

Spreading of HIV-Specific CD8⁺ T-Cell Repertoire in Long-Term Nonprogressors and Its Role in the Control of Viral Load and Disease Activity

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ABSTRACT: Long-term non-progressors (LTNP) represent a minority of human immunodeficiency virus (HIV) infected individuals characterized by stable or even increasing CD4⁺ T-cell count and by stronger immune responses against HIV than progressors. In this study, HIV-specific effector CD8⁺ T cells, as detected by both a sensitive *ex vivo* enzyme-linked immunospot (ELISPOT) assay and specific major histocompatibility complex (MHC) peptide tetramers, were at a low frequency in the peripheral blood of LTNP, and recognized a lower number of HIV peptides than their memory resting cell counterparts. Both factors may account for the lack of complete HIV clearance by LTNP, who could control the viral spread, and displayed a higher magnitude of cytotoxic T lymphocyte (CTL) responses than progressors. By combining cell purification and ELISPOT assays this study

demonstrates that both effector and memory resting cells were confined to a CD8⁺ population with memory CD45RO⁺ phenotype, with the former being CD28[−] and the latter CD28⁺. Longitudinal studies highlighted a relatively stable HIV-specific effector repertoire, viremia, and CD4⁺ T-cell counts, which were all correlated with maintenance of nonprogressor status. In conclusion, the analysis of HIV-specific cellular responses in these individuals may help define clear correlates of protective immunity in HIV infection. *Human Immunology* 62, 561–576 (2001). © American Society for Histocompatibility and Immunogenetics, 2001. Published by Elsevier Science Inc.

KEYWORDS: HIV type-1; long-term nonprogressors; CD8⁺ T lymphocytes; ELISPOT; HLA-A2.1 tetramers

ABBREVIATIONS

CTL cytotoxic T lymphocytes
HIV human immunodeficiency virus
MHC major histocompatibility complex
LTNP long-term nonprogressors
ELISPOT enzyme-linked immunospot
LDA limiting dilution analysis
HAART highly active antiretroviral therapy
HBV Hepatitis B virus
P BMC peripheral blood mononuclear cells
EBV Epstein-Barr virus

HCV Hepatitis C virus
BSP BirA substrate peptide
β₂-m β₂-microglobulin
PE phycoerythrin
IFN interferon
APC antigen-presenting cells
IL interleukin
HLA human leucocyte antigens
CD8_{ME} memory effector CD8⁺ T cells
CD8_{MR} memory resting CD8 T cells

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INTRODUCTION

Cytotoxic T lymphocytes (CTL) are known to play a central role in the immune response against the vast majority of viruses [1]. Although their participation in human immunodeficiency virus (HIV) infection is still not completely elucidated, several studies suggest that CTL play a crucial role in controlling HIV infection [2–4]. In agreement with these observations, a recent study based on the newly developed major histocompatibility complex (MHC) peptide tetramer technology, reported an inverse correlation between HIV-specific CTL frequency and plasma viral load [5]. Strong CTL responses versus HIV peptides have been detected in infected patients, and perhaps the most intriguing feature of cell-mediated immunity to HIV is that the CTL response can be maintained for long periods of time in long-term nonprogressors (LTNP) with relatively low levels of plasma viremia [6–11]. Low levels of HIV load and replication in peripheral blood have been demonstrated to be the strongest correlates of nonprogression in a cohort of LTNP hemophiliacs [12]. Also, associations of human MHC genes with HIV disease progression have been reported [13, 14], indirectly implying an antiviral effect of HIV-specific immunity. Unfortunately, after several years of infection, a failure of the CTL response occurs and a shift from the nonprogressor to the progressor status is observed [15]. At this stage of the disease a high level of HIV replication along a steeper decrease of circulating CD4⁺ T cells results in the severe impairment of the immune system. Death usually occurs by the establishment of opportunistic infections. However, what causes CTL responses to be unable to eliminate HIV is unclear, although several strategies of immune evasion have been proposed [16–19].

To better elucidate the role of CTL in the control of HIV infection, HIV-specific, CD8⁺ T-cell responses in a cohort of LTNP and progressors were studied. Using several different analytic methods, T cells were tested for their ability to recognize a panel of well-defined, HLA-A2 and -A3 supertype restricted HIV peptides [20]. First, they were tested for their capability to perform specific cytotoxic function in a classical ⁵¹Chromium (⁵¹Cr) release assay. Second, responses of *in vivo* activated CD8⁺ T cells, capable of rapid effector function within few hours of contact, were analyzed with class I binding HIV peptides and without previous restimulation or addition of a particular cytokine *in vitro*. To accomplish the latter, we have used a sensitive *ex vivo* enzyme-linked immunospot (ELISPOT) assay, allowing the enumeration of individual cytokine-secreting T cells, which are generally too rare to be detected by conventional limiting dilution analysis (LDA) [21–23]. Finally,

HLA-A2 tetrameric molecules were also utilized to detect T cells specific for HIV peptides [5, 23–27]. Techniques, such as MHC tetramers and ELISPOT, because of their greater sensitivity compared to CTL assay or to LDA, have greatly enhanced our understanding of the complex interplay between CTL responses and the dynamics of viral infections in a variety of different experimental and disease systems [21, 23, 25, 28].

Our results illustrate how sensitive strategies based on these techniques can be utilized to investigate the mechanisms involved in the immune control of HIV infection.

MATERIAL AND METHODS

Study Populations

Ten LTNP patients were selected on the basis of the following criteria. Patients had been infected for 7 or more years, presented no HIV-related symptoms during this period, had never received any antiretroviral treatment, were in healthy condition, and had their CD4⁺ T-cell count persistently higher than 500 cells/ μ l of blood (Table 1). We defined these patients LTNP because, at study entry, they had documented seropositivities ranging from 9 to 15 years. Later on, two patients (patients 6 and 10) progressed from the LTNP condition to seroconversion after 15 and 13 years, respectively, and initiated HAART (zidovudine, lamivudine, and indinavir) shortly after the second analysis of HIV-specific CD8⁺ T-cell activities (Table 1). Moreover, seven progressors (Table 1), as well as 20 healthy donors (not shown), were chosen as a control in our study. Notably, progressors 1 and 2, although illustrating some clinical characteristics that resembled those of LTNP, were included in the progressor category because they, as well as the typical progressors 3 and 4, were started on HAART (zidovudine, lamivudine, and indinavir or efavirenz) shortly after the first study of their HIV-specific CD8⁺ T cells, as a result of persistently elevated viremia (Table 1). On the contrary, progressors 6 and 7 were studied (in 1998) even though they were already under therapy with zidovudine and lamivudine, since 1992 and 1993, respectively. None of the 20 healthy donors, used as controls, had clinical or biologic signs of HIV, hepatitis B virus (HBV), hepatitis C virus (HCV) infections, nor were they exposed to the common risk factors for these viral infections. HIV-RNA was measured in plasma samples by HIV-1 Amplicor Monitor assay (Roche Diagnostic Systems, Milan, Italy), with a sensitivity of 400 copies of HIV-RNA/ml.

Cell Preparation

Peripheral blood mononuclear cells (PBMC) were isolated on Lymphoprep cushions (LSM, Organon Teknika

TABLE 1 Clinical characteristics of LTNP and progressors

Patients	Age (years)/sex	1st Anti-HIV ⁺ (year)	CDC group ^a	CD4 ⁺ T cells/ μ l					HIV-RNA copies/ml				
				1995	1996	1997	1998	1999	1995	1996	1997	1998	1999
LTNP													
1	38/M	1986		932	956	993^{a,b}	775	839	<400	<400	<400^a	<400	<400
2	40/M	1987		808	870	697^a	938	568	5700	7700	2500^a	4100	ND
3	52/M	1985		809	651	762	543	729^a	3900	5000	6700	7500	12,000^a
4	35/F	1988		ND	1236	1203^a	986	ND	<400	<400	<400^a	<400	<400
5	22/M	1984		1135	739	700^a	650	769	686	ND	1580^a	2685	960
6	35/M	1983		600^a	517	308	160^c	174	3147^a	9831	17,700	159,664^c	<400
7	31/M	1984		1047	972	1146	907^a	608	9867	ND	21,368	29,765^a	ND
8	28/M	1983		ND	669^a	ND	ND	ND	ND	<200^a	ND	ND	ND
9	47/M	1985		619	585	642	609^a	731	ND	4675	5380	3977^a	11,300
10	20/M	1985		630^a	394	470	362^c	311	419^a	ND	5955	28,903^c	89,200
Progressors													
1	34/M	1992	A1	1325	1648	695^{a,c}	1127	1006	6000	93,000	62,000^{a,c}	<400	ND
2	41/M	1994	A1	1510	1496	1206	1120	1024^{a,c}	4000	5800	33,000	66,000	180,000^{a,c}
3	24/M	1983	C2	139^{a,c}	105	178	143	173	64,145^{a,c}	162,100	31,660	64,900	100,000
4	29/M	1985	B2	127^{a,c}	14	NA ^e	NA ^e	NA ^e	667,123^{a,c}	176,946	NA ^e	NA ^e	NA ^e
5	40/M	1990	B1	ND	ND	ND	ND	170^a	ND	ND	ND	ND	81,293^a
6 ^d	39/F	1990	A2	384	ND	ND	1031^a	ND	ND	ND	ND	1000^a	ND
7 ^d	37/M	1992	B2	400	ND	ND	707^a	ND	ND	ND	ND	650^a	ND

^a At study entry.^b CD4 cell count and HIV-RNA determinations performed at the time of HIV-specific CD8⁺ T-cell studies are indicated in bold.^c Antiretroviral therapy started shortly after these CD4/HIV-RNA determinations.^d Patients studied 5 years after HAART started.^e NA = not applicable (death in 1997).

ND = not determined.

Corp., Durham, NC, USA) and frozen in liquid nitrogen. Then, cells from each vial were serially thawed and tested for either ELISPOT assay, cytotoxicity assay, or tetramer assays. CD8⁺ T cells were isolated by immunomagnetic separation with anti-CD8 monoclonal antibody attached to Dynabeads (Dyna, Oslo, Norway), as previously described [20, 29, 30]. Briefly, bead-coated cells (positively selected cells) were removed by a magnet (Dyna) and detached from magnetic beads by incubation with Detachabead (Dyna) according to the manufacturers' instructions. The purity of the positively selected CD8⁺ T cells displayed was routinely higher than 98%, as detected by flow cytometry. In some experiments, either CD45RO⁺, CD45RA⁺, or CD28⁺ populations were positively isolated by incubating PBMC with anti-CD45RO (IgG2a, UCHL1; Pharmingen, San Diego, CA, USA), anti-CD45RA (IgG2b, HI100; Pharmingen), or anti-CD28 monoclonal antibodies (IgG1, CD28.2; Pharmingen), respectively. Cells were incubated for 30 min on ice, washed four times, and then incubated with goat antimouse IgG mAb attached to Dynabeads. Purification of positively selected CD45RO⁺, CD45RA⁺, CD28⁺ cells, and CD28-depleted population were confirmed by flow cytometry analysis.

Synthetic Peptides and HLA-A2/HIV Peptide Tetramers

Peptides were synthesized as previously described [20], purified to >95% homogeneity by reverse-phase high-performance liquid chromatography, and analyzed by amino acid analysis and/or mass spectrometry analysis. MHC molecules were purified from appropriate Epstein-Barr virus (EBV) transformed B-cell lines by affinity chromatography as previously described [20]. Quantitative assays for binding of peptides to soluble class I molecules based on the inhibition of binding of a radio-labeled standard probe peptide to detergent solubilized MHC molecules have been described previously [20]. In the inhibition assays, peptide inhibitors were typically tested at concentrations ranging from 120 μ g/ml to 1.2 ng/ml. The data were then plotted and the dose yielding 50% inhibition was measured. Peptides were tested in two to four completely independent experiments.

Peptides refolded with MHC class I molecules were obtained in tetrameric complexes in our laboratory, as previously described [5, 24]. Briefly, purified HLA-A2 molecules, containing the BirA substrate peptide (BSP) for enzymatic biotinylation and β_2 -microglobulin (β_2 -m) were produced in a prokaryotic expression system

[31]. Heavy chain, β_2 -m and peptide of interest were refolded by the dilution method [32]. The refolded product was first concentrated and then incubated with recombinant BirA in the presence of biotin (Avidity, Denver, CO, USA). The biotinylated complex was purified by gel filtration followed by ion-exchange chromatography. Lastly, extravidin-phycoerythrin (PE) conjugate (Sigma, St. Louis, MO, USA) was added at 1:4 molar ratio and tetrameric complexes concentrated to 1 mg/ml.

ELISPOT Assay

Interferon (IFN)- γ secreting effector T cells were detected at levels of highly purified peripheral CD8⁺ T cells by ELISPOT, as previously described [20]. Briefly, 96-well nitrocellulose-backed plates (MAHA S4510; Millipore, Bedford, MA, USA) were coated with 5 μ g/ml of mouse antihuman IFN- γ monoclonal antibody (Pharmingen). Plates were washed with PBS supplemented with 0.25% Tween 20 (PBS/0.25% Tween20; Sigma) and blocked with PBS/10% human serum (HS). Purified CD8⁺ T cells were deposited in the well in duplicate at 5 and 2.5×10^4 cells/well, and incubated in the presence of irradiated (3000 rads) autologous CD8-depleted PBMC or autologous EBV-B cells (12000 rads) used as antigen-presenting cells (APC) (2×10^5 /well), which were previously pulsed or not with peptide (10 μ g/ml). After 4- to 6-h incubation at 37°C, plates were washed with PBS/0.25% Tween 20, and 100 μ l of 2- μ g/ml biotinylated mouse antihuman IFN- γ (Pharmingen) was added to the well. After 2-h incubation at room temperature, plates were washed five times, 50- μ l streptavidin horseradish peroxidase (dilution 1:500; Pharmingen) was added to the wells, and the plates were incubated for a further 90 min at room temperature. Colorimetric reaction was obtained using aminoethyl carbazole solution (Sigma) as a substrate. Spots were quantified using the AID ELISPOT Reader (AID GmbH, Strassberg, Germany), a computer-based system allowing the semiautomatic interpretation of ELISPOT microtiter plates. The spots were quantitated by three parameters: spot intensity (measured in brightness units), overall spot surface (three-dimensional size, as measured in square millimeters), and ratio between spot surface and spot area (two-dimensional size). Once these parameters were set up, eliminating background or artifacts, the counting software quantitated the number of spots in the well, and the data were exported for analysis.

In order to detect HIV-specific responses by memory resting T cells, ELISPOT assays were performed with short-term CD8⁺ T-cell lines. Briefly, fresh CD8⁺ T cells (5×10^4 /well) were incubated with the single HLA-A2 or -A3 binding HIV peptides (10 μ g/ml) in the presence of autologous irradiated CD8-depleted PBMC, as APC (10^5 /well). After 5 days, cultures were supple-

mented with 50 U/ml human recombinant interleukin (rIL)-2 (Proleukin; Eurocetus, Emeryville, CA, USA). After an additional 5-day culture, 50-U/ml rIL-2 was added, and on day 15 the cells were tested in an antigen-specific ELISPOT assay. In particular, autologous irradiated PBMC samples, used as APC, were pulsed with the different peptides (10 μ g/ml) for 2 h. Pulsed or non-pulsed APC were then plated onto precoated plates (5×10^4 /well), in the presence of 5×10^4 T cells from each peptide-stimulated culture.

⁵¹Cr-release Assay and LDA

CTL assay was performed upon two rounds of antigen stimulation *in vitro*, as previously described [20, 30]. Briefly, PBMC were plated into 200 μ l RPMI-10% HS onto 96-well round-bottomed plates (2×10^5 /well) in the presence of HIV peptides (10 μ g/ml, with at least three wells for each peptide). After 5 days of culture, 50-U/ml rIL-2 (Proleukin; Eurocetus) was added and, after another 5 days, cells were restimulated with autologous irradiated PBMC (5×10^4 /well), peptide, and IL-2. Then, 50-U/ml rIL-2 was seeded 5 days later, and after another 5-day culture, each sample was tested in CTL-specific assay, using ⁵¹Cr-labeled allogeneic as targets, HLA-matched EBV-B cells pulsed or not with the relevant peptide. LDA was carried out setting up replicate microcultures (12 wells for each peptide) in 96-well round-bottomed plates with whole PBMC plated in 200- μ l RPMI-10%HS at dilutions ranging from 12,500 to 200,000 cells/well, using the same procedures of antigen-stimulation described above.

Tetramer Cell Staining

PBMC (10^6 cells) samples were incubated with 1- μ g/ml tetramer solution at 37°C for 15 min. Cells were washed twice with FACS buffer (PBS one time, 2% FCS, 0.01% sodium azide) and incubated with both mouse antihuman CD8 tricolor monoclonal antibody (Caltag Laboratories, San Francisco, CA, USA) and fluoresceinated mouse antihuman leucocyte antigens-DR (Becton Dickinson, San Jose, CA, USA) at 4°C for 30 min. After incubation cells were washed twice with FACS buffer, acquired with a FACScan flow cytometer (Becton Dickinson), and analyzed using CellQuest software (Becton Dickinson).

Statistical Analysis

Statistical analysis of data was performed by the Student *t*-test for independent samples. Statistical significance of $p < 0.05$ was assumed.

TABLE 2 HLA class I binding capacity of potential HIV epitopes

Potential A2 supertype epitopes					
Protein	1st position	Sequence	Sequence conservancy ^a	A2 binding (IC ₅₀ nM)	Alleles bound
POL	306	YTAFTIPSI	93	26.3	5
POL	764	MASDFNLPPV	68	62.5	5
GAG	397	VLAEMSQV	74	66.6	5
POL	175	CTLNFPISPI	100	147.0	5
NEF	158	LTFGWCFKL	74	294.1	5
NEF	158*	LTFGWCFKL	74	35.7	5
ENV	731	LLQLTVWGI	63	9.8	4
POL	438	KLVGKLNWA	95	59.5	4
ENV	134	KLTPLCVTL	95	102.0	4
VPR	59	AIIRILQQI	74	333.3	4
POL	156	LVGPTPVNI	100	454.5	4
VPR	62	RILQQLLFI	74	19.2	3
POL	176	TLNFPISPI	100	75.7	3
GAG	282	MTNNPIPIV	89	166.6	3
POL	125	KMIGGIGGFI	95	172.4	3
POL	476	ILKEPVHGV	79	192.3	3
POL	762	RAMASDFNL	79	217.3	3
POL	869	KAACWWAGI	79	277.7	3
Potential A2 supertype epitopes					
Protein	1st position	Sequence	Sequence conservancy ^a	A3/A11 binding (IC ₅₀ nM)	Alleles bound
GAG	174	MVHQAI SPR	58	61.1/89.6	5
POL	921	AVFIHNFKR	100	64.7/3.3	5
POL	714	YLA WVP AHK	95	142.9/105.3	5
POL	961	KIQNFRVYYR	95	343.8/28.6	5
POL	919	QMAVFIHNFK	100	9.2/8.5	5
GAG	173	QMVHQAI SPR	58	423.1/6000	4
POL	343	MTKILEPFR	84	13750/375	4
POL	337	AIFQSSMTK	79	10/10	3
NEF	94	QVPLRPMTYK	79	18/9.5	3
ENV	49	TVYYGVPVWK	95	11/3.5	3
POL	92	TIKIGGQLK	63	40.7/181.8	3
ENV	63	TLFCASDAK	84	118.3/11.3	3
ENV	62	TTLFCASDAK	84	119.6/27.3	3
POL	91	VTIKIGGQLK	63	297.3/28.6	3
POL	236	NTPVFAIKK	94.7	333.3/100	3
POL	920	MAVFIHNFK	100	647.1/20	3

^a Conservation in clade B isolates, based on analysis of 1999 Los Alamos HIV molecular immunology database.

* Represents?

RESULTS

Selection of Potential HLA-A2- and HLA-A3-Supertype HIV Peptides

HIV peptides with totally conserved sequences among different isolates present in the Los Alamos database ($\geq 79\%$) [2] that contain HLA binding motifs were identified, synthesized, and tested for their class I binding capacity, as previously described [20] (Table 2). The binding data is presented as 50% inhibitory nanomolar concentration (IC₅₀) nanomolar values, peptides with binding affinity ≤ 50 nM are classified as “good” bind-

ers, and peptides in the 50–500 nM range are classified as “intermediate” binders. Peptides that are good or intermediate binders for the common HLA class I alleles HLA-*0201, -*0301, or -*1101 are shown in Table 2. The capacity of these peptides to bind other molecules included in either the HLA-A2-supertype (A*0201, A*0202, A*0203, A*0206, A*6802) or the HLA-A3-supertype (A*0301, A*1101, A*3101, A*3301, A*6801) were measured again in order to indicate cross-binding capacity [20]. All peptides selected for this study were characterized by their capacity to bind three

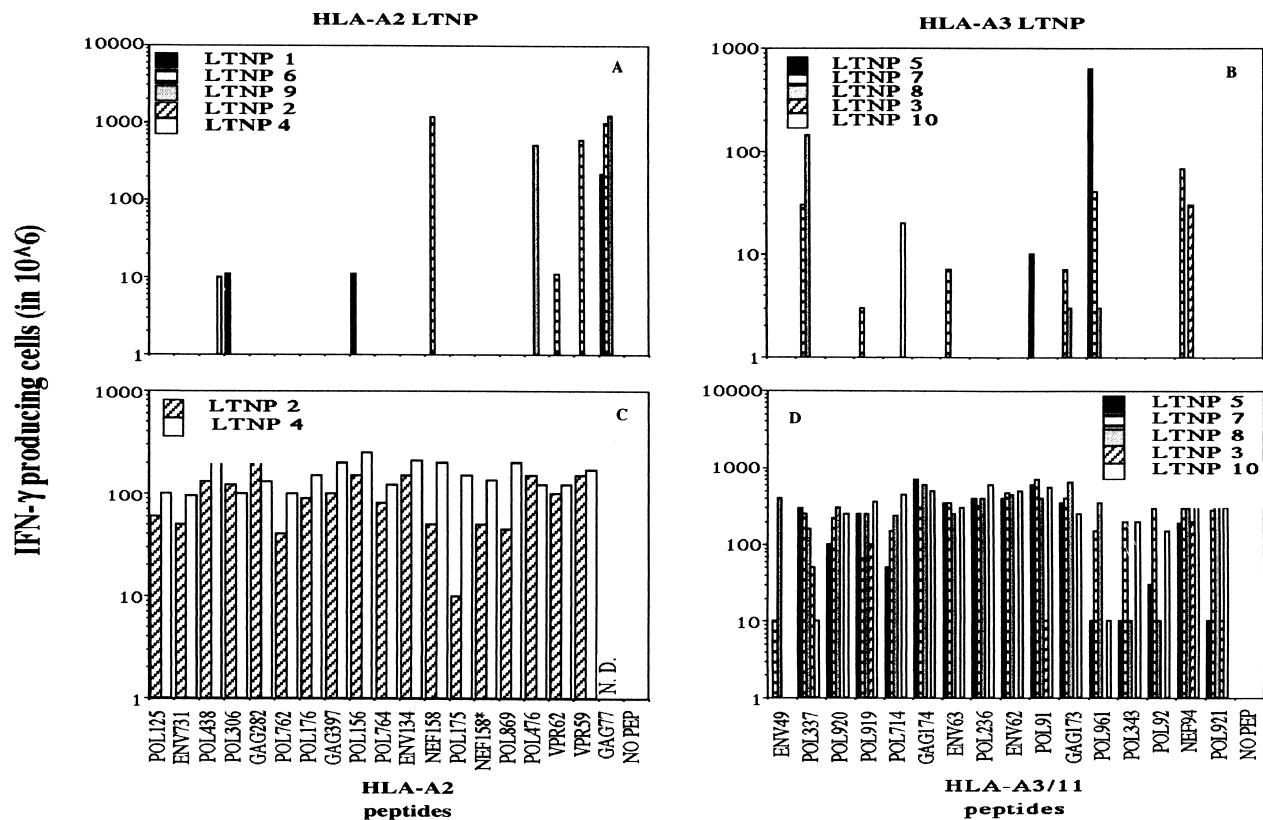


FIGURE 1 Comparison between HIV peptide-specific CD8_{ME} and CD8_{MR} responses in LTNP. Panels A and B illustrate CD8_{ME} responses were assessed by ELISPOT assay for the capability to form IFN- γ spots within 6 h of contact with HIV peptides presented by autologous APC. For panels C and D, CD8_{MR} responses and CD8⁺ T cells were previously stimulated with peptides presented by autologous APC and IL-2. Then, T-cell lines were tested in the ELISPOT assay. The CD8_{MR} responses were detected only in two of five HLA-A2 patients for lacking of sufficient number of PBMC. Each bar represents the value of a single patient. Data represent mean of duplicate 2.5 and 1.25×10^5 cells/well determinations. Because no spot was detected in the absence of the single peptides for each cell line, only one bar representing the values without peptide is shown.

of five most common molecules of the relevant HLA supertype. In addition, the gag₍₇₇₋₈₅₎ peptide (SLYNT VATL) was tested because of its known immunodominance in HIV-infected individuals [5].

HIV-Specific Effector CD8⁺ T Cells

Low frequencies of CD8⁺ T cells displaying prompt effector function in response to at least one HIV peptide were detected in the peripheral blood of nine of ten LTNP (Figure 1A and 1B), but only in three of seven progressors (Figure 2A). Effectors from LTNP responded

to a higher number of peptides than progressors ($p = 0.038$). The effector function of these cells was underlined by the fact that a significant number of these cells formed spots within 6 h of contact with HIV peptides presented by autologous APC, without previous antigen restimulation or IL-2 addition *in vitro* [20]. The effectors, as well as the memory resting cells, were confined in any case in the CD8⁺ population, because only highly purified CD8⁺ T cells were used in the ELISPOT assay. Furthermore, CD4⁺ cells formed only negligible HIV-specific spots in response to the different HIV peptides (not shown). No CD8_{MR} spot was obtained in response to irrelevant HLA-A2 or A3 binder HCV peptides (NS4₁₈₅₁₋₁₈₆₀: ILAGYGAGV; core₁₃₂₋₁₄₀: DLMGYI PLV) in the study populations (not shown). Interestingly, the majority of the responding cells was CD8⁺ CD45RO⁺ CD28⁻, a phenotype recently related to memory CD8⁺ T cells with effector function (CD8_{ME}) [20, 33]. Indeed, cells forming highest amounts of IFN- γ spots within 6 h of contact with HIV peptide were predominantly represented in the highly-purified CD8⁺, CD45RO⁺, or CD28⁻ populations (Figure 3A). In contrast, no substantial response was observed when the CD28⁻ cell subset was previously stimulated with peptides and IL-2, and then tested in the ELISPOT assay

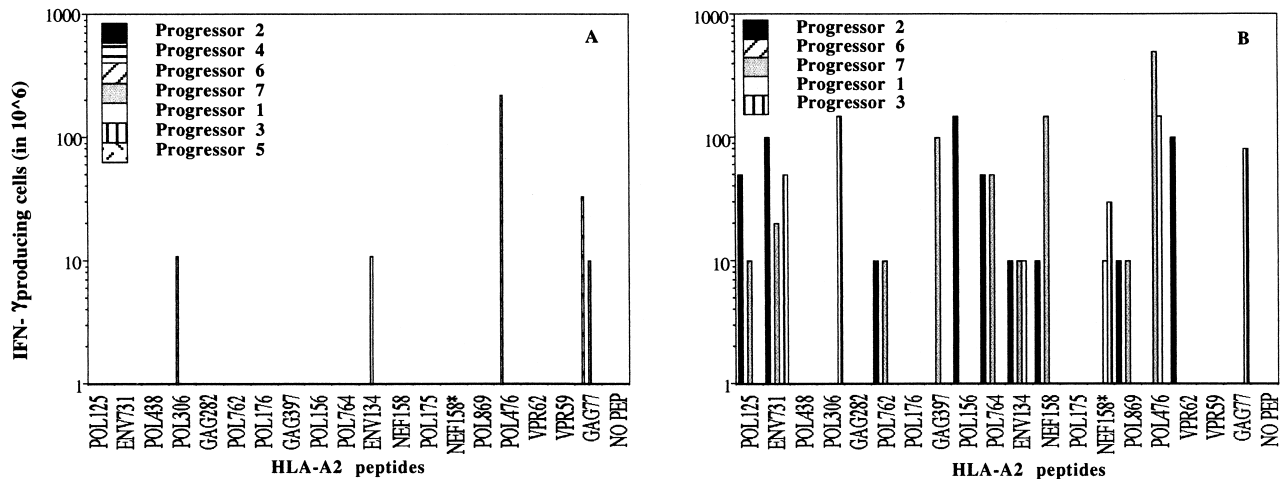


FIGURE 2 Comparison between HIV peptide-specific CD8_{ME} and CD8_{MR} responses in progressor patients. Both HIV-specific CD8_{ME} (A) and CD8_{MR} (B) responses in seven progressors are shown. Each bar represents the value of a single patient. Data represents mean values of duplicate determinations. Because no spots were detected in the absence of peptides for each cell line, only one bar representing the values without peptide is shown.

(Figure 3B) [34]. No correlation was found between the frequency of HIV-specific CD8_{ME} cells, as detected by ELISPOT, and either the levels of plasma viremia or the CD4⁺ T-cell number in both LTNP and progressors (not shown). None of the purified CD8⁺ T cells from 20 healthy controls studied under identical conditions formed IFN- γ spots (not shown).

HIV-Specific Memory Resting CD8⁺ T Cells

To detect memory resting responses by ELISPOT assay, freshly-isolated CD8⁺ T cells were previously expanded by one round of stimulation with autologous APC, peptide and IL-2. The resulting short-term T-cell lines were then tested in the ELISPOT assay to enumerate HIV-specific, cytokine-producing cells. This analysis was performed in seven of ten LTNP and five of seven progressors whose PBMC number was not a limiting factor. Strong memory resting CD8⁺ T-cell responses (CD8_{MR}) directed against the majority of HIV peptides (Figures 1C and 1D) were detected in all seven LTNP, whereas the response of four of five progressors tested was directed against a more limited number of peptides ($p = 0.001$) (Figure 2B). The finding that the number of IFN- γ spots to a nonspecific stimulus, such as PHA (not shown) was virtually similar between LTNP and progressors, rules out the possibility that PBMC from the latter may die quicker than those of LTNP during culture. Notably, the memory resting CTL from LTNP re-

sponded to much higher numbers of HIV peptides than those recognized by the memory effectors ($p < 0.001$) (Figure 1). These cells were defined as "memory resting" because phenotypic analysis revealed that they were confined to a CD45RO⁺ CD28⁺ population, which formed spots only after antigen restimulation and IL-2 addition *in vitro* (Figure 3B). Indeed, cells forming spots upon a previous antigen-stimulation *in vitro* were predominantly contained in both the highly-purified CD45RO⁺ and CD28⁺ populations (Figure 3B).

HIV-Specific CTL Assay

Antigen-specific cytotoxic function was tested in the LTNP and progressor subgroups, whose peripheral blood was tested by ELISPOT, as described above, for the presence of HIV-specific CD8_{MR} cells. Again, LTNP showed significantly stronger cytotoxicity values ($p = 0.035$) against a broader panel of peptides ($p = 0.049$) than progressors (Figure 4). The finding that the majority of the cytotoxic responses was directed to HIV epitopes also recognized by CD8_{MR} cells forming IFN- γ spots, which further supports the antigen-specific activities detected in our tests. No correlation was found between the responsiveness of HIV-specific CD8_{MR} cells, as detected by ELISPOT, and either the levels of plasma HIV-RNA or the number of CD4⁺ T cells in both LTNP and progressors (not shown). The CD8⁺ T cells derived from 20 healthy controls tested did not respond against any of the HIV peptides when tested directly *ex vivo* or upon antigen restimulation *in vitro* (not shown).

Quantitation of HLA-A2/HIV-Specific T Cells by Using HLA-Tetramers

Next, we constructed tetramers of HLA-A2 molecules [24], refolded with the β_2 -m and either HIVgag(77-85: SLYNTVATL), HIVpol(438-446: KLVGKLNWA), or HIVnef(158-166: LTFGWCFKL) peptides, to directly

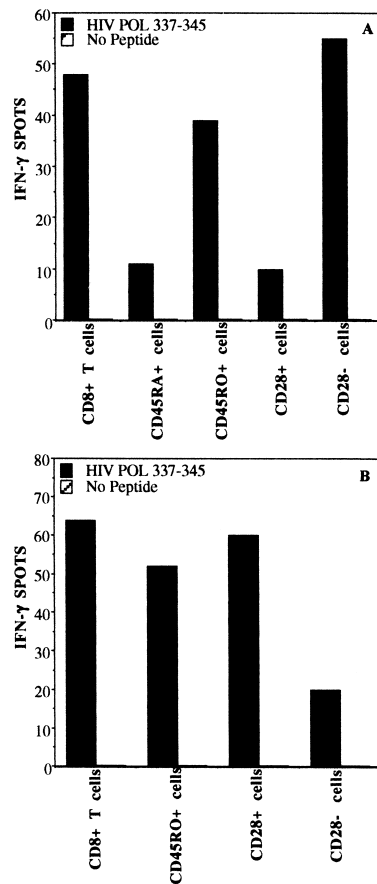


FIGURE 3 The majority of HIV-specific CD8⁺ T cells performing either prompt effector or recall responses *in vitro* are both confined into a memory CD45RO⁺ population with either a CD28⁺ or CD28⁺ phenotype, respectively. CD45RO⁺, CD45RA⁺, or CD28⁺ cells purified by immunomagnetic separation (as described in Materials and Methods), as well as PBMC depleted from the CD28⁺ cells (CD28⁻ cells), were tested in ELISPOT (5×10^5 /well) for their capability to form spots within 6 h in response to the HLA-A3 binding HIVpol(337-345) peptide presented by autologous APC (A), or upon previous stimulation with peptide in IL-2 conditioned medium for 10 days (B). FACS analysis demonstrated >95% of purity in the positively-sorted cells (CD45RO⁺, CD45RA⁺, or CD28⁺ cells), and about 80% depletion of CD28⁺ cells in CD28-depleted PBMC. Data represents means of duplicate determinations. Reproducible results were obtained in three different patients.

enumerate *ex vivo* HIV-specific CD8⁺ T cells freshly-isolated from PBMC. In preliminary experiments, the tetramer binding specificity was verified by the staining of CD8⁺ T-cell lines specific for relevant HIV peptides, previously derived from PBMC of chronically HIV-infected patients (not shown). In the series of experiments, summarized in Table 3, PBMC from 11 patients (6 HLA-A2⁺ LTNP and 5 HLA-A2⁺ progressors) were double-stained with PE-tetramer/HIV peptide com-

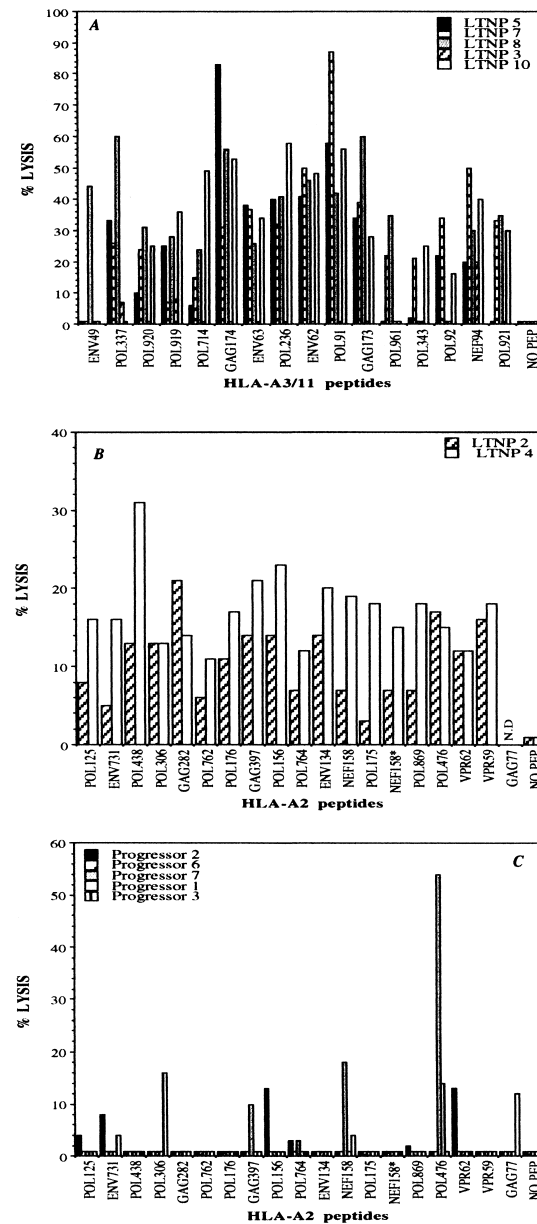


TABLE 3 HIV-specific CD8⁺ T-cell frequencies enumerated by ELISPOT, tetramers, or LDA in HLA-A2⁺ LTNP or progressors

Patients	Date of sample	Gag	Gag	Gag	Nef	Nef	Nef	Nef	Pol	Pol	Pol	Pol
		77-85	77-85	77-85	158-166	158-166	158-166	158-166	438-446	438-446	438-446	438-446
		ELISPOT	tetramer	tetramer-DR	LDA ^a	ELISPOT	tetramer	tetramer-DR	LDA	ELISPOT	tetramer	tetramer-DR
LTNP												
1	11-04-97	1/4545	1/1500	Negative	ND	Negative	1/500	Negative	ND	Negative	Negative	Negative
1	12-10-98	1/4545	1/1430	ND	ND	Negative	ND	ND	ND	Negative	ND	ND
2	11-04-97	Negative	ND	ND	ND	Negative	ND	ND	ND	Negative	ND	ND
4	05-03-97	Negative	Negative	Negative	ND	Negative	Negative	Negative	ND	1/100000	Negative	Negative
6	07-11-96	1/1000	1/280	1/5000	ND	Negative	Negative	Negative	ND	Negative	1/2500	Negative
6	21-12-98	1/2500	1/200	1/2500	ND	1/60000	1/6670	1/25900	ND	Negative	Negative	Negative
9	14-12-98	1/1000	1/126	ND	ND	Negative	Negative	ND	ND	Negative	ND	ND
9	04-06-99	1/10000	1/300	1/3333	ND	ND	Negative	Negative	ND	Negative	ND	ND
Progressors												
1	12-11-97	Negative	Negative	Negative	Negative	Negative	1/8330	Negative	Negative	Negative	ND	Negative
1	12-10-98	Negative	ND	ND	Negative	Negative	ND	ND	Negative	Negative	ND	Negative
2	12-11-97	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative	Negative	Negative
2	03-03-99	Negative	Negative	Negative	Negative	Negative	ND	ND	Negative	Negative	1/10000	Negative
3	07-11-96	1/100000	1/6670	ND	1/280000	Negative	1/6670	1/4950	Negative	Negative	Negative	Negative
3	30-11-98	1/100000	1/7500	Negative	1/370000	Negative	1/6670	ND	Negative	Negative	Negative	Negative
3	10-06-99	Negative	Negative	Negative	ND	1/33000	1/3300	1/25500	Negative	Negative	1/50000	Negative
4	28-03-95	Negative	Negative	Negative	ND	Negative	ND	ND	Negative	Neg	ND	ND
6	09-12-98	1/33000	1/1666	ND	Negative	Negative	ND	ND	Negative	Negative	ND	Negative
7	14-01-99	Negative	ND	ND	Negative	Negative	Negative	Negative	ND	Negative	ND	Negative

^a For LDA, cells were considered positive when specific lysis was 15% above background.

ND = not determined; Negative = frequencies undetectable by either ELISPOT, or tetramer, or LDA.

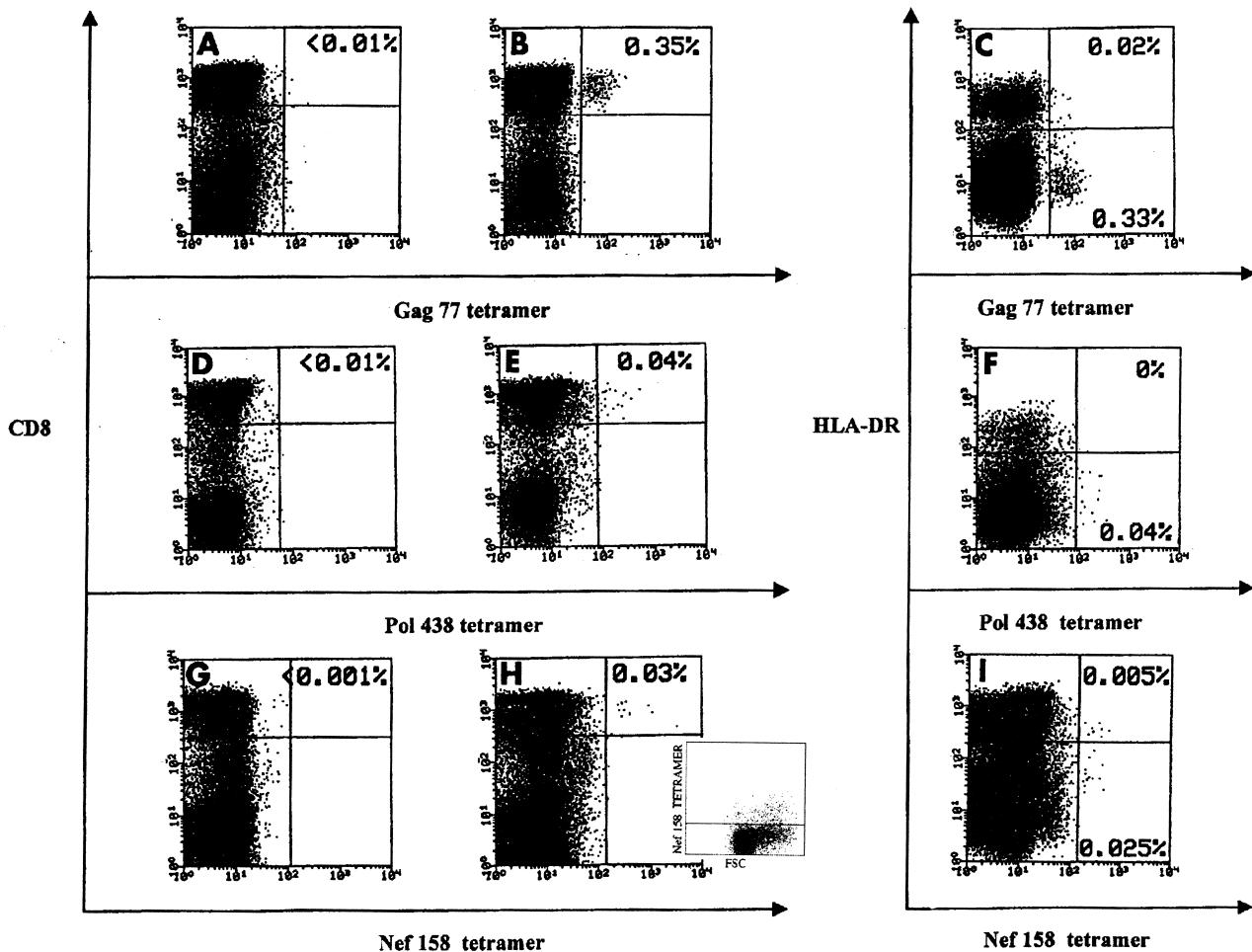


FIGURE 5 Staining of HIV-specific CD8⁺ T cells with tetramers of HLA-A2.1 molecules complexed with different HIV peptides. PBMC from a representative HLA-A2.1⁺ LTNP were stained with mAbs specific for CD8 and tetramers of HLA-A2 molecules, refolded with the β_2 -m and either HIV_{gag}(77-85) (B), HIV_{pol}(438-446) (E), or HIV_{nef}(158-166) peptide (H). Cells were also stained with anti-HLA-DR mAb and gag- (C), or pol- (F), or nef-tetramer (I). HLA-A2.1⁺ PBMC from healthy individuals (A, D, and G) and HLA-A2.1⁺ PBMC from LTNP (not shown), tested as controls for each staining, were constantly negative for each tetramer. The representative experiment into the inset shows that nef-specific cells, although hardly detectable in fresh PBMC, became well detectable with the corresponding tetramer and anti-CD8 mAb after *in vitro* peptide-stimulation of the same PBMC. Same results were obtained with the gag- or the pol-specific CD8⁺ T-cell lines (not shown).

cutoff between specific and nonspecific T-cell responses. As predicted, tetramers detected more HIV-specific cells in LTNP than in progressors (Table 3). Cells positive for triple staining with tetramer/HIV peptide, anti-CD8, and anti-HLA-DR antibodies (activated cells) represented only a minority of the entire HIV-specific T-cell

population, as detected with tetramer and anti-CD8 monoclonal antibody alone (Table 3). Furthermore, it should also be noted that the frequency of ELISPOT CD8_{ME} correlated more closely with the frequency of tetramer-positive, activated/effector cells rather than with that of the total tetramer-positive population (Table 3). These observations support the notion that effector cells are present at relatively low frequency in both LTNP and progressors. However, the frequencies of HIV-specific CD8⁺ T cells, as detected by both tetramers and ELISPOT, were severalfold higher than those detected by LDA in all individuals tested (Table 3). This may be related to the lower sensitivity of the LDA assays, which detects only precursors capable of *in vitro* proliferation and differentiation into effectors, but does not detect nonproliferating effector T cells [21]. Finally, once again, no correlation was found between the frequency of HIV-specific CD8⁺ T cells, as detected by tetramers, and either the plasma viremia or the number of circulating CD4⁺ T cells in both LTNP and progressors (not shown). None of the 20 control PBMC (10 randomly

selected HLA-A2⁺ HIV-infected patients with a clinical status comparable with the HLA-A2⁺ study population, and 10 HLA-A2⁺ healthy individuals) was positive for staining for the tetramers, nor was HIV-PBMC colored by control tetramers expressing HCV peptide (not shown).

Longitudinal Studies

In order to probe the potential role of CTL in control HIV infection, we correlated the frequencies of HIV-specific CD8⁺ T cells, as detected by ELISPOT or tetramers, with the natural evolution of disease in the context of a given individual during a long-term follow-up. The parameters taken into consideration were HIV load, CD4⁺ T-cell number, CD8⁺ T-cell responses. Interestingly, in the majority of LTNP (patients 1, 5, 6, and 10), the frequencies and the breadth of HIV-specific CD8_{ME} cell responses, as detected by ELISPOT, tended to be positively related to the levels of viremia and inversely to the number of CD4⁺ T cells (Figure 6). In particular, LTNP 5 and 1 demonstrated a spreading of the effector responses, in concomitance with relatively stable parameters over the course of the study (Figure 6), and maintained their LTNP condition for more than 1 year after the last analysis (Figure 6 and Table 1). On the contrary, LTNP 6 and 10, showing moderate alterations of clinical parameters since the beginning of the follow-up (Table 1), presented severe erosion of CD4⁺ T-cell number and increase of viremia, in concomitance with either a spreading of the effector responses or an increase of the pol-specific cell frequency alone, respectively (Figure 6). Both these patients switched from LTNP to the progressor state and began to take antiretrovirals following our last CD8⁺ T cell response analysis (Table 1).

These results were further supported by tetramer analysis performed in the HLA-A2⁺ patients followed in our study. Indeed, LTNP 6, who underwent the progressor state, exhibited an increase of both HIV_{gag}(77-85)- and HIV_{nef}(158-166)-specific CD8⁺ T-cell frequencies, which were related to an important increase of viremia and to a drastic abatement of the number of CD4⁺ T cells (Figure 7). On the contrary, the steady-state of the HIV_{gag}(77-85)-specific CD8⁺ T-cell frequency shown in LTNP 1, belonging to the group with stable LTNP progression, was related to a slight increase of viremia and to a negligible decrease of CD4⁺ T-cell number (Figure 7). Only one patient (LTNP 9), 6 months after the first determination, had a slight increase of both viremia and CD4⁺ T-cell count, concomitant to the decreased frequencies of both HIV_{pol}(476-484)- and HIV_{gag}(77-85)-specific CD8⁺ T cells, as detected by both ELISPOT (Figure 6) and tetramer (Figure 7) analyses. Further follow-up of this individual is planned to extend

the correlation between viremia and CD8⁺ T-cell responses.

It is also interesting that two controls, studied before and during the antiretroviral therapy, exhibited divergent CTL response behaviors according to the different response to the therapy. In particular, we followed the progressor 1, who presented clinical parameters similar to those of LTNP, but, however, started antiretroviral therapy because of a sustained increase of viremia (Table 1). This patient responded well to the therapy (Table 1), revealing a monospecific CD8⁺ T-cell response against the HIV_{env}(134-142) epitope, as detected by ELISPOT, which became undetectable after 11 months from the start of therapy, and was associated with significant reductions in viremia and preservation of the CD4⁺ T-cell population (Figure 6). On the contrary, the typical progressor 3 (Table 1) mounted a sustained response of HIV_{gag}(77-85)-specific CD8_{ME} cells, as detected by both ELISPOT (Figure 6) and tetramer (Figure 7) assays, and a widening of CD8⁺ T-cell repertoire after 2 years from the start of therapy. In particular, he exhibited a sustained high viremia and very low CD4⁺ T-cell number even before the therapy was started, which remained constant or even decreased further after 1 year, in association with loss of the HIV_{gag}-specific response (Figures 6 and 7), while displaying a higher level of CTL multispecific response (Figure 6).

DISCUSSION

The data presented in this study illustrates two main points. First, LTNP, although displaying a higher magnitude of CTL responses than progressors, revealed low frequencies of HIV-specific CD8_{ME} and a CD8_{MR} repertoire that recognized a much larger panel of peptides than CD8_{ME} cells. Altogether these findings may account for the LTNP capability of controlling viral spreading, but, on the other hand, are not fully HIV-infected cells. It is tempting to hypothesize that the establishment of viral persistence in LTNP, in spite of the CTL control, may take place in part as a result of insufficient numbers of effectors arising from the HIV-specific, memory resting T-cell pool. This view is supported by recent observations presenting that the resolution of several viral infections is related to the presence of high numbers of effectors; capable of killing infected cells and releasing antiviral cytokines, while chronic infections are related to a drastic abatement of the effector T-cell pool [5, 22-27, 35-37]. The majority of CD8_{MR}, which formed IFN- γ spots, were also efficient antigen-specific cytotoxic cells, suggesting that both direct killing of infected cells and a potential bystander effect of antiviral cytokines may contribute to suppress HIV propagation [20, 38, 39]. Because CD8_{ME} cells

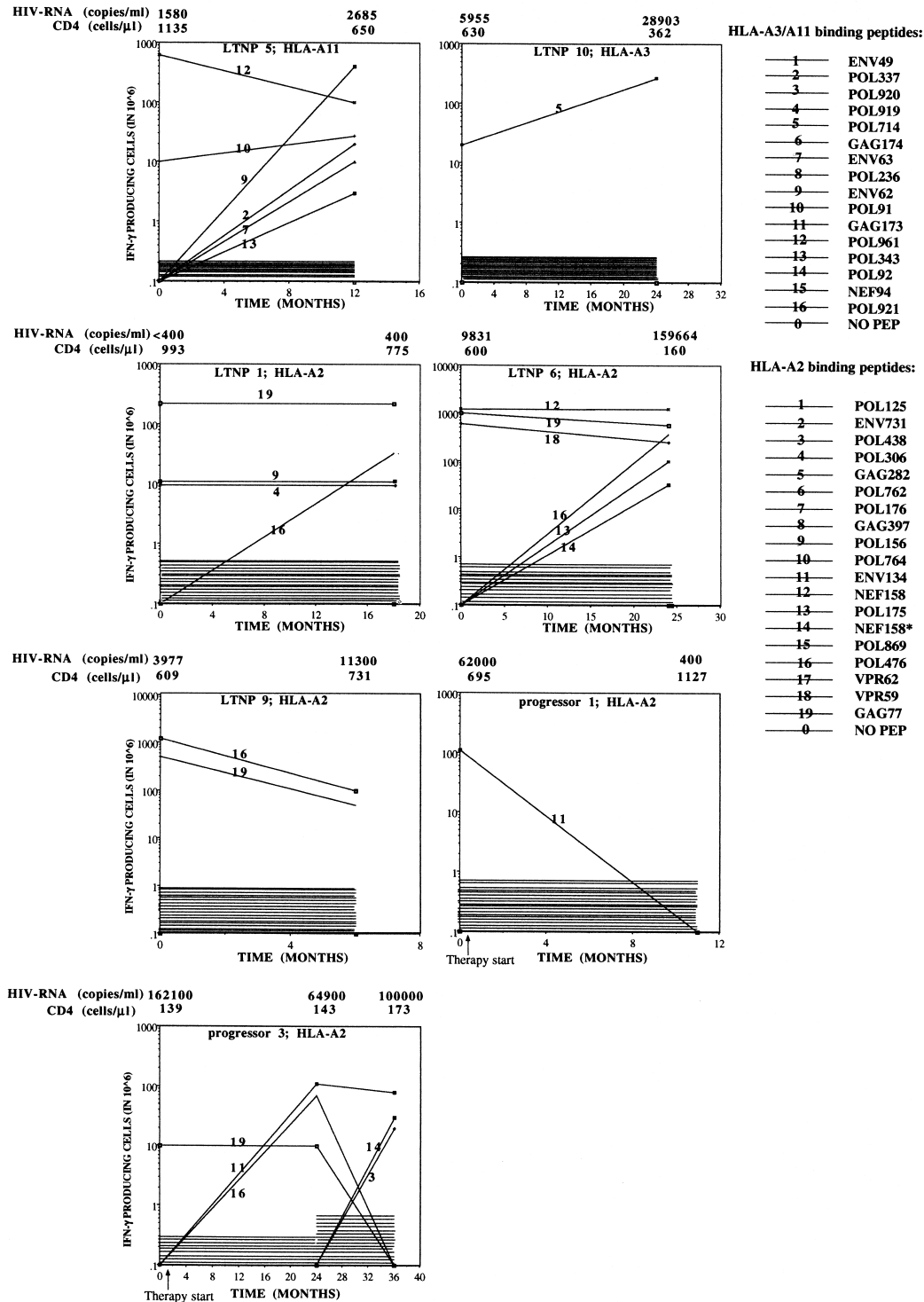


FIGURE 6 Longitudinal study of frequencies of HIV-specific effector CD8⁺ T cells, as detected by ELISPOT. Results of CD8⁺ T cell responses obtained at the time of the first determination and at the time, in which they were related to significant variations of viremia and/or CD4⁺ T-cell count are shown in four LTNP (Patients 5, 10, 1, 6). Patients 5 and 1 remained in the LTNP category more than 1 year from the last CD8⁺ T-cell response determinations, whereas Patients 10

and 6 underwent the progressor state and the necessity of antiretroviral therapy, soon after the last HIV-specific T-cell determinations. Also, two progressor patients (Patients 1 and 3) were studied, as controls, before and during antiretroviral therapy. Only detectable responses are signed, each with a number corresponding to a single peptide, although negative responses are all grouped below the value 1 of the x-axis.

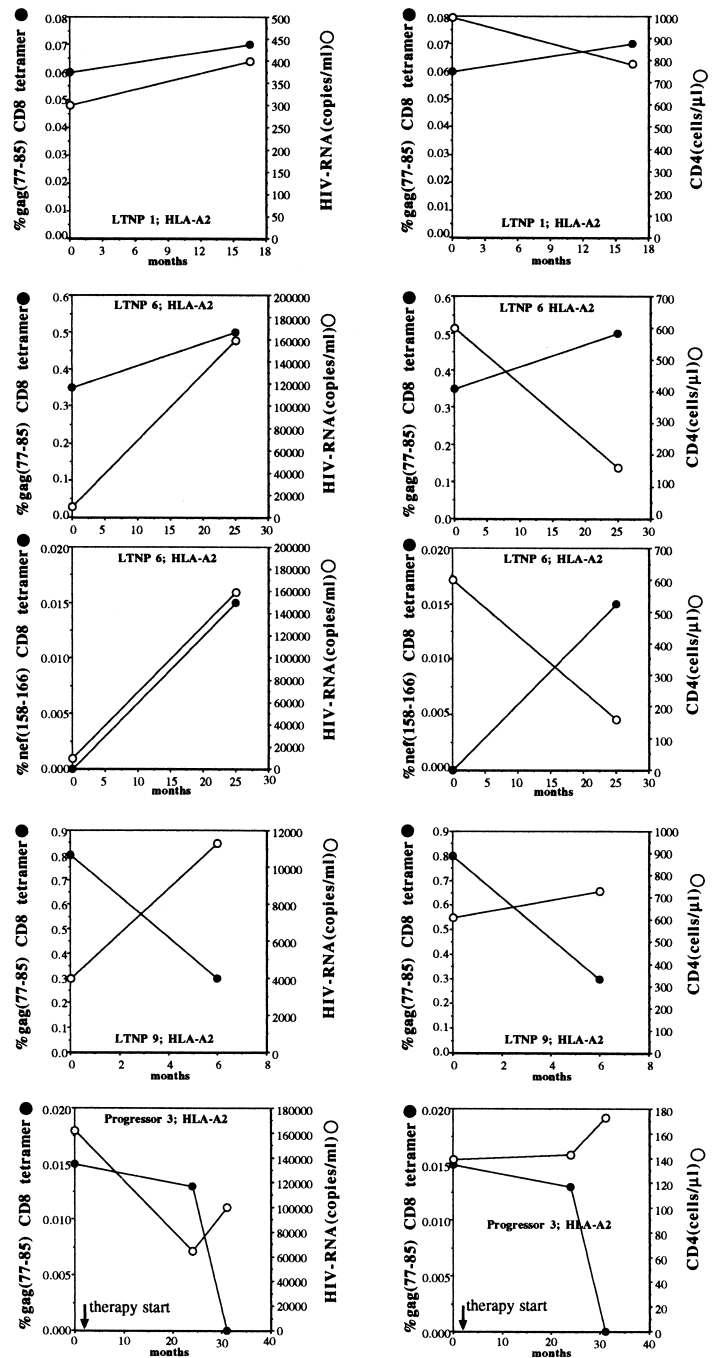


FIGURE 7 Longitudinal study of frequencies of HIV-specific CD8⁺ T cells, as detected by HLA-A2/HIV tetramers. Four representative patients have been followed for the CD8⁺ T-cell frequencies detected by tetramers, which confirmed results obtained by ELISPOT (Figure 6). LTNP 1 with a steady-state among viremia, CD4⁺ T-cell number and gag-specific CD8⁺ T-cell frequency, maintained the LTNP status more than 1 year after the last immunologic determinations. LTNP 6 showing a drastic abatement of CD4⁺ T cells, and a concomitant increase of both viremia and frequencies of gag- and nef-specific cells, underwent progressor state. Patient 9 is the only LTNP showing the frequency of the HIV-specific CD8⁺ T cells inversely related to viremia. The tetramer study in progressor 3, not responding to the antiretroviral therapy, confirmed that HIVgag₍₇₇₋₈₅₎-specific CD8⁺ T-cell effectors disappeared, in concomitance with the increased viremia and a sustained low CD4⁺ T-cell number. However, the lack of a large panel of tetramers did not allow to determine the spreading of HIV-specific cell responses, as detected by ELISPOT (see Figure 6).

required neither restimulation nor the addition of particular cytokines *in vitro* for performing their functions, they should be capable of HIV-specific effector function *in vivo*, possibly providing antiviral immunity [5, 22, 23, 25]. These are bona fide memory effector cells because they belonged to a CD8⁺ CD45RO⁺ CD28⁻ population, phenotype recently related to memory CD8⁺ T cells with effector function [20, 33]. In contrast, CD8_{MR} cells were confined to a CD45RO⁺ CD28⁺ population,

and exploited effector functions only upon antigen-induced proliferation *in vitro* [20, 33, 34].

The second main observation of this study is that no correlation was found between CD8⁺ T-cell frequencies, as detected by ELISPOT or tetramers, and the levels of either plasma HIV-RNA or peripheral CD4⁺ T cells, both in LTNP and in progressors, when crosssections were examined. In this regard, previous studies presented an inverse correlation between HIV-specific CD8⁺ T-

cell frequencies and viral load in primary infection or asymptomatic patients [3, 5, 40], whereas others did not [41–43]. One possible explanation of these discrepancies may lie in the fact that CD8⁺ T cells, even if sharing the same specificities and studied with similar assays, may control the virus with different efficiencies in different phases of disease. Furthermore, the steady-state between viral load and the immune response established in LTNP may hinder detection of an inverse correlation between these parameters. The possible presence of undetected viral reservoirs [44, 45] may further complicate accurate measures of correlations between viremia and CTL functions.

In contrast, in a longitudinal study of some LTNP, the number of effector CD8⁺ T cells recognizing multiple peptides, as detected by both ELISPOT and tetramer assays, tended to be positively related to the levels of viremia and inversely related to the number of circulating CD4⁺ T cells in all (patients 1, 5, 6 and 10) but one (patient 9) LTNP studied. Although the number of LTNP followed in the longitudinal analysis was not sufficient to allow statistical analysis, these data are in apparent contrast to previous reports, indicating that the survival after HIV infection is dependent on an inverse correlation between CTL strength and viral loads [3, 5, 40]. This may further explain the fact that CTL responses were capable of controlling HIV replication, but not clearing the virus, in our LTNP, which illustrates a constant equilibrium among CTL responses, HIV load, and CD4⁺ T-cell number. Therefore, we suggest that recurrent waves of new CTL responses are generated in LTNP in order to control the HIV spreading and the possible rising of viral variants, which would in turn be generated to escape from the strong CTL pressure [46, 47], and so on. However, in the long run, an unbalance of this homeostatic state may occur, allowing the possibility that the protective immune responses become dangerous. Indeed, in the advanced stages of HIV infection, the virus-specific CTL, on the one hand, may lose the capacity of controlling the viral spread, and may, on the other hand, participate in the progressive CD4⁺ T-cell depletion [2–5, 8, 10, 11, 15]. This hypothesis is supported by the finding that LTNP 6 and 10, exhibiting lower CD4⁺ T-cell numbers than those observed in the other patients since the beginning of the follow-up, presented a notable worsening of the clinical parameters, although the CD8⁺ T-cell responses, as detected by ELISPOT or tetramer analyses, were as a consequence of the increased viral load. In this situation, the antiviral response may assume immunopathologic features associated with the development of the progressor state and the requirement of antiretroviral therapy.

Furthermore, the antiretroviral therapy could supply beneficial effects according to the clinical status of treated patients. Indeed, they were evident in progressor

1, revealing a moderate alteration of CD4⁺ T-cell number, but not in progressor 3, who exhibited a very low CD4⁺ T-cell number and a very high viremia before therapy began.

In conclusion, our results suggest that LTNP are capable of controlling disease progression because of their higher CTL responses than progressors. Moreover, we propose that the inability of LTNP to clear HIV may be, in part, dependent on an insufficient generation of effectors from the HIV-specific CD8_{MR} pool. This defect may be caused by the failure of CD4⁺ T cells, which represents the most important immunologic characteristic of HIV-infected patients [16]. In the long run, this situation may allow the establishment of distinctive marks of immunopathology and the shift to the progressor status, as observed in some of our LTNP during the long-term follow-up.

Finally, the study of HIV-specific effector T cells in LTNP may provide an important rationale for the design of innovative vaccine priming for antigen-specific CTL responses. In this respect it is worth noting that all epitopes used in the present study were with totally conserved sequences among different isolates, and, therefore, should induce effective immunity against most HIV isolates. Because of their broad crossreactivity among different HLA molecules, they should also provide effective coverage of patient populations of different ethnic backgrounds.

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