CTL-Mediated Selective Pressure Influences Dynamic Evolution and Pathogenic Functions of HIV-1 Nef[1]

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HIV-1 Nef plays multiple roles in modulating immune responses, even though it is a dominant CTL target itself. How Nef accomplishes the balance between such conflicting selective pressures remains elusive. By genetic and functional studies, we found that Arg^{75}Thr and Tyr^{85}Phe mutations, located in a well-conserved proline-rich region in Nef, were differently associated with escape from CTL responses specific for two overlapping HLA-B35-restricted epitopes. CTLs specific for an epitope, that selected Tyr^{85}Phe, were elicited earlier and had more potent functional avidities than did those that selected Arg^{75}Thr. Although the double mutant could escape from both CTLs, the mutations are rarely observed in combination naturally. Introduction of both mutations reduced Nef's HLA class I down-regulation activity and increased the susceptibility of virus-infected cells to recognition by CTLs targeting other epitopes. Moreover, the mutant Nef was impaired in the association with activated cellular kinases and in the enhancement of viral replication. These results highlight CTL immunosurveillance as important modulators of Nef's biological activity in the infected host. ***The Journal of Immunology,*** **2008, 180: 1107–1116.**

The accessory gene product Nef is a critical determinant for the pathogenesis of the primate lentiviruses, HIV-1, HIV-2, and SIV. The importance of Nef in viral pathogenesis was first shown in rhesus macaques, where a large deletion of the *nef* gene severely reduced SIV pathogenicity (1). This finding was supported by the fact that a cohort consisting of one blood donor and eight transfusion recipients infected with Nef-defective HIV-1 demonstrated dramatically decreased rates of disease progression (2, 3). The impact on the outcome of HIV/SIV infection likely results from the synergy of multiple functions exerted by Nef that may be differentially regulated over time (4). Nef enhances viral replication and virion infectivity (5–7) and affects cells in many ways, including altering T cell activation and maturation (6, 8–11), subverting the apoptotic machinery, and down-regulating a number of cell surface receptors including CD4 and HLA class I (7, 12, 13). The down-regulation of MHC class I (MHC-I)[3] by SIV Nef in rhesus macaques limits CD8 T cell-mediated killing and contributes to the pathogenic effect of Nef in vivo, highlighting the importance of Nef-mediated immunoevasion to facilitate disease progression (14).

The initial peak of viral replication after primary HIV infection begins to decline simultaneously with the appearance of HIV-specific CD8 T lymphocytes (15, 16) that can eliminate HIV-infected cells directly by MHC-I-restricted cytolysis or indirectly through the production of soluble factors such as cytokines and chemokines (17, 18). The biological relevance of HIV-specific CTLs in HIV infection is also supported by the results of in vivo studies demonstrating a dramatic rise of viremia and an accelerated clinical disease progression in SIV-infected macaques after the artificial depletion of CD8$^+$ cells (19, 20). Among HIV proteins targeted by HIV-specific CTLs, HIV Nef protein is expressed at high levels early in an HIV infection (21) and elicits a strong CTL response in a number of subjects (22, 23). Most antigenic determinants are located within a multirestricted, immunodominant central region spanning residues 73–94 and 113–147 (22, 24), including a highly conserved proline-rich region containing an Src homology 3 (SH3)-binding motif, PxxP (Nef$_{73-82}$: PVRPQVPLRP) critical for several but not all Nef functions (6, 7, 25–27). In particular, HIV-infected subjects expressing the HLA-B*3501 molecule, which prefers a proline residue on the second position of its antigenic peptides, show vigorous HLA-B35-restricted CTL responses toward the proline-rich region of Nef (22, 28, 29).

In the present study, we focused on HLA-B35-restricted CD8 T cell responses toward the functionally important PxxP region of HIV-1 Nef to ask whether CTL responses can impose constraints on Nef activity. Remarkably, sequence analysis of autologous viruses revealed the association of two different mutations with patients carrying HLA-B*35, one of which was earlier shown to be a naturally occurring variation that can modulate Nef functions (25). Further detailed analyses of CTL responses and Nef functions demonstrated that Nef balances between the conflicting selective pressures during the course of an HIV-1 infection. These findings suggest an important role of HIV-1 Nef-specific CTL responses in the control of Nef activity during the progression of an HIV-1 infection.

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[3] Abbreviations used in this paper: MHC-I, MHC class I; SH3, Src homology 3; 7-AAD, 7-aminoactinomycin D; wt, wild type; IVKA, in vitro kinase assay.

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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).

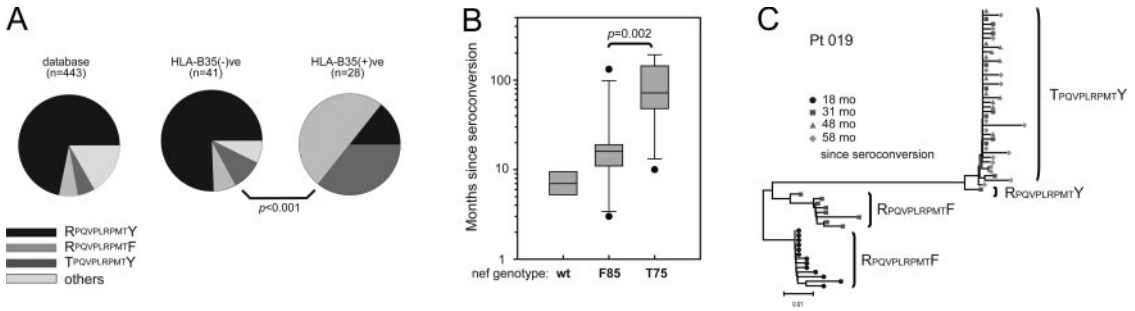

FIGURE 1. Dynamic evolution of autologous Nef sequences in HIV-infected individuals expressing HLA-B*35. *A*, Frequency of clones representing the HIV-1 Nef amino acid sequence at the RY11 epitope region as indicated in pie charts, based on the results from the Los Alamos database (*left*). The frequencies 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

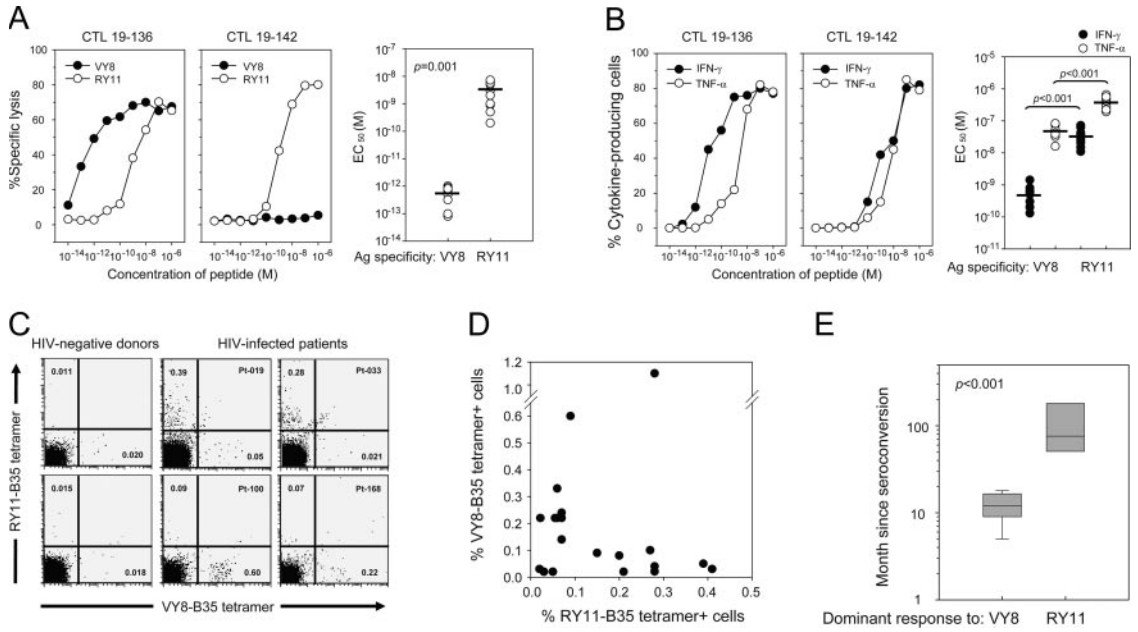

FIGURE 2. HLA-B35-restricted CTL responses toward PxxP region of Nef. *A* and *B*, Cytotoxic activity (*A*) and cytokine-producing activity (*B*) of VY8 or RY11-specific CTL clones generated from multiple donors (patients 01, 03, 19, and 136) were analyzed by using C1R-B3501 cells pulsed with various 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., ***T***PQVPLRPMT***F*** (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 $HLA\text{-}B35^+$ 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 $HLA\text{-}B35^+$ 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 $0.0153 \pm 0.0022\%$. 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. 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

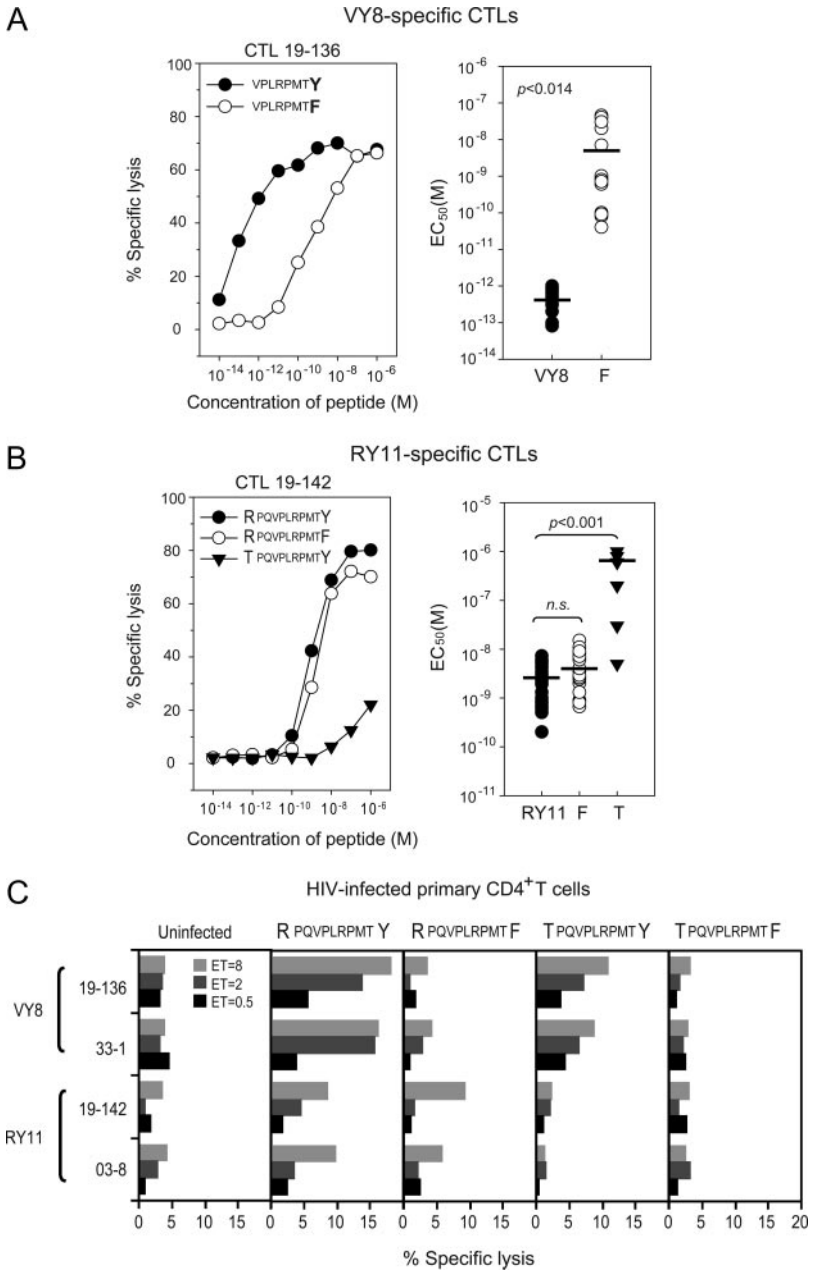

FIGURE 3. CTL responses to variant Ags. *A* and *B*, VY8 and RY11-specific CTL clones (same clones as in Fig. 2, *A* and *B*) were tested for their ability to respond to variant peptides by using C1R-B3501 cells pulsed with various concentrations of the wt or variant peptides (ET = 2). Representative 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.

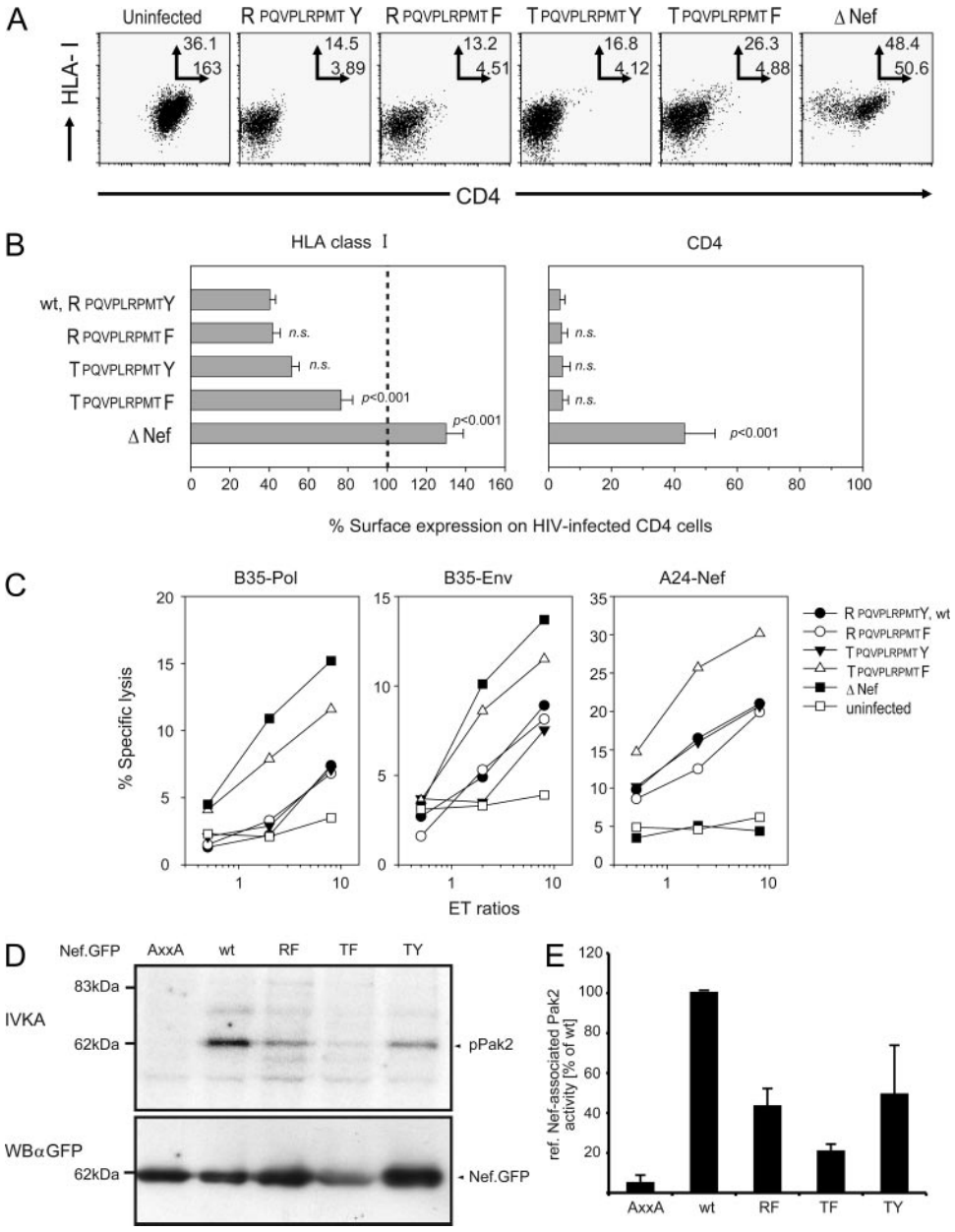

FIGURE 4. Functional consequences of CTL escape Nef mutations. *A*, Freshly isolated primary $CD4^+$ cells from an HIV-negative donor ($HLA\text{-}B35^+$) were activated by PHA for 3 days and then infected with wt or various variants for 5 days. The cells were stained with anti-HLA-Bw6 mAb (clone: SFR8-B6) 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),

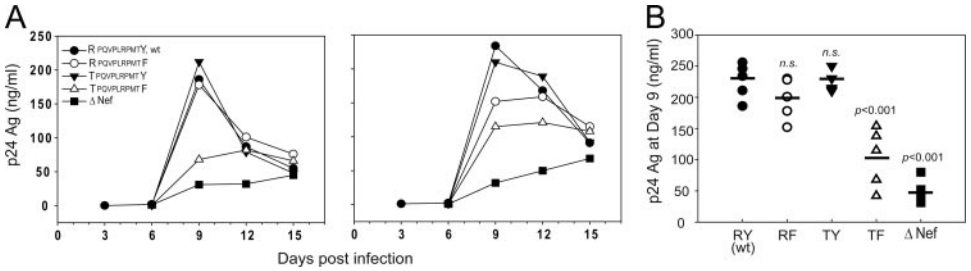

FIGURE 5. Effects of Nef mutations on viral replication in PBMC. *A*, Freshly isolated PBMC samples from two HIV-negative donors were first infected with wt or various variant HIV-1s and 3 days later cells were activated by PHA. For monitoring viral replication, culture supernatants were collected every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