Table I. *Summary of HLA-B35$^+$ subjects used in this study*[a]

Pt.	HLA Class I Allele	Months since Seroconversion	Viral Load (log_{10}/ml)	CD4 (mm^{-3})	Antiretoviral Therapy	Nef Sequence	PBMC Availability
001	*A2402/A2603, B3501/B4002*	132	ND	227	+	RPQVPLRPMT**F**	−
		192	3.9	223	+	**T**PQVPLRPMTY	+
003	*A2402/A2601, B3501/B5101*	72	ND	480	−	RPQVPLRPMT**F**	−
		144	ND	252	+	**T**PQVPLRPMTY	+
006	*A24/A26, B35/B52*	48	ND	102	+	RPQVPLRPMT**F**	−
015	*A11/A24, B35/B54*	147	BD	383	+	**T**PQVPLRPMTY	+
016	*A26/A33, B35/B44*	7	ND	43	−	RPQVPLRPMT**F**	−
017	*A2/A24, B35/B48*	192	BD	254	+	**T**PQVPLRPMTY	−
019	*A2402/−, B3501/B5201*	18	4.7	524	−	RPQVPLRPMT**F**	−
		80	BD	1574	+	**T**PQVPLRPMTY	+
025	*A24/A31, B35*	26	ND	50	+	**T**PQVPLRPMTY	−
027	*A24/A26, B35/B44*	4	ND	84	+	RPQVPLRPMT**F**	−
033	*A0207/A3101, B3501/B4601*	72	5.3	326	−	**T**PQVPLRPMTY	+
034	*A2402/A2601, B3501/B4801*	48	4.4	201	−	**T**PQVPLRPMTY	+
042	*A24/A31, B35/B60*	59	3.8	311	−	**T**PQVPLRPMTY	+
046	*A2, B35/B61*	48	BD	263	+	**T**PQVPLRPMTY	+
099	*A2402/−, B3501/B61*	12	3.9	984	−	RPQVPLRPMT**F**	+
100	*A2601/−, B3501/B4001*	16	5.0	614	−	RPQVPLRPMT**F**	+
102	*A2402/A0206, B3501/B0702*	17	2.8	482	−	RPQVPLRPMT**F**	+
131	*A2402/A0207, B3501/B4601*	10	1.9	563	+	RPQVPLRPMT**F**	+
136	*A2402/A2601, B3501/B5201*	15	4.4	308	−	RPQVPLRPMT**F**	+
141	*A0201/A3101, B3501/B5401*	10	5.3	382	−	RPQVPLRPMTY	+
		20	5.1	360	+	RPQVPLRPMT**F**	+
145	*A0207/A2601, B3501/B5101*	6	BD	645	−	RPQVPLRPMTY	−
		18	4.6	685	−	RPQVPLRPMT**F**	+
161	*A2402/A2601, B3501/B5401*	13	2.3	955	−	RPQVPLRPMT**F**	+
168	*A2601/−, B3501/−*	5	2.3	408	+	RPQVPLRPMTY	+
178	*A2601/A3101, B3501/B4601*	8	2.7	568	+	RPQVPLRPMTY	+

[a] ND, Not determined; BD, below detection limit. Bold, underlined letters in the sequences represent mutations.

Materials and Methods

Subjects

A total of 23 individuals (HLA-B35$^+$) with HIV infection followed at the AIDS Clinical Center (International Medical Center of Japan) were enrolled for functional analysis of HIV-specific CD8 T cells and autologous HIV-1 sequence analysis in this study. Subjects were selected based on the availability of plasma and PBMC samples as well as HLA-B*35 expression. Clinical data of all subjects are listed in Table I. Patients 01, 03, and 17 are hemophiliacs who had been infected with HIV-1 through contaminated blood products. Because the time of HIV-1 infection or the time of seroconversion was not known for these subjects, we suspect that their infection occurred in 1983 based on a survey done on Japanese hemophiliacs. In addition, 41 individuals (negative for HLA-B*35) with HIV infections were enrolled for autologous HIV-1 sequence analysis. The study was conducted in accordance with the human experimentation guidelines of the International Medical Center of Japan and Kumamoto University.

Sequence analysis of autologous HIV-1

HIV-1 particles were precipitated by ultracentrifugation (50,000 rpm, 30 min) of patients' plasma, after which the viral RNA was extracted from them. DNA fragments encoding Nef proteins were amplified by a nested PCR, gel purified, and sequenced directly as described (29). The fragments were cloned into a plasmid and then sequenced for phylogenetic tree analysis.

For phylogenetic tree analysis of intrapatient evolution of the *nef* gene (HXB2 coordinate, 8932–9555), nucleotide sequences were initially aligned by using Clustal W and then manually adjusted to maximize alignment of codon triplet as needed. Regions that could not be unambiguously aligned were removed from subsequent phylogenetic analysis. The MEGA3 package of sequence analysis programs was used for detailed phylogenetic analysis (30). Pairwise evolutionary distances were calculated by using the Kimura 2-parameter model for estimation of distances, and phylogenetic trees were constructed by the neighbor-joining method.

Generation of T cell clones

CTL clones or lines were established by stimulation of PBMC with a synthetic peptide, as previously described (31). 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-B3501 cells pulsed with 1 μM peptide in RPMI 1640 with 10% FCS and 100 U/ml rIL-2). Two weeks later, cells showing substantial Ag-specific cytolytic activity were maintained in the medium with peptide stimulation weekly.

Preparation of HIV-1 variants

The full-length HIV-1 pNL43 derivatives in which the *nef* gene was completely removed (pNL43ΔNef) or replaced with SF2 *nef* (pNL43SF2Nef) were created earlier (32). The Arg^{75} to Thr and Tyr^{85} to Phe mutations were achieved by site-directed mutagenesis based on SF2 *nef*. 293T cells were transfected with each of the constructs, and the infectious HIV-1 virions released into the medium were collected 48 h later. The p24 Ag concentrations of virus stocks were determined by p24 Ag ELISA.

Flow cytometric analysis

HLA stabilization assay. Peptide-binding activity for HLA-B*3501 was assessed by an HLA stabilization assay using RMA-S cells expressing HLA-B*3501 as described earlier (31).

HLA tetramer analysis. The HLA-B3501 tetramers in complex with the VY8 and RY11 peptides were prepared as previously described (31). Cryopreserved PBMC of HIV-positive (2×10^6) or -negative donors (3×10^6) were stained with the PE- and allophycocyanin-labeled tetramers at 37°C for 15 min followed by anti-CD8-PerCP (BD Biosciences/BD Pharmingen) and anti-CD3-FITC (DakoCytomation) at 4°C for 15 min. The $CD3^+CD8^+$ cells were gated and then analyzed for binding with the tetramers by flow cytometry (FACSCalibur; BD Biosciences).

Intracellular cytokine staining assay. Intracellular cytokine staining of Ag-specific CTL clones was done as previously described (33). Briefly, CTL clones (4×10^4 cells) were incubated with C1R-B3501 cells (4×10^4 cells) alone or pulsed with various concentrations of peptides for 6 h at 37 °C in the presence of brefeldin A (10 μg/ml). The cells were stained first with anti-CD8 mAb and 7-aminoactinomycin D (7-AAD), permeabilized in a detergent buffer, and then stained with mAb specific for IFN-γ or TNF-α (BD Biosciences/BD Pharmingen).

Cytotoxic assays

Toward peptide-loaded cells. The cytotoxic activity of the CTL clones was determined by a standard ^{51}Cr-release assay as described previously (31).

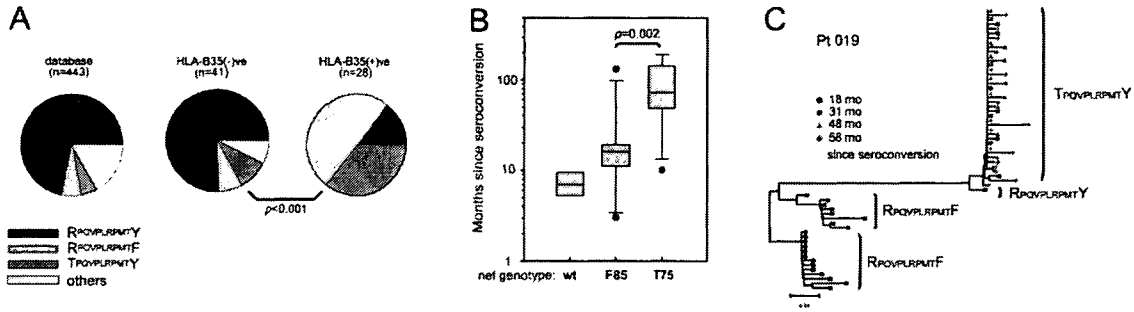

FIGURE 1. Dynamic evolution of autologous Nef sequences in HIV-infected individuals expressing HLA-B*35. *A*, Frequency of clones representing the HIV-1 Nef amino acid sequence at the RY11 epitope region as indicated in pie charts, based on the results from the Los Alamos database (*left*). The frequencies of individuals whose autologous viruses had the Nef amino acid sequences indicated when the plasma samples were collected from HIV-infected individuals negative (*middle*) or positive (*right*) for HLA-B*35 are shown. Statistical analysis was performed by using the χ^2 test. *B*, Differences in the duration of HIV infection (months since seroconversion) and the autologous *nef* genotypes, wt, $Tyr^{85}Phe$ (F85) or $Arg^{75}Thr$ (T75) in HLA-$B35^+$ patients. Boxes indicate values between 25th and 75th percentiles. Horizontal lines across boxes indicate the median value ± SD. Lines extend from the box to the highest and lowest values. Data include outliers (●). Statistical analysis was performed by use of the Mann-Whitney *U* test. *C*, A neighbor-joining phylogenetic tree analysis of intrahost evolution of autologous *nef* gene. Plasma HIV-1 RNA samples were collected from patient 19 at the indicated time points. The *nef* gene segment was PCR-amplified, cloned into a plasmid, and sequenced (n = 61). The amino acid sequences of the epitopic region are indicated at the *right* of the tree.

Toward HIV-infected primary CD4⁺ cells. $CD4^+$ cells were purified from PBMC taken freshly from HIV-negative donors expressing HLA-B*3501 by using a magnetic cell separation system (Miltenyi Biotec) and stimulated with PHA (3 μg/ml; Sigma-Aldrich) for 4 days. The activated $CD4^+$ cells were then infected at relatively high titers (1 μg of p24 Ag per 10^6 cells) with wild-type (wt) or various variant HIV-1 for 6 h, and incubated for an additional 3–5 days. The HIV-infected $CD4^+$ cells (4000 cells/well) were then mixed with CTL clones at various ET ratios for 6 h at 37°C after having been labeled with ^{51}Cr. It should be noted that 30 ± 5% of the cells were p24 Ag^+ as revealed by intracellular flow cytometric analysis of HIV-infected $CD4^+$ cells.

HIV-1 replication assay

PBMC samples freshly isolated from HIV-seronegative donors were first infected with wt or various variant HIV-1s at 5 ng of p24 Ag in 5×10^5 cells for 4 h. The cells were washed, suspended in a culture medium (RPMI 1640, 10% FCS), and seeded in a 96-well plate at 10^5 cells/well. Three days later, the cells were stimulated with PHA at 2 μg/ml. Culture supernatants were collected and replaced with a fresh medium supplemented with human rIL-2 every 3 days. To monitor viral replication, we determined the amount of p24 Ag in the culture supernatant by a specific ELISA.

In vitro kinase assay (IVKA)

IVKA was performed as described earlier (34). Briefly, Jurkat cells (10^7) expressing wt or various variant Nef-GFP fusion proteins were lysed in KEB (50 mM Tris-HCl (pH 8), containing 137 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, Na_3VO_4, protease inhibitor mixture) at 24 h postelectroporation. Cleared lysates were immunoprecipitated with anti-GFP polyclonal Ab and the immunoprecipitates were resuspended in KAB (50 mM HEPES (pH 8), containing 150 mM NaCl, 5 mM EDTA, 0.02% Triton X-100, 10 mM $MgCl_2$) with 10 μCi of [γ-^{32}P]ATP (Amersham) for 5 min. Bound proteins were then separated by SDS-PAGE and subjected to phosphorimager (Bio-Rad) visualization and quantification. Levels of immunoisolated Nef-GFP were determined by Western-blotting of the IVKA reactions and subsequent quantification by LICOR Odyssee.

Statistical analysis

Statistical analysis and graphical presentations were done by using a computer program, SigmaPlot, with a statistical package (Hulinks). Unless otherwise indicated, results were given as median or mean ± SD. Statistical analysis of significance (p values) was based on the χ^2, Mann-Whitney rank sum, or two-tailed t test, or a one-way ANOVA, where applicable, and $p < 0.05$ was considered to be significant.

Results

*Evolution of PxxP region of Nef associated with HLA-B*35*

We previously reported that the Arg^{75} to Thr mutation (T75, amino acid numbers based on SF2 strain) in Nef was functionally associated with escape from a CTL response specific for the RY11 epitope (Nef_{75-85}: RPQVPLRPMTY) presented by HLA-B*35 in patients in the chronic phase of an HIV-1 infection (29). When we recruited more subjects including some in the early phase of infection and analyzed their autologous *nef* genotypes, another mutation, Tyr^{85} to Phe (F85), was also found in some of these HLA-$B35^+$ patients (Table I). As a result, ~50 and 40% of autologous *nef* alleles encoded the F85 and T75 mutation, respectively, in patients with HLA-B*35 (Fig. 1*A*), whereas either mutation was found in only ~5% of patients negative for HLA-B*35 as well as in all sequences from the Los Alamos HIV database (www.hiv.lanl.gov/). These data demonstrate that both T75 and F85 single mutations in Nef were differently associated with autologous viruses in patients with HLA-B*35 expression.

Because the F85 mutation was seemingly found in HLA-$B35^+$ HIV-infected subjects <2-year since seroconversion, we next analyzed the correlations between the duration of HIV infection and autologous *nef* genotypes in HLA-$B35^+$ subjects (Fig. 1*B*). The median (±SD) number of months since seroconversion in subjects with autologous wt, F85, and T75 Nef sequences was 7.0 ± 1.1, 16.0 ± 9.4, and 72.0 ± 19.5, respectively (Fig. 1*B*). This cross-sectional analysis demonstrated that HIV-1 acquired the F85 mutation earlier and the T75 mutation later concomitant with the reversion of the F85 mutation to the wt during an HIV-1 infection in subjects with HLA-B*35 expression.

*Intrahost evolution of Nef mutations associated with HLA-B*35*

To ask whether these mutations and reversions occurred sequentially within a subject, we collected plasma viral RNA samples at additional time points from three subjects, patients 001, 003, and 019. The amino acid sequence in the epitopic region sequentially changed from RPQVPLRPMTF to TPQVPLRPMTY (different amino acid residues are underlined; referred to as RF and TY, respectively, hereafter), within each subject (Table I).

To further characterize the intrapatient evolution in this region, the *nef* genotypes of plasma HIV-1 RNA of patient 19 were determined at several time points. The neighbor-joining phylogenetic tree showed that successive fixation of advantageous mutations and the extinction of unfavorable lineages had occurred, suggesting that the focus of the CTL response and/or the balance between the selective pressures that were at work on the epitope had changed over time (Fig. 1*C*). It is of interest to note that when

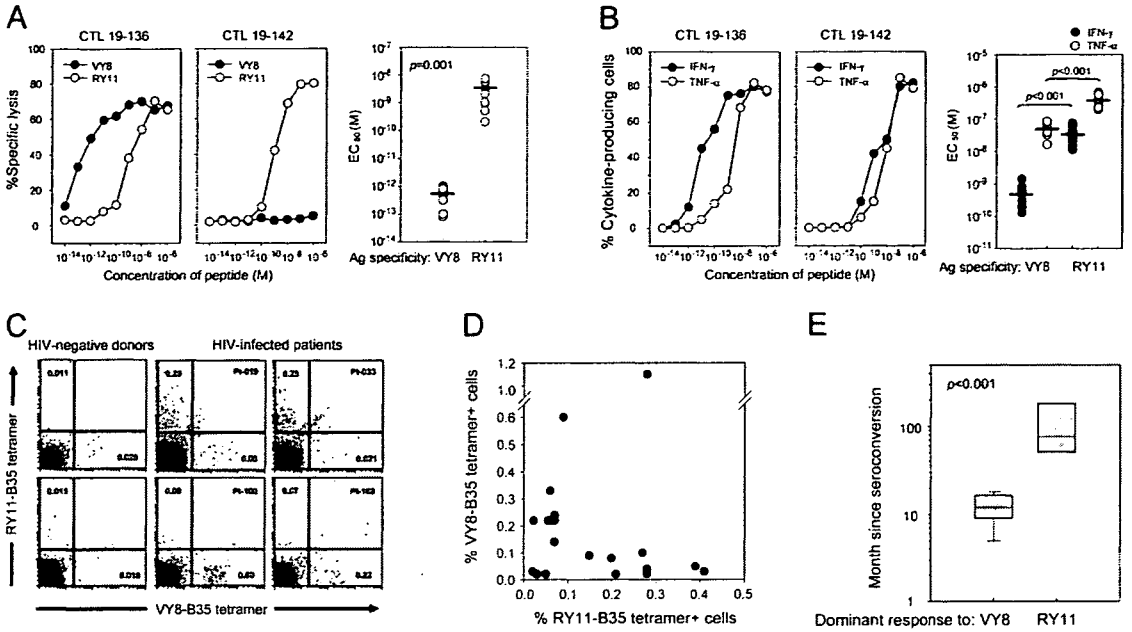

FIGURE 2. HLA-B35-restricted CTL responses toward PxxP region of Nef. *A* and *B*, Cytotoxic activity (*A*) and cytokine-producing activity (*B*) of VY8 or RY11-specific CTL clones generated from multiple donors (patients 01, 03, 19, and 136) were analyzed by using C1R-B3501 cells pulsed with various concentrations of the indicated peptides (Effector to target cell ratio = 2). Representative peptide-titration data obtained by CTL clones 19-136 and 19-142 (generated from PBMC of patient 19) specific for VY8 and RY11, respectively, are shown (*left* and *middle panels*). EC_{50} values thus obtained from an additional nine clones (total 10 clones each) generated from multiple donors are shown (*right panels*). Horizontal bars indicate means, and statistic analysis were performed by using the two-tailed *t* test. *n.s.*, not significant. Cytotoxic activity and cytokine-producing activity in the absence of the cognate peptide were always below 3 and 0.1%, respectively. *C* and *D*, PBMC samples isolated from 5 HIV-negative and 19 HIV-positive donors were analyzed by flow cytometry by using HLA-B*3501 tetramers in complex with VY8 or RY11 peptides. Cells that were $CD3^+CD8^+$ were gated and then analyzed for their frequency of HLA-$tetramer^+$ cells. Some representative dot plots of 2 HIV-negative and 4 HIV-positive donors are shown with frequencies of HLA-$tetramer^+$ cells in each dot plot (*C*). The frequencies of HLA-$tetramer^+$ cells for VY8 and RY11 epitopes in each individual subject are shown (*D*). It should be noted that reversing the fluorochromes of the tetramers gave identical results and that the background level of HLA-tetramer staining was 0.022%, as determined by the data from 5 HIV-negative donors (mean + 3 SD). *E*, Differences in months since seroconversion between the subject groups who showed dominant CD8 T cell responses to VY8 or RY11 epitope. Boxes indicate values between 25th and 75th percentiles. Horizontal lines across boxes indicate the median value ± SD. Lines extend from the box to the highest and lowest values. Statistical analysis was performed by using the Mann-Whitney *U* test.

the type of Nef variants changed from RF to TY by two amino acid substitutions, the Nef variant with two mutations, i.e., TPQVPLRPMTF (referred to as TF), was not apparently selected. Rather, the T75 mutation appeared to arise from a different lineage of viral quasispecies in this host (Fig. 1*C*). In addition, the TF double mutation was barely found in Los Alamos HIV database (1 of 443 entries), suggesting that the combination of these two mutations causes a significant fitness cost in viral replication in vivo.

Fine epitope mapping of HLA-B35-restricted CD8 T cells to PxxP region of Nef

We next examined HLA-B35-restricted CD8 T cell responses toward the PxxP region of Nef. Although HLA-B*35 prefers proline at position 2 in its binding peptide and this region can provide various candidate peptides for CTL epitopes, only two peptides, VY8 (Nef_{78-85}: VPLRPMTY) and RY11 (Nef_{75-85}: RPQVPLRPMTY), showed substantial CTL responses in the HLA-$B35^+$ subjects (data not shown), confirming previous observations (29, 35, 36). However, it is possible that VY8 is the minimum epitope for CTL, because VY8 is entirely contained within RY11. To clarify this issue, we generated CTL clones by stimulating PBMC of HLA-$B35^+$ HIV-infected patients with either VY8 or RY11 peptide and then analyzed their Ag specificity by cytotoxic assays. CTL clone 136 generated from subject patient 19 (designated CTL 19-136) with VY8 stimulation showed cytolytic activities toward target cells pulsed with either peptide, although VY8 was a ~1000-fold more sensitive ligand than RY11 (Fig. 2*A*). In contrast, another CTL clone, CTL 19-142, which had been stimulated with RY11, showed cytolytic activity toward C1R-B3501 cells pulsed with RY11 but not toward those pulsed with VY8 (Fig. 2*A*). Furthermore, when staining CTL clones with HLA-B*3501 tetramers in complex with VY8 and RY11, CTL 19-136 and 19-142 exclusively bound the VY8- and RY11-B35 tetramers, respectively (data not shown). These data indicate that VY8 and RY11 were different optimal epitopes presented by HLA-B3501 and are recognized by a different set of CTLs.

During the peptide-titration analysis, we noticed that CTL 19-136 had much higher functional avidity for its cognate peptide than CTL 19-142, with the EC_{50} values toward the cognate Ags of CTL 19-136 and 19-142 being 2.81×10^{-13} and 7.50×10^{-10} M, respectively (Fig. 2*A*). We further generated CTL clones from PBMC of three additional subjects, patients 001, 003, and 033, and determined their functional avidity toward each cognate Ag. Although the functional avidity of these CTL clones were different even within the same specificity (~30-fold), VY8-specific CTL clones had more potent functional avidity than RY11-specific ones (~5000-fold), as the mean EC_{50} values of VY8- and RY11-specific CTL clones were $5.29 \pm 1.13 \times 10^{-13}$ and $3.14 \pm 0.82 \times 10^{-9}$ M, respectively (Fig. 2*B*).

Furthermore, evaluating the CTL sensitivity by Ag-specific IFN-γ and TNF-α production revealed that VY8-specific CTLs also showed more potent functional avidity than RY11-specific ones, as mean EC_{50} values for IFN-γ secretion were $5.30 \pm 1.21 \times 10^{-10}$ and $3.50 \pm 0.61 \times 10^{-8}$ M, and those for TNF-α

secretion, $5.02 \pm 0.69 \times 10^{-8}$ M and $3.75 \pm 0.48 \times 10^{-7}$ M, for VY8- and RY11-specific clones, respectively (Fig. 2*B*). However, it is interesting to note that the difference in avidity for cytokine production between VY8- and RY11-specific CTLs was smaller than that observed in cytotoxic activity (Fig. 2, *A* and *B*).

Analysis of HLA-B35-restricted CD8 T cell responses to the PxxP region of Nef ex vivo

We next examined the frequency of VY8- and RY11-specific $CD8^+$ cells in patients' PBMC ex vivo by using HLA-B35 tetramers in complex with VY8 and RY11 as shown in the representative data in Fig. 2*C*. The background level of the HLA-tetramer analysis was considered to be 0.022% (mean + 3 SD) as the overall frequency of HLA-tetramer$^+$ cells in HIV-negative donors ($n = 6$) was 0.0153 ± 0.0022%. The frequency of HLA-tetramer$^+$ cells in HIV-infected subjects ($n = 19$) was 0.198 ± 0.060 and 0.160 ± 0.029 for VY8 and RY11 epitopes, respectively, and both responses were not statistically different overall ($p = 0.58$, paired t test).

Interestingly, looking at the frequencies of HLA-tetramer$^+$ cells in each individual subject, every subject showed a response to either the VY8 or RY11 epitope but not to both epitopes simultaneously (Fig. 2*D*). The median ($\pm$SD) number of months since seroconversion in subjects who had dominant response to VY8 or RY11 was 13.0 ± 1.4 or 76.0 ± 19, respectively (Fig. 2*E*), suggesting an immunological shift from VY8 to RY11 in HLA-B35-restricted CD8 T cell responses during the course of their HIV infection.

Effects of antigenic variations on VY8- and RY11-specific CTLs

We next asked whether Nef mutations affected the binding between epitope peptides and HLA-B*3501. The HLA-I stabilization assay using RMA-S cells expressing HLA-B*3501 showed that the VY8 and RY11 peptides bound HLA-B*3501 comparably, as the EC_{50} values for their binding activities were $20.4 \pm 7.55 \times 10^{-5}$ and $4.65 \pm 1.63 \times 10^{-5}$ M, respectively. Although the Phe substitution at the C terminus of either peptide (VY8-8F and RY11-11F) did not change their binding activities, the Thr substitution at the N terminus of RY11 (RY11-1T) resulted in ~10-fold increased binding activity. These data indicate that the binding activity of all peptides tested were within the range of HLA-B3501-restricted CTL epitopes (29, 31, 36, 37).

We then tested the cytotoxic activity of CTL clones toward C1R-B3501 cells pulsed with the variant peptides. A VY8-specific CTL clone, CTL 19-136, showed ~1000-fold decreased sensitivity toward VY8-8F (Fig. 3*A*). A similar trend was also observed in a panel of nine additional VY8-specific CTL clones as used in Fig. 2*A*, with mean EC_{50} of $4.43 \pm 0.63 \times 10^{-13}$ and $8.23 \pm 3.08 \times 10^{-9}$ M for VY8 and VY8-8F, respectively (Fig. 3*A*). In contrast, a RY11-specific CTL clone, CTL 19-142, showed preserved sensitivity toward RY11-11F, whereas it showed >100-fold decreased sensitivity toward RY11-1T (Fig. 3*B*). Again, a panel of nine additional RY11-specific clones showed similar results, with mean EC_{50} of $2.75 \pm 0.46 \times 10^{-9}$, $4.32 \pm 0.81 \times 10^{-9}$, and $8.47 \pm 3.28 \times 10^{-7}$ M for RY11, RY11-11F, and RY11-1T, respectively (Fig. 3*B*). These data indicate that VY8- and RY11-specific CTLs had different patterns of Ag fine specificity toward naturally arising variants, suggesting a direct association between the epitope evolution in autologous Nef proteins (Fig. 1*B*) and the kinetic change of CTL immunodominance in vivo (Fig. 2*E*).

Cytotoxic activity of VY8- and RY11-specific CTLs toward HIV-infected primary CD4 T cells

As HIV-infected CD4 T cells are the predominant target of HIV-specific CTLs in vivo, we next examined the cytotoxic activity of CTL clones toward primary CD4 T cells infected with wt or variant HIV-1. CD4 T cells prepared from HIV-negative donors (HLA-B3501$^+$) were first stimulated with PHA and then infected with wt or various variant viruses. Four days later, ~30% of the cells appeared to be infected with all viruses, as revealed by intracellular flow cytometry for p24 Ag (data not shown), suggesting that all viruses had comparable replicative capacity when primary CD4 T cells were preactivated before infection (see below). Both CTLs specific for VY8 (CTL 19-136 and 33-1) and RY11 (CTL 19-142 and 03-8) were cytotoxic toward CD4 T cells infected with wt HIV-1 (Fig. 3*C*). However, the cytolytic activity of VY8-specific CTLs was more potent than that of RY11-specific ones, suggesting a link between potent functional avidity (Fig. 2*A*) and antiviral activity (Fig. 3*C*) of VY8-specific CTLs. VY8 and RY11-specific CTLs failed to kill primary CD4 T cells infected with F85 and T75 virus variants, respectively (Fig. 3*C*), consistent with the data obtained from the peptide-pulse experiments (Fig. 3, *A* and *B*). In contrast, although VY8-specific CTLs were cytotoxic toward primary CD4 T cells infected with the T75 variant virus, the activity toward the T75 variant was less than that toward the wt virus (Fig. 3*C*), suggesting that the T75 mutation, located in the region flanking the N terminus of VY8, could modulate the Ag-processing pathway for the generation of the VY8 epitope in these cells. Moreover, these data clearly show that the double mutant virus could escape from both types of CTLs (Fig. 3*C*). The fact that the mutations in autologous viruses is very rare in combination (Fig. 1*A*) suggests that the combination of both mutations imposes functional constraints on Nef.

Effects of Nef mutations on down-regulation of surface receptors

We next sought to identify such functional constraints of variants carrying these CTL escape mutations. Because Nef helps HIV-infected cells to evade CTL lysis by down-modulating cell surface HLA-I and the PxxP motif is critical for this activity (12, 17, 26, 38), we first examined whether the mutations affected the HLA-I down-regulation activity by Nef. Down-regulation of cell surface CD4, that is mediated by a different cellular pathway and does not involve the PxxP motif (27), was analyzed in parallel.

We isolated primary CD4 T cells from an HIV-negative donor, activated them with PHA, and infected them with wt or various variant viruses. In flow cytometric analysis, the surface levels of HLA-I were reduced to 40.1% of normal (uninfected cells) in cells infected with wt HIV-1, and no HLA-I down-regulation was observed in ΔNef virus-infected cells (Fig. 4*A*). In contrast, the TF double variant showed diminished down-regulation activity, as the TF variant-infected cells retained 73.1% of the normal level of HLA-I, whereas F85 and T75 variants showed HLA-I down-regulation activity comparable to that of the wt, with their surface levels being 36.3 and 46.5%, respectively (Fig. 4*A*). The same experiments using CD4 T cells isolated from three different HIV-negative donors reproducibly showed the TF variant to have a diminished activity in terms of HLA-I down-regulation (Fig. 4*B*). In stark contrast, all cells infected with variant viruses except for ΔNef showed down-regulation activity for CD4 comparable to that of the wt (Fig. 4, *A* and *B*). In addition, Western blot analysis of virus-producing cells for Nef proteins showed that all variant viruses except for ΔNef had expression levels of Nef comparable to that of the wt (data not shown). These data demonstrate that the

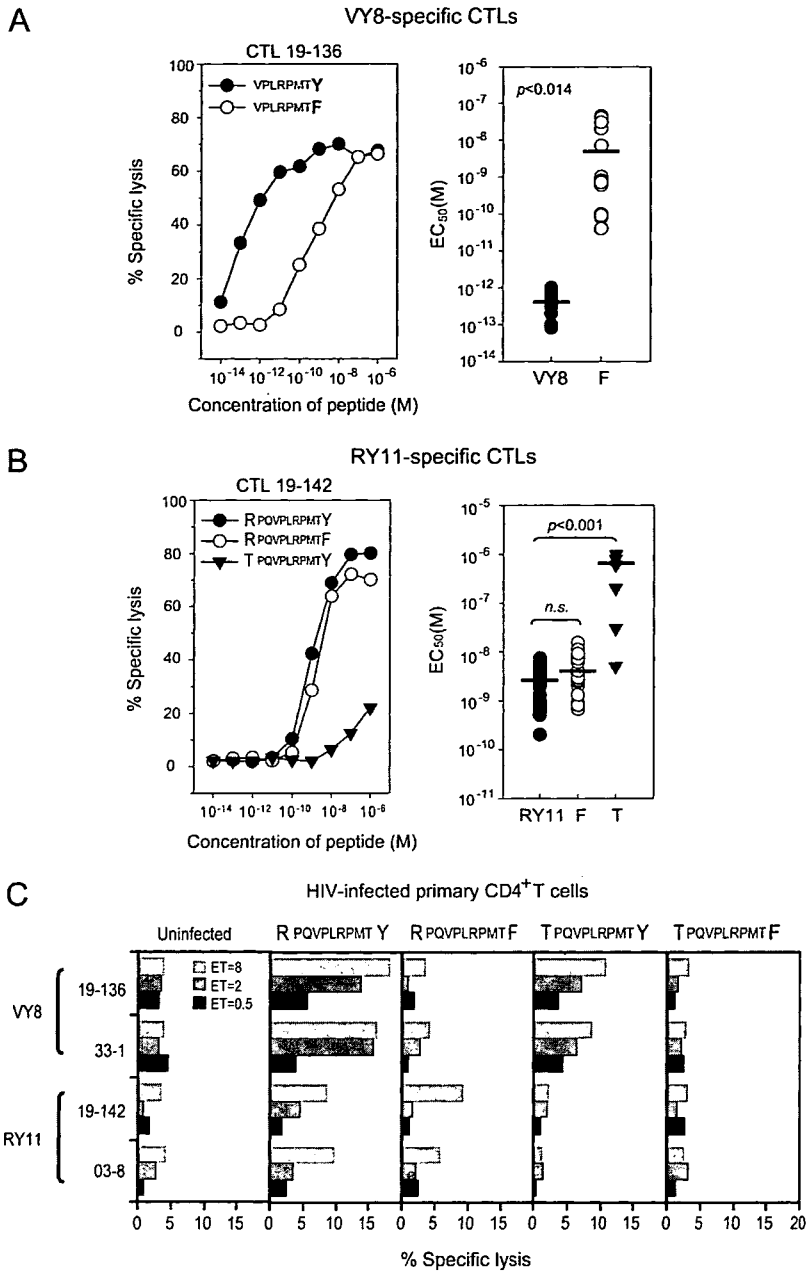

FIGURE 3. CTL responses to variant Ags. *A* and *B*, VY8 and RY11-specific CTL clones (same clones as in Fig. 2, *A* and *B*) were tested for their ability to respond to variant peptides by using C1R-B3501 cells pulsed with various concentrations of the wt or variant peptides (ET = 2). Representative peptide-titration data obtained for CTL 19-136 and 19-142 are shown (each *left panel*). EC_{50} values thus obtained for an additional 9 clones (total 10 clones) are also shown (each *right panel*). Horizontal bars indicate means, and statistic analysis was performed by using the paired *t* test. Cytotoxic activity in the absence of the peptide was always <3%. *C*, The VY8- and RY11-specific CTL clones were analyzed for their cytolytic activity toward target cells at ET = 0.5, 2, and 8 as indicated. The target cells were primary $CD4^+$ T cells that had been isolated from an HIV-negative donor (HLA-$B3501^+$), activated by PHA, and infected with wt or various variant viruses. The frequency of HIV-infected cells among target cells as determined by intracellular p24 Ag expression was 31.5, 33.2, 34.5, and 29.8% for wt, RF, TY, and TF variants, respectively. An additional experiment conducted by using a different blood donor (HLA-$B3501^+$) showed similar results.

combination of both mutations selectively diminishes the HLA-I down-regulation activity by Nef.

Effects of Nef mutations on cytolytic activity of CTL clones with other specificity

To test whether the observed differences in HLA-I down-regulation affect the susceptibility of HIV-infected cells to recognition by CTLs, we assessed the cytolytic activity of CTL clones with specificity to HIV-1 gene products other than Nef and other restriction toward primary CD4 T cells infected with wt and Nef variant viruses.

Freshly isolated CD4 T cells from an HIV-negative donor (HLA-$B35^+$ and HLA-$A24^+$) were infected with various HIV-1 as above and mixed with CTL clones specific for Pol and Env epitopes presented by HLA-B*3501 as well as with a clone specific for another Nef epitope presented by HLA-A*2402 (designate as B35-Pol, B35-Env, and A24-Nef, respectively). Although the amino acid sequences in the epitope regions of B35-Pol, B35-Env, and A24-Nef were the same among the wt and variant viruses tested, CTL-mediated killing activity appeared to be different among target cells infected with these viruses (Fig. 4*C*). Both B35-Pol and B35-Env CTLs showed most potent cytotoxic activity toward target cells infected with the ΔNef variant, whereas the same CTLs showed weak cytolytic activity toward wt virus-infected cells (Fig. 4*C*). Interestingly, CTLs markedly killed cells infected with the TF double mutant virus, whereas they weakly killed cells infected with either T75 or F85 single mutant virus (Fig. 4*C*). Moreover, in A24-Nef CTL-mediated cytotoxic activity, we also observed that the TF double mutant virus-infected cells were more potently killed than cells infected with wt or single mutant viruses (Fig. 4*C*). These data suggest that the diminished HLA-I down-regulation (i.e., increased level of cell surface HLA-I) in CD4 T cells infected with the TF double mutant virus resulted in increased susceptibility to killing by CTLs, leading to a possible selective disadvantage for the variant virus in vivo.

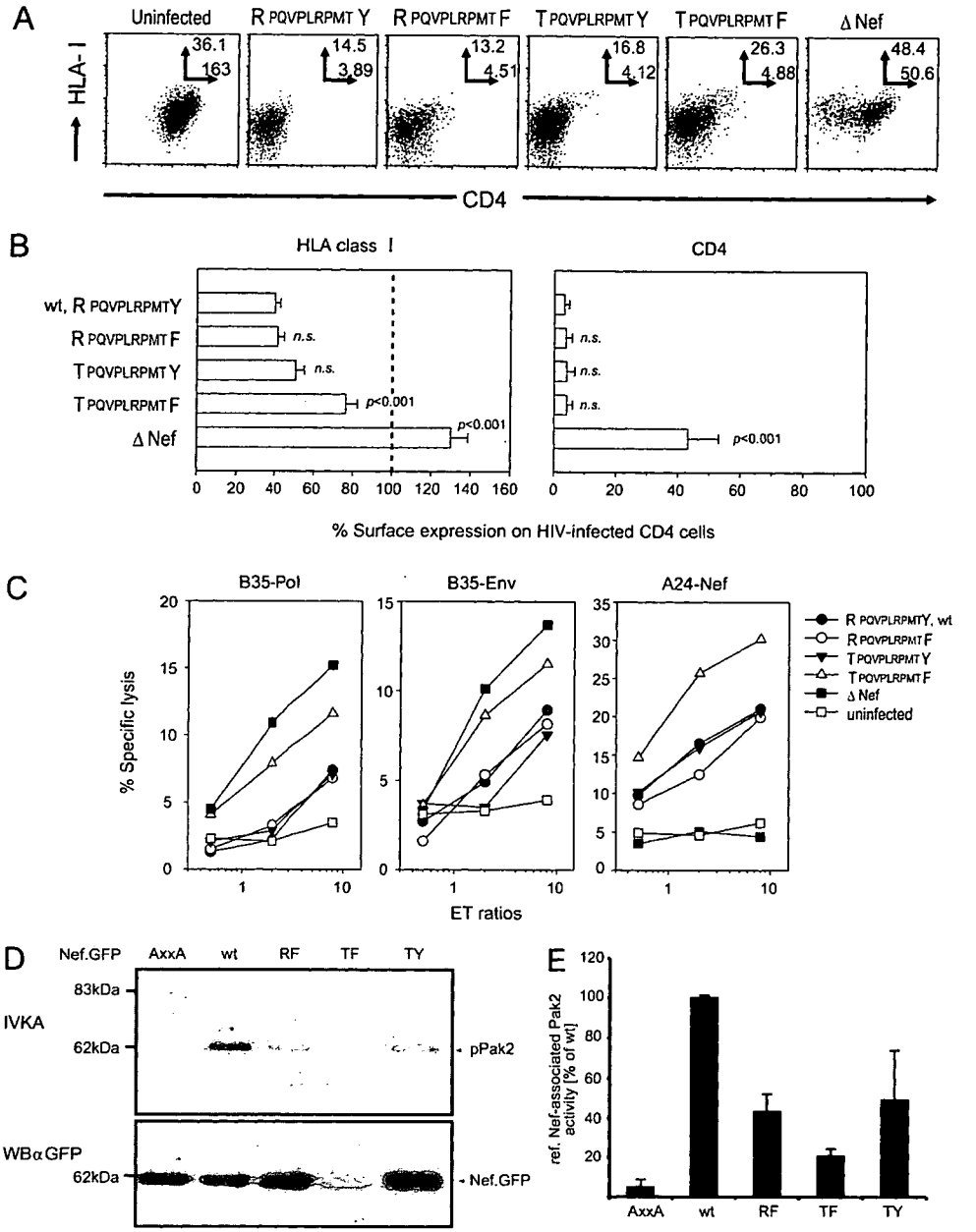

FIGURE 4. Functional consequences of CTL escape Nef mutations. *A*, Freshly isolated primary $CD4^+$ cells from an HIV-negative donor ($HLA\text{-}B35^+$) were activated by PHA for 3 days and then infected with wt or various variants for 5 days. The cells were stained with anti-HLA-Bw6 mAb (clone: SFR8-B6) and anti-CD4 mAb, and 7-AAD followed by intracellular staining for p24 Ag. In flow cytometric analysis, cells negative for 7-AAD and positive for p24 Ag were gated and analyzed for their fluorescence intensity for HLA-Bw6 and CD4. The frequency of infected cells was 29.6, 34.3, 30.5, 31.9, and 26.2% for HIV-1 wt, RF, TY, TF, and ΔNef variants, respectively. The mean fluorescence intensities (MFI) for HLA-Bw6 and CD4 are shown in the *right upper corner* of the dot plots. *B*, The same experiment as above was done by using three additional HIV-negative donors. The Ab specific for HLA-I allotypes used was either SFR8-B6 or A11,1M as appropriate for each donor. The MFI level of HLA-I and CD4 on uninfected cells was set to 100% and indicated by the dotted vertical line in the graph. Statistical analysis was performed by ANOVA with multiple comparisons vs wt. *n.s.*, not significant. *C*, Primary $CD4^+$ cells infected with wt or various variant HIV-1s as in Fig. 3 (the donor carries both HLA-A*2402 and HLA-B*3501) were used as target cells for cytolysis by CTL clones specific for HLA-B3501-restricted Pol ($Pol_{273-282}$: VPLDKDFRKY), Env (Env_{77-85}: DPNPQEVVL), or HLA-A2402-restricted Nef epitope ($Nef_{138-147}$: RYPLTFGWCF). An additional experiment using a different blood donor (positive for both HLA-A*2402 and HLA-B*3501) showed similar results. *D*, Nef-associated Pak2 activity. Jurkat cells were electroporated with plasmid DNAs encoding the indicated Nef-GFP fusion proteins. Total cell lysates were immunoprecipitated with anti-GFP Ab, and the resultant immunoprecipitates were analyzed by IVKA for Pak2 autophosphorylation (pPak) (*upper panel*). The same IVKA reactions were directly separated by SDS-PAGE and analyzed for immunoisolated Nef-GFP levels by Western blotting with anti-GFP Ab (*lower panel*). *E*, Quantification of Nef-Pak2 association. The indicated values represent the Nef-associated Pak2 activity after the levels of pPak2 had been normalized to the amounts of immunoisolated Nef-GFP. Values presented are the mean of at least three independent experiments with the indicated SEM expressed relative to the wt control that was arbitrarily set to 100%.

Effects of Nef mutations on the association of Nef with the cellular kinase Pak2

Given this reduced ability to down-modulate cell surface HLA-I, we also wanted to assess whether other Nef activities that depend on the interaction of the PxxP motif with SH3 domain-containing ligands are affected by the CTL escape mutations. To this end, we analyzed the association of Nef with cellular Pak2 kinase activity. This interaction is conserved among a variety of lentiviruses (39),

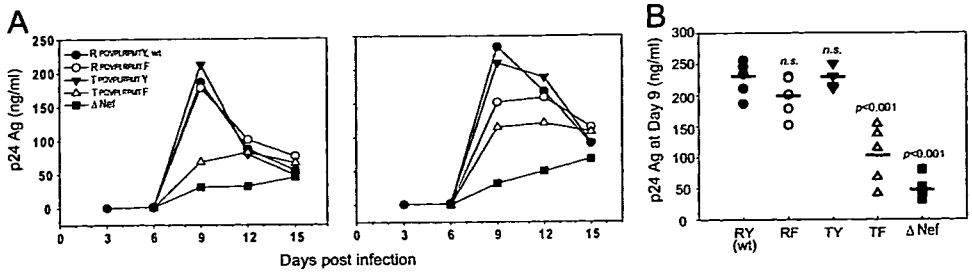

FIGURE 5. Effects of Nef mutations on viral replication in PBMC. *A*, Freshly isolated PBMC samples from two HIV-negative donors were first infected with wt or various variant HIV-1s and 3 days later cells were activated by PHA. For monitoring viral replication, culture supernatants were collected every 3 days and replaced with fresh medium containing rIL-2. *B*, The same experiment as above was done by using three additional HIV-negative donors. The level of p24 Ag obtained at day 9 postinfection was plotted and statistically analyzed based on ANOVA with multiple comparisons vs the wt. *n.s.*, not significant. Horizontal bars indicate means of data obtained for the five different PBMC donors.

strictly depends on the integrity of the PxxP motif and has multiple functional consequences that may optimize virus production (10, 40, 41). Expectedly (34, 39), wt Nef from HIV-1 SF2 (wt) showed robust association with phosphorylated Pak2 (pPak2) while the AxxA mutant (both Pro^{76} and ^{80} to Ala) did not show substantial association with pPak2 (Fig. 4*D*). Pak2 association was substantially reduced but not abrogated for the F85 (RF) and T75 (TY) single variants. According to phosphorimager quantification of the Nef-associated Pak2 signal and normalization to the levels of Nef present in the IVKA (Fig. 4*E*), Pak2 association was more than two-fold reduced for these two Nef variants relative to wt. The TF double mutant displayed an even stronger reduction to ~20% Pak2 association relative to wt Nef (Fig. 4, *D* and *E*). These data suggest that the T75 and F85 mutations in the PxxP region of Nef affect its ability to interact with SH3 domain-containing ligands.

Effects of Nef mutations on viral replication in PBMC

Nef significantly enhances virus replication in primary CD4 T cells, particularly if these cells are exposed to HIV-1 before activation with mitogens (42, 43). Because amino acid substitutions from prolines to alanines in the PxxP region have been shown to decrease this activity (25, 26, 32), we asked whether the T75 and F85 mutations would have similar effects.

Freshly isolated PBMC from two HIV-negative donors were first exposed to wt or various variant viruses for 3 days and subsequently activated by PHA. In both donors' PBMC, the wt and T75 variant viruses showed comparable replication kinetics, whereas the replication of the ΔNef virus was substantially delayed (Fig. 5*A*). The replication of the F85 variant virus was comparable to that of the wt virus in PBMC of a donor and was partially impaired with PBMC of another one (Fig. 5*A*). In contrast, the double TF variant virus showed delayed replication kinetics in PBMC from both donors (Fig. 5*A*).

To account for this donor variability, results from a total of five donors are summarized in Fig. 5*B*. As all PBMC samples showed a peak on day 9 after infection with the wt virus, the amounts of p24 Ag at day 9 after infection with the variant viruses were measured and statistically analyzed by multiple comparisons vs the wt (Fig. 5*B*). The ΔNef virus showed reproducibly the weakest replicative capacity under this assay condition, in good agreement with previous reports (26, 32). In addition, the TF double variant virus showed diminished capacity for viral replication compared with the wt; whereas each type of single variant virus did not show much difference in replication capacity (Fig. 5*B*). These data demonstrate that, even in the absence of HIV-specific CTL responses, the combination of T75 and F85 mutations is disadvantageous for Nef's ability to enhance virus replication.

Discussion

It is thought that the *nef* gene has higher levels of mutational plasticity in response to selective pressures compared with genes exhibiting structural or functional constraints (e.g., Gag, protease, reverse transcriptase, or integrase), because it exhibits considerable sequence diversity in vivo. In fact, some CTL escape variants of Nef, such as those with the mutations located in the CTL epitopes restricted by HLA-B*57 and HLA-A*24, have been suggested to have minimum fitness cost on the virus. This is because, in such a region, reversions are not often observed after transmission of the virus to new hosts who are negative for that particular *HLA-I* allele and the mutations are readily fixed in the population in the meantime (44, 45). In contrast, we show in the present study that the naturally arising mutations in the well-conserved PxxP region of HIV-1 Nef are selected under active CTL-mediated selective force at work and these mutations alone or in combination can modulate the pathogenic function by HIV-1 Nef including HLA-I down-regulation, enhancement of viral replication, and association with an activated cellular kinase, strongly suggesting that these mutations can impose functional constraints on the Nef activity and viral replication in vivo. Considering that various Nef activities substantially vary during the course of an infection at different stages of disease progression (4) and that there are substantial numbers of HLA-I-associated sequence variations in Nef (46–48), immunosurveillance by the Nef-specific CTLs plays additional roles in modulating the pathogenic potential of HIV-1 through selection of CTL-escape mutations in Nef particularly those in a well-conserved functional region.

It is obvious that HLA-B35-restricted CTL responses were shifted in patients during the early to chronic phase of an HIV-1 infection in our study, as the Nef VY8 epitope was dominantly recognized by CTLs relatively early in the infection, whereas the N-terminal extended RY11 epitope was recognized by CTLs in the chronic phase. This observation is in line with previous reports showing that CTL epitope specificity is different during the course of an HIV infection (23, 49, 50). Particularly, an immunodominant response directed against the HIV Gag p17-derived, HLA-A0201-restricted SL9 epitope (SLYNTVATL) was not detected early in an infection (50). Although the mechanisms underlying this phenomenon are not yet known, one possible explanation is that the responses detected in the early stage of an infection could have "mutated away," opening the field for a second wave of CTL specificities taking over in their place. The CTLs induced by a second or third waves of CTL specificities may have decreased antiviral effectiveness as predicted in the mathematical antigenic oscillation

model proposed by Nowak et al. (51). Our data support this scenario that the highly active VY8-specific CTLs elicited early in an infection were rendered ineffective apparently due to the acquisition of the F85 Nef mutation by the virus and that subsequently the cross-reactive RY11-specific CTLs, yet having moderate antiviral activity, became dominant. It is interest to note that the T75 variant, which had been selected by RY11-specific CTLs during the chronic phase, can induce de novo variant-specific CTLs with less effective Ag-specific proliferative capacity, further reducing antiviral activity of CTLs in vivo (29).

HLA-B*35 has been documented to be associated with rapid disease progression to AIDS (52). However, a further detailed study showed that individuals having HLA-B*35 allelic variants, including B3502/3503/3504, progress more rapidly to AIDS than do those with HLA-B*3501 (53). All the HLA-B35$^+$ subjects in this study were considered to carry HLA-B*3501, as the HLA-B*3501 is highly prevalent in the HLA-B35$^+$ Japanese population, though we have not yet done the genotypic analysis of *HLA-B* loci of all of the subjects. Further studies are needed to clarify whether CTL responses toward the PxxP region of Nef may be associated with the difference in the disease progression among HIV-infected patients having different HLA-B35 allelic variants.

Although the TF double mutation provided the best CTL escape of the Nef variants tested here, this variant was barely selected in HLA-B35$^+$ patients. This suggested that important functional constrains imposed by these combinatorial mutations precluded selection of these variants. The present study revealed at least two independent possible reasons for such a counterselection. First, the T75 and F85 double mutation in HIV-1 Nef significantly reduced the down-regulation activity of HLA-I and resulted in increased recognition by Pol- and Env-specific CTLs. Because down-regulation of MHC-I by SIV Nef in rhesus macaques limits CD8 T cell-mediated killing and contributes to the pathogenic effect of Nef in vivo (14), these results suggest that the sustained HLA-I down-regulation activity by HIV-1 Nef is required for efficient viral replication in vivo. This observation is in line with a previous report demonstrating that Nef mutations selected by Nef-specific CTLs in vitro, although most mutations disrupted *nef* reading frames in their study, leads to progeny virions that are increased in their susceptibility to CTLs with specificities for proteins other than Nef (54). However, the mutations in that report are different from representative naturally arising variations (54) as the *nef* reading frame is highly maintained intact in vivo (55) and large deletions or frame shifts are rarely observed. In contrast, the current study focused on the naturally arising mutations that are selected under Nef-specific CTL responses in vivo.

Second, the double mutation also affected PxxP-dependent activities of Nef in the absence of HIV-specific CTLs and significantly impaired Nef's ability to boost HIV-1 replication in primary human T lymphocytes. Because the individual mutations caused no significant impairment to HIV replication in the experimental system used, these results also help to explain why the double mutant is counterselected in HIV-infected patients. On the molecular level, Nef's effects on viral replication are likely mediated by a number of yet to be fully defined protein interactions. Among others, its association with Pak2 activity has also been implicated in the Nef-mediated enhancement of virus infectivity and replication (40, 56, 57). In this scenario, our results suggest that the reduction of Nef-Pak2 below a certain threshold activity may contribute to the reduction of Nef's ability to boost HIV spread. More importantly, the reduction of Pak2 association indicates that CTL escape Nef variants are impaired in their interaction with SH3 domains, which is expected to have select functional consequences in various cellular environments.

Together, these results demonstrate that CTL escape has severe consequences on the functionality of the PxxP motif in Nef, both for its role in immunoevasion and intrinsic replicative potential of the virus. Thus, a vaccine regimen that can elicit CTL responses targeting the regions involved in HLA-I down-regulation activity by Nef could be a potent candidate for future vaccine design.

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Disclosures

The authors have no financial conflict of interest.

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Different Abilities of Escape Mutant-Specific Cytotoxic T Cells To Suppress Replication of Escape Mutant and Wild-Type Human Immunodeficiency Virus Type 1 in New Hosts▽

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There is much evidence that in human immunodeficiency virus type 1 (HIV-1)-infected individuals, strong cytotoxic T lymphocyte (CTL)-mediated immune pressure results in the selection of HIV-1 mutants that have escaped from wild-type-specific CTLs. If escape mutant-specific CTLs are not elicited in new hosts sharing donor HLA molecules, the transmission of these mutants results in the accumulation of escape mutants in the population. However, whether escape mutant-specific CTLs are definitively not elicited in new hosts sharing donor HLA molecules still remains unclear. A previous study showed that a Y-to-F substitution at the second position (2F) of the Nef138-10 epitope is significantly detected in HLA-A*2402$^+$ hemophilic donors. Presently, we confirmed that this 2F mutant was an escape mutant by demonstrating strong and weak abilities of Nef138-10-specific CTL clones to suppress replication of the wild-type and 2F mutant viruses, respectively. We demonstrated the existence of the 2F-specific CTLs in three new hosts who had been primarily infected with the 2F mutant. The 2F-specific CTL clones suppressed the replication of both wild-type and mutant viruses. However, the abilities of these clones to suppress replication of the 2F virus were much weaker than those of wild-type-specific and the 2F-specific ones to suppress replication of the wild-type virus. These findings indicate that the 2F mutant is conserved in HIV-1-infected donors having HLA-A*2402, because the 2F-specific CTLs failed to completely suppress the 2F mutant replication and effectively prevented viral reversion in new hosts carrying HLA-A*2402.

Cytotoxic T lymphocytes (CTLs) play an important role in the control of human immunodeficiency virus type 1 (HIV-1) replication during acute and chronic phases of HIV-1 infections (9, 28, 34). However, CTLs cannot completely eradicate HIV-1 because HIV-1 escapes from the host immune system by various mechanisms, including mutations of immunodominant CTL epitopes (10–12, 40). A substitution of one amino acid within CTL epitopes is crucial for binding to HLA class I molecules or for the interaction between the T-cell receptors (TCRs) of specific CTLs and the peptide-HLA class I complex. Both mechanisms result in the loss of CTL activities against target cells infected with HIV-1 and contribute to the selection of a virus able to escape from CTLs (10, 13, 23, 26, 35). There are many studies demonstrating that CTL-mediated immune pressure selects CTL escape variants during both acute and chronic HIV-1 and simian immunodeficiency virus (SIV) infections (2, 15, 31) and that selection of the escape mutants could result in the loss of immune control and disease progression (6, 16, 23). The escape of HIV-1 from CTL responses has been proposed to be an important obstacle for HIV-1 vaccine development (7, 16, 39).

HIV-1 mutations that allow escape from HIV-1-specific CTLs are HLA dependent because HIV-1-specific T-cell responses are restricted by HLA alleles. This means that an HIV-1 escape mutant can adapt in populations sharing some dominant HLA alleles (33). An escape mutant can be transmitted vertically from mother to child (21, 22) and horizontally between individuals through unprotected sexual intercourse (USI) (3, 20, 21, 29). A study on HIV-1 evolution has provided direct evidence that an escape mutation of an HLA-B57/5801-restricted CTL epitope is stable after transmission to individuals who did not share HLA-B57/5801 and suggested the accumulation of the escape mutation in the population (29). On the other hand, a recent study demonstrated that an escape mutant selected by the CTLs specific for the wild-type (WT) virus can elicit the escape mutant-specific CTLs in the same donors (4), suggesting the possibility that these escape mutant-specific CTLs are elicited in new donors carrying the same restriction allele. If these escape mutant-specific CTLs are elicited in the donors, it is likely that the escape mutant cannot adapt in them. However, it is well known that in both HIV-1 and SIV infections, common escape mutations are poorly recognized in new hosts who share the same HLA alleles with a donor (17, 32).

In a Japanese population infected with HIV-1 through USI, mutant viruses with Y-to-F substitutions at the second position (2F) in the HLA-A*2402-restricted, Nef138-10 WT CTL epitope (RYPLTFGWCF) were shown to accumulate in HLA-A*2402-positive and even HLA-A*2402-negative patients (20). Nef138-10-specific CTLs are frequently detected in

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chronically HIV-1-infected, HLA-A*2402-positive Japanese individuals (25), suggesting that the Nef138-10 CTL epitope is an immunodominant CTL epitope in the population. On the other hand, the 2F mutation of this epitope impaired the cytotoxic activity of the Nef138-10-specific CTLs, suggesting this mutation to be an escape one (20). We found that Nef138-10 WT tetramer$^+$CD8$^+$ T cells frequently exist, even in HLA-A*2402-positive Japanese patients with primary infections (unpublished data). As most of these Japanese patients were infected with the 2F mutant virus, we speculated that 2F-specific CTLs would be elicited in new hosts having HLA-A*2402.

The present study addressed the following three questions. Do Nef138-10-specific CTLs have strong abilities to suppress HIV-1 replication, but fail to suppress replication of the 2F mutant? Can the 2F escape mutant elicit 2F mutant-specific CTLs in a new host? Can the 2F-specific CTLs suppress replication of 2F mutant and WT viruses? The answers to these are expected to clarify the mechanisms of accumulation of escape mutants in the population.

MATERIALS AND METHODS

Patient samples. This study was approved by the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects, according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. Patient HLA types were determined by standard sequence-based genotyping. Donors with structured treatment interruption belonged to the clinical trial designed as a prospective study of the AIDS Clinical Center, International Medical Center of Japan.

Patients with early HIV infection, with or without acute retroviral symptoms, were recruited. Early HIV infection was confirmed within 6 months before recruitment by a documented history of seroconversion detected by enzyme-linked immunosorbent assay or by a longitudinal increase in band density on Western blots. Patients with active opportunistic infections or psychological disorders, or those treated with immunomodulatory agents, were excluded.

Sequence of autologous virus. Viral RNA was extracted from samples of plasma from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (QIAGEN), and cDNA was synthesized from the RNA with SuperScript RNase H reverse transcriptase and random primer (Invitrogen). The Nef region was amplified by nested PCR using *Taq* DNA polymerase (Promega). Proviral DNA was extracted from HIV-1-infected patient PBMCs by using a QIAamp DNA blood mini kit (QIAGEN). We prepared the following *nef*-specific primer sets: 5′-AGCAGCAGATGGGGTGGGAGC-3′ and 5′-AGCATCTGAGGGA CGCCACTCCC-3′ for the first PCR primer set 1, 5′-TCGAGACCTGGAAA AACATGGAGC-3′ and 5′-AAAGTCCCCAGCGGAAAGTCCC-3′ for the second PCR primer set 1, 5′-TCGAGACCTGGAAAAACATGGAGC-3′ and 5′-TAACCAGAGAGACCCAGTACAGGC-3′ for the first PCR primer set 2, and 5′-GGCTAGAAGCACAAGAGGAGG-3′ and 5′-AGCATCTGAGGGAC GCCACTCCC-3′ for the second PCR primer set 2. The PCR cycling conditions were 94°C for 30 s, followed by 30 cycles of 30 s at 94°C, 40 s at 55 or 60°C, and 1 min at 72°C, with a final extension of 72°C for 7 min or 94°C for 1 min for the first PCR, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C, with a final extension of 72°C for 7 min for the second PCR. The PCR products were then agarose gel purified and sequenced directly or cloned by the use of a TOPO TA cloning kit (Invitrogen). All DNA sequencing was performed by using a BigDye Terminator (version 1.1) cycle sequencing kit (Applied Biosystems) and an ABI PRISM 310 genetic analyzer.

HIV-1-specific CTL clones. For the generation of KI-158-derived and KI-144-derived CTL clones, PBMCs which were obtained at weeks 69 and 64, respectively, after the first visit were used. Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding cells of this population into U-bottom, 96-well microtiter plates together with 200 μl cloning mixture (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human interleukin-2, irradiated allogeneic PBMCs from a healthy donor, and irradiated C1R-A*2402 cells prepulsed with the corresponding peptide), as previously described (25). Nef138-10-specific and Nef138-10-2F-specific CTLs were generated by using WT (RYPLTFGWCF) and 2F (RFPLT FGWCF) peptides, respectively. All CTL clones were cultured in RPMI 1640-10% FCS supplemented with 200 U/ml recombinant human interleukin-2 and stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

HIV-1 clones. Infectious proviral clones of HIV-1, pNL-432 and its Nef mutant pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), reported previously, were used (1). For pNL-432-10F, pNL-432-2F10F, and pNL-M20A-10F, mutations were introduced by site-directed mutagenesis based on overlap extension.

Flow cytometric analysis for surface expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells. HLA-A*2402-positive CD4$^+$ T cells infected with HIV-1 clone were stained to assess the expression of HLA class I in HIV-1-infected CD4$^+$ T cells, as previously described (38). Briefly, the cells were stained with anti-A11 and -A24 monoclonal antibody (MAb) A11.1 M, following staining with phycoerythrin (PE)-conjugated anti-mouse immunoglobulin (Ig) (Dako-Cytomation, Glostrup, Denmark) and thereafter were fixed and permeabilized for intracellular HIV-1 p24 staining with fluorescein isothiocyanate (FITC)-conjugated anti-p24 MAb KC-57 (Beckman Coulter, Miami, FL). The expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells was analyzed by using a flow cytometry.

Peptide-binding assay. The binding of peptides to HLA-A*2402 molecules was tested as previously described (25). HLA-A*2402 RMA-S transfectants, which were transfected with HLA-A*2402 and human β_2-microglobulin, lacked functional TAP2. They express a very low level of HLA class I molecules on the cell surfaces when they are cultured at 37°C, while empty HLA class I molecules are stably expressed if they are cultured at 26°C, because empty HLA class I molecules are unstable on cell surfaces at 37°C but stable at 26°C. The stabilization of HLA class I molecules is dependent on peptide binding affinity (24, 30, 36). Briefly, RMA-S-A*2402 cells were cultured at 26°C for 14 to 18 h. The cells were incubated at 26°C for 1 h with WT (RYPLTFGWCF) or 2F (RFPLTFG WCF) peptide at various concentrations and then at 37°C for 3 h. After two washes with phosphate-buffered saline (PBS) supplemented with 20% FCS (PBS-20% FCS), they were incubated for 30 min on ice with an appropriate dilution of MAb TP25.99. After two washes with PBS-20% FCS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated sheep IgG with anti-mouse Ig specificity (Silenus Laboratories, Hawthorn, Australia). Finally, they were washed three times with PBS-20% FCS, after which the fluorescence intensity was measured by using a flow cytometer (Becton Dickinson, Mountain View, CA).

HLA-peptide tetrameric complexes. The tetrameric complexes were synthesized as previously described (5). Briefly, an ectodomain of HLA class I proteins and human β_2 microglobulin, produced in *Escherichia coli* that had been transformed with the relevant expression plasmids, were first solubilized in denaturing buffer containing 8 M urea and refolded in refolding buffer in the presence of a synthesized peptide for 48 h at 4°C. The resultant 45-kDa complex was purified by size exclusion (Superdex G75; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England) and anion exchange chromatographies (MonoQ column; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). Purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C terminus of the heavy chain and were mixed with PE-conjugated avidin (ExtrAvidin-PE; Sigma-Aldrich, St. Louis, MO) at a molar ratio of 4:1.

Tetramer analysis. CTL clones were first stained with either PE-conjugated WT or allophycocyanin (APC)-conjugated mutant (2F) tetramer (final concentration, 300 nM) at 37°C for 30 min. For the competitive assay, the clones were stained with WT and 2F tetramer (final concentration, 300 nM for each tetramer) at the same time at 37°C for 30 min. After two washes with RPMI 1640 medium supplemented with 10% FCS (RPMI 1640-10% FCS), the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min. For ex vivo analysis, thawed PBMCs were stained with PE-conjugated WT and APC-conjugated 2F tetramers (final concentration, 300 nM for each tetramer) at 37°C for 30 min. Following two washes with RPMI 1640-10% FCS, the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min and subsequently analyzed by using flow cytometry. The percentage of tetramer-positive cells among CD8-positive cells was then calculated.

Replication suppression assay. The abilities of HIV-1-specific CTLs to suppress HIV-1 replication were examined as previously described (37). Briefly, CD4$^+$ T cells were incubated with a given HIV-1 clone for 6 h at 37°C. After three washes with RPMI 1640-10% FCS, the cells were cocultured with HIV-1-specific CTL clones. From day 3 to day 9 postinfection, 10 μl of culture supernatant was collected and the concentration of p24 antigen (Ag) was measured by use of an enzyme immunoassay (HIV-1 p24 Ag enzyme-linked immunosorbent assay kit; ZeptoMetrix Corporation, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1 − concentration of p24 Ag in the supernatant of HIV-1-infected CD4$^+$ T cells

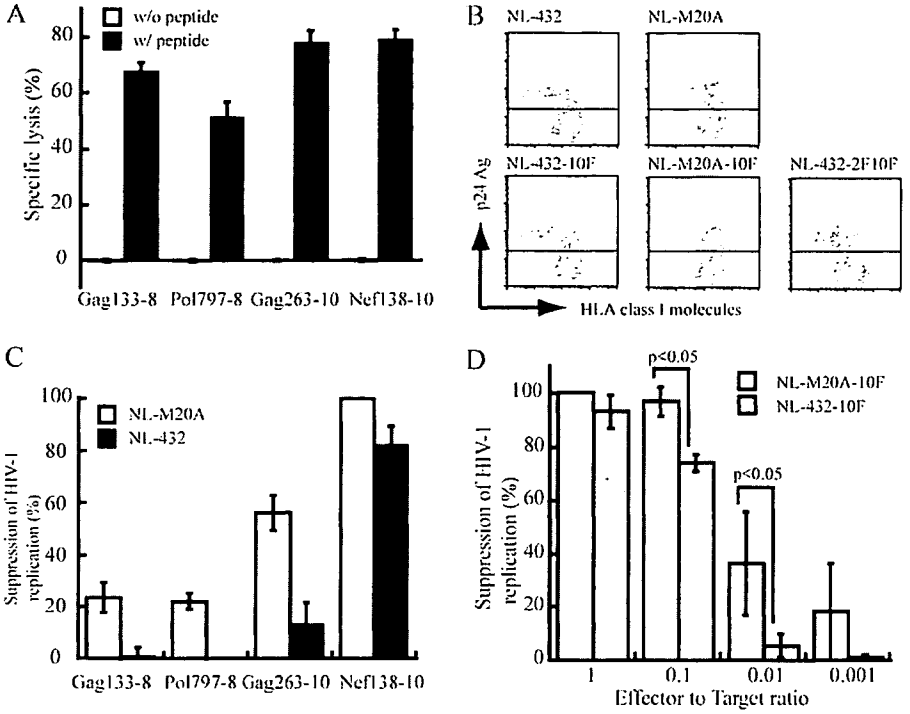


FIG. 1. Suppression of HIV-1 replication in HIV-1-infected CD4$^+$ T cells by HLA-A*2402-restricted HIV-1-specific CTLs. (A) Cytolytic activities of HLA-A*2402-restricted HIV-1-specific CTLs (four Gag133-8-specific, five Pol797-8-specific, four Gag263-10-specific, and four Nef138-10-specific CTL clones) toward HLA-A*2402$^+$ cells pulsed with (w) the indicated peptides (1,000 nM). w/o, without. The cells were tested at an E-to-T ratio of 2:1. Values represent averages ± standard deviations (SD) (error bars) of results from triplicate assays. (B) CD4$^+$ T cells derived from healthy donors were infected with NL-432, NL-M20A, NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cultured for 3 to 5 days. The cultured CD4$^+$ T cells were stained with anti-p24 and A11.1 M anti-A11 and anti-A24 MAbs. (C) Suppression of HIV-1 replication by HLA-A*2402-restricted CTLs. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432 or NL-M20A and then cocultured with HIV-1-specific CTLs at an E-to-T ratio of 1:1. HIV-1 p24 Ags in the supernatant were measured on day 6 postinfection by conducting an enzyme immunoassay. The percentage of suppression of HIV-1 replication was calculated. Values represent averages ± SD (error bars) for four Gag133-8-specific, three Pol797-8-specific, four Gag263-10-specific, and three Nef138-10-specific CTL clones. (D) The ability of Nef138-10-specific CTL clone 189 to suppress NL-432-10F and NL-M20A-10F replication in CD4$^+$ T cells. HIV-1-infected HLA-A*2402$^+$CD4$^+$ T cells were cocultured with Nef138-10-specific CTLs at various E-to-T ratios. Values represent averages ± SD (error bars) of results from two independent experiments. The *P* values were determined by nonparametric Mann-Whitney test.

cultured with HIV-1-specific CTLs/concentration of p24 Ag in the supernatant of HIV-1-infected CD4$^+$ T cells cultured without the CTLs) × 100.

^{51}Cr release assay. The cytotoxicity of CTL clones against C1R-A*2402 prepulsed with appropriate peptide at various concentrations or HIV-1-infected 221-CD4$^+$-A*2402 cells was determined as previously described (37, 38). Briefly, target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline. Effector cells were cocultured with target cells (2×10^3/well) for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release was determined by measuring the counts per minute (cpm) in the supernatant in the wells containing only target cells (cpm spn). Maximum release (cmp max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100. Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where cpm exp is the cpm in the supernatant in the wells containing both the target and effector cells at an effector-to-target ratio of 2:1. The peptide concentration providing half of the maximum percentage of specific lysis (LL_{50}) was calculated by using KaleidaGraph (Hulinks, Inc., Tokyo, Japan).

RESULTS

Complete suppression of HIV-1 replication by HLA-A*2402-restricted Nef138-10-specific CTLs. Since Nef138-10-specific CTLs are frequently detected in HLA-A*2402-positive HIV-1-infected individuals, it has been speculated that Nef138-10 (RYPLTFGWCY) is an immunodominant epitope and that these specific CTLs can strongly suppress HIV-1 replication. To clarify the abilities of Nef138-10-specific CTLs to suppress HIV-1 replication, we compared the abilities of four epitope-specific, HLA-A*2402-restricted CTL clones to suppress HIV-1 replication by assaying for the suppression of HIV-1 replication. All CTL clones specific for the Gag133-8, Pol797-8, Gag263-10, or Nef138-10 epitope showed similar cytotoxic activities against target cells prepulsed with the corresponding peptide (Fig. 1A). Gag133-8, Pol797-8, and Gag263-10 epitopes are conserved in both NL-432 and NL-M20A strains, the latter of which has the ability to down-regulate the expression of CD4 molecules, but not the expression of HLA class I molecules, on the cell surface, whereas the Nef138-10 epitope is not conserved in these strains. The Nef138-10 epitope has a tyrosine instead of phenylalanine at position 10, but Nef138-10 epitope-specific CTLs effectively recognized both peptides, RYPLTFGWCF and RYPLTFGWCY. We investigated HLA-A*2402 down-regulation on CD4$^+$ cells infected with NL-432 or NL-M20A. The down-regulation of HLA-A*2402 was found on CD4$^+$ cells infected with NL-432, but not on those infected with NL-M20A (Fig. 1B). We measured the abilities of these CTL clones to suppress HIV-1 replication in primary CD4$^+$ T cells infected with either HIV-1 clone NL-432 or HIV-1 clone NL-M20A. Nef138-10-specific CTL clones com-

pletely suppressed both NL-432 and NL-M20A replication at an effector-to-target (E-to-T) ratio of 1:1, whereas CTL clones specific for the Gag133-8, Pol797-8, or Gag263-10 epitope partially suppressed NL-M20A replication but failed to suppress NL-432 replication (Fig. 1C), indicating that Nef138-10-specific CTLs have strong abilities to suppress NL-432 replication. We generated mutant viruses, NL-432-10F and NL-M20A-10F, carrying the SF2 strain-derived Nef138-10 epitope because the CTL clones specific for the SF2 strain-derived Nef138-10 epitope (RYPLTF GWCF) had been established. Down-regulation of HLA-A*2402 was found on $CD4^+$ cells infected with NL-432-10F, whereas it did not occur on those infected with NL-M20A-10F (Fig. 1B). The strong ability of Nef138-10-specific CTL clone 189 to suppress the replication of both viruses was also found at an E-to-T ratio of 0.1:1 (Fig. 1D). These results indicate that Nef138-10-specific CTLs had strong abilities to suppress HIV-1 replication, regardless of Nef-mediated down-regulation of HLA class I molecules. A significant difference between the abilities of the Nef138-10-specific CTL clone to suppress NL-432-10F and NL-M20A-10F replication was found at E-to-T ratios of 0.1:1 and 0.01:1, but not at an E-to-T ratio of 1:1 (Fig. 1D), suggesting that the suppressive ability of this CTL clone was minimally affected by Nef-mediated HLA class I molecules.

A previous study demonstrated that the 2F substitution of this epitope is associated with HLA-A*2402$^+$ Japanese hemophiliacs (20). We therefore investigated HLA-A*2402-associated mutations of the Nef138-10 epitope in chronically HIV-1-infected Japanese hemophiliacs and nonhemophiliacs. We sequenced the Nef138-10 epitope and its flanking region from HIV-1 RNA from plasma samples from 41 HLA-A*2402-positive and 22 HLA-A*2402-negative Japanese patients (Fig. 2A). We found mainly three types of mutation at the epitope region: a Y-to-F substitution at the second position (2F), a T-to-C substitution at the fifth position, and an F-to-Y substitution at the tenth position. Only the 2F substitution was significantly associated with HLA-A*2402 (Fig. 2B). Although we detected an I-to-T or I-to-V substitution at the -1 position in the N-terminal flanking region, we could not find any association with HLA-A*2402 or other HLA alleles (data not shown). We also analyzed the 2F substitution in two groups, hemophiliacs and nonhemophiliacs who had become infected through USI. In both groups, the 2F substitution was significantly associated with HLA-A*2402 (Fig. 2C). A previous study showed that the 2F substitution was significantly found in the HLA-A*2402-positive Japanese hemophiliacs but not in HLA-A*2402-positive patients infected through USI (20). Our results confirmed the association of the 2F substitution with HLA-A*2402 in Japanese hemophiliacs but suggested a different conclusion for the Japanese patients infected through USI. The frequency of 2F substitutions was much higher in HLA-A*2402$^-$ patients infected through USI than that in HLA-A*2402$^-$ hemophiliacs (31% versus 0%), suggesting that this mutation had accumulated in the HLA-A*2402$^-$ population.

Antiviral activities of Nef138-10-specific CTLs toward NL-432 and Nef138-10-2F mutant virus. To investigate the effect of the 2F substitution in the Nef138-10 epitope on the antiviral activities of Nef138-10-specific CTLs, we first measured the cytotoxic activity of these cells against WT or mutant epitope peptide-prepulsed cells (Fig. 3A). Four CTL clones derived from an HIV-1-infected Japanese patient (KI-158) showed

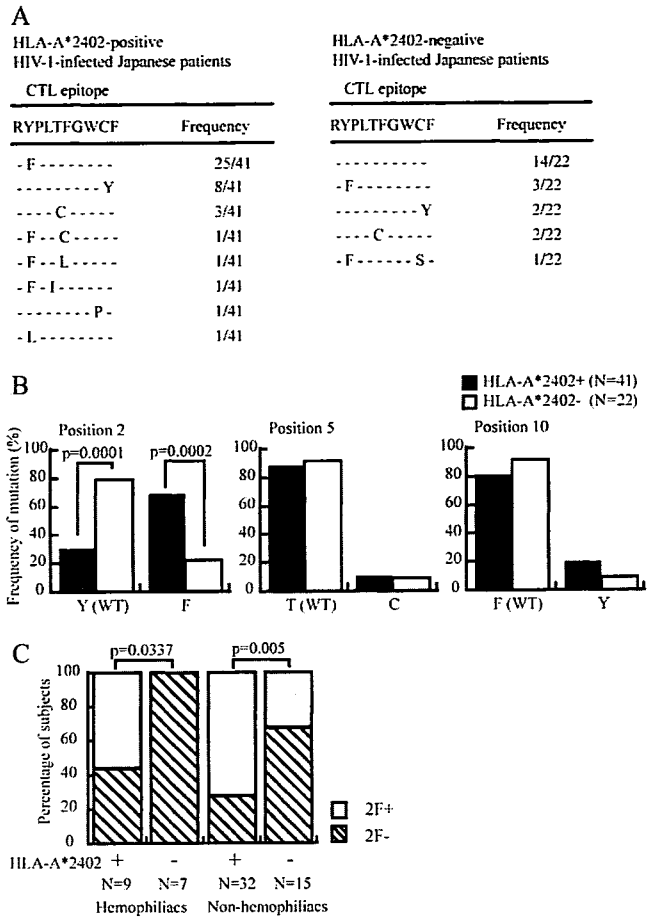


FIG. 2. Frequency of mutation in the Nef138-10 epitope and flanking region. (A) Alignment of the amino acid sequences from 41 HLA-A*2402$^+$ and 22 HLA-A*2402$^-$ patients. (B) Frequency of the substitutions in the Nef138-10 epitope. The Y-to-F substitution at position 2 was more frequently found in the HIV-1-infected Japanese population expressing HLA-A*2402 ($P = 0.0002$). The vertical axis shows the frequency (%) of mutation among 41 HLA-A*2402$^+$ and 22 HLA-A*2402$^-$ patients. The horizontal axis shows the amino acid at each position. (C) Y-to-F substitutions between hemophiliacs and nonhemophiliacs (more than 2 years after infection). Bars indicate the percentages of individuals whose viral sequences are 2F (shaded part of bar) or not (striped part of bar) in subjects either expressing HLA-A*2402 (2F+) or not expressing it (2F−). The P values were determined by Fisher's exact test.

higher cytotoxic activities toward HLA-A*2402$^+$ target cells prepulsed with WT peptide (LL_{50}, 0.16 ± 0.07 nM) than toward those prepulsed with the mutant epitope peptide (LL_{50}, 5.03 ± 6.23 nM). The difference in CTL activity between the two targets varied among the four clones. The binding affinity of the WT peptide for HLA-A*2402 molecules was higher than that of the mutant peptide, but the difference between these peptides was minimal (Fig. 3B). These results indicate that TCRs of Nef138-10-specific CTLs could effectively recognize the 2F mutant epitope. To investigate the effects of the 2F substitution on the abilities of Nef138-10-specific CTLs to recognize target cells infected with HIV-1, we measured the activities of Nef138-10-specific CTL clones to kill HIV-1-infected cells and to suppress HIV-1 replication in HIV-1-infected

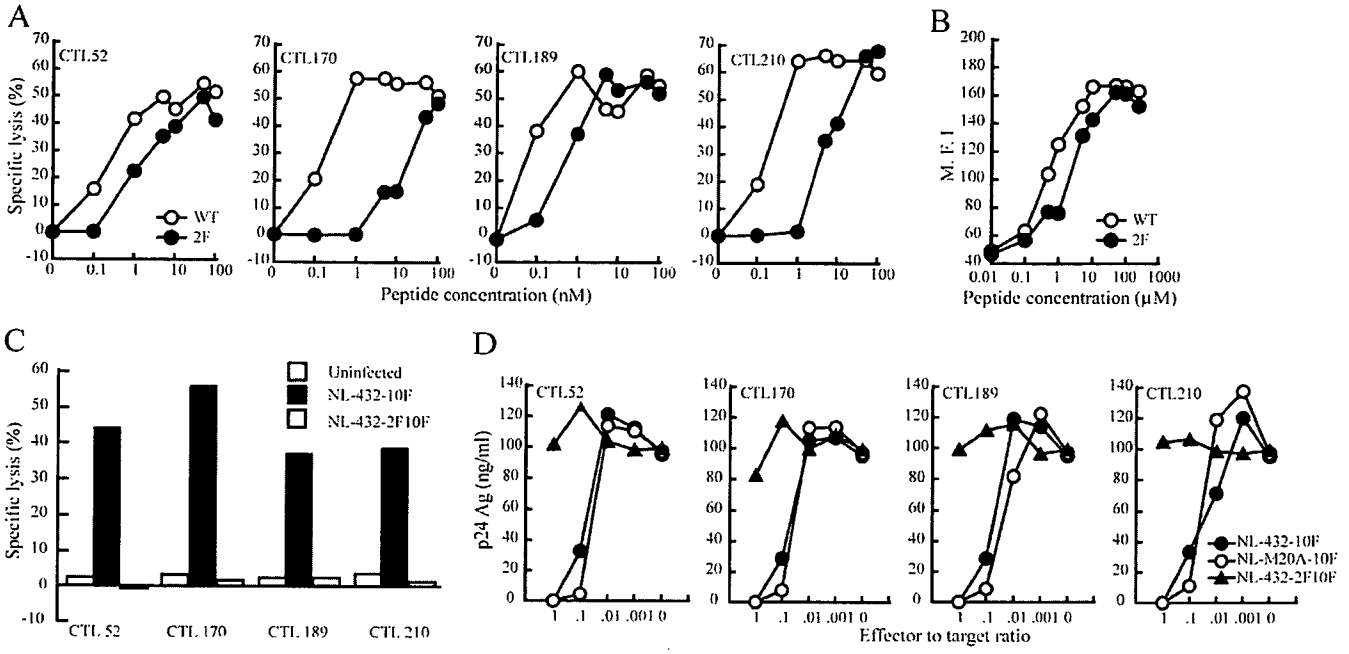


FIG. 3. Abilities of Nef138-10-specific CTLs to suppress replication of NL-432-2F10F. (A) Cytolytic activity of HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402$^+$ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). They were tested at an E-to-T ratio of 2:1. (B) Binding of WT and 2F peptides to HLA-A*2402 molecules was quantified by the HLA-A*2402 stabilization assay. (C) Cytolytic activity of HLA-A*2402-restricted Nef138-10-specific CTLs toward NL-432-10F-infected or NL-432-2F10F-infected HLA-A*2402$^+$ cells (40% and 50% p24-positive cells, respectively) used as targets. Cytolytic activity of the CTLs was tested at an E-to-T ratio of 2:1. (D) Abilities of HIV-1-specific CTLs to suppress NL-432-2F10F replication in CD4$^+$ T cells. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cocultured with Nef138-10-specific CTLs at various E-to-T ratios. The HIV-1 p24 Ag level in the supernatant was measured on day 6 postinfection by enzyme immunoassay.

CD4$^+$ T cells (Fig. 3C and D). For the assay, NL-432-2F10F, carrying a Y-to-F substitution at the second position, had been established. All four CTL clones strongly lysed NL-432-10F-infected cells but not NL-432-2F10F-infected cells (Fig. 3C). In addition, those CTL clones strongly suppressed the replication of both NL-432-10F and NL-M20A-10F, but not that of NL-432-2F10F (Fig. 3D). Taken together, these results indicate that the 2F substitution is a mutation permitting escape from Nef138-10-specific CTLs.

Antiviral response to the 2F mutant of CTLs having high-affinity TCRs for the 2F mutant epitope. Among the established Nef138-10-specific CTL clones, we found three CTL clones (from HIV-1-infected patient KI-144) to have much higher cytotoxic activity toward cells prepulsed with mutant peptide than toward those prepulsed with the WT peptide (Fig. 4A). The cytotoxic activities of these CTL clones toward the former were approximately 100-fold higher than those toward the latter (Fig. 4A), suggesting that these CTL clones carried TCRs that more effectively recognized the mutant epitope than that of the WT. To test this possibility, we generated HLA-A*2402 tetramers with Nef138-10 and its 2F mutant and then tested the abilities of these tetramers to bind to the CTL clones at different concentrations of the tetramers. Clone 82 exhibited stronger affinity for the 2F tetramer than for the WT tetramer, whereas clone 189 showed weaker affinity for the former tetramer than for the latter one (Fig. 4B). These results indicate that clone 82 had a TCR with higher affinity for the 2F mutant than for the WT.

It is speculated that CTLs carrying high-affinity TCRs for the 2F mutant would have the ability to recognize cells infected with the 2F virus. Therefore, we investigated the activities of clones 82, 98, and 108 against HIV-1-infected cells by measuring their cytotoxic activities toward HIV-1-infected cells. These CTL clones killed NL-432-2F10F-infected cells (Fig. 4C), indicating that the Nef138-2F mutant epitope was presented in HIV-1-infected cells. Interestingly, they killed NL-432-10F-infected cells more effectively than they did NL-432-2F10F-infected cells (Fig. 4C). This finding indicates that the presentation of the 2F epitope was much weaker than that of the WT one. The results may not reflect Ag presentation of the 2F mutant epitope in HIV-1-infected CD4$^+$ T cells in vivo, because 221 transfectants highly expressing HLA-A*2402 were used as target cells in this assay. We therefore tested the abilities of these CTL clones to suppress HIV-1 replication. They significantly suppressed the replication of NL-432-2F10F at an E-to-T ratio of 1:1 (Fig. 4D and E) and more effectively suppressed the replication of NL-432-10F and NL-M20A-10F than that of NL-432-2F10F (Fig. 4D), supporting the idea that the 2F mutant epitope was presented more weakly than the WT epitope was. Since KI-144-derived CTL clones had higher-affinity TCRs for the 2F mutant and suppressed the replication of NL-432-2F10F, we assume that the 2F mutant-specific CTLs had been elicited by priming with the mutant epitope in this patient.

Ex vivo analysis of the 2F mutant-specific CTLs in HIV-1-infected individuals who had been infected with the 2F mutant virus. Since the 2F mutant-specific CTL clones were established from patient KI-144, we assumed that the 2F mutant-

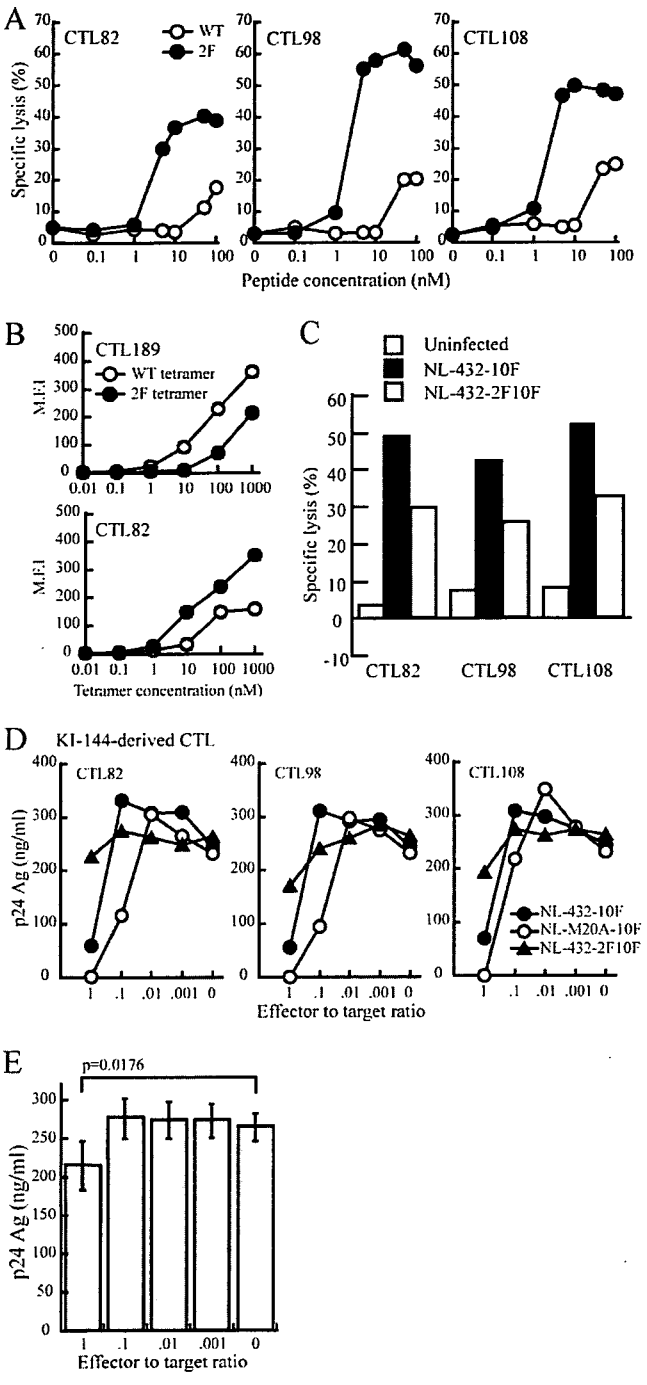
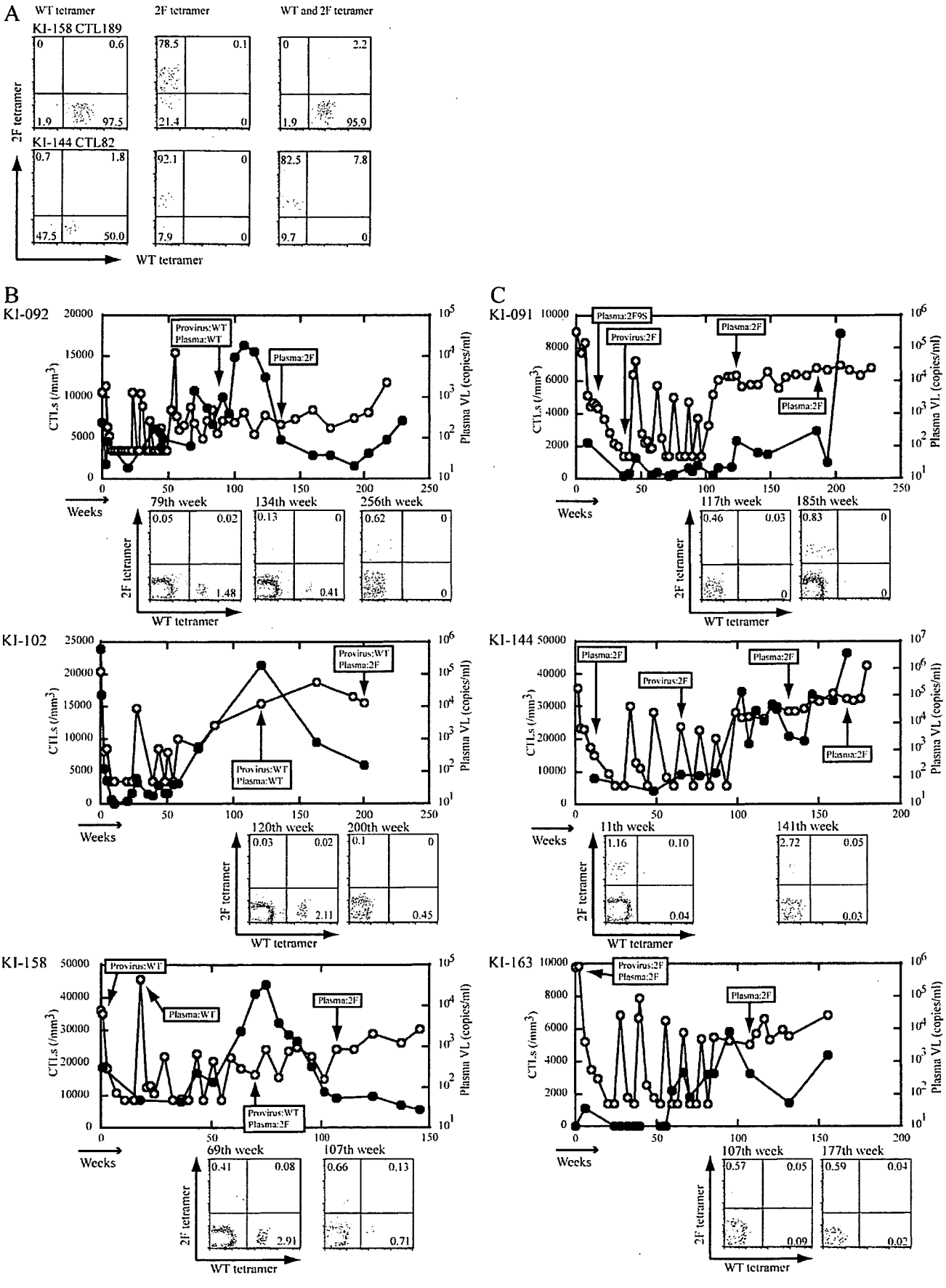


FIG. 4. Distinct antiviral activities toward NL-432-2F10F of KI-144-derived Nef138-10-specific CTL clones. (A) Cytolytic activity of KI-144-derived HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402$^+$ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). The clones were tested at an E-to-T ratio of 2:1. (B) WT and 2F tetramer binding activities of CTL clones 189 (top) and 82 (bottom) clones were determined. (C) Cytolytic activity of clones in panel A toward NL-432-10F-infected or NL-432-2F10F-infected HLA-A*2402$^+$ cells (45% and 55% p24-positive cells, respectively) used as target cells. Cytolytic activity was tested at an E-to-T ratio of 2:1. (D) Abilities of HIV-1-specific CTLs to suppress NL-432-2F10F replication in CD4$^+$ T cells. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cocultured with KI-144-derived CTLs dominantly binding 2F tetramers. The various E-to-T ratios are indicated. The HIV-1 p24 Ag level in the supernatant was measured on day 6 postinfection by using the enzyme immunoassay. (E) Replication of NL-432-2F10F at each E-to-T ratio. Values represent the averages for the three CTL clones ± standard deviations (error bars). Significant differences between cultures with and without CTLs are shown (nonparametric Mann-Whitney test).

specific CTLs were effectively elicited in this patient. To clarify this point, we investigated when and how many 2F-specific CTLs were elicited in this patient. First we established a competitive tetramer binding assay using two HLA-A*2402 tetramers carrying the 2F and the WT peptides to detect the respective 2F-specific and the WT-specific CD8$^+$ T cells. When the CTL clones were stained with either tetramer at different concentrations, clone 82 from patient KI-144 exhibited stronger affinity for the 2F tetramer than for the WT one, whereas clone 189 from patient KI-158 showed weaker affinity for the former tetramer than for the latter (Fig. 4B). Therefore, we expected that each CTL clone would bind to only its higher-affinity tetramer when both tetramers were used at the same concentration. Indeed, when clones 189 and 82 were stained using these tetramers, clones 189 and 82 bound to only the WT and the 2F tetramers, respectively (Fig. 5A). By employing this assay, we investigated the appearance of Nef138-10-specific or 2F-specific CTLs in patients with early HIV-1 infection (Fig. 5B and C). Ex vivo analysis of PBMCs from three patients, KI-092, KI-102, and KI-158, who had been infected with the WT virus, showed that they had mainly the WT-specific CTLs when the WT was found, and then 2F-specific CTLs became predominant after the 2F mutant took the place of the WT-specific CTLs (Fig. 5B). WT virus was found in KI-092 and KI-102 for approximately 2 years after infection, indicating that the 2F mutant had been slowly selected by the specific CTLs. On the other hand, ex vivo analysis of PBMCs from patients KI-091, KI-144, and KI-163 revealed that they had the 2F-specific CTLs only (Fig. 5C). In addition, these patients had 2F sequences in both plasma RNA and proviral DNA throughout their clinical course (Fig. 5C). These findings strongly suggest that these patients had been infected with the 2F mutant. Thus, the 2F mutant-specific CTLs were effectively elicited in donors who had been primarily infected with the 2F mutant virus. However, it remains possible that the number of WT-specific CTLs was too low to be detected by the ex vivo tetramer binding assay. To exclude this possibility, we investigated whether the patients had the WT-specific memory CD8$^+$ T cells. We stimulated their PBMCs with either WT or mutant peptide and cultured the PBMCs for 2 weeks. The WT tetramer-binding CD8$^+$ T cells were then counted by using the competitive tetramer binding assay. The WT-specific CD8$^+$ T cells were not detected among the cultured cells (data not shown), indicating that these patients did not have WT-specific CTLs. These results indicate that when the 2F mutant virus infects a new HLA-A*2402$^+$ host, this host recognizes the 2F mutant epitope and elicits the 2F mutant-specific CTLs.

DISCUSSION

A previous study showed that the 2F mutant was frequently detected in chronically HIV-1-infected individuals having


A
WT tetramer
2F tetramer
WT and 2F tetramer
KI-158 CTL189
KI-144 CTL82
2F tetramer
WT tetramer
B
KI-092
Provirus:WT
Plasma:WT
Plasma:2F
CTLs (/mm3)
Plasma VL (copies/ml)
Weeks
79th week
134th week
256th week
KI-102
Provirus:WT
Plasma:2F
Provirus:WT
Plasma:WT
120th week
200th week
KI-158
Provirus:WT
Plasma:WT
Plasma:2F
Provirus:WT
Plasma:2F
69th week
107th week
C
KI-091
Plasma:2F9S
Provirus:2F
Plasma:2F
Plasma:2F
117th week
185th week
KI-144
Plasma:2F
Provirus:2F
Plasma:2F
Plasma:2F
11th week
141th week
KI-163
Provirus:2F
Plasma:2F
Plasma:2F
107th week
177th week

HLA-A*2402 and that Nef138-10-specific CTLs failed to kill target cells infected with HIV-1 recombinant Sendai virus containing the 2F mutant; data suggested that the 2F is a mutation for escape from the specific CTLs (20). However, the question remained as to whether Nef138-10-specific CTLs can mediate strong immune pressure on HIV-1 replication so that they select the 2F mutant in vivo. In the present study, we clarified this question by investigating the abilities of Nef138-10-specific CTL clones to suppress replication of the WT and the 2F mutant viruses. Each Nef138-10-specific CTL clone exhibited a strong ability to suppress replication of Nef^+ HIV-1 at E-to-T ratios of 1:1 and 0.1:1. This ability is much stronger than that of most HIV-1-specific CTLs (19, 37, 38), suggesting that these CTLs can mediate strong immune pressure in vivo. In addition, our study using the HIV-1 2F mutant showed that Nef138-10-specific CTLs failed to kill target cells infected with the 2F mutant and to suppress replication of the mutant, confirming that 2F is an escape mutant.

Escape mutations occur at sites within CTL epitopes, where the substitution of an amino acid abrogates HLA binding, reduces the recognition of the TCR, and/or interferes with efficient Ag processing (14, 41). The 2F mutant peptide bound to HLA-A*2402 molecules with an efficiency similar to that of the WT peptide. Both the WT and the 2F tetramers bound to Nef138-10-specific CTL clones. In addition, Nef138-10-specific CTL clones killed target cells prepulsed with Nef138-10-2F peptide. These findings suggest that the escape mechanism of the 2F mutant involves the disruption of cellular processing of the 2F mutant peptide. However, since Nef138-10-2F-specific CTL clones effectively killed the target cells infected with the 2F mutant virus and suppressed the replication of the mutant virus, the 2F peptide can be naturally processed and presented by HLA-A*2402. The 2F-specific CTL clones could recognize the 2F peptide much more effectively than the WT peptide, whereas the clones showed much stronger abilities to suppress replication of the WT virus than that of the 2F mutant virus. These observations indicate that the change from Tyr to Phe remarkably reduced the presentation of the epitope peptides in Ag processing but that the 2F mutant could still be presented in the cells infected with the mutant.

It is well known that in both HIV-1 and SIV infections, escape mutations are poorly recognized in new hosts who share the same HLA alleles with donors (17, 32). If escape mutant peptides fail to bind to HLA class I restriction molecules or the mutation critically affects the Ag processing, these escape mutants are hardly recognized and fail to elicit the specific CTLs in new hosts sharing the same HLA alleles. On the other hand, if escape mutant peptides can bind to HLA class I restriction molecules and can be processed and presented, it remains possible that the mutant epitope is recognized in new hosts. The 2F mutant peptide effectively bound to HLA-A*2402 (Fig. 3B), suggesting the possibility that the 2F mutant peptide is presented by HLA-A*2402. We therefore selected the three donors who were infected with the 2F mutant virus at an early phase (within 10 weeks before the first visit) and investigated whether the 2F-specific $CD8^+$ T cells were elicited in these donors. It was strongly suggested that these patients had been infected with the 2F virus, since in the donors who had been infected with the WT virus, the 2F mutation was selected approximately 2 years after infection. The 2F-specific $CD8^+$ T cells were elicited in these three donors, although this mutant epitope was very weakly presented by HLA-A*2402. Thus, escape mutant-specific CTLs can be elicited in new hosts even if the mutant epitope peptide is very weakly presented.

The reversion of a CTL escape mutation to the WT occurs when the mutant virus is transmitted to a new host not sharing HLA class I alleles (18, 29) and even to a new host sharing HLA class I alleles with the monkey donors before the specific CTL is elicited (8, 27). Although the reversion of the 2F epitope to the WT one was reported for chronically HIV-1-infected individuals having no HLA-A*2402, the rate of reversion was very low (20), suggesting that the Y-to-F substitution does not inflict a large fitness cost on HIV-1. A previous study showed that the 2F mutant was still detectable in 56% of $HLA\text{-}A^*2402^-$ Japanese patients infected through USI (20). In contrast, in the present study, it was found in only 31% of the patients. This difference between these two studies may have resulted from the difference in the time when the sequence was analyzed after the infection. Although the frequency of the 2F mutant in $HLA\text{-}A^*2402^-$ Japanese individuals is different between the two studies, the studies indicate that 2F mutant did accumulate in $HLA\text{-}A^*2402^-$ Japanese individuals infected through USI. The reversion of this epitope should occur but may be very slow in $HLA\text{-}A^*2402^-$ donors. It is thought that the reversion does not occur in $HLA\text{-}A^*2402^+$ individuals, because the 2F-specific CTLs can strongly suppress replication of the WT virus. Indeed, the reversion was not found in three patients who had been primarily infected with the 2F mutant virus and monitored for 2 to 3 years. Thus, the 2F mutant is accumulating in the Japanese population, of which 70% carry HLA-A*2402.

The competitive tetramer binding assay using the two tetramers could distinguish $CD8^+$ T cells carrying high-affinity TCRs for the WT epitope from those carrying high-affinity ones for the 2F epitope. By using this assay, we found that patients who had been infected with the WT virus first produced WT-specific $CD8^+$ T cells and then 2F-specific $CD8^+$ T cells approximately 6 to 12 months after the 2F mutant had become predominant. In those patients, the 2F mutant virus appeared more than 12 months after the WT virus infection. These findings support our contention that 2F is an escape mutant and that the three donors who had 2F sequences in

FIG. 5. Detection of Nef138-10-2F-specific CTLs in HIV-1-infected patients who had been infected with 2F mutant virus. (A) Tetramer binding of Nef138-10-specific CTLs. KI-158-derived CTL clone 189 (top) and KI-144-derived CTL clone 82 (bottom) were stained with either WT (left panels) or 2F (middle panels) tetramers or both (right panels). The percentage of tetramer-positive cells among $CD8^+$ cells was measured. (B and C) Ex vivo analysis of Nef138-10-specific and Nef138-10-2F-specific CTLs. Nef138-10-specific CTLs in PBMCs derived from $HLA\text{-}A^*2402^+$ HIV-1-infected individuals were measured by using both WT and mutant (2F) tetramers. Symbols: ●, WT tetramer-positive cells; ○, plasma viral loads. The epitope sequences from viral RNA (plasma) or provirus DNA (PBMCs) during the clinical course are shown. The *x* axis represents weekly course from the first visit. The frequency of $CD8^+$ cells positive for each or both tetramers is given in the quadrants below the graphs. VL, viral load.

their plasma RNA and proviral DNA during the early phase were primarily infected with 2F virus.

In the present study, we demonstrated that new hosts could effectively produce the 2F escape mutant-specific CTLs, even though the 2F mutant epitope was very weakly presented by HLA-A*2402 in HIV-1-infected cells. The 2F-specific CTLs could suppress replication of the 2F mutant virus, but this ability was much weaker toward the 2F mutant than toward the WT virus. The reversion from 2F to WT was not found in the three patients who had been infected primarily with the 2F mutant virus and monitored for 2 to 3 years. This lack of reversion is explained by the fact that the 2F-specific CTLs could effectively suppress replication of the WT virus. This mutant accumulated in HLA-A*2402$^-$ USI patients. Since HLA-A*2402 is a common allele found in approximately 70% of the Japanese population, the 2F mutant can accumulate in the Japanese AIDS population.

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HIV-1–specific CTLs effectively suppress replication of HIV-1 in HIV-1–infected macrophages

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Both CD4+ T cells and macrophages are major reservoirs of HIV-1. Previous study showed that HIV-1–specific cytolytic T lymphocytes (CTLs) hardly recognize HIV-1–infected CD4+ T cells because of Nef-mediated HLA class I down-regulation, suggesting that HIV-1 escapes from HIV-1–specific CTLs and continues to replicate in HIV-1–infected donors. On the other hand, the CTL recognition of HIV-1–infected macrophages and the effect of Nef-mediated HLA class I down-regulation on this recognition still remain unclear. We show a strong HIV-1 antigen presentation by HIV-1–infected macrophages. HIV-1–specific CTLs had strong abilities to suppress HIV-1R5 virus replication in HIV-1–infected macrophages and to kill HIV-1R5–infected macrophages. Nef-mediated HLA class I down-regulation minimally influenced the recognition of HIV-1–infected macrophages by HIV-1–specific CTLs. In addition, HIV-1–infected macrophages had a stronger ability to stimulate the proliferation of HIV-1–specific CTLs than HIV-1–infected CD4+ T cells. Thus, the effect of Nef-mediated HLA class I down-regulation was less critical with respect to the recognition by HIV-1–specific CTLs of HIV-infected macrophages than that of HIV-1–infected CD4+ T cells. These findings support the idea that the strong HIV-1 antigen presentation by HIV-1–infected macrophages is one of the mechanisms mediating effective induction of HIV-1–specific CTLs in the acute and early chronic phases of HIV-1 infection. (Blood. 2007;109:4832-4838)

Introduction

HIV-1–specific CD8+ T cells play a critical role in the control of HIV-1 infections.[1,2] However, HIV-1–infected individuals develop AIDS if they are not treated with antiretroviral therapy. HIV-1 escape occurs during acute and chronic phases of an HIV-1 infection.[3] Several hypotheses concerning HIV-1 mechanisms affording escape from the host immune system have been proposed.[4-6] One of these is impaired activity of HIV-1–specific cytotoxic T lymphocytes (CTLs) to kill HIV-1–infected CD4+ T cells and to suppress HIV-1 replication by Nef-mediated down-regulation of HLA class I molecules. Previous studies reported that HIV-1–specific CTL clones failed to kill CD4+ T cells infected with Nef+ HIV-1.[7,8] Our previous studies using NL-432 X4 clone and NL-M20A lacking Nef function for HLA class I molecules showed that most HIV-1–infected CTLs failed to kill NL-432–infected CD4+ T cells and partially suppressed NL-432 replication but that they could effectively kill NL-M20A–infected CD4+ T cells and completely suppress NL-M20A replication,[8,9] indicating that Nef-mediated HLA class I down-regulation critically affects recognition of HIV-1–infected CD4+ T cells by HIV-1–specific CTLs. The effects of Nef-mediated HLA class I down-regulation on these antiviral activities of HIV-1–specific CTLs varied among CTLs specific for various HIV-1 epitopes.[9]

CD4+ T cells and macrophages are major targets of HIV-1.[10,11] Macrophages are persistently infected with HIV-1 and serve as a reservoir of the M-tropic/R5 strain of HIV-1.[11,12] HIV-1–infected macrophages are detected in the various tissues of individuals infected with HIV-1, disseminating HIV-1 throughout the body.[13] Therefore, the ability of CTLs to suppress HIV-1 replication in macrophages may be an important factor in the control of HIV-1 infections. Previous studies showed that HIV-1–specific CTLs can kill HIV-1–infected alveolar macrophages derived from HIV-1–infected individuals.[14,15] However, it still remains unclear whether such CTLs effectively suppress HIV-1 replication in macrophages and whether Nef-mediated HLA class I down-regulation critically affects HIV-1–specific CTL recognition of HIV-1–infected macrophages as it does that of HIV-1–infected CD4+ T cells.

The X4 virus infects CD4+ T cells and weakly infects macrophages, whereas the R5 virus infects both macrophages and CD4+ T cells. The X4 virus dominantly appears in late phase of HIV-1 infection, whereas the R5 virus involves in the transmission and replicates in the early phase. Analysis of CTL responses to X4 virus-infected CD4+ T cells, R5 virus-infected CD4+ T cells, and R5 virus-infected macrophages is important to understand CTL-mediated immune responses in both early and late phases of HIV-1 infection.

In this study, we tested the ability of HIV-1–specific CD8+ T cells to kill HIV-1R5 virus-infected macrophages and to suppress the replication of HIV-1R5 virus in macrophages, and we also investigated the effect of Nef-mediated HLA class I down-regulation on the recognition by HIV-1–specific CD8+ T cells of HIV-1R5 virus-infected macrophages. In addition, we compared the antiviral activities of these cells against HIV-1R5 virus-infected macrophages with those against HIV-1-R5 virus-infected or X4 virus-infected CD4+ T cells. Finally, we investigated the mechanisms underlying the effective recognition of HIV-1–infected macrophages by HIV-1–specific CTLs.

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