Weak anti-HIV CD8+ T-cell effector activity in HIV primary infection

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HIV-specific CD8+ T cells play a major role in the control of virus during HIV primary infection (PI) but do not completely prevent viral replication. We used IFN-γ enzyme-linked immunospot assay and intracellular staining to characterize the ex vivo CD8+ T-cell responses to a large variety of HIV epitopic peptides in 24 subjects with early HIV PI. We observed HIV-specific responses in 71% of subjects. Gag and Nef peptides were more frequently recognized than Env and Pol peptides. The number of peptides recognized was low (median 2, range 0–6). In contrast, a much broader response was observed in 30 asymptomatic subjects with chronic infection: all were responders with a median of 5 peptides recognized (range 1–13). The frequency of HIV-specific CD8+ T cells among PBMC for a given peptide was of the same order of magnitude in both groups. The proportion of HIV-specific CD8+CD28– terminally differentiated T cells was much lower in PI than at the chronic stage of infection. The weakness of the immune response during HIV PI could partially account for the failure to control HIV. These findings have potential importance for defining immunotherapeutic strategies and establishing the goals for effective vaccination.

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Introduction

HIV primary infection (PI) is characterized by an intense viral replication and the subsequent induction of an immune response (1, 2). The cellular immune response appears to be the main effector involved in controlling viral replication at this stage, and several studies have reported that the rapid induction of an HIV-specific, cytotoxic T lymphocyte (CTL) response occurs at the same time as the decline in viral titers (3, 4). Direct evidence of the role of CD8+ T cells in controlling viral replication in vivo was provided recently in the primary simian immunodeficiency virus (SIV) infection of Rhesus macaques (5). However, the immune response fails to eliminate the virus from the host during acute infection, which is then followed by persistent viral replication and chronic HIV infection.

The reasons for this incomplete control of viral replication are not clearly understood. A steady-state is gradually achieved when the production of new viral particles is offset by the control of infected cells. Whether this control results either from the cytolytic destruction of infected cells or from the noncytolytic suppressive antiviral activity of CD8+ T cells is not clearly established (6). The resulting viral replication then influences the prognosis of HIV infection (7). The factors that determine this virologic set point may be linked to quantitative or qualitative differences in the immune response (8).

The mobilization of a broad CD8+ T-cell repertoire early in HIV PI is associated with slower progression of disease. This suggests that the ability to recruit a wide spectrum of HIV-specific CTL responses during PI leads to better control of viral replication (8). However, even in these subjects, the viral load usually remains readily detectable in the absence of therapy, despite the persistence of a broad and intense CD8+ T-cell response. This suggests that the induction of the cellular immune response has been inadequate in most subjects. Thus, a thorough analysis of the immune response during early HIV PI is essential for a clear understanding of the mechanisms responsible for its lack of effectiveness.

Therefore, we characterized the HIV-specific CD8+ T-cell effector response in 24 subjects during HIV-1 PI using IFN-γ enzyme-linked immunospot (ELISPOT) assay and intracellular staining. We evaluated the breadth of the CD8+ T-cell repertoire induced by using a wide variety of optimum HLA class-I epitopic peptides derived from the sequences of the *env*, *gag*, *pol*, and *nef* genes of HIV-1 as stimulator antigens. The antiviral repertoire of each subject was defined by the multispecificity of the response (which was assessed as the number

of epitopic peptides recognized) and the response intensity (assessed as the frequency of IFN-γ–secreting cells). Expression of CD28 on HIV-specific $CD8^+$ T cells was also analyzed. These values were then compared with those obtained for asymptomatic subjects during the chronic stage of infection.

Methods

Study population. We studied 24 subjects included in the multicenter French PRIMO cohort. The study was approved by the local Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (Paris, France).

After giving informed consent, subjects were included whether the HIV PI was symptomatic or not. The HIV-1 PI was diagnosed by (a) an incomplete Western blot (i.e., a lack of anti-p68 and anti-p34 antibodies), or (b) a positive p24 antigenemia with a negative or indeterminate ELISA or negative Western blot. The date of infection was estimated using the date of the onset of symptoms minus 15 days, or, in case of asymptomatic PI, the date of incomplete Western blot minus 1 month. No subject had been given any antiretroviral therapy when included in the study.

For comparison, 30 untreated, asymptomatic HIV-seropositive individuals in the chronic stage of infection were also studied.

Cells and peptides. PBMC were isolated by density gradient centrifugation (Ficoll-Paque; Pharmacia Biotech AB, Uppsala, Sweden). Subjects were HLA-typed by genotype analysis (ACTGene, Evry, France). The sequences of HIV-1 LAI epitopes used may be consulted on the National Institutes of Health (Bethesda, Maryland, USA) HIV molecular immunology database (9). Peptides were synthesized by Neosystem (Strasbourg, France) and supplied by the Agence Nationale de Recherches sur le SIDA (Paris, France). Lyophilized peptides were diluted to 1 mg/mL in water plus 10% DMSO, aliquoted, and then stored at –20°C. They were used at a final concentration of 1 μg/mL.

ELISPOT assay. IFN-γ secretion by virus-specific $CD8^+$ T cells was quantitated by ELISPOT assay. Ninety-six–well nitrocellulose plates (Millipore Corp., Bedford, Massachusetts, USA) were coated with 1 μg/mL capture mouse anti-human IFN-γ mAb (Mabtech AB, Nacka, Sweden). PBMC were plated in triplicate at serial dilutions (3×10^5 to 10^4 cells per well). Appropriate stimuli were then added, and the plates were incubated for 20 hours at 37°C and 5% CO_2. Wells were then washed, filled with 100 μL biotinylated mouse monoclonal anti-human IFN-γ (Mabtech), and then filled with alkaline phosphatase–labeled extravidin (Sigma Chemical Co., St. Louis, Missouri, USA). Spots were developed by adding chromogenic alkaline phosphatase conjugate substrate (Bio-Rad Laboratories Inc., Hercules, California, USA). Colored spots were counted using a stereomicroscope. Positive controls consisted of 6 wells containing 300–1,000 cells with 50 ng/mL PMA and 500 ng/mL ionomycin. Negative controls consisted of cells cultured in medium alone or with irrelevant peptides (10). In most experiments, negative controls yielded 0–1 spot per well. In experimental wells, the signal was considered positive if (a) the number of spots was greater than the mean ± 3 standard deviations observed with the negative controls, and (b) the number of spots obtained was proportional to the number of plated cells. Frequencies of IFN-γ spot-forming cells (SFC) were then calculated after subtracting negative control values.

Cell surface labeling and flow cytometry. The FACS analyses were performed using a FACScan cytofluorometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) after staining of whole blood samples with the following mAb's: FITC-labeled anti-CD8 mAb and phycoerythrin (PE)-labeled anti-CD38, -CD28, -CD45RO, or –HLA-DR mAb's (all from Becton Dickinson Immunocytometry Systems).

Intracellular staining of IFN-γ. Cells (1.8×10^6) were cultured for 6 hours in 300 μL complete medium with 10 μg/mL brefeldin A in the presence of various stimuli. Several HIV, Ebstein-Barr virus (EBV), and influenza peptides were tested for each individual. For some subjects, autologous EBV-transformed B-lymphoblastoid cell line (B-LCL), which were infected with recombinant vaccinia viruses expressing the *env* or *pol* HIV-1 LAI genes, were used as stimulators, as described previously (10). Positive control consisted of activation with 25 ng/mL PMA and 1 μg/mL ionomycin. Negative controls consisted of medium alone, peptides that were negative in the ELISPOT assay, or autologous B-LCL infected with the wild-type vaccinia virus.

Table 1
Clinical and biological characteristics of the 24 subjects with primary HIV infection

Subject	Age	Symptoms[A]	Duration of symptoms (days)	Estimated time from infection[B] (days)	T-cell counts[C]		Log plasma HIV RNA[D]
					CD4	CD8	
HIL	66	++	8	24	217	308	6.0
KKK	51	++	10	26	329	785	6.5
BKC	45	++	21	32	439	2,136	5.9
DOX	47	++	17	34	156	751	5.9
NUY	40	+++	36	34	582	1,197	5.9
COH	22	++	12	35	292	569	5.3
EOD	27	0		35	548	973	5.5
KXO	34	+	5	35	954	744	3.0
LEU	28	++	7	37	734	1,205	4.8
FQQ	25	+++	7	39	788	913	4.6
ADB	29	+++	15	44	550	596	4.0
OVU	43	+++	30	44	783	910	2.7
OUI	38	++	30	46	637	5,712	5.8
BRI	49	0		52	600	537	5.2
KOA	35	+	7	57	945	703	4.5
OFP	43	0		57	453	821	4.7
ASS	28	0		58	624	761	3.8
IRO	28	+++	20	60	696	1,183	5.6
GVT	26	++	16	61	1,240	4,883	5.2
KVG	25	++	15	63	600	1,340	4.7
AWA	31	++	10	66	1,004	939	3.3
GFR	28	++	8	83	554	1,224	4.9
LLV	49	++	15	93	521	1,587	5.7
KJA	26	0		97	760	615	3.5
Median	33		15	45	600	910	4.9

[A]The classification of symptom severity is based on the number of different symptoms harbored by the patients: 1–3: +; 4–6: ++; ≥7: +++. The following suggestive symptoms of acute HIV-1 infection were considered: fever, fatigue, rash, headache, lymphadenopathy, pharyngitis, myalgia or arthralgia, nausea, vomiting or diarrhea, oral and genital ulcers. [B]See Methods for calculation of the estimated date of infection. [C]Values are given per microliter. [D]Values are given in $\log_{10}$ of viral titers in copies per milliliter.

Table 2
Characteristics of the ex vivo functional repertoire of antiviral CD8+ T lymphocytes in the 24 subjects with primary infection

Subject	HLA	No. of peptides positive/tested					positive	Nb SFC[B]		
		Env	Gag	Pol	Nef	Total[A]	peptides (%)	Mean[C]	Range[D]	Total[E]
HIL	A1/24 B8/18	1/3	1/2	0/1	1/4	**3/10**	30	1,233	400–2,200	3,700
KKK	A11/24 B8/15	0/2	0/6	0/4	2/4	**2/16**	13	340	30–650	680
BKC	A2/28 B18/44	0/1	0/2	0/4	0/1	**0/8**	0	.		
DOX	A2/23 B44/49	0/1	0/2	0/3	0/1	**0/7**	0	.		
NUY	A3/25 B8/51	0/3	2/6	0/2	1/2	**3/13**	23	328	25–560	985
COH	A2/2 B8/–	0/2	1/3	0/4	1/2	**2/11**	18	1130	380–1,880	2,260
EOD	A2/28 B8/49	0/1	1/3	0/5	1/3	**2/12**	16	365	30–700	730
KXO	A2/24 B22/40	0/2	0/1	0/5	1/2	**1/10**	10	420	420	420
LEU	A2/68 B35/51	0/1	1/2	3/5	0/4	**4/12**	33	338	30–1,200	1,350
FQQ	A1/26 B35/63	0/0	0/1	0/1	0/3	**0/5**	0	.		
ADB	A2/23 B40/44	0/1	0/2	0/4	0/1	**0/8**	0	.		
OVU	A1/24 B27/57	0/2	1/2	0/1	1/4	**2/9**	22	65	30–100	130
OUI	A2/3 B35/60	0/2	0/5	0/6	1/6	**1/19**	5	1,500	1,500	1,500
BRI	A29/66 B35/60	0/0	0/1	1/1	0/3	**1/5**	20	25	25	25
KOA	A2/33 B7/14	1/3	1/1	0/3	1/3	**3/10**	30	533	30–1,500	1,600
OFP	A30/33 B45/53	0/0	1/1	0/0	0/1	**1/2**	50	800	800	800
ASS	A2/3 B7/18	1/4	1/4	0/4	1/4	**3/16**	18	617	200–1,300	1,850
IRO	A1/2 B44/57	0/1	0/2	0/4	0/2	**0/9**	0	.		
GVT	A2/24 B51/–	0/2	0/1	0/5	0/3	**0/11**	0	.		
KVG	A1/24 B8/35	1/2	1/5	1/3	3/7	**6/17**	35	425	10–1,000	2,550
AWA	A1/– B8/52	0/1	1/3	0/1	1/3	**2/8**	25	550	100–1,000	1,100
GFR	A1/24 B18/37	0/1	1/1	0/1	1/2	**2/5**	40	765	540–990	1,530
LLV	A2/23 B7/49	0/3	0/1	0/3	0/3	**0/10**	0	.		
KJA	A26/30 B8/63	0/1	3/4	0/1	2/2	**5/8**	63	1,550	215–3,630	7,752

[A]Number of peptides recognized by a given individual per number of peptides tested for this individual. [B]Number of IFN-γ SFC per 10^6 PBMC expressed as mean[C] and range[D] for each individual. [E]Total intensity defined for each subject as the sum of the numbers of SFC per 10^6 PBMC for all viral peptides recognized.

Cells were stained directly in the culture tubes with 15 μL anti-CD8 PercP and 15 μL anti-CD28 PE, or with is type-matched negative control reagents. Cells were then permeabilized and incubated with 30 μL anti-human IFN-γ–FITC or with an isotype-matched negative control reagent. Finally, cells were washed, resuspended in 100 μL buffer containing 1% PFA, and then stored at 4°C in the dark before flow cytometry analysis.

Five-parameter analysis (using foward scatter [FSC], SSC, FITC, PE, and peridinin chlorophyll protein [PerCP]) was performed on a FACScan cytofluorometer using the Cellquest software (Becton Dickinson Immunocytometry Systems). For each sample 125,000–500,000 events were acquired, gated on CD8 expression and a scatter gate designed to include only viable lymphocytes.

Statistical analyses. Data analyses were performed with the StatView 4.5 software (Abacus Concepts, San Francisco, California, USA). Comparisons between variables were performed by using ANOVA or the Mann-Whitney *U*-test. Correlations were identified by using simple linear regression analysis and Spearman rank test. $P \leq 0.05$ was considered significant.

Results

Study population. The 24 subjects with HIV PI included 21 men and 3 women, with a median age of 33 years. They had been infected recently (median 45 days since the estimated date of infection). HIV PI was symptomatic in 19 of them (79%). The clinical, immunologic, and virological characteristics of these subjects are shown in Table 1 and are similar to those of cohorts described previously (11–13).

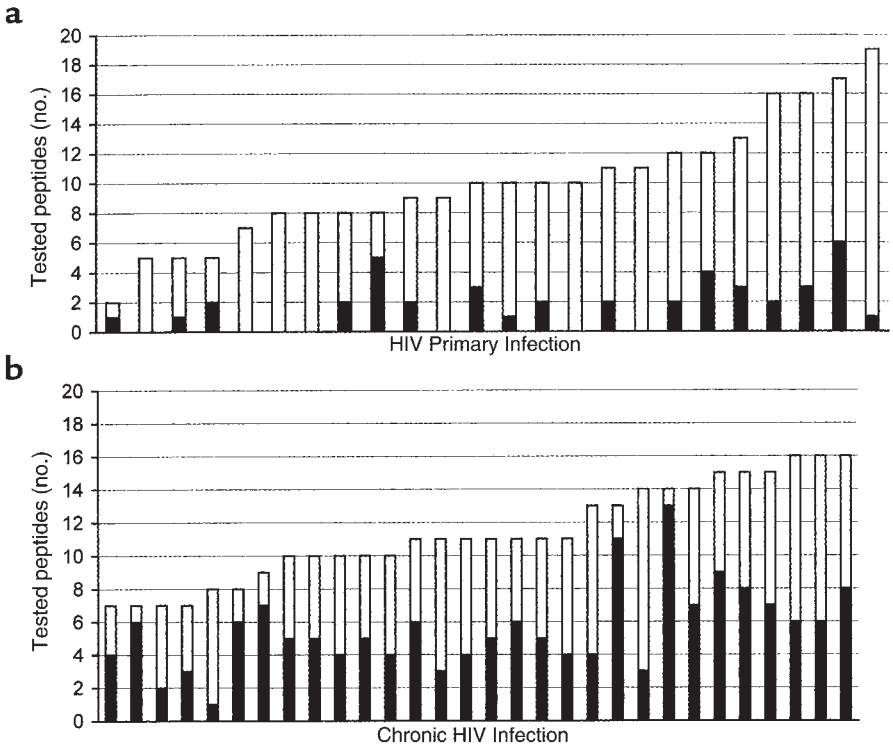
The 30 asymptomatic subjects included 26 men and 4 women. At the time of the test, these individuals had been diagnosed HIV-positive for a median of 5 years (ranging from 9 months to 12 years). Their blood median $CD4^+$ and $CD8^+$ T-cell counts were 340 and 958 per μL, respectively. Median plasma viral load was 4.5 log. Tissue HLA-typing data are shown in Table 2.

HIV-specific IFN-γ–secreting CD8+ T cells. HLA-restricted $CD8^+$ T-cell responses were evaluated by IFN-γ ELISPOT assays using a wide range of synthetic HIV peptides derived from the Env, Gag, Pol, and Nef proteins. A median of 10 peptides were tested per individual (range 2–19) depending on HLA type.

IFN-γ–secreting cells were detected in 17 subjects (71%) (Table 2). Studies performed with purified cells indicated that $CD8^+$ T cells were responsible for IFN-γ synthesis (data not shown). Gag and Nef peptides were more frequently recognized (in 54% and 58% of subjects, respectively) than Env and Pol peptides (in 19% and 13% of subjects, respectively). To evaluate whether the low recognition of Env and Pol peptides could be due to a bias in the selection of peptides tested, HIV-specific $CD8^+$ T-cell responses were evaluated in 6 nonresponder subjects by stimulating effector cells with autologous B-LCL expressing the entire *env* and *pol* genes (individuals DOX, EOD, GVT, IRO, KXO, OFP). Only 1 of these individuals (subject OFP) expressed a reactivity against *pol*-expressing targets. However, no Pol peptide had been tested in this individual because of the lack of available Pol peptides known to be restricted by his HLA alleles.

All subjects recognized only a few peptides (median 2, range 0–6). However, because the numbers of peptides tested for each individual varied depending on their HLA haplotype, the percentage of peptides tested that were recognizable by each individual is a more accurate indication of the breadth of HIV recognition; these percent-

Figure 1
Comparison of anti-HIV CD8+ T-cell responses of HIV-infected subjects in PI (**a**) and during chronic infection (**b**). The Y axis represents the numbers of tested peptides; black bars show numbers of recognized peptides; and white bars show numbers of peptides not recognized. Each bar on the X axis represents the data obtained for 1 individual.



ages ranged from 0% to 62% (mean: 19%; median: 18.5%).

These data are remarkably different from those obtained in the 30 asymptomatic subjects tested in the chronic stage of infection in whom HIV peptides elicited a much broader response (Figure 1). All asymptomatic individuals were responders recognizing at least 1 peptide. All viral proteins were frequently recognized: Env, Gag, Pol, and Nef peptides giving positive responses in 69%, 90%, 77%, and 90% of subjects, respectively ($P < 0.0001$, comparing chronically infected subjects and individuals with PI for each protein) (Table 3). Although the numbers of peptides tested were similar in both groups, a higher frequency of recognition was observed in chronically infected subjects; a median of 5 peptides induced IFN-γ synthesis with a mean percentage of recognized peptides equaling 49% ± 19%, range 13%–93% ($P < 0.0001$ compared with PI subjects).

In contrast, the frequencies of CD8+ T cells responding to a given peptide were of the same order of magnitude at both stages of infection ranging from 10 to 3,630 per 10^6 PBMC (30–1,500, 10th–90th percentile) during PI and from 6 to 7,260 (50–2,100, 10th–90th percentile) during chronic infection (Tables 2 and 3).

The total SFC count, as an index of the total reactivity against HIV peptides, ranged from 25 to 7,552 per 10^6 cells in responding subjects with PI (median 1,350). During chronic infection, these values ranged from 230 to 16,413, with a significantly higher median value of 4,476 ($P < 0.0001$). This higher global reactivity reflected the broader response observed during the chronic stage.

We then looked for a relationship between HIV-induced IFN-γ synthesis and the virological and immunological status of the subjects with PI. We first analyzed the relationship between the 3 parameters of the CD8+ T-cell response (percentage of peptides recognized, mean SFC, and total SFC counts) and the viral load; no statistical correlation was found. Similarly, we found no correlation between the parameters of the CD8+ T-cell response and the immunological status, assessed by CD4+ T-cell counts (data not shown).

IFN-γ–inducing HIV peptides. We then analyzed the data at the peptide level to look for different patterns of reactivity

Table 3
Comparison between the ex vivo functional repertoire of antiviral CD8+ T lymphocytes in chronic and acute HIV-1 infection

Subjects	Frequency of subjects responding to HIV-1 proteins No. of responders/No. tested (%)					% positive peptides[A]	Mean SFC number[B]		Total SFC number[C]	
	Env	Gag	Pol	Nef	Total	median	median	range	median	range
Primary infection	4/21	13/24	3/23	14/24	**17/24**	19%	533	10–3,630	1,350	25–7,552
n = 24	(19%)	(54%)	(13%)	(58%)	**(71%)**					
Chronic infection	20/29	26/29	23/30	27/30	**30/30**	49%	727	6–7,260	4,476	230–16,413
n = 30	(69%)	(90%)	(77%)	(90%)	**(100%)**					

[A]For each individual, percent of positive peptides was calculated as described in Table 2; median of these values is given for each group. [B]For each individual, mean SFC number was calculated as described in Table 2; median and range of these values is given for each group. [C]For each individual, total SFC number was calculated as described in Table 2; median and range of these values is given for each group.

against the different HIV peptides. A total of 42 peptides were tested in PI subjects, 21 of them (50%) eliciting a positive response. In contrast, 43 out of the 45 peptides (96%) elicited a positive response in the chronic stage (Table 4).

In subjects with HIV PI, we observed a positive correlation between the frequency at which a given peptide was recognized (among the individuals bearing the corresponding HLA class I molecule) and the intensity of the response it induced, as expressed in SFC ($R = 0.73$; $P = 0.0006$).

Table 4
Frequencies of peptide recognition according to the HLA restriction molecule

Peptides	Restriction	Chronic infection				Primary infection			
	Molecules[A]	Responding subjects[B]		SFC number/peptide		Responding subjects[B]		SFC number/peptide	
		Frequency	%	Mean	Range	Frequency	%	Mean	Range
Nef 121-128	A1	1/5	20%	10	—	0/5	0%	—	—
Nef 184-191	A1	1/5	20%	800	—	0/4	0%	—	—
Env 121-129	A2	4/14	29%	247	25–435	0/13	0%	—	—
Gag 77-85	A2	10/14	71%	2,264	300–7,260	2/13	15%	615	30–1,200
Pol 200-208	A2	2/12	17%	726	250–1,202	0/13	0%	—	—
Pol 476-484	A2	10/14	71%	738	55–1,979	1/13	8%	30	—
Pol 588-596	A2	1/12	8%	120	—	1/13	8%	60	—
Pol 683-692	A2	0/14	0%	—	—	1/9	11%	60	—
Nef 82-91	A2	0/3	0%	—	—	1/3	33%	1,500	—
Nef 136-145	A2	4/14	29%	309	70–630	0/13	0%	—	—
Env 775-785	A3	1/6	17%	106	—	0/3	0%	—	—
Gag 18-26	A3	1/7	14%	15	—	0/3	0%	—	—
Gag 20-28	A3	6/7	86%	286	59–450	2/3	67%	300	200–400
Gag 266-275	A3	1/4	25%	6	—	0/3	0%	—	—
Pol 325-333	A3	1/3	33%	63	—	0/3	0%	—	—
Nef 73-82	A3	5/6	83%	1016	38–3,000	1/3	33%	350	—
Gag 83-91	A11	1/5	20%	106	—	0/1	0%	—	—
Gag 349-359	A11	2/3	67%	141	50–232	0/1	0%	—	—
Pol 325-333	A11	5/5	100%	224	70–610	0/1	0%	—	—
Pol 507-517	A11	5/5	100%	385	70–863	0/1	0%	—	—
Nef 73-82	A11	5/5	100%	1413	105–3,106	0/1	0%	—	—
Nef 84-92	A11	3/5	60%	231	32–420	1/1	100%	650	—
Env 590-598	A24	4/8	50%	791	255–1,516	0/7	0%	—	—
Gag 77-85	A24	—	—	—	—	1/5	20%	540	—
Pol 508-517	A24	6/8	75%	681	149–1,534	1/7	14%	40	—
Nef 134-143	A24	6/8	75%	467	28–1,345	4/7	57%	702	400–1,000
Gag 20-28	A30	1/2	50%	60	—	2/2	100%	2,215	800–3,630
Nef 73-82	A30	0/1	0%	—	—	1/2	50%	1,005	—
Env 775-785	A31	3/3	100%	406	130–743	—	—	—	—
Gag 20-28	A31	1/3	33%	1,177	—	—	—	—	—
Gag 83-91	A31	1/1	100%	607	—	—	—	—	—
Nef 84-92	A31	1/5	20%	106	—	—	—	—	—
Env 424-432	A32	3/5	60%	310	52–650	—	—	—	—
Env 774-782	A32	1/5	20%	306	—	—	—	—	—
Pol 559-568	A32	4/5	80%	450	60–1,172	—	—	—	—
Env 303-312	B7	3/8	38%	476	119–1,060	0/3	0%	—	—
Env 848-856	B7	6/8	75%	942	8–1,884	2/3	67%	1,400	1,300–1,500
Nef 128-137	B7	6/8	75%	397	17–1,017	0/3	0%	—	—
Nef 68-76	B7	3/8	38%	106	19–250	1/3	33%	70	—
Env 591-598	B8	3/8	38%	636	200–1,454	1/7	14%	10	—
Env 791-800	B8	—	—	—	—	1/1	100%	1,100	—
Env 851-859	B8	2/6	33%	418	85–751	0/1	0%	—	—
Gag 24-32	B8	2/7	29%	1310	370–2,250	1/6	17%	215	—
Gag 259-267	B8	7/8	88%	1591	163–3,000	7/8	88%	867	30–2,500
Gag 329-337	B8	2/6	33%	96	40–153	0/6	0%	—	—
Pol 185-193	B8	2/6	33%	256	120–392	0/6	0%	—	—
Nef 90-97	B8	8/8	100%	900	32–2,976	7/8	88%	719	25–1,880
Nef 135-143	B18	1/1	100%	6,700	—	—	—	—	—
Env 791-800	B27	2/2	100%	558	510–606	0/1	0%	—	—
Gag 263-272	B27	3/3	100%	821	100–1,824	1/1	100%	100	—
Nef 134-141	B27	2/2	100%	465	330–660	1/1	100%	30	—
Gag 254-262	B35	1/4	25%	142	—	0/5	0%	—	—
Pol 342-350	B35	3/4	75%	405	100–1,015	1/5	20%	25	—
Nef 68-76	B35	1/3	33%	51	—	0/5	0%	—	—
Nef 74-81	B35	1/4	25%	4,879	—	0/5	0%	—	—
Nef 135-143	B35	2/4	50%	2,708	2,326–3,090	1/5	20%	200	—
Gag 178-186	B44	7/11	64%	1,368	106–6,455	0/4	0%	—	—

[A]Some peptides may be restricted by several related HLA molecules such as the A3 superfamily (A3; A30; A31; A33); thus they were mentioned in the Table according to the HLA haplotype of the responding patients. [B]Number of subjects recognizing the peptide per number of subjects bearing the HLA restriction molecule who have been tested with this peptide.

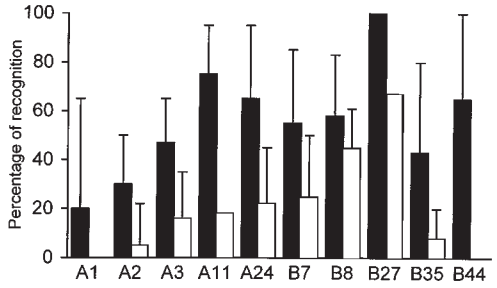


Figure 2
Comparison of the frequencies of anti-HIV CD8+ T-cell responses for different HLA restriction elements. The mean percentages (and standard deviation) of viral epitopic peptides recognized are shown for each HLA restriction element for HIV-infected individuals during PI (open bars) or chronic infection (filled bars).

The peptides recognized in more than 50% of subjects during PI induced positive responses in 84% of subjects with chronic infection. The peptides that were rarely recognized in PI elicited responses in only 46% of chronically infected subjects. Peptides not recognized at all in PI elicited responses in 36% of chronically infected subjects. Most of these peptides were recognized with relatively high intensities during the chronic stage (Table 4).

We then determined whether the low response rate to HIV peptides during PI was correlated with particular HLA haplotypes. Whichever HLA-A or HLA-B allele was considered, peptide recognition frequencies were lower in PI than in the chronic stage (Figure 2). Thus, the weaker response observed in subjects with PI was related with the stage of infection rather than with the HLA haplotype. However, there were some differences when we focused on individual HLA-peptide relationships (Table 4). Interestingly, peptides restricted by HLA-A*0201 were seldom recognized in PI. This is even the case for the 2 peptides Gag 77-85 and Pol 476-484 frequently described as immunodominant (14, 15). In contrast, 2 HLA-B8–restricted peptides (Gag 259-267 and Nef 90-97) were frequently recognized at both stages of HIV infection. Within peptides restricted by the HLA-A24 molecule, Nef 134-143 was frequently recognized in both groups, whereas Env 590-598 and Pol 508-517 were seldom recognized by subjects with PI (0% and 14% of responders, respectively, versus 50% and 75% for chronically infected individuals; Table 4). Similar patterns were observed with other HLA alleles that are less frequently represented in the study population. Peptides restricted by the HLA-A11 and HLA-B44 alleles were seldom recognized during PI. In contrast, peptides restricted by HLA-B27 were frequently recognized at both stages of infection. The patterns for HLA-B7 and HLA-B35 were similar to what we observed with HLA-A24; some peptides restricted by these molecules being frequently recognized in both groups of subjects, whereas others were seldom recognized by subjects with PI when compared with chronically infected individuals (Table 4).

Activation and differentiation markers of HIV-specific CD8+ T cells. The fact that HIV-specific CD8+ T-cell activity was lower during PI than in the chronic stage prompted us to evaluate the global level of CD8+ T-cell activation as indicated by phenotypic alterations. There were increases in the percentages of CD8+ T-cell subsets expressing HLA-DR (42% ± 17%), CD38 (65% ± 17%), or CD45RO (49% ± 17%) during PI when compared with values observed in an HIV-seronegative control group (Figure 3). However, the fraction of CD8+-activated T cells was lower than in the chronic stage where these percentages were 74% ± 13%, 86% ± 9%, and 59% ± 16%, respectively. In contrast, the proportion of CD8+CD28+ T cells was lower in PI (53% ± 15%) than in the control group (71% ± 14%), but higher than in chronically infected subjects (25% ± 9%), therefore indicating a moderate increase in terminally differentiated CD8+CD28– T cells during PI.

We have previously shown that CD28 expression on virus-specific CD8+ T cells is different between HIV-specific and EBV- or influenza-specific CD8+ T cells in subjects with chronic HIV infection (16). We therefore evaluated CD28 expression on CD8+ T cells that secrete IFN-γ in response to HIV, EBV, or influenza peptides in PI. Whereas the CD8+ T cells directed against influenza or EBV peptides were predominantly CD28+ in all groups, a significant part of the HIV-specific CD8+ T cells expressed CD28 (53% ± 13%) during PI, although this percentage was only 16% ± 13% in the chronic stage ($P < 0.0001$) (Figure 4).

Discussion

There is evidence that HIV-specific CD8+ T cells play a major role in the control of viral replication during HIV PI. However, most studies have followed small numbers of subjects (3, 4, 13, 17) or have focused only on CTL activity (11). To investigate the CD8+ T-cell effector response directed against a large variety of HIV epitopic peptides, we performed sensitive IFN-γ ELISPOT assay in 24 subjects in the early stage of HIV PI.

The most striking fact emerging from this study is that the magnitude of the HIV-specific CD8+ T-cell activity is much lower in the PI than in the chronic stage of infection. Furthermore, our results also suggest that the response in HIV PI is only moderate when compared with acute EBV or measles infections (18–22).

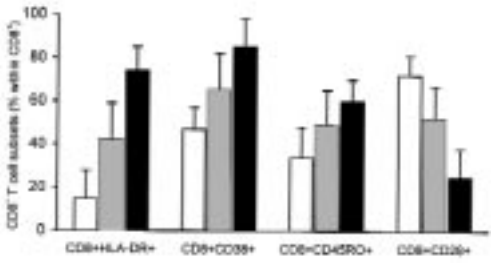


Figure 3
Level of CD8+ T-cell activation in peripheral blood. Bars represent percentages (±SD) of CD8+HLA-DR+, CD8+CD38+, CD8+CD45RO+, and CD8+CD28+ in PBMC of HIV-infected subjects during PI (gray bars) and chronic infection (black bars) as compared with control HIV-seronegative individuals (white bars).

There was an HIV-specific response in 71% of subjects, which is consistent with the 74% responder rate reported by Musey et al. (11). The HIV-specific activity was much lower than that observed in subjects at the chronic stage, where 100% of individuals responded to at least 1 HIV peptide. In addition, responder subjects in PI only reacted to a limited number of peptides, whereas those at the chronic stage had a broad, specific response directed at several peptides in the various HIV proteins.

We used a large variety of HIV epitopic peptides. However, these peptides do not encompass entire sequences of all HIV proteins, and they have been defined largely from subjects with chronic HIV infection. As we cannot exclude the possibility that subjects with PI recognize epitopes distinct from those identified in the chronic stage, we may have underestimated the breadth of their anti-HIV $CD8^+$ T-cell repertoire. This may explain why we observed a low reactivity against Env even though most responders recognized this protein in the study reported by Musey et al. (11). However, experiments performed with B-LCL infected with recombinant vaccinia viruses and still showing a low T-cell reactivity against Env and Pol do not confirm this hypothesis. In addition, these approaches would only detect conserved epitopes between HIV-1 LAI and autologous isolates. Finally, HIV regulatory proteins may be preferential targets of the $CD8^+$ T-cell response in the early phase of infection, as already suggested by the high recognition frequency of Nef peptides. We are currently investigating whether Tat, Rev, and the other HIV regulatory proteins are targets of response in the PI. In this context, it must be noted that most peptides recognized in acute EBV infection are derived from the immediate early and early proteins of the lytic cycle of EBV (21).

The low magnitude of the $CD8^+$ T-cell response in blood may also be due to the sequestration of HIV-specific $CD8^+$ T cells at sites of virus replication in the lymphoid organs. However, this seems unlikely in the light of recent studies using the SIV simian model of HIV infection (23, 24).

Differences in the breadth of the response between the 2 stages of infection vary according to the HLA haplotype. HLA-A*0201, HLA-A11, and HLA-B44 restricted peptides were seldom recognized during PI. In contrast, peptides restricted by HLA-B8 or HLA-B27 were frequently recognized at both stages of infection. More variable patterns were observed with HLA-A24, HLA-B7, and HLA-B35. Such differences between HLA molecules have already been observed and the great efficiency of HLA-B8 and HLA-B27 alleles in presenting antigen has been described, particularly during acute EBV infection (19, 25). These data need to be confirmed in experiments on larger numbers of subjects sharing the same HLA alleles to evaluate the relationship between these findings and the reported prognostic value of HLA profiles.

Contrasting with differences observed in the numbers of peptides recognized between the 2 stages of infection, the frequencies of $CD8^+$ T cells specific for a given peptide were of the same order of magnitude in subjects with acute or chronic HIV infection. However, because acute viral infections are usually characterized by a large expansion of antigen-specific cells, the frequencies observed in PI should be considered low. In subjects with PI, we observed a positive correlation between the frequency at which a given peptide was recognized and the mean SFC count induced by this peptide. This suggests that the peptides frequently recognized early in infection may be immunodominant epitopes. Conversely, in chronic infection, peptides recognized in a small proportion of individuals may have high SFC counts. This may suggest a delayed expansion of cells recognizing subdominant epitopes in response to chronic viral stimulation.

The frequencies of HIV-specific cells are of the same order of magnitude as those found using a similar technique with EBV peptides in the latent phase of EBV

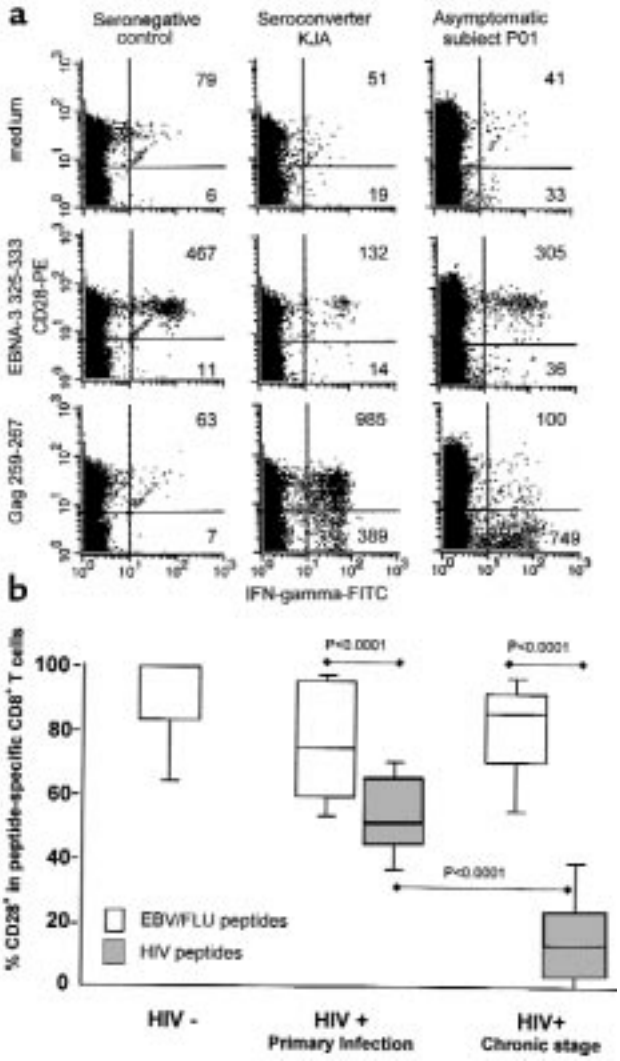


Figure 4

Comparison of the proportion of $CD8^+CD28^+$ T cells specific for HIV or other viral peptides among $CD8^+$ T cells. (**a**) Representative experiments from 3 HLA-B8–expressing individuals where PBMC were incubated in medium alone or stimulated with peptides EBNA-3 325-333 derived from EBV and Gag 259-267 from HIV-1. IFN-γ intracellular production (x axis) and CD28 expression (y axis) were analyzed on gated $CD8^+$ T lymphocytes. 200,000 events were acquired for subject KJA and 400,000 for the other individuals. Absolute numbers of events are indicated in the right quadrants of each dot-plot. (**b**) Boxes representing the fraction of $CD8^+$ $CD28^+$ T cells (25th–75th percentile) specific for HIV peptides (gray boxes) or for influenza or EBV peptides (white boxes) in subjects with PI (n = 14) or chronic HIV infection (n = 14), and seronegative controls (n = 4).

infection (26). In contrast, the frequencies of EBV-specific $CD8^+$ T cells are much higher in acute EBV infection. These frequency values ranged from 0.5% to 6.6% for HLA-A2–restricted T cells and from 29% to 44% for HLA-B8–restricted T cells (18). However, these frequencies have been assayed by tetramer staining, and it has been recently shown that this method gives higher frequencies than those obtained by IFN-γ ELISPOT (26). Tetramer analysis may detect either cells without functional activity or cells that secrete cytokines other than IFN-γ (27, 28). In acute measles infection, polyclonal direct cytotoxic responses have been reported in 77% of subjects, which also suggests a high frequency of specific $CD8^+$ effector T cells (22).

The relative contribution of $CD8^+CD28^+$ T cells and $CD8^+CD28^-$ T cells to the control of HIV is still unclear but remains a major issue. In chronic HIV infection, the $CD8^+CD28^+$ T cells have noncytolytic suppressive activity and have been associated with a good prognosis (29–31), whereas $CD8^+CD28^-$ T cells are cytotoxic and increase in parallel with the evolution towards terminal immunodeficiency (29, 32). However, the $CD8^+CD28^-$ T cells may be a major player in early and efficient control of viruses during acute infections. It has been reported that expression of CD28 is very low on the highly reactive $CD8^+$ T cells during acute EBV infection (18). In this context, the weak expansion of HIV-specific $CD8^+CD28^-$ T cells that we observed on HIV-specific $CD8^+$ T cells in subjects with HIV PI is remarkable.

As a whole, there is a marked discrepancy between the large global increase of $CD8^+CD28^-$ T cells observed in blood and the relatively small numbers of HIV-specific $CD8^+$ T cells detected, particularly of the $CD28^-$ phenotype. Several hypotheses may explain this discrepancy. First, some of the expanded $CD8^+CD28^-$ T cells may be directed at untested epitopes, derived from autologous HIV-1 sequences different from that of HIV-1 LAI, or present within some regulatory proteins, as already discussed. Second, an accumulation of nonfunctional $CD8^+$ T cells could occur during HIV PI as suggested by tetramer analysis in chronically HIV-infected individuals (27), and as demonstrated in lymphocytic choriomeningitis virus–infected mice (28) and patients with melanoma (33). Further evidence for impaired in vivo functionality of HIV-specific $CD8^+$ T cells has been provided recently (14, 34, 35). Bystander activation and expansion of $CD8^+$ T cells directed against viruses other than HIV is unlikely because most of these cells express the CD28 molecule, at least in absence of acute or recurrent infection (16).

During HIV PI and in chronically infected subjects, we found no correlation between the total anti-HIV $CD8^+$ T-cell activity and plasma viral RNA (10). First, in PI it may be difficult to demonstrate any relationship between the viral load and the immune response before a steady-state has been achieved. Koup et al. (3) demonstrated that CTL levels increase as viral load declines to a set-point, and Musey et al. (11) found a correlation between the high frequencies of HIV-specific CTLp and low plasma HIV RNA only after 6 months of infection. Second, at both stages of infection, $CD8^+$ T cells with different specificities may control the virus with different efficiencies. This may account for the lack of correlation between viral load and the aggregate $CD8^+$ T-cell responses directed at all recognized epitopes. In this context, asymptomatic patients exhibit an inverse correlation between the viral load and the number of $CD8^+$ T cells directed at 2 HLA-A*0201 restricted, immunodominant epitopes (Gag 77-85 and Pol 476-484) (36). This suggests that these $CD8^+$ T cells are highly efficient, although the functional activity of cells binding to tetrameric complexes containing these 2 peptides has recently been questioned (27, 34). In chronic infection, among the 10 HLA-A*0201–positive individuals whose immune response recognized these epitopes, only 4 had available viral load determinations that precluded statistical analysis. It is interesting to note that these 2 immunodominant peptides were rarely recognized in PI.

There could be several explanations for the weak immune response observed in HIV PI. A defect in $CD4^+$ T helper cells may be involved. The role of these cells has been recently underscored in long-term nonprogressor subjects (37–39) and in individuals treated with highly active antiretroviral treatment (HAART) during PI (37). Such responses are generally weak or absent before therapy (manuscript in preparation). A qualitative or quantitative defect of dendritic cells, particularly those bearing the CD11c molecule (40), could be responsible for $CD4^+$ and $CD8^+$ T-cell defects, because these cells play an important role in inducing primary T-cell responses (41). Finally, HIV, like other viruses, may develop different strategies to avoid immune response. Various mechanisms have been postulated including a nef-mediated downregulation of MHC class I expression that would directly hamper the $CD8^+$ T-cell response (42, 43).

We conclude that a clear understanding of the immune mechanisms responsible for controlling HIV replication during PI is important for understanding the pathogenesis of AIDS. We have described particular quantitative and qualitative features of the immune response in PI that may be responsible for the lack of control in HIV infection. In particular, the number of CTL specificities may be a critical factor for the resolution of infection, as recently demonstrated in an acute model of hepatitis C virus infection in chimpanzees (44). In addition, the immune response further decreases during antiretroviral therapy (12, 13, 27, 45, 46). This decreased response could also be responsible for the failure to eradicate the virus even when the viral load is extremely low because of effective therapy. This response could perhaps be strengthened to control the virus more effectively. Serial interruptions of antiviral therapy have been shown to achieve this goal (47, 48). However, controlled immunotherapeutic protocols associated with HAART may be a safer option. The observations made on the immune response during PI should help to improve the design of these protocols.

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