

Altering Effects of Antigenic Variations in HIV-1 on Antiviral Effectiveness of HIV-Specific CTLs¹

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The mutational escape of HIV-1 from established CTL responses is becoming evident. However, it is not yet clear whether antigenic variations of HIV-1 may have an additional effect on the differential antiviral effectiveness of HIV-specific CTLs. Herein, we characterized HIV-specific CTL responses toward Pol, Env, and Nef optimal epitopes presented by HLA-B*35 during a chronic phase of HIV-1 infection. We found CTL escape variants within Pol and Nef epitopes that affected recognition by TCRs, although there was no mutation within the Env epitope. An analysis of peptide-HLA tetrameric complexes revealed that CD8 T cells exclusively specific for the Nef variant were generated following domination by the variant viruses. The variant-specific cells were capable of killing target cells and producing antiviral cytokines but showed impaired Ag-specific proliferation *ex vivo*, whereas wild-type specific cells had potent activities. Moreover, clonotypic CD8 T cells specific for the Pol variant showed diminished proliferation, whereas Env-specific ones had no functional heterogeneity. Taken together, our data indicate that antigenic variations that abolished TCR recognition not only resulted in escape from established CTL responses but also eventually generated another subset of variant-specific CTLs having decreased antiviral activity, causing an additional negative effect on antiviral immune responses during a chronic HIV infection. *The Journal of Immunology*, 2007, 178: 5513–5523.

Virus-specific CD8⁺ CTLs play a critical role in the control of persistent virus infections including those by HIV-1. However, recent studies show that HIV-specific CD8 T cell responses, measured by their ability to bind with peptide-HLA class I tetrameric complexes (HLA tetramers) or to secrete IFN- γ Ag specifically, are not correlated with the control of viremia in chronic HIV-1 infections (1, 2), suggesting a progressive functional defect in HIV-specific CTLs during a chronic infection that is not measurable by these assays. Accordingly, HIV-specific CD8 T cells in individuals with a primary infection and individuals with a long-term nonprogressive infection exhibit strong Ag-dependent *ex vivo* proliferative capacity, whereas those from patients with a progressive disease course lose such capacity (3–5). In addition, recent reports show that various degrees of impairment of the effector functions of virus-specific CD8 T cells are influenced by Ag persistence and Ag levels in mice and humans (5–8), suggesting that antiviral effectiveness of HIV-specific CD8 T cells can be impaired through repeated stimulation by the same cognate Ags. In contrast, it is reported that significant differences also exist in the effectiveness of HIV-specific CTLs among different specificities and restricting elements (9, 10), as well as among

TCR clonotypes within the same specificity (11, 12). Therefore, different Ags or a set of amino acid substitutions within an Ag may be involved differently in the generation of the altered antiviral effectiveness of HIV-specific CTLs.

It is becoming evident that the mutational escape of HIV from established CTL responses occurs in individual human hosts (13, 14). CTL escape mutations occur at critical sites in the CTL epitopes in the viral genome or in the flanking sequences encoding these epitopes, leading to altered Ag processing (15, 16), loss of peptide-HLA binding, or loss of TCR recognition. The two former consequences of these mutations result in the ultimate loss of epitopes to be presented on the surface of virus-infected cells for recognition by CTLs. In contrast, the latter consequence is thought to provide a relatively weak selective advantage for HIV, because this type of mutation results in the loss of recognition by some existing CTL lines while maintaining recognition by other cross-recognizing CTL subsets. It is not clear why mutations that affect TCR recognition are selected in the virus under CTL-mediated immune pressure even though they can provide only moderate selective advantage for the virus.

In the present study, we focused on CD8 T cell responses specific for the HIV-1 Pol, Env, and Nef optimal epitopes presented by HLA-B*35 in patients at the chronic phase of HIV-1 infection to ask whether and how antigenic variations of HIV-1 have an additional effect on the altered antiviral activity of HIV-specific CTLs. Sequence analysis of autologous viruses showed the existence of HLA-B*35-associated mutations within Pol and Nef epitopes that affected TCR recognition. HLA tetramer analysis revealed that the Nef variant-specific CD8 T cells were generated following domination by the variant viruses. The variant-specific cells had the ability to kill target cells and secrete antiviral cytokines but, interestingly, they showed impaired proliferation activity *ex vivo*. Similar defects in proliferative capacity were also observed in the variant Pol-specific CD8 T cells.

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Materials and Methods

Subjects

A total of seven individuals (HLA-B*35⁺) with chronic HIV infection (>2 years) followed at the AIDS Clinical Center, International Medical Center of Japan (Tokyo, Japan) were enrolled for functional analysis of HIV-specific CD8 T cells in this study. All subjects except patient (Pt)³ 42 (Pt-42) had been receiving antiretroviral therapy (see Table I for details). For autologous HIV-1 sequence analysis, a total of 42 individuals with chronic HIV infection (>2 years) followed at the same hospital as above were enrolled. Among them, 12 individuals expressed HLA-B*35 and the other 30 individuals did not express it. Thirty-eight of the total (11 for HLA-B*35⁺ and 27 for HLA-B*35⁻) had been receiving antiretroviral therapy. The study was conducted in accordance with the human experimentation guidelines of the International Medical Center of Japan and Kumamoto University (Kumamoto, Japan).

Sequence analysis of autologous HIV-1 and TCR-encoding genes

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm for 30 min) of patients' plasma, after which the viral RNA was extracted from them. A nested PCR was conducted by using sets of primers specific for the *pol*, *env*, and *nef* genes of HIV-1, as described earlier (17). PCR-amplified DNA fragments were gel purified and sequenced directly or cloned into a plasmid and then sequenced.

TCR-encoding genes of HIV-specific CD8 T cells were cloned and sequenced as previously described (18). Briefly, total RNA was prepared from T cell clones or FACS-sorted tetramer⁺ CD8⁺ cells, and cDNA encoding α and β TCRs were obtained by using a SMART PCR cDNA synthesis kit (Clontech Laboratories). Alignment of the V and J regions of α and β TCR genes was performed by using the ImMunoGeneTics database (<http://imgt.cines.fr>) created by M.-P. Lefranc (Institut de Génétique Humaine, Montpellier, France) (19).

Construction of Nef-expressing target cells

For target cells endogenously expressing Nef-GFP fusion proteins, DNA fragments encoding the Nef protein (HIV-1 NL43) and GFP were cloned into plasmid pcDNA3.1 (Invitrogen Life Technologies). A mutation, Met²⁰ to Ala, was introduced to abolish HLA class I down-regulation activity by Nef (20), and the Thr⁷⁵ to Arg mutation was achieved by site-directed mutagenesis. The m⁷GpppG-capped and poly(A)-tailed mRNAs were prepared in vitro by using a mMessage mMachine T7 Ultra kit (Ambion).

The mRNA samples were delivered to target cells by electroporation. Briefly, cells were suspended in a serum-free medium (Opti-MEM; Invitrogen Life Technologies) at the cell density of 4×10^6 cells/ml, mixed with 10 μ g of mRNA, and electroporated by using a Gene Pulser device (Bio-Rad). The cells were immediately transferred to medium (RPMI 1640 and 10% FBS), incubated at 37°C for 16 h, and then used as target cells. It should be noted that 15 \pm 5% of the cells had died (positive for 7-aminoactinomycin D (7-AAD) staining) by 16 h and that 85 \pm 5% cells of the viable cells expressed GFP as revealed by flow cytometric analysis.

Generation of T cell clones

CTL clones or lines were established by the stimulation of PBMCs with a synthetic peptide as previously described (18). Briefly, a bulk CTL culture was seeded at a density of 0.8 or 5 cells/well with a cloning mixture (irradiated allogeneic PBMC and C1R-B*3501 cells pulsed with 1 μ M peptide in RPMI 1640 with 10% FCS and 100 U/ml rIL-2). Two weeks later, cells positive for growth were tested for cytolytic activity by the ⁵¹Cr-release assay described below.

HLA stabilization assay

Peptide-binding activity for HLA-B*3501 was assessed by an HLA stabilization assay. RMA-S cells expressing HLA-B*3501 were cultured for 16 h at 26°C and then pulsed with various concentrations of peptide for 3 h at 26°C. The cells were then incubated at 37°C for 3 h and subsequently stained with an anti-HLA class I mAb (TP25.99). The surface expression level of HLA-B*3501 was evaluated by flow cytometry.

HLA tetramer analysis

The HLA-B*3501-tetramers in complex with a series of wild-type and variant peptides were prepared as previously described (18). Cryopreserved

PBMCs of HIV-positive (2×10^6) or -negative donors (3×10^6) were stained with the tetramer at 37°C for 15 min followed by anti-CD8-PerCP (BD Biosciences) or anti-TCR Abs at 4°C for 15 min. The anti-V δ 1 mAb (BD Biosciences) was kindly provided by L. Moretta (University of Genova, Genova, Italy) and the other anti-TCR mAbs were purchased from Pierce Endogen and Beckman Coulter. They were then washed twice and analyzed by flow cytometry. Dead cells were gated out by 7-AAD staining as needed.

Cytotoxic assay

The cytotoxic activity of the CTL clones was determined by a standard ⁵¹Cr-release assay as described previously (18). For peptide-pulsed target cells, ⁵¹Cr-labeled C1R-B*3501 cells (2×10^3 cells/well) were pulsed with various concentrations of the peptide and incubated with T cells for 4 h at 37°C. For virus-infected target cells, autologous EBV-transformed B cell lines were infected with vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 HXB2D. It should be noted that ~75% of the cells were positive for the intracellular p24 Gag Ag when HIV-infected cells were used for CTL assays. The cells were incubated with T cells for 6 h at 37°C after having been labeled with ⁵¹Cr.

For cytotoxic assays ex vivo, cryopreserved PBMCs that had been preincubated for 2 h at 37 °C in RPMI 1640 containing 20% FCS were separated in CD8⁺ and CD8⁻ subsets by using anti-CD8 mAb-conjugated magnetic beads (Miltenyi Biotec), and the resultant CD8⁺ and CD8⁻ cells were used for effector and target cells, respectively. Autologous EBV-transformed B cells were also used for target cells as needed. The effector cells were incubated at various ET ratios in RPMI 1640 supplemented with 10% FCS and 100 U/ml rIL-2 for 6 h and then mixed with the ⁵¹Cr-labeled target cells (3000 cells/well) that had been pulsed with the wild-type and variant peptides. Cells were incubated for additional 12 h at 37°C.

Intracellular cytokine staining assay

Cryopreserved PBMC (2×10^6) of HIV-positive or -negative individuals were first incubated overnight in RPMI 1640 supplemented with 10% FCS and 200 U of IL-2. They were then incubated in the absence or presence of 1 μ M Nef75 peptide (RPQVPLRPMTY or TPQVPLRPMTY) for 2 h at 37°C. Brefeldin A (10 μ g/ml) was then added and the cells were incubated for an additional 4 h. Cells were permeabilized and stained with mAbs specific for IFN- γ , TNF- α , and IL-2 (BD Biosciences) as previously described (21).

Ex vivo proliferation assay

To analyze Ag-specific expansion of HLA-tetramer⁺ cells, cryopreserved PBMCs (2×10^6) of the HIV-positive or -negative donors were first incubated as described above. They were then stimulated with irradiated EBV-transformed B cells expressing HLA-B*3501 that had been pulsed with 1 μ M Nef75 peptide (RPQVPLRPMTY or TPQVPLRPMTY) or transfected with mRNA encoding GFP alone, Nef-GFP, or Nef variant-GFP fusion proteins. The cells were cultured at 37°C for 12 days in the same medium. A portion of the stimulated cells (5×10^5) were stained with HLA tetramers and anti-CD8 and anti-CD3 mAbs as previously described (12).

To further analyze proliferating cells in response to Ag stimulation, cryopreserved PBMC samples of HIV-positive donors were first incubated in RPMI 1640 containing 20% FCS for 2 h and then labeled with CFSE (Molecular Probes) as directed by the manufacturer's recommendation. Aliquots of cells (2×10^6 each) were separately incubated in RPMI 1640 with 10% FCS and 100 U/ml rIL-2 and stimulated with IL-2 alone or in combination with a cognate peptide at 1 μ M concentration. After 6 days, the resultant cells were collected, stained with PE-conjugated HLA tetramers followed by anti-CD8 and anti-CD3 mAbs, and analyzed by flow cytometry. The CD3⁺CD8⁺ subsets were gated and the fluorescence intensity of CFSE within the tetramer⁺ cells were analyzed.

Statistical analysis

Results were given as the mean \pm SD. Statistical analysis of significance (*p* values) was based on paired or unpaired two-tailed *t* tests.

Results

HLA-B*35-restricted CD8 T cell responses to HIV-1

We first examined cross-sectionally the CD8 T cell responses of chronic HIV-infected patients toward HIV-1 optimal epitopes (refer to the database: <http://www.hiv.lanl.gov>) restricted by HLA-B*35. PBMCs isolated from HLA-B*35⁺ patients (*n* = 7; see Table I for the clinical state of the subjects) were analyzed with

³ Abbreviations used in this paper: Pt, patient; 7-AAD, 7-aminoactinomycin D; PD-1, programmed death 1.

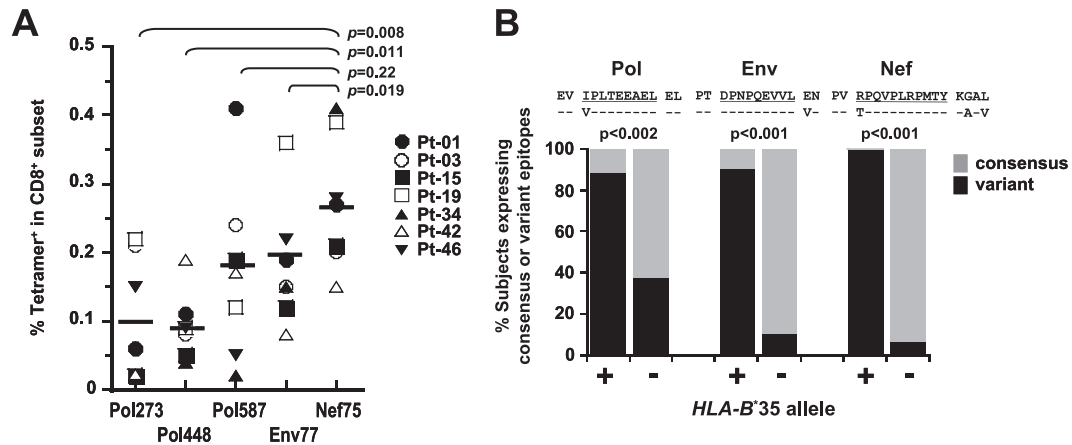


FIGURE 1. HLA-B*35-restricted CD8 T cell responses to HIV-1 and HIV-1 variants associated with *HLA-B*35* at the population level. **A**, Cryopreserved PBMC of seven HIV-infected patients were stained with HLA-B*35 tetramers in complex with the indicated peptides. The PBMC samples used for each patient were taken at the following times: Pt-01, September 1999; Pt-03, June 2002; Pt-15, June 2001; Pt-19, May 2001; Pt-34, April 2001; Pt-42, August 2001; and Pt-46, April 2001 (see Table I). The frequency of tetramer⁺ CD8⁺ in the total CD8⁺ subset was plotted in the graph. Bars indicate the mean for each group. It should be noted that the background level of staining was 0.025% for all tetramers used as determined by the data from at least five HIV-negative donors (mean + 3 SD). **B**, Autologous viral RNA was prepared from chronic HIV-infected patients positive ($n = 12$) or negative ($n = 30$) for HLA-B*35. The Pol-, Env-, and Nef-encoding regions within and flanking the HLA-B*35-restricted CTL epitopes were specifically amplified by PCR and directly sequenced. Amino acid sequences indicated are clade B consensus (*upper line*) and variants associated with HLA-B*35 (*lower line*). Epitope regions are underlined and dashes denote amino acids identical with those of the clade B consensus sequence.

HLA-B35 tetramers in complex with a series of epitope peptides having clade B consensus sequences. The frequencies of tetramer⁺ CD8⁺ cells among the total CD8⁺ cells were shown in Fig. 1A and as follows: Pol273 (VPLDKDFRKY), $0.10 \pm 0.013\%$; Pol448 (IPLTEEAEL), $0.093 \pm 0.007\%$; Pol587 (EPIVGAETF), $0.17 \pm 0.019\%$; Env77 (DPNPQEVVL), $0.18 \pm 0.013\%$; and Nef75 (RPQVPLRPMTY), $0.26 \pm 0.09\%$. The immunodominant Nef75 epitope, subdominant Pol448 epitope, and intermediate Env77 epitope were selected for further analysis in this study.

Evolution of HIV-1 variants associated with the *HLA-B*35* allele

The analysis of long-standing changes in autologous virus sequences of the HLA-B*35⁺ chronic HIV-infected patients ($n = 7$) showed Ile to Val and Arg to Thr changes at Pol-448 and Nef-75, respectively, and Gly to Ala and Leu to Val changes in the flanking region of the Nef epitope (Table I). Despite the absence of a sequential change within the Env77 epitope, there was a substantial difference at Env86 (Table I).

Further cross-sectional analysis of the autologous virus sequence by using 42 chronic patients (12 HLA-B*35⁺ and 30 HLA-B*35⁻) clearly showed that the amino acid changes observed in

Table I were all significantly associated with *HLA-B*35* (Fig. 1B), suggesting that these sequence variations were selected by CD8 T cell-mediated immune responses restricted by HLA-B*35.

Interestingly, replication-competent HIV-1 NL43 carries amino acid residues identical with those of *HLA-B*35*-associated mutations, suggesting the minimal effects of these variants on the virus replication. It is also noteworthy that we found mutations in autologous viruses of HLA-B*35⁺ patients even though most of them (11 of 12) had been receiving antiretroviral therapy, confirming a previous report showing the evolution of CTL escape mutations even when virus replication was suppressed by antiretroviral therapy (22).

Thr⁷⁵ of Nef as a CTL escape mutation that abolished TCR recognition

By conducting an HLA stabilization assay, we first examined whether the variant peptide (Thr⁷⁵ peptide: TPQVPLRPMTY) had lost its ability to bind with HLA-B*3501. Interestingly, the binding activity between HLA-B*3501 and the variant Thr⁷⁵ peptide was ~10-fold higher than that of the wild-type peptide (Arg⁷⁵ peptide: RPQVPLRPMTY), as the concentrations of the Arg⁷⁵ and Thr⁷⁵

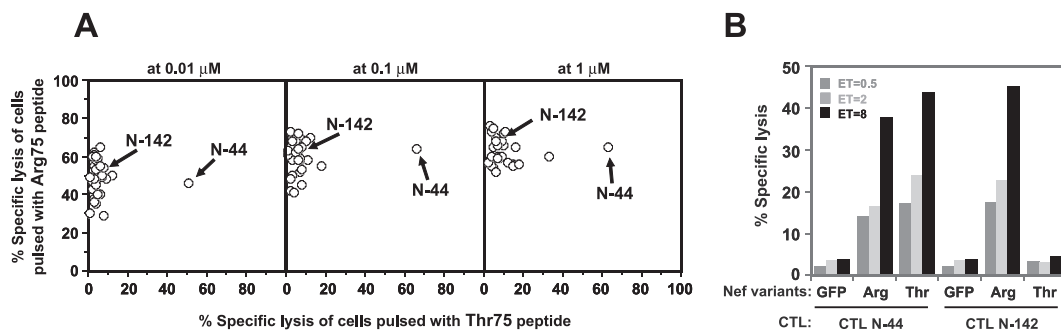


FIGURE 2. Generation of CTL clones specific for Nef and its variant in vitro. **A**, Cytotoxic activity of a representative set of 50 CTL clones toward C1R-B*3501 cells pulsed with the indicated concentrations (0.01, 0.1, and $1 \mu\text{M}$) of the Arg⁷⁵ or Thr⁷⁵ peptide at an E:T ratio of 2:1. The data obtained for CTL N-44 and N-142 are indicated. Specific lysis in the absence of peptide was $<5\%$. **B**, Cytotoxic activity of CTL clones CTL N-44 and N-142 toward C1R-B*3501 cells expressing the Arg⁷⁵-Nef-GFP protein, the Thr⁷⁵-Nef-GFP protein, or GFP alone. Of the target cells, $85 \pm 5\%$ were GFP⁺.

Table II. Summary of representative TCRs specific for HIV-1 antigens presented by HLA-B*35

Specificity	Clonotypic mAb	CTL Clone	TCR Usage		
			V Region	J Region	CDR 3
Pol448	Vδ 1	CTL 55	TRDV1*01	TRAJ54*01	CALGEGGAQKLVF CASRTGGTLIEQYF
	Vα 12	CTL 589	TRBV6-1*01 TRAV19*01 TRBV5-4*01	TRAJ53*01 TRBJ2-5*01	CALSHNSGGSNYKLTFGKG CASSFRGGKTQYFPGP
Env77	None	CTL E-113	TRAV26-1*01 TRBV15*02	TRAJ40*01 TRBJ1-1*01	CIVRERGTYKYIF CATRGGGLNTEAFF
	Vβ 5(c)	CTL E-118	TRAV13-2*01 TRBV5-1*01	TRAJ13*01 TRBJ2-1*01	CAETPNSGGYQKVTF CASSLFPGLAGLSSYNEQFF
Nef75	Vβ 9	CTL N-27	TRAV12-3*01 TRBV3-1*01	TRAJ49*01 TRBJ2-3*01	CAMSEGTGNQYFYF CASSQTMGLDLTDTQYF
	Vβ 7	CTL N-44	TRAV21*02 TRBV4-1*01	TRAJ21*01 TRBJ2-1*01	CAVRGTSYGKLTFF CASSQGPWTGVDNEQFF
	Vβ 3	CTL N-117	TRAV21*02 TRBV28*01	TRAJ24*01 TRBJ2-2*01	CAVLKSDSWGKLFQF CASSSSTGLETGTGELFF
	Vβ 3	CTL N-142	TRAV1-1*01 TRBV28*01	TRAJ3*01 TRBJ2-5*01	CAVRGKYSSASKIIF CASSKNRERETQYF

peptides that yielded 50% of the maximum binding level were 56.2 ± 4.6 and $5.70 \pm 0.27 \mu\text{M}$, respectively.

We then sought to generate CTL clones specific for the Arg⁷⁵ or Thr⁷⁵ peptide in vitro. By a 1 μM Arg⁷⁵ peptide stimulation of PBMC (Pt-01, Pt-03, and Pt-19), >50 CTL clones were generated. The cytotoxic activity of these CTL clones toward cells pulsed with the Arg⁷⁵ or Thr⁷⁵ peptide showed that all clones except one were exclusively specific for the Arg⁷⁵ peptide (Fig. 2A). The Vβ³⁺ T cells showed ~50% frequency among the Arg⁷⁵-specific CTL clones, and TCR usage of representative Arg⁷⁵-specific CTL clones (CTL N-27, N-117, and N-142) is shown in Table II. Remarkably, only one of >50 CTL clones (CTL N-44; Fig. 2A), which had Vβ⁷⁺ TCR (Table II), showed cytotoxic activity toward cells pulsed with either Arg⁷⁵ or Thr⁷⁵ peptides, although an additional CTL clone showed partially cross-reactive capacity at the high concentrations of the Thr⁷⁵ peptide (Fig. 2A). These data

suggested an extremely low frequency of precursors of such cross-reactive CD8 T cells in these subjects (see below).

Next, we found that the cross-reactive CTL N-44 had comparable killing activity toward C1R-B*3501 cells expressing either the Arg⁷⁵- or Thr⁷⁵-Nef-GFP fusion protein (Fig. 2B), indicating that the Thr⁷⁵ peptide was endogenously processed and extracellularly presented by HLA-B*3501 for CTL recognition. Moreover, a representative Arg⁷⁵-specific CTL clone, CTL N-142, showed killing activity toward cells expressing the Arg⁷⁵-GFP fusion protein but not toward those expressing the Thr⁷⁵-GFP one (Fig. 2B), indicating again that Thr⁷⁵ was a CTL escape mutation that abolished TCR recognition.

It should be noted, however, that despite a number of attempts we failed to generate Thr⁷⁵-specific CTL clones or lines by stimulating the PBMCs of all seven patients with the Thr⁷⁵ peptide although the samples were taken when their autologous HIV-1 had

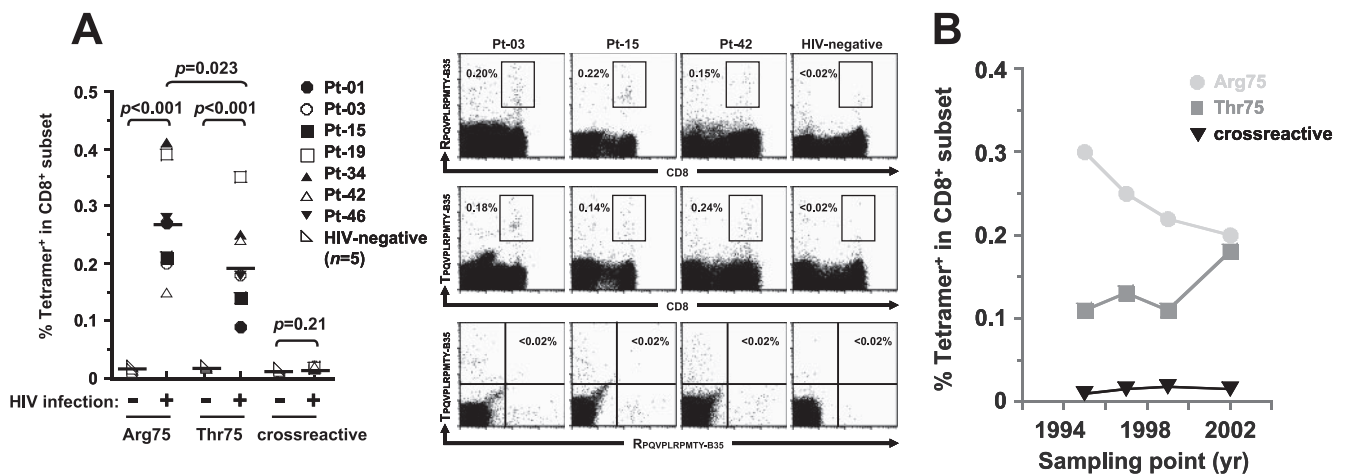


FIGURE 3. CD8 T cell responses toward Nef and its variant ex vivo. A, PBMCs of seven HIV-infected patients, the same as those used in Fig. 1A, and five HIV-negative donors were stained with PE and allophycocyanin-conjugated HLA-B*35 tetramers in complex with the Arg⁷⁵ and Thr⁷⁵ peptides, respectively. After the dead cells had been gated out by staining with 7-AAD, the remaining cells were analyzed for their binding to each of the tetramers and for their cross-reactivity. The frequency of tetramer⁺CD8⁺ in the total CD8⁺ subset was plotted in the graph. Bars indicate the mean for each group. Representative dot plots for three HIV-infected subjects (Pt-03, Pt-15, and Pt-42) and a HIV-negative donor are shown with tetramer⁺ frequency values in each dot plot. It should be noted that identical results were obtained when the fluorochromes of HLA tetramers were reversed. B, PBMCs of Pt-03 taken at several different time points were stained with HLA-B*35 tetramer in complex with the Arg⁷⁵ (RPQVPLRPMTY) or Thr⁷⁵ (TPQVPLRPMTY) Nef peptide and subsequently with anti-CD8 mAb and 7-AAD. The frequency of tetramer⁺CD8⁺ in the total CD8⁺ subset was calculated and plotted in the graph.

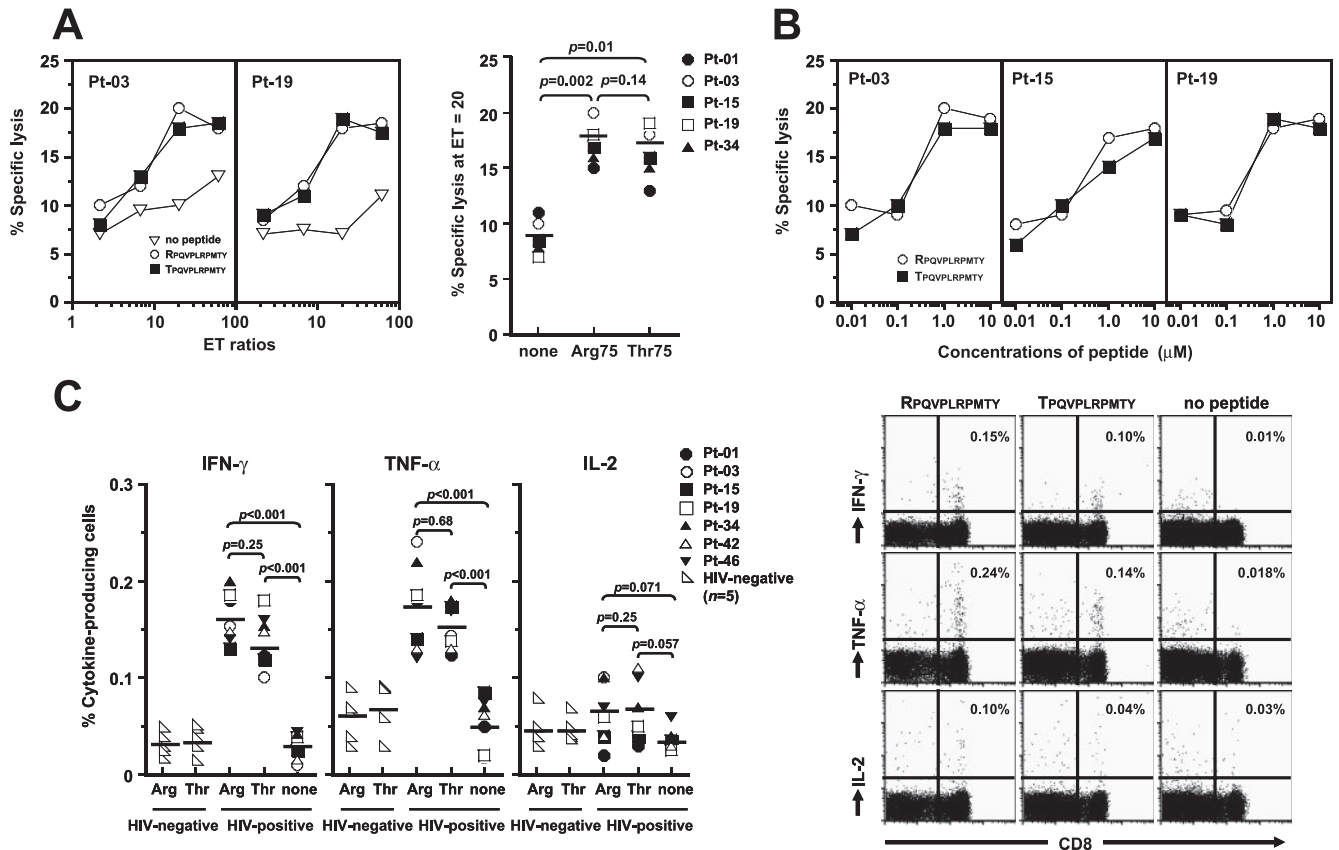


FIGURE 4. Antiviral effector functions of CD8 T cells specific for Nef epitopes. **A**, The cytotoxic activity of CD8⁺ cells isolated from PBMC samples of HIV-positive donors (Pt-01, Pt-03, Pt-15, Pt-19, and Pt-34) was examined by ⁵¹Cr-release assay directly ex vivo. The autologous target cells alone or pulsed with 1 μM Arg⁷⁵ or Thr⁷⁵ peptide were incubated with the effector CD8⁺ cells at E:T ratios of 2.2, 6.7, 20, and 60 for 12 h. At the left, a representative set of data for Pt-03 and Pt-19 is shown. At the right, cytotoxic activity obtained at an E:T ratio of 20 is shown. Horizontal bars in the graphs indicate the mean for each group. It should be noted that the cytotoxic activity of CD8⁺ cells was not examined for Pt-42 and Pt-46 because of the insufficient number of cells available for this assay. **B**, The cytotoxic activity of CD8⁺ cells isolated from PBMC samples of HIV-positive donors (Pt-03, Pt-15, and Pt-19) was examined by the ⁵¹Cr-release assay with the autologous target cells pulsed with the indicated concentrations of the Arg⁷⁵ or Thr⁷⁵ peptide at an E:T ratio of 20. Specific lysis in the absence of peptide is shown in **A**. **C**, PBMC samples, the same as those used in Fig. 1A, were stimulated or unstimulated with 1 μM Arg⁷⁵ or Thr⁷⁵ peptide for 6 h and then stained intracellularly with anti-IFN- γ , TNF- α , or IL-2 mAbs. The frequencies of the subsets exhibiting cytokine⁺CD8⁺ cells within the CD8⁺ cell population are indicated in the graphs. Horizontal bars in the graphs indicate the mean for each group. At the right a representative set of dot plots for Pt-03 is shown with cytokine⁺ frequency values at the upper right corner of each dot plot.

Thr⁷⁵ (Table I), suggesting a lack of Thr⁷⁵-specific T cell precursors due to original antigenic sin or a lack of proliferation capacity in these subjects.

HLA tetramer analysis of CD8 T cells specific for Nef and its variant ex vivo

To see whether the Thr⁷⁵ variant epitope was recognized by CD8 T cells, we obtained PBMC samples from seven chronically infected patients (Table I) after the variant viruses had become dominant and analyzed them ex vivo by using HLA tetramers. The frequencies of the RPQVPLRPMTY-B35 and TPQVPLRPMTY-B35 tetramer⁺ subsets within the CD8⁺ cells of the seven HIV-infected patients were significantly above the background level, being 0.261 ± 0.094 and $0.186 \pm 0.081\%$, respectively; whereas those of five HIV-negative donors were 0.015 ± 0.0045 and $0.018 \pm 0.009\%$, respectively (Fig. 3A). The frequencies of the cross-reactive fractions were $<0.02\%$ in all samples tested (Fig. 3A). These data indicate that the Thr⁷⁵ and Arg⁷⁵ peptides were immunogenic and exclusively recognized by a different subset of CD8 T cells widely in chronic HIV-infected patients having HLA-B*35.

Longitudinal analysis of Pt-03 PBMC showed that the CD8 T cells specific for the Arg⁷⁵ peptide decreased in frequency and

those specific for the Thr⁷⁵ peptide increased (Fig. 3B). The increase in the frequency of Thr⁷⁵-specific CD8 T cells appeared to occur following the dominance of the Thr⁷⁵ variant over the autologous virus (Table I). However, the question of whether Thr⁷⁵-specific CD8 T cells were absent before the autologous virus developed the Thr⁷⁵ mutation could not be examined because PBMC samples during the primary infection were not available in this study. However, considering the following three points, namely that Thr⁷⁵ (22 of 390; 5.6%) was rarely found in the Los Alamos database compared with Arg⁷⁵ (302 of 390; 77.4%), the autologous virus from five of seven patients developed the Arg to Thr mutation during chronic infection (Table I), and cross-reactive CD8 T cell subsets were barely found in all subjects, it is most likely that Thr⁷⁵-specific CD8 T cells were newly generated in vivo in response to the Thr⁷⁵ epitope after the autologous virus had undergone the Thr⁷⁵ mutation.

Cytotoxic activity of CD8 T cells specific for Nef and its variant ex vivo

We then examined the Arg⁷⁵- or Thr⁷⁵-specific CD8 T cells for their Ag-specific killing activity. Because the variant Thr⁷⁵-specific CTL lines or clones could not be established in vitro (Fig. 2A) although the Thr⁷⁵-specific CD8 T cells were found ex vivo by the

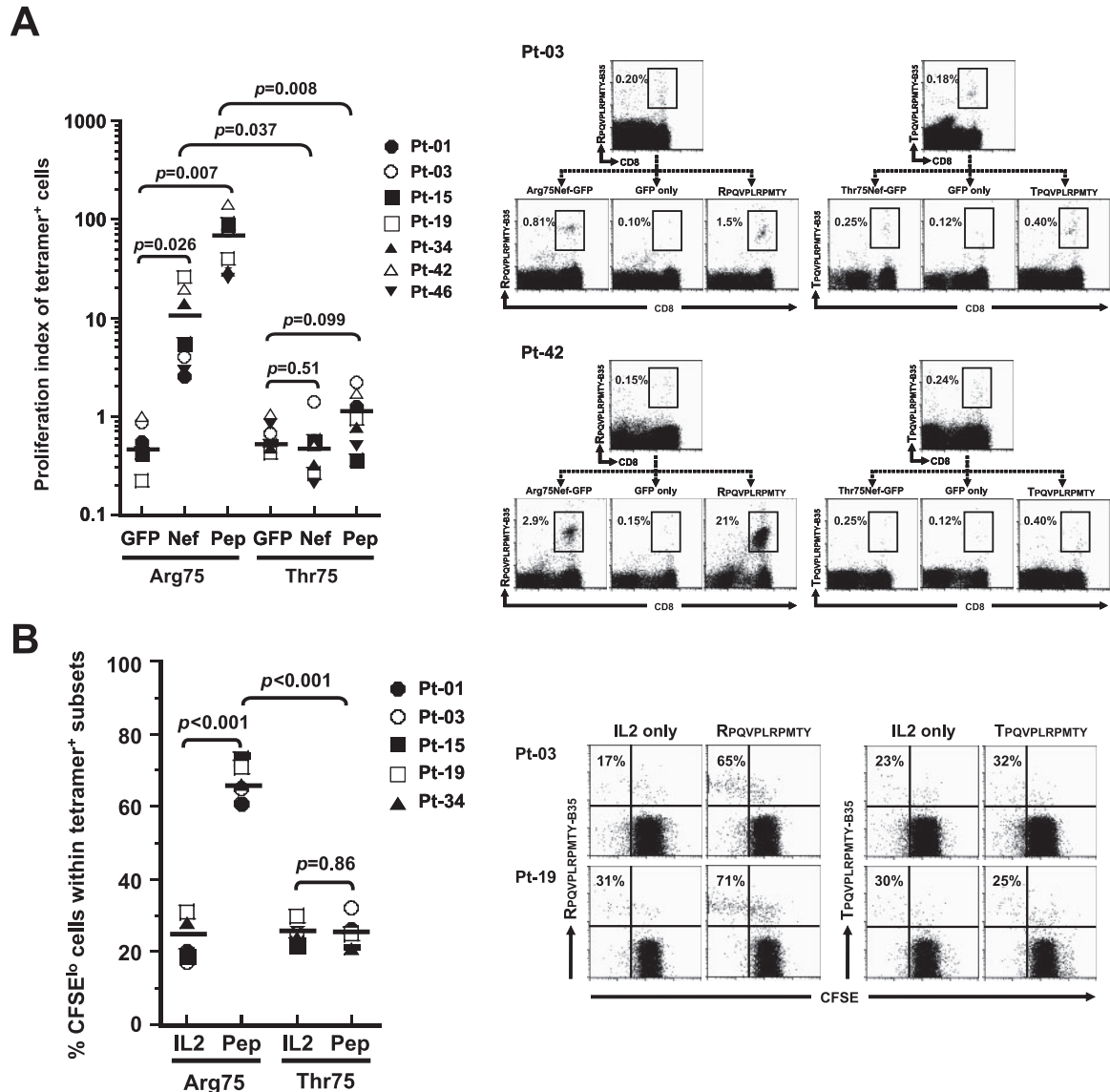


FIGURE 5. Proliferative capacity of CD8 T cells specific for Nef epitopes. *A*, The PBMC samples, the same as those used in Fig. 1*A*, were stimulated with irradiated C1R-B*3501 cells or autologous EBV-transformed B cells that had been pulsed with 1 μ M Arg⁷⁵ or Thr⁷⁵ peptide or had been transduced with mRNA encoding GFP or Nef-GFP fusion proteins carrying Arg⁷⁵ or Thr⁷⁵. Twelve days after the stimulation, the cells were analyzed with the HLA-B*35 tetramers. As shown in the graph at the *left*, the proliferation index was obtained as the ratio of tetramer⁺ frequencies after and before stimulation. Horizontal bars in the graphs indicate the mean for each group. At the *right*, a representative set of dot plots for Pt-03 and Pt-42 is shown with tetramer⁺ frequency values. *B*, The PBMC samples of HIV-positive donors (Pt-01, Pt-03, Pt-15, Pt-19, and Pt-34) were first labeled with CFSE and incubated in a medium containing human rIL-2 (100 U/ml). The cells were stimulated with IL-2 alone or in combination with 1 μ M Arg⁷⁵ or Thr⁷⁵ peptide. After 6 days of culture, the cells were then stained with indicated HLA-tetramers and anti-CD8 and -CD3 Abs. The CD3⁺ CD8⁺ subsets were gated and analyzed for their fluorescence intensity of CFSE. The frequency of CFSE^{lo} cells within the tetramer⁺ subset is shown in the graph at the *left*. Horizontal bars in the graph indicate the mean for each group. At the *right*, a representative set of dot plots for Pt-03 and Pt-19 is shown with CFSE^{lo} frequency values. It should be noted that CFSE dilution assay was not conducted for the subjects Pt-42 and Pt-46 because of the insufficient number of cells available for this assay.

tetramer analysis (Fig. 3*A*), we sought to analyze the Ag-specific cytolytic activity of CD8 T cells by a ⁵¹Cr-release assay directly *ex vivo*. CD8⁺ and CD8⁻ cells were first isolated from the PBMCs of HIV-positive donors by a magnetic bead separation system and used as effector and target cells, respectively, for the cytolytic assay. The CD8⁺ cells of the subjects tested showed cytotoxic to target cells pulsed with Arg⁷⁵ and Thr⁷⁵ peptides (1 μ M) at an E:T ratio of 20 with specific lysis of 17.2 \pm 1.9% and 16.2 \pm 2.4%, respectively (Fig. 4*A*), whereas the background level of their specific lysis in the absence of the peptide was 8.9 \pm 1.6% (Fig. 4*A*). As also shown in the representative data for Pt-03 and Pt-19 with various E:T ratios, the cytolytic activity of CD8⁺ cells specific for the Arg⁷⁵ and Thr⁷⁵ peptides was not substantially different (Fig. 4*A*).

Peptide titration experiments were also performed using CD8⁺ cells of Pt-03, Pt-15, and Pt-19, as these subjects showed relatively high cytolytic activity *ex vivo* (Fig. 4*A*). As shown in Fig. 4*B*, the cytolytic activity of CD8⁺ cells toward cells pulsed with the Arg⁷⁵ and Thr⁷⁵ peptides was not much different in any range of the peptide concentration tested, suggesting the comparable functional avidity of both CD8 T cell subsets toward the given Ag.

Production of antiviral cytokines of CD8 T cells specific for Nef and its variant ex vivo

We further examined the ability of Nef-specific CD8 T cells to produce antiviral cytokines. The data show that IFN- γ and TNF- α responses were all significantly above the background level, being

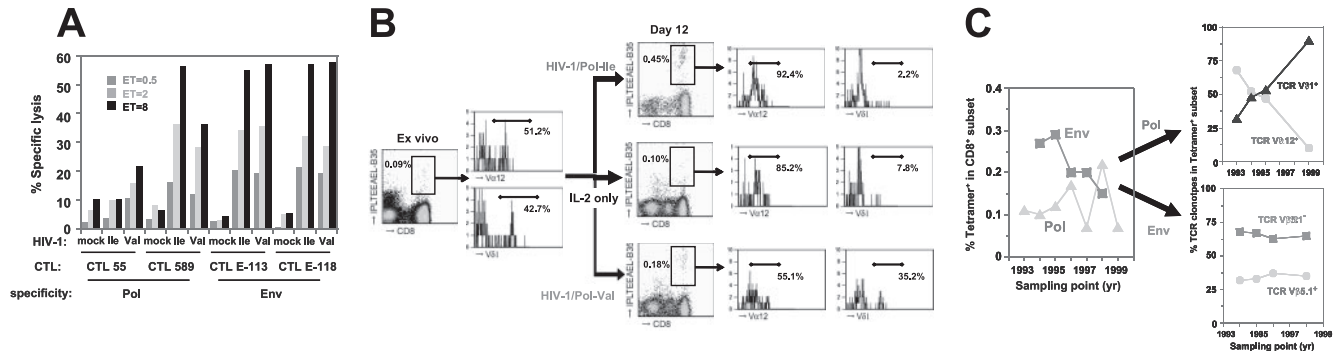


FIGURE 6. Functional analysis of CD8 T cells specific for Pol and Env epitopes. *A*, Cytotoxic activity of four different CTL clones, CTL 55, CTL 589, E-113, and E-118, toward autologous EBV-transformed B cells infected with mock or vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 expressing Ile or Val at Pol 448. It should be noted that $\sim 75\%$ of the target cells were positive for p24 Gag protein expression. *B*, Pt-03 PBMCs were stimulated with IL-2 alone or with autologous EBV-transformed B cells infected with the same viruses as above. After 12 days in culture, the cells were stained with a HLA-B*35 tetramer in complex with Pol-Ile peptide and then additionally with anti-CD8 and anti-TCR mAbs. The CD8⁺ tetramer⁺ subsets were gated and analyzed for their TCR usage. *C*, PBMCs of Pt-03 taken at several different time points were separately stained with the HLA-B*35 tetramer in complex with the Pol (IPLTEEAEL) or Env (DPNPQEVVL) peptide and subsequently with anti-CD8 and anti-TCR mAbs. The CD8⁺tetramer⁺ subsets were gated and analyzed for their TCR usage.

0.161 ± 0.025 and $0.171 \pm 0.047\%$, respectively, in response to the Arg⁷⁵ peptide and 0.140 ± 0.027 and $0.150 \pm 0.023\%$, respectively, in response to the Thr⁷⁵ peptide (Fig. 4*B*). In contrast, the IL-2 response was not significant, being 0.061 ± 0.031 and $0.063 \pm 0.031\%$ in response to the Arg⁷⁵ and Thr⁷⁵ peptides, respectively (Fig. 4*B*). Also, the extent of generation of cytokine-producing cells (IFN- γ and TNF- α) in response to Arg⁷⁵ and Thr⁷⁵ peptides was not significantly different (Fig. 4*B*), indicating that CD8 T cells specific for the Arg⁷⁵ and Thr⁷⁵ peptides were comparably functional in terms of Ag-specific cytokine production.

Proliferation capacity of CD8 T cells specific for Nef and its variant ex vivo

We next tested the Nef-specific CD8 T cells for their Ag-specific proliferation capacities ex vivo as assessed by both the expansion of tetramer⁺ cells and the dilution of CFSE fluorescence intensity, because recent reports showed that HIV-specific CD8 T cells with progressive infection lose their ability to proliferate in response to Ag (3, 4).

PBMC samples were mixed with autologous EBV-transformed B cell lines or C1R-B*3501 cells expressing GFP or Nef-GFP fusion proteins carrying Arg⁷⁵ or Thr⁷⁵ in the absence or presence of the synthetic Arg⁷⁵ or Thr⁷⁵ peptide, respectively, and then analyzed by using HLA tetramers on days 0, 6, and 12. Because continuous expansion was observed even between days 6 and 12 (data not shown), the proliferation index was calculated by dividing the frequency of tetramer⁺ cells at day 12 after stimulation with a given Ag by the frequency of tetramer⁺ cells ex vivo (Fig. 5*A*). We considered a proliferation index of <1.0 to indicate no proliferation capacity of the subset. All subjects tested showed significant expansion of Arg⁷⁵-specific CD8 T cells upon stimulation with Arg⁷⁵-Nef-GFP and the Arg⁷⁵ peptide with an index value of 11.4 ± 7.11 and 62.4 ± 41.9 , respectively, whereas they did not show Ag-specific expansion upon stimulation with GFP alone, Thr⁷⁵-Nef-GFP, or the Thr⁷⁵ peptide because the index value was 0.635 ± 0.166 , 0.554 ± 0.279 or 1.11 ± 0.66 , respectively (Fig. 5*A*). Also, the proliferation index of untreated PBMC (IL-2 alone) was 0.742 ± 0.203 (data not shown).

To further confirm the difference in proliferative capacity between Arg⁷⁵- and Thr⁷⁵-specific CD8 T cells, we analyzed them by monitoring CFSE fluorescence of tetramer⁺ cells upon stimulation with the cognate peptides for 6 days. Upon stimulation with IL-2 alone, the frequencies of proliferating cells as measured by

CFSE^{low} cells within the tetramer⁺ cells were 23.0 ± 6.1 and $25.8 \pm 3.2\%$ for Arg⁷⁵ and Thr⁷⁵-specific cells, respectively (Fig. 5*B*). Stimulation with the Arg⁷⁵ peptide resulted in a significant increase of proliferating CD8 T cells specific for the Arg⁷⁵ peptide, as the frequency of CFSE^{low} cells within the tetramer⁺ cells was $67.2 \pm 4.8\%$ (Fig. 5*B*). In contrast, the Thr⁷⁵ peptide stimulation showed virtually no change in the frequency of CFSE^{low} cells within the tetramer⁺ cells ($25.4 \pm 4.1\%$) compared with stimulation with IL-2 alone (Fig. 5*B*). Thus, the proliferative capacity of CD8 T cell subsets obtained by a CFSE dilution assay (Fig. 5*B*) was in good agreement with the data obtained by quantification of the tetramer⁺ cell expansion assay (Fig. 5*A*). These data indicate that CD8 T cells specific for the Arg⁷⁵ peptide retained their Ag-dependent proliferative capacity in patients with chronic HIV-1 infection, whereas those specific for the variant Thr⁷⁵ peptide did not have such capacity (Fig. 5, *A* and *B*) although both cells showed comparable Ag-specific cytolytic activity (Fig. 4*A*) and cytokine secretion activity (Fig. 4*B*). Also, this observation may explain the failure to generate CTL clones or lines specific for the Thr⁷⁵ peptide in vitro (see above).

Functional analysis of CTL clones specific for Pol, Env, and variant epitopes

Next we examined CD8 T cell responses to Pol, Env, and their variant epitopes. The binding activity between HLA-B*3501 and the wild-type (Pol-Ile; IPLTEEAEL) or the variant epitope (Pol-Val; VPLTEEAEL) was comparable, and analysis of Pt-03 PBMC with tetramers in complex with either peptide showed that all tetramer⁺ cells were cross-reactive for both tetramers (data not shown) and that they consisted of two different clonotypes, V $\delta 1$ ⁺ and V $\alpha 12$ ⁺ (Table II). The representative clones, which had been generated by stimulation with the Pol-Ile peptide, were CTL55 (21) and CTL589 (18), respectively. CTL589 was cytotoxic toward cells infected with HIV-1 expressing Pol-Ile or Pol-Val (HIV-1_{Pol-Ile} and HIV-1_{Pol-Val}, respectively), whereas CTL55 killed HIV-1_{Pol-Val}-infected cells but not HIV-1_{Pol-Ile}-infected cells (Fig. 6*A*), indicating that CTL55 was exclusively specific for the variant Pol-Val epitope in terms of virus-infected cells as a target.

TCR analysis of a number of CTL clones specific for the Env epitope generated from Pt-03 PBMCs revealed that they also consisted of two different TCR clonotypes (Table II). Representative CTL clones CTL E-113 and E-118 were comparably cytotoxic

toward cells infected with HIV-1_{Pol-Ile} or HIV-1_{Pol-Val} (Fig. 6A), suggesting no functional difference between Env-specific CTLs. Taken together, the data suggest that differential functional cytotoxicity of CTL clonotypes toward wild-type and variant HIV-1 was caused by antigenic variations of the Pol epitope in autologous HIV-1.

Impaired proliferation of CD8 T cells specific for the Pol variant epitope ex vivo

We then tested the Pol-specific CD8 T cells for their proliferation activity in response to HIV-1_{Pol-Ile} and HIV-1_{Pol-Val} ex vivo. Pt-03 PBMCs were cocultured with cells infected with mock (IL-2 alone), HIV-1_{Pol-Ile}, or HIV-1_{Pol-Val} for 12 days and then stained with HLA-B35 tetramers. The proliferation index of the V α 12⁺tetramer⁺ subset was ~10, 2, and 1 when the cells were stimulated with HIV-1_{Pol-Ile}, HIV-1_{Pol-Val}, and IL-2 alone, respectively (Fig. 6B), indicating that the V α 12⁺ CD8 T cells had potent proliferation activity in response to the Pol-Ile epitope. However, the proliferation index of the V δ 1⁺ tetramer⁺ subset was <1.5 in response to both viruses (Fig. 6B), indicating the impaired Ag-specific proliferation of this subset of CD8 T cells specific for the variant Pol-Val epitope. Thus, not only CD8 T cells specific for the variant Nef epitope but also those specific for the variant Pol epitope showed impaired Ag-specific proliferation activity ex vivo (Figs. 5, A and B, and 6B).

Finally, we longitudinally analyzed the changes in tetramer⁺ frequencies specific for Pol and Env epitopes and their constituent T cell clonotypes of subject Pt-03. TCR clonotypes within the Pol-specific CD8⁺ cell population showed an increase in the frequency of the functionally impaired V δ 1⁺ subset over time (Fig. 6C), suggesting that the sum of antiviral effector functions of Pol-specific CD8 T cells had declined. The time course of this change seemed to have occurred after the appearance of the Ile to Val mutation in the autologous HIV-1 (Table I). In contrast, an analysis of the Env-specific CD8 T cell response revealed that the frequency of the V β 5(c)⁺ T cell clonotypes within the Env-specific tetramer⁺ subset was virtually constant (Fig. 6C). These data suggest that T cell clonotypes within the same specificity had not basically changed over time in the absence of significant variations within the epitope, thus highlighting the substantial changes in specificity and clonotypes observed in CD8 T cells specific for Pol and Nef epitopes with mutations at TCR contact sites.

Discussion

We showed herein that the CTL escape mutations that abolished TCR recognition had the ability to recruit variant-specific CTLs using different TCR clonotypes after the autologous HIV-1 had become dominated by the variant during the chronic phase of an HIV-1 infection in patients with HLA-B*35. However, because these variant-specific CTLs did not have potent Ag-specific proliferation capacity, this recruitment of CTLs barely correlated with the increased antiviral effectiveness of HIV-specific CTLs, although the breadth or magnitude of HIV-specific CTL responses was apparently maintained. Thus, these data demonstrate that CTL escape mutations that abolished TCR recognition not only led to escape from established wild-type-specific CTL responses but also could eventually have the additional effect of generating variant-specific CTLs with impaired proliferative capacity. Apparently, this observation provides evidence that supports the paradigm in CTL escapology in which CTL escape virus variants can persist only if the host is unable to mount an immune response against the variant epitopes or if the newly generated variant-specific immune responses are not as effective as the established wild type-specific ones.

It has recently been reported that the functional heterogeneity and loss of proliferative activity of virus-specific CD8 T cell responses is influenced by Ag persistence and Ag levels in mice and humans (5–7). The data obtained in this study showing a loss of proliferative capacity of variant-specific CTLs despite their having an IFN- γ -producing activity are surprisingly similar to those data showing virus-specific CTLs functionally impaired by chronic Ag exposure in mice chronically infected with the lymphocytic choriomeningitis virus (6). Thus, our data can be interpreted to indicate that when the CTL escape virus variants become dominant, the variant-specific CD8 T cells are repeatedly stimulated by the variant Ags, which can lead to a loss of functions by the variant-specific subsets, whereas the wild type-specific CD8 T cells see little Ag, which can lead to the restoration of functions by the wild type-specific ones. In this regard, it is interesting to ask whether CD8 T cells specific for the wild-type and the variant Nef epitopes differently express a receptor programmed death 1 (PD-1), because several recent reports show that functionally impaired virus-specific CD8 T cells express PD-1 in mice and humans chronically infected with lymphocytic choriomeningitis virus (8) and HIV-1 (23, 24), respectively. However, we observed no significant difference in the level of PD-1 expression between CD8 T cells specific for the wild-type and variant Nef epitopes or even for other epitopes in this setting (data not shown), most likely because only a limited number of subjects was tested or the subjects tested had been receiving antiretroviral therapy in this study. Because relatively large variations were demonstrated in the level of PD-1 expression in virus-specific human CD8 T cells among individuals and specificities within the same individual and because antiretroviral therapy also influenced PD-1 expression (23, 24), it could be difficult to estimate the level of functional impairment of CD8 T cells from the absolute level of PD-1 expression when a limited number of subjects and epitopes are tested. Further studies are needed to clarify the effect of antigenic variations of HIV-1 on the differential levels of PD-1 expression in HIV-1-specific CD8 T cells in untreated subjects with acute and chronic phase of HIV-1 infection.

Alternatively, a report showing the restoration of a proliferative response by some fractions of HIV-specific CD8 T cells through the addition of exogenous IL-2 highlights the importance of IL-2-secreting CD4 helper T cells for the maintenance of effective antiviral CD8 T cell responses in a chronic infection (4). Another report showing that HIV-specific CD8 T cell proliferation is supported by IL-2-secreting CD8 T cells in vitro suggests the importance of autocrine help to maintain CD8 T cell effectiveness during the CD4-diminished chronic phase of an infection (5). In our study, the difference in the proliferative capacity of CD8 T cells in patients with a chronic infection was primarily related to their epitope specificity. CD8 T cells specific for the variant epitopes had diminished proliferative capacity even in the presence of exogenous IL-2. Considering that the variant epitopes were selected and became dominant late after the primary infection, it is possible that the variant-specific CD8 T cells could have been primed during the CD4-diminished chronic phase of infection, resulting in impaired function of the variant-specific CD8 T cells.

It is also thought that wild-type and variant epitopes have a different inherent property for inducing CD8 T cell responses, as the nature of the Ags determines helper requirement for CTL priming in vivo (25). The variant-specific CTLs may not be fully functional due to the low avidity interactions between their TCRs and the variant Ags. Indeed, the variant Pol-specific CTL clone (CTL55) showed weak killing activity toward cells infected with the variant virus in our study, suggesting the generation of low avidity CD8 T cells in response to antigenic variations of the virus.

However, this was not the case in the Nef-specific CD8 T cells, as a cytolytic assay *ex vivo* showed that wild-type and variant Nef-specific CD8 T cells had comparable functional avidity in our study, suggesting that the variant epitope has immunogenic potential sufficient for CTL recognition, although the newly arising variant-specific CD8 T cells showed impaired proliferative capacity. Given that such a variant epitope is potentially immunogenic, it is possible that the variant Ag could induce fully functional CTL responses when individuals are primary infected with the variant virus. This issue needs to be further addressed as such information will be important for therapeutic vaccine design.

Original antigenic sin, which was originally described in the humoral response to influenza virus, has been applied to cellular responses for limiting the ability of the immune system to generate new responses to escape variants (26, 27). In this scenario, variant-specific CTLs are not generated but the variant epitope can continue to stimulate the proliferation of CTLs specific for the wild-type epitope (28). In our study, the *ex vivo* analysis of patients' PBMCs by HLA tetramers as well as IFN- γ assays clearly showed the generation of CD8 T cells exclusively specific for the variant epitopes, indicating that original antigenic sin could be overcome in the case of some HLA-B*35-restricted epitopes. This finding is consistent with recent reports showing that, based on *ex vivo* analysis by IFN- γ assays, the human immune system is capable of mounting novel CD8 T cell responses against CTL escape variants of Gag epitopes restricted by HLA-A*11 (29) and HLA-B*57 (30). However, considering that HLA-B*35 is an HLA class I allele associated with rapid disease progression while HLA-A*11 and HLA-B*57 are associated with slow disease progression (10, 31), it is conceivable that the failure to generate functionally effective, variant-specific CTLs restricted by HLA-B*35, as observed in this study, could result in relatively insufficient virus containment by HLA-B*35-restricted CTL responses *in vivo*, leading to a consequent association between HLA-B*35 and rapid disease progression. In this regard, further comprehensive analysis of the wild-type and variant-specific CTL responses restricted by HLA-B*35 and certain B*35 subtypes in treatment-naïve subjects is becoming intriguing.

It is of note that the frequency of the wild type Pol-specific CD8 T cell subsets was much reduced after the emergence of CTL escape variants in the Pol epitope, whereas the wild type Nef-specific ones persisted following CTL escape in the Nef epitope in our study. Because we obtained autologous virus sequence data from viral RNAs in plasma, it is conceivable that a small number of cells latently infected with the variant viruses yet having a wild-type Nef sequence remained *in vivo* and eventually reactivated the wild type Nef-specific CD8 T cell subsets. Alternatively, a transient reversion of variant viruses to the wild-type Nef sequence arose, although such wild-type viruses could be rapidly controlled by wild type-specific CD8 T cells to undetectable levels. In any event, these scenarios can be possible when a virus variant has replicative disadvantage over the wild-type virus *in vivo*. Interestingly, it is reported (32) that the Thr mutation in Nef (corresponding to Thr⁷⁵ in this study although a different numbering system was used therein) resulted in much decreased capacity for supporting viral replication, although the replication-competent strain NL43 has Thr at the same position. Further analysis to clarify whether CTL-mediated selective pressure can modulate the pathogenic functions of Nef and lead to long-term favorable effects on HIV-infected individuals against disease progression would be intriguing.

Recent studies have demonstrated that mutational escape from HIV-specific CTL is caused by interference with the intracellular processing of virus-derived proteins (15, 16). We also found HLA-B*35-associated mutations flanking the epitopic regions in Env

and Nef. However, Env-specific CTL clones showed comparable cytolytic activity toward cells expressing wild-type and mutant proteins (T. Ueno, unpublished observations), suggesting that altered Ag processing was not involved in this setting. Further studies are needed to determine whether and, if so, how antigenic variations causing altered Ag processing affect HLA-B*35-restricted CTL responses and the functional heterogeneity of HIV-specific CTLs in patients with a chronic infection.

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Disclosures

The authors have no financial conflict of interest.

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