

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, Tyr⁸⁵Phe (F85) or Arg⁷⁵Thr (T75) in HLA-B35⁺ patients. Boxes indicate values between 25th and 75th percentiles. Horizontal lines across boxes indicate the median value \pm SD. Lines extend from the box to the highest and lowest values. Data include outliers (\bullet). 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 μ g/ml; Sigma-Aldrich) for 4 days. The activated CD4⁺ cells were then infected at relatively high titers (1 μ g of p24 Ag per 10⁶ 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 ⁵¹Cr. It should be noted that 30 \pm 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 \times 10⁵ 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 10⁵ cells/well. Three days later, the cells were stimulated with PHA at 2 μ g/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 (10⁷) 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, Na₂VO₄, 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 MgCl₂) with 10 μ Ci of [γ -³²P]ATP (Amersham) for 5 min. Bound proteins were then separated by SDS-PAGE and subjected to phosphorimager (Bio-Rad) visualization and quantification. Levels of immunoprecipitated Nef-GFP were determined by Western-blotting of the IVKA reactions and subsequent quantification by LICOR Odyssey.

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 \pm 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 Arg⁷⁵ to Thr mutation (T75, amino acid numbers based on SF2 strain) in Nef was functionally asso-

ciated 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, Tyr⁸⁵ 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. 1A), 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. 1B). The median (\pm SD) number of months since seroconversion in subjects with autologous wt, F85, and T75 Nef sequences was 7.0 \pm 1.1, 16.0 \pm 9.4, and 72.0 \pm 19.5, respectively (Fig. 1B). 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 RPQVPLRPMTY 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. 1C). It is of interest to note that when

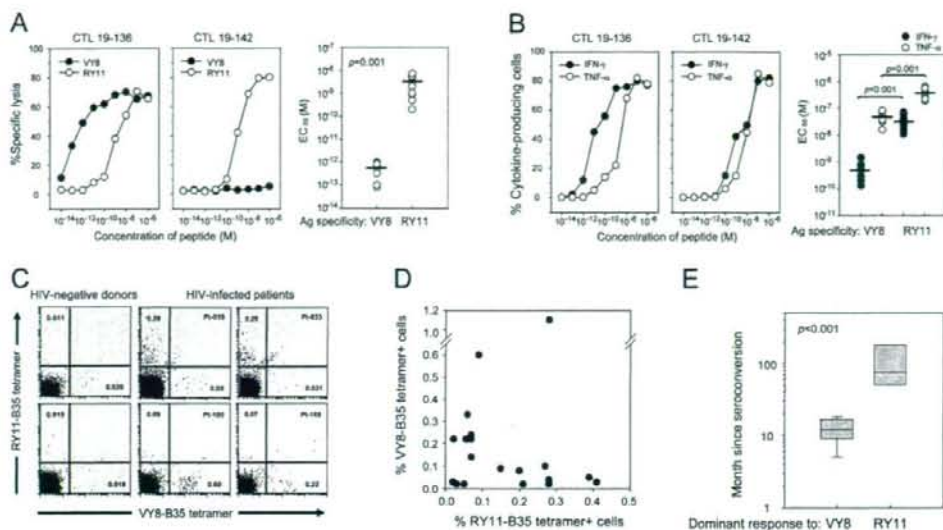


FIGURE 2. HLA-B35-restricted CTL responses toward PxxP region of Nef. **A** and **B**, Cytotoxic activity (**A**) and cytokine-producing activity (**B**) of VY8 or RY11-specific CTL clones generated from multiple donors (patients 01, 03, 19, and 136) were analyzed by using C1R-B3501 cells pulsed with 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 statistical 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 \pm 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., **T**PQVPLRPM**T**E (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. 1C). 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 (Nef₇₈₋₈₅: VPLRPMTY) and RY11 (Nef₇₅₋₈₅: 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. 2A). In con-

trast, 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. 2A). 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. 2A). 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. 2B).

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. 2B). 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. 2C. 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 $0.0153 \pm 0.0022\%$. 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. 2D). 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. 2E), 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 EC₅₀ 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 CIR-B3501 cells pulsed with the variant peptides. A VY8-specific CTL clone, CTL 19-136, showed ~1000-fold decreased sensitivity toward VY8-8F (Fig. 3A). A similar trend was also observed in a panel of nine additional VY8-specific CTL clones as used in Fig. 2A, with mean EC₅₀ 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. 3A). 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. 3B). Again, a panel of nine additional RY11-specific clones showed similar results, with mean EC₅₀ 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. 3B). 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. 1B) and the kinetic change of CTL immunodominance in vivo (Fig. 2E).

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

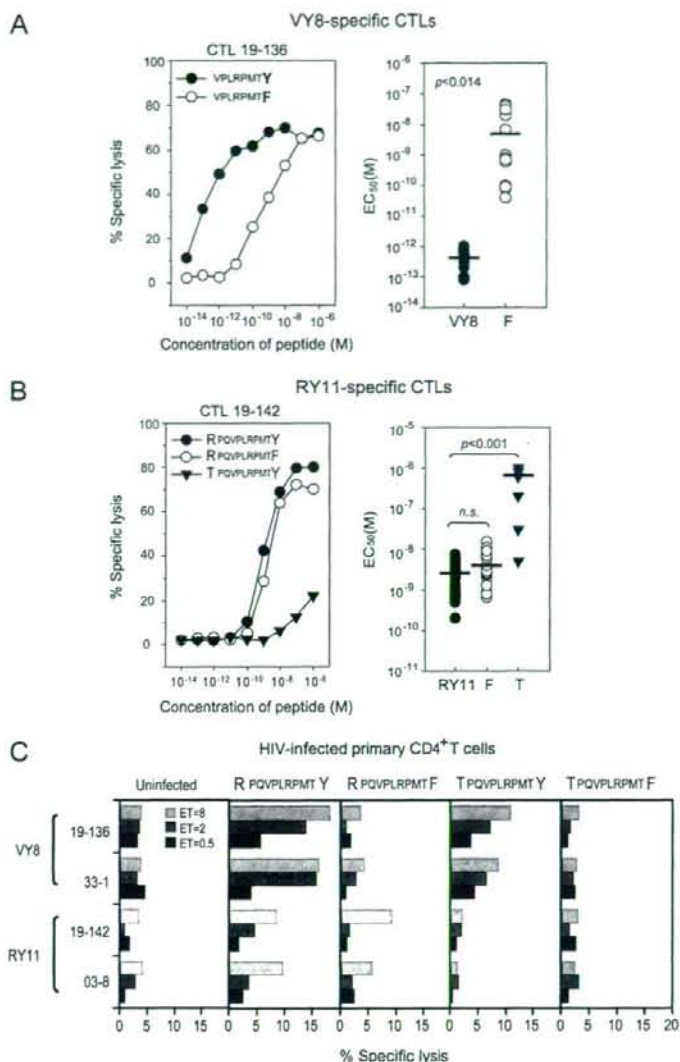
As HIV-infected CD4 T cells are the predominant target of HIV-specific CTLs in vivo, we next examined the cytotoxic activity of CTL clones toward primary CD4 T cells infected with wt or variant HIV-1. CD4 T cells prepared from HIV-negative donors (HLA-B3501⁺) were first stimulated with PHA and then infected 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. 3C). 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. 2A) and antiviral activity (Fig. 3C) 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. 3C), 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. 3C), 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. 3C). The fact that the mutations in autologous viruses is very rare in combination (Fig. 1A) 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 Δ Nef virus-infected cells (Fig. 4A). 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. 4A). 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. 4B). In stark contrast, all cells infected with variant viruses except for Δ Nef 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 Δ Nef 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 CIR-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). EC₅₀ 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. 4C). Both B35-Pol and B35-Env CTLs showed most potent cytotoxic activity toward target cells infected with the Δ Nef variant, whereas the same CTLs showed weak cytolytic activity toward wt virus-infected cells (Fig. 4C). 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. 4C). 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. 4C). 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.

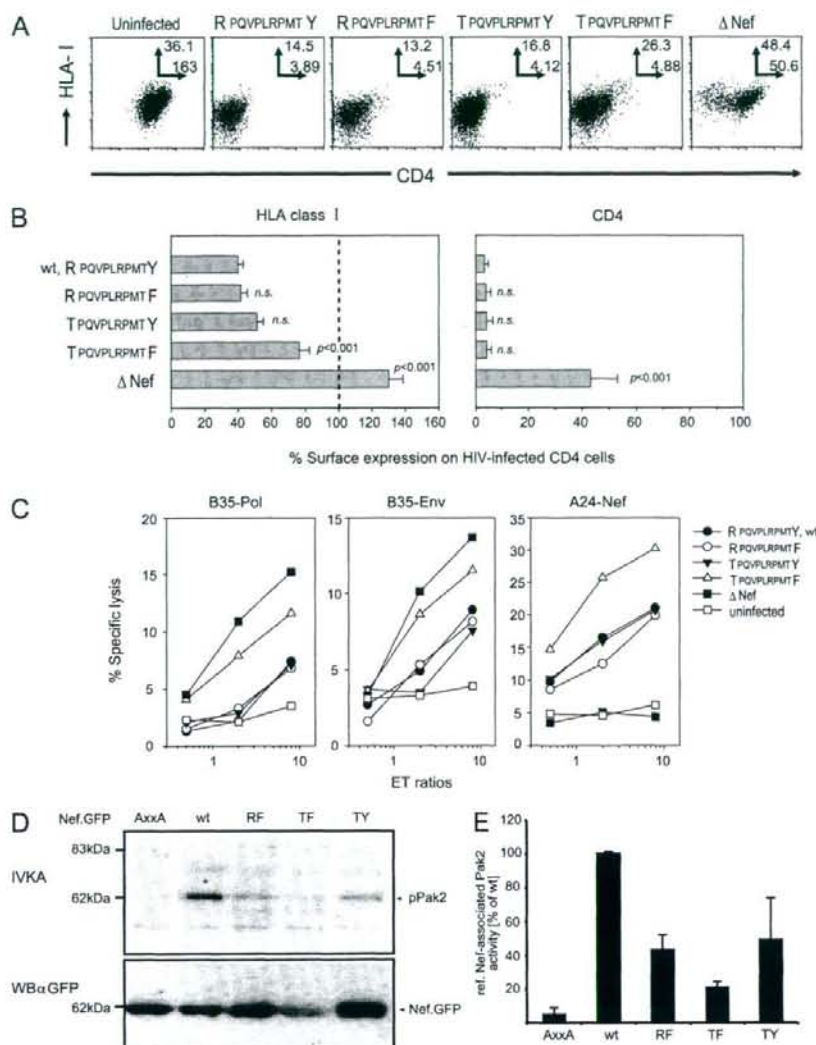


FIGURE 4. Functional consequences of CTL escape Nef mutations. **A**, Freshly isolated primary CD4⁺ cells from an HIV-negative donor (HLA-B35⁺) were activated by PHA for 3 days and then infected with wt or various variants for 5 days. The cells were stained with anti-HLA-Bw6 mAb (clone: SFR8-B6) and 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 Δ Nef 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 cytotoxicity by CTL clones specific for HLA-B3501-restricted Pol (Pol₂₇₃₋₂₈₂: VPLDKDFRKY), Env (Env₇₇₋₈₅: DPNPQEVVL), or HLA-A2402-restricted Nef epitope (Nef₁₃₈₋₁₄₇: 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 immunoprecipitated 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 immunoprecipitated 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),

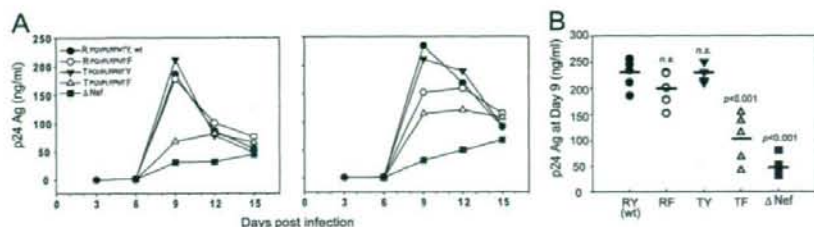


FIGURE 5. Effects of Nef mutations on viral replication in PBMC. *A*, Freshly isolated PBMC samples from two HIV-negative donors were first infected with wt or various variant HIV-1s and 3 days later cells were activated by PHA. For monitoring viral replication, culture supernatants were collected every 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 Pro⁷⁶ 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 Δ Nef 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 Δ Nef 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 interesting 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 constraints 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.

Acknowledgments

We thank Y. Idegami and T. Akahoshi for their technical help. We also thank Drs. A. Kawana-Tachikawa, Zabrina Brumme, and Chanson Brumme for helpful discussion.

Disclosures

The authors have no financial conflict of interest.

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Different immunodominance of HIV-1-specific CTL epitopes among three subtypes of HLA-A*26 associated with slow progression to AIDS

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Received 1 November 2007

Available online 21 November 2007

Abstract

It is speculated that HLA-A*26-restricted HIV-1-specific CTLs can control HIV-1, since HLA-A*26 is associated with a slow progression to AIDS. In three major HLA-A*26 subtypes, HLA-A*2601-restricted, and HLA-A*2603-restricted HIV-1 epitopes have been identified, but HLA-A*2602-restricted ones have not. We here identified HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and compared the immunodominance of the epitopes among the three subtypes. Out of 110 HIV-1 peptides carrying HLA-A*26 anchor residues, only the Gag169-177 peptide, which had been previously identified as an HLA-A*2601- and HLA-A*2603-restricted immunodominant epitope, induced Gag169-177-specific CD8⁺ T cells from only two of six HLA-A*2602⁺ HIV-1-infected individuals. No difference in affinity of this epitope peptide was found among these three HLA-A*26 subtypes, indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected HLA-A*2602⁺ individuals. These findings indicate different immunodominance of Gag169-177 epitope among 3 HLA-A*26 subtypes.

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Keywords: HLA-A*26; HLA-A*2602; HIV-1; Epitope; CTL

Human immunodeficiency virus type-1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) responses play an important role in the control of HIV-1 infections [1–5]. However, it is thought that HIV-1 can escape from the host immune system, since it fails to completely eradicate HIV-1 from infected individuals. There are several proposed mechanisms that would allow HIV-1-infected cells to escape from being killed by HIV-1-specific CD8⁺ T cells [6–11]. A mutation within the viral epitopes recognized by CTLs is one of these mechanisms [6,7]. Identification and characterization of HIV-1 CTL epitopes are therefore necessary for studies on the immunopathogenesis of AIDS. In addition, since HIV-1-specific CTLs are expected to suppress HIV-1

replication *in vivo*, characterization of these epitopes is also necessary for studies aimed at developing HIV-1 vaccines and immunotherapy to induce HIV-1-specific CTLs, either of which might be expected to prevent HIV-1 infection and the progression to AIDS.

HLA-A*26 is one of the alleles associated with a slow progression to AIDS [12]. Therefore, identification and characterization of HIV-1-specific epitopes presented by this allele are necessary for studies on the immunopathogenesis of AIDS and vaccine development. Three HLA-A*26 subtypes, i.e., HLA-A*2601, HLA-A*2602, and HLA-A*2603, are found at a gene frequency of 7.7%, 2.3%, and 1.5%, respectively, in the Japanese population [13]. We previously identified four HLA-A*2601- and two HLA-A*2603-restricted HIV-1 epitopes by using reverse immunogenetics [14,15]. Both HLA-A*2601 and -A*2603 presented one immunodominant epitope, Gag169-177

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(EVIPMFSA), which overlaps with the HLA-B*57-restricted immunodominant epitope KF11 (KAF-SPEVIMPF) [16]. Gag169-177-specific CTLs have been speculated to control HIV-1 replication.

In the present study, we sought to identify HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and to compare them with HLA-A*2601- and HLA-A*2603-restricted ones [14,15].

Materials and methods

Cells. CIR and TAP-defective cells of mouse cell line RMA-S were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). CIR cells expressing HLA-A*2601, -A*2602 or -A*2603 (CIR-A*2601, CIR-A*2602 or CIR-A*2603, respectively) were generated by transfecting the CIR cells with the HLA-A*2601, -A*2602 or -A*2603 gene, respectively [17]. RMA-S transfectants expressing HLA-A*2602 (RMA-S-A*2602) were previously generated [18]. CIR-A*2601, -A*2602 and -A*2603 were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml neomycin; and RMA-S-A*2602, in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Synthetic peptides. Sequences derived from four proteins of the human immunodeficiency virus type-1 SF2 strain (HIV-1: Env, Gag, Pol, and Nef) were screened for HLA-A*2602-binding motifs. Peptides were prepared by utilizing an automated multiple peptide synthesizer, with the Fmoc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry. Peptides with more than 90% of purity were used in the present study.

HLA-stabilization assay. Binding of HIV-1-derived peptides to HLA-A*2602 was measured as previously described by using RMA-S-A*2602 cells [18]. RMA-S-A*2602 cells were cultured for about 16 h. Then they were incubated with peptides at 26 °C for 1 h and subsequently at 37 °C for 3 h. Peptide-pulsed cells were stained with the HLA class I α_3 domain-specific mAb TP25.99 [19] and the FITC-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity (MFI) was measured by using a FACS Calibur (BD Bioscience, San Jose, CA, USA). HLA-A*2602-binding peptides were defined as those which at a concentration of 10^{-3} M caused a >25% increase in MFI compared with the MFI of control RMA-S-A*2602 cells cultured at 26 °C. The peptide concentration that yielded the half-maximal levels of the MFI was calculated and was reported as the BL50 value.

Patients. Blood samples were collected with informed consent from six HIV-1 clade B-infected patients with HLA-A*2602 (KO-003, KI-021, KI-030, KI-082, KI-382, and KI-478), 11 those with HLA-A*2601, and eight those with HLA-A*2603 at the AIDS Medical Center, National Hospital Organization, Osaka National Hospital or the AIDS Clinical Center, International Medical Center of Japan. Clinical stage of all patients tested was chronic one. Significant difference of CD4 count was not found among three subtype groups (HLA-A*2601: 458 ± 257 , HLA-A*2602: 564 ± 299 , HLA-A*2603: 314 ± 109). This study was approved by the ethical committees of Kumamoto University, International Medical Center of Japan, and Osaka National Hospital. Informed consent was obtained from all subjects, according to the Declaration of Helsinki.

Intracellular cytokine staining (ICC assay). After CIR-A*2601, CIR-A*2602 or CIR-A*2603 cells had been incubated for 60 min with each peptide (1 μ M) or each peptide cocktail (1 μ M concentration of each peptide), they were washed twice with RPMI-1640 containing 10% FCS. These CIR-A*26 cells and cultured PBMCs were incubated at 37 °C for 6 h at an effector-to-stimulator ratio of 1:4 after the addition of Brefeldin A (10 μ g/ml). Next, the cells were stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde at 4 °C for 20 min, and then permeabilized at 4 °C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and then stained with anti-IFN- γ mAb (BD Bioscience Pharmingen, San Diego, CA). The cells were finally resuspended in PBS containing 2% para-

maldehyde, and then the percentage of CD8⁺ cells positive for intracellular IFN- γ was determined by using the FACSCalibur.

ICC assay using CIR-A*2602 cells infected with recombinant HIV-1 vaccinia. CIR-A*2602 cells were infected for 1 h at 37 °C with 10 plaque-forming units (per target cell) of recombinant vaccinia virus expressing HIV-1 SF2 Gag protein or of WT vaccinia virus and cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and then incubated with cultured effector cells at 37 °C for 6 h after the addition of Brefeldin A (10 μ g/ml). The ability of the effector cells to produce IFN- γ was tested at an E:S ratio of 1:4. The cells were then stained with anti-CD8 mAb and anti-IFN- γ mAb.

Results and discussion

HLA-A*2602-binding peptides have two anchor residues, Val, Phe, Ile, Leu or Thr at position 2 and Tyr, Phe, Met or Leu at the C-terminus [17]. A previous study demonstrated that acidic amino acids (Asp and Glu) and a broad range of amino acids with the exception of positively charged amino acids function as an anchor at position 1 and the C-terminus, respectively [18]. Therefore, to identify HLA-A*2602-binding HIV-1 peptides, 8-mer to 11-mer sequences containing the anchor residues Asp or Glu at position 1, Val, Thr, Ile, Leu or Phe at position 2, and any amino acids except positively charged ones at the C-terminus were selected from the sequence of Gag, Pol, Nef, and Env proteins in the HIV-1 SF2 strain; and then 110 peptides matching these sequences were synthesized. The binding affinity of these synthesized peptides for the HLA-A*2602 molecule was tested by using the HLA-stabilization assay [18,20]. Representative results are shown in Fig. 1. Thirty-two out of these 110 peptides bound to HLA-A*2602 (Table 1). The frequency of HLA-A*2602-binding HIV-1 peptides was similar to that of HLA-A*2601- or HLA-A*2603-binding HIV-1 peptides previously identified by using the same 110 peptides [14,15].

PBMCs from three HLA-A*2602⁺ HIV-1-infected individuals (KO-003, KI-030 and KI-082) were stimulated

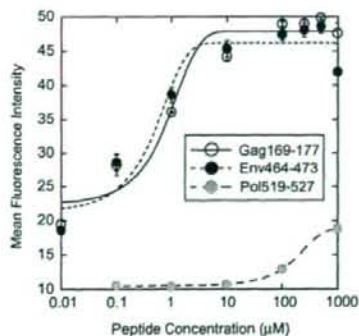


Fig. 1. Binding to HLA-A*2602 of HIV-1 peptides carrying HLA-A*2602 motif. Binding of the peptides carrying A*2602 anchors was measured by a stabilization assay using RMA-S-A*2602 cells. Representative results showing high-affinity peptides (Gag169-177 and Env464-473) and a very low-affinity peptide (Pol519-527) are given in this figure.

Table 1
HLA-A*2602-binding peptides

Sequence	Position	BL50 ^a
EVFRPGGDM	Env464-473	3.98×10^{-7}
EVIPMFSAL	Gag169-177	5.84×10^{-7}
ELKKIIGQV	Pol872-880	9.88×10^{-6}
EVNIVTDSQY	Pol647-656	1.21×10^{-5}
EVVIRSDNF	Env272-280	4.1×10^{-5}
ELNKRITQDF	Pol234-242	1.02×10^{-4}
EIKGEIKNCSF	Env147-157	1.62×10^{-4}
DIVIIQYMDLL	Pol332-342	1.94×10^{-4}
DTTNQKTEL	Pol626-634	2.78×10^{-4}
EIVASCDKQCL	Pol750-760	3.50×10^{-4}
ETVPVKLPGM	Pol161-171	4.26×10^{-4}
ETWEAWWMEYW	Pol551-561	6.28×10^{-4}
ETWEAWWMEY	Pol551-560	$>1 \times 10^{-3}$
EICGHKAIGTV	Pol121-131	$>1 \times 10^{-3}$
EVVLGNVTENF	Env82-92	$>1 \times 10^{-3}$
DLRSLCLFSY	Env758-767	$>1 \times 10^{-3}$
DLWIYHTQGYF	Nef115-125	$>1 \times 10^{-3}$
EVIPLTEEA	Pol446-454	$>1 \times 10^{-3}$
ETPGIRYQY	Pol293-301	$>1 \times 10^{-3}$
EVYYDPSKDLV	Pol471-481	$>1 \times 10^{-3}$
EVYYDPSKDL	Pol471-480	$>1 \times 10^{-3}$
ETKLGKAGYV	Pol604-613	$>1 \times 10^{-3}$
EVHNVWATHAC	Env63-73	$>1 \times 10^{-3}$
EVQLGIPHPA	Pol244-253	$>1 \times 10^{-3}$
ELYPLTSLRS	Gag484-493	$>1 \times 10^{-3}$
DLNNTMLNTV	Gag185-193	$>1 \times 10^{-3}$
DVKQLTEAV	Pol519-527	$>1 \times 10^{-3}$
ELYPLTSLRSL	Gag484-494	$>1 \times 10^{-3}$
DIQKLVGKGL	Pol411-419	$>1 \times 10^{-3}$
DIAGTTSTL	Gag235-245	$>1 \times 10^{-3}$
ELRQHLLRW	Pol359-367	$>1 \times 10^{-3}$
DTKEALEKI	Gag96-104	$>1 \times 10^{-3}$

^a The half maximal binding level was calculated as the peptide concentration yielding the half-maximal MFI.

in vitro for 14 days with a cocktail of HLA-A*2602-binding peptides containing 5–7 peptides (cocktail 1: Env464-473, Gag169-177, Pol647-656, Pol872-880, and Pol551-560; cocktail 2: Env272-280, Pol121-131, Env82-92, Pol551-561, Env758-767, Pol234-242, and Nef115-125; cocktail 3: Pol446-454, Pol293-301, Pol471-481, Pol471-480, Pol604-613, Env147-157, and Env63-73; cocktail 4: Pol244-253, Gag484-493, Gag185-193, Pol626-634, Pol519-527, Gag484-494, and Pol411-419; and cocktail 5: Gag235-243, Pol359-367, Pol161-171, Gag96-104, Pol750-760, and Pol332-342). IFN- γ production by each bulk culture in response to C1R-A*2602 prepulsed with the corresponding peptide cocktail was assessed by intracellular IFN- γ staining. All five cocktails failed to induce specific CD8⁺ T cells among the cells in bulk culture obtained from patients KI-030 and KI-082. On the other hand, only cocktail 1 induced specific CD8⁺ T cells among the cells in bulk culture obtained from patient KO-003 (Fig. 2A). To determine which peptides in the cocktail induced the specific CD8⁺ T cells, we re-stimulated the cells of this bulk culture with C1R-A*2602 cells prepulsed with each single peptide in this cocktail to detect the specific CD8⁺ T cells. Only the Gag169-177 peptide induced CD8⁺ T cells producing IFN- γ (Fig. 2B).

To clarify whether Gag169-177 was a naturally occurring peptide, we investigated the ability of these peptide-specific CD8⁺ T cells to produce IFN- γ after having stimulated them with C1R-A*2602 cells infected with recombinant HIV-1 vaccinia virus (r-HIV vaccinia). IFN- γ -producing cells were induced in the Gag169-177-specific CD8⁺ T cell culture after stimulation with r-HIV vaccinia-infected C1R-A*2602 cells, whereas they were not detected in that stimulated with WT vaccinia-infected C1R-A*2602 cells or r-HIV vaccinia-infected C1R cells (Fig. 2C). These results indicate that Gag169-177 is indeed a naturally occurring HIV-1 epitope peptide presented by HLA-A*2602.

Gag169-177-specific CD8⁺ T cells were induced from only 1 of the 3 HLA-A*2602⁺ HIV-1-infected individuals; whereas two HLA-A*2601 epitopes, Pol647-656 and Env464-473, which were also HLA-A*2602-binding peptides, failed to induce specific T cells in these individuals. To address lower frequency of these peptide-specific CD8⁺ T cells, we investigated the induction of the specific CD8⁺ T cells by stimulating PBMC from three additional donors carrying HLA-A*2602 with these three peptides. Only Gag169-177 peptide induced the specific CD8⁺ T cells in one donor. Thus, Gag169-177-specific CD8⁺ T cells were induced in 2 of 6 HLA-A*2602⁺ HIV-1-infected individuals whereas they were induced in 8 of 11 HLA-A*2601⁺ and 7 of 8 HLA-A*2603⁺ HIV-1-infected ones (Fig. 2D). These results indicate that Gag169-177 is a subdominant epitope in the HLA-A*2602⁺ donors.

Gag169-177-specific CD8⁺ T cells were previously detected in five of seven HLA-A*2601⁺ donors and in all four HLA-A*2603⁺ ones [14,15], suggesting that Gag169-177 is a dominant epitope in HIV-1-infected individuals carrying either of these HLA-A*26 alleles. Additional experiments in the present study confirmed the immunodominance of this epitope in HLA-A*2601⁺ and HLA-A*2603⁺ donors (Fig. 2D). In contrast, they were detected in only two of six HLA-A*2602⁺ donors, indicating Gag169-177 to be a subdominant epitope in HLA-A*2602⁺ individuals. Interestingly, they were elicited in only a long-term non-progressor and a controller having low viral load. HLA-A*1101-restricted Gag349-, Nef73-, and/or Nef84-specific CD8⁺ T cells were induced in two of four HLA-A*1101⁺/A*2602⁺ donors who did not have Gag169-177-specific CD8⁺ T cells (data not shown), supporting that these HLA-A*2602⁺ donors maintain HIV-1-specific cellular immunity. The affinity of Gag169-177 for HLA-A*2602 was similar to that for HLA-A*2601 and -A*2603 (Table 2), indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected individuals carrying HLA-A*2602. Pol604-612 and Env63-72 are HLA-A*2601 and HLA-A*2603 epitopes, respectively [14,15]. These epitope peptides failed to bind to HLA-A*2602 (data not shown). Since only one amino acid, at residue 116, differs between HLA-A*2602 and the other two subtypes (Asp

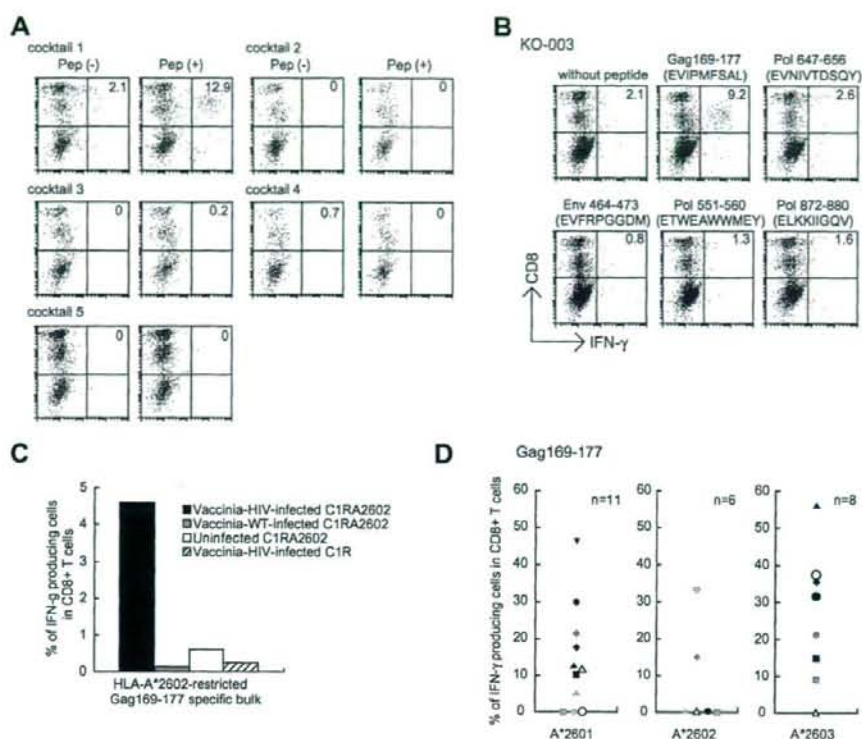


Fig. 2. Identification and recognition of Gag169-177-specific CD8 $^{+}$ T cells. (A) PBMCs from HIV-1-infected individuals with HLA-A*2602 (KO-003) were cultured for 10–14 days after they had been stimulated with the indicated cocktails of HLA-A*2602-binding peptides. The cultured cells were then tested for IFN- γ production by CD8 $^{+}$ T cells after stimulation with C1R-A*2602 cells prepulsed with the peptide cocktails. (B) Induction of Gag169-177-specific CD8 $^{+}$ T cells. PBMCs from KO-003 were stimulated with peptide cocktail 1 and cultured for 10–14 days. The cultured cells were stimulated with C1R-A*2602 cells prepulsed with each single peptide included in cocktail 1. The population of IFN- γ -producing CD8 $^{+}$ T cells was determined by using flow cytometry. The percentage of IFN- γ -producing CD8 $^{+}$ T cells is presented at the right of the upper right-hand quadrant. (C) Presentation of Gag169-177 by HLA-A*2602 on r-HIV-1 vaccinia-infected cells. Bulk cultures containing Gag169-177-specific CD8 $^{+}$ T cells were examined for IFN- γ production after they had been stimulated with C1R-A*2602 cells infected with wild-type vaccinia (Vaccinia-WT) or with C1R-A*2602 cells or with C1R cells infected with r-HIV-1Gag-vaccinia (Vaccinia-HIV-1), or uninfected C1R-A*2602 cells (Uninfected). The percentage of IFN- γ -producing CD8 $^{+}$ T cells was measured by using flow cytometry. (D) Percentage of Gag169-177-specific CD8 $^{+}$ T cells in HIV-1-infected individuals having three HLA-A*26 subtypes. The percentage of IFN- γ -producing cells among CD8 $^{+}$ T cells from each individual was plotted in the graph. The percentage of IFN- γ -producing cells in the cultures was measured by using flow cytometry after they had been stimulated with the corresponding C1R-A*26 cells prepulsed with Gag169-177 peptide.

Table 2

Comparison of binding affinity of HLA-A*26 epitope peptides among three HLA-A*26 subtypes and induction of the peptide-specific CD8 $^{+}$ T cells

	Binding affinity (BL50)			Comparison of binding affinity		Frequency ^a		
	A*2601	A*2602	A*2603	A*2602/A*2601	A*2602/A*2603	A*2601	A*2602	A*2603
Gag169-177	7.5×10^{-7}	5.8×10^{-7}	2.1×10^{-6}	0.77	0.28	8/11	2/6	7/8
Env63-72	1.1×10^{-4}	No binding	7.6×10^{-7}	—	—	0/11	NT	3/8
Pol604-612	6.5×10^{-5}	No binding	No binding	—	—	10/11	NT	NT
Pol647-656	6.3×10^{-5}	1.2×10^{-5}	6.6×10^{-4}	0.19	0.02	1/11	0/6	0/8
Env464-473	1.5×10^{-6}	4.0×10^{-7}	3.7×10^{-5}	0.27	0.01	1/11	0/6	0/8

NT, not tested.

^a The number of individuals in whom peptide specific CD8 $^{+}$ T cells were induced/the number of tested individuals.

for HLA-A*2601 and -A*2603, but Asn for HLA-A*2602), this substitution in the floor of the peptide binding groove is thought to affect the binding of these peptides.

In summary, Gag169-177 was not an HIV-1 immunodominant epitope in HIV-1-infected individuals carrying HLA-A*2602, whereas it was one in those carrying HLA-

A*2601 or -A*2603. These findings imply the possibility that HLA-A*2602 is not an allele associated with a slow progression to AIDS. However, it still remains unknown that Gag169-177-specific CTLs can control HIV-1. A further study using a cohort of a large number of subjects will clarify the association of these HLA-A*26 subtypes or Gag169-177-specific CTLs with the progression of AIDS.

Acknowledgments

The authors thank Dr. S. Ferrone for the gift of mAb TP25.99, Dr. H. Gatanaka for proving clinical data, and Sachiko Sakai for her secretarial assistance. This research was supported by a grant-in aid for scientific research from the Ministry of Health, Labour, and Welfare of the government of Japan, and by a grant from the Japan Health Science Foundation.

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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^V

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Received 4 July 2007/Accepted 15 October 2007

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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^V Published ahead of print on 24 October 2007.

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 CGCACTCCC-3' for the first PCR primer set 1, 5'-TCGAGACCTGGAAA AACATGGAGC-3' and 5'-AAAAGTCCCGAGCGGAAAGTCCC-3' for the second PCR primer set 1, 5'-TCGAGACCTGGAAAACATGGAGC-3' and 5'-TAACCAGAGAGACCCAGTACAGGC-3' for the first PCR primer set 2, and 5'-GGTAGAAGCACAAGAGGAGG-3' and 5'-AGCATCTGAGGAGC 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 μ l 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 (RFPLTFGWCF) 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 cytometer.

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 (RFPLTFGWCF) 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 (Mono Q 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 (ExtraAvidin-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 μ l 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

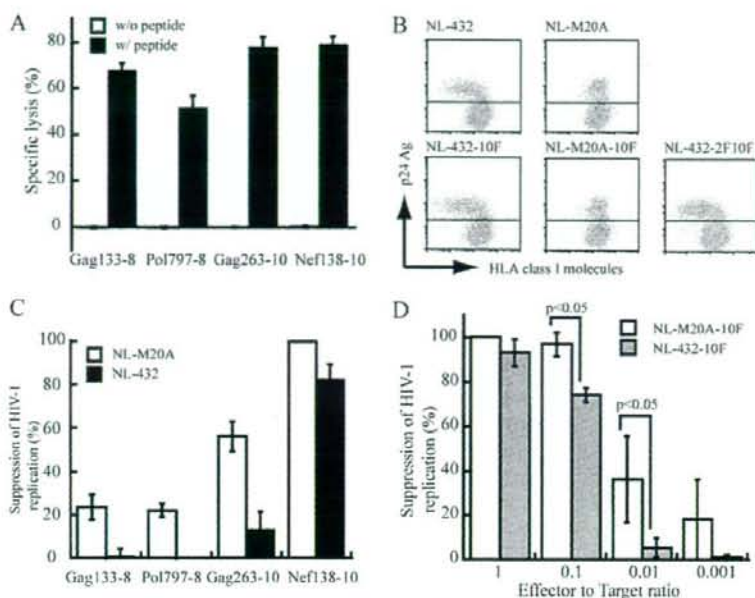


FIG. 1. Suppression of HIV-1 replication in HIV-1-infected CD4⁺ T cells by HLA-A*2402-restricted HIV-1-specific CTLs. (A) Cytolytic activities of HLA-A*2402-restricted HIV-1-specific CTLs (four Gag133-8-specific, five Pol797-8-specific, four Gag263-10-specific, and four Nef138-10-specific CTL clones) 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 \pm 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 \pm 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 \pm 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) \times 100.

⁵¹Cr 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 μ Ci of Na₂⁵¹CrO₄ 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 ⁵¹Cr 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 ⁵¹Cr from the target cells in the presence of 2.5% Triton X-100. Specific lysis was defined as (cpm exp - cpm spn)/(cmp max - cpm spn) \times 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 (LL₅₀) 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 (RY PLTFGWY) 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 RYPLTFGWY. 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 (RYPLTFGWCF) 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

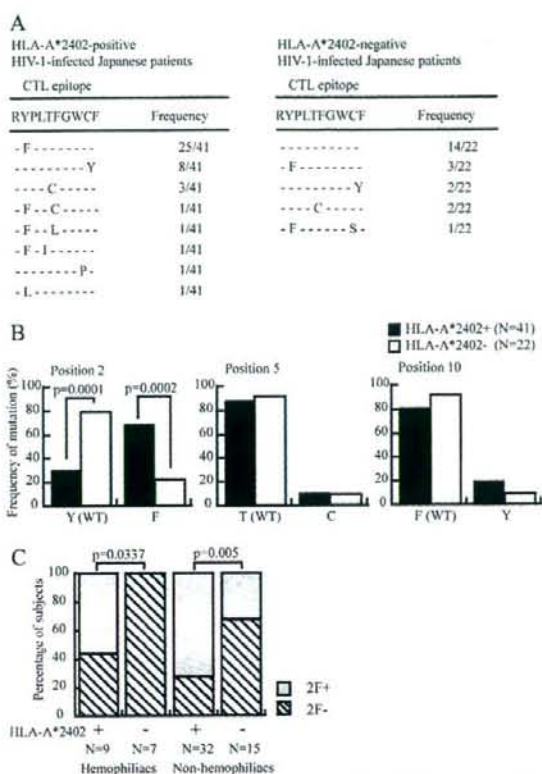


FIG. 2. Frequency of mutation in the Nef138-10 epitope and flanking region. (A) Alignment of the amino acid sequences from 41 HLA-A*2402⁺ and 22 HLA-A*2402⁻ patients. (B) Frequency of the substitutions in the Nef138-10 epitope. The Y-to-F substitution at position 2 was more frequently found in the HIV-1-infected 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

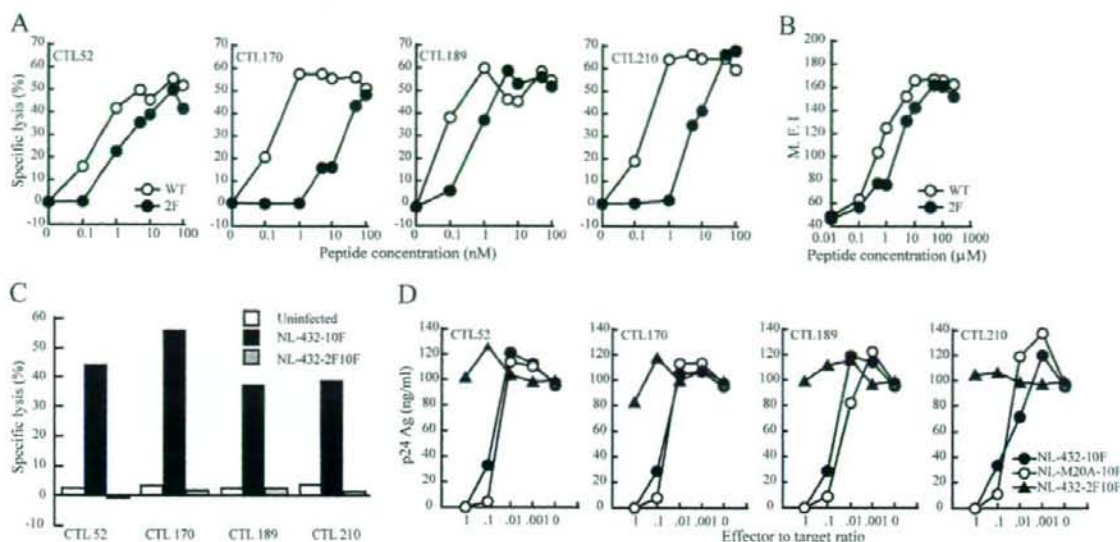


FIG. 3. Abilities of Nef138-10-specific CTLs to suppress replication of NL-432-2F10F. (A) Cytolytic activity of HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402⁺ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). They were tested at an E-to-T ratio of 2:1. (B) Binding 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-

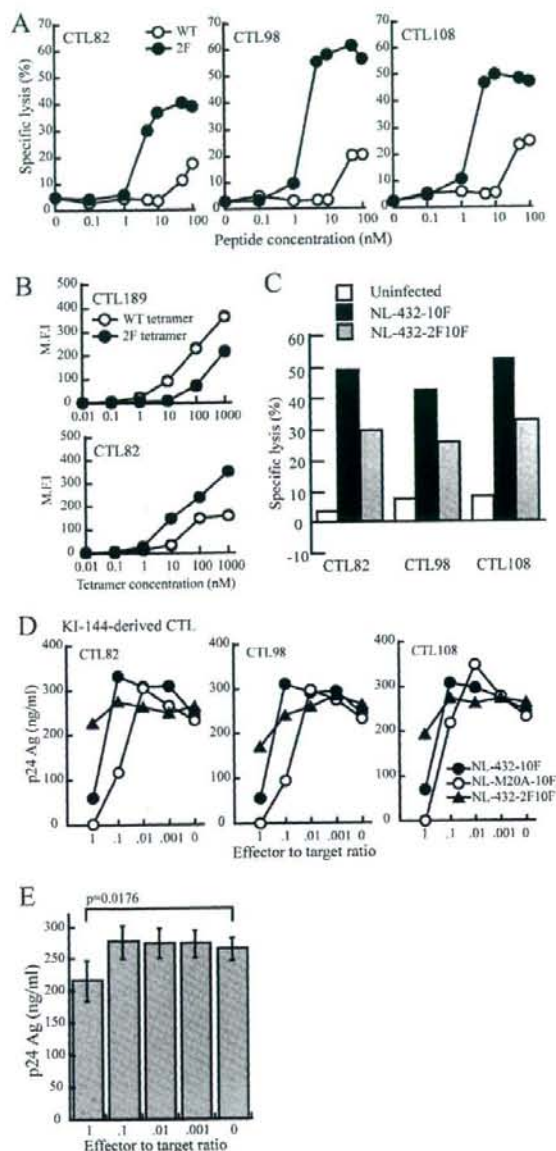


FIG. 4. Distinct antiviral activities toward NL-432-2F10F of KI-144-derived Nef138-10-specific CTL clones. (A) Cytolytic activity of KI-144-derived HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402+ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). The clones were tested 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

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

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).

