

Fig. 2. Identification of HIV-1 integration site in the lymphoma cells with genome walking. (A) Sequence of the HIV-1 5'-LTR (upper panel) and 3'-LTR (lower panel) insertion site in the lymphoma genome. Whole sequences of PCR products are registered as GenBank DQ355432 (5'-LTR, 190 bp) and DQ117603 (3'-LTR, 1.5 kbp), respectively. The sequence of the lymphoma genome is shown in the lower line in black letters. The upper colored line indicates the HIV-1 LTR sequence (blue, GenBank K03455 or AF538307) and STAT3 genomic sequence (violet, GenBank AY572796). HIV-1 intervening sequence between 5'LTR and gag is indicated by green. Duplication of the cellular 5 bp (GAATC) and additional dinucleotides (TG in 5'-LTR and CA in 3'-LTR) by HIV-1 integrase are underlined. *DraI* site is indicated by italics. (B) PCR for the junction region of 3'LTR and STAT3 gene using HIV3LTR-F and Stat3intron-R primers (see Fig. 3D). 1, PBMCs from a healthy donor; 2, HIV-1-positive Molt4 cell line; 3, lymphoma cells with HIV-1-integration; 4, KS lesion from the patient; 5, AIDS-related lymphoma from an unrelated patient; 6, lymphoma from a non-HIV-1-infected patient; 7, BCBL-1 (KSHV-positive B cell line); 8, No DNA. The lower panel shows the results of an internal control ( $\beta$ -globin gene). (C) PCR of genomic DNA with a STAT3-intron forward primer (F in this figure, Stat3-intronF2) in combination with 5' LTR reverse primers (lanes 1–6, 55R, 78R, 348R, 495R, 563R and 612R), and a reverse primer positioning between 5'LTR and gag (lane 7, 676R). The upper panel shows the positions of these primers. A 188 bp product was identified when the 676R primer was used with the STAT3 intron primer (lane 7). If the 5'LTR was intact, the predicted size of this amplicon would have been 777 bp. (D) Map of the defective HIV-1 insertion site in the STAT3 gene. Violet numbers indicate the number in GenBank AY572796 (STAT3). Blue boxes are HIV-1 genomes.

of the HIV-1-integrated lymphoma cells (Fig. 4D). To know the phosphorylation status of STAT3, we immunostained the slide using an anti-pSTAT3 (Tyr-705) antibody as a primary antibody. However, any signal was not found in the lymphoma cells (data not shown). We also examined STAT3 expression in

24 cases of lymphoma, including nine cases of AIDS-related lymphoma and 15 of non-AIDS-related lymphoma, normal tonsillar tissues and lymph nodes derived from unrelated patients. The nine cases of AIDS-related lymphoma contained seven of EBV-positive diffuse large B cell lymphoma

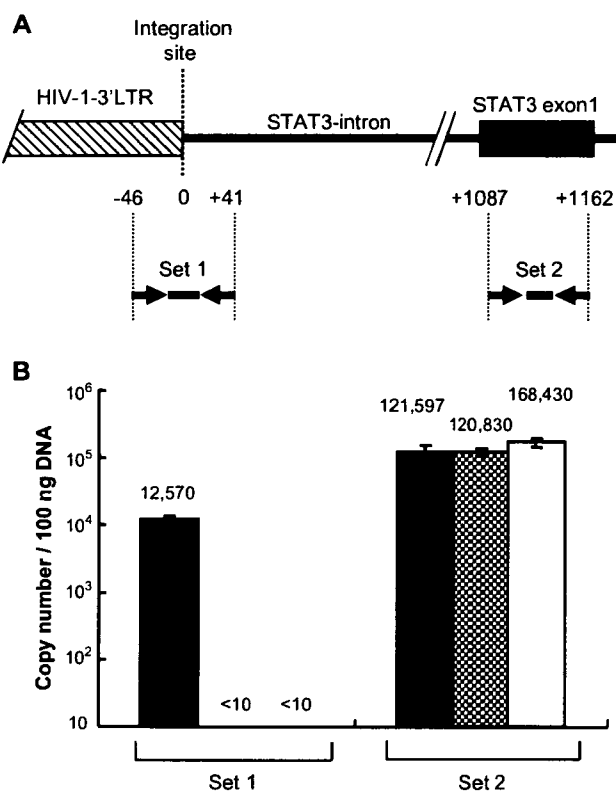


Fig. 3. Quantitative analysis of genes for HIV-1 integration site. (A) Probe-primer sets for real time PCR. The top line with boxes is a genome map around HIV-1 integration site of HIV-1 3'LTR. Numbers with plus and minus under the genome map indicate distances (bp) from the integration site. Arrows and heavy lines are probe-primer sets of real time PCR. (B) Copy numbers of HIV-1 integration site and STAT3 gene. Black, gray and white bars indicate mean copy number per 100 ng DNA of this case, HIV-1-positive Molt4 cell line, and TY-1 (HIV-1-negative, KSHV-positive B cell line), respectively. Copy numbers per 100 ng DNA are indicated on the top of each bar. Error bars indicate standard errors of triplicate samples.

(DLBCL), and two cases of Hodgkin's disease. The 15 cases of non-AIDS-related lymphoma contained 12 EBV-positive or EBV-negative DLBCL and three cases of Hodgkin's disease. Immunohistochemistry revealed that several cases of AIDS-related lymphoma and one of HIV-unrelated lymphoma expressed STAT3 predominantly in the cytoplasm (Fig. 4E); however, no case expressed STAT3 predominantly in the nucleus (Table 1). STAT3 expression was not found, or was weak, in other cases examined (Fig. 4F). These data suggest that the integration of HIV-1 induced high expression of STAT3 in the lymphoma cells of the patient.

### 3.6. Transfection of STAT3 expression plasmid to primary B cells in vitro

To investigate if expression of STAT3 induces cell growth, we constructed an expression plasmid for STAT3 and transfected the plasmid to B cells. At first, to confirm expression of STAT3 by Nucleofector transfection, His-tagged STAT3 was expressed in TY-1, a KSHV-positive B cell line.

Immunofluorescence assay using anti-STAT3 and anti-6x His antibodies revealed that transfection efficiency to lymphocytes was 30–40% in this experiment (Fig. 5A). Addition of IL-6 to culture medium of transfected TY-1 altered the localization of STAT3 from the cytoplasm to the nucleus, suggesting that the transfected STAT3 reacted with IL-6 stimulation (Fig. 5B). Then, we investigated the proliferation of STAT3-transfected primary B cells. Cell proliferation assay after 48 h transfection showed that the proliferation of STAT3-transfected primary B cells were slightly higher than that of vector-transfected primary B cells (Fig. 5C, Mann–Whitney test,  $p < 0.01$ ). However, 4 days after transfection, the difference was not statistically significant (data not shown). The transfection of STAT3 to B cells was repeated 4 times with similar results. These data suggested that transfection of STAT3 might induce a transient proliferation in the primary B cells in vitro.

## 4. Discussion

In the present study, we present a case of AIDS-related B cell lymphoma with HIV-1 integration. HIV-1 with defective 5'LTR integrated into the upstream region of the first STAT3 coding exon. The 3' LTR had strong promoter activity, resulting in increased expression of STAT3 in the nuclei of lymphoma cells. This is the first case report describing dysregulation of STAT3 by HIV-1 integration, resulting in B cell lymphoma development.

STAT3 is an important molecule for IL-6-type cytokines that signal and stimulate proliferation and terminal differentiation of B cells [20]. STAT3 also plays some oncogenic roles. Activated and phosphorylated STAT3 has been observed in a variety of experimental and numerous human malignancies [21–23]. A recent study reveals that high expression of unphosphorylated STAT3 results in up-regulation of oncogenes, suggesting that overexpression of either form of STAT3, phosphorylated and unphosphorylated, might induce cancer [24]. Although we failed to detect phosphorylated STAT3, high expression of STAT3 in the nucleus implies that activated STAT3 may bind to DNA and activate some genes constitutively. Alternatively, it implies that overexpression of unphosphorylated STAT3 in the nucleus might induce various oncogenes such as *cdc2*, *cyclin B1* and *myc* [24]. However, our transfection study of STAT3 resulted in transient cell proliferation in the primary B cells (Fig. 5), suggesting that additional factors other than STAT3 expression might be required for complete transformation of primary B cells. HIV-1 integrated into *c-fes/fps* in other reported cases of AIDS-related lymphoma [6], and it has been demonstrated that *c-fes* activates STAT3 [25]. Thus, STAT3 may play some roles in the lymphomagenesis in the cases of HIV-1-integrated lymphoma.

This case was B cell lymphoma. HIV-1 usually infects and integrates into T cells or macrophages, and it is uncommon for HIV-1 to infect B cells. In the report by other group, HIV-1 provirus was frequently detected in macrophages infiltrating lymphomas, not in lymphoma cells [6]. However, in our case, we concluded that the HIV-1 integration occurred in the lymphoma cells, not in T cells or macrophages infiltrating

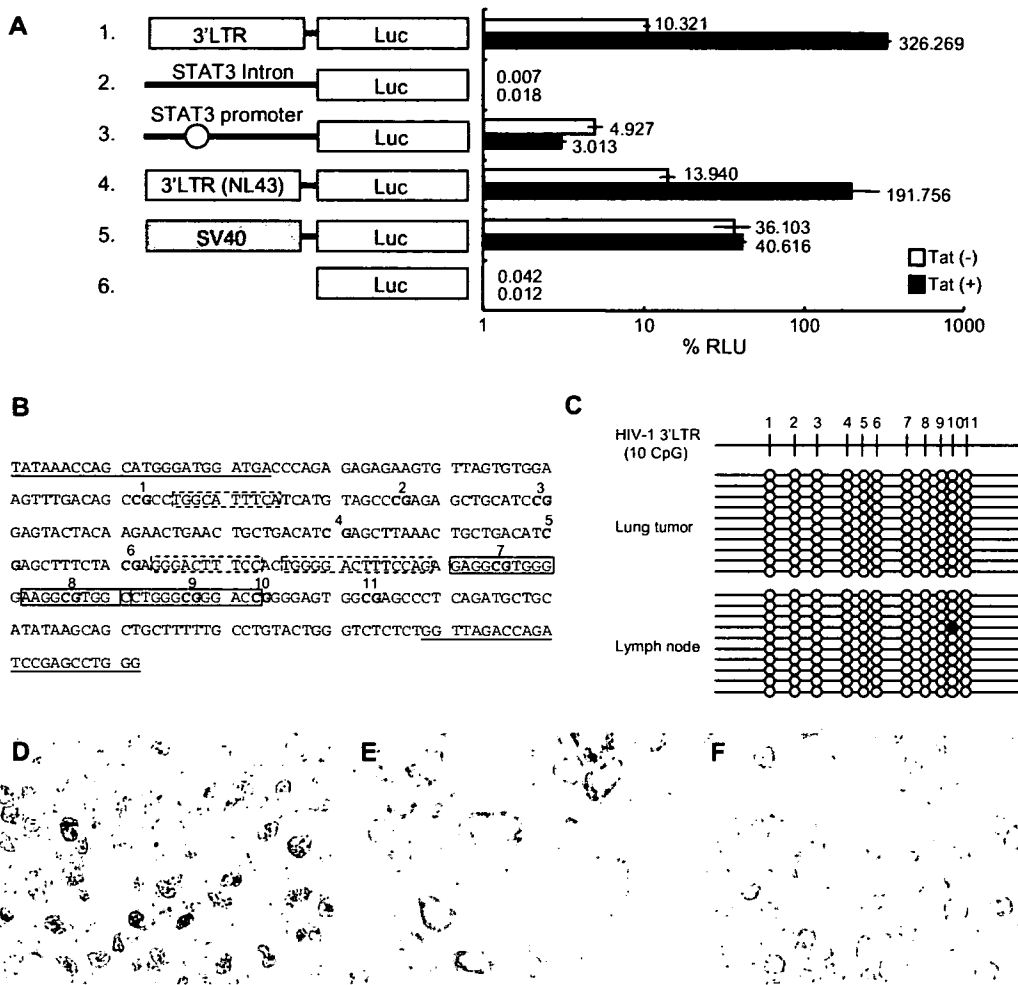


Fig. 4. Promoter activity of HIV-1 3'LTR and STAT3 expression in the lymphoma. (A) Promoter activity of HIV-1 3'LTR by reporter assay. Schematic representation of promoter constructs used in transient transfection assays is shown on the left. Forty-eight hours after transfection, cells were collected and the luciferase activity was measured. The percentage relative luminescence units (RLU) were calculated by dividing firefly activity by renilla activity. Horizontal bars indicate standard deviations of three independent experiments. (B and C) No methylation in a promoter enhancer region of HIV-1 3'LTR in the HIV-1-integrated lymphoma. (B) CpG sites in the promoter enhancer region of 3'LTR of the HIV-1 provirus in the patient with HIV-1-integrated lymphoma (218–529 in GenBank DQ117603). CpG sites are in boldface and numbered from the 5' end of the LTR (1–11). Nuclear factor- $\kappa$ B and Sp1 sites identified with Motif Search (Kyoto University Bioinformatics center, Kyoto, Japan, <http://motif.genome.jp/>) at a 75% cut-off value are indicated by boxes with broken and solid lines, respectively; Sequences used for primers are indicated by underlining. (C) Levels of CpG methylation of the promoter enhancer region of HIV-1 3'LTR in the HIV-1-integrated lymphoma and lymph nodes in the patient. Results of bisulfite genomic sequencing coupled with TA cloning are shown. The methylation status of 10 clones for each sample is presented; methylation of each CpG site is expressed as a filled circle, and unmethylated sites are shown as open circles. Top, schematic description of CpG sites in the 3'LTR of (B). (D–F) Immunohistochemistry of STAT3. The HIV-1-integrated lymphoma cells expressed STAT3 predominantly in the nucleus (D), however, signals of STAT3 were weak and localized in the cytoplasm in the other case of KSHV-positive, AIDS-related lymphoma (E), and were very weak in a case of EBV-positive, AIDS-related lymphoma (F). Original magnification is  $\times 400$ .

in the lymphoma, because of following reasons: (1) there were few T cells in the lymphoma tissue by immunohistochemistry for CD3 (data not shown); (2) HIV-1 DNA was detected in the lymphoma at a high copy number, that is very rare or none in AIDS-related lymphoma [26]; (3) HIV-1 sequences suggested

the possibility of X4 viruses, which leads the integrated HIV-1 sequences are usually not found in the macrophages; (4) some different HIV-1 V3 sequences were identified between the lymphoma and lymph node; and (5) the titer of HIV-1 DNA in the lymphoma were higher than that in the lymph node (Fig. 1G). Then, how did HIV-1 infect B cells in the patient? Although detail mechanism of HIV-1 infection to B cells in this case was still unknown, we presume that KSHV played an important role in HIV-1 infection to B cells. This case of lymphoma was positive for KSHV and EBV by PCR, however, KSHV and EBV did not play a direct role in the oncogenesis of the lymphoma because of the low or absent expression of

Table 1  
STAT3 expression in AIDS-related and unrelated lymphoma

STAT3 expression	Nucleus	Cytoplasm	No expression	Total
AIDS-related lymphoma	1*	7	2	10
Non-AIDS-related lymphoma	0	1	14	15

\*HIV-integrated lymphoma reported in the present study.

LANA and EBERs. It is possible that KSHV infection might increase susceptibility of B cells expressing CD4 and CXCR4 to infection with the X4 genotype of the HIV-1 [27]. Moreover, it is demonstrated that KSHV-encoded ORF50 protein increases susceptibility of B cells to infection with HIV-1 [28]. Although ORF50, CD4 and CXCR4 were not detected in the lymphoma cells by immunohistochemistry (data not shown), it is possible that KSHV-infected B cells might be infected and integrated by HIV-1 in the early stage of lymphoma development.

Although an intensive study revealed that there were many hot spots of HIV-1 integration [29], the STAT3 gene was not included in the list of hot spots. Thus, the STAT3 gene is a novel target of HIV-1 integration. Since HIV-1 DNA has not been detectable in DNAs extracted from AIDS-related

lymphoma cases by Southern blot hybridization, so far [26], HIV-1 integration should be rare in AIDS-related lymphoma. A recent study demonstrates a decrease in EBV-positive lymphoma among patients with AIDS because of introduction of highly active antiretroviral therapy (HAART) [30]. Therefore, novel mechanisms other than oncogenesis by EBV or KSHV may have been involved in the lymphomagenesis of AIDS-related lymphoma recently. There is no report describing a frequency of HIV-1 integration among AIDS related lymphoma. HIV-1 usually infects T cells or macrophages in AIDS patients, however, T cell lymphoma is still rare among AIDS-related lymphoma in the HAART era [30]. In addition, HIV-1 infection to B cells would occur in a very special condition, such as under KSHV infection. Taken together, although the case we described in the present study contained an important scientific phenomenon on STAT3, HIV-1-integrated lymphoma should be very rare among AIDS-related lymphoma.

### Acknowledgments

This study was supported by Health and Labor Sciences Research Grants on HIV/AIDS from the Ministry of Health, Labor and Welfare (grants H15-AIDS-005 to H.K.), a Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant 19590485 to H.K.), a grant for Research on Health Sciences focusing on Drug Innovation from Japan Health Sciences Foundation (grant SA14831 to H.K.), NIH MO1 RR00096 (to W.N.R.), NIH HL57879 (to M.D.W.), NIH HL 59832 (to M.D.W.), NIH DA022162 (to Y.H.), American Lung Association (to M.D.W.), Japanese Foundation for AIDS Prevention (to Y.H.), Uehara Memorial Foundation (to Y.H.) and the New York University Center for AIDS Research (to Y.H.). The authors declare that they have no competing financial interests.

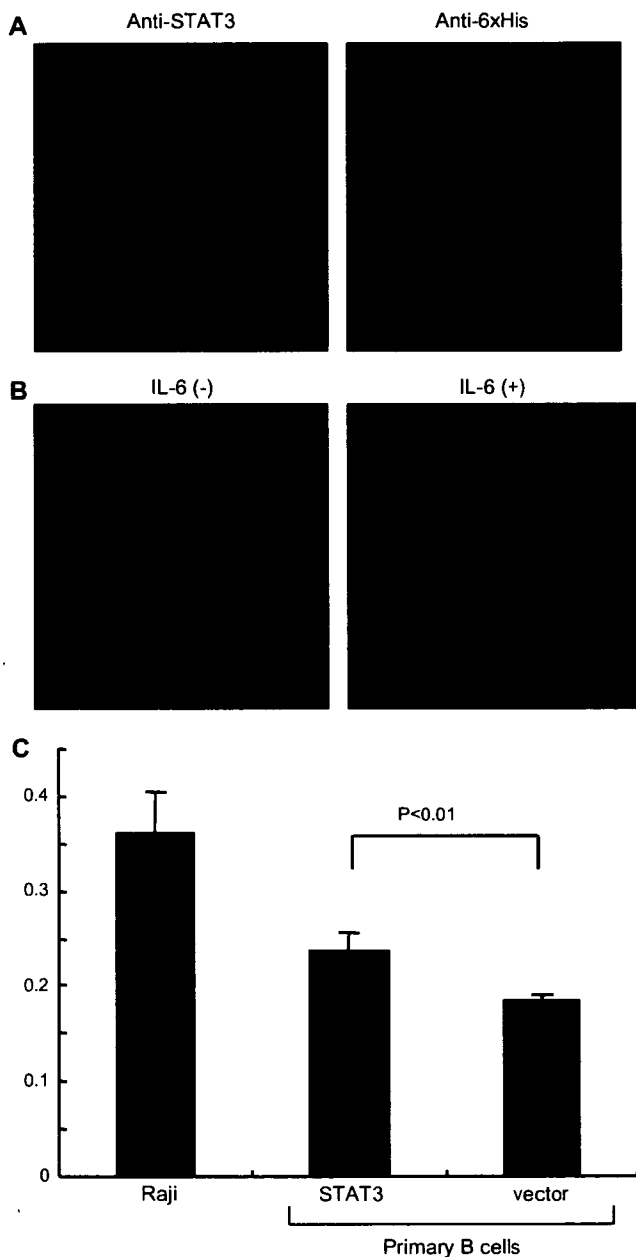


Fig. 5. Transfection of STAT3 into B cells in vitro. (A) STAT3 expression in the STAT3-transfected TY-1, a KSHV-positive B cell line. The cells were transfected with STAT3 expression vector by Nucleofector (Amaxa, Cologne, Germany) using O-06 program. STAT3 expression was detected by anti-STAT3 mouse monoclonal antibody (green in left panel) and anti-6xHis antibody, followed by Alexa 488-conjugated anti-mouse IgG antibody (molecular probe, green in right panel). Red color indicates nuclear counterstaining of propidium iodide. (B) Localization of transfecting STAT3 in TY-1. His-tagged STAT3 was detected by anti-6x His antibody in the cytoplasm of B cells (left panel). In the presence of IL-6 (Peprotech, Rocky Hill, NJ, 0.1 ng/ml), transfecting STAT3 localizes in the nucleus predominantly (right panel). (C) Cell proliferation assay for STAT3-transfected primary B lymphocytes. Primary B cells were isolated from PBMC. The purity of B cell (CD19+) was >95%. The cells were transfected with STAT3 expression vector expressing STAT3 and CD4 by Nucleofector using U-15 program. Transfection efficiency to primary B cells was around 20%. To increase the proportion of transfected cells, the transfected B cells were separated with CD4 microbeads after 16 h of the transfection (Miltenyl Biotec, Auburn, CA). 48 h after transfection of STAT3 or vector to primary B cells, the proliferation rate was measured with BrdU ELISA (Roche). Raji is an EBV-positive Burkitt lymphoma cell line (untransfected). Numbers in Y-axis indicates absorbance in ELISA. Error bars indicate standard errors of 8 independent experiments.

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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<sup>∇</sup>

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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<sup>+</sup> 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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<sup>∇</sup> 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<sup>+</sup>CD8<sup>+</sup> T cells frequently exist, even in HLA-A\*2402-positive Japanese patients with primary infections (unpublished data). As most of these Japanese patients were infected with the 2F mutant virus, we speculated that 2F-specific CTLs would be elicited in new hosts having HLA-A\*2402.

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

#### MATERIALS AND METHODS

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

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

**Sequence of autologous virus.** Viral RNA was extracted from samples of plasma from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (QIAGEN), and cDNA was synthesized from the RNA with SuperScript RNase H reverse transcriptase and random primer (Invitrogen). The Nef region was amplified by nested PCR using *Taq* DNA polymerase (Promega). Proviral DNA was extracted from HIV-1-infected patient PBMCs by using a QIAamp DNA blood mini kit (QIAGEN). We prepared the following *nef*-specific primer sets: 5'-AGCAGCAGATGGGGTGGGAGC-3' and 5'-AGCATCTGAGGGA CGCCACTCCC-3' for the first PCR primer set 1, 5'-TCGAGACCTGGAAA AACATGGAGC-3' and 5'-AAAGTCCCCAGCGGAAAAGTCCC-3' for the second PCR primer set 1, 5'-TCGAGACCTGGAAAACATGGAGC-3' and 5'-TAACCAGAGAGACCCAGTACAGGC-3' for the first PCR primer set 2, and 5'-GGCTAGAAGCACAAGAGGAGG-3' and 5'-AGCATCTGAGGGA 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  $\mu$ 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<sup>+</sup> T cells.** HLA-A\*2402-positive CD4<sup>+</sup> T cells infected with HIV-1 clone were stained to assess the expression of HLA class I in HIV-1-infected CD4<sup>+</sup> 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<sup>+</sup> T cells was analyzed by using a flow cytometry.

**Peptide-binding assay.** The binding of peptides to HLA-A\*2402 molecules was tested as previously described (25). HLA-A\*2402 RMA-S transfectants, which were transfected with HLA-A\*2402 and human  $\beta_2$ -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  $\beta_2$  microglobulin, produced in *Escherichia coli* that had been transformed with the relevant expression plasmids, were first solubilized in denaturing buffer containing 8 M urea and refolded in refolding buffer in the presence of a synthesized peptide for 48 h at 4°C. The resultant 45-kDa complex was purified by size exclusion (Superdex G75; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England) and anion exchange chromatographies (MonoQ column; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). Purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C terminus of the heavy chain and were mixed with PE-conjugated avidin (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<sup>+</sup> 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  $\mu$ 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<sup>+</sup> T cells

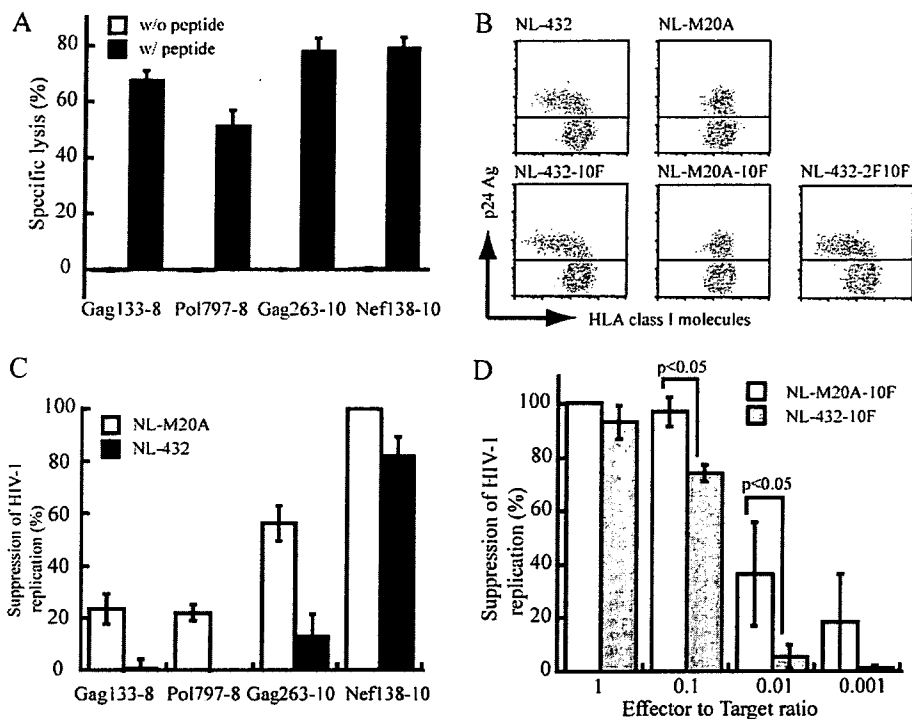


FIG. 1. Suppression of HIV-1 replication in HIV-1-infected CD4<sup>+</sup> T cells by HLA-A\*2402-restricted HIV-1-specific CTLs. (A) Cytolytic activities of HLA-A\*2402-restricted HIV-1-specific CTLs (four Gag133-8-specific, five Pol797-8-specific, four Gag263-10-specific, and four Nef138-10-specific CTL clones) toward HLA-A\*2402<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells derived from an HLA-A\*2402<sup>+</sup> 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<sup>+</sup> T cells. HIV-1-infected HLA-A\*2402<sup>+</sup> CD4<sup>+</sup> 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<sup>+</sup> T cells cultured without the CTLs)  $\times$  100.

<sup>51</sup>Cr release assay. The cytotoxicity of CTL clones against C1R-A\*2402 pre-pulsed with appropriate peptide at various concentrations or HIV-1-infected 221-CD4<sup>+</sup>-A\*2402 cells was determined as previously described (37, 38). Briefly, target cells ( $2 \times 10^5$ ) were incubated for 60 min with 100  $\mu$ Ci of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> in saline. Effector cells were cocultured with target cells ( $2 \times 10^5$ /well) for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous <sup>51</sup>Cr release was determined by measuring the counts per minute (cpm) in the supernatant in the wells containing only target cells (cpm spn). Maximum release (cmp max) was determined by measuring the release of <sup>51</sup>Cr 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 (LL<sub>50</sub>) was calculated by using KaleidaGraph (Hulinks, Inc., Tokyo, Japan).

## RESULTS

**Complete suppression of HIV-1 replication by HLA-A\*2402-restricted Nef138-10-specific CTLs.** Since Nef138-10-specific CTLs are frequently detected in HLA-A\*2402-positive HIV-1-infected individuals, it has been speculated that Nef138-10 (RYPLTFGWCY) is an immunodominant epitope and that these specific CTLs can strongly suppress HIV-1 replication. To clarify

the abilities of Nef138-10-specific CTLs to suppress HIV-1 replication, we compared the abilities of four epitope-specific, HLA-A\*2402-restricted CTL clones to suppress HIV-1 replication by assaying for the suppression of HIV-1 replication. All CTL clones specific for the Gag133-8, Pol797-8, Gag263-10, or Nef138-10 epitope showed similar cytotoxic activities against target cells pre-pulsed with the corresponding peptide (Fig. 1A). Gag133-8, Pol797-8, and Gag263-10 epitopes are conserved in both NL-432 and NL-M20A strains, the latter of which has the ability to down-regulate the expression of CD4 molecules, but not the expression of HLA class I molecules, on the cell surface, whereas the Nef138-10 epitope is not conserved in these strains. The Nef138-10 epitope has a tyrosine instead of phenylalanine at position 10, but Nef138-10 epitope-specific CTLs effectively recognized both peptides, RYPLTFGWCF and RYPLTFGWCY. We investigated HLA-A\*2402 down-regulation on CD4<sup>+</sup> cells infected with NL-432 or NL-M20A. The down-regulation of HLA-A\*2402 was found on CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>-</sup> patients infected through USI than that in HLA-A\*2402<sup>-</sup> hemophiliacs (31% versus 0%), suggesting that this mutation had accumulated in the HLA-A\*2402<sup>-</sup> 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

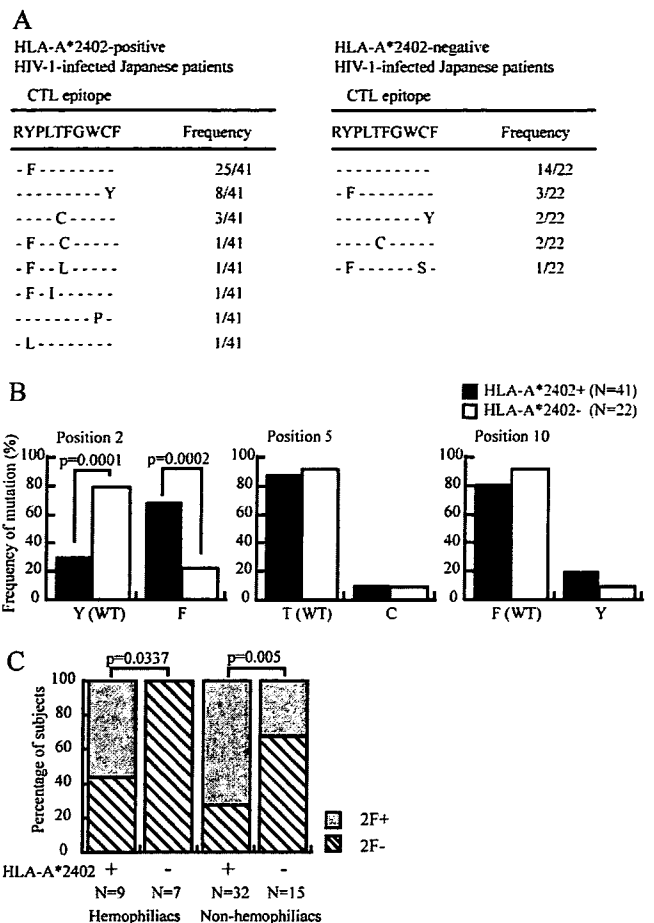


FIG. 2. Frequency of mutation in the Nef138-10 epitope and flanking region. (A) Alignment of the amino acid sequences from 41 HLA-A\*2402<sup>+</sup> and 22 HLA-A\*2402<sup>-</sup> patients. (B) Frequency of the substitutions in the Nef138-10 epitope. The Y-to-F substitution at position 2 was more frequently 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<sup>+</sup> and 22 HLA-A\*2402<sup>-</sup> 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<sup>+</sup> target cells prepulsed with WT peptide ( $LL_{50}$ ,  $0.16 \pm 0.07$  nM) than toward those prepulsed with the mutant epitope peptide ( $LL_{50}$ ,  $5.03 \pm 6.23$  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

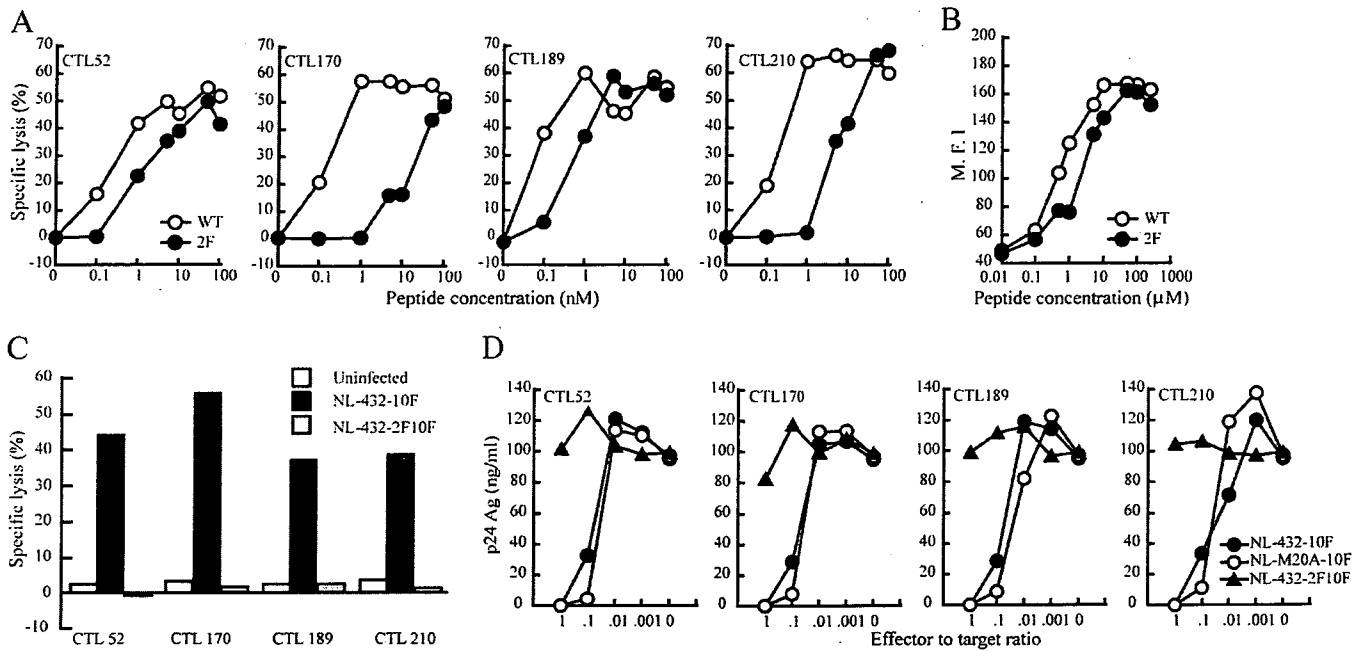


FIG. 3. Abilities of Nef138-10-specific CTLs to suppress replication of NL-432-2F10F. (A) Cytolytic activity of HLA-A\*2402-restricted Nef138-10-specific CTL clones toward HLA-A\*2402<sup>+</sup> cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). They were tested at an E-to-T ratio of 2:1. (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<sup>+</sup> 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<sup>+</sup> T cells. Cultured CD4<sup>+</sup> T cells derived from an HLA-A\*2402<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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-

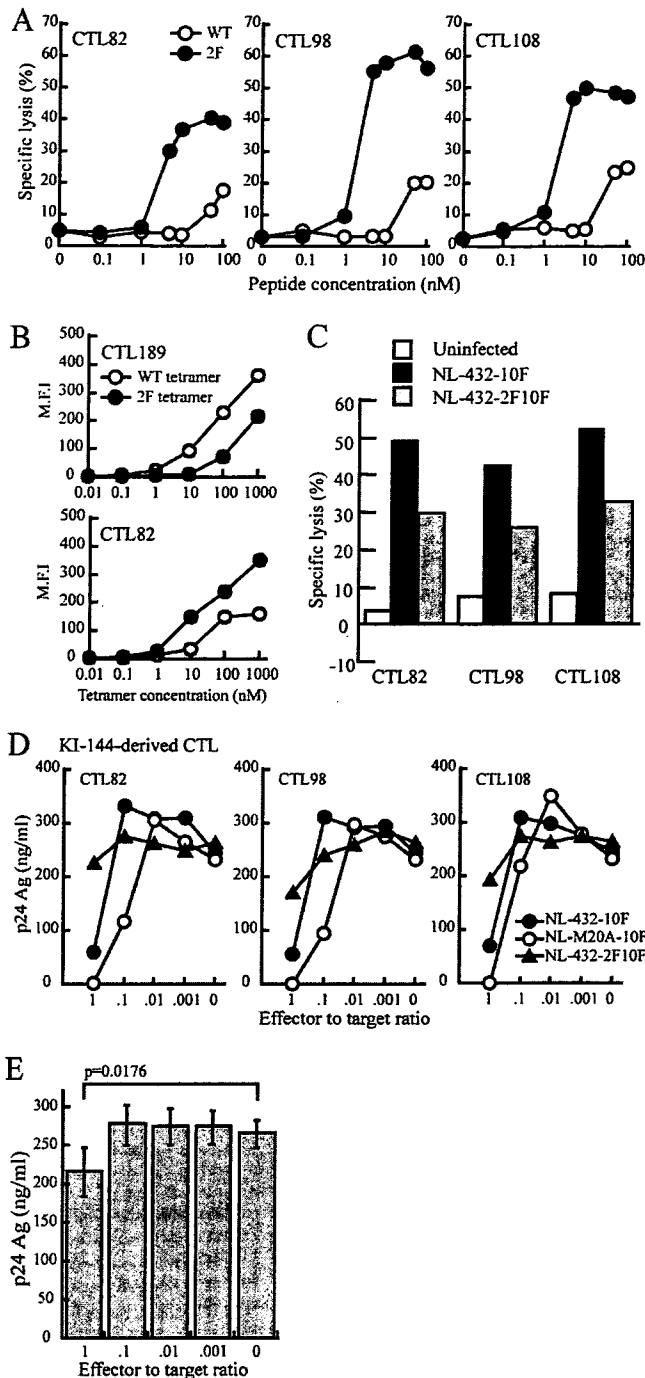


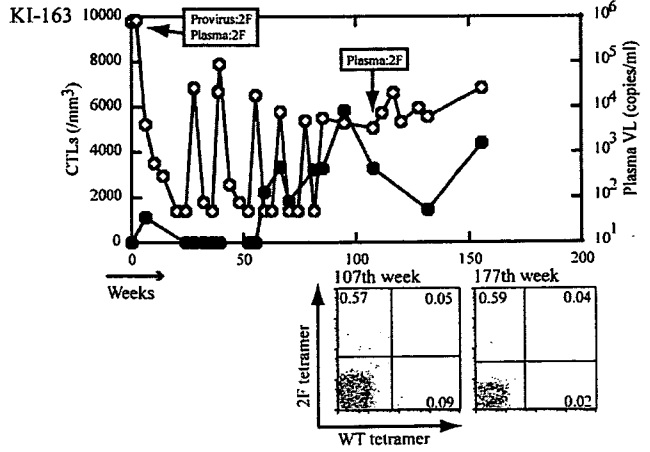
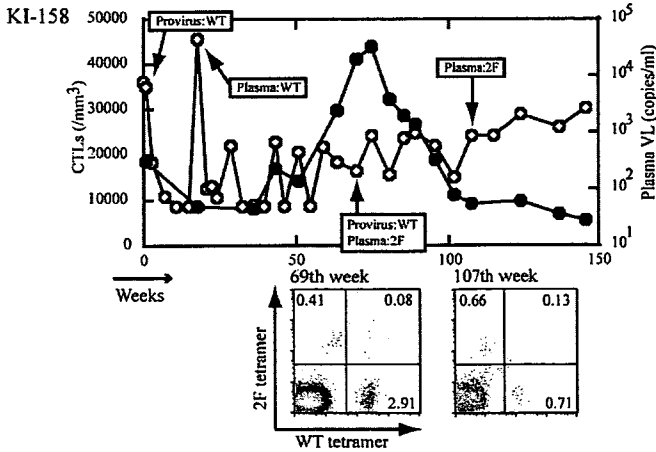
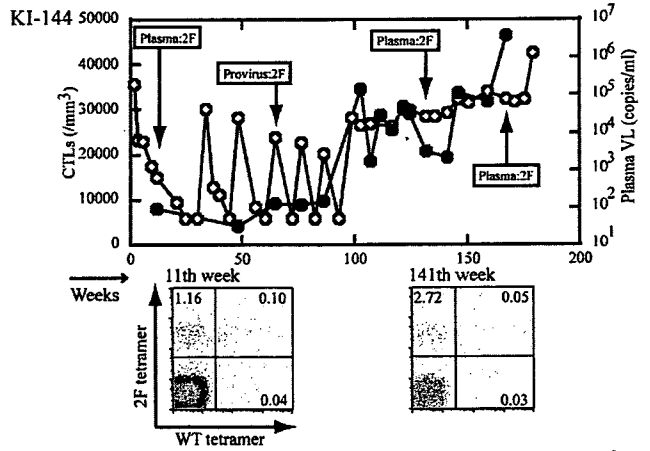
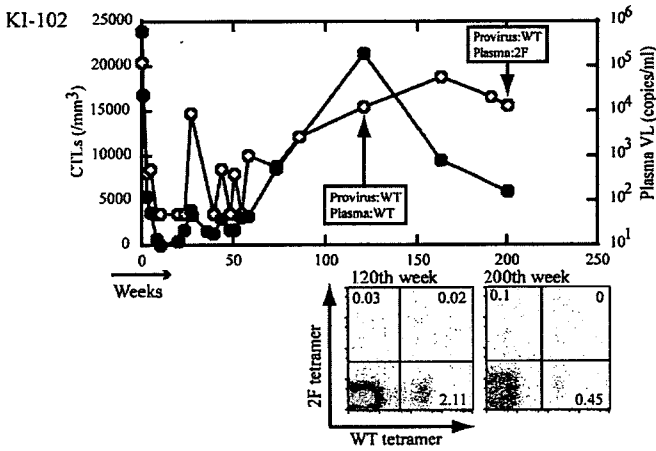
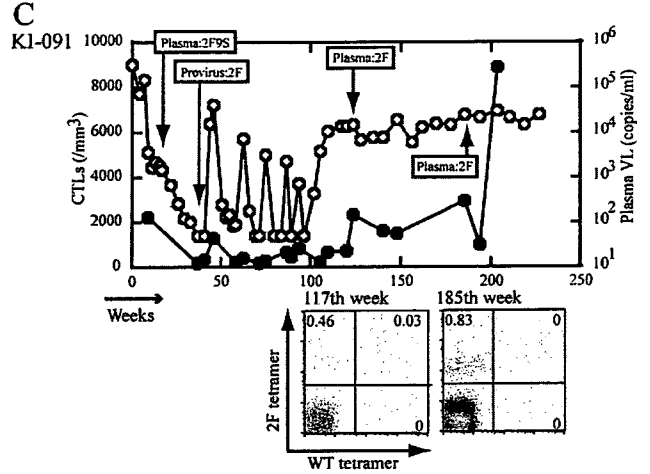
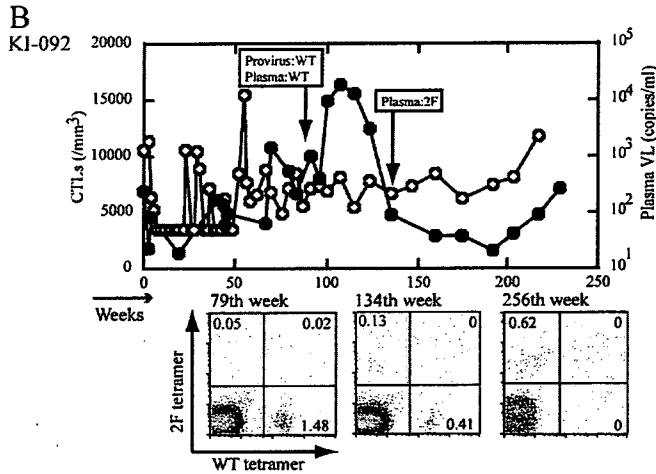
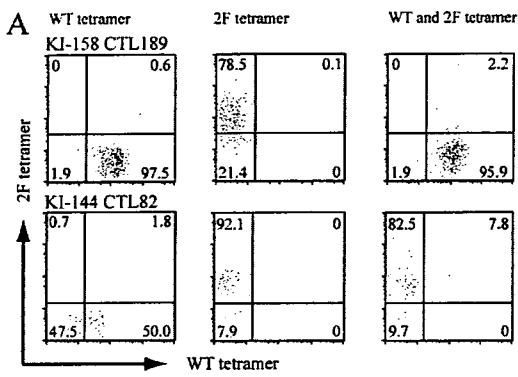
FIG. 4. Distinct antiviral activities toward NL432-2F10F of KI-144-derived Nef138-10-specific CTL clones. (A) Cytolytic activity of KI-144-derived HLA-A\*2402-restricted Nef138-10-specific CTL clones toward HLA-A\*2402<sup>+</sup> cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). The clones 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<sup>+</sup> 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<sup>+</sup> T cells. Cultured CD4<sup>+</sup> T cells derived from an HLA-A\*2402<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells. We stimulated their PBMCs with either WT or mutant peptide and cultured the PBMCs for 2 weeks. The WT tetramer-binding CD8<sup>+</sup> T cells were then counted by using the competitive tetramer binding assay. The WT-specific CD8<sup>+</sup> 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<sup>+</sup> 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).



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

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

It is well known that in both HIV-1 and SIV infections, escape mutations are poorly recognized in new hosts who share the same HLA alleles with donors (17, 32). If escape mutant peptides fail to bind to HLA class I restriction molecules or the mutation critically affects the Ag processing, these escape mutants are hardly recognized and fail to elicit the specific CTLs in new hosts sharing the same HLA alleles. On the other hand, if escape mutant peptides can bind to HLA class I restriction molecules and can be processed and presented, it remains possible that the mutant epitope is recognized in new hosts. The 2F mutant peptide effectively bound to HLA-A\*2402 (Fig.

3B), suggesting the possibility that the 2F mutant peptide is presented by HLA-A\*2402. We therefore selected the three donors who were infected with the 2F mutant virus at an early phase (within 10 weeks before the first visit) and investigated whether the 2F-specific CD8<sup>+</sup> T cells were elicited in these donors. It was strongly suggested that these patients had been infected with the 2F virus, since in the donors who had been infected with the WT virus, the 2F mutation was selected approximately 2 years after infection. The 2F-specific CD8<sup>+</sup> T cells were elicited in these three donors, although this mutant epitope was very weakly presented by HLA-A\*2402. Thus, escape mutant-specific CTLs can be elicited in new hosts even if the mutant epitope peptide is very weakly presented.

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

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

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

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

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

#### ACKNOWLEDGMENTS

We thank Sachiko Sakai for her secretarial assistance.

This research was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases launched as a project commissioned by the Ministry of Education, Science, Sports, and Culture, Japan; by a grant-in-aid for scientific research from the Ministry of Health, Japan; by a grant-in-aid (no. 18390141) for scientific research from the Ministry of Education, Science, Sports, and Culture, Japan; and by a grant from the Japan Health Science Foundation. M.F. is a JSPS Research Fellow.

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# CTL-Mediated Selective Pressure Influences Dynamic Evolution and Pathogenic Functions of HIV-1 Nef<sup>1</sup>

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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<sup>75</sup>Thr and Tyr<sup>85</sup>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<sup>85</sup>Phe, were elicited earlier and had more potent functional avidities than did those that selected Arg<sup>75</sup>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.

**T**he 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)<sup>3</sup> 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 cytotoxicity 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<sup>+</sup> 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<sub>73–82</sub>: PVR-PQVPLRP) 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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Received for publication September 28, 2007. Accepted for publication November 8, 2007.

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<sup>1</sup> This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (to T.U.), by a Grant-in-Aid for AIDS Research from the Ministry of Health, Labor, and Welfare of Japan (to T.U., S.O., and M.T.), and by Deutsche Forschungsgemeinschaft Grant SFB 638 (Project A11, to O.T.F.).

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<sup>3</sup> 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<sup>+</sup> subjects used in this study<sup>a</sup>

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

<sup>a</sup> 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<sup>+</sup>) 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 (ir-

radiated 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<sup>75</sup> to Thr and Tyr<sup>85</sup> 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

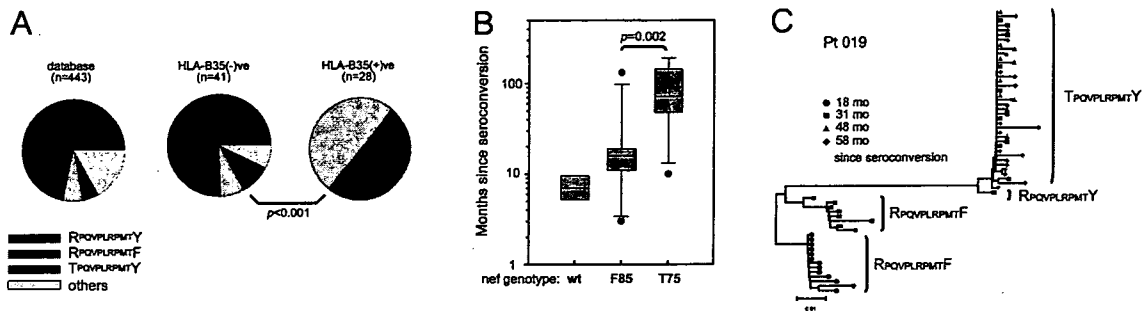
**HLA stabilization assay.** Peptide-binding activity for HLA-B\*3501 was assessed by an HLA stabilization assay using RMA-S cells expressing HLA-B\*3501 as described earlier (31).

**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 ( $2 \times 10^6$ ) or -negative donors ( $3 \times 10^6$ ) 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<sup>+</sup>CD8<sup>+</sup> 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 ( $4 \times 10^4$  cells) were incubated with C1R-B3501 cells ( $4 \times 10^4$  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 <sup>51</sup>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  $\chi^2$  test. *B*, Differences in the duration of HIV infection (months since seroconversion) and the autologous *nef* genotypes, wt, Tyr<sup>85</sup>Phe (F85) or Arg<sup>75</sup>Thr (T75) in HLA-B35<sup>+</sup> 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<sup>+</sup> cells.** CD4<sup>+</sup> 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  $\mu$ g/ml; Sigma-Aldrich) for 4 days. The activated CD4<sup>+</sup> cells were then infected at relatively high titers (1  $\mu$ g of p24 Ag per 10<sup>6</sup> 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<sup>+</sup> 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 <sup>51</sup>Cr. It should be noted that 30  $\pm$  5% of the cells were p24 Ag<sup>+</sup> as revealed by intracellular flow cytometric analysis of HIV-infected CD4<sup>+</sup> 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  $\times$  10<sup>5</sup> 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<sup>5</sup> cells/well. Three days later, the cells were stimulated with PHA at 2  $\mu$ 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 (10<sup>7</sup>) 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<sub>2</sub>VO<sub>4</sub>, 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<sub>2</sub>) with 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>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 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  $\chi^2$ , 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<sup>75</sup> to Thr mutation (T75, amino acid numbers based on SF2 strain) in Nef was functionally asso-

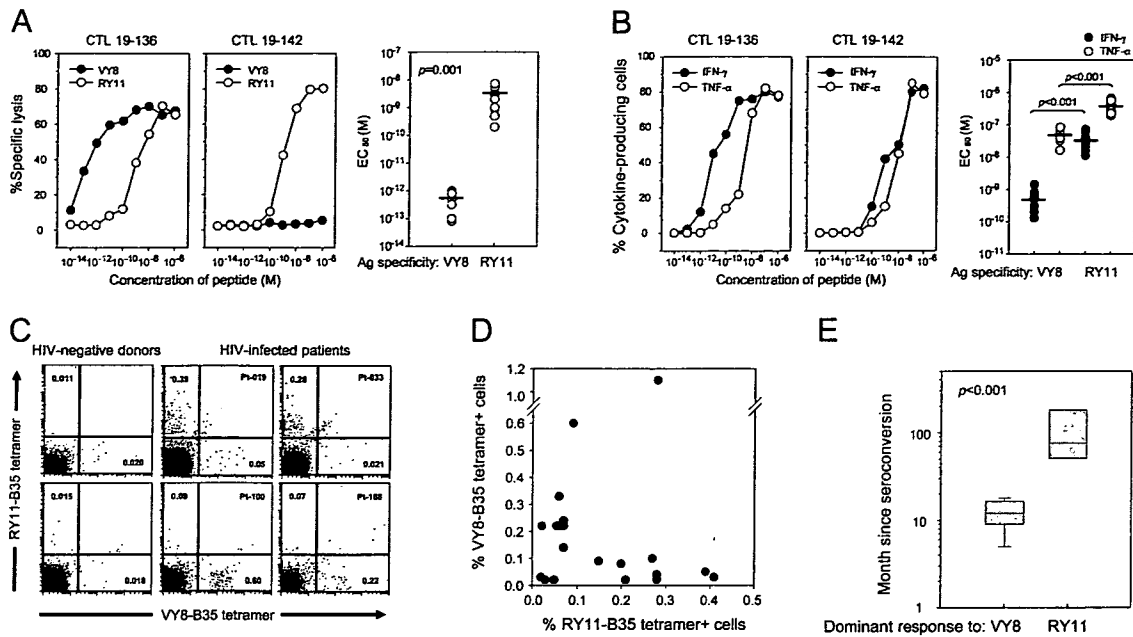
ciated with escape from a CTL response specific for the RY11 epitope (Nef<sub>75–85</sub>: RPQVPLRPMTY) presented by HLA-B\*35 in patients in the chronic phase of an HIV-1 infection (29). When we recruited more subjects including some in the early phase of infection and analyzed their autologous *nef* genotypes, another mutation, Tyr<sup>85</sup> to Phe (F85), was also found in some of these HLA-B35<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 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 intrapatent 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<sup>+</sup> cells. Some representative dot plots of 2 HIV-negative and 4 HIV-positive donors are shown with frequencies of HLA-tetramer<sup>+</sup> cells in each dot plot (**C**). The frequencies of HLA-tetramer<sup>+</sup> 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., TPQVPLRPMTE (referred to as TF), was not apparently selected. Rather, the T75 mutation appeared to arise from a different lineage of viral quasiespecies 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 (Nef<sub>78-85</sub>: VPLRPMTY) and RY11 (Nef<sub>75-85</sub>: RPQVPLRPMTY), showed substantial CTL responses in the HLA-B35<sup>+</sup> 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<sup>+</sup> 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  $2.81 \times 10^{-13}$  and  $7.50 \times 10^{-10}$  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- $\gamma$  and TNF- $\alpha$  production revealed that VY8-specific CTLs also showed more potent functional avidity than RY11-specific ones, as mean  $EC_{50}$  values for IFN- $\gamma$  secretion were  $5.30 \pm 1.21 \times 10^{-10}$  and  $3.50 \pm 0.61 \times 10^{-8}$  M, and those for TNF- $\alpha$

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<sup>+</sup> 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<sup>+</sup> cells in HIV-negative donors ( $n = 6$ ) was  $0.0153 \pm 0.0022\%$ . The frequency of HLA-tetramer<sup>+</sup> cells in HIV-infected subjects ( $n = 19$ ) was  $0.198 \pm 0.060$  and  $0.160 \pm 0.029$  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<sup>+</sup> 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  $13.0 \pm 1.4$  or  $76.0 \pm 19$ , 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 EC<sub>50</sub> 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  $\sim$ 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  $\sim$ 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 EC<sub>50</sub> 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 EC<sub>50</sub> 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<sup>+</sup>) were first stimulated with PHA and then infected with wt or various variant viruses. Four days later,  $\sim$ 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  $\Delta$ 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  $\Delta$ 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  $\Delta$ Nef had expression levels of Nef comparable to that of the wt (data not shown). These data demonstrate that the