Frequency of Class I HLA-Restricted Anti-HIV CD8$^+$ T Cells in Individuals Receiving Highly Active Antiretroviral Therapy (HAART)[1]

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Peptide/MHC tetrameric complexes were used to enumerate the frequency of HLA class I-restricted epitope-specific CD8$^+$ T cells in 18 HLA-A*0201 HIV type 1-infected asymptomatic patients. HLA-A*0201 molecules were complexed to HIV Gag p17 (amino acids 77–85) and reverse transcriptase (amino acids 464–472) peptides, biotinylated, and bound to streptavidin-phycoerythrin to form tetramers. We show in this study that 17 of 18 HIV-1-infected asymptomatic patients have circulating frequencies of 1/50–1/1000 CD8$^+$ T cells that recognize both Gag and Pol CTL epitopes or either epitope alone. The functional nature of these cells is open to interpretation, as we show that despite relatively high frequencies of fresh epitope-specific CD8$^+$ T cells, variant epitope sequences in viral plasma progeny were rare. In addition, the majority of tetramer-positive cells did not display discernible fresh CTL activity; only after restimulation with specific peptide in culture was there an expansion of epitope-specific CD8$^+$ cells, correlating with high CTL activity. These data suggest that fresh tetramer-stained cells probably represent memory precursors; we demonstrate, with the application of highly active antiretroviral therapy, that the interruption of chronic antigenic stimulation causes significant reductions in the frequency of these cells in five of six patients. In conclusion, this study provides evidence that persistently replicating viral populations are probably required to maintain high frequencies of HIV-1 epitope-specific CD8$^+$ T cells in asymptomatic chronically infected individuals *The Journal of Immunology,* **1999, 162: 1780–1788.**

With the advent of highly active antiretroviral drugs, one of the major questions is whether immune integrity in HIV-1-infected patients receiving highly active antiretroviral therapy (HAART)[3] can be improved. Previous reports have suggested that improvement may be possible, and that the immune system has the flexibility to respond positively to the removal or reduction of high levels of replicating HIV-1 (1–3). Data from different studies have shown, using Vβ repertoire analysis, that oligoclonally expanded CD4$^+$ (4) or CD8$^+$ (3) T cells are significantly diminished during HAART. We have shown recently that a skewed CD8$^+$ T cell repertoire, as measured by Vα and Vβ TCR expression on CD8$^+$ cells, was significantly reduced after 8 wk of HAART (3). This finding provides indirect evidence that lowering persistently high levels of viral replication reduces the stimulus that maintains expanded CD8$^+$ clones. However, a recent study has shown that a highly perturbed CD8$^+$ TCR repertoire is not influenced by a reduction in viral burden in patients receiving HAART (4). These data may reflect persistent CTL expansion despite the suppression of virus, although the Ag specificity of these cells was never examined. We have decided to address this issue and to extend our earlier observation (3) by investigating the frequency of epitope-specific CD8$^+$ cells using peptide/MHC tetrameric complexes (5). We wished to explore the hypothesis that high levels of replicating HIV-1 are required to maintain anti-HIV CD8$^+$ cells. As it has been shown functionally that a restricted anti-HIV CTL clonal repertoire exists in infected individuals (6, 7), it is possible that a reduced clonal repertoire during HAART could potentially result in broader CTL responses to other Ags.

Since the use of peptide/MHC tetramers was first reported (5), we have been investigating the relationship between the occurrence of these cells with CTL function in relation to HAART. Recent reports have shown that there is a good relationship between IL-7-driven in vitro-expanded CTL and tetramer staining (8), and that a significant relationship exists between freshly stained tetramer and fresh CTL lysis (9). This latter study also indicated that tetramer-positive cells had an inverse correlation with viral load in natural infection and concluded that the maintenance of CTL was driven by virus.

In this study, we have used peptide/MHC complexes to focus our investigation on CD8$^+$ T cells that recognize two HLA-A*0201-restricted HIV-1 CTL epitopes. We have related the frequency of epitope-specific CD8$^+$ cells with viral CTL epitope sequence changes, CTL function, and the effect of HAART; consequently, we were able to examine the effect of removing persistent HIV-1 replication. Our data suggest that the frequencies of CD8$^+$ T cells binding peptide/MHC tetramers are likely to be memory CTLs rather than active effector

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[3] Abbreviations used in this paper: HAART, highly active antiretroviral therapy; B-LCL, B lymphoblastoid cell line; DC, dendritic cell; SQV, saquinavir.

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Table I. *Treatment history and clinical parameters of 18 HIV-1-infected HLA-A*0201 individuals used for measuring Gag and Pol peptide/MHC tetramer binding and viral epitope sequence analysis*[a]

Patient (P)	Treatment History Prior to Study	Date of p24 Ag	CDC Stage of Disease	CD4 Count	RNA Copies/ml
Treatment-Naive					
P1	None	1994	A1	568	3,221
P2	None	1996	A1	506	33,891
P3	None	1997	A2	498	11,300
P4	None	1998	A2	419	4,883
P5	None	1997	A2	230	3,570
P6	None	1997	A3	16	68,864
Drug-Naive					
P7	gp160 vaccine or placebo	1989	A1	845	10,670
P8	gp160 vaccine	1991	A1	620	15,000
P9	gp160 vaccine or placebo	1995	A1	598	23,982
P10	gp160-pulsed allogeneic DC	1989	A2	478	132,291
P11	gp 160 vaccine or placebo	1990	A2	411	3,862
P12	Allogeneic DC	1990	A2	342	40,941
Drug-experienced					
P13	IND+3TC+AZT	1986	A1	724	392
P14	IND+3TC+AZT; Gag/Pol/gp160-pulsed allo- and auto-DC	1992	A1	709	7,500
p15	IND+3TC+AZT	1993	A1	591	707
P16	IND+SQV+3TC+AZT; gp160-pulsed DC	1984	A2	259	110,489
P17	IND+3TC+AZT	1989	A2	226	42,034
P18	IND+SQV+3TC and allogeneic CTL	1985	A3	196	53,548

[a] IND, indinavir; 3TC, lamuvidine; AZT, azuvidine.

cells. In turn, the relatively high frequency of these cells appears to be maintained by the presence of HIV-1 replication, because only when viral turnover is potently suppressed does the frequency of epitope-specific $CD8^+$ T cells decline.

Materials and Methods

Patients

A total of 18 HLA-A*0201 patients were analyzed in a cross-sectional manner to explore the relationship between the frequency of Gag- and Pol-specific $CD8^+$ T cells and the variation in viral CTL epitope sequences. Table I shows the details of treatment history before the current investigation, estimated time of HIV-1 diagnosis, Centers for Disease Control and Prevention (CDC) disease stage, $CD4^+$ counts, and RNA copies per milliliter at the time of investigation. The estimated time of HIV-1 diagnosis was determined according to the first recorded p24 Ag. The cohort was subdivided into three groups: six patients who never received any form of treatment (treatment-naive); six patients who received either glycoprotein 160 vaccine/placebo or allogeneic dendritic cell (DC) therapy (10) that ended 12 mo before the current study (drug-naive), and six patients who received combinations of antiretroviral drug therapy with or without adjunctive immunotherapy. A selection of these patients was further assessed for fresh and in vitro-cultured CTL function and correlated to peptide/MHC tetramer binding. Table II indicates which patients were selected for this analysis.

Of the patients in the study, 9 of 18 were then selected for further study in a longitudinal substudy to assess the effects of HAART on the frequency of $CD8^+$ T cells binding peptide/MHC tetramers (Table II). Five of six patients in the first group were selected (treatment-naive) to assess the effect of this regimen on patients receiving drug treatment for the first time. The effect of HAART on this group was compared with four other patients: P12, who had received DC immunotherapy (drug naive), and P13, P15, and P17, who had received protease inhibitor monotherapy followed by a triple HAART rescue regimen (11).

HLA typing

The selection of patients for study was made on the basis of HLA-A*0201 type, which was identified using standard class I serological methods.

PBMC preparation

Blood was collected in tubes containing acid citrate dextrose to prevent coagulation and PBMCs were isolated using Ficoll-Hypaque (Pharmacia, Piscataway, NJ). After washing to remove excess platelets, cells were resuspended in PBS at a concentration of 1×10^6 cells/ml.

Monoclonal Abs

The following FITC-conjugated mAbs were purchased: anti-CD69 and anti-CD45RA (Becton Dickinson, San Jose, CA); anti-CD28, anti-CD38, and anti-HLA-DR (Immunotech-Coulter, Hialeah, FL); and CyChrome-conjugated anti-CD8 (clone RPA-T8) (PharMingen, Cupertino, CA).

Tetrameric peptide/MHC complexes

The synthesis of the HLA-A*0201 tetrameric complexes used in this study has been described elsewhere (5) and was folded to express one epitope in

Table II. *Patients used in a substudy for analysis of CTL function and effects of HAART*

Patient (P)	CTL Function	Effect of HAART
Treatment-naive		
P1		X
P2		X
P3		X
P4	X	X
P5		
P6		X
Drug-naive		
P7	X	
P8	X	
P9	X	
P10	X	
P11	X	
P12	X	X
Drug-experienced		
P13		X
P14	X	
P15	X	X
P16		
P17		X
P18		

the p17 region of Gag (SLYNTVATL) and the other in reverse transcriptase (ILKEPVHGV).

Cell staining and flow cytometry

Either freshly isolated, thawed from frozen, or peptide-stimulated cells (500,000) were resuspended in 15 μl of PBS plus 2.5% FCS, supplemented with 2 mM sodium azide, and incubated on ice for 45–60 min along with 25 μl of phycoerythrin-labeled HLA-A*0201 tetramers (4 μg). In addition, 5 μl of anti-CD8-CyChrome stock and one of a panel of FITC-conjugated Abs (5 μl) were added along with the tetramer complexes. Consequently, the total volume per stain was 50 μl, making the final tetramer concentration 2 μg/stain. Stained cells were washed twice in cold PBS/2.5% FCS and once with cold PBS and fixed in PBS plus 2% formaldehyde. After staining, cells were analyzed within 24 h using a FACScan flow cytometer. A CD8$^+$ lymphocyte gate was made and 50,000 events were collected. Subset analysis was performed using either CellQuest (Becton Dickinson) or FlowJo software (TreeStar, Cupertino, CA). Color compensation settings were made with each round of staining using patient cells labeled singly with anti-CD8-FITC, -phycoerythrin, and -CyChrome.

Generation of EBV B lymphoblastoid cell line (B-LCL)

For each patient that was measured for CTL activity, EBV-B-LCLs were generated ≥6–8 wk before assay. These cells were used as autologous targets in all CTL assays. EBV-B-LCLs were generated by infecting 2× 10^6 PBMCs with EBV stock supernatants from B95-8 cell lines (American Type Culture Collection, Manassas, VA) and were cultured in the presence of cyclosporin A (14 μg/ml) in media supplemented with 10% FCS. Transformation usually occurred after 2–3 wk, and lines were established after 6–8 wk.

CTL assays

Fresh CTL activity was assessed using a standard 6-h ^{51}Cr release assay employing radioactively labeled autologous EBV-B-LCL as targets pulsed with Gag or Pol peptides. In vitro peptide-stimulated cells were assessed for CTL activity in a 4-h assay using EBV-B-LCL pulsed with peptides (4 μM) (10). Background ^{51}Cr release was always <25%. Specific CTL lysis was calculated as follows: ([E − M]/[T − M]) × 100%, where E is the experimental release, M is the minimum release in the presence of media alone, and T is the maximum release after targets were lysed with 10% Triton X-100 detergent.

In vitro CTL cultures

Freshly isolated patient PBMCs (3–5 × 10^6) were incubated with 2 μmol/ml of either Gag or Pol peptide for 24 h, after which 50 U/ml rIL-2 (Life Technologies, Gaithersburg, MD) was added. The peptides SLYNTVATL and ILKEPVHGV were purchased from Quality Controlled Biochemicals (Hopkinton, MA) and were purified once by HPLC. Cultured cells were then fed with fresh media plus 50 U/ml rIL-2 every 3 days, and CTL activity was measured after 14 days.

Viral load

Plasma viral load was assessed using the Roche Amplicor kit (Burlington, NC), according to the manufacturer's instructions.

Epitope sequencing

RNA was extracted from plasma using Qiagen Viral RNA Prep kits (Chatsworth, CA) according to the manufacturer's instructions. The purified RNA was reverse-transcribed and amplified using Superscript One-Step reagent (Life Technologies) according to the manufacturer's instructions. The primers for the Gag epitope were MAW-5 (GTG CGA GAG CGT CGG TA) and SK39 (TTT GGT CCT TGT CTT ATG TCC AGA ATG C); primers for the Pol epitope were B (GGA TGG AAA GGA TCA CC) and MAW-19 (GCT GGC TAC TAT TTC TTT TGC). The cycling parameters were 45°C for 30 min and 95°C for 2 min followed by 40 cycles at 94°C for 15 s, 55°C for 20 s, and 72°C for 2 min. A second-round PCR was performed using 5 μl of the first PCR reaction with 1× PCR buffer, 2.5 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Life Technologies), 150 μM of deoxynucleoside triphosphate (Pharmacia), and 10 pmol of primer (Operon Technologies, Alameda, CA). The second-round primers for the Gag epitope were SK431 (TGC TAT GTC AGT TCC CCT TGG TTC TCT) and MAW-29 (AAC ATA TAG TAT GGG CAA G); second-round primers for the Pol epitope were MAW-15 (TTC CTT TGG ATG GGT TAT GA) and MAW-20 (TTC TTG GGC CTT ATC CTA TTC C). The cycling parameters were 35 cycles at 94°C for 15 s, 55°C for 20 s, and 72°C for 2 min. PCR products were then diluted 1/2–1/4 with water, and 10 μl was used in dichloro-rhodamine terminator reactions (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The sequencing primers for the Gag epitope were 77seq (AGC CTT CTC TTC TAC TAC TTT TAC) and MAW-7 (ACA ACC ATC CCT TCA GAC); sequencing primers for the Pol epitope were RT21 (CTG TAT TTC TGC TAT TAA GTC TTT TGA TGG G) and MAW-17 (TTG GGC AAG

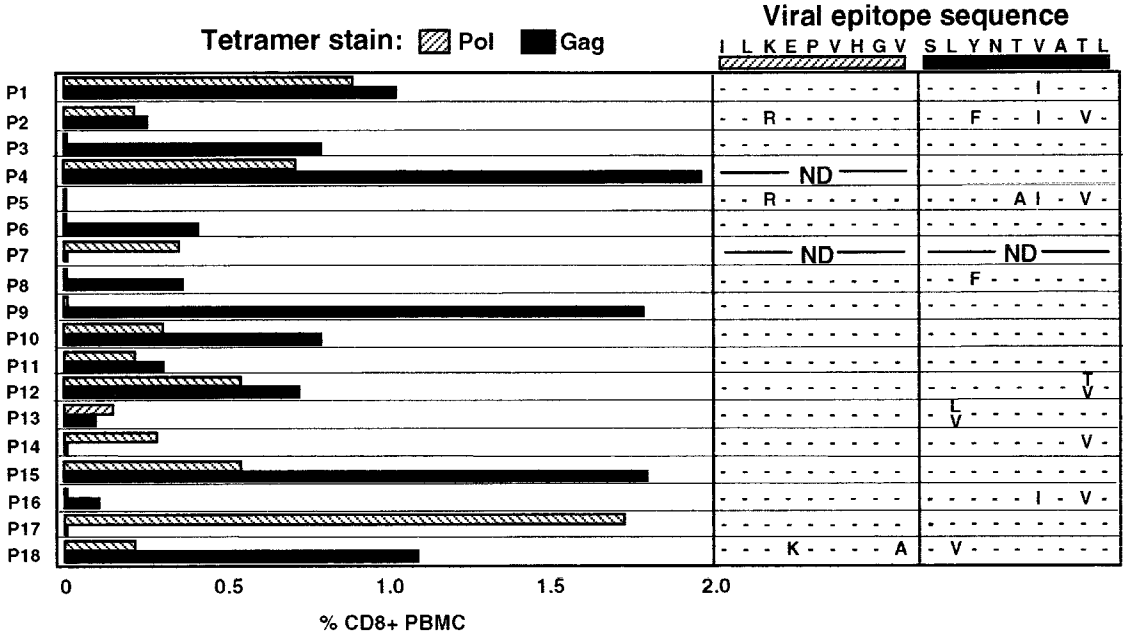

FIGURE 1. Pol and Gag peptide/MHC tetramer stains in 18 HLA-A*0201 patients in relation to plasma HIV-1 epitope sequence. Tetramer staining was assessed on gated CD8$^+$ lymphocytes after collecting 50,000 events within the gate. Viral epitope sequencing was performed from extracted plasma RNA using dideoxyterminator sequencing (see *Materials and Methods*) and was compared with reference samples using Sequence Navigator software (Applied Biosystems).

TCA GAT TTA CG). Data were collected on a model 377 DNA sequencer (Applied Biosystems) and edited manually. Mixtures were reported when a minority peak was 30% of the majority peak.

Statistical analysis

The strength of association between variables was measured using Spearman rank order correlation. Significant differences between values were measured using the paired or unpaired Student *t* test or the Wilcoxon test for unpaired data sets.

Results

Frequency of Gag- and Pol-specific CD8$^+$ cells

The frequency of peptide/MHC Gag and Pol tetramer-positive CD8$^+$ cells in the 18 HLA-A*0201 patients is shown in Fig. 1. The objective of this part of the study was to observe the frequency of epitope-specific CD8$^+$ T cells in a spread of patients. The Spearman rank correlation between the frequency of either Gag or Pol tetramer binding and plasma viral load showed a lack of association ($r = -0.12$ for Gag; $r = -0.17$ for Pol); this finding is in contrast to the observations of Ogg et al. (9), who showed a significant negative correlation between viral load and pooled Gag and Pol tetramer staining. It is likely that the heterogeneity of the treatment regimens given to the patients before analysis in the current study may have obscured any potential relationship between tetramer-positive CD8$^+$ cells and viral load.

A total of 13 of 18 patients possessed a greater proportion of CD8$^+$ cells recognizing the Gag epitope, and 4 individuals possessed CD8$^+$ cells that showed higher recognition of the Pol epitope. Of the 13 patients who displayed binding to both Gag and Pol tetramers, 11 had higher frequencies of CD8$^+$ cells recognizing the Gag epitope (range: 0.26–1.98%).

Viral epitope sequences

To determine whether frequencies of epitope-specific CD8$^+$ T cells were exerting anti-HIV pressure, plasma virus was isolated from each patient and sequenced in regions of Gag and Pol spanning the epitope sequences corresponding to the peptides folded into each tetramer. Sequence analysis of the two epitopes showed that 50% (9 of 18) of infected individuals had variant Gag epitopes and 17% (3 of 18) had variant Pol epitopes when compared with the peptides used in the construct (consensus sequence for subtype B) Fig. 1. Two patients (P2 and P8) showed a tyrosine for phenylalanine amino acid substitution at position 3. Another two patients (P12 and P13) had mixed viral populations shown by threonine + valine and leucine + valine and at positions 8 and 2, respectively.

Relationship between tetramer binding and CTL function

To evaluate whether the frequency of tetramer binding related to CTL function, we performed tetramer staining and measured CTL activity from fresh PBMCs and after in vitro culture with peptide. The relationship between the frequency of Gag and Pol tetramer-positive CD8$^+$ cells with fresh CTL activity failed to show a significant correlation (Fig. 2, *A* and *B*). Despite positive tetramer binding to either Gag or Pol (ranging from 0 to 1.81% for Gag and from 0 to 0.72% for Pol), the corresponding fresh peptide-specific lysis was often below the 5% ^{51}Cr release cut-off; lysis was not detectable in three individuals. Two of seven individuals displayed fresh lysis above 5% ^{51}Cr release against the Gag epitope (Fig. 2*A*) and a further two against the Pol epitope (Fig. 2*B*). Stimulation of cells in vitro with specific peptide and rIL-2 showed that tetramer-positive CD8$^+$ cells could be expanded, corresponding to peptide-specific CTL activity. Fig. 2*C* shows that a significant correlation ($r = 0.76$, $p = 0.006$) existed between the percentage of Pol CTL lysis (25:1 E:T ratio) after in vitro culture and fresh Pol tetramer stain before culture. Likewise, there was a highly significant association between the percentage of Pol-specific CTL lysis and corresponding tetramer staining after in vitro culture ($r = 0.96$, $p < 0.001$; Fig. 2*D*). Correlation between fresh and in vitro-cultured Pol tetramer staining (Fig. 2*E*) revealed a significant linear relationship ($r = 0.83$, $p = 0.005$). Similar restimulation experiments with the Gag epitope revealed that only one of nine patients (P4) showed an expansion of Gag tetramer-positive CD8$^+$ cells that yielded high peptide-specific lysis (Fig. 2*F*). In those patients in which Gag tetramer-positive cells could not be expanded, alternative stimulation strategies were employed. The addition of rIL-7 and a different source of peptide (Bachem California, Torrance, CA) yielded similar results (data not shown), and indicated that Gag-specific cells could not always be propagated in culture despite being detectable in fresh PBMCs.

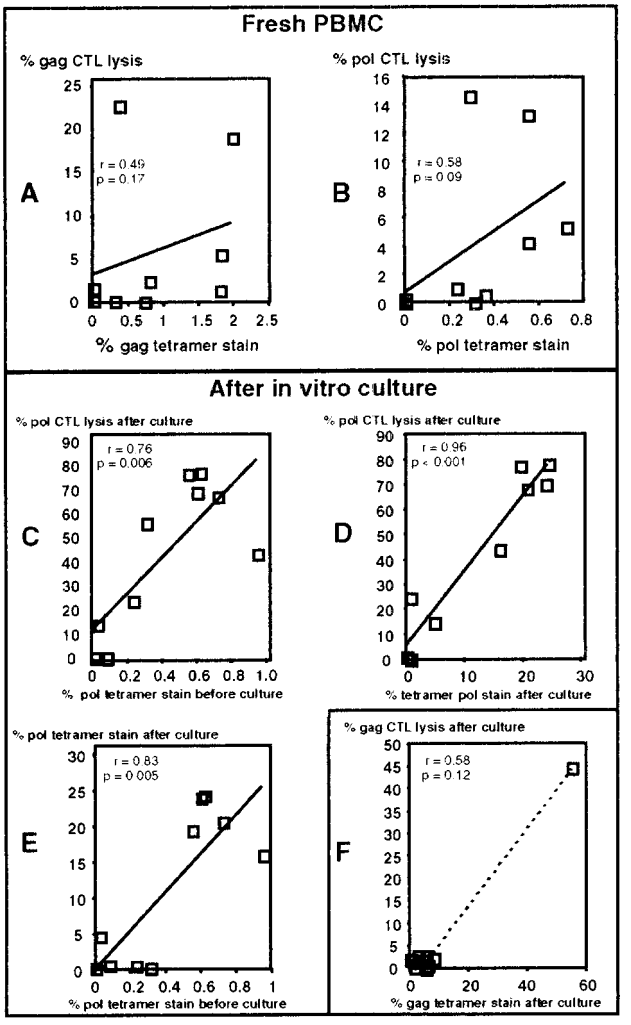

FIGURE 2. Correlation between functional CTL and peptide/MHC tetramer binding. Correlations between fresh Gag and Pol CTL lysis and tetramer staining (*A* and *B*), between Pol CTL lysis after culture and fresh Pol stain (*C*) and Pol stain after culture (*D*), and between Pol stain after culture and Pol stain before culture (*E*) are shown. The correlation between Gag CTL lysis and the percentage of CD8$^+$Gag$^+$ T cells after in vitro culture is shown in *F*. The strength of association between parameters for all correlations was measured using Spearman rank order correlation.

Phenotypes of resting vs activated tetramer-binding cells

Fresh CD8$^+$ cells binding peptide/MHC tetramers were shown previously to display surface phenotypes characteristic of memory

Table III. *Phenotype of CD8$^+$Gag$^+$ or CD8$^+$Po1$^+$ tetramer-positive cells that coexpress (+) or do not coexpress (−) CD45RA, CD28, CD38, HLA-DR, and CD69 surface Ags*[a]

	% CD45RA		% CD28		% HLA-DR		% CD38		% CD69	
	−	+	−	+	−	+	−	+	−	+
% CD8$^+$Gag$^+$ (n = 11)										
Median	0.75	0.18	0.39	0.26	0.25	0.32	0.4	0.11	0.76	0.03
25%	0.27	0.08	0.18	0.21	0.13	0.12	0.21	0.07	0.31	0.01
75%	1.08	0.28	0.80	0.37	0.32	0.4	0.84	0.3	0.91	0.07
% CD8$^+$Po1$^+$ (n = 8)										
Median	0.59	0.09	0.12	0.39	0.18	0.37	0.28	0.11	0.57	0.06
25%	0.18	0.03	0.07	0.08	0.1	0.13	0.1	0.07	0.47	0.04
75%	0.78	0.13	0.16	0.44	0.22	0.39	0.32	0.16	0.66	0.06

[a] Data are shown as % median values and interquartile ranges from the analysis of treatment- and drug-naive patients.

cells (5). We have extended these observations to include the coexpression of activation markers. Table III shows median values of the percent expression of CD45RA, CD28, CD38, HLA-DR, and CD69 Ags on gated CD8$^+$ cells coexpressing either Gag or Pol tetramers. We selected treatment- and drug-naive patients for analysis to avoid the influence of antiretroviral drug therapy, which has been associated with lower activation marker expression on CD8$^+$ cells (1, 3). Of 12 patients (Table I), 11 showed Gag tetramer binding and 8 showed Pol binding (Fig. 1) and thus represented patient numbers for phenotype analysis (Table III). Large proportions of Gag- or Pol-expressing CD8$^+$ cells were devoid of the CD45RA naive cell marker, confirming that these cells were predominantly memory cells (5). Coexpression of CD28 and HLA-DR on fresh cells appeared to be more variable between patients, with CD28 coexpression found on both Gag$^+$ and Gag$^-$ CD8$^+$ populations. A greater proportion of CD8$^+$Pol$^+$ cells coexpressed CD28 and HLA-DR (not significant); a representative example is shown in Fig. 3. Coexpression of activation markers CD38 and CD69 on either CD8$^+$Gag$^+$ or CD8$^+$Pol$^+$ cells revealed a lack of expression, indicating that fresh tetramer-positive cells were not acutely activated in these patients.

Representative tetramer staining in P12 is shown in Fig. 3, where fresh CD8$^+$Gag$^+$ and CD8$^+$Pol$^+$ cells expressing costimulatory and activation markers are shown. In this patient, there was differential expression of CD28 between Gag and Pol CD8$^+$ cells; discernible fresh CTL activity was apparent only against autologous targets pulsed with the Pol peptide. After stimulation with peptide in vitro, the frequency of CD8$^+$Pol$^+$-binding cells increased 24-fold and appeared to lose CD28 expression and gain expression of CD38 and intermediate CD69 expression. Increased expression of these markers corresponded to an enhanced Pol-specific CTL activity of 84% at a 25:1 E:T ratio.

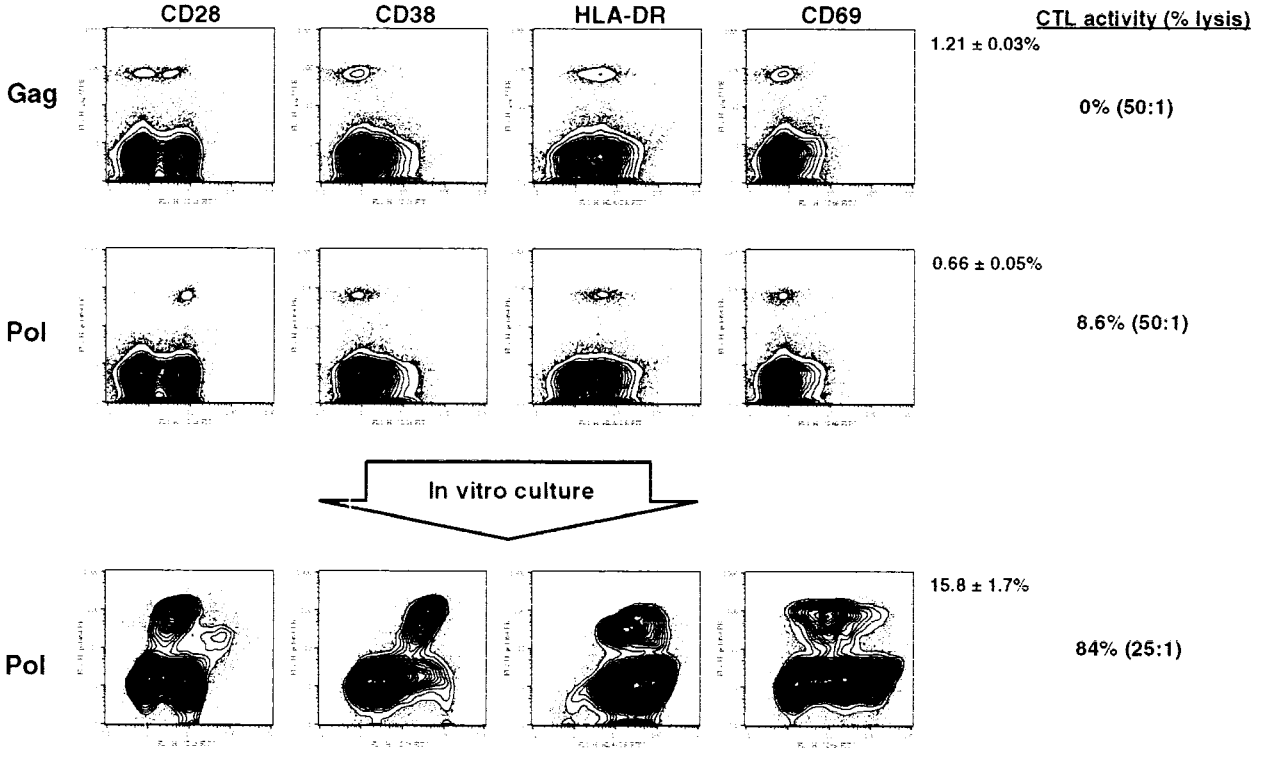

FIGURE 3. Surface Ag expression of CD8$^+$ cells binding peptide/MHC tetrameric complexes. Representative FACS contour plots of gated CD8$^+$ lymphocytes indicating the coexpression of CD28, CD38, HLA-DR, and CD69 in fresh Gag- and Pol-specific cells and subsequently after stimulation with the Pol peptide in vitro are shown. The corresponding CTL activity also is shown. FACS data were acquired using a FACScan (Becton Dickinson) flow cytometer and analyzed using FlowJo software set at 2% probability contour levels and displaying outlier events.

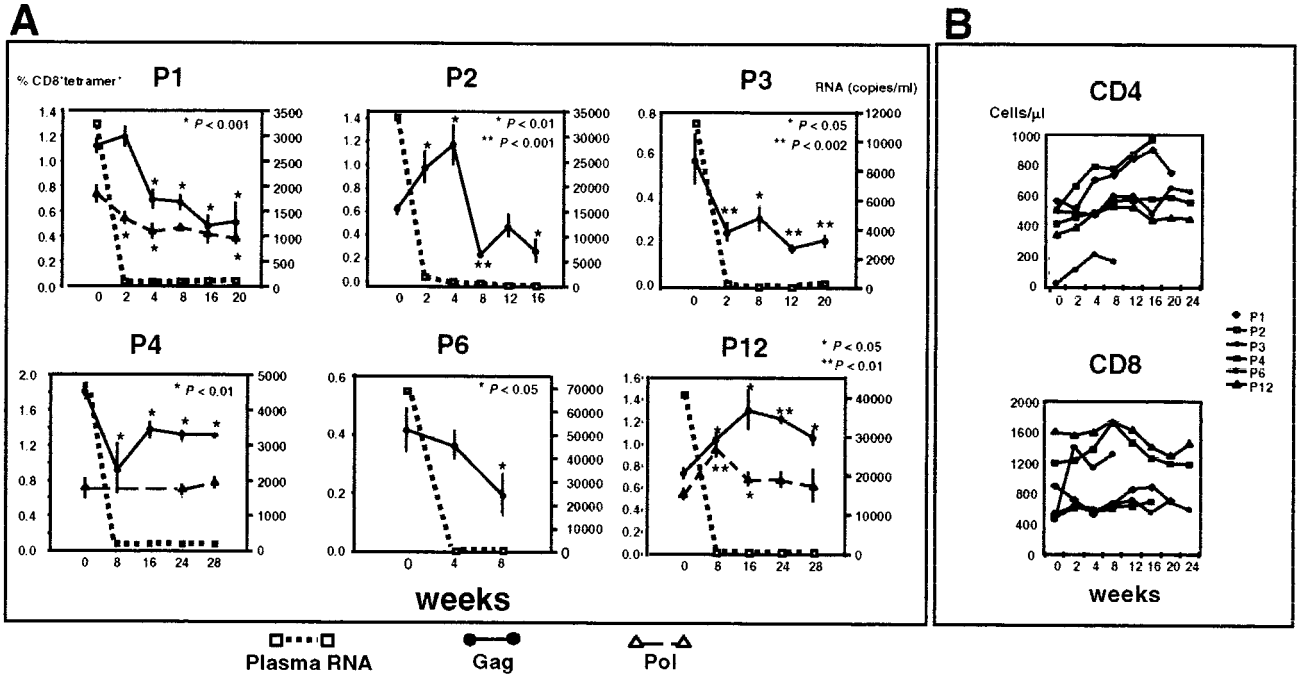

FIGURE 4. (*A*) Changes in $CD8^+$ T cells binding peptide/MHC tetrameric complexes in treatment-naive patients receiving HAART. The course of $CD4^+$ and $CD8^+$ cell changes in these patients is shown (*B*). Viral load was measured in plasma using the Roche Amplicor kit. Each datapoint is the mean ± SD of five replicate staining experiments; a comparison between timepoints was made using Wilcoxon's signed rank test.

Changes in the frequency of tetramer-binding T cells during HAART

Fig. 4*A* shows changes in the frequency of $CD8^+Gag^+$ or $CD8^+Pol^+$ cells in six patients receiving HAART for the first time over variable periods of ≤28 wk. Consistent for all patients was the sharp decline in plasma RNA copy numbers to below detectable limits, irrespective of the initial starting viral load at baseline. After 8 wk of HAART, five of six patients showed a significant decline in the frequency of $CD8^+Gag^+$ cells. P12 did not show this trend, and there was a persistent and significant increase in the frequency of $CD8^+Gag^+$ cells lasting for ≤28 wk (representative FACS data is shown in Fig. 3 at 24 wk). The frequency of $CD8^+Pol^+$ cells showed a similar trend in this patient, but never to the same magnitude, indicating a significant increase from 0.55 ± 0.06% to 0.95 ± 0.09% after 8 wk. P1 displayed an increased frequency of $CD8^+Gag^+$ cells in the first 2 wk of HAART; by 4 wk the frequency was significantly reduced. Likewise, in P2 there was a significant increase of $CD8^+Gag^+$ (0.66 ± 0.05% to 1.22 ± 0.14%) in the first 4 wk; by 8 wk the frequency of $CD8^+$ cells was significantly reduced (0.28 ± 0.02%) (Fig. 4*A*).

Fig. 4*B* shows changes in the numbers of $CD4^+$ and $CD8^+$ cells during HAART for these patients. All patients showed increased $CD4^+$ counts from a mean of 322 ± 206 cells/μl at baseline (n = 6) to 677 ± 219 cells/μl at wk 16 (n = 5). Likewise, $CD8^+$ counts increased from a mean of 841 ± 429 cells/μl at baseline to 943 ± 328 cells/μl at wk 16.

Changes in $CD8^+Gag^+$ subsets during HAART

Closer analysis of the increased frequency of $CD8^+Gag^+$ cells in P2 over the first 4 wk of HAART is shown in Fig. 5. There was an increase in the frequency of $CD8^+Gag^+$ cells coexpressing CD38, HLA-DR, and CD69 (Fig. 5*A*). By 8 wk, coexpression of these markers was lost. Fig. 5*B* shows the emergence of these activated Gag-specific $CD8^+$ cells as contour FACS plots at 2 and 4 wk, with their disappearance at 16 wk.

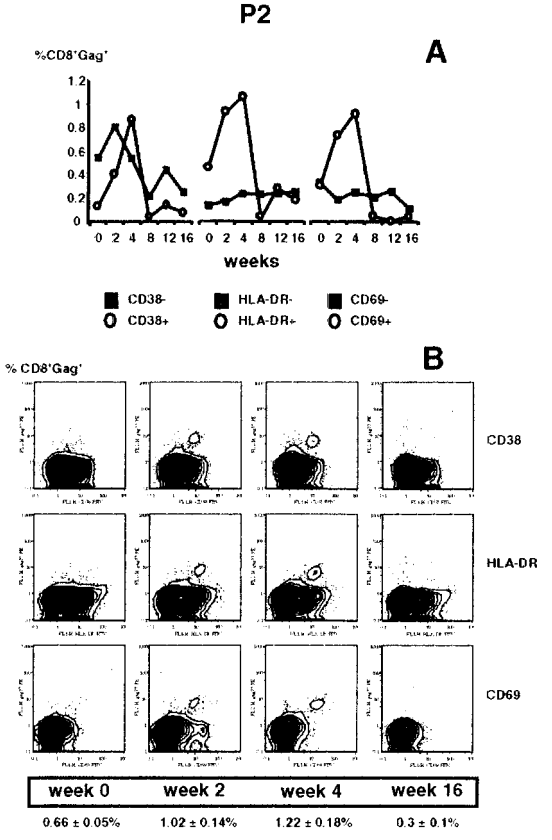

FIGURE 5. Expression of surface activation markers on $CD8^+$ cells binding peptide/MHC tetramers after commencement of HAART. *A*, Coexpression of CD38, HLA-DR, and CD69 on $CD8^+Gag^+$ cells in the first 16 wk of HAART in P2. *B*, FACS contour plots showing distinct populations of activated $CD8^+Gag^+$ cells during the first 4 wk after the commencement of therapy.

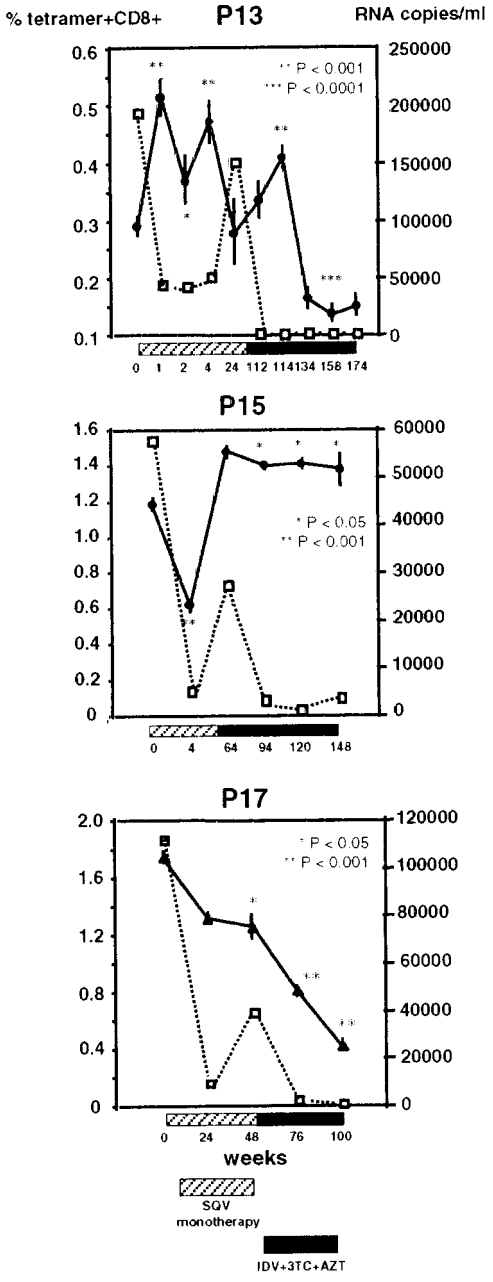

FIGURE 6. Longer-term follow-up of changes in the frequency of peptide/MHC tetramer staining in patients over 2–3 years. Initial treatment consisted of SQV monotherapy (hatched bars) that was switched to a triple HAART of indinavir plus lamuvidine (3TC) plus azuvidine (AZT) (solid bars). Each datapoint is the mean ± SD of five replicate staining experiments; a comparison between timepoints was made using Wilcoxon's signed rank test.

Long-term follow-up of CD8$^+$ tetramer-positive cells

To assess changes of CD8$^+$ tetramer-positive frequencies over longer-term viral load changes, we analyzed three patients who initially failed saquinavir (SQV) monotherapy (11) and were provided with HAART as a rescue regimen. The frequencies of CD8$^+$Gag$^+$ cells (P13 and P15) and CD8$^+$Pol$^+$ cells (P17) are shown in Fig. 6, where each patient displayed individualistic responses to the switch in treatment to triple-drug HAART (represented by solid bars). Two patients showed a significant reduction in the frequency of CD8$^+$Gag$^+$ cells (P13) and CD8$^+$Pol$^+$ cells (P17) only when HAART had commenced. One of these patients (P13) showed a significant elevation of CD8$^+$Gag$^+$ cell frequencies that oscillated for 24 wk during SQV monotherapy despite an initial reduction in viral load (Fig. 6). After the commencement of HAART, there was a further significant increase in CD8$^+$Gag$^+$ frequencies after 2 wk, which then fell to a nadir of 0.12% after 20 wk. There appeared to be more of a consistent decline in the frequency CD8$^+$Pol$^+$ cells in P17, although only limited timepoints were available for analysis. Contrary to these observations, P15 displayed an initial fall in the frequency of CD8$^+$Gag$^+$ cells after 4 wk, but values were significantly elevated above baseline during the HAART regimen.

Discussion

Until very recently, it was not possible to directly measure and visualize HIV epitope-specific CD8$^+$ cells. With the introduction of peptide/MHC tetrameric complexes (5), it became apparent that the frequency of Ag-specific cells was consistently higher than that shown by limiting dilution analysis. Indeed, recent data have shown that there is a 10- to 50-fold gap between the data obtained using limiting dilution analysis and tetramer staining for LCMV-specific CD8$^+$ T cells (12). Therefore, functional assays may well underestimate CTL frequencies in response to viral infections, perhaps due to cell death during culture (13). The present study has shown that 72% of the HIV-infected individuals studied have CD8$^+$ cells recognizing an epitope in Gag (SLYNTVATL) and to a lesser extent Pol (ILKEPVHGV). The lower proportion of CD8$^+$ cells recognizing the Pol epitope most likely reflects fewer reverse transcriptase epitopes being expressed by infected cells (14–16) and possibly with less efficiency than Gag (17). Despite the lower Pol frequencies, half of the patients ($n = 9$) possessed CD8$^+$ cells recognizing both epitopes; this finding differs from the exclusivity of epitope recognition that was reported recently (9). Despite the relatively high frequencies of epitope-specific cells in at least three patients (ranging from 1/50 to 1/70 CD8$^+$ cells that recognize Gag), there was no evidence for epitope variation in replicating viral populations. This would suggest that these cells were not imparting any selection pressure (18), especially within the more variable Gag region. However, sequencing did reveal that half of the Gag epitopes were variant from the index sequence folded into the tetrameric complex. Most of the amino acid substitutions, however, would not necessarily prevent epitope expression or TCR engagement, except in two patients (P2 and P8) whose viral populations showed phenylalanine to tyrosine substitution at position 3, which is a secondary anchor change that is known to prevent CTL recognition (19). The change from lysine to arginine in two Pol sequences may reduce binding efficiency (20) and may explain why P5 has CD8$^+$ cells that do not recognize the Pol tetramer. Overall, these data show that despite the existence of epitope-specific CD8$^+$ cells, there were no meaningful epitope variations in replicating viral populations, suggesting that selection pressure leading to CTL escape is not a common phenomena during chronic infection. This may well be in agreement with a recent study by Brander et al. (21); alternatively, these cells may not be active CTL effectors.

The lack of a significant correlation between tetramer-positive cells and fresh discernible CTL activity suggests that the majority of tetramer-binding T cells are more likely nonactive effectors. Although the magnitude of fresh CTL lysis cannot be discounted (9), the possibility that tetramer-binding cells are memory cells is supported by restimulation experiments. Pol-specific CD8$^+$ cells are expanded by $\leq$20-fold when restimulated with peptide in culture, which correlates significantly with CTL activity (Fig. 2). Phenotypically, Pol-specific CD8$^+$ cells gained expression of CD38

and CD69, with a maintenance of HLA-DR and a loss of CD28 expression (Fig. 3); this finding is compatible with activated CTL effectors (22, 23). Consequently, we interpret the lack of substantial CD38 and CD69 Ag expression on fresh circulating epitope-specific $CD8^+$ cells (Table III) as being compatible with a memory phenotype, thus providing further support that these cells are not active effector CTLs.

When we measured the frequency of epitope-specific cells in patients receiving potent antiretroviral therapy for the first time, five of six showed a significant loss of tetramer-positive $CD8^+$ cells in parallel with a suppression of replicating HIV (Fig. 4). This observation suggests that the maintenance of HIV-1-specific clones is dependent upon the persistence of replicating virus. A reduction in cell frequency was independent of gross $CD4^+$ and $CD8^+$ changes (Fig. 4*B*) and was also not likely due to mutations occurring in Gag or Pol epitopes that were measured in the longer-term patient group receiving triple HAART: P13, P15, and P17 (data not shown). As most epitope-specific $CD8^+$ T cells were not acutely activated before drug therapy (Table III), a loss of circulating tetramer-stained cells in response to HAART was unlikely due to a decline in the gross activated $CD8^+$ T cell pool. There is also a possibility that a migration or redistribution of Ag-specific $CD8^+$ cells occurs from the periphery to reservoir sites of HIV during HAART. It has been shown in natural HIV-1 infection that skewed $CD8^+$ TCR repertoires are found within lymphoid sites (24, 25), where intense viral replication and infection are taking place. Conversely, it has also been shown that HIV-specific CTL clones preferentially accumulate in the blood as opposed to the lymph node (26). Therefore, it seems unlikely that such a redistribution would take place, especially as the viral burden is also reduced in lymphoid tissue during HAART (27). One patient in our study (P12) was the only subject to show a continuous increase in $CD8^+Gag^+$ cells, and to a lesser extent $CD8^+Pol^+$ cells, during HAART. It is of interest that this patient received DC immunotherapy 12 mo before receiving a drug where some infusions of DCs were pulsed with both the Gag and Pol epitopes (10). Intuitively, it would seem that expanded Gag-specific cells during HAART may be potentially beneficial and that combined immunotherapy with HAART may prove a useful approach (28). However, these data may merely reflect an enrichment of a dominant clone that may ultimately reduce the pool of functional T cells available to control other infections and variations in HIV. Additional studies would need to be designed that would critically evaluate this possibility.

The observed increase in $CD8^+Gag^+$ cells in the first few weeks after HAART initiation in some patients (P1, P2, and P13) may reflect a redistribution of cells from lymphoid tissue sites into the periphery. A detailed analysis of one patient (P2) revealed that these cells were acutely activated, expressed CD38 and CD69, and were found in the circulation in the first 4 wk. It is likely that viral load suppression is accompanied by reduced inflammatory signals and more normal cytokine levels (2), allowing sequestered $CD8^+$ CTLs to appear in the circulation (29, 30).

Collectively, these data may possibly reflect a scenario whereby a removal of Ag persistence reduces the requirement for protective immune responses (31, 32). For the maintenance of T cell memory and, hence, potential protective responses, a source of consistent Ag stimulation is thought to be crucial (31). This view has been upheld in studies in which adoptively transferred primed $CD8^+$ CTLs appear to die unless rechallenged with specific Ag (33). Although there are opposing views for the maintenance of T cell memory (34), our findings are consistent with the notion that a reduction of viral antigenic stimulus is associated with decreased numbers of activated $CD8^+$ cells (1, 3); without constant stimulation, Ag-specific CTLs disappear (35).

The data in this study may therefore support the view that high frequencies of Ag-specific $CD8^+$ cells in asymptomatic HIV-1-infected individuals may merely reflect viral turnover rather than being determinants in the control of viral replication (35, 36).

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