Materials and methods

This study was approved by Kumamoto University Ethics Committee. Informed consent was obtained from all subjects, in accordance the Declaration of Helsinki.

Isolation and culture of macrophages and CD4+ T cells

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of HLA-B*5101+ or HLA-B*3501+ healthy donors by an adherence method as previously described.[16] CD4+ T cells were purified from nonadherent cells by means of anti–human CD4 monoclonal antibody (mAb)–coated magnetic beads (magnetic-activated cell sorting [MACS] beads; Miltenyi Biotec, Bergisch Galdbach, Germany). These cultured macrophages and CD4+ T cells were infected with HIV-1 clones as previously described.[8]

HIV-1–specific CTL clones

HIV-1–specific CTL clones (HLA-B*5101-restricted CTL clones: Pol743-8-40, Pol283-8-237, -240, -320, and -340; Gag327-9-131, -142, -148, and -287; Rev71-11-8, -17, and -55; and HLA-B*3501-restricted CTL clones: Env77-9-110, Pol273-9-2; and an HLA-A*1101-restricted CTL clone: Gag349-11-18 and -22 as mismatched CTL clone) were generated as previously described.[17-19] These CTL clones predominantly showed $CD27^-CD28^-CD45RA^-$ phenotype (data not shown).

HIV-1 clones

An infectious proviral clone of HIV-1, pNL-432, and its Nef mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously.[20] pJRFL and its Nef-defective mutant, pJR-Xh, which has a frame shift at a *Xho*I site (44th amino acid of Nef protein), were kindly donated by Dr Koyanagi (Kyoto University, Kyoto, Japan). $pJRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ were constructed by exchanging the Nef region of JRFL for that of NL-432 or NL-M20A.

CTL assay

The cytotoxicity of CTL clones against HIV-1–infected macrophages or CD4+ T cells (40-50% p24 antigen-positive cells) was determined by a standard ^{51}Cr-release assay, as previously described.[8]

Flow cytometric analysis

Cells infected with HIV-1 clone were stained to assess the expression of HLA class I in HIV-1–infected macrophages or CD4+ T cells, as previously described.[9] For detection of intracellular cytokines, HIV-1–specific CTL clones were cocultured with HIV-1–infected cells for 6 hours at an effector-stimulator (E/S) ratio of 1:4. Then, brefeldin A was added (10 μg/mL). After a 6-hour incubation, the cells were stained with FITC-labeled anti–human IFN-γ, PE-labeled anti–human MIP-1β, PerCp-labeled anti–human CD8, or APC-labeled anti–human TNF-α mAbs (BD Biosciences, San Jose, CA), as previously described.[21]

Suppression of HIV-1 replication by HIV-1–specific CTLs

The ability of HIV-1–specific CTLs to suppress HIV-1 replication was examined as previously described.[8] Briefly, CD4+ T cells or macrophages were incubated with a given HIV-1 clone for 12 hours at 37°C. After several washes with R10 medium, the cells were cocultured with HIV-1–specific CTL clones. From day 3 to 12 after infection, 10 μL culture supernatant was collected, and the concentration of p24 antigen was measured by use of an enzyme immunoassay (HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA] kit; ZeptMetrix, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1 − concentration of p24 antigen in the supernatant of HIV-1–infected CD4+ T cells cultured with HIV-1–specific CTLs/concentration of p24 antigen in the supernatant of HIV-1–infected CD4+ T cells culture without the CTLs) × 100.

Western blot analysis

Cells were lysed in lysis buffer (1% Triton X-100, 50 mM Tris HCl, pH 7.6, 1 mM $MgCl_2$, 150 mM NaCl, and 0.1 mM EDTA) containing a mixture of protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Samples were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-10% polyacrylamide gel electrophoresis (PAGE), and transferred to an Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA). Protein detection was performed after incubation with appropriate first and secondary antibodies by using a Chromogenic Western Blot Immunodetection Kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The first antibodies for p24 and Nef were purchased from ZeptoMetrix and Advanced Biotechnologies, respectively. Quantification was performed by using National Institutes of Health Image.

Proliferation assay

HIV-1–infected CD4+ T cells or macrophages were irradiated and cocultured with thawed HIV-1–specific CTL clones (5×10^3 cells/well) for 3 days in triplicate in 96-well plates at an E/S ratio of 1:4. Then 0.5 μCi/well (0.0185 MBq) of ^3H [thymidine] was added, and the cells were subsequently incubated for an additional 16 hours. The incorporation was measured by a scintillation counter.

Results

Strong abilities of HIV-1–specific CTL clones to suppress HIV-1 replication in macrophages and to kill HIV-1–infected macrophages

To investigate CTL recognition of HIV-1–infected macrophages, we measured the ability of HIV-1–specific CTLs to suppress the replication of HIV-1 R5 strain JRFL and X4 strain NL-432 in HIV-1–infected macrophages and CD4+ T cells, respectively. We used CTL clones specific for 4 HLA-B*5101-restricted epitopes (Pol743-9, Pol283-8, Gag327-9, and Rev71-11) and 2 HLA-B*3501-restricted epitopes (Env77-9 and Pol273-9). Previous studies using these epitope-specific CTL clones demonstrated that the 2 B*5101-restricted Pol-specific CTL clones completely suppressed the replication of HIV-1 X4 strain NL-432 but that other CTL clones only partially suppressed it.[8,9] We measured the ability of these 6 CTL clones to suppress the replication of JRFL and its Nef-defective mutant JR-Xh in HIV-1–infected macrophages. The surface expression of HLA-B*5101 molecules was down-regulated in JRFL-infected macrophages and NL-432–infected CD4+ T cells but not in JR-Xh–infected macrophages and NL-M20A–infected CD4+ T cells (Figure 1). The down-regulation of HLA-B*3501 molecules was also found in only JRFL-infected macrophages and NL-432–infected CD4+ T cells (data not shown). SF2-Rev71-11-8 partially suppressed the replication of JRFL, whereas the other 5 clones completely suppressed the replication of both JRFL and JR-Xh (Figure 2A). On the other hand, only Pol743-9– and Pol283-8–specific CTLs completely suppressed the replication of both NL-432 and NL-M20A in HIV-1–infected CD4+ T cells (Figure 2B). These CTL clones showed similar effects in terms of their cytolytic activity toward HIV-1–infected macrophages and CD4+ T cells (Figure 2C-D). These results of the CTL clones for JRFL-infected macrophages contrast with those for NL-432–infected CD4+ T cells.[8,9]

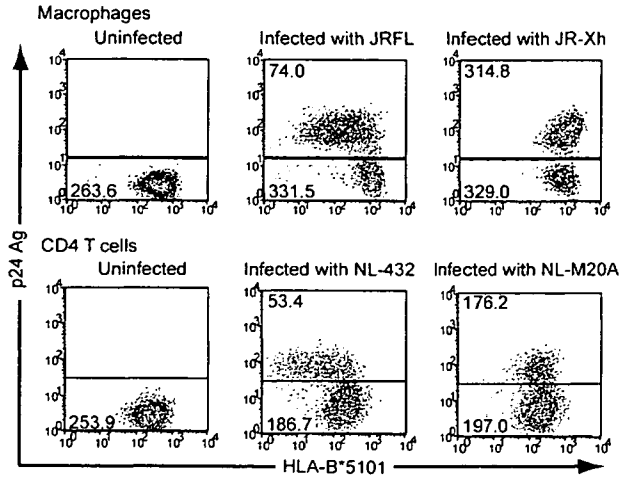

Figure 1. Expression of HLA class I molecules on macrophages or CD4⁺ T cells infected with Nef⁺ or Nef⁻ HIV-1. Macrophages established from monocytes and CD4⁺ T cells of an HLA-B*5101⁺ donor were infected with HIV-1 JRFL or JR-Xh and NL-432 or NL-M20A, respectively, and then cultured for 6 days. The cultured macrophages and CD4⁺ T cells were stained with anti-p24 and 4D12 anti–HLA-B5 mAbs. The surface expression of HLA-B*5101 on p24⁺ or p24⁻ cells is shown as the mean fluorescence intensity (MFI) in each figure.

Comparison between abilities of HIV-1–specific CTLs to suppress R5 virus replication in CD4⁺ T cells and macrophages

It remains possible that the strong ability to suppress the replication of JRFL and the effect of Nef-mediated HLA class I down-regulation were a strain-dependent effect. To exclude this possibility, we generated 2 R5 chimera viruses, specifically, $JRFL_{NL\text{-}432Nef}$ (JRFL carrying NL432-derived Nef) and $JRFL_{NL\text{-}M20ANef}$ (JRFL carrying NL-M20A–derived Nef), and then investigated the ability of 4 HLA-B*5101-restricted CTL clones to suppress the replication of these chimera viruses in HIV-1–infected CD4⁺ T cells and macrophages. The down-regulation of HLA class I molecules was found to occur in $JRFL_{NL\text{-}432\ Nef}$-infected cells, but not in $JRFL_{NL\text{-}M20A\ Nef}$-infected cells (data not shown). The Rev71-11-8 CTL clone suppressed the replication of $JRFL_{NL\text{-}M20A\ Nef}$ in $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages more strongly than that of $JRFL_{NL\text{-}432\ Nef}$ in $JRFL_{NL\text{-}432\ Nef}$-infected macrophages, whereas other CTL clones showed the same ability to suppress the replication of the 2 JRFL chimera viruses (Figure 3A).

These CTL clones revealed more than 10- to 100-fold stronger ability to suppress the replication of the chimera viruses in HIV-1–infected macrophages than in HIV-1–infected CD4⁺ T cells (Figure 3A). The replication kinetics of $JRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ between these 2 cell types were similar, as shown in Figure 3B, thus indicating that the difference in the ability of the specific CTL clones to suppress the chimera virus replication was unrelated to replication kinetics. Thus, these results indicate that HIV-1–specific CTLs could recognize HIV-1–infected macrophages more effectively than HIV-1–infected CD4⁺ T cells.

Ability of HIV-1–infected macrophages to stimulate HIV-1–specific CTLs

We further analyzed the cytokine production from the HLA-B*5101-restricted CTL clones after having stimulated them with either HIV-1–infected CD4⁺ T cells or HIV-1–infected macrophages. IFN-γ–, MIP-1β–, or TNF-α–producing CTL clones were much more detectable after the clones had been stimulated with HIV-1 chimera virus-infected macrophages than after stimulation with HIV-1 chimera virus-infected CD4⁺ T cells (Figure 4A-B). We considered p24⁻ cells to be HIV-1–uninfected cells because only p24⁺ cells showed down-regulation of CD4 (data not shown). HIV-1–infected cells might exist in p24⁻ cells, but they should express very low level of HIV-1 proteins and can hardly stimulate HIV-1–specific CTLs. Therefore we counted p24⁺ cells as HIV-1–infected cells. Frequencies of Pol283-8-340 CTL clones producing

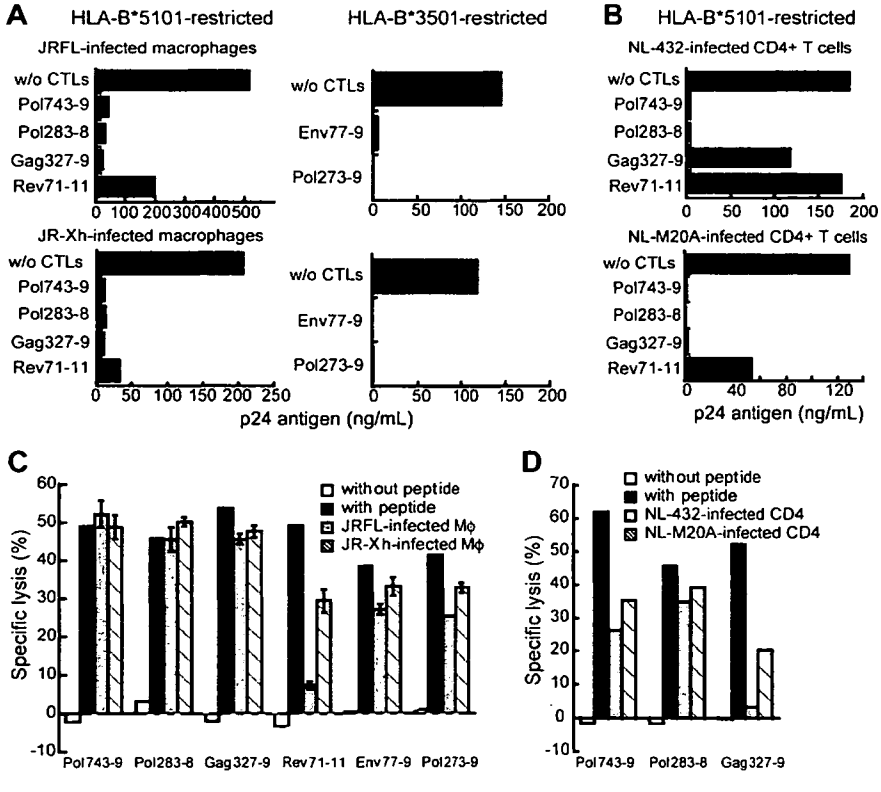

Figure 2. Strong abilities of HIV-1–specific CTLs to suppress HIV-1 replication in HIV-1–infected macrophages and to kill them. (A-B) Ability of HIV-1–specific CTL clones to suppress HIV-1 replication in HIV-1–infected macrophages and in HIV-1–infected CD4⁺ T cells. Macrophages and CD4⁺ T cells from an HLA-B*5101⁺ donor and an HLA-B*3501⁺ donor were infected with JRFL or JR-Xh and NL-432 or NL-M20A, respectively, and then cocultured with each HIV-1–specific CTL clone at an E/T ratio of 1:1. HIV-1 p24 antigens in the supernatant were measured on day 9 after infection by use of an enzyme immunoassay. Data shown in the figure are averages of triplicate assays for each HIV-1–specific CTL clone. (C) Cytotoxic activity against HIV-1–infected macrophages. Macrophages from an HLA-B*5101⁺ donor and an HLA-B*3501⁺ donor were infected with JRFL or JR-Xh. JRFL-infected (56% of total cells were p24 antigen-positive), JR-Xh–infected (48% of total cells were p24 antigen-positive) macrophages were used as target cells at an E/T ratio of 2:1. Data shown in the figure are averages ± SD of triplicate assays for each HIV-1–specific CTL clones. (D) Cytotoxic activity against HIV-1–infected CD4⁺ T cells. CD4⁺ T cells from an HLA-B*5101⁺ donor were infected with NL-432 or NL-M20A. NL-432–infected (81.6% of total cells were p24 antigen-positive) and NL-M20A–infected (79.1% of total cells were p24 antigen-positive) were used as target cells at an E/T ratio of 2:1.

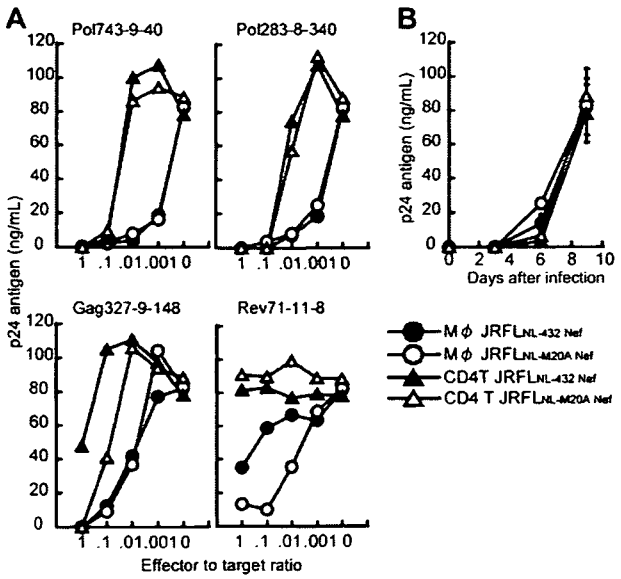

Figure 3. Comparison between abilities of HIV-1–specific CTLs to suppress HIV-1 replication in CD4⁺ T cells and macrophages infected with HIV-1 R5 strain. (A) The ability of HIV-1–specific CTL clones to suppress $JRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ replication in CD4⁺ T cells and macrophages infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$. CD4⁺ T cells and macrophages from HLA-B*5101⁺ donor were infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$ and then cocultured with HLA-B*5101-restricted HIV-1–specific CTL clones at various E/T ratios. The amount of HIV-1 p24 antigen in the supernatant on day 9 after infection was measured by using an enzyme immunoassay. (B) Kinetics of $JRFL_{NL\text{-}432\ Nef}$ and $JRFL_{NL\text{-}M20A\ Nef}$ replication in CD4⁺ T cells and macrophages infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$. The amount of HIV-1 p24 antigen in the supernatant on days 3 to 9 after infection was measured by the enzyme immunoassay. Data shown in the figure are averages of triplicate assays for each time point. The experiments shown in panels A and B were performed simultaneously.

at least one cytokine were 69.6% and 72.5%, after the clones had been stimulated with $JRFL_{NL\text{-}432\ Nef}$-infected and $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages, respectively, whereas they were 29.7% and 43.8% after stimulation with $JRFL_{NL\text{-}432\ Nef}$-infected and $JRFL_{NL\text{-}M20A\ Nef}$-infected CD4⁺ T cells, respectively. HIV-1–specific CTL clones stimulated with HIV-1–infected CD4⁺ T cells predominantly produced a single or 2 cytokines, whereas cells producing 3 cytokines were more frequently found among those stimulated with HIV-1–infected macrophages (Figure 4B). Similar results were found for other CTL clones tested (Figure 4A). These results suggest that HIV-1–infected macrophages can stimulate HIV-1–specific CD8⁺ T cells more strongly than HIV-1–infected CD4⁺ T cells in vivo.

HIV-1–specific CTLs are frequently found in HIV-1–infected individuals, although their number decreases in the late chronic phase of an HIV-1 infection.[22] Since we found the ability of HIV-1 antigen presentation by HIV-1–infected CD4⁺ T cells to be much weaker than that by HIV-1–infected macrophages and macrophages are well known to be professional antigen-presenting cells, we speculated that HIV-1–infected macrophages would induce the proliferation of HIV-1–specific CD8⁺ T cells more effectively than HIV-1–infected CD4⁺ T cells. To test this possibility, we analyzed the capacities of HIV-1–infected macrophages and HIV-1–infected CD4⁺ T cells to induce the proliferation of HLA-B*5101-restricted HIV-1–specific CTL clones (Pol283-8-340, Gag327-9-148, Rev71-11-17, and HLA-mismatched CTL clones; Figure 5A). All 3 HLA-B*5101-restricted CTL clones cocultured with $JRFL_{NL\text{-}M20A\ Nef}$-infected or $JRFL_{NL\text{-}432\ Nef}$-infected macrophages or $JRFL_{NL\text{-}M20A\ Nef}$-infected CD4⁺ T cells showed significantly higher proliferation than those cocultured with uninfected macrophages or uninfected CD4⁺ T cells, respectively, whereas when the clones were cocultured with $JRFL_{NL\text{-}432}$–infected CD4⁺ T cells, only Pol283-8-40 showed significantly higher proliferation compared with that obtained with uninfected CD4⁺ T cells. The proliferation abilities of these CTL clones stimulated with $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages were significantly higher than those of the clones stimulated with $JRFL_{NL\text{-}M20A\ Nef}$-infected CD4⁺ T cells. Similar results were found for the proliferation of all 3 HLA-B*5101-restricted HIV-1–specific CTL clones stimulated with $JRFL_{NL\text{-}432\ Nef}$-infected macrophages or $JRFL_{NL\text{-}432\ Nef}$-infected CD4⁺ T cells. To confirm these

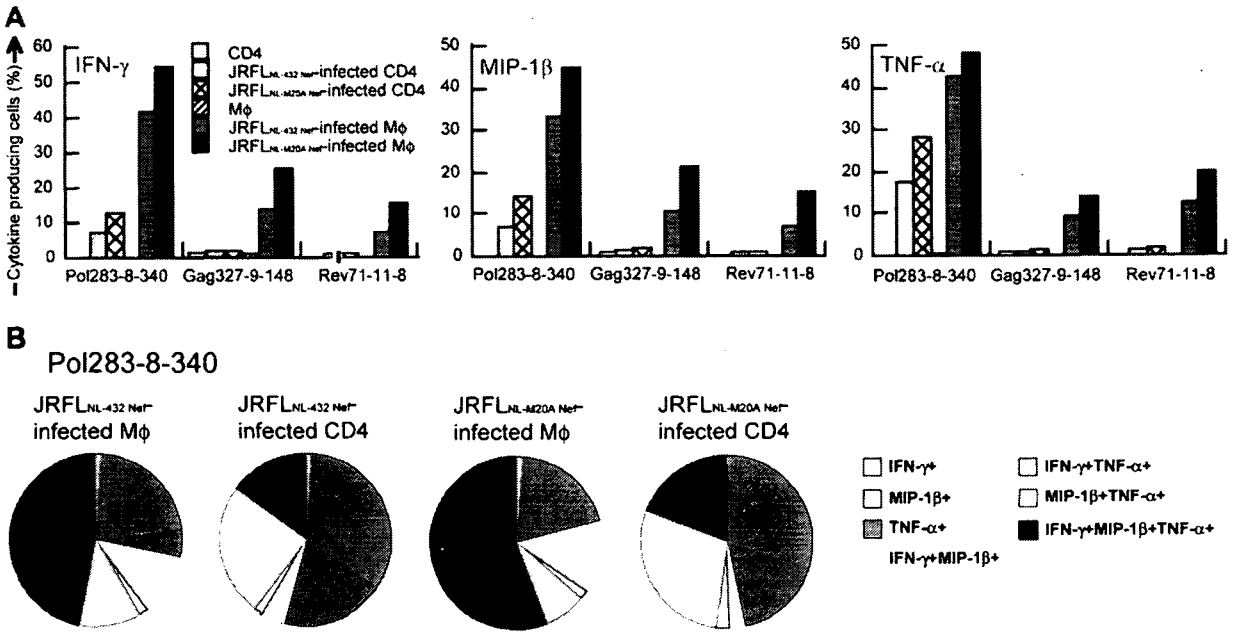

Figure 4. Ability of HIV-1–infected CD4⁺ T cells and HIV-1–infected macrophages to stimulate cytokine production by HIV-1–specific CTLs. Cultured CD4⁺ T cells and macrophages were infected with $JRFL_{NL\text{-}432\ Nef}$ or $JRFL_{NL\text{-}M20A\ Nef}$. $JRFL_{NL\text{-}432\ Nef}$-infected macrophages (22.7% p24 antigen-positive) and CD4⁺ T cells (19.9% p24 antigen-positive), as well as $JRFL_{NL\text{-}M20A\ Nef}$-infected macrophages (21.5% p24 antigen-positive) and CD4⁺ T cells (22.4% p24 antigen-positive) were used to stimulate 3 HLA-B*5101-restricted CTL clones at an effector-stimulator (E/S) ratio of 1:4. (A) The frequency of cells expressing each cytokine is shown as a percentage of the total number of CD8⁺ cells. (B) The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown in this pie chart.

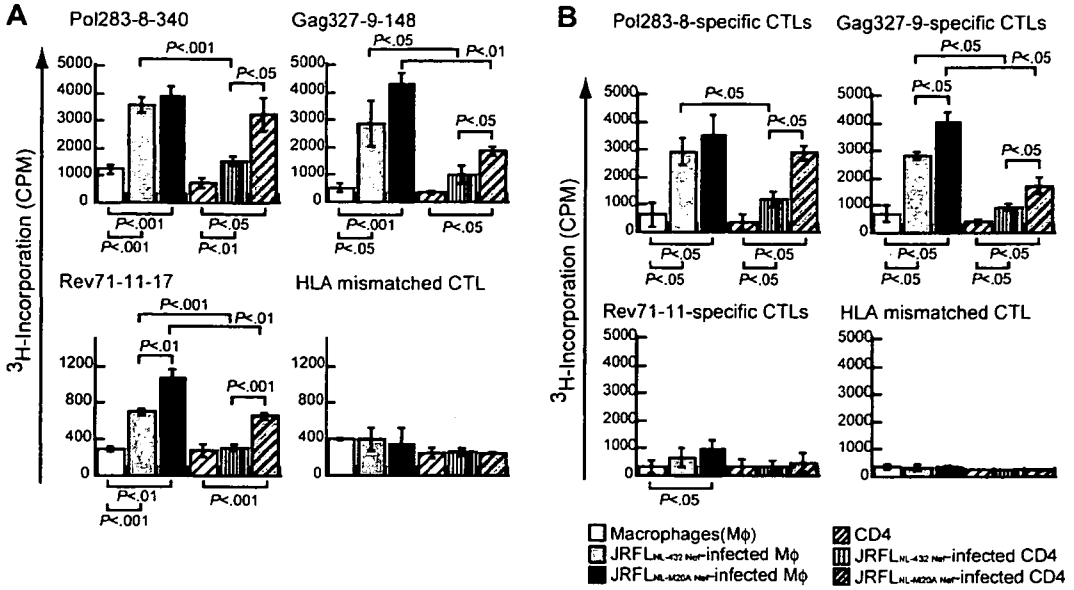

Figure 5. Ability of HIV-1–infected CD4$^+$ T cells and HIV-1–infected macrophages to induce proliferation of HIV-1–specific CTLs. Eleven HLA-B*5101-restricted CTL clones (Pol283-8-340, -320, -237, and -240; Gag327-9-148, -142, -287, and -131; Rev71-11-8, -55, and -17) were cocultured for 96 hours with uninfected macrophages, irradiated JRFL$_{NL\text{-}432\ Nef}$-infected macrophages (17.8% p24 antigen-positive), JRFL$_{NL\text{-}M20A\ Nef}$-infected macrophages (23.2% p24 antigen-positive), uninfected CD4$^+$ T cells, JRFL$_{NL\text{-}432\ Nef}$-infected CD4$^+$ T cells (20.8% p24 antigen-positive), or JRFL$_{NL\text{-}M20A\ Nef}$-infected CD4$^+$ T cells (24.8% p24 antigen-positive) at an E/S ratio of 1:4. The incorporation was measured after an additional 16-hour incubation. (A) Typical example of ^{3}H-incorporation in HLA-B*5101-restricted CTL clones (Pol283-8-340, Gag327-9-148, and Rev71-11-17), and HLA-mismatched CTL clone. Data shown in this figure are averages ± SD of triplicate assays. (B) Average ± SD of proliferation in triplicate assays for 4 Pol283-8–, 4 Gag327-9–, or 3 Rev71-11–specific CTL clones.

results, we measured the proliferation of other CTL clones with the same specificity (Figure 5B). A higher proliferation of the CTL clones stimulated with HIV-1–infected macrophages than with HIV-1–infected CD4$^+$ T cells was confirmed for Pol283-8– and Gag327-9–specific CTL clones but not for the Rev71-11–specific CTL clones. Furthermore, they showed a higher proliferation when they were stimulated with JRFL$_{NL\text{-}M20A\ Nef}$-infected cells than with JRFL$_{NL\text{-}432\ Nef}$-infected cells, but the influence of Nef-mediated down-regulation of HLA class I molecules was less crucial for the stimulation with HIV-1–infected macrophages than for that with HIV-1–infected CD4$^+$ T cells. These results strongly suggest that HIV-1–infected macrophages can much more effectively induce proliferation of HIV-1–specific CTLs than can HIV-1–infected CD4$^+$ T cells in vivo and support our idea that HIV-1–specific CTLs are strongly induced by HIV-1–infected macrophages in the acute and early chronic phases but that they are weakly induced in the late chronic phase, since the X4 virus predominantly replicates in this phase.

High expression of HIV-1 proteins in HIV-1–infected macrophages

We speculated that the difference in the suppressive effect of HIV-1–specific CTLs on JRFL replication between macrophages and CD4$^+$ T cells may have resulted from a difference in the amount of surface expression of HLA class I molecules between these HIV-1–infected cells. Flow cytometric analysis using mAb specific for HLA-B*5101 revealed that the surface expression of HLA-B*5101 on CD4$^+$ T cells was approximately 2-fold lower than that on macrophages (data not shown). To investigate the effect of this difference in surface expression of HLA-B*5101 on the recognition by HIV-1–specific CTLs, we measured the killing activity of 3 HLA-B*5101–restricted CTL clones toward HLA-B*5101$^+$CD4$^+$ T cells or macrophages prepulsed with the appropriate epitope peptides (Figure 6A). The ability of CTLs to kill the

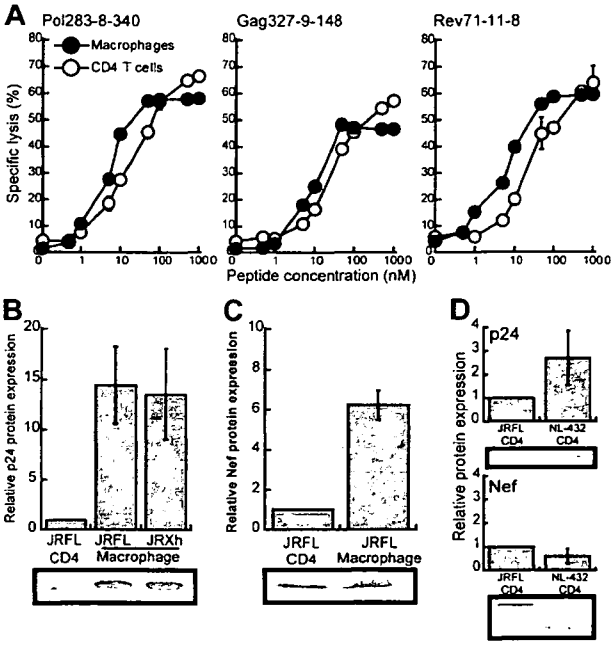

Figure 6. Different expression of HLA class I molecules and HIV-1 proteins between CD4$^+$ T cells and macrophages infected with HIV-1. (A) Comparison of the susceptibility between CD4$^+$ T cells and macrophages for cytotoxic activity of HIV-1–specific CTL clones. Cytotoxic activity of HLA-B*5101-restricted CTL clones was examined for CD4$^+$ T cells and macrophages prepulsed with each epitope peptide at an E/T ratio of 2:1. Data shown in the figure are averages of triplicate assays for each CTL clone. (B-C) The expression of p24 and Nef proteins in JRFL-infected CD4$^+$ T cells and macrophages. After p24$^+$ cells had become 20% to 30% of the total cell population, these cells were lysed. The cell lysates (6 μg) were analyzed by Western blotting with anti-p24 or anti-Nef mAb. Relative protein expression indicates the ratio of the amount of the p24 and Nef proteins in JRFL or JR-Xh–infected macrophages to that in JRFL-infected CD4$^+$ T cells per equal cell number. Data are shown as the average for 3 independent experiments. (D) The expression of p24 and Nef proteins in CD4$^+$ T cells infected with either NL-432 or JRFL. Data are shown as the average ± SD for 3 independent experiments.

peptide-pulsed macrophages (LL_{50}, peptide concentration providing a half of maximum percent specific lysis) was approximately 3-fold lower (2.67 ± 0.53) than that to kill the peptide-pulsed $CD4^+$ T cells. These results suggest that the difference in surface expression of HLA-B*5101 molecules between macrophages and $CD4^+$ T cells may partially influence the recognition of these cells by HIV-1–specific $CD8^+$ T cells. Another possibility is that HIV-1 antigens are much more expressed in HIV-1–infected macrophages than in HIV-1–infected $CD4^+$ T cells. To examine this possibility, we measured the amount of p24 and Nef proteins in HIV-1–infected macrophages and HIV-1–infected $CD4^+$ T cells. The amount of p24 was approximately 13-fold larger in either JRFL- or JR-Xh–infected macrophages than in JRFL-infected $CD4^+$ T cell (Figure 6B), and that of Nef protein was more than 7-fold larger in JRFL-infected macrophages than in JRFL-infected $CD4^+$ T cells (Figure 6C). There was no difference in the amount of p24 or Nef protein between NL-432–infected $CD4^+$ T cell and JRFL-infected $CD4^+$ T cells (Figure 6D). Such results indicate that HIV-1–infected macrophages can synthesize much more HIV-1 protein than HIV-1–infected $CD4^+$ T cells. Thus, it is likely that the difference in HIV-1 antigen presentation between the 2 cells resulted from the difference in the production of HIV-1 epitope peptide, because the difference in HIV-1 protein expression was much larger than that in HLA class I expression. These results suggest that HIV-1–infected macrophages can present a sufficient amount of peptide-MHC class I complexes for CTL recognition in spite of Nef-mediated down-regulation of HLA class I molecules.

Discussion

Previous studies showed that HIV-1–specific CTLs can kill HIV-1–infected alveolar macrophages derived from HIV-1–infected individuals but that they failed to kill HIV-1–infected $CD4^+$ T cells.[7,14,15] These results imply that HIV-1–infected macrophages can present HIV-1 antigens more effectively than HIV-1–infected $CD4^+$ T cells. Our previous studies using NL-432 X4 clone and NL-M20A lacking Nef function for HLA class I molecules showed that most HIV-1–infected CTLs failed to kill NL-432–infected $CD4^+$ T cells and partially suppressed NL-432 replication but that they could effectively kill NL-M20A–infected $CD4^+$ T cells and completely suppress NL-M20A replication,[8,9] indicating that Nef-mediated HLA class I down-regulation critically affects recognition of HIV-1–infected $CD4^+$ T cells by HIV-1–specific CTLs. These studies together suggest that the assay measuring the ability of HIV-1–specific CTLs to suppress HIV-1 replication is more sensitive than the cytotoxic assay and imply that the effect of Nef-mediated HLA class I down-regulation is much stronger on the recognition by HIV-1–specific CTLs of HIV-1–infected $CD4^+$ T cells than that of HIV-1–infected macrophages. In fact, we here demonstrated that HIV-1–specific CTLs much more strongly suppressed JRFL replication in the culture of HIV-1–specific CTLs with JRFL-infected macrophages than that in those of HIV-1–specific CTLs with JRFL-infected $CD4^+$ T cells. Thus, the present study indicates that Nef-mediated HLA class I down-regulation only partially affected recognition of HIV-1–infected macrophages by HIV-1–specific CTLs.

The difference in the suppressive effect of HIV-1–specific CTLs on HIV-1 replication between macrophages and $CD4^+$ T cells may be explained by several mechanisms such as differences of HLA class I surface expression and HIV-1 protein expression between macrophages and $CD4^+$ T cells. The present study demonstrated that the surface expression of HLA class I molecules on macrophages was approximately 2-fold higher than that on $CD4^+$ T cells and that this difference weakly influenced ability of HIV-1–specific CTL clones to kill these cells prepulsed with the epitope peptides. These results suggest that the difference in HLA class I surface expression between these 2 cells only partially influenced that in the suppressive effect of HIV-1–specific CTLs on HIV-1 replication. On the other hand, we demonstrated that HIV-1 antigens were much more expressed in HIV-1–infected macrophages than in HIV-1–infected $CD4^+$ T cells. Thus, it is likely that the difference in HIV-1 protein expression between the 2 cells resulted in the difference in HIV-1 antigen presentation since the difference in HIV-1 protein expression was much larger than that in HLA class I expression. Because macrophages are also known to carry costimulatory molecules and function as professional antigen-presenting cells, HIV-1–specific CTLs can effectively proliferate when stimulated by HIV-1–infected macrophages.

A previous study showed that most HIV-1–specific CTLs partially suppress NL-432 replication in NL-432–infected $CD4^+$ T cells,[8,9] whereas the present study exhibited that they also had similar ability to suppress JRFL replication in JRFL-infected $CD4^+$ T cells, indicating that HIV-1–specific CTLs fail to suppress HIV-1 replication in $CD4^+$ T cells in early and late phases of HIV-1 infection. In contrast, HIV-1–specific CTLs strongly suppressed HIV-1 replication in macrophages. These observations imply that HIV-1 replication is more controlled by the CTLs in the early phase than in the late stage.

It is well known that dendritic cells (DCs) play an important role in the transmission of HIV-1 to $CD4^+$ T cells and in antigen presentation.[23] DCs can present antigens to naïve T cells, whereas macrophages present antigens only to memory and effector T cells.[24-27] These findings suggest that DCs present HIV-1 antigens to naïve T cells, so that HIV-1–specific effector and memory T cells are induced in the early stage of an HIV-1 infection. On the other hand, HIV-1–infected macrophages may play a role in maintenance of HIV-1–specific memory and effector T cells, because macrophages can stimulate memory and effector T cells but not naïve T cells. In fact, the present study demonstrated that HIV-1–infected macrophages stimulated HIV-1–specific CTL clones much more strongly than did HIV-1–infected $CD4^+$ T cells, indicating that HIV-1–specific $CD8^+$ T cells are maintained in HIV-1–infected donors due to stimulation by HIV-1–infected macrophages but not due to that by HIV-1–infected $CD4^+$ T cells. In HIV-1–infected individuals, the number of DCs is decreased and their functional impairment is observed,[28,29] suggesting that HIV-1–specific memory and effector T cells may be maintained by antigen presentation by HIV-1–infected macrophages rather than by DCs. Since the X4 virus, which infects only $CD4^+$ T cells, dominantly appears in the late phase, the antigen presentation by HIV-1–infected macrophages would not be expected in this phase. In addition to the loss of HIV-1–specific helper T cells and DCs,[28-32] this may be one of the mechanisms that mediates the reduction in the number of HIV-1–specific T cells and failure of suppression of HIV-1 replication in the late phase.

In the present study, we demonstrated a strong HIV-1 antigen presentation by HIV-1–infected macrophages and less effect of Nef-mediated HLA class I down-regulation on the recognition of HIV-1–infected macrophages by HIV-1–specific $CD8^+$ T cells. HIV-1 R5 virus-infected macrophages could induce higher proliferation of HIV-1–specific CTLs. Antigen presentation by HIV-1–infected macrophages and DCs are major pathways for the induction of HIV-1–specific T cells in HIV-1–infected donors.

Because HIV-1–infected macrophages are frequently detected in various tissues,[33-36] they may be considered to be involved in the maintenance of HIV-1–specific acquired immunity in acute and early chronic phases of an HIV-1 infection. However, because HIV-1 expression depends on the activation statuses of the cells, it still remains unclear that HIV-1–infected macrophages can strongly express HIV-1 proteins and can strongly stimulate HIV-1–specific CTLs in vivo. Further studies of HIV-1–infected macrophages in vivo are necessary to clarify whether HIV-1–infected macrophages are strong professional antigen-presenting cells in vivo.

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Authorship

Contribution: M.F. performed experiments, analyzed data, and helped to write the manuscript; M.T. designed experiments and helped to write the manuscript.

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Altering Effects of Antigenic Variations in HIV-1 on Antiviral Effectiveness of HIV-Specific CTLs[1]

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

Virus-specific $CD8^+$ CTLs play a critical role in the control of persistent virus infections including those by HIV-1. However, recent studies show that HIV-specific CD8 T cell responses, measured by their ability to bind with peptide-HLA class I tetrameric complexes (HLA tetramers) or to secrete IFN-γ Ag specifically, are not correlated with the control of viremia in chronic HIV-1 infections (1, 2), suggesting a progressive functional defect in HIV-specific CTLs during a chronic infection that is not measurable by these assays. Accordingly, HIV-specific CD8 T cells in individuals with a primary infection and individuals with a long-term nonprogressive infection exhibit strong Ag-dependent ex vivo proliferative capacity, whereas those from patients with a progressive disease course lose such capacity (3–5). In addition, recent reports show that various degrees of impairment of the effector functions of virus-specific CD8 T cells are influenced by Ag persistence and Ag levels in mice and humans (5–8), suggesting that antiviral effectiveness of HIV-specific CD8 T cells can be impaired through repeated stimulation by the same cognate Ags. In contrast, it is reported that significant differences also exist in the effectiveness of HIV-specific CTLs among different specificities and restricting elements (9, 10), as well as among TCR clonotypes within the same specificity (11, 12). Therefore, different Ags or a set of amino acid substitutions within an Ag may be involved differently in the generation of the altered antiviral effectiveness of HIV-specific CTLs.

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

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

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Materials and Methods

Subjects

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

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

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

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

Construction of Nef-expressing target cells

For target cells endogenously expressing Nef-GFP fusion proteins, DNA fragments encoding the Nef protein (HIV-1 NL43) and GFP were cloned into plasmid pcDNA3.1 (Invitrogen Life Technologies). A mutation, Met^{20} to Ala, was introduced to abolish HLA class I down-regulation activity by Nef (20), and the Thr^{75} to Arg mutation was achieved by site-directed mutagenesis. The m^{7}GpppG-capped and poly(A)-tailed mRNAs were prepared in vitro by using a mMessage mMachine T7 Ultra kit (Ambion).

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

Generation of T cell clones

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

HLA stabilization assay

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

HLA tetramer analysis

The HLA-B*3501-tetramers in complex with a series of wild-type and variant peptides were prepared as previously described (18). Cryopreserved PBMCs of HIV-positive (2×10^6) or -negative donors (3×10^6) were stained with the tetramer at 37°C for 15 min followed by anti-CD8-PerCP (BD Biosciences) or anti-TCR Abs at 4°C for 15 min. The anti-Vδ1 mAb A13 was kindly provided by L. Moretta (University of Genova, Genova, Italy) and the other anti-TCR mAbs were purchased from Pierce Endogen and Beckman Coulter. They were then washed twice and analyzed by flow cytometry. Dead cells were gated out by 7-AAD staining as needed.

Cytotoxic assay

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

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

Intracellular cytokine staining assay

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

Ex vivo proliferation assay

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

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

Statistical analysis

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

Results

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

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

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

Table I. *Longitudinal analysis of autologous virus sequence in regions within or flanking HLA-B35-restricted CTL epitopes in patients with chronic HIV infection*

Patient Identification	HLA Type	Date	CD4 (mm^{-3})	Viral Load[a]	Antiviral Therapy[b]	Pol448[c]	Frequency	Env 77[c]	Frequency	Nef75[c]	Frequency
Pt-01	A2402/A2603, B3501/B4002	Oct 1995	47	ND	AZT, ddI	EVIPLTEEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
		Apr 1997	146	4.5	AZT, ddC, IDV	EVVPLTEEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
		Sep 1999	223	3.9	D4T, 3TC, RTV, SQV	EVVPLTEEAELEL	Direct	PTDPNPQEVVLVN	6/12	PVTPQVPLRPMTYKAAV	Direct
								PTDPNPQEVELTN	6/12		
Pt-03	A2402/A2601, B3501/B5101	May 1989	480	ND	IFN-α	EVIPLTEEAELEL	12/18	PTDPNPQEVVLVN	14/14	PVRPQVPLRPMTFKGAV	5/15
						EVVPLTEEAELEL	6/18			PVTPQVPLRPMTYKAAV	10/15
		Mar 1993	289	ND	AZT	EVIPLTEEAELEL	1/14	PTDPNPQEVVLVN	Direct	PVRPQVPLRPMTFKGAV	1/15
						EVVPLTEEAELEL	13/14			PVTPQVPLRPMTYKAAV	14/15
		May 1995	252	ND	AZT, ddI	EVVPLTEEAELEL	11/11	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	12/12
		Jul 1997	320	4.2	AZT, ddI, SQV	EVVPLTEEAELEL	12/12	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
		Jun 2002	203	3.1	ABC, TDF, LPV	EVVPLTEEAELEL	14/14	PTDPNPQEVVLVN	12/12	PVTPQVPLRPMTYKAAV	Direct
Pt-15	A11/A24, B35/B54	Jul 1996	193	3.3	3TC, d4T, NFV	EVVPLTAEAELEL	12/12	PTDPNPQEVVLVN	Direct	PVRPQVPLRPMTRRAAI	12/12
		Dec 1998	162	4.6	3TC, d4T, NFV	EVVPLTAEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	12/12
		Jun 2001	383	BL	ABC, EFV, LPV	EVVPLTAEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
Pt-19	A2402, B3501/B5201	Jun 1996	524	4.7	ND	EVIPLTEEAELEL	7/12	PTDPNPQEVVLVN	Direct	PVRPQVPLRPMTYKGAF	3/13
						EVVPLTEEAELEL	5/12			PVRPQVPLRPMTFKGAF	10/13
		Jul 1997	714	4.3	ND	EVVPLTEEAELEL	14/14	PTDPNPQEVVLVN	Direct	PVRPQVPLRPMTYKAAV	1/16
										PVRPQVPLRPMTFKGAF	5/16
										PVTPQVPLRPMTYKAAV	10/16
		Dec 1999	601	2.8	3TC, d4T, IDV	EVVPLTEEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	14/14
		May 2001	1574	BL	d4T, 3TC, IDV	EVVPLTEEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
Pt-34	A2402/A2601, B3501/B4801	May 1999	393	3.7	d4T, 3TC	EVIPLTEEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVRPQVPLRPMTFKGAV	4/12
										PVTPQVPLRPMTYKAAV	8/12
		Apr 2001	201	4.4	ND	EVVPLTAEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
Pt-42	A24/A31, B35/B60	Apr 1999	400	3.5	ND	EVVPLTAEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVRPQVPLRPMTFKGAV	2/10
										PVTPQVPLRPMTYKAAV	8/10
		Aug 2001	311	3.8	ND	EVVPLTAEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
Pt-46	A2, B35/B61	May 1999	231	BL	AZT, 3TC, IDV	EVVPLTEEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct
		Apr 2001	263	BL	d4T, 3TC, IDV, RTV	EVVPLTEEAELEL	Direct	PTDPNPQEVVLVN	Direct	PVTPQVPLRPMTYKAAV	Direct

[a] Viral load is log 10 copies/ml. BL, Below detection level; ND, not done.

[b] ABC, Abacavir; AZT, azidothymidine; ddI, dideoxyinosine; ddC, dideoxycytidine; d4T, didehydrothymidine; EFV, efavirenz; IDV, indinavir; LPV, lopinavir; RTV, ritonavir; SQV, saquinavir; 3TC, dideoxythiacytidine; TDF, tenofovir.

[c] Boldface letters in sequences are mutations. Epitope regions are underlined. Numbers in "Frequency" column indicate a clonal frequency of plasmid clones sequenced following the cloning of PCR-amplified DNA fragments into plasmid vectors. "Direct" indicates that the data were obtained by direct sequencing of PCR-amplified DNA fragments.

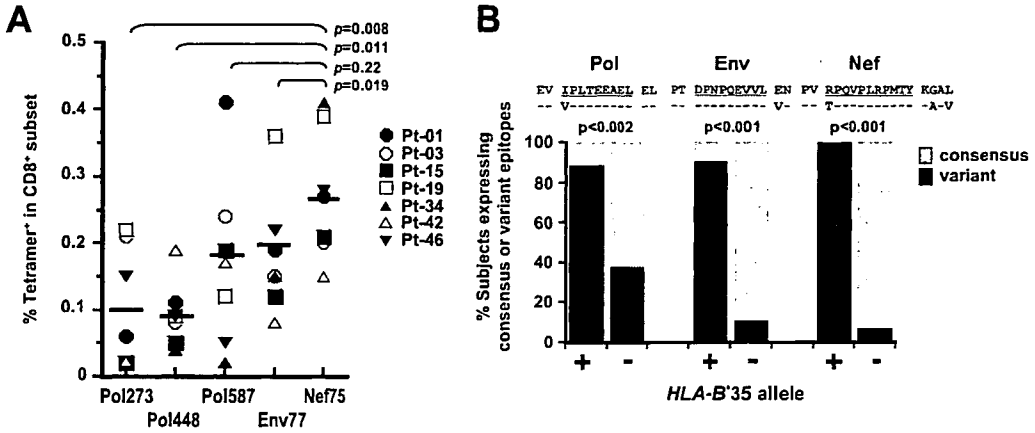

FIGURE 1. HLA-B*35-restricted CD8 T cell responses to HIV-1 and HIV-1 variants associated with *HLA-B*35* at the population level. *A*, Cryopreserved PBMC of seven HIV-infected patients were stained with HLA-B*35 tetramers in complex with the indicated peptides. The PBMC samples used for each patient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

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

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

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

Further cross-sectional analysis of the autologous virus sequence by using 42 chronic patients (12 HLA-B*35$^+$ and 30 HLA-B*35$^-$) clearly showed that the amino acid changes observed in Table I were all significantly associated with *HLA-B*35* (Fig. 1*B*), suggesting that these sequence variations were selected by CD8 T cell-mediated immune responses restricted by HLA-B*35.

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

Thr^{75} of Nef as a CTL escape mutation that abolished TCR recognition

By conducting an HLA stabilization assay, we first examined whether the variant peptide (Thr^{75} peptide: TPQVPLRPMTY) had lost its ability to bind with HLA-B*3501. Interestingly, the binding activity between HLA-B*3501 and the variant Thr^{75} peptide was ~10-fold higher than that of the wild-type peptide (Arg^{75} peptide: RPQVPLRPMTY), as the concentrations of the Arg^{75} and Thr^{75}

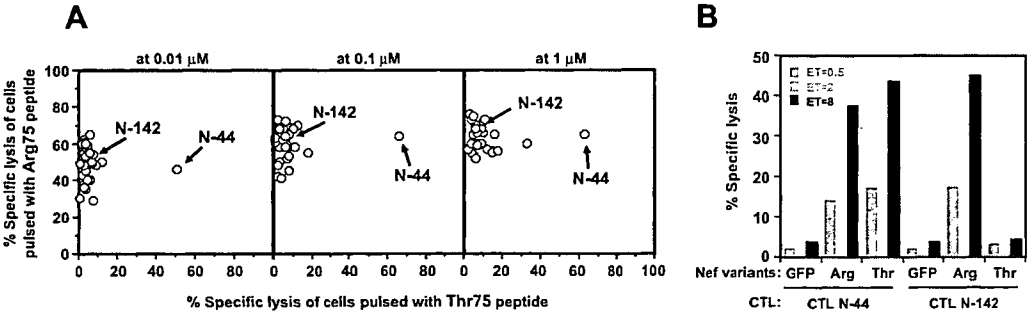

FIGURE 2. Generation of CTL clones specific for Nef and its variant in vitro. *A*, Cytotoxic activity of a representative set of 50 CTL clones toward C1R-B*3501 cells pulsed with the indicated concentrations (0.01, 0.1, and 1 µM) of the Arg^{75} or Thr^{75} peptide at an E:T ratio of 2:1. The data obtained for CTL N-44 and N-142 are indicated. Specific lysis in the absence of peptide was <5%. *B*, Cytotoxic activity of CTL clones CTL N-44 and N-142 toward C1R-B*3501 cells expressing the Arg^{75}-Nef-GFP protein, the Thr^{75}-Nef-GFP protein, or GFP alone. Of the target cells, 85 ± 5% were GFP$^+$.

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

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

peptides that yielded 50% of the maximum binding level were 56.2 ± 4.6 and 5.70 ± 0.27 μM, respectively.

We then sought to generate CTL clones specific for the Arg^{75} or Thr^{75} peptide in vitro. By a 1 μM Arg^{75} peptide stimulation of PBMC (Pt-01, Pt-03, and Pt-19), >50 CTL clones were generated. The cytotoxic activity of these CTL clones toward cells pulsed with the Arg^{75} or Thr^{75} peptide showed that all clones except one were exclusively specific for the Arg^{75} peptide (Fig. 2*A*). The $V\beta3^+$ T cells showed ~50% frequency among the Arg^{75}-specific CTL clones, and TCR usage of representative Arg^{75}-specific CTL clones (CTL N-27, N-117, and N-142) is shown in Table II. Remarkably, only one of >50 CTL clones (CTL N-44; Fig. 2*A*), which had $V\beta7^+$ TCR (Table II), showed cytotoxic activity toward cells pulsed with either Arg^{75} or Thr^{75} peptides, although an additional CTL clone showed partially cross-reactive capacity at the high concentrations of the Thr^{75} peptide (Fig. 2*A*). These data suggested an extremely low frequency of precursors of such cross-reactive CD8 T cells in these subjects (see below).

Next, we found that the cross-reactive CTL N-44 had comparable killing activity toward C1R-B*3501 cells expressing either the Arg^{75}- or Thr^{75}-Nef-GFP fusion protein (Fig. 2*B*), indicating that the Thr^{75} peptide was endogenously processed and extracellularly presented by HLA-B*3501 for CTL recognition. Moreover, a representative Arg^{75}-specific CTL clone, CTL N-142, showed killing activity toward cells expressing the Arg^{75}-GFP fusion protein but not toward those expressing the Thr^{75}-GFP one (Fig. 2*B*), indicating again that Thr^{75} was a CTL escape mutation that abolished TCR recognition.

It should be noted, however, that despite a number of attempts we failed to generate Thr^{75}-specific CTL clones or lines by stimulating the PBMCs of all seven patients with the Thr^{75} peptide although the samples were taken when their autologous HIV-1 had

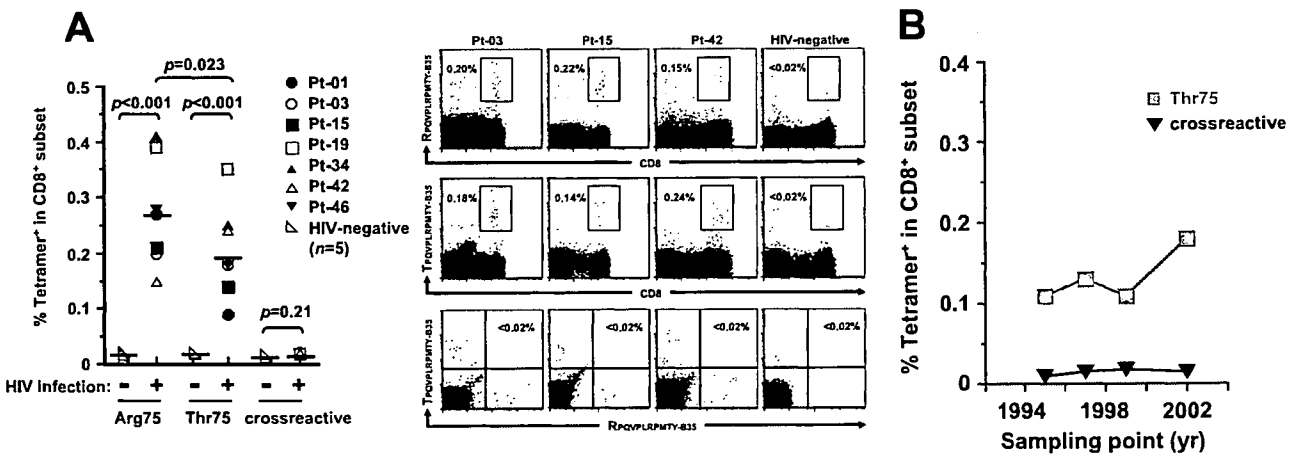

FIGURE 3. CD8 T cell responses toward Nef and its variant ex vivo. *A*, PBMCs of seven HIV-infected patients, the same as those used in Fig. 1*A*, and five HIV-negative donors were stained with PE and allophycocyanin-conjugated HLA-B*35 tetramers in complex with the Arg^{75} and Thr^{75} peptides, respectively. After the dead cells had been gated out by staining with 7-AAD, the remaining cells were analyzed for their binding to each of the tetramers and for their cross-reactivity. The frequency of $tetramer^+CD8^-$ in the total $CD8^+$ subset was plotted in the graph. Bars indicate the mean for each group. Representative dot plots for three HIV-infected subjects (Pt-03, Pt-15, and Pt-42) and a HIV-negative donor are shown with $tetramer^+$ frequency values in each dot plot. It should be noted that identical results were obtained when the fluorochromes of HLA tetramers were reversed. *B*, PBMCs of Pt-03 taken at several different time points were stained with HLA-B*35 tetramer in complex with the Arg^{75} (RPQVPLRPMTY) or Thr^{75} (TPQVPLRPMTY) Nef peptide and subsequently with anti-CD8 mAb and 7-AAD. The frequency of $tetramer^+CD8^+$ in the total $CD8^+$ subset was calculated and plotted in the graph.

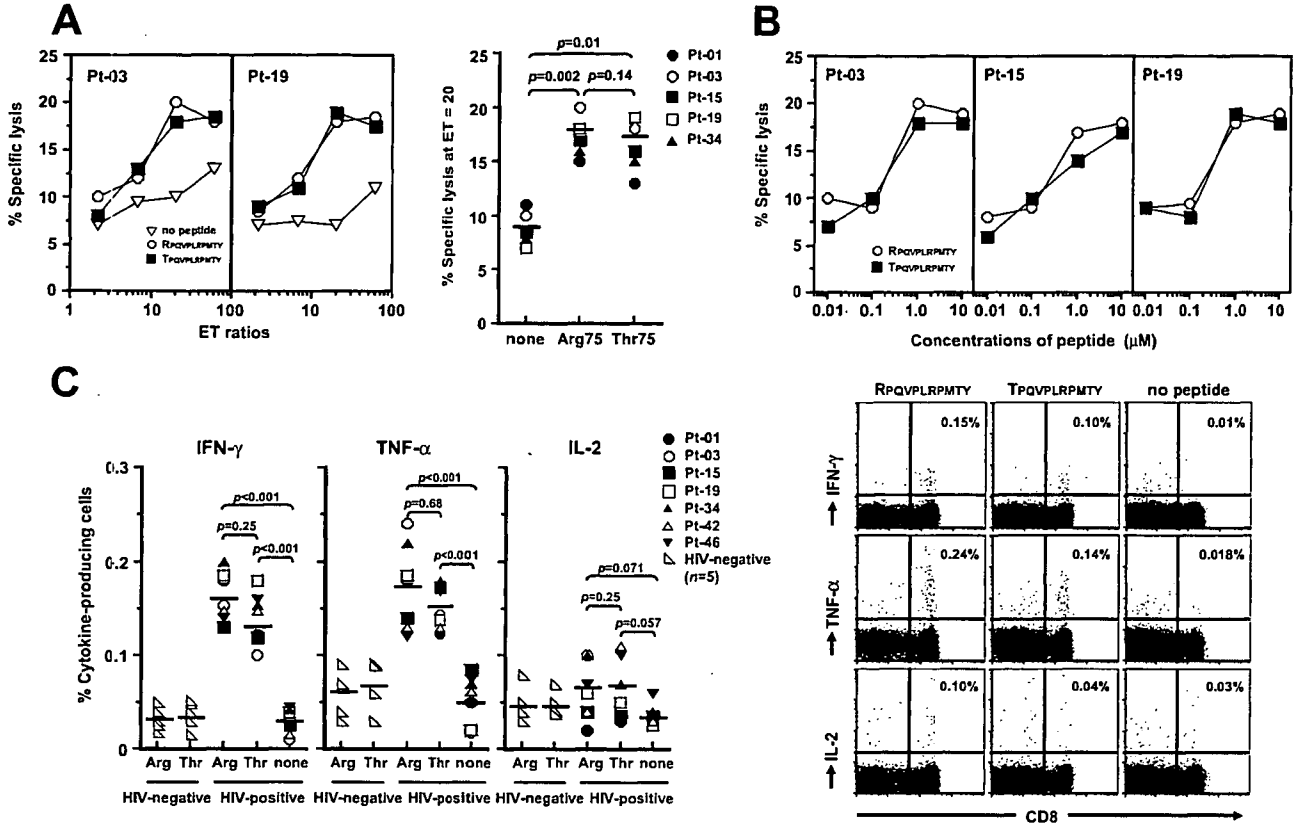

FIGURE 4. Antiviral effector functions of CD8 T cells specific for Nef epitopes. *A*, The cytotoxic activity of $CD8^+$ cells isolated from PBMC samples of HIV-positive donors (Pt-01, Pt-03, Pt-15, Pt-19, and Pt-34) was examined by ^{51}Cr-release assay directly ex vivo. The autologous target cells alone or pulsed with 1 μM Arg^{75} or Thr^{75} peptide were incubated with the effector $CD8^+$ cells at E:T ratios of 2.2, 6.7, 20, and 60 for 12 h. At the *left*, a representative set of data for Pt-03 and Pt-19 is shown. At the *right*, cytotoxic activity obtained at an E:T ratio of 20 is shown. Horizontal bars in the graphs indicate the mean for each group. It should be noted that the cytotoxic activity of $CD8^+$ cells was not examined for Pt-42 and Pt-46 because of the insufficient number of cells available for this assay. *B*, The cytotoxic activity of $CD8^+$ cells isolated from PBMC samples of HIV-positive donors (Pt-03, Pt-15, and Pt-19) was examined by the ^{51}Cr-release assay with the autologous target cells pulsed with the indicated concentrations of the Arg^{75} or Thr^{75} peptide at an E:T ratio of 20. Specific lysis in the absence of peptide is shown in *A*. *C*, PBMC samples, the same as those used in Fig. 1*A*, were stimulated or unstimulated with 1 μM Arg^{75} or Thr^{75} peptide for 6 h and then stained intracellularly with anti-IFN-γ, TNF-α, or IL-2 mAbs. The frequencies of the subsets exhibiting cytokine$^+CD8^+$ cells within the $CD8^+$ cell population are indicated in the graphs. Horizontal bars in the graphs indicate the mean for each group. At the *right* a representative set of dot plots for Pt-03 is shown with cytokine$^+$ frequency values at the *upper right corner* of each dot plot.

Thr^{75} (Table I), suggesting a lack of Thr^{75}-specific T cell precursors due to original antigenic sin or a lack of proliferation capacity in these subjects.

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

To see whether the Thr^{75} variant epitope was recognized by CD8 T cells, we obtained PBMC samples from seven chronically infected patients (Table I) after the variant viruses had become dominant and analyzed them ex vivo by using HLA tetramers. The frequencies of the RPQVPLRPMTY-B35 and TPQVPLRPMTY-B35 tetramer$^+$ subsets within the $CD8^+$ cells of the seven HIV-infected patients were significantly above the background level, being 0.261 ± 0.094 and 0.186 ± 0.081%, respectively; whereas those of five HIV-negative donors were 0.015 ± 0.0045 and 0.018 ± 0.009%, respectively (Fig. 3*A*). The frequencies of the cross-reactive fractions were <0.02% in all samples tested (Fig. 3*A*). These data indicate that the Thr^{75} and Arg^{75} peptides were immunogenic and exclusively recognized by a different subset of CD8 T cells widely in chronic HIV-infected patients having HLA-B*35.

Longitudinal analysis of Pt-03 PBMC showed that the CD8 T cells specific for the Arg^{75} peptide decreased in frequency and those specific for the Thr^{75} peptide increased (Fig. 3*B*). The increase in the frequency of Thr^{75}-specific CD8 T cells appeared to occur following the dominance of the Thr^{75} variant over the autologous virus (Table I). However, the question of whether Thr^{75}-specific CD8 T cells were absent before the autologous virus developed the Thr^{75} mutation could not be examined because PBMC samples during the primary infection were not available in this study. However, considering the following three points, namely that Thr^{75} (22 of 390; 5.6%) was rarely found in the Los Alamos database compared with Arg^{75} (302 of 390; 77.4%), the autologous virus from five of seven patients developed the Arg to Thr mutation during chronic infection (Table I), and cross-reactive CD8 T cell subsets were barely found in all subjects, it is most likely that Thr^{75}-specific CD8 T cells were newly generated in vivo in response to the Thr^{75} epitope after the autologous virus had undergone the Thr^{75} mutation.

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

We then examined the Arg^{75}- or Thr^{75}-specific CD8 T cells for their Ag-specific killing activity. Because the variant Thr^{75}-specific CTL lines or clones could not be established in vitro (Fig. 2*A*) although the Thr^{75}-specific CD8 T cells were found ex vivo by the

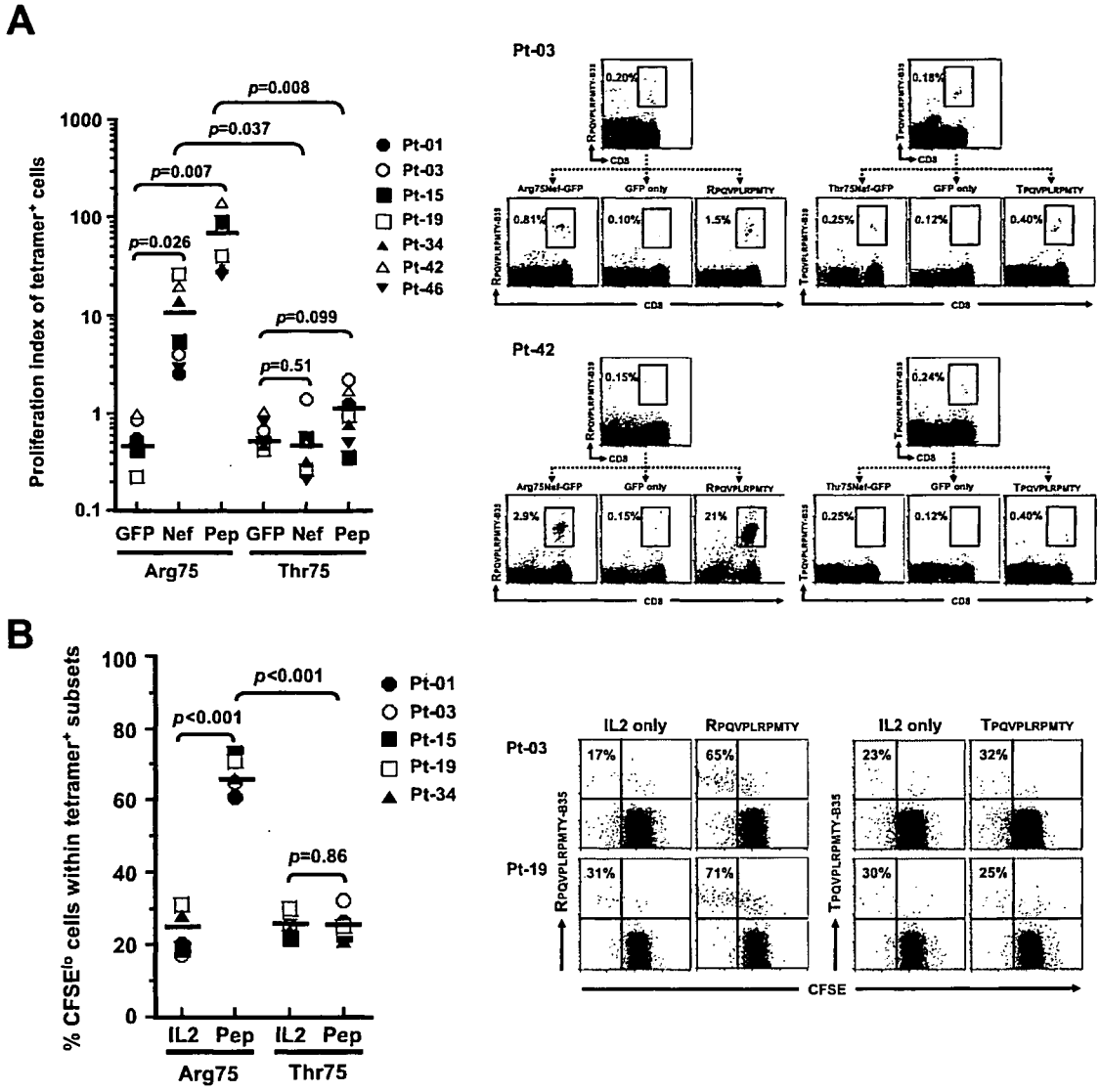

FIGURE 5. Proliferative capacity of CD8 T cells specific for Nef epitopes. *A*, The PBMC samples, the same as those used in Fig. 1*A*, were stimulated with irradiated C1R-B*3501 cells or autologous EBV-transformed B cells that had been pulsed with 1 μM Arg^{75} or Thr^{75} peptide or had been transduced with mRNA encoding GFP or Nef-GFP fusion proteins carrying Arg^{75} or Thr^{75}. Twelve days after the stimulation, the cells were analyzed with the HLA-B*35 tetramers. As shown in the graph at the *left*, the proliferation index was obtained as the ratio of $tetramer^+$ frequencies after and before stimulation. Horizontal bars in the graphs indicate the mean for each group. At the *right*, a representative set of dot plots for Pt-03 and Pt-42 is shown with $tetramer^+$ frequency values. *B*, The PBMC samples of HIV-positive donors (Pt-01, Pt-03, Pt-15, Pt-19, and Pt-34) were first labeled with CFSE and incubated in a medium containing human rIL-2 (100 U/ml). The cells were stimulated with IL-2 alone or in combination with 1 μM Arg^{75} or Thr^{75} peptide. After 6 days of culture, the cells were then stained with indicated HLA-tetramers and anti-CD8 and -CD3 Abs. The $CD3^+$ $CD8^+$ subsets were gated and analyzed for their fluorescence intensity of CFSE. The frequency of $CFSE^{low}$ cells within the $tetramer^+$ subset is shown in the graph at the *left*. Horizontal bars in the graph indicate the mean for each group. At the *right*, a representative set of dot plots for Pt-03 and Pt-19 is shown with $CFSE^{low}$ frequency values. It should be noted that CFSE dilution assay was not conducted for the subjects Pt-42 and Pt-46 because of the insufficient number of cells available for this assay.

tetramer analysis (Fig. 3*A*), we sought to analyze the Ag-specific cytolytic activity of CD8 T cells by a ^{51}Cr-release assay directly ex vivo. $CD8^+$ and $CD8^-$ cells were first isolated from the PBMCs of HIV-positive donors by a magnetic bead separation system and used as effector and target cells, respectively, for the cytolytic assay. The $CD8^+$ cells of the subjects tested showed cytotoxic to target cells pulsed with Arg^{75} and Thr^{75} peptides (1 μM) at an E:T ratio of 20 with specific lysis of 17.2 ± 1.9% and 16.2 ± 2.4%, respectively (Fig. 4*A*), whereas the background level of their specific lysis in the absence of the peptide was 8.9 ± 1.6% (Fig. 4*A*). As also shown in the representative data for Pt-03 and Pt-19 with various E:T ratios, the cytolytic activity of $CD8^+$ cells specific for the Arg^{75} and Thr^{75} peptides was not substantially different (Fig. 4*A*).

Peptide titration experiments were also performed using $CD8^+$ cells of Pt-03, Pt-15, and Pt-19, as these subjects showed relatively high cytolytic activity ex vivo (Fig. 4*A*). As shown in Fig. 4*B*, the cytolytic activity of $CD8^+$ cells toward cells pulsed with the Arg^{75} and Thr^{75} peptides was not much different in any range of the peptide concentration tested, suggesting the comparable functional avidity of both CD8 T cell subsets toward the given Ag.

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

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

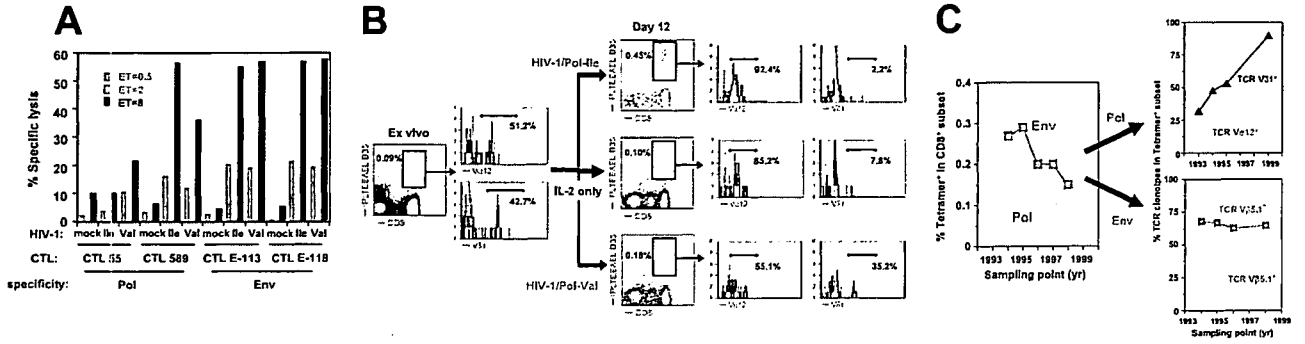

FIGURE 6. Functional analysis of CD8 T cells specific for Pol and Env epitopes. *A*, Cytotoxic activity of four different CTL clones, CTL 55, CTL 589, E-113, and E-118, toward autologous EBV-transformed B cells infected with mock or vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 expressing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

0.161 ± 0.025 and 0.171 ± 0.047%, respectively, in response to the Arg^{75} peptide and 0.140 ± 0.027 and 0.150 ± 0.023%, respectively, in response to the Thr^{75} peptide (Fig. 4*B*). In contrast, the IL-2 response was not significant, being 0.061 ± 0.031 and 0.063 ± 0.031% in response to the Arg^{75} and Thr^{75} peptides, respectively (Fig. 4*B*). Also, the extent of generation of cytokine-producing cells (IFN-γ and TNF-α) in response to Arg^{75} and Thr^{75} peptides was not significantly different (Fig. 4*B*), indicating that CD8 T cells specific for the Arg^{75} and Thr^{75} peptides were comparably functional in terms of Ag-specific cytokine production.

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

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

PBMC samples were mixed with autologous EBV-transformed B cell lines or C1R-B*3501 cells expressing GFP or Nef-GFP fusion proteins carrying Arg^{75} or Thr^{75} in the absence or presence of the synthetic Arg^{75} or Thr^{75} peptide, respectively, and then analyzed by using HLA tetramers on days 0, 6, and 12. Because continuous expansion was observed even between days 6 and 12 (data not shown), the proliferation index was calculated by dividing the frequency of $tetramer^+$ cells at day 12 after stimulation with a given Ag by the frequency of $tetramer^+$ cells ex vivo (Fig. 5*A*). We considered a proliferation index of <1.0 to indicate no proliferation capacity of the subset. All subjects tested showed significant expansion of Arg^{75}-specific CD8 T cells upon stimulation with Arg^{75}-Nef-GFP and the Arg^{75} peptide with an index value of 11.4 ± 7.11 and 62.4 ± 41.9, respectively, whereas they did not show Ag-specific expansion upon stimulation with GFP alone, Thr^{75}-Nef-GFP, or the Thr^{75} peptide because the index value was 0.635 ± 0.166, 0.554 ± 0.279 or 1.11 ± 0.66, respectively (Fig. 5*A*). Also, the proliferation index of untreated PBMC (IL-2 alone) was 0.742 ± 0.203 (data not shown).

To further confirm the difference in proliferative capacity between Arg^{75}- and Thr^{75}-specific CD8 T cells, we analyzed them by monitoring CFSE fluorescence of $tetramer^+$ cells upon stimulation with the cognate peptides for 6 days. Upon stimulation with IL-2 alone, the frequencies of proliferating cells as measured by $CFSE^{low}$ cells within the $tetramer^+$ cells were 23.0 ± 6.1 and 25.8 ± 3.2% for Arg^{75} and Thr^{75}-specific cells, respectively (Fig. 5*B*). Stimulation with the Arg^{75} peptide resulted in a significant increase of proliferating CD8 T cells specific for the Arg^{75} peptide, as the frequency of $CFSE^{low}$ cells within the $tetramer^+$ cells was 67.2 ± 4.8% (Fig. 5*B*). In contrast, the Thr^{75} peptide stimulation showed virtually no change in the frequency of $CFSE^{low}$ cells within the $tetramer^+$ cells (25.4 ± 4.1%) compared with stimulation with IL-2 alone (Fig. 5*B*). Thus, the proliferative capacity of CD8 T cell subsets obtained by a CFSE dilution assay (Fig. 5*B*) was in good agreement with the data obtained by quantification of the $tetramer^+$ cell expansion assay (Fig. 5*A*). These data indicate that CD8 T cells specific for the Arg^{75} peptide retained their Ag-dependent proliferative capacity in patients with chronic HIV-1 infection, whereas those specific for the variant Thr^{75} peptide did not have such capacity (Fig. 5, *A* and *B*) although both cells showed comparable Ag-specific cytolytic activity (Fig. 4*A*) and cytokine secretion activity (Fig. 4*B*). Also, this observation may explain the failure to generate CTL clones or lines specific for the Thr^{75} peptide in vitro (see above).

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

Next we examined CD8 T cell responses to Pol, Env, and their variant epitopes. The binding activity between HLA-B*3501 and the wild-type (Pol-Ile; IPLTEEAEL) or the variant epitope (Pol-Val; VPLTEEAEL) was comparable, and analysis of Pt-03 PBMC with tetramers in complex with either peptide showed that all $tetramer^+$ cells were cross-reactive for both tetramers (data not shown) and that they consisted of two different clonotypes, $V\delta1^+$ and $V\alpha12^+$ (Table II). The representative clones, which had been generated by stimulation with the Pol-Ile peptide, were CTL55 (21) and CTL589 (18), respectively. CTL589 was cytotoxic toward cells infected with HIV-1 expressing Pol-Ile or Pol-Val ($HIV\text{-}1_{Pol\text{-}Ile}$ and $HIV\text{-}1_{Pol\text{-}Val}$, respectively), whereas CTL55 killed $HIV\text{-}1_{Pol\text{-}Val}$-infected cells but not $HIV\text{-}1_{Pol\text{-}Ile}$-infected cells (Fig. 6*A*), indicating that CTL55 was exclusively specific for the variant Pol-Val epitope in terms of virus-infected cells as a target.

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

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

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

We then tested the Pol-specific CD8 T cells for their proliferation activity in response to HIV-$1_{Pol\text{-}Ile}$ and HIV-$1_{Pol\text{-}Val}$ ex vivo. Pt-03 PBMCs were cocultured with cells infected with mock (IL-2 alone), HIV-$1_{Pol\text{-}Ile}$, or HIV-$1_{Pol\text{-}Val}$ for 12 days and then stained with HLA-B35 tetramers. The proliferation index of the $V\alpha12^+$tetramer$^+$ subset was ~10, 2, and 1 when the cells were stimulated with HIV-$1_{Pol\text{-}Ile}$, HIV-$1_{Pol\text{-}Val}$, and IL-2 alone, respectively (Fig. 6*B*), indicating that the $V\alpha12^+$ CD8 T cells had potent proliferation activity in response to the Pol-Ile epitope. However, the proliferation index of the $V\delta1^+$ tetramer$^+$ subset was <1.5 in response to both viruses (Fig. 6*B*), indicating the impaired Ag-specific proliferation of this subset of CD8 T cells specific for the variant Pol-Val epitope. Thus, not only CD8 T cells specific for the variant Nef epitope but also those specific for the variant Pol epitope showed impaired Ag-specific proliferation activity ex vivo (Figs. 5, *A* and *B*, and 6*B*).

Finally, we longitudinally analyzed the changes in tetramer$^+$ frequencies specific for Pol and Env epitopes and their constituent T cell clonotypes of subject Pt-03. TCR clonotypes within the Pol-specific CD8$^+$ cell population showed an increase in the frequency of the functionally impaired $V\delta1^+$ subset over time (Fig. 6*C*), suggesting that the sum of antiviral effector functions of Pol-specific CD8 T cells had declined. The time course of this change seemed to have occurred after the appearance of the Ile to Val mutation in the autologous HIV-1 (Table I). In contrast, an analysis of the Env-specific CD8 T cell response revealed that the frequency of the $V\beta5(c)^+$ T cell clonotypes within the Env-specific tetramer$^+$ subset was virtually constant (Fig. 6*C*). These data suggest that T cell clonotypes within the same specificity had not basically changed over time in the absence of significant variations within the epitope, thus highlighting the substantial changes in specificity and clonotypes observed in CD8 T cells specific for Pol and Nef epitopes with mutations at TCR contact sites.

Discussion

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

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

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

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

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

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

It is of note that the frequency of the wild type Pol-specific CD8 T cell subsets was much reduced after the emergence of CTL escape variants in the Pol epitope, whereas the wild type Nef-specific ones persisted following CTL escape in the Nef epitope in our study. Because we obtained autologous virus sequence data from viral RNAs in plasma, it is conceivable that a small number of cells latently infected with the variant viruses yet having a wild-type Nef sequence remained in vivo and eventually reactivated the wild type Nef-specific CD8 T cell subsets. Alternatively, a transient reversion of variant viruses to the wild-type Nef sequence arose, although such wild-type viruses could be rapidly controlled by wild type-specific CD8 T cells to undetectable levels. In any event, these scenarios can be possible when a virus variant has replicative disadvantage over the wild-type virus in vivo. Interestingly, it is reported (32) that the Thr mutation in Nef (corresponding to Thr^{75} in this study although a different numbering system was used therein) resulted in much decreased capacity for supporting viral replication, although the replication-competent strain NL43 has Thr at the same position. Further analysis to clarify whether CTL-mediated selective pressure can modulate the pathogenic functions of Nef and lead to long-term favorable effects on HIV-infected individuals against disease progression would be intriguing.

Recent studies have demonstrated that mutational escape from HIV-specific CTL is caused by interference with the intracellular processing of virus-derived proteins (15, 16). We also found HLA-B*35-associated mutations flanking the epitopic regions in Env and Nef. However, Env-specific CTL clones showed comparable cytolytic activity toward cells expressing wild-type and mutant proteins (T. Ueno, unpublished observations), suggesting that altered Ag processing was not involved in this setting. Further studies are needed to determine whether and, if so, how antigenic variations causing altered Ag processing affect HLA-B*35-restricted CTL responses and the functional heterogeneity of HIV-specific CTLs in patients with a chronic infection.

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Disclosures

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A haplotype of the human CXCR1 gene protective against rapid disease progression in HIV-1$^+$ patients

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Chemokines and their receptors are key factors in the onset and progression of AIDS. Among them, accumulating evidence strongly indicates the involvement of IL-8 and its receptors, CXCR1 and CXCR2, in AIDS-related conditions. Through extensive investigation of genetic variations of the human *CXCR1–CXCR2* locus, we identified a haplotype of the CXCR1 gene (*CXCR1-Ha*) carrying two nonsynonymous single nucleotide polymorphisms, *CXCR1_300* (Met to Arg) in the N terminus extracellular domain and *CXCR1_142* (Arg to Cys) in the C terminus intracellular domain. Transfection experiments with *CXCR1* cDNAs corresponding to the *CXCR1-Ha* and the alternative *CXCR1-HA* haplotype showed reduced expression of CD4 and CXCR4 in *CXCR1-Ha* cells in human osteosarcoma cells as well as in Jurkat and CEM human T lymphocytes. Furthermore, the efficiency of X4-tropic HIV-1_{NL4-3} infection was significantly lower in *CXCR1-Ha* cells than in *CXCR1-HA* cells. The results were further confirmed by a series of experiments using six HIV-1 clinical isolates from AIDS patients. A genetic association study was performed by using an HIV-1$^+$ patient cohort consisting of two subpopulations of AIDS with extreme phenotypes of rapid and slow progression of the disease. The frequency of the *CXCR1-Ha* allele is markedly less frequent in patients with rapid disease onset than those with slow progression (P = 0.0003). These results provide strong evidence of a protective role of the *CXCR1-Ha* allele on disease progression in AIDS, probably acting through modulation of CD4 and CXCR4 expression.

AIDS | SNP | chemokine receptor | genotyping

A principal feature of AIDS is progressive depletion of CD4$^+$ cells, leading to multiple immune-related symptoms (1). Rates of CD4$^+$ depletion and subsequent disease progression are highly variable among HIV-1-seropositive individuals (2). A small portion of patients maintains the normal range of CD4$^+$ cell counts and is free of disease symptoms for many years, whereas some others have a contrasting host response characterized by rapid loss of CD4$^+$ and onset of symptoms (3). The role of the chemokine–chemokine receptor system has been extensively investigated after the discovery of the anti-HIV-1 activity of the CC-chemokines RANTES, MIP-1α, and MIP-1β (4) and the identification of CXCR4 and CCR5 as the major coreceptors of HIV-1 (5–7). A genetic variant of *CCR5* (*delta-32*) has been shown to associate with low risk of HIV-1 infection and slow disease progression (SP) in Caucasians (8–10). Although variants in other chemokine and receptor genes may also affect the disease progression, their association with HIV infection and disease progression have not been elucidated.

IL-8 is the best-characterized proinflammatory C-X-C chemokine (11). IL-8 activates and attracts neutrophils, T cells, and basophils and is believed to be a key mediator in inflammatory disorders (12–14). We could not find association of variants of *IL-8* with risk of HIV-1 seroconversion or disease progression (A.V., M.L., and F.M., unpublished work). However, dysregulation and elevated IL-8 production (15, 16) and reduced expression of IL-8 receptors, CXCR1 and CXCR2, were reported in HIV-1-infected patients (17). HIV-1 replication was shown to be up-regulated by IL-8 in macrophages and T lymphocytes, and inhibited by IL-8 antagonists and GRO-α (18). Reduced CXCR1 activity upon HIV-1 infection due to cross-receptor-mediated internalisation with the major coreceptors CCR5 and CXCR4 has been shown (19). These observations suggest that CXCR1 and CXCR2 could affect AIDS-related conditions.

Results

Identification and Characterization of the CXCR1 and CXCR2 Polymorphisms. *CXCR1* and *CXCR2* form a single locus spanning a region of ≈26 kb on chromosome 2q35. By sequencing we determined genetic polymorphisms in 471 French Caucasian volunteers [control (CTR) series] (see *Materials and Methods*). Among 93 polymorphisms identified, 21 had minor allele frequencies of >1% (Fig. 1 and Table 1). Two of these involved nonsynonymous amino acid substitutions: a change of methionine to arginine at position 31 in the N terminus extracellular domain of CXCR1 (*CXCR1_300*) and a change of arginine to cysteine at position 335 in the C terminus intracellular domain (*CXCR1_142*). Strong linkage disequilibrium (LD) was observed across *CXCR1-CXCR2*. The 21 frequent variants formed 10 haplotypes with estimated frequencies of >1%. In particular, the alleles at the two nonsynonymous variant sites in *CXCR1* exhibited complete LD on 942 chromosomes. Four other polymorphisms, *CXCR1_200*, *CXCR1_219*, *CXCR2_7222390* and *CXCR2_7222360*, were also in complete LD with these. The minor

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Abbreviations: CTR, control; HOS, human osteosarcoma; LD, linkage disequilibrium; N.S.I., nonsyncytia-inducing; RP, rapid disease progression; S.I., syncytia-inducing; SP, slow disease progression.

Data deposition: The data reported in this paper have been deposited in the National Center for Biotechnology Information dbSNP database (ID nos. ss69355493–ss69355497).

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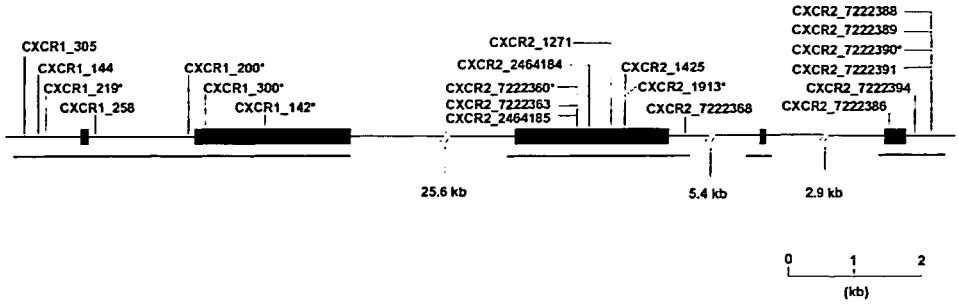

Fig. 1. Physical map of the human CXCR1 and CXCR2 genes. Coding and untranslated regions are indicated by black and gray bars, respectively. The location of polymorphisms with frequencies of >1% is indicated. The asterisk corresponds to polymorphisms that are in strong LD. A horizontal line below each gene shows the regions that have been sequenced.

alleles at these six sites constituted a single haplotype with an estimated frequency of 3.9% (*CXCR1–2_H3* in Table 2). Nearly complete LD was also observed with *CXCR2_1913*.

Reduction of Cell-Surface Expression of CD4 and CXCR4 in *CXCR1-Ha* Cells. To evaluate biological function of the two *CXCR1* nonsynonymous variants, we established transfectants of the *CXCR1-HA* and *CXCR1-Ha* haplotypes, in which HA and Ha incorporate the major and minor alleles, respectively, at both the *CXCR1_300* and the *CXCR1_142* sites (Table 2). Cell-surface expression of the HIV receptor CD4 and coreceptors CXCR4 and CCR5 was assessed on human osteosarcoma (HOS) cells after introduction of *CXCR1* variant cDNAs (see *Materials and Methods*). Flow cytometric analysis showed a reduction of cell-surface expression of CD4 and CXCR4 in *CXCR1-Ha* cells compared with *CXCR1-HA* cells (Fig. 2*A*). Western blotting and RT-PCR showed that the expression of CD4 protein and the amount of CD4 mRNA were markedly lower in *CXCR1-Ha* compared with *CXCR1-HA* cells, whereas CCR5 was unaffected (Fig. 2 *B* and *C*). Reduced expression of CXCR4 was also confirmed at a protein level by Western blotting, although no clear difference of mRNA amount was detected by RT-PCR. (Fig. 2 *B* and *C*). We then examined whether variant cDNAs also affects levels of endogenous CD4 and CXCR4 molecules by transfection experiments using Jurkat human T lymphocytes. Reduced expression of CD4 was observed in Jurkat *CXCR1-Ha* cells compared with Jurkat *CXCR1-HA* cells by flow cytometry, Western blotting and RT-PCR (Fig. 2 *D–F*). Endogenous CD4 and CXCR4 showed similar patterns in another T lymphocyte cell line, CEM cells, transfected with variant cDNAs (results not shown).

The cell-surface expression levels of *CXCR1-HA* and *CXCR1-Ha* were found to be similar (Fig. 3*A*). The intracellular [Ca^{2+}] mobilization, receptor endocytotic activity, and chemotactic activity were slightly reduced in *CXCR1-Ha* cells as compared with *CXCR1-HA* cells (Fig. 3 *B–D*). Biological consequences of these phenomena still remain to be investigated. Immunohistochemical analysis revealed that the distribution of CD4 at the plasma

Table 1. Summary of genetic polymorphisms in the human CXCR1 and CXCR2 genes

Polymorphisms						Frequencies A1			*P* values for statistical tests (when <0.01)		
Gene	CNG ID	Position	Location	A1	A2	CTR	SP	RP	CTR vs. SP vs. RP	RP vs. NP	dbSNP ID
CXCR1	305	−2668	Promoter	G	A	0.95	0.95	0.95	—	—	rs2671222
	144	−2423	Promoter	G	A	0.98	0.98	0.99	—	—	rs17838611
	219	−2329	Promoter	C	T	0.96	0.94	1.00	0.0008	0.0003	rs16858841
	258	−1566	Intron	C	G	0.94	0.95	0.95	—	—	rs3138060
	200	−143	Intron	C	T	0.96	0.94	1.00	0.0008	0.0003	rs16858816
	300	92	Exon (Met/Arg)	T	G	0.96	0.94	1.00	0.001	0.0003	rs16858811
	142	1003	Exon (Arg/Cys)	C	T	0.96	0.94	1.00	0.001	0.0002	rs16858808
CXCR2	7222388	−9203	Promoter	A	G	0.57	0.59	0.55	—	—	rs3890158
	7222389	−9191	Promoter	—	T	0.57	0.59	0.56	—	—	ss69355493
	7222390	−9185	Promoter	T	G	0.96	0.93	1.00	0.0004	0.0003	rs3890157
	7222391	−9179	Promoter	T	—	0.52	0.59	0.57	—	—	ss69355494
	7222394	−8909	Promoter	T	C	0.52	0.52	0.54	—	—	rs4674258
	7222386	−8490	Exon (5′-UTR)	A	G	0.57	0.59	0.55	—	—	rs4674259
	7222368	−270	Intron	G	A	0.53	0.54	0.54	—	—	ss69355495
	1913	768	Exon (Val/Val)	C	T	0.95	0.93	0.99	0.0015	0.0006	rs11574750
	1425	786	Exon (Leu/Leu)	C	T	0.52	0.51	0.51	—	—	rs2230054
	1271	936	Exon (Leu/Leu)	C	T	0.99	0.99	0.99	—	—	ss69355496
	2464184	1209	Exon (3′-UTR)	C	T	0.57	0.58	0.51	—	—	rs1126579
	7222360	1420	Exon (3′-UTR)	A	G	0.96	0.94	1.00	0.0004	0.0002	rs13306441
	7222363	1437	Exon (3′-UTR)	C	T	0.01	0.01	0.01	—	—	ss69355497
	2464185	1441	Exon (3′-UTR)	G	A	0.53	0.53	0.54	—	—	rs1126580

The position of each SNP was counted on the reference sequence (NT_005403.10) from the first nucleotide of the initiation codon as +1. A1 represents the nucleotide identical to that of the reference sequence. Single Nucleotide Polymorphism Database (dbSNP) IDs are also given for those that are already reported and registered on dpSNP (www.ncbi.nlm.nih.gov/SNP). *P* values for comparisons of the allele frequencies in the specified series are shown when <0.05. For these comparisons, adjustment for multiple testing (see *Methods*) gave $P < 0.01$ in all instances except for the CTR vs. SP vs. RP comparison of *SNP_7222390* in *CXCR2* ($P = 0.02$). CNG, Centre National de Génotypage.

Table 2. Haplotypes of *CXCR1* and *CXCR2* sites that are associated with disease progression

	Polymorphisms								Haplotype distribution						*P* value	
									CTR		SP		RP		CTR vs. SP vs. RP	SP vs. RP
Haplotypes	219	200	300	142	7222390	7222391	1913	7222360	Counts	Freq	Counts	Freq	Counts	Freq	Corrected	
CXCR1_HA	C	C	T	C	—	—	—	—	903/942	0.958	496/526	0.943	172/172	1.000	0.00083	0.00029
CXCR1_Ha	T	T	G	T	—	—	—	—	39/942	0.041	30/526	0.057	0/172	0.000	0.00083	0.00029
CXCR2_H1	—	—	—	—	T	—	C	A	446/940	0.474	271/524	0.516	96/170	0.565	0.053	0.290
CXCR2_H2	—	—	—	—	T	T	C	A	445/940	0.474	217/524	0.415	73/170	0.429	0.077	0.721
CXCR2_H3	—	—	—	—	G	—	T	G	39/940	0.041	31/524	0.059	0/170	0.000	0.00064	0.00018
CXCR2_H4	—	—	—	—	T	—	T	A	10/940	0.010	5/524	0.009	1/170	0.006	0.923	1.000
CXCR1–2_H1	C	C	T	C	T	—	C	A	447/938	0.474	272/522	0.517	97/172	0.564	0.054	0.334
CXCR1–2_H2	C	C	T	C	T	T	C	A	444/938	0.472	218/522	0.415	74/172	0.430	0.096	0.790
CXCR1–2_H3	T	T	G	T	G	—	T	G	37/938	0.039	29/522	0.055	0/172	0.000	0.00108	0.00029
CXCR1–2_H4	C	C	T	C	T	—	T	A	10/938	0.010	3/522	0.006	1/172	0.006	0.839	1.000

Freq, frequency.

membrane coincided with *CXCR1-HA*, but not with *CXCR1-Ha* (Fig. 3*E*).

We then examined whether the *CXCR1-Ha* haplotype correlates lower CD4 expression under physiological conditions. Of ≈2,800 healthy Thai volunteers, we chose 8 subjects heterozygous for the *CXCR1* allele (*CXCR1-HA/Ha*) and 11 wild-type homozygotes (*CXCR1-HA/HA*). Because of a low frequency (5.3%) of the *CXCR1-Ha* allele in Thai population, we couldn't include individuals homozygous for the *CXCR1-Ha* allele. Expression of the cell surface CD4 was performed by using flow cytometry by measuring the mean fluorescence intensity (MFI) of $CXCR1^+/CD4^+$ fraction of peripheral blood leukocytes. Although a tendency of lower CD4 expression levels was observed in individuals carrying *CXCR1-HA/Ha* (MFI: 224.57 ± 41.13) than in those with *CXCR1-HA/HA* (MFI: 234.82 ± 29.53), the difference was not statistically significant, probably due to a limited number of individuals examined.

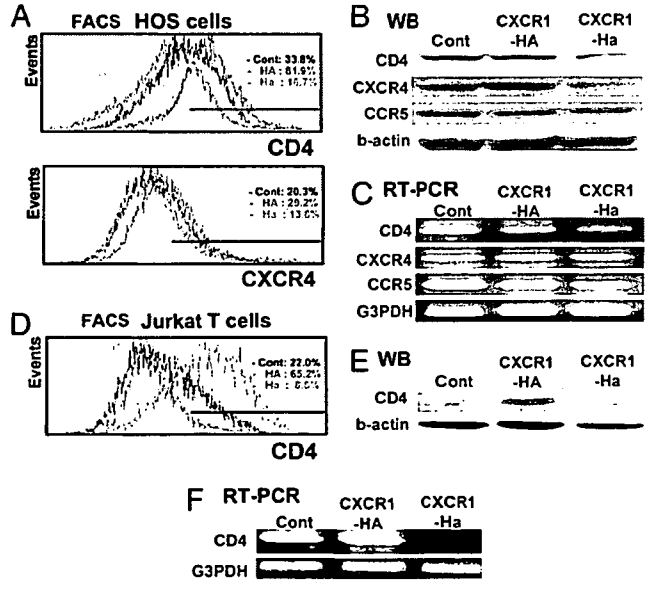

Fig. 2. Expression of HIV receptor/coreceptor on HOS cells (*A–C*) or Jurkat cells (*D–F*). (*A*) Flow cytometric analysis of HIV receptor CD4 and coreceptor CXCR4. (*B* and *C*) Western blot analysis (*B*) and RT-PCR analysis (*C*) of HIV receptor CD4, and coreceptors CXCR4 and CCR5. (*D*) Flow cytometric analysis of HIV receptor CD4. (*E* and *F*) Western blot analysis (*E*) and RT-PCR analysis (*F*) of HIV receptor CD4. A representative result from three independent experiments is shown.

Future examinations employing individuals who are homozygous for the *CXCR1-Ha* allele, a rare subset of the population, will provide a conclusive answer to this issue.

***CXCR1-Ha* Has an Inhibitory Effect on HIV Infection.** It is of interest to examine whether CXCR1 variability influences the efficiency of HIV-1 infection *in vitro*. We first analyzed the infection efficiency of X4-tropic HIV-1_{NL4-3} strain to HOS *CXCR1-HA* and *CXCR1-Ha* transfectants by using HIV p24 expression as a marker. After HIV-1_{NL4-3} exposure, *CXCR1-Ha* cells showed lower HIV p24 expression compared with control cells (transfected only with empty vector), whereas *CXCR1-HA* cells exhibited enhanced p24 levels (Fig. 4*A*). There was no significant difference in p24 levels between *CXCR1-Ha* and *CXCR1-HA* cells when R5-tropic HIV was used (data not shown). These results were further confirmed by a series of experiments with HIV-1 clinical isolates obtained from AIDS patients with or without syncytia formation in MT-2 cells (termed S.I. or N.S.I., respectively). *CXCR1-Ha* transfectants showed significantly lower p24 expression compared with *CXCR1-HA* transfectants in all of the isolates (Fig. 4), demonstrating an inhibitory effect of the Ha-variant allele on HIV-1 infection. Interestingly, p24 levels in *CXCR1-HA* cells were consistently similar to control cells in the experiments with HIV-1 isolates from patients without syncytia formation (Fig. 4*C*), whereas they were consistently increased in HIV-1 isolates from patients with syncytia (Fig. 4*B*) and X4-tropic HIV-1_{NL4-3} strain (Fig. 4*A*). This finding may reflect the content of X4-tropic HIV-1 in isolates; S.I. isolates are from patients in later stages of disease and thus richer in X4-tropic HIV-1. Increased cell-surface CXCR4 expression in *CXCR1-HA* cells may result in higher p24 levels. When using N.S.I. isolates with a lesser content of X4-tropic HIV-1, the difference of p24 expression became smaller between control cells and *CXCR1-HA* cells. The possibility of the involvement of R5-tropic HIV-1 in reduction of infection efficiency in *CXCR1-Ha* cells is also conceivable. In all instances, *CXCR1-Ha* cells had lower p24 expression than control cells.

Predominant Role of *CXCR1_142* Over *CXCR1_300*. Additional transfection experiments were undertaken with HOS cells by using artificial constructs of *CXCR1* cDNA that carry a single variation, either at *CXCR1_142* or *CXCR1_300*. These experiments allowed us to map the effect on CD4 expression on the cell surface to the *CXCR1_142* site and exclude a major effect of *CXCR1_300* (results not shown). Other data also support a predominant role for *CXCR1_142* over *CXCR1_300*. The minor allele at *CXCR1_142* introduces a cysteine residue in the C terminus intracellular domain, which is a target of palmitoylation in most chemokine receptors. Palmitoylation influences receptor trafficking and signal