The effect of epitope variation on the profile of cytotoxic T lymphocyte responses to the HIV envelope glycoprotein

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Abstract

To address the relationship between viral and host factors during HIV infection, we analyzed the effect of viral mutations on T cell responses in seropositive, asymptomatic HLA-A2[+] individuals using four envelope (env)-specific peptides with the HLA-A*0201 binding motif. We showed that the natural sequence variation was frequent within epitopes located in the C-terminal region of the env glycoprotein and was largely responsible for a lower env-specific cytotoxic T lymphocyte (CTL) activity in the peptide-stimulated cultures. The highest CTL responses *in vitro* were induced with conserved epitopes D1 and 4.3 that mapped to the N-terminal region of the env glycoprotein. These peptides exhibited high binding affinity for HLA-A*0201 molecules and stimulated CD8^{+} T cells of relatively limited TCR V_β chain repertoire. Decreased CTL activities to the D1 epitope were observed in the absence of any detectable viral mutation, and were associated with lower proliferative responses and expression of the CD28 antigen. Results of this study demonstrate that the degree of sequence variation within a stimulatory epitope of the viral quasispecies, as well as proliferative potential of the effector cells, are among the factors underlying decreased CTL activity in HIV-infected patients. These experiments also provide evidence that the D1 peptide might be useful for the development of vaccines and immune-based therapy.

Introduction

Over the past several years, much progress has been made in the understanding of the mechanisms by which cytotoxic T lymphocytes (CTL) recognize virally infected cells with the use of naturally processed peptides (generally 8–10 amino acids long) containing sequence motifs critical for high-affinity binding of peptide to specific class I molecules (1,2). Because mutation and consequent virus variation is a characteristic feature of HIV infection, the unequivocal demonstration of CTL escape mutations arising specifically within regions of the virus encoding immunodominant CTL epitopes has been suggested to contribute to viral spread and the inability of natural anti-HIV immunity to prevent the onset of AIDS (3–6).

Two recent studies of the evolution of CTL responses to immunodominant HLA-B27 and HLA-B44 epitopes provided direct evidence of the substantial pressure exerted by the early $CD8^+$ CTL responses on HIV replication *in vivo* (7,8). These results also demonstrated the potential effectiveness of CTL activity on outgrowth of the virus, which has been compared to that of anti-retroviral drugs (9).

From the immunological perspective, the presence of the early CTL response predominantly focused on a highly immunodominant viral epitope may explain why CTL-mediated selection of escape-conferring mutants was more evident in these patients than in those with polyvalent CTL responses directed to multiple epitopes. In the latter type of responses, the outgrowth of virus variants with escape-conferring mutations in only one epitope may be controlled by CTL directed to other epitopes. These responses may represent a series of CTL escapes to a succession of immunodominant epitopes (6,7) and small decreases in the CTL responses could give mutant viruses an advantage even in the face of CTL responses to several epitopes. Consequently, the CTL escape mutants, through uncontrolled growth, would lead rapidly to immune collapse and AIDS.

Although many examples of mutations in the epitopes which rendered the mutant virus resistant to the CTL-mediated lysis have been described, accumulating evidence indicates that the CTL response mounted to a virus infection can be modified by the host. Cellular factors at the level of antigen processing, HLA binding affinity and TCR repertoire can account for the apparent hierarchy of immunogenicity observed among the various peptides (10). However, the relative contributions of some of these factors to specificity and breath of the response *in vivo* remain controversial. For example, some studies suggested that a broadly directed response is favorable, whereas others argued that a narrowly directed response to a dominant epitope is advantageous (6,11). Among additional unresolved questions in the virus–host interaction is the role of increased turnover in $CD8^+$ T cells in the course of HIV infection, possibly associated with expansion of $CD28^-CD8^+$ T cells with shortened telomere length and replicative senescence (12–14). A possible consequence of the changes of the dynamics of $CD8^+$ cells might be selective loss of HIV-specific CTL precursors during progression of HIV disease despite retention of memory cells specific for other antigens (15).

We have extended the previous analysis on the genetic pathway of virus escape from CTL pressure in a single immunodominant epitope (7,8) by investigating the effect of sequence variation on CTL responses to HLA-A*0201-restricted epitopes that mapped to conserved regions of the envelope (env) glycoprotein. In parallel, we examined expression of the CD28 antigen and TCR V_β gene segment usage by the env peptide-specific T cells. We showed that the natural sequence variation was frequent within epitopes located in the C-terminal region of the env glycoprotein and was associated with decreased CTL activities. Peptides corresponding to the N-terminal region of gp120 were characterized by a high degree of conservation and induced CTL with relatively limited TCR V_β chain usage. Decreased CTL responses to the conserved epitope located within the N-terminal region of gp120 were observed in the absence of any detectable viral mutation, and were associated with lower growth rate and expression of the CD28 antigen on the $CD8^+$ effector cells.

Methods

Patients

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood of HIV-infected adults followed in the Clinical Office of the Division of Infectious Diseases at Thomas Jefferson University Hospital, Philadelphia, PA, who had given written informed consent. The HIV-infected individuals were at early stages of disease and were undergoing antiretroviral therapy with a protease inhibitor (Crixivan®) in combination with nucleoside reverse transcriptase inhibitors in random-ized, controlled clinical trials which were followed by open extension.

All HIV-infected patients expressed HLA-A2 antigen deter-mined by the standard microlymphocytotoxicity assay (One Lambda, Canoga Park, CA; Biotest Diagnostics, Denville, NJ) according to the manufacturer's directions.

mAb and immunofluorescence analysis

The mAb comprised the following: OKT-3 (anti-CD3), OKT-4 (anti-CD4), OKT-8 (anti-CD8), 4B10 (anti-CD28), 3G8 (anti-CD16), BB7.2 (anti-HLA-A2), UCHL1 (CD45RO), and anti-bodies directed to TCR $V_\beta 1$, $V_\beta 2$, $V_\beta 3$, $V_\beta 5.1$, $V_\beta 6.7$, $V_\beta 8$, $V_\beta 9$, $V_\beta 11$, $V_\beta 12$, $V_\beta 13.1$, $V_\beta 14$, $V_\beta 16$, $V_\beta 17$, $V_\beta 18$, $V_\beta 20$, $V_\beta 21.3$, $V_\beta 22$ and $V_\beta 23$. Cells were incubated with 20 µl of unconjug-ated mAb (1:400 dilution of ascites fluid), washed in PBS containing 2% FCS and 0.1% sodium azide, and stained with 20 ml of 1:40 dilution of FITC-conjugated F(ab')$_2$ fragment of goat anti-mouse Ig (Organon Teknika, West Chester, PA). Cells were analyzed by flow cytometry on a Coulter Cytofluorograf System, Profile II. Control cells were incubated with mouse IgG followed by staining with FITC-conjugated F(ab')$_2$ fragment of goat anti-mouse Ig. For two-color immunofluorescence, cells were incubated with 20 µl of unconjugated mAb (1:400 dilution of ascites fluid), stained with 20 µl of 1:40 dilution of FITC-conjugated F(ab')$_2$ fragment of goat anti-mouse Ig, followed by 20 µl of biotin-coupled mAb and 20 µl of phycoerythrin-conjugated streptavidin. Antibodies OKT3, OKT4, OKT8 and BB7.2 were produced from cells purchased from ATCC (Rockville, MD); antibody 3G8 was produced in G. Trinchieri's laboratory; antibody 4B10 was purchased from Coutler/Immunotech (Westbrook, ME); antibody UCHL1 was pur-chased from Dako (Carpenteria, CA); and antibodies specific for TCR V_β chains were purchased from Coutler/Immunotech and T Cell Sciences (Cambridge, MA).

Synthetic peptides

The env peptides 4.3 (QMHEDIISL, amino acids 102–110), D1 (KLTPLCVTL, amino acids 120–128), 5.3 (RLRDLLLIV, amino acids 769–777) and D2 (LLNATAIAV, amino acids 815–823) were synthesized by standard Fmoc methodology (16), purified by reverse-phase HPLC and characterized by amino acid analysis and laser-desorption mass spectroscopy at The Wistar Institute (Philadelphia, PA).

Peptide binding assay

Direct binding of env peptides to HLA-A*0201 molecules was quantitated by flow cytometry analysis of RMA-S cells transfected with the HLA-A*0201 (RMA-S-A*0201 cells) (17). Briefly, RMA-S-A*0201 cells were cultured at 26°C for 18–24 h. Cells (6×10^5/50 µl of PBS supplemented with 20% FCS) were incubated with 50 µl of PBS containing different concentrations of peptides at 26°C for 1 h and transferred to 37°C for 3 h. After washing with PBS, cells were incubated with anti-HLA-A2 mAb followed by FITC-conjugated $(Fab')_2$ fragment of goat anti-mouse Ig (Organon Teknika). The up-regulation of surface expression of the HLA-A*0201 molecule after binding with specific peptides was shown as a fluorescence ratio (FR), defined as mean fluorescence of the sample to mean fluorescence of the background (i.e. RMA-S-A*0201 cells cultured without peptide at 26 and 37°C, and stained with anti-HLA-A2 mAb) (18). Peptide 6.1 (LKIPEHVVG) without the HLA-A2 anchor motif was included as a control peptide.

Target cell lines

Autologous B lymphoblastoid cell lines (LCL) were established for use as target cells by incubation of PBMC with supernatant from the Epstein–Barr virus-producing marmoset cell line B95.8 (ATCC) as described (19).

Stimulation of env-specific CTL responses

PBMC isolated by centrifugation over Ficoll-Hypaque ($d = 1.077$ g/cm^3) were cryopreserved at different time points during the study. For induction of env-specific CTL responses, cryopreserved PBMC were thawed and expanded *in vitro* after stimulation with peptide-specific or anti-CD3 mAb (20,21). Briefly, cells were adjusted to concentration 3×10^6 cells/ml and cultured in flat-bottom wells of 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) in RPMI 1640 medium supplemented with 10% FCS along with one of the following: anti-CD3 mAb (OKT3, 100 ng/ml), or HIV env peptides 4.3, D1, 5.3 or D2 (0.5 µM). For some experiments, CD8-enriched cells were obtained by removal of $CD4^+$ T cells, NK cells and B cells by the panning technique (20) before peptide-specific stimulation. All cultures were maintained in medium containing human rIL-2 (50 U/ml) for approximately 2 weeks and tested for cytotoxicity against autologous LCL cells infected with vaccinia virus (VV) expressing the env gene products.

Cytotoxicity assays

Standard ^{51}Cr-release assays were performed as described (21–23). Briefly, autologous LCL were infected overnight with 10 m.o.i. of the CR19 VV (vac) alone or with VV expressing the full-length HIV-1BH10 env (vPE16) gene products and labeled with $Na_2^{51}CrO_4$ (DuPont NEN, Boston, MA). After washings, 10^4 target cells were combined with *in vitro* stimulated T cell lines established from each patient. After 4 h, supernatants were harvested and radioactivity was measured in a 1470 Wizard γ-counter (Wallac, Gaithersburg, MD). Spontaneous ^{51}Cr-release was always <15% of maximum release. Specific lysis was calculated as: 100×([experimental release – spontaneous release]/[maximum release – spontaneous release]). Percent HIV-specific lysis was calculated by sub-

tracting the percent specific lysis against vac-infected target cells from percent specific lysis against VV-env-infected target cells.

Proliferation assays

Proliferation assays were performed on CD8-enriched cultures of T lymphocytes (3×10^5 cells/well) stimulated with the D1 peptide (0.5 µM) or cultured in medium alone. On day 4, [^{3}H]thymidine was added to each culture (1 µCi/well) and cells were harvested 8 h later. The [^{3}H]thymidine uptake was measured with a scintillation counter.

RT-PCR and sequence analysis

Natural viral sequences within regions corresponding to the env peptides 4.3, D1, 5.3 and D2 were determined by RT-PCR using cellular RNA isolated from PBMC of HIV-infected patients and two sets of primers. One set of primers amplified a region from nucleotide 1 to 1095 (5′-ACA GAA TTC ATG AGA GTG AAG GAG AAA TAT-3′, sense; 5′-GGT CTA GAC CTG AGG ATT GCT TAA AGA TT-3′, antisense) and a second set of primers amplified a region from nucleotide 2248 to 2570 (5′-AAC GGA TCC TTA GCA CTT ATC TGG-3′, sense; 5′-ACG TCG ACC TCG AGT TAT AGC AAA ATC CTT TC-3′, antisense). The obtained PCR fragments were cloned into pBluescript II SK± vector and sequenced. Relative proportions of wild-type and mutant sequences were determined independently by subjecting the individual clones to double-stranded automated DNA sequencing. The sequence analysis was performed in the Nucleic Acid Facility, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, using an automated 377 DNA sequencer ABI Prism and dichlororo-damine reaction mix (Perkin Elmer).

Results

*Binding of env peptides to HLA-A*0201 molecules*

For induction of epitope-specific and HLA-A*0201-restricted CTL in PBMC of HIV-infected patients, we selected four peptides that mapped in the conserved regions of the env glycoprotein. Two of these peptides, D1 (amino acids 120–128) and D2 (amino acids 815–823), have been previously described as co-dominant HLA-A*0201-restricted cytotoxic T cell epitopes (21), whereas antigenicity of peptides 4.3 and 5.3 has not been tested before. All peptides possess the HLA-A*0201 motif with L or M at position 2 and V or L at position 9 as anchor residues (1). The HLA-A*0201 binding ability of each peptide was measured by stabilization of HLA-A*0201 molecules on transporter-deficient RMA-S-A*0201 cells by immunofluorescence staining with anti-HLA-A2 mAb followed by flow cytometry analysis. The binding affinity for HLA-A*0201, expressed as a ratio of the mean fluorescence of cells incubated with peptides to the mean fluorescence of control cells, revealed that 4.3 and D1 represent the most abundant peptide ligands of HLA-A*0201 (Fig. 1). Both peptides induced the highest increases of HLA-A*0201 antigen expression and stabilized HLA-A*0201 molecules on the cell surface at concentrations that were 10- to 100-fold lower than those required by peptides D2 and 5.3 to up-regulate HLA-A*0201 expression above the control level. The effect was
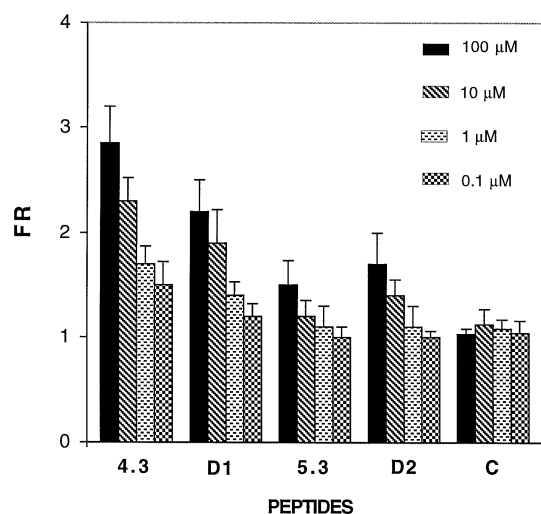
Fig. 1. HLA-A*0201 binding characteristics of the env peptides. Up-regulation of the HLA-A*0201 molecules on the surface of RMA-S-A*0201 cells is shown as a FR. The FR is defined as mean fluorescence of experimental sample to mean fluorescence of background (i.e. RMA-S-A*0201 cells cultured without peptide at 26°C and 37°C and stained with anti-HLA-A2 antibody). Peptide 6.1 (LKIPEHVVG) missing the HLA-A*0201 anchor motif was included as a control peptide.

Table 1. Clinical and laboratory data of HIV-infected patients

Patients	HLA class I[a]	HIV RNA (copies/ ml plasma)[b]	CD4 (%)	Absolute CD4 (cells/mm³)
510	A2; B49, 51	1259 (500)[c]	31 (32)	805 (936)
409	A2, 33; B14, 27; Cw1	4298 (1079)	23 (21)	510 (630)
416	A2. 3; B14, 44	5757 (3935)	27 (26)	370 (580)
539	A2; B18, 35	2211 (940)	22 (24)	464 (547)
427	A2, 3; B7, 44	31 307 (2292)	14 (19)	550 (510)
529	A2, 3; B27, 44; CW2	19 544 (527)	22 (29)	766 (897)

[a]HLA class I typings were performed at the American Red Cross using standard serological methods.
[b]Plasma HIV RNA levels were measured after reverse transcription and PCR amplification using a commercial assay (Roche Diagnostic System, Branchburg, NJ).
[c]HIV viral load and the number of CD4+ T cells are shown at the beginning and at the end (in parentheses) of the study.

specific since surface expression of HLA-A*0201 on RMA-S-A*0201 cells loaded with the unrelated peptide 6.1 (LKI-PEHVVG) was similar to that of HLA-A*0201 molecules on RMA-S-A*0201 cells cultured at 37°C.

CTL activity of PBMC stimulated with env-encoded peptides

Responses to the env peptides were analyzed in asymptomatic HIV-infected and HLA-A2+ individuals. For the analysis, HIV-infected patients were first examined for the env-specific CTL activity in bulk cultures stimulated with a CD3-specific mAb and screened against ^{51}Cr-labeled autologous LCL infected with the vPE16 recombinant VV expressing the env gene products. Responses that were >10% specific lysis in repeated experiments were considered positive. Six patients who exhibited env-specific CTL activity after polyclonal stimulation were further analyzed for responses to the env peptides 4.3, D1, 5.3 and D2. The patients' HIV viral load ranged from 1259 to 31,307 HIV RNA copies/ml of plasma (Table 1), and the number of CD4+ T cells varied between 370 and 805 cells/mm³. During the ~1.5-year follow-up period, all patients were undergoing antiretroviral therapy and exhibited small increases in CD4+ T cell numbers that were concomitant with decreases in viral load.

To examine CTL responses to the env peptides, PBMC of the HIV-infected patients were incubated with each peptide for ~2 weeks and analyzed for CTL activity against autologous ^{51}Cr-labeled LCL expressing the env gene products. Consistent with previous findings (21), the CTL responses in the env peptide-stimulated cultures differed among HIV-infected patients (Fig. 2). In patient 510, the magnitudes of CTL activities induced by peptides D1 and D2 were comparable and higher than those detected in cultures stimulated with

the 4.3 and 5.3 peptides. Responses to peptide D2 were also detectable in patients 409 and 416, but were at background levels in the remaining patients. In the majority of patients, the responses to peptides D1 and 4.3 were consistently higher than those induced by peptides D2 over a relatively broad range of E:T ratios. The antigenicity of the 5.3 peptide was low and no CTL activity was detected in cultures stimulated with the control 6.1 peptide (not shown). Among the analyzed HIV-infected individuals, only in patient 529 were responses to all peptides <20% specific lysis at an E:T ratio of 24:1.

All env peptide-induced cultures were homogeneous for CD3 and CD45RO antigen expression, and consisted of 60–78% of CD8+ T cells (not shown). The highest CTL activities against VV-env-infected autologous LCL were detected in D1-stimulated and CD8-enriched cultures of patients 510 and 409, suggesting that the responses were mediated by CD8+ cells. The CTL responses were inhibited by mAb to MHC class I and CD3, demonstrating MHC class I restriction and TCR-mediated recognition (not shown).

Sequence variation of env-specific CTL epitopes

Because the differences in the ability of the env peptides to stimulate CTL responses could be related to the amino acid sequence variability at these loci in the viral genomes, we analyzed autologous provirus sequences from activated T cells that encode the env peptide epitopes. Analysis of 10 independent cDNA clones derived from cellular RNA of each patient revealed no mutation in the D1 peptide (Table 2). Furthermore, usually one mutation at position 1, 3, 5 or 8 could be detected in ~10% of HIV isolates from clades A, B C and E (Table 3) (24). The 4.3 peptide exhibited low rates of variation, manifested by no more than two amino acid substitutions in 10–20% of cDNA clones, which could con-
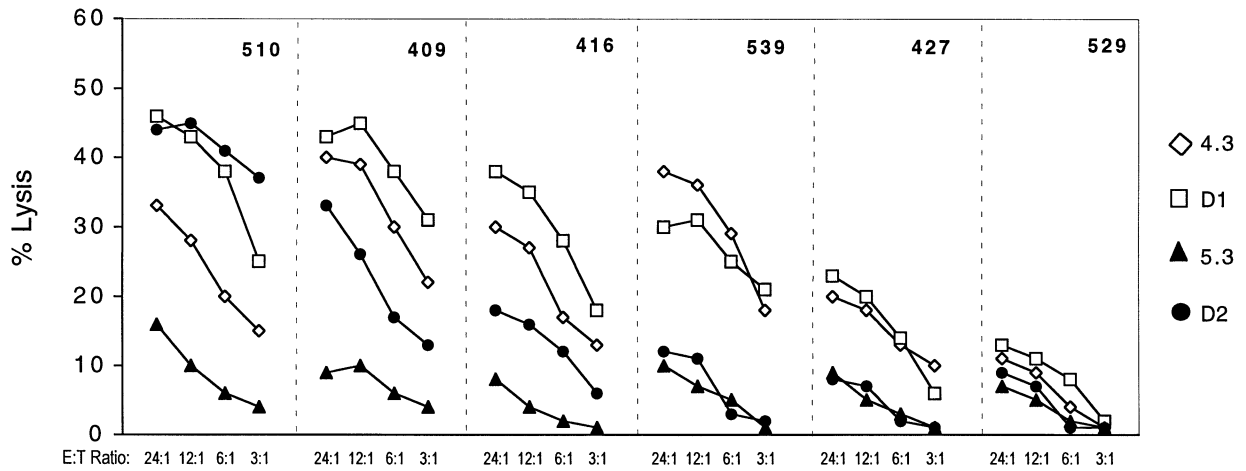
Fig. 2. CTL activity of env peptide-induced PBMC derived from HIV-infected patients against VV-env-infected autologous LCL. PBMC of HIV-infected individuals were stimulated with env-specific peptides (0.5 µM) and tested on day 14 in a ^{51}Cr-release assay against target cells expressing the env gene products. Cells infected with VV were used as a negative control and CTL activity was computed as differences between CTL responses to env versus the control. Results are representative of three separate experiments.

Table 2. Autologous sequence variation within env-specific epitopes with the HLA-A2.1 binding motif

Patients	4.3		D1		5.3		D2	
	Viral RNA sequence[a]	(%)	Viral RNA sequence[a]	(%)	Viral RNA sequence[a]	(%)	Viral RNA sequence[a]	(%)
510	Q M H E D I I S L	100	K L T P L C V T L	100	R L R D L L L I V	100	L L N A T A I A V	100
409	- - - - - - - - - - - - - - - - N -	90	- - - - - - - - -	100	- - - - - - - - -	100	- - - - - - - - -	100
416	H - - - - - - - - H - D - - - - - -	80 20	- - - - - - - - -	100	- - - - - - - - - - - I - - - - - -	80 20	- - - T I - - - - - - - - - - - - -	80 20
539	- - - - - - - - - - - - V - - - - -	80 20	- - - - - - - - -	100	- - - - - - - - - - - G - - - - - -	80 20	- - - T I - - - - - - - - I - - - - - - - - - - - - -	60 20 20
427	- - - - - - - - - - - P G - - - - -	80 20	- - - - - - - - -	100	- - - - - - - - - H - - - - - - - -	80 20	- - - T I S - S L - - - T I - - - L - - - T I S - - -	60 20 20
529	- - - - - - - - -	100	- - - - - - - - -	100	- - - - - - - - - - - K - - - - - - - - K - - - S - -	20 40 20	- - - T I - - - - - - - T I - - - -	80 20

[a]Natural viral sequences within regions corresponding to the env peptides 4.3, D1. 5.3 and D2 were determined by RT-PCR using cellular RNA isolated from PBMC of HIV-infected patients and env-specific primers. Relative proportions of wild-type and mutant seqences were determined independently by subjecting individual cDNA clones to double-stranded automated DNA sequencing. A total of 10 independent cDNA clones were analyzed from each patient.

tribute to the small decreases in CTL responses relative to the D1 peptide-induced activity in some patients. A similar rate of mutation was detected in the 5.3 epitope. This, together with a weak binding affinity of the 5.3 epitope to HLA-A*0201 molecules, could explain the low responses to this peptide in the HIV-infected patients. Among the analyzed peptide, the highest rate of sequence variation was detected in the D2 epitope (Table 2). Substitutions of 4A/T and 5T/I were most frequent, and were observed in four out of six HIV-infected patients. They represented 80% of the viral quasispecies in patients 416 and 539, and were present in every sequence derived from patients 427 and 529.

To investigate to what extent the 4A/T and 5T/I mutations could affect the ability of the D2 peptide to stimulate CTL responses *in vitro*, we analyzed env-specific CTL activities in D2- and D2-4A/T, 5T/I mutant-stimulated cultures. In patient 409, the CTL responses to the D2-4A/T, 5T/I variant were reduced by ~70% over a broad range of E:T ratios compared to those stimulated with the D2 epitope (Fig. 3A). Similar profile of responses were observed in patient 510 (not shown). Although env-specific CTL responses to the mutated D2 epitope were not entirely abrogated in these patients, results of this study suggest that the D2-4AT, 5T/I variant is less immunogenic than its wild-type counterpart.

Table 3. Sequence variation within the D1 and D2 epitopes in primary HIV isolates

Clade	No. of HIV isolates	Amino acid substitutions[a]	
		D1 [KLTPLCVTL]	D2 [LLNATAIAV]
Consensus **A**	23	(2) 1K/E (2) 1K/Q (1) 3T/P	(3) 3N/D, 4A/T, 5T/I (2) 2L/F, 3N/D, 4A/T, 5T/I, 8A/V (2) 2L/V, 3N/D, 4A/T, 5T/I (1) 2L/F, 3N/D, 4A/T, 5T/I, 7I/V, 8A/V, 9V/I (1) 3N/D, 4A/T, 5T/I, 8A/V (1) 3N/D, 4A/T, 5T/I, 7I/L (1) 3N/D, 5T/V, 7I/V (1) 3N/D, 4A/T, 5T/V (1) 3N/D
Consensus **B**	92	(6) 8T/S (1) 5L/I (1) 3T/A (1) 3T/P (1) 1K/Q (1) 1K/T, 2L/I	(5) 4A/T (4) 7I/V (3) 5T/I (2) 4A/V (1) 2L/F, 3N/D, 4A/T, 5T/I (1) 2L/F, 3N/D, 5T/I (1) 2L/F, 4A/T, 5T/I (1) 2L/F, 5T/I (1) 3N/D, 5T/V (1) 4A/T, 8A/V (1) 2L/V, 4A/V (1) 2L/F, 4A/T (1) 8A/V (1) 3N/D (1) 3N/S (1) 2L/F
Consensus **C**	44	(1) 1K/V (1) 2L/M (1) 8T/I	(4) 3N/D, 4A/T (2) 2L/F, 3N/D, 4A/T, 5T/I (2) 3N/D, 4A/T, 5T/I, 8A/T (2) 3N/D (1) 1L/I, 2L/V, 3N/D, 4A/T, 5T/L (1) 2L/V, 3N/D, 4A/T, 5T/I (1) 2L/V, 3N/D, 5T/I, 8A/T (1) 3N/D, 4A/T, 5T/I (1) 3N/D, 4A/T, 8A/T (1) 3N/D, 4A/T, 5T/V (1) 4A/T, 8A/T (1) 4A/T (1) 4A/I
Consensus **E**	20	(2) 1K/R (1) 5L/P	(1) 3N/D, 5T/A, 9V/I (1) 3N/D, 8A/I (1) 2L/F, 3N/D (1) 3N/D, 9V/A (1) 4A/T (1) 2L/F (1) 3N/D

[a]The number in parenthesis denotes numbers of isolates with the indicated mutation. Based on the Los Alamos database.

TCR V$_\beta$ usage by env peptide-induced CTL

To establish the effect of the autologous sequence variation on TCR V$_\beta$ chain usage, the env peptide-specific T cells were analyzed by two-color immunofluorescence staining with V$_\beta$ chain- and CD8-specific mAb. In most cultures with highly inducible env-specific CTL activities, the expansion of CD8$^+$ T cell lines was dominated by approximately three V$_\beta$ families with usual predominance of only one V$_\beta$ chain (Fig. 4). For example, in D1-, D2-, and 4.3 peptide-induced cultures derived from patient 510, the respective proportions of the dominant V$_\beta$17$^+$CD8$^+$, V$_\beta$9$^+$CD8$^+$ and V$_\beta$2$^+$CD8$^+$ cells were

45, 39 and 32%. A similar profile of T cell responses was detected in D1- and 4.3-stimulated cultures of patient 416. In contrast, env peptide-specific T cell lines of patient 529 with low CTL activities consisted of cells expressing several V$_\beta$ gene segments (Fig. 4). All V$_\beta$ gene families were expressed by cells stimulated with soluble anti-CD3 mAb, suggesting that the profile of V$_\beta$ chain usage in the env peptide-specific cultures could not reflect deletions in the TCR V$_\beta$ chain repertoire (25). In addition, the higher numbers of V$_\beta$ families in D2-4A/T, 5T/I mutant-stimulated cultures of patient 409 compared to those in D2-specific T cell lines (Fig. 3B) suggest
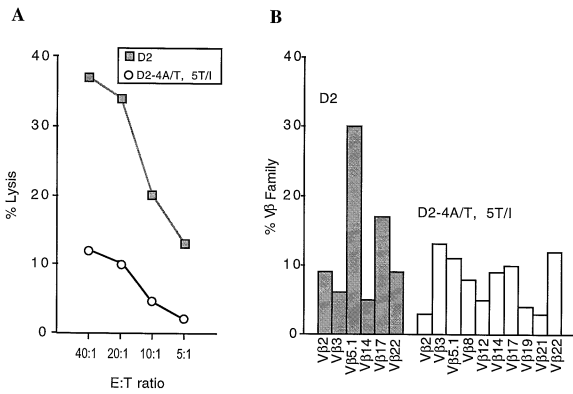
Fig. 3. Effect of autologous sequence variation on CTL responses and TCR V_β gene segment usage. PBMC of patient 409 were stimulated with peptide D2 or the D2-4A/T, 5T/I mutant and analyzed for env-specific CTL activity (A) or expression of TCR V_β chains by two-color immunofluorescence staining using mAb specific for TCR V_β chains and CD8 (B). Control cells were stained with FITC- and phycoerythrin-conjugated mouse Ig.
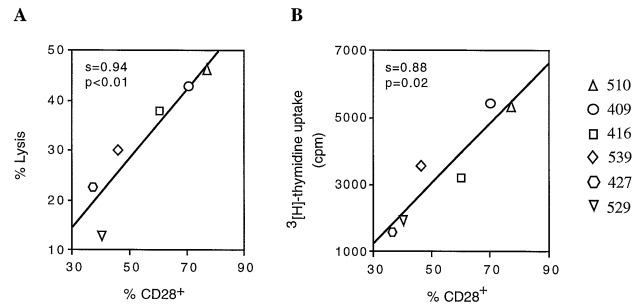

Fig. 5. Linear association between expression of the CD28 antigen and env-specific CTL activity (A) or proliferative responses (B) in D1-induced cultures of six HIV-infected individuals. Analyses of CTL activities against VV-env-infected autologous LCL (E:T ratio of 20:1), expression of CD28 antigen and proliferative responses were carried out in D1 peptide-stimulated cultures of CD8-enriched PBMC. The proliferative responses were computed as differences between [³H]thymidine uptake in D1-stimulated versus control cultures. The lines were generated using least squares regression analysis.

Fig. 4. TCR V_β gene repertoire in CD8⁺ cell subsets of anti-CD3 mAb- or env peptide-stimulated PBMC of HIV-infected patients. PBMC were stimulated with anti-CD3 mAb (100 ng/ml) or env-specific peptides (0.5 µM) and analyzed by two-color immunofluorescence staining using mAb specific for TCR V_β chains and CD8. Control cells were stained with FITC- and phycoerythrin-conjugated mouse Ig.

that the restricted V_β gene segment usage in cultures with high CTL responses might be due to the oligoclonal expansion of the env peptide-specific CTL precursors.

Expression of the CD28 antigen in D1-induced CTL lines

The increased sequence variation within the D2 epitope was associated with lower CTL responses to this peptide in the majority of analyzed patients. However, the sequence variation could not explain the heterogeneity of responses to the D1 peptide among HIV-infected patients because no mutation was detected within this epitope. Accumulating evidence suggests that the loss of CTL-mediated control of viral replication during HIV infection can be related to the increased turnover of the CD8⁺ effector cells, possibly leading to expansion of a non-proliferative subset of cells that lack expression of the CD28 antigen (12,13). This, together with the results of previous studies which showed that cytolytic activity is confined to the CD28⁺ subset of CD8⁺ cells (26), prompted us to examine the relationship between CD28 antigen expression and env-specific CTL responses in D1-induced cultures.

The level of CD28 expression in CD8-enriched T cell lines stimulated with the D1 peptide was analyzed in parallel with [³H]thymidine uptake. As shown in Fig. 5(A), there was a significant association between the number of CD28⁺ cells in D1-stimulated cultures and the level of env-specific CTL responses ($s = 0.94$, $P < 0.01$). For example, D1-stimulated cell lines established from patient 510, with high env-specific CTL activities, were among those exhibiting increased numbers (>80%) of CD28⁺ cells. A similar profile was detected in patients 409 and 416, whereas the number of CD28⁺ cells and the level of the CD28 antigen expression were reduced in D1-stimulated cultures with low env-specific CTL responses. Consistent with the previous findings on the relationship between replicative capability and the proportion of CD28⁺ cells (12), decreased CD28 expression in D1-induced cultures was associated with lower proliferative responses ($s = 0.88$, $P = 0.02$; Fig. 5B).
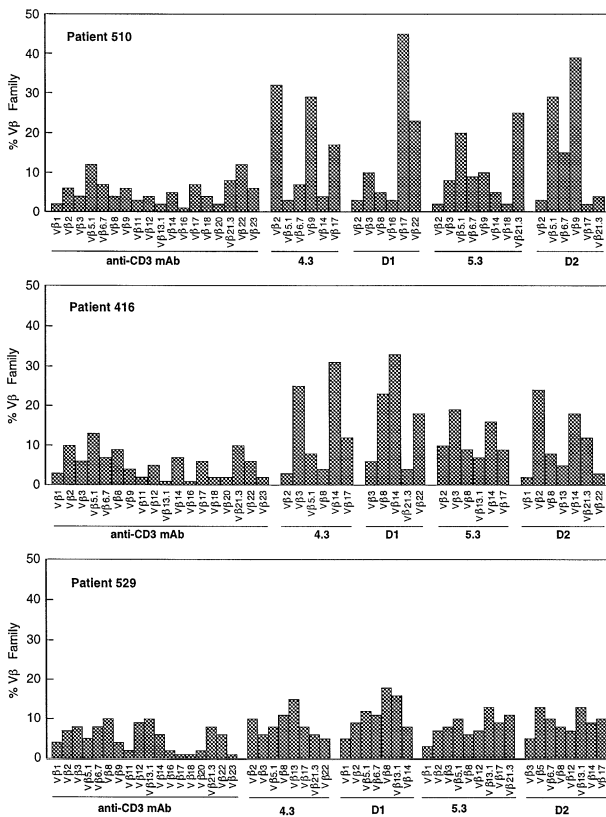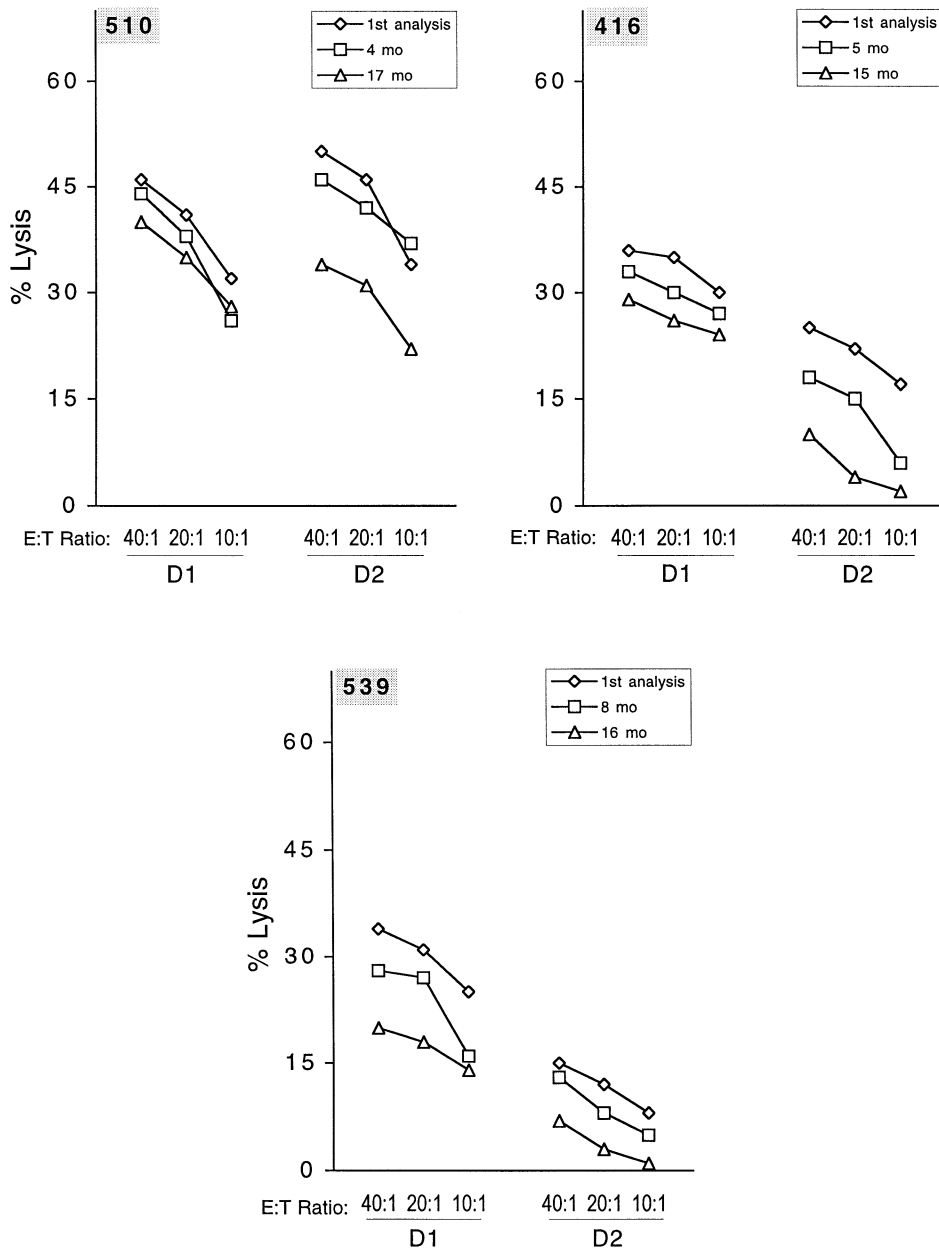
Fig. 6. Analysis of CTL responses in cultures stimulated with peptides D1 and D2 during prospective studies in patients 510, 416 and 539. PBMC of HIV-infected patients were stimulated with the D1 or D2 peptide (0.5 μM) at the indicated time points of the study and tested in a ^{51}Cr-release assay against target cells expressing the env gene products. Cells infected with VV were used as a negative control and CTL activity was computed as differences between CTL responses to env versus the control.

Prospective studies of env peptide-specific CTL responses

Results of the cross-sectional study revealed that responses to the epitopes D1 and D2 were highly heterogeneous among the HIV-infected patients. To investigate further the relation between HLA-A2-restricted CTL responses and the CD28 antigen expression or epitope variation, we studied longitudinally patients 510, 416 and 539. These patients were selected for the study because they exhibited high responses to the D1 peptide and demonstrated fluctuations in the recognition of the D2 epitope associated with sequence variation in the

viral quasispecies. The total follow-up period was 16 ± 0.8 months, during which the patients remained clinically stable. As shown in Fig. 6, the env-specific responses to peptide D1 were similar at different time points of the analysis in patient 510 and showed ~20% decreases in patient 416. The D1 peptide-stimulated cultures derived from these patients exhibited comparable levels of the CD28 antigen expression (Fig. 7A and B). Only in patient 539, responses to the D1 epitope were ~2-fold lower at the end of the study (Fig. 6). In this patient, the number of CD28$^+$ cells in D1-stimulated

A

Patient 510

Patient 416

Patient 539
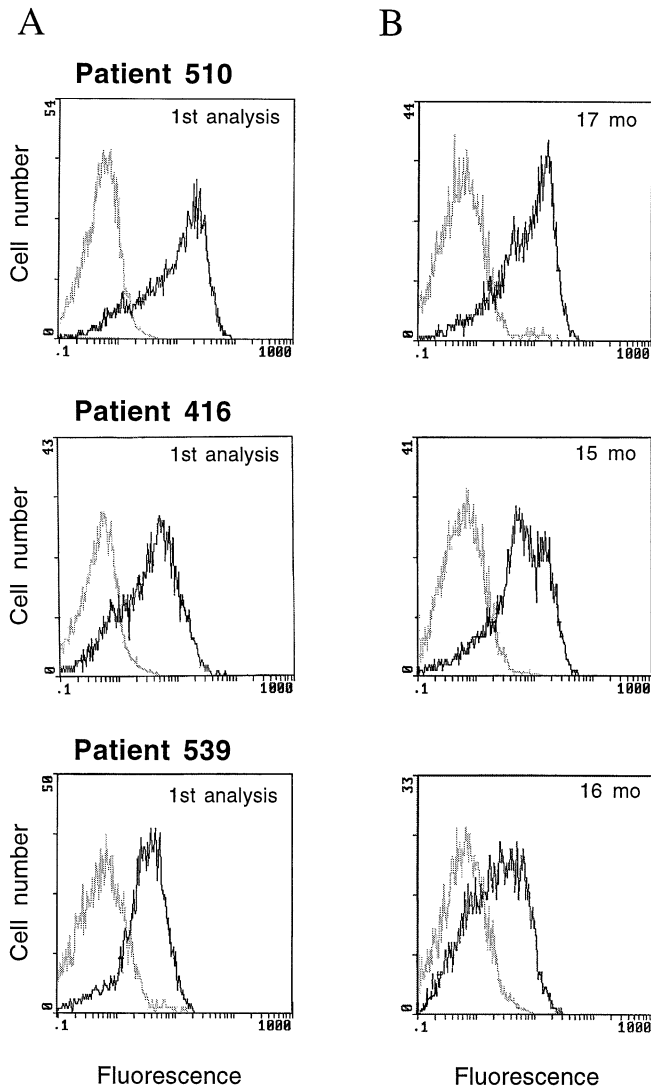

Fluorescence

B

Fluorescence

Fig. 7. Expression of the CD28 antigen in CD8-enriched cultures stimulated with the D1 peptide at the beginning and at the end of the study (A and B respectively). CD8-enriched PBMC of HIV-infected individuals were analyzed for expression of CD28 antigen (black line) after stimulation with the D1 peptide. Cells stained with FITC-conjugated mouse Ig served as negative controls (gray line).

cultures decreased from 68 to 46% in the absence of any mutation within the D1 epitope in the viral genome (Fig. 7A and B).

The env-specific CTL activity in cultures stimulated with the D2 peptide decreased in all analyzed patients (Fig. 6). In patient 510, responses to the D2 peptide were reduced by ~30% during the 17 month follow-up period, possibly due to the appearance of the 2L/V substitution at the anchor position 2 in 10% of the total viral quasispecies. In patient 416, the env-specific CTL responses in cultures stimulated with the D2 peptide exhibited the most prominent changes. In this patient, the responses to the D2 epitope decreased almost to a background level at the end of the study. This could be related to increases in the numbers of the autologous provirus

with 4A/T, 5T/I mutations from 80 to 100%, so that all of the proviral DNA sequences encoded the mutated D2 epitope. In patient 539, who exhibited a high rate of mutations within the D2 epitope, responses to this peptide were relatively low at the beginning of the analysis and showed additional decreases throughout the study.

Discussion

The development of vaccines and immune-based therapy to control HIV replication and disease progression has been hampered by the lack of understanding of the role played by HIV-specific CTL in HIV infection and the ultimate failure of CTL to eradicate the infection. The description of restricting HLA antigens and optimal CTL epitopes created the opportunity to examine in depth the virus–CTL interaction in HIV-infected patients. In this study, we analyzed the molecular basis for differential env-specific CTL responses in HIV-infected individuals by characterizing the natural sequence variation, TCR V_β chain usage and phenotype of env peptide-stimulated T cell lines established from six HIV-infected individuals. We showed that epitopes D1 and D2 exhibited the most striking differences in the mutation rate and stimulatory capabilities of CTL responses *in vitro*. Consistent with the previous findings that antiviral pressure exerted by HIV epitope-specific CTL induced a rapid selection of CTL escape virus (7,8), the high rate of mutations in the D2 epitope was associated with decreased env-specific CTL activity in the majority of HIV-infected patients. On the other hand, peptide D1 was characterized by a remarkable degree of conservation and was capable of stimulating env-specific CTL responses in a large proportion of the HIV-infected individuals. With respect to the vaccine design, results of these experiments suggest that the D1 epitope may induce more stable CTL responses in HLA-A2$^+$ individuals than peptide D2.

According to the multiple epitope model (6), the most important consequence of the antigenic variation *in vivo* is a shift of the immunodominant response to other, generally weaker, epitopes. In view of this concept, it is possible that the selection pressure by patients' CTL on the D2 epitope resulted in numbers of mutations and might have induced a shift in the dominant response from the D2 to the D1 peptide. For example, the reduction of D2-specific responses in patients 416 and 510 who maintained relatively high levels of D1-specific activity argues for this possibility. However, because the 4A/T and 5T/I substitutions in the D2 epitope are highly represented among primary HIV isolates from all clades (Table 3), and the mutated peptide is weakly immunogenic, it is possible that some patients infected with the mutant virus never develop significant levels of D2-specific responses. Additionally, the host's prior immune experience with unrelated pathogens could also influence responses to peptides D1 and D2 in HIV-infected patients (27). For example, the recruitment of D1- and D2-specific CTL from a pool of naive cells or from a population of memory cells that cross-react on these epitopes could affect the magnitude of the CTL activity.

Although the immunogenic potential of D1 and D2 peptides during HIV infection remains to be elucidated, it was of interest to find out that decreased env-specific CTL activity induced

by the D1 peptide was not associated with any mutation within this epitope. Similarly, a high degree of conservation within the D1 epitope, despite mutations in its flanking sequences, was observed in HIV isolates listed in the Los Alamos database. These results, together with the observed association between D1-induced CTL and proliferative responses, suggest that diminished CTL activity might result from a gradual loss of the HIV-specific CTL memory cells *in vivo*. It is unknown to what extent the decreases in CTL activity are related to an increased turnover of CD8$^+$ cells due to chronic antigenic stimulation (12,13) or unbalanced cytokine production observed during progression of HIV infection (20,28). Although long-lasting, monospecific CTL responses that decreased due to mutations within the immuno-dominant epitopes have been described in HIV-infected patients (7, 8), we and others have demonstrated the influence of HIV peptide-specific T$_h$ cell activity on the level of HIV-specific CTL responses in HIV-infected patients (28,29). Thus, it is possible that dysfunction of T$_h$ cells, which are essential for the maintenance of effective immunity during chronic infection (30), may contribute to the selective loss of CTL activity in HIV-infected patients. Further in-depth analysis of virus–host interactions by continued longitudinal study of the phenotypic changes of epitope-specific CTL, together with natural sequence variations within the viral genome and HIV-specific T$_h$ responses, will help the understanding of the fate of these cells during progression of HIV infection.

Acknowledgements

Abbreviations

CTL	cytotoxic T lymphocyte
env	HIV envelope glycoprotein
FR	fluorescence ratio
LCL	lymphoblastoid cell line
PBMC	peripheral blood mononuclear cells
vac	vaccinia
VV	vaccinia virus

References

1 Falk, K., Rotzschke, O., Stevanovic, S., Jung, G. and Rammensee, H.-G. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351:290.
2 Rammensee, H.-G., Friede, T. and Stevanovic, M. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics* 41:178.
3 Phillips, R. E., Rowland-Jones, S., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R. and Rizza, C. R. 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* 354:453.
4 Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D., Koppe, B., Rosenberg, W., Boyd, D., Edwards, A., Giangrande, P., Phillips, R. E. and McMichael, A. J. 1994. Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature* 369:403.
5 Klenerman, P., Meier, U. C., Phillips, R. E. and McMichael, A. J. 1995. The effects of natural altered peptide ligands on the whole blood cytotoxic T lymphocyte response to human immunodeficiency virus. *Eur. J. Immunol.* 25:1927.
6 Nowak, M. A., May, R. M., Phillips, R. E., Rowland-Jones, S., Lalloo, D. G., McAdam, S., Klenerman, P., Koppe, B., Sigmund, K., Bangham, C. R. M. and McMichael, A. J. 1995. Antigenic oscillations and shifting immunodominance in HIV-1 infections. *Nature* 375:606.
7 Goulder, P. J. R., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A. J. and Rowland-Jones, S. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nature Med.* 3:212.
8 Borrow, P., Lewicki, H., Wei X., Horwitz, M. S., Peffer, N., Meyers, H., Nelson, J. A., Gairin, J. E., Hahn, B. H., Oldstone, M. B. A. and Shaw, G. M. 1997. Antiviral pressure excerpted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Med.* 3:205.
9 Zagury, D. 1997. A naturally unbalanced combat. *Nature Med.* 3:156.
10 Johson, R. P. and Walker, B. D. 1994. CTL in HIV-1 infection: responses to structural proteins. *Curr. Top. Microbiol. Immunol.* 189:35M.
11 Kalams, S. A., Johnson, R. P., Trocha, A. K., Dynan, M. J., Ngo, H. S., D'Aquila, R. T., Kurnick, J. T. and Walker, B. D. 1994. Longitudinal analysis of T cell receptor (TCR) gene usage by human immunodeficiency virus 1 envelope-specific cytotoxic T lymphocyte clones reveals a limited TCR repertoire. *J. Exp. Med.* 179:1261.
12 Effros, R. B., Allsopp, R., Chiu, C.-P., Hausner, M. A., Hirji, K., Wang, L., Harley, C. B., Villeponteau, B., West, M. D. and Giorgi, J.V. 1996. Shortened telomeres in the expanded CD28⁻CD8$^+$ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. *AIDS* 10:F17.
13 Wolthers, K. C., Wisman, G. B. A., Otto, S. A., de Roda Husman A., Schaft, N., de Wolf, F., Goudsmit, J., Coutinho, R. A., van der Zee, A. G. J., Meyaard, L. and Miedema, F. 1996. T cell telomere length in HIV-1 infection: no evidence for increased CD4$^+$ T cell turnover. *Science* 274:1543.
14 Palmer, L. D., Weng, N., Levine, B. L., June, C. H., Lane, H. C. and Hodes, R. J. 1997. Telomere length, telomerase activity, and replicative potential in HIV infection: analysis of CD4$^+$ and CD8$^+$ T cells from HIV-discordant monozygotic twins. *J. Exp. Med.* 185:1381.
15 Carmichael, A., Jin, X., Sissons, P. and Borysiewicz, L. 1993. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) responses at different stages of HIV-1 infection: differential CTL responses to HIV and Epstein–Barr virus in late disease. *J. Exp. Med.* 177:249.
16 Otvos, L. Jr., Urge, L., Xiang, Z. Q., Krivulka, G. R., Nagy, L., Szenderei, G. I. and Ertl, H. C. 1994. Glycosylation of synthetic T-helper cell epitopic peptides influences their antigenic potency and conformation in a sugar location-specific manner. *Biochim. Biophys. Acta.* 1224:68.
17 Takamiya, Y., Schonbach, C., Nokihara, K., Yamaguchi, M., Ferrone, S., Kano, K., Egawa, K. and Takiguchi, M. 1993. HLA-B*3501–peptide interactions: role of anchor residues of peptides in their binding to HLA-B*3501 molecules. *Int. Immunol.* 6:255.
18 Nietfeld W., Bauer, M., Fevrier, M., Maier, R., Holzwarth, B., Frank Maier B. R., Riviere, Y. and Meyerhans, A. 1994. Sequence constrains and recognition by CTL of an HLA-B27-restricted HIV-1 gag epitope. *J. Immunol.* 154:2188.
19 Kozbor, D. and Roder, J. C. 1981. Requirements for the establishment of high-titered human monoclonal antibodies against tetanus toxoid using the Epstein–Barr virus technique. *J. Immunol.* 127: 1275.
20 Hyjek, E., Lischner, H. W., Hyslop, T., Bartkowiak, J., Kubin, M., Trinchieri, G. and Kozbor D. 1995. Cytokine patterns during progression to AIDS in children with perinatal HIV infection. *J. Immunol.* 155:4060.
21 Dupuis, M., Kundu, S. M. and Merigan, T. 1995. Characterization of HLA-A*0201-restricted cytotoxic T cell epitopes in conserved regions of the HIV type 1 gp160 protein. *J. Immunol.* 155:2232.

22 Koup, R. A., Pikora, C. A., Luzuriaga, K., Brettler, D. B., Day, E. S., Mazzara, G. P. and Sullivan, J. L. 1991. Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigens in infected persons: *in vitro* quantitation of effector cell populations with p17 and p24 specificities. *J. Exp. Med.* 174:1593.

23 Safrit, J. T., Andrews, C. A., Zhu, T., Ho, D. D. and Koup, R. A. 1994. Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: recognition of autologous virus sequences within a conserved immunodominant epitope. *J. Exp. Med.* 179:463.

24 Myers, G., Korber, S., Wain-Hobson, S., Smith, R. F. and Pavlakis, G. N. 1993. *Human Retroviruses and AIDS*. Los Alamos National Laboratory, Los Alamos, NM.

25 Posnett, D. N., Kabak, S., Hodtsev, A. S., Goldberg, E. A. and Asch, A. 1993. T-cell antigen receptor V$_\beta$ subsets are not preferentially deleted in AIDS. *AIDS* 7:625.

26 Damle, N. K., Mohagheghpour, N., Hansen, J. A. and Engelman, E. G,. 1983. Alloantigen-specific cytotoxic and suppressor T lymphocytes are derived from phenotypically distinct precursors. *J. Immunol.* 131:2296.

27 Selin, L. K., Nahill, S. R. and Welsh, R. M. 1994. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. *J. Exp. Med.* 179:1933.

28 Wasik, T. J., Jagodzinski, P. P., Hyjek, E. M., Lischner, H. W. and Kozbor, D. 1996. Diminished HIV-specific CTL activity associated with enhanced type 2 responses to HIV-specific peptides during perinatal HIV infection. *J. Immunol.* 158:6029.

29 Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A. and Walker, B. D. 1997. Vigorous HIV-1-specific CD4^{+} T cell responses associated with control of viremia. *Science* 278:1447.

30 Giovarelli, M., Santoni, A., Jemma, C., Musso, T., Giuffrida, A. M., Cavallo, G, Landolfo, S. and Forni, G. 1988. Obligatory role of IFN-γ in induction of lymphokine-activated and T lymphocyte killer activity, but not in boosting of natural cytotoxicity. *J. Immunol.* 141:2831.