypes/total sequences (t0)	CDR3 sequences (t36)	No. clonotypes/total sequences (t36)
CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG CASSQDRR-----RRFFGSG	15/64	CASSQDR-----RRRFFGSG CASSQDR-----RRRFFGSG CASSQDR-----RRRFFGSG	3/70
CASSHDRA-----EQYFGPG CASSHDRA-----EQYFGPG CASSHDRA-----EQYFGPG CASSHDRA-----EQYFGPG	4/64	CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG CASSHDR-----AEQYFGPG	15/70
CASSQERSGA----NVLTFGAG CASSQERSGA----NVLTFGAG CASSQERSGA----NVLTFGAG	3/64	CASSQERSGA----NVLTFGAG	1/70
CASSLS-----GQSPLHFGNG CASSLS-----GQSPLHFGNG	2/64	CASSLSGQ-----SPLHFGNG CASSLSGQ-----SPLHFGNG	2/70

FIGURE 4. TCRBV CDR3 spectratyping profiles and sequence analysis of TCR V(D)J coding joints from CD4 BV16 of a representative patient (patient 3) before (t₀) and after 36 months of therapy (t₃₆). CDR3 sequences detected more than once are presented. At t₀ the repertoire diversity, expressed as the percentage ratio between the number of different sequences obtained and the total number of sequences, was 63%, compared with 98% observed in the normal control (not shown). According to results obtained with CDR3 spectratyping at t₃₆, a general reduction of the size of previously expanded clones was observed (repertoire diversity, 70%). However, a further broadening of previously expanded clones was also detected.

significant changes in the relative BV usage, but no deletions of TCRBV families, even in patients with advanced disease and very low CD4 cell counts.^{31,39–42} Our data are consistent with these latter observations because all BV families we investigated, covering the major part of the TCR repertoire, were expressed in all patients irrespective of CD4 counts, sometimes in the form of expansions both in CD4⁺ and in CD8⁺ cells. These expansions were particularly prominent within the CD8 subset, where they probably reflect the massive immune response to HIV infection. In this regard, it has been calculated that during the acute T-cell response, up to 10% of CD8⁺ T lymphocytes are HIV specific²; meanwhile, during chronic HIV infection, up to 14% of the circulating CD8⁺ T cells would be HIV specific,^{4,43,44} percentages that rise up to 18% in long-term nonprogressors.⁴⁴ However, BV expansions in CD4⁺ cells were mostly observed in patients with more advanced disease, probably as a consequence of lower CD4⁺ cell counts, and were, in general, less extensive. Interestingly, in both CD4⁺ and CD8⁺ cells, the BV expansions were not randomly dispersed through all the repertoires but tended to cluster to limited BV families. As in the present study, the preferential expansion of particular BV families has been repeatedly shown in numerous as well as contrasting reports,^{33–38} but the difficulties in interpreting such different data have precluded, up to now, definitive conclusions on the role of superantigen-versus antigen-driven T-cell expansion and deletion.

Due to the lack of reliable techniques for assessing the antigen specificity of expanded clones, the mechanisms underlying CD4 depletion and skewing of TCR repertoire have yet to be fully elucidated. In particular, it is still unclear whether CD4 depletion occurs by clonal or random deletion of CD4⁺ cells, and there is controversy on the role of clonal expansions versus deletion in determining skewing of TCR repertoire.

However, suppression of viral replication through HAART, along with the production of new naive cells, should result in the generation of more diverse T-cell repertoires. According to this hypothesis, we found a broadening of T-cell repertoires during HAART. However, although disruptions of CD4 and CD8 TCRBV repertoires were mitigated, a normal repertoire was not yet restored after 36 months of fully suppressive therapy. On the contrary, some new expansions, whose oligoclonal nature was demonstrated by the sequence analysis of the CDR3 region, could be detected throughout the study period, both in CD4⁺ and in CD8⁺ T cells.

At present, the mechanisms underlying the partial recovery of the T-cell defects after prolonged suppression of viral load remain to be elucidated. Although a number of hypotheses can be proposed, the more fitting explanation for the incomplete T-cell restoration during HAART would be the persistence of low level of viral replication, Sharkey et al⁴⁵ recently demonstrated, using episome-specific PCR, ongoing virus replication in a large percentage of infected individuals on HAART despite sustained undetectable levels of plasma

viral RNA. This report confirmed the results of previous studies suggesting ongoing replication in aviremic patients on the basis of viral sequence evolution⁴⁶ and the presence of HIV-1-specific transcripts.⁴⁷ These findings clearly explain, in our opinion, the appearance of new oligoclonal expansions during HAART that are likely driven by HIV proteins with newly acquired antigenic specificities.

The chronic antigen stimulation resulting from residual viral replication would determine a persistent upregulation of T-cell activation, as shown in our study, preventing a complete immune restoration. Consistent with this idea, a number of recent findings have suggested that a strong and persistent immune activation would be responsible for the elevated proliferation rate and cell death in HIV infection.⁴⁸ In a study of T-cell dynamics during HAART, Deeks et al⁴⁹ have demonstrated that even a partial virus suppression can reduce CD4⁺ cell turnover and activation, thereby resulting in sustained CD4⁺ cell gains.⁴⁹ In our patients, the number of peripheral CD4⁺ T cells increased throughout the study period, although at a progressively declining rate of growth. The major effect of HAART on the recovery of CD4⁺ T-cell counts occurred in the first 2 years of therapy, with a steep rise in the CD4⁺ T-cell count in the first 3 months followed by a slower, though significant, increase up to the end of the 2nd year. In the 3rd year of therapy, CD4⁺ cell count showed only minor changes approaching a plateau level despite undetectable plasma HIV-1 RNA levels. Interestingly, CD4⁺ T-cell counts, although significantly increased as compared with baseline, remained below the normal value after 3 years of therapy; this raises the question of whether all subjects on HAART will reach normal levels of CD4⁺ T lymphocytes again.

A more detailed analysis of CD4⁺ T-cell repopulation showed a similar rate of growth for both naive and memory subsets. These data indicate that HIV-1 infection affects both naive and memory peripheral blood CD4⁺ T cells to a similar degree and are in good agreement with previous results demonstrating that in untreated HIV-1 infection, the number of both apoptotic and proliferating cells is, within the CD4 population, equally distributed over all subsets.⁴⁸ However, a delay in the HAART-induced recovery of CD4⁺ naive T cells has been also shown.¹³ Previous reports have suggested that the biphasic kinetics of naive and memory T cells repopulation eventually observed in chronically HIV-1-infected patients correlated with the stage of the disease. In fact, it has been demonstrated that the magnitude of recovering naive T cell reflects the numbers of these cells present at baseline.^{48,50} Therefore, in patients with more advanced disease the clone-wise or random deletion of naive T cells and, consequently, the disruption of the TCRBV repertoire might be more difficult to correct than in patients with moderate immunodeficiency.

The preferential decrease in the number of naive CD4⁺ T cells could reflect interference of the virus with T-cell renewal capacity rather than with peripheral production of these

cells.^{51,52} To this regard, we have recently demonstrated in chronic HIV-infected patients an impairment of bone marrow function.⁵³ This was seen in association with increased levels of the cytokine interleukin-7 (IL-7), which is known to be important in both bone marrow and thymic function, stimulating proliferation and inhibiting apoptosis of lineage-restricted progenitor cells.⁵⁴ These data were in agreement with those reported by Napolitano et al⁵⁵ showing increased levels of IL-7 in lymph nodes of CD4⁺ T-cell-depleted patients. However, high levels of IL-7 were also observed by Mussini et al⁵⁶ in a group of HAART-treated patients so-called CD4 exploders characterized by a relevant increase in their CD4 count and by a normalization of T-cell repertoires.

Although, from a clinical point of view, data on changes of TCR repertoires during HAART may appear less influential, they nevertheless elicit noteworthy considerations. Because most HAART-treated patients fail to fully restore T-cell repertoires and numbers despite a prolonged and complete suppression of viral load, the decision on when to start therapy should be carefully evaluated. Starting therapy as soon as possible would not only preserve the anti-HIV cytotoxic activity by preventing the normally occurring phenomena of viral escape and CTL exhaustion but would also limit the erosion of the CD4⁺ T-cell pool as well as the impairment of T-cell renewal capacity.

In addition, the impact of HAART on HIV-associated malignancy remains to be established. Although recent studies indicate that the incidence of Kaposi sarcoma and primary brain lymphoma has decreased,⁵⁷ they suggest that current therapies have not had a proportionate effect on systemic non-Hodgkin lymphoma (NHL).^{58,59} Because major risk factors for HIV-associated NHL are immune deficiency and chronic B-cell stimulation in the absence of effective T-cell control,⁶⁰ the partial HAART-induced recovery of the immune system may be insufficient to prevent systemic NHL.

In conclusion, our data indicate that even in patients starting HAART at moderate levels of immunodeficiency have a fully suppressed viral load, a complete restoration of the diversity of the TCRBV repertoire is unlikely. Therefore, if the goal of the therapy is the *restitutio ad integrum* of the disrupted T-cell pools, this should be started as soon as possible (ie, before the occurrence of those events, such as viral escape, exhaustion of CTL clones, shift CCR5-CXCR4, and thymic failure), impressing a negative turning point to the HIV disease.

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