$CD27^{low}CD28^{-}CD45RA^{+/-}$ phenotypes (Fig 2*A*). Approximately 60% of the $CD27^{-}CD28^{-}CD45RA^{+/-}$ cells expressed CXCR1, whereas only 20% of $CD27^{low}CD28^{-}CD45RA^{+/-}$ cells expressed this receptor (Fig. 2*B*). Because these subsets have cytotoxic activity (4, 6), these results suggest that CXCR1 is expressed only on the subsets with cytolytic effector function.

Because $CD27^{-}CD28^{-}CD45RA^{+/-}$ subsets express a much higher level of perforin than other CD27CD28CD45RA subsets (4), we suspected that CXCR1 expression would be positively correlated with perforin expression in $CD8^{+}$ T cells. Therefore, we analyzed the correlation between CXCR1 and perforin expression on/in $CD8^{+}$ T cells from five healthy individuals. Almost all $CXCR1^{+}$ cells were perforin-positive cells, although perforin-positive cells included both $CXCR1^{+}$ and $CXCR1^{-}$ cells (Fig. 3*A*). To analyze semiquantitatively the coexpression of CXCR1 and perforin in $CD8^{+}$ T cells, we divided the perforin-positive population into 4–8 fractions according to the level of perforin expression. The frequency of $CXCR1^{+}$ cells (Fig. 3*B*) and expression level of CXCR1 (data not shown) were positively related to perforin expression, demonstrating that CXCR1 expression is strongly associated with perforin expression in $CD8^{+}$ T cells. These findings support the idea that CXCR1 is a marker for cytolytic effector $CD8^{+}$ T cells.

Because CCR5 is expressed on memory and effector $CD8^{+}$ T cells (16, 17), it may be assumed that $CXCR1^{+}CCR5^{+}$ cells would exist among $CD8^{+}$ T cells. In fact, $CXCR1^{+}CCR5^{+}$ cells were found mostly in $CD27^{-}CD28^{-}CD45RA^{+/-}$ subsets (data not shown). These $CXCR1^{+}CCR5^{+}$ cells expressed lower levels of CXCR1 and CCR5 than the $CXCR1^{+}CCR5^{-}$ cells or $CXCR1^{-}CCR5^{+}$ cells, suggesting that $CXCR1^{+}CCR5^{+}$ cells are an intermediate type between the $CXCR1^{+}CCR5^{-}$ cells and $CXCR1^{-}CCR5^{+}$ cells in these effector subsets.

HCMV-specific $CD8^{+}$ T cells have the $CD27^{-}CD28^{-}CD45RA^{+/-}$ effector phenotype or $CD27^{low}CD28^{-}CD45RA^{+/-}$ memory/effector phenotype and the ability to kill target cells (4), whereas EBV-specific $CD8^{+}$ T cells have the $CD27^{+}CD28^{+}CD45RA^{-}$ memory phenotype and fail to kill target cells (6), implying that HCMV-specific $CD8^{+}$ T cells would express CXCR1 and EBV-specific ones would not. Therefore, we investigated the expression of CXCR1 on HCMV-specific and EBV-specific $CD8^{+}$ T cells. PBMCs from individuals U14 and U17 were stained with the HLA-A*0201/

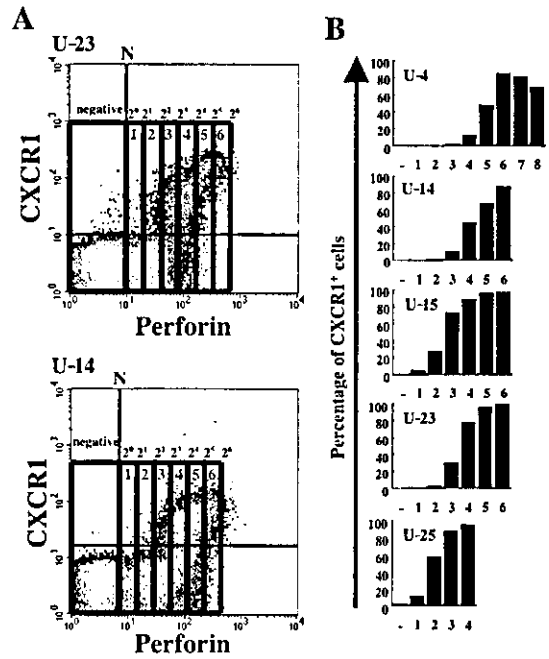

FIGURE 3. Coexpression of CXCR1 and perforin in $CD8^{+}$ T cells. PBMC from five individuals were stained with anti-CD8, anti-perforin, and anti-CXCR1 or mouse IgG1 mAb. The $CD8^{+}$ subset was gated and then analyzed for CXCR1 and perforin expression. N, Mean fluorescence intensity (MFI) between perforin-positive and perforin-negative populations. The perforin-positive population was further divided according to the difference in MFI level as follows: fraction 1, MFI of $N \times 2^0$ to $N \times 2^1$; fraction 2, MFI of $N \times 2^1$ to $N \times 2^2$; fraction 3, MFI of $N \times 2^2$ to $N \times 2^3$; fraction 4, MFI of $N \times 2^3$ to $N \times 2^4$; fraction 5, MFI of $N \times 2^4$ to $N \times 2^5$; fraction 6, MFI of $N \times 2^5$ to $N \times 2^6$; fraction 7, MFI of $N \times 2^6$ to $N \times 2^7$; and fraction 8, MFI of $N \times 2^7$ to $N \times 2^8$. *A*, Coexpression of CXCR1 and perforin in $CD8^{+}$ T cells from individuals U23 and U14. *B*, Percentage of CXCR1-positive cells in the perforin-negative population and in each fraction of the perforin-positive population from five individuals.

HCMV and the HLA-A*1101/EBV tetramers, respectively, together with anti-CD8 and anti-CXCR1 mAb. HCMV-specific $CD8^{+}$ T cells expressed CXCR1, whereas very few EBV-specific $CD8^{+}$ T cells expressed this receptor (Fig. 4*A*). Because HCMV-specific and EBV-specific $CD8^{+}$ T cells expressed effector and memory phenotypes, respectively (Fig. 4*A*), the results indicate that viral epitope-specific $CD8^{+}$ T cells

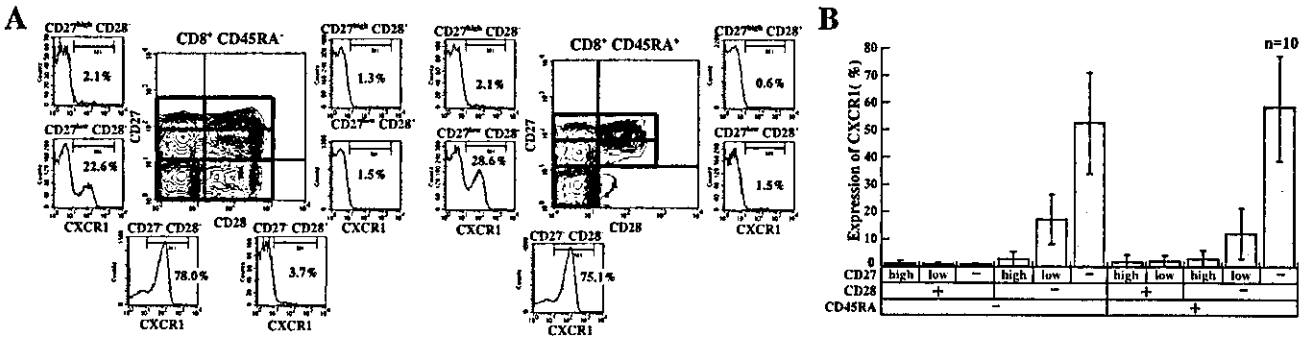

FIGURE 2. Surface expression of CXCR1 on $CD8^{+}$ T cells with effector and memory/effector phenotypes. *A*, Frequency of $CXCR1^{+}$ cells in each CD27CD28CD45RA subset of $CD8^{+}$ T cells. $CD8^{+}$ T cells were isolated from a given individual (U25) and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR1 mAbs. Each CD27CD28CD45RA subset was gated and then analyzed for CXCR1 expression. The percentage of $CXCR1^{+}$ cells in each subset is shown in each plot. *B*, Frequency of $CXCR1^{+}$ cells in each CD27CD28CD45RA subset of $CD8^{+}$ T cells from 10 individuals. The mean percentage and SD of $CXCR1^{+}$ cells in each subset are indicated.

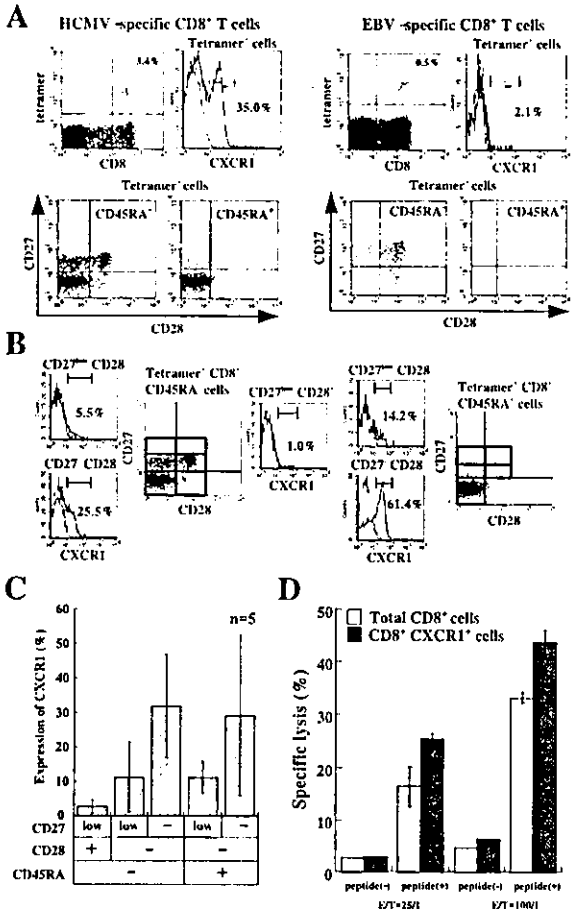

FIGURE 4. Surface expression of CXCR1 on HCMV-specific and EBV-specific $CD8^+$ T cells. *A*, Surface expression of CXCR1 on HCMV-specific and EBV-specific $CD8^+$ T cells. PBMCs from HLA-A*0201$^+$ individual (U14) and HLA-A*1101$^+$ individual (U17) were stained with the HLA-A*0201 tetramer and HLA-A*1101 tetramer, respectively, as well as with anti-CD8 and anti-CXCR1 mAbs. $CD8^+$tetramer$^+$ cells were gated and then analyzed for the expression of CXCR1. $CD8^+$ T cells from the same individual were also stained with anti-CD27, anti-CD28, and anti-CD45RA mAbs as well as with the HLA-A*0201 tetramer or the HLA-A*1101 tetramer. The expression of CD27, CD28, and CD45RA on tetramer$^+$ cells is shown. *B*, Surface expression of CXCR1 on different CD27CD28CD45RA subsets of HCMV-specific $CD8^+$ T cells. $CD8^+$ T cells were isolated from individual U14 and then stained with anti-CD27, anti-CD28, anti-CD45RA, and anti-CXCR1 mAbs, as well as with the HLA-A*0201 tetramer. Each CD27CD28CD45RA subset of tetramer$^+$ cells was gated and then analyzed for CXCR1 expression. The percentage of CXCR1$^+$ cells in each subset is indicated on each plot. *C*, Frequency of CXCR1$^+$ cells in different CD27CD28CD45RA subsets of HCMV-specific $CD8^+$ T cells from 5 HLA-A*0201/-A*0206$^+$ individuals. The mean percentage and SD of CXCR1$^+$ cells in each subset are shown. *D*, Enriching HCMV-specific CTL by sorting CXCR1$^+$$CD8^+$ T cells. Cytotoxic activity of sorted CXCR1$^+$$CD8^+$ T cells (96.6% purity) and total $CD8^+$ T cells for C1R-A*0201 cells prepulsed with HCMV-1 $pp65_{495-503}$ peptide were measured at E:T ratios of 25:1 and 100:1.

with the effector phenotype expressed CXCR1. Indeed, six-color flow cytometric analysis showed that ~30% of HCMV-specific $CD8^+$ T cells with the $CD27^-CD28^-CD45RA^{+/-}$ phenotype expressed CXCR1, but that only 10% of those with the $CD27^{low}CD28^-CD45RA^{+/-}$ phenotypes expressed it (Fig. 4, *B* and *C*). The ability of sorted CXCR1$^+$$CD8^+$ T cells to kill HCMV epitope peptide-pulsed cells was ~2-fold higher than that of total $CD8^+$ T cells (Fig. 4*D*), suggesting that a considerable number of HCMV-specific $CD8^+$ T cells with cytotoxic activity expressed CXCR1.

To determine whether chemotaxis of CXCR1$^+$$CD8^+$ T cells is induced by IL-8, we examined the in vitro chemotaxic activity of $CD8^+$ T cells purified from a healthy individual. The chemotaxic activity of the cells represents as IL-8 induced migratory cells (percentage of migrated cells in absence of IL-8 was subtracted from that in presence of IL-8). Migration of purified $CD8^+$ T cells was dose-dependently induced by IL-8 with 11.4% of these cells being CXCR1$^+$. In contrast, chemotaxis of CXCR1$^-$$CD8^+$ T cells, which were sorted by a cell sorter, was not induced by IL-8 (Fig. 5). Taken together, these results indicate that chemotaxis of CXCR1$^+$$CD8^+$ T cells is induced by IL-8. These results were confirmed in an experiment using a different individual (data not shown). Thus, CXCR1 on $CD8^+$ T cells has a definite biological function.

There are some differences in the ligand specificity between the two IL-8 receptors. CXCR1 binds IL-8 and granulocyte chemotactic protein (GCP)-2 with high affinity, and growth-related oncogene and neutrophil-activating peptide-2 with low affinity, whereas CXCR2 binds IL-8, GCP-2, growth-related oncogene, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 with high affinity (16). Thus, CXCR1 is the more specific receptor for IL-8. These findings suggest that $CD8^+$ T cells can respond only to IL-8 and GCP-2 in vivo, implying that effector $CD8^+$ T cells may be specifically attracted to inflammatory sites where IL-8 is produced.

Recent studies in mice showed that leukotriene B_4, which is produced from mast cells, is involved in the homing of effector $CD4^+$ and $CD8^+$ T cells, which express BLT1, a receptor for leukotriene B_4 (17–19), thus suggesting a crucial role for the leukotriene B_4-BLT1 pathway in allergic inflammation. Our present data also imply a role for the IL-8-CXCR1 pathway in the homing of effector $CD8^+$ T cells in various kinds of inflammation. Further studies on effector $CD8^+$ T cell homing via IL-8-CXCR1 pathway may be expected to clarify in the role of this pathway in various infectious diseases.

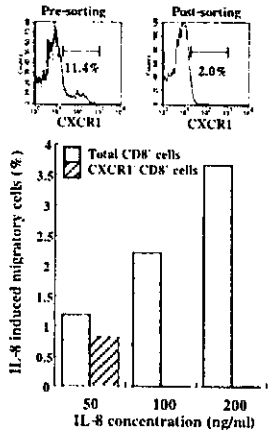

FIGURE 5. Induction of CXCR1$^+$$CD8^+$ T cell migration by IL-8. Migration of total $CD8^+$ T cells including 11.4% CXCR1$^+$ cells (presorting $CD8^+$ T cells) and of sorted CXCR1$^-$$CD8^+$ T cells (after sorting $CD8^+$ T cells) were induced by different concentrations of IL-8. IL-8 induced migratory cells were calculated as follows: percentage of migrated cells in presence of IL-8 minus that in absence of IL-8.

Acknowledgments

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Functionally Impaired HIV-Specific CD8 T Cells Show High Affinity TCR-Ligand Interactions[1]

Takamasa Ueno,* Hiroko Tomiyama,* Mamoru Fujiwara,* Shinichi Oka,† and Masafumi Takiguchi[2]*

We eventually isolated two different clonotypic CD8 T cell subsets recognizing an HIV Pol-derived epitope peptide (IPLTEEAEL) in association with HLA-B35 from a chronic HIV-infected patient. By kinetic analysis experiments, the subsets showed a >3-fold difference in half-lives for the HLA tetramer in complex with the Pol peptide. In functional assays in vitro and ex vivo, both subsets showed substantial functional avidity toward peptide-loaded cells. However, the high affinity subset did not show cytolytic activity, cytokine production, or proliferation activity toward HIV-infected cells, whereas the moderate affinity one showed potent activities. Furthermore, using ectopic expression of each of the TCR genes into primary human CD8 T cells, the CD8 T cells transduced with the high affinity TCR showed greater binding activity toward the tetramer and impaired cytotoxic activity toward HIV-infected cells, corroborating the results obtained with parental CD8 T cells. Taken together, these data indicate that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions, providing us further insight into the immune evasion mechanisms by HIV. *The Journal of Immunology,* **2004, 173: 5451–5457.**

Our understanding of how HIV avoids control by the human immune system remains incomplete. Although $CD8^+$ CTL are believed to have an important role in the immunopathogenesis of HIV-1 infection, it is not completely clear why viral replication persists and progressive immunodeficiency generally ensues (see recent reviews, 1–5). The findings of several studies show that HIV-specific CTL taken ex vivo can have functional defects that could undermine their control of the virus. For example, whereas most HIV-specific CD8 T cells in patients with chronic HIV disease produced antiviral cytokines on contact with cognate Ag, these cells showed diminished perforin expression and capacity for proliferation compared with CMV-specific T cells (6) and T cells in long term nonprogressors (7), respectively. Such different functional outcomes in T cells can be caused by the quality of T cell activation, such as the strength of TCR engagement and costimulatory or inhibitory interactions (8–10). The kinetics and affinity of interaction between TCR and peptide-MHC complex (pMHC)[3] are the basis of T cell activation. For the most part, longer half-lives of TCR-pMHC interaction correspond to higher T cell activation (11–14). However, in the case of some peptide variants as well as mutations in MHC and/or TCR, a longer half-life was reported to weaken T cell reactivity (15–18). Moreover, it remains unclear what are the functional roles of peripheral T cell subsets that bear TCR with high affinity for a MHC ligand in association with a foreign peptide, because T cells with high affinity for a foreign pMHC appear to be negatively selected in the thymus and not exported to the periphery (19).

In contrast, we and others have generated many CTL lines and clones from HIV-infected patients that were cytotoxic toward HIV-infected cells in vitro in the course of experiments to identify HIV-derived CTL epitopes in previous studies (20). Given that only T cells that were positive for epitope-specific cytolytic activity were reported in these studies, we hypothesized that T cells with negative cytolytic activity toward HIV-infected cells, which may reflect the loss of antiviral effector functions of HIV-specific CTLs in vivo, were concurrently generated, but not further examined, due to their negative activity. Therefore, to examine cell-based mechanisms involved with impaired functions of HIV-specific CD8 T cells, we have again been testing CD8 T cell clones isolated from HIV-infected patients for their lack of killing activity toward HIV-infected cells even though they retained their specificity toward HIV Ags.

In the present study we focused on remarkable functional differences in two different CD8 $\alpha\beta$ T cell subsets (TCR $V\alpha12^+$ and $V\delta1^+$) specific for an HIV Pol-derived epitope peptide (IPLTEEAEL) from a chronic HIV-infected patient. Interestingly, the subsets showed a >3-fold difference in binding activity toward the HLA tetramer in complex with the Pol peptide. The high affinity subset ($V\delta1^+$) showed impaired reactivity toward HIV-infected cells in vitro and ex vivo, whereas the moderate affinity subset ($V\alpha12^+$) had potent reactivity. Additional genetic transfer of each of these TCR genes into human primary CD8 T cells clearly indicated that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions.

Materials and Methods

Tetramer binding assay

The CTL lines (5×10^4 cells; >60% $tetramer^+$ $CD8^+$ cells) generated by repeated stimulation of the patient's lymphocytes (*HLA-A**2402/*A**2601, *HLA-B**3501/*B**5101) with the Pol peptide (IPLTEEAEL) were first stained with various concentrations of the tetramer at 4 or 37°C for 15 min. The cells were subsequently stained at 4°C for 15 min with anti-CD8-PerCP (BD Pharmingen, San Diego, CA), FITC-conjugated anti-$V\alpha12$

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[3] Abbreviations used in this paper: pMHC, peptide-MHC complex; IRES, internal ribosome entry site; MFI, mean fluorescence intensity.

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TCR (Serotec, Oxford, U.K.), and anti-Vδ1 TCR mAbs. The anti-Vδ1 TCR mAb (A13; provided by L. Moretta, Istituto di Istologia ed Embriologia Generale, Genova, Italy) (21) had been labeled with PE-conjugated Fab specific for the Fc portion of mouse IgG1 (Molecular Probes, Eugene, OR). For the kinetic analysis of tetramer binding, the CTL line was first incubated with 5 μM tetramer at 4°C. A portion of the reaction was removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with anti-CD8 and anti-TCR mAbs as described above. For kinetic analysis of the tetramer dissociation, a CTL line was stained with 5 μM tetramer for 60 min at 4°C. Then cells were rapidly washed twice and suspended in 1.5 ml of a buffer (2% BSA in PBS) supplemented with a blocking Ab. A portion of the reaction was then removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with the anti-CD8 and anti-TCR Abs. For the flow cytometric analysis, $V\alpha12^+$ or $V\delta1^+$ $CD8^+$ cells were gated and then assessed for their tetramer binding level.

Cytotoxic assay

The cytotoxic activity of CTL clones generated previously (22, 23) was determined by a standard ^{51}Cr release assay as previously described (22). For Pol peptide-pulsed target cells, ^{51}Cr-labeled C1R-B*3501 cells were pulsed with the peptide for 1 h, then incubated with the effector T cells for an additional 4 h at 37°C. For virus-infected target cells, C1R-B*3501 cells or .221-B*3501 cells expressing human CD4 Ag were infected with HIV-1 GagPol-expressing vaccinia virus, HIV-1 LAI, or vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 HXB2D. Note that all these viruses have the same epitope sequence as that used for synthetic Pol peptide (IPLTEEAEL). The cells were subsequently labeled with ^{51}Cr and incubated with the effector T cells for 6 h at 37°C. It should be noted that >70% cells expressed the p24 Gag Ag, as revealed by intracellular flow cytometric analysis of target cells.

Cytokine secretion assay

CTL clones were cocultured with .221-CD4-B*3501 cells, either pulsed with various concentrations of the Pol peptide or infected with HIV-1 LAI for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h. Then the cells were permeabilized, stained with anti-IFN-γ and TNF-α mAbs (BD Pharmingen), and analyzed by flow cytometry as previously described (23).

Ex vivo activation assay

Cryopreserved PBMC of HIV-positive (1×10^6) or negative donors (5×10^6) were stained with the tetramer at 37°C for 15 min, followed by anti-CD8 and anti-TCR Abs at 4°C for 15 min. The cryopreserved PBMC of the HIV-positive patient were stimulated, or not, with irradiated .221-CD4-B*3501 cells, either pulsed with 100 nM Pol peptide or infected with HIV-1 LAI (>70% p24 Gag^+). The cells were cultured at 37°C for 12 days in RPMI 1640 supplemented with 10% FCS and 200 U of IL-2. A portion of the stimulated cells (2×10^5) was stained as described above.

Construction of retroviral vectors and gene transfer

The genes encoding full-length α and β TCR of CTL 55 (23) and 589 (22) were subcloned into the pGC-based retroviral vector (pGCDNsap[MSCV]; provided by M. Onodera, Tsukuba University, Ibaragi, Japan) (24). The sequence data of the TCR genes are available from DDBJ under accession numbers AB164056, AB164057, AB164620, and AB164621. The genes encoding a murine heat-stable Ag (CD24) or a GFP were also incorporated into the constructs with an internal ribosome entry site (IRES) following the α or β TCR gene to facilitate monitoring of the expression of the α or β TCR gene, respectively, in the transduced cells.

Human primary CD8 T cells were isolated from PBMC of an HIV-negative healthy donor with *HLA-B*3501* using anti-CD8 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant CD8 T cells were activated over a 3-day period by anti-CD3 mAb (OKT3) coated on the culture dish, then transferred to recombinant fibronectin-coated plates (Takara Shuzo, Otsu, Japan) and incubated for 72 h with the retrovirus supernatant containing the TCR α-IRES-CD24 gene. Transduced T cells expressing CD24 Ags were isolated using PE-labeled anti-CD24 mAb (BD Pharmingen) and anti-PE magnetic beads (Miltenyi Biotec). The isolated cells (>80% of the cells were $CD8^+$ $CD24^+$) were subsequently transduced with another construct containing TCR β-IRES-GFP as described above.

Results

Functional difference in CTL clones in response to HIV-infected cells in vitro

An HIV Pol peptide (IPLTEEAEL) is a CTL epitope endogenously presented by HLA-B*3501 (20). In Pol peptide-stimulated lymphocytes from a patient with chronic HIV infection, we generated two $CD8^+$ αβ T cell clones, designated CTL 55 and 589, that were shown to express TCR Vδ1.1/Vβ13.3 and Vα12.1/Vβ5.6, respectively, on their cell surface (22, 23). It is of note that the genes encoding a Vδ1 variable segment are expressed in ~0.5% of peripheral $CD8^+$ αβ T cells in human healthy individuals and that the Vδ1-bearing TCR αβ recognizes a peptide presented by HLA class I molecules (21, 23). The functional properties of both T cell clones were first tested for their cytotoxic and cytokine production activities in response to cells either pulsed with the Pol peptide or infected with viruses expressing HIV Pol proteins. Peptide titration experiments showed that both clones had substantial cytotoxic activities (Fig. 1*A*). It should be noted that in repeated experiments, CTL 589 appeared to

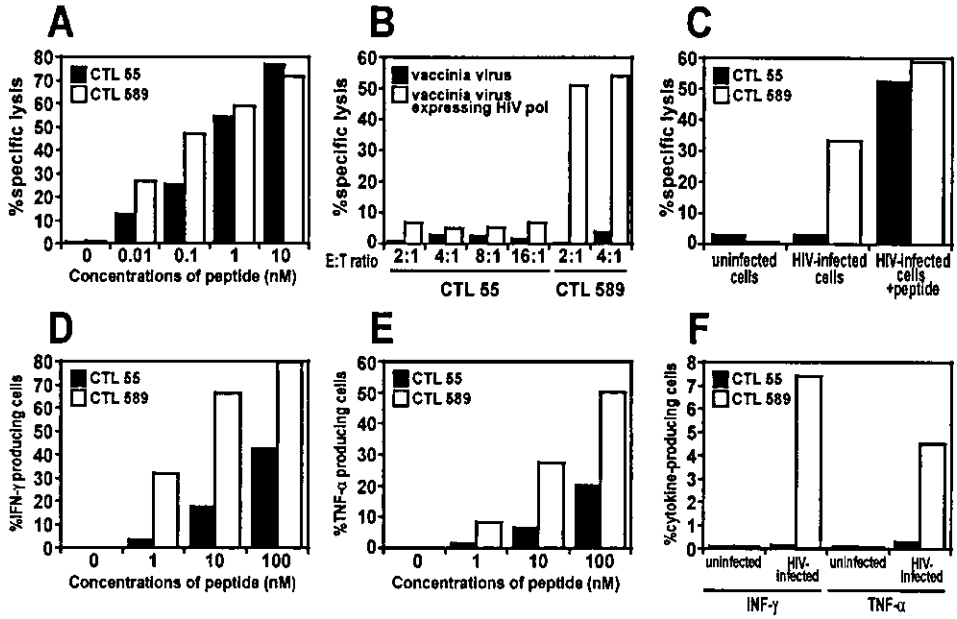


FIGURE 1. Analysis of effector functions of the CTL clones. *A* and *B*, Cytotoxic activity of CTL 55 and 589 toward C1R-B*3501 cells either pulsed with the indicated concentrations of the Pol peptide (*A*) or infected with vaccinia virus expressing HIV-1 GagPol polyproteins at the indicated E:T cell ratios (*B*). *C*, Cytotoxic activity of CTL 55 and 589 toward .221-B*3501 cells uninfected or infected with HIV-1 in the absence or the presence of 100 nM Pol peptide at an E:T cell ratio of 2:1. *D–F*, Intracellular staining for IFN-γ and TNF-α of CTL 55 and 589 in response to .221-B3501 cells either pulsed with the indicated concentrations of the Pol peptide (*D* and *E*, respectively) or infected with HIV-1 at an E:T cell ratio of 1:1 (*F*). Data are shown as the means of duplicate assays for at least three independent experiments.

have more profound cytotoxic activity at low concentrations of the Pol peptide than CTL 55, although the difference was modest under the assay conditions tested (Fig. 1*A*). CTL 589 also showed substantial cytotoxic activities toward cells infected with vaccinia virus expressing HIV-1 Gag-Pol polyproteins (Fig. 1*B*) as well as toward those infected with HIV-1 (Fig. 1*C*). In sharp contrast, CTL 55 was not cytotoxic toward cells infected with either viruses, even at increased E:T cell ratios (Fig. 1, *B* and *C*). Addition of the Pol peptide to the culture medium restored the cytotoxic activity of CTL 55 toward target cells (Fig. 1*C*), indicating the cytotoxic potential of CTL 55 during the assay.

As observed in cytotoxic assays, intracellular cytokine staining experiments showed that both clones had substantial ability to produce IFN-γ (Fig. 1*D*) and TNF-α (Fig. 1*E*) in response to the Pol peptide-loaded cells, although CTL 55 responded to a lesser extent than CTL 589. In response to HIV-infected cells, CTL 55 did not show production of IFN-γ or TNF-α (Fig. 1*F*), whereas CTL 589 produced both cytokines, confirming the impaired responsiveness of CTL 55 toward HIV-infected cells. Moreover, the other Pol peptide-specific CTL clones, 349 and 562, that had TCRs identical with CTL 55 and 589, respectively, showed a similar pattern of functional differences as that observed for CTL 55 and 589 (data not shown), suggesting that the impaired responsiveness toward HIV-infected cells was an inherent property of certain T cell subsets specific to the Pol peptide, rather than of a particular CTL clone.

Different proliferation capacities between the tetramer$^+$ CD8 T cell subsets in response to HIV-infected cells ex vivo

To further investigate the functional difference between these CD8$^+$ T cells toward HIV-infected cells, we examined the ex vivo proliferation capacity of these CD8 T cells in response to HIV-infected cells, because the Pol peptide-specific T cell clonotypes corresponding to CTL 55 and 589 can be exclusively stained by anti-TCR Vδ1 (21) and Vα12 Abs in association with the tetramer. In addition, TCR analysis of CD8$^+$tetramer$^+$ cells of the patient, followed by cell sorting, revealed that all TCR α transcripts that carried either Vδ1- or Vα12-encoded region had the CDR 3α sequence identical with that of TCR 55α or 589α, respectively (data not shown), confirming the T cell clonality and Ag specificity of tetramer$^+$ cells in the flow cytometric analysis.

Firstly, direct analysis of unstimulated peripheral lymphocytes of the HIV-infected patient ex vivo revealed that the frequency of tetramer$^+$ CD8 T cells was ~0.1% of the total CD8 T cell population and that the Vδ1$^+$ and Vα12$^+$ subsets were 75 and 8%, respectively, within this tetramer$^+$ fraction (Fig. 2*B* and Table I), whereas analysis of peripheral lymphocytes of the HIV-negative donors ex vivo revealed that the frequency of tetramer$^+$ CD8 T cells was ~0.02% of the total CD8 T cell population, and that the Vδ1$^+$ and Vα12$^+$ subsets were <5% within this tetramer$^+$ fraction (Fig. 2*A*).

Peripheral lymphocytes of the HIV-infected patient were then stimulated with cells that had been either pulsed with the Pol peptide or infected with HIV-1 and cultured for 12 days. When stimulated with the Pol peptide, frequencies of the Vδ1$^+$ and Vα12$^+$tetramer$^+$ CD8 T cell subsets in the total CD8 T cells were increased 7- and 30-fold (Table I and Fig. 2*D*), respectively, indicating that both subsets had substantial proliferation capacity in response to the Pol peptide-loaded cells. This observation was consistent with our previous finding that CTL 55 and 589 had been generated by repeated stimulation of the patient's lymphocytes by the Pol peptide. In contrast, when stimulated with HIV-infected cells, the frequency of the Vα12$^+$tetramer$^+$ CD8 T cell subset was increased >70-fold, whereas the frequency of the Vδ1$^+$tetramer$^+$ CD8 T cell subset was decreased (Fig. 2*E* and Table I), indicating that the Vδ1$^+$tetramer$^+$ CD8 T cell subset could not respond to HIV-infected cells. Noticeably, the frequency of the tetramer$^+$ CD8 T cell subset, without any Ag stimulation, was virtually constant after a 12-day culture period (Fig. 2*C* and Table I), confirming the Ag-specific proliferation response of the Vα12$^+$tetramer$^+$ subset. It is also of interest that the Vα12$^+$ cells showed more profound proliferation when stimulated by cells infected with HIV than when pulsed with the Pol peptide (Fig. 2 and Table I), suggesting a qualitative difference in Ag presentation to T cells between infected cells and peptide-loaded cells.

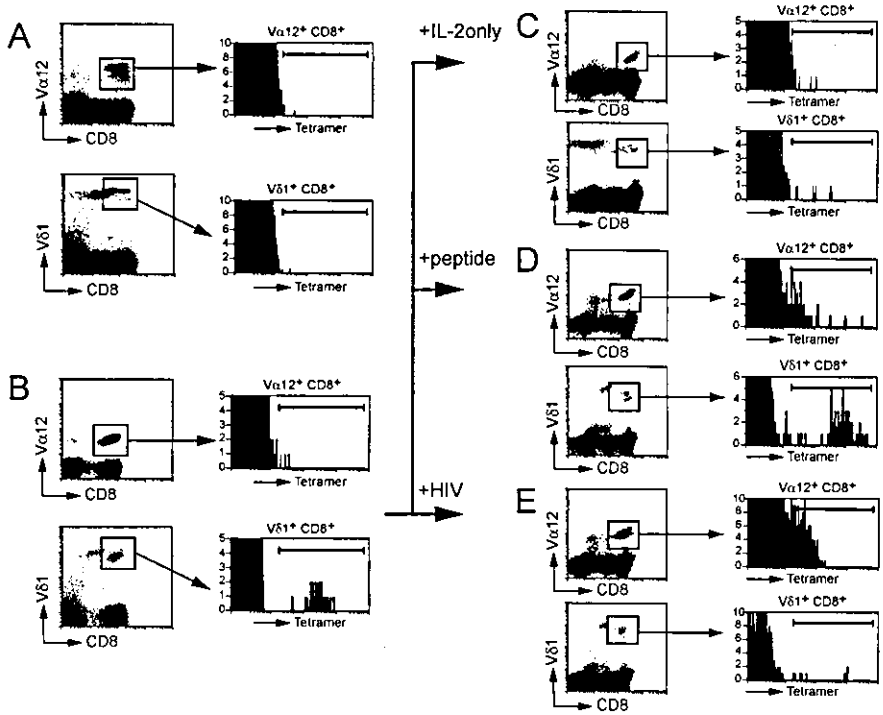


FIGURE 2. Ex vivo analysis of the tetramer$^+$ CD8 T cells in the peripheral lymphocytes in response to Ag stimulation. Cryopreserved PBMC of a representative HIV-negative donor (5×10^6; *A*) and the HIV-infected patient (1×10^6; *B*) were stained with the tetramer and anti-CD8 and anti-TCR Abs. The PBMC of the HIV-infected patient after stimulation with IL-2 alone (*C*) or with .221-B*3501 cells either pulsed with the Pol peptide (*D*) or infected with HIV-1 (*E*) were stained as described above. The Vδ1$^+$ and Vα12$^+$ CD8$^+$ subsets were gated, then analyzed for their levels of tetramer binding. Similar data were obtained in a separate independent experiment. The frequencies and MFI values of the tetramer$^+$ cells in each subset of the HIV-infected patient are summarized in Table I.

Table I. *Summary of ex vivo flow cytometric analysis of tetramer$^+$ CD8$^+$ cells*[a]

Subset	Before Stimulation		+IL-2 Only		+Peptide-Pulsed Cells		+HIV-Infected Cells	
	Cell number	MFI	Cell number	MFI	Cell number	MFI	Cell number	MFI
CD8$^+$Tet$^+$	113	173	46	83.9	1025	525	863	128
CD8$^+$Tet$^+$Vα12$^+$	10	50.9	13	51.9	310	123	755	97.2
CD8$^+$Tet$^+$Vδ1$^+$	86	291	20	105	502	710	12	705

[a] Cell number is the number of cells in the indicated subset per 1×10^5 total CD8 T cells. Mean fluorescence intensity (MFI) for the tetramer in each tetramer$^+$ (Tet$^+$) subset is also shown.

Distinct binding kinetics in HIV-specific CD8 T cell subsets to tetramer

During the flow cytometric analysis, we noticed that the Vα12$^+$ and Vδ1$^+$ subsets had different binding activities toward the tetramer (Fig. 2*D*, for example). Staining CTL clones with the tetramer showed that Vδ1$^+$ CTL 55 had >2-fold greater binding activity than Vα12$^+$ CTL 589 (Fig. 3), whereas both clones showed comparable surface expression of the CD3 Ags (Fig. 3), indicating that the apparent difference in tetramer binding was not due to the surface density of the TCR/CD3 complex. Consistent with this, when the tetramer$^+$ cells were divided into two subsets in the ex vivo flow cytometric analysis, the Vδ1$^+$ cells were mostly found in the tetramer^{high} subset, whereas the Vα12$^+$ cells were in the tetramer^{low} subset (Fig. 4*A*). These observations are interesting, because the extent of T cell activation is known to be sensitive to subtle differences in the duration of TCR-ligand interactions (12, 13, 17).

Because large temperature effects on TCR-pMHC interactions have been noted (12, 25, 26), we first examined the effect of reaction temperature on binding activity toward the tetramer. Pol peptide-stimulated lymphocytes were stained with various concentrations of the tetramer at 4 or 37°C, and the mean fluorescence intensity (MFI) of both subsets was determined by flow cytometry. At either temperature, the Vδ1$^+$CD8$^+$tetramer$^+$ cells showed ~3-fold higher MFI for the tetramer than the corresponding Vα12$^+$ cells (Fig. 4*B*), indicating that both T cell subsets could bind the tetramer with sufficient sensitivity and specificity.

We next examined the kinetic interactions of the tetramer with the Vδ1$^+$ or Vα12$^+$ subset at 4°C, because the level of tetramer binding and its half-life were previously shown to correlate with monomer affinity and off-rate, respectively (13, 27). Although the association of the tetramer with the two subsets was not significantly different (Fig. 4*C*), the dissociation of the tetramer from Vδ1$^+$ cells substantially delayed compared with that from Vα12$^+$ cells (Fig. 4*D*). The half-lives of the tetramer dissociation from the Vδ1$^+$ and Vα12$^+$ subsets were calculated to be 30.0 ± 1.6 and 8.1 ± 0.4 min, respectively. These results suggest that the >3-fold longer half-life of the Vδ1$^+$ subset for interaction with the tetramer than that of the Vα12$^+$ subset was a cause of the impaired antiviral effector functions of the Vδ1$^+$ subset.

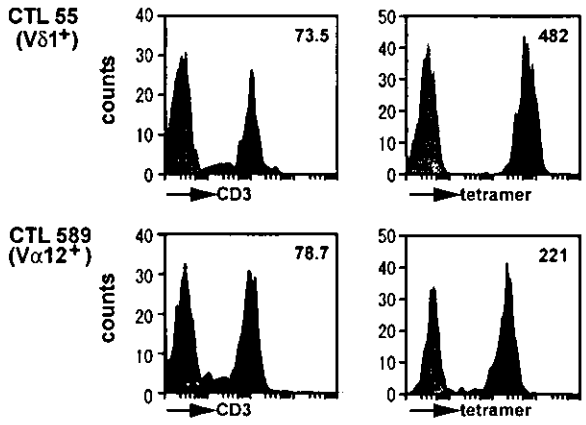


FIGURE 3. Flow cytometric analysis of CTL clones. Two CTL clones, CTL 55 and 589, selected from the Vδ1$^+$ and Vα12$^+$ CD8 T cell subsets, respectively, were stained (■) or not stained (□) with anti-CD3 mAb or the tetramer at 4°C, and analyzed by flow cytometry. MFI values are shown in the *upper right* corners.

Impaired responsiveness of CD8 T cells to HIV-infected cells solely caused by TCR

To clarify whether the high affinity interaction with the tetramer and the impaired responsiveness to HIV-infected cells observed in the Vδ1$^+$tetramer$^+$CD8 T cell subset were caused by the ligand recognition property of the TCR, we transduced human primary CD8 T cells with both TCR genes separately.

Both α and β TCR genes for CTL 55 (Vδ1.1/Vβ13.3) and 589 (Vα12.1/Vβ5.6) were cloned into a pGC-based retrovirus vector with the gene encoding murine CD24 or GFP downstream of IRES for bicistronic expression of α or β TCR genes, respectively (Fig. 5*A*). Human primary CD8 T cells prepared from an HIV-negative donor carrying *HLA-B*3501* were sequentially transduced with α and β TCR genes and analyzed by flow cytometry. As shown in Fig. 5*B*, tetramer$^+$CD8$^+$ subsets reached 15 and 34% of the total lymphocytes for 55 TCR and 589 TCR-transduced cells, respectively, whereas the tetramer$^+$CD8$^+$ subset remained 0.05% of the total lymphocyte population in the case of mock-transduced cells. As measured by the MFI values for the tetramer, the tetramer$^+$CD8$^+$ fraction (gated cells in Fig. 5*B*) of 55 TCR-transduced cells appeared to have 2.8-fold greater binding activity toward the tetramer than that of the 589 TCR-transduced ones. In contrast, both tetramer$^+$CD8$^+$ fractions showed comparable MFI values for CD24 and GFP (within 1.5-fold; Fig. 5, *B* and *C*), which should reflect the expression levels of α and β TCR genes, respectively. These data indicate that the difference in tetramer binding activity observed between the Vδ1$^+$ and Vα12$^+$tetramer$^+$CD8 T cells was solely due to their TCR.

The tetramer$^+$CD8$^+$ fractions of the TCR-transduced cells were then sorted and tested for their cytotoxic activity toward cells either pulsed with the Pol peptide or infected with HIV-1. Both 55 and 589 TCR-transduced cells showed substantial cytotoxic activity toward peptide-loaded cells (Fig. 6*A*), and the activities were as potent as those of their parental CTL clones (cf., Figs. 1*A* and 6*A*). Also, both transduced cells showed modest differences in cytotoxic activities at low concentrations of the Pol peptide (Fig. 6*A*), consistent with the observations made on parental CTL clones (Fig. 1*A*). In contrast, the 55 TCR-transduced cells did not show cytotoxic activity toward HIV-infected cells, whereas 589 TCR-transduced ones killed cells infected with HIV-1 (Fig. 6*B*), again in agreement with the observations made on the parental CTL clones (Fig. 1*C*). These data strongly support our conclusion that the different cytotoxic activities toward HIV-infected cells observed in

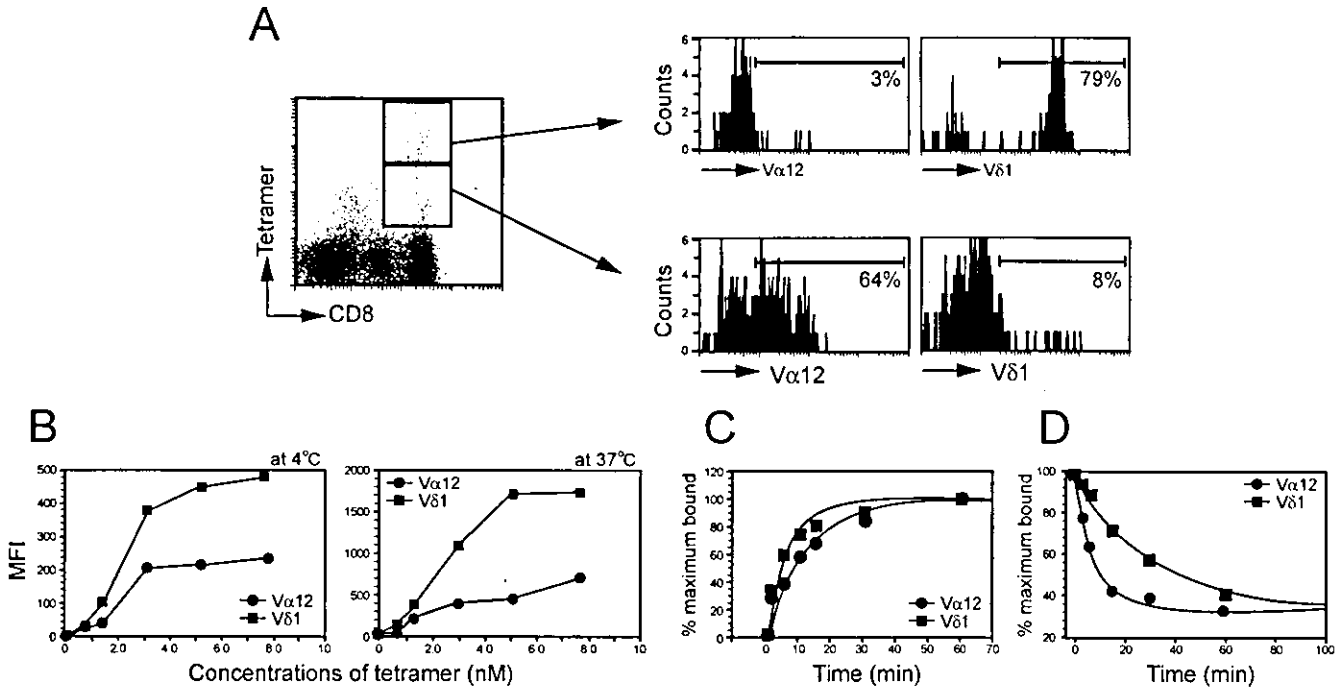


FIGURE 4. Flow cytometric analysis of kinetic interaction between the tetramer and CD8 T cell subsets. *A*, TCR analysis of tetramer^{high} and tetramer^{low} CD8 T cell subsets in Pol peptide-stimulated peripheral lymphocytes of the HIV-infected patient. *B*, Tetramer binding activities of V$\delta1^+$ and V$\alpha12^+$ CD8 T cell subsets determined at 4 or 37°C. *C* and *D*, Association (*C*) and dissociation (*D*) kinetic analysis of interaction between the tetramer and the V$\delta1^+$ and V$\alpha12^+$ CD8 T cell subsets at 4°C. Data are shown as the means of duplicate assays. An independent experiment gave similar results.

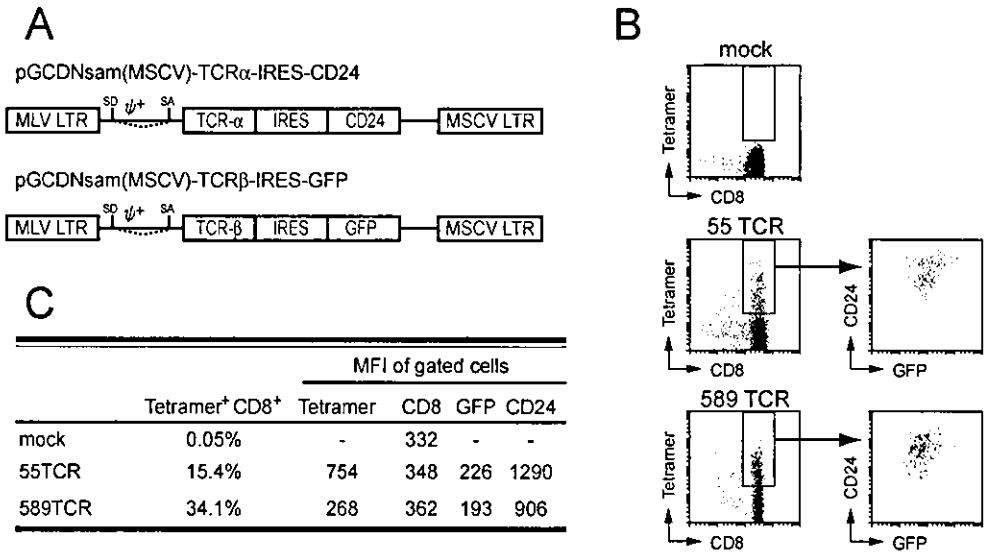
the V$\delta1^+$ and V$\alpha12^+$tetramer$^+$CD8 T cells were solely due to the difference in the TCR.

Discussion

We showed in this study that CD8 T cells with relatively high affinity TCR for an HIV-derived peptide were present in vivo and that these T cells had substantially diminished functional outcomes in response to cells infected with HIV. The difference in functional outcome of the two CD8$^+$tetramer$^+$ T cell subsets (V$\alpha12^+$ and V$\delta1^+$) was evaluated in terms of both the cytotoxic and cytokine production activities of T cell clones in vitro and their proliferation capacity, as assessed by direct ex vivo assays. The results revealed the impaired responsiveness of the higher affinity T cell subset (V$\delta1^+$) to virus-infected cells. In contrast, the V$\delta1^+$ T cells showed functional avidity to cells pulsed with the HIV-derived epitope peptide comparable to that of the other HIV-specific CD8 T cell subset (V$\alpha12^+$), which was competent for effector functions toward HIV-infected cells, indicating that the functional impairment observed in this study did not result from T cell anergy, skewed maturation of CD8 T cells in the periphery, or any defect in signal transduction machinery reported previously (1, 2, 6). There are a myriad of studies that focused on functional differences among Ag-specific (tetramer$^+$) CD8 T cells for HIV (6, 7, 28–30) as well as for other viruses and tumors (31–33), and such studies indicated functional heterogeneity of tetramer$^+$ CD8 T cells. In our study we demonstrated that the duration of TCR-pMHC interactions could have considerable effects on the antiviral effector functions and proliferation capacity of HIV-specific CD8 T cells.

It is known that the T cell is sensitive to subtle differences in the duration of TCR-ligand interactions and that, in general, a longer duration of TCR-pMHC interactions corresponds to higher T cell activation (14, 34, 35). However, Kalergis et al. (18) showed that T cell hybridoma cells transfected with a mutant TCR displayed impaired T cell activation and had an ~2-fold longer half-life for interaction with the tetramer than cells transfected with the wild-type TCR. The present study focused on the human primary CD8 T cells specific for an HIV Ag and showed that a >3-fold increased duration of the TCR-pMHC interaction resulted in impaired, rather than increased, T cell reactivity toward HIV-infected cells, supporting the findings by Kalergis et al. (18). It is likely that HIV-specific T cells have a means to improve their functional avidity to virus-infected cells because only limited numbers of the cognate epitope peptide could be presented on the surface of HIV-infected cells. A serial triggering model (36), based on the T cell integrating the number of TCRs that have interacted with pMHC, predicts that too long an interaction of TCR-pMHC slows the dissociation of TCR from pMHC, so that fewer TCRs are triggered; this may explain our observation. Indeed, we reproducibly observed the different cytotoxic activities of CTL 55 and 589 at low concentrations of the Pol peptide. If the amount of the Pol peptide presented on the surface of virus-infected cells was close to this range, their functional difference in contact with virus-infected cells could be simply explained by their different avidity to low density Ags. However, considering that CTL 55 and 589 showed significant difference in specific killing activity toward HIV-infected cells (<5 and >30%, respectively), the HIV-derived Ag would be preferentially presented at certain sites on the surface of HIV-infected cells with a local density sufficient to activate CTL 589, but not CTL 55. In this regard, it may be helpful to analyze the density and localization of the presenting epitopes on the surface of virus-infected and peptide-pulsed cells using a reagent such as a recombinant Fab specific to the peptide-MHC class I complex.

One of the CTL clones showing the higher affinity TCR-ligand interaction, CTL 55 demonstrated significant differences between their response to peptide-pulsed and virus-infected target cells in this study. It is conceivable that T cells bearing the high affinity TCR can be unduly susceptible to an inhibitory mechanism of HIV-infected cells. Alternatively, a longer duration of TCR-ligand



C

		MFI of gated cells			
	Tetramer$^+$CD8$^+$	Tetramer	CD8	GFP	CD24
mock	0.05%	-	332	-	-
55TCR	15.4%	754	348	226	1290
589TCR	34.1%	268	362	193	906

FIGURE 5. Retrovirus-mediated transduction of human primary CD8 T cells with TCR genes. *A*, Linear representations of the retroviral vector constructs used for transduction with α and β TCR genes. SD, splicing donor; SA, splicing acceptor; ψ^+, packaging signal; MLV, murine leukemia virus; MSCV, murine stem cell virus; LTR, long terminal repeat. *B*, Human primary CD8 T cells isolated from an HIV-negative donor, mock-transduced or transduced with 55 TCR or 589 TCR, and analyzed by flow cytometry. The tetramer$^+$CD8$^+$ subsets were gated, and their expression levels of GFP and CD24 were analyzed. *C*, Summary of frequencies and MFI values of the gated cells. Data shown are similar to those obtained in separate independent experiments.

interaction can lead to recruiting inhibitory receptors or molecules to the sites where the ligand-engaged TCR/CD3 signaling complexes were in action. However, similar different outcomes were observed in CTL 55 when the target cells were infected with vaccinia virus expressing HIV Pol protein, implying the existence of a general inhibitory mechanism of virus-infected cells, rather than an HIV-specific one. It is therefore interesting to see the differences in gene expression profiles of CTL 55 in contact with cells pulsed with the peptide and infected with viruses that had the epitope sequence.

We used an HLA class I tetramer for HIV-specific CD8 T cell subsets to analyze the relationship between affinity and cellular responses. The avidity of tetramer binding gives relative values that can be used as a surrogate for true affinity measurements, such as by surface plasmon resonance (19, 27). Tetramer binding more closely reflects the avidity of the T cell, rather than the intrinsic affinity of the TCR, because the tetramer binding to TCR is measured on the surface of the T cell in the presence of CD3 elements, coreceptors, and other molecules. In our study retrovirus-mediated transfer of TCR genes into human primary CD8 T cells showed that differences in the two CD8 T cells in terms of tetramer binding activity and effector functions toward HIV-infected cells solely depended on the TCR used for transduction. This experimental result clearly indicates that the affinity difference between the two TCRs interacting with the same pMHC ligand is the cause of their distinct responsiveness to HIV-infected cells.

It is reported that the Pol peptide was presented by both HLA-B*3501 and HLA-B*5101, and that CTL 589 cross-recognized the Pol peptide in complex with both HLA molecules (22). Although HLA-B*5101 bound the Pol peptide to a >50-fold lesser extent than HLA-B*3501, it is still possible that CTL 55 and 589 were differently restricted by either HLA-B*3501 or HLA-B*5101, yet specific to the same Pol peptide, because the patient has both *HLA-B*3501 and *HLA-B*5101. However, this is unlikely because CTL 55 did not respond to HLA-B*5101-expressing cells pulsed with the Pol peptide or to those infected with viruses expressing the epitope (H. Tomiyama and T. Ueno, unpublished observations), indicating that the different functional outcomes between CTL 55 and 589 were not due to the difference in their restriction elements.

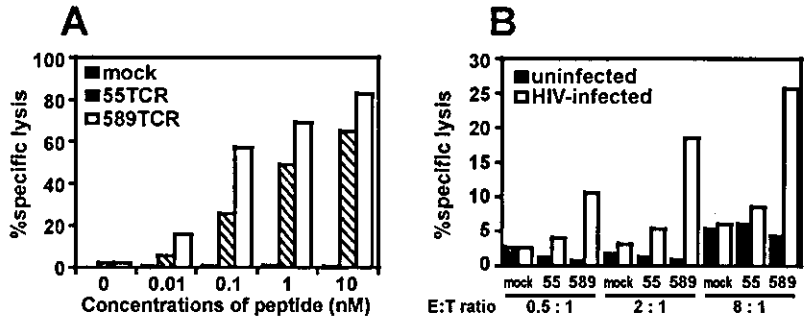


FIGURE 6. Cytotoxic activity of the 55 and 589 TCR-transduced CD8 T cells. The CD8$^+$tetramer$^+$ subsets of the 55 and 589 TCR-transduced cells were sorted (see Fig. 5) and analyzed for their cytotoxic activity toward CIR-B*3501 cells pulsed with the indicated concentrations of the Pol peptide at an E:T cell ratio of 1:1 (*A*). The same set of TCR-transduced cells was assessed for their cytotoxic activity toward CIR-B*3501 cells, infected or not with vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 HXB2D at the indicated E:T cell ratio (*B*). Data are shown as the means of duplicate assays for three independent experiments.

It remains unclear how such a high affinity TCR was generated in the periphery of the patient with chronic HIV infection. It was reported that T cells with high affinity TCR for foreign pMHC appear to be present in the thymus, but that these T cells are eliminated and not exported to the periphery (19). This scenario is most likely because TCRs with high affinity for foreign pMHC show considerable self-reactivity and therefore are negatively selected in the thymus (37). Considering the HIV-infected thymus, a significant increase in the expression of MHC class I molecules was reported, with this MHC class I up-regulation resulting in decreased surface expression of CD8 Ags on thymocytes (38, 39). This could lead to a decrease in the overall avidity of the TCR-CD8 signaling complex for self-pMHC ligands, allowing T cells with high affinity TCR to escape negative selection in the thymus and be exported to the periphery. Although we have only tested limited numbers of T cells, we provide evidence that the high affinity TCR-pMHC interactions can cause an additional level of functional defect in HIV-specific CD8 T cells. Also, it is strongly suggested that such defective CD8 T cells could undermine their control of HIV in vivo.

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Reconstitution of anti-HIV effector functions of primary human CD8 T lymphocytes by transfer of HIV-specific αβ TCR genes

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We redirected the antigen specificity of primary human CD8 T cells by retrovirus-mediated transduction of genes encoding αβ TCR specific to HIV-1 Pol protein. A large polyclonal population of TCR-transduced CD8 T cells showed substantial cytotoxic and cytokine production activities toward target cells either pulsed with the peptide or infected with HIV-1, and their functional activities were comparable to those of the parental CTL clone. Peptide fine-specificity and promiscuous recognition of HLA class I supertypes of the parental CTL clone were also preserved in the TCR-transduced cells. There were no signs of allogeneic responses in these cells, although hybrid TCR dimers consisting of transduced TCR and endogenous TCR were suspected to have been formed in these cells, as the effect of transgene expression on the surface expression of the desired TCR was limited. Moreover, the TCR-transduced cells showed potent inhibitory activity against HIV-1 replication *in vitro*, although the differential surface expression of the desired TCR resulted in differential functional avidity of individual TCR-transduced cells toward the peptide-pulsed target cells. These data suggest that the reconstitution of HIV-specific immunoreactive T cells engineered by genetic transfer of HIV-specific TCR is a potential alternative to immunotherapeutic applications against HIV infections.

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1 Introduction

The transfer of immunoreactive cells into patients, termed adoptive immunotherapy, for the control of viral infections as well as for the treatment of some tumors is an area of considerable interest both in basic research and clinical practice. Adoptive immunotherapy with $CD8^+$ cytotoxic T lymphocytes (CTL) had direct clinical impact on the management of patients at risk for cytomegalovirus and Epstein-Barr virus diseases [1–5]. However, the transfer of HIV-specific CTL lines and clones into HIV-infected patients, although safe, has so far produced only modest results [6–9], despite a line of evidence demonstrating that HIV-specific CTL have an important role in the immunopathogenesis of HIV infection [10].

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Abbreviations: GFP: Green fluorescent protein **IRES:** Internal ribosome entry site **MSCV:** Murine stem cell virus **MFI:** Mean fluorescence intensity

Such poor responses of autologous HIV-specific CTL in patients may be explained by recent findings showing that HIV-specific CTL taken *ex vivo* can have functional defects due to their impaired maturation in the periphery [10–13]. For example, whereas most HIV-specific CD8 T cells in patients with chronic HIV disease produced antiviral cytokines on contact with cognate antigen, these cells showed diminished perforin expression and capacity for proliferation compared with cytomegalovirus-specific T cells [14] and T cells in long-term nonprogressors [15], respectively. Moreover, the evidence that structured treatment interruption-mediated control of viral replication was associated with expansion of virus-specific CD8 T cells with a fully differentiated effector phenotype in patients with treated acute HIV-1 infection [16] strongly supports the importance of functionally mature CD8 T cells in the control of HIV-1 replication.

Redirecting the antigen specificity of T lymphocytes by the transfer of antigen-specific T cell receptor (TCR) genes to T lymphocytes has recently been described as

a potential method of generating large numbers of tumor antigen-reactive T lymphocytes with appropriate effector phenotypes [17, 18]. Using this approach, various investigators demonstrated that retroviral transfer of high-avidity TCR into T lymphocytes is accompanied by the maintenance of the parental T cell avidity and that TCR-transferred T cells preserved peptide fine-specificity in comparison to the parental CTL clone [19, 20]. Furthermore, in a murine model, T cells retrovirally transduced with gene-encoding TCR could expand *in vivo* upon antigen challenge, efficiently home into effector sites, and mediate the rejection of antigen-expressing tumors [21–24].

Although all of these studies were done using TCR specific for tumor antigens, adoptive transfer of TCR-transduced T cells into patients for the control of HIV infections could also have considerable potential. In this regard, it was reported that T cells transduced with the gene encoding a TCR specific for the p17 Gag peptide presented by HLA-A3 into T cells showed cytolytic activity toward both target cells pulsed with the epitope peptide and those infected with HIV-1 [25]. However, such cytolytic activity was observed only after cloning the transduced T cells having the highest surface expression of the transduced TCR [25]. In addition, analysis of the anti-HIV effector function of TCR-transduced T cells, including their cytokine secretion activity and antiviral replication capacity, has not yet been performed.

In the present study, we redirected the antigen specificity of primary human CD8 T cells by sequential transduction of genes encoding αβ TCR specific for an HIV-1 Pol peptide (IPLTEEAEL) using a retrovirus vector system that allows highly sustained transgene expression in the transduced T cells. A large polyclonal population of TCR-transduced CD8 T cells showed peptide fine-specificity and promiscuous recognition of HLA class I supertypes as well as cytotoxic and cytokine production activities that were all comparable to those of the parental CTL clone. Moreover, although the single-cell-sorted TCR-transduced CD8 T cells had a distributed pattern of functional avidity toward the peptide-pulsed target cells, all of the TCR-transduced T cells tested showed comparably potent inhibitory activity toward HIV-1 replication *in vitro*.

2 Results and discussion

2.1 Creation of functional TCR retroviral vectors

CTL clone 589, specific for an HIV Pol peptide ($Pol_{448-456}$; IPLTEEAEL) presented by HLA-B*3501, was previously found to express one β (Vβ5.6) and two in-frame α (Vα10 and Vα12.1) transcripts of TCR [26]. In addition, the transfer of individual α and β chains of TCR to TCR-negative mouse T cell hybridoma cells by use of a Moloney murine leukemia virus (MLV)-based retroviral vector construct pMX [27] revealed that the complex of Vα12.1 TCR and Vβ5.6 was exclusively expressed on the cell surface and solely responsible for the peptide-specific responses [26]. In the present study, both α and β TCR genes of TCR 589 (Vα12.1/Vβ5.6) were cloned into a pGC-based retrovirus vector with the gene encoding mouse CD24 or green fluorescent protein (GFP) downstream of internal ribosome entry site (IRES) for bicistronic expression of α or β TCR genes, respectively (Fig. 1A), in order to facilitate monitoring the expression levels of transduced TCR genes without the need for anti-TCR antibodies. We used a retroviral vector, designated pGCsap(MSCV) [28], in which the 3′-long-terminal repeat (LTR) of pGCsap had been replaced with that of murine stem cell virus (MSCV), allowing sustained expression of the transgene in the transduced T cells [28].

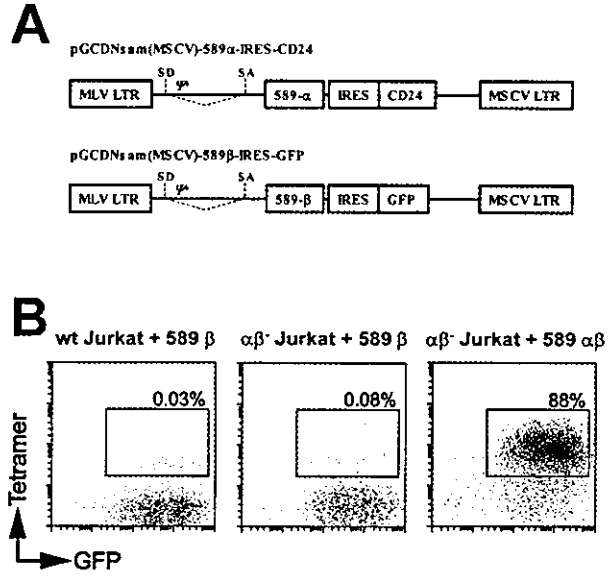

Fig. 1. Creation of functional retroviral vectors for TCR transduction. (A) Linear representation of retroviral vector constructs for transduction of αβ TCR 589 genes. SD and SA, splicing donor and acceptor, respectively; Ψ^+, packaging signal. (B) Wild-type (wt) and TCR-negative variant ($\alpha\beta^-$) of Jurkat cells were transduced with TCR 589 β-IRES-GFP alone, stained with HLA tetramer, and analyzed by flow cytometry. Other $\alpha\beta^-$ Jurkat cells that had been transduced with TCR 589 α-IRES-CD24 were further transduced with TCR 589 β-IRES-GFP and analyzed as above. GFP^+ $tetramer^+$ fractions were gated, and their frequencies within the total cell population are shown. The transduction efficiency for Jurkat cells was >90% as measured by the frequency of the GFP^+ fraction.

In order to confirm the potency of the retroviral vectors, we transduced wild-type Jurkat cells and a Jurkat variant that harbors neither α nor β TCR ($\alpha\beta^-$ Jurkat) with these constructs, stained them with HLA tetramer, and analyzed them by flow cytometry (Fig. 1B). The $\alpha\beta^-$ Jurkat variant transduced with both α and β TCR 589 genes appeared to be stained by the HLA tetramer, whereas the Jurkat cells and the $\alpha\beta^-$ Jurkat variant transduced with the β gene alone were not stained by the HLA tetramer (Fig. 1B). These results clearly indicate that the TCR 589 complex was expressed on the surface of the transduced cells and that the TCR had antigen specificity identical to that of the parental CTL 589.

2.2 TCR gene transfer to primary human CD8 T cells

Primary human CD8 T cells prepared from an HIV-negative donor carrying *HLA-B*3501* were activated by plate-coated OKT3 mAb and sequentially transduced with α and β genes of TCR 589. The transduced CD8 T cells were selected for their expression of GFP and CD24 antigen using fluorescence-activated cell sorting and stimulated again with the plate-coated OKT3 mAb. A fraction representing 9.5% of the resultant CD8 T cells bound the HLA tetramer in complex with the Pol peptide, whereas only 0.05% of the cells were stained by the tetramer in the case of the mock-transduced cells (Fig. 2A). We then stimulated these cells with the Pol peptide and cultured them for 10 days in the presence of rIL-2. Of great interest, the tetramer$^+$ fraction increased to 35% of the TCR-transduced cells, whereas the level of GFP and CD24 expression remained comparable (Fig. 2A), suggesting preferential proliferation of the tetramer$^+$ phenotype in response to antigen stimulation.

We then tested the TCR-transduced cells for their cytotoxic activity toward HLA-B*3501-expressing cells either pulsed with the Pol peptide or infected with HIV-1. The TCR-transduced cells showed substantial cytotoxic activity toward the peptide-loaded cells even at a peptide concentration down to 0.01 nM (Fig. 2B). Also, the cells showed cytotoxic activity toward HIV-infected cells, whereas they were not cytotoxic toward uninfected cells (Fig. 2C), indicating the antigen-specific and high-avidity nature of the TCR-transduced T cells.

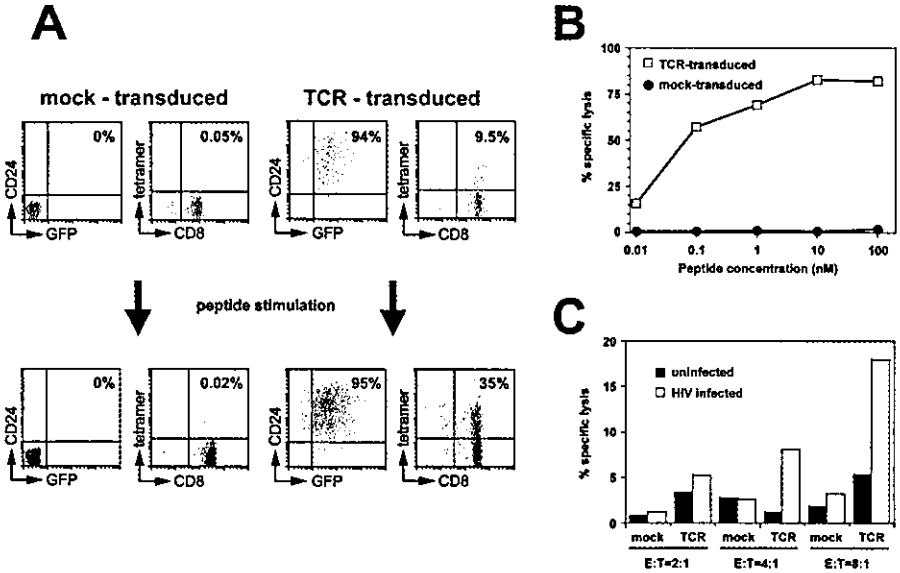

Fig. 2. Flow-cytometric and functional analysis of TCR-transduced primary CD8 T cells. (A) Primary human CD8 T cells that had been sorted into the GFP$^+$ CD24$^+$ fraction after transduction with TCR-encoded vectors were stained with anti-CD24-PE, anti-CD8-PerCP, and tetramer-allophycocyanin, and analyzed by flow cytometry before or after antigen stimulation. Mock-transduced cells were analyzed as above. Frequencies of GFP$^+$ CD24$^+$ or CD8$^+$ tetramer$^+$ fractions within the total cell population are indicated in the upper right corners of each dot plot. (B, C) The mock- or TCR-transduced cells were analyzed for their cytotoxic activity toward 221-CD4-B*3501 cells (2×10^4) either pulsed with the indicated concentrations of Pol peptide at an E/T ratio of 4:1 (B) or infected with HIV-1 LAI at the indicated E/T ratios (C). Data are shown as the means of duplicate assays in one experiment; an additional experiment gave similar results.

2.3 Maintenance of ligand specificity and functional avidity of TCR-transduced CD8 T cells

We next asked whether the ligand specificity and functional avidity of the parental CTL clone 589 would be preserved in the TCR-transduced T cells. The tetramer$^+$ subset of the TCR-transduced T cells was sorted and cultured with autologous feeder cells pulsed with the Pol peptide. A fraction comprising 82% of the resulting tetramer$^+$-sorted cells were CD8$^+$ tetramer$^+$ with a mean fluorescence intensity (MFI) value for the tetramer of 225; whereas >98% of the parental CTL 589 were CD8$^+$ tetramer$^+$ with an MFI for the tetramer of 411 (Fig. 3A).

Effector functions of the tetramer$^+$-sorted cells and parental CTL 589 appeared to be comparable as assessed by their cytokine production activity (Fig. 3B) and their cytotoxic activity (Fig. 3C) in response to the Pol peptide-pulsed target cells, indicating that the functional avidity of parental CTL 589 was maintained in the TCR-transduced T cells. In addition, the antigen specificity of both cells was tested using a series of naturally occurring mutations in the epitope region found in a database (http://www.hiv.lanl.gov/content/index). Both cells showed a similar pattern of reactivity toward the mutant peptides tested (Fig. 3C), indicating that the peptide fine-specificity of CTL 589 was preserved in the TCR-transduced T cells.

We also tested the TCR-transduced T cells for their ligand specificity toward HLA class I molecules, since CTL clone 589 was previously found to exhibit dual specificity, recognizing the Pol peptide presented by both HLA-B*3501 and HLA-B*5101 [26]. The TCR-transduced T cells showed a peptide-specific response to cells expressing either HLA-B*3501 or HLA-B*5101, and their cytotoxic activities were comparable to those of parental CTL 589 (Fig. 3D), indicating that the TCR-transduced

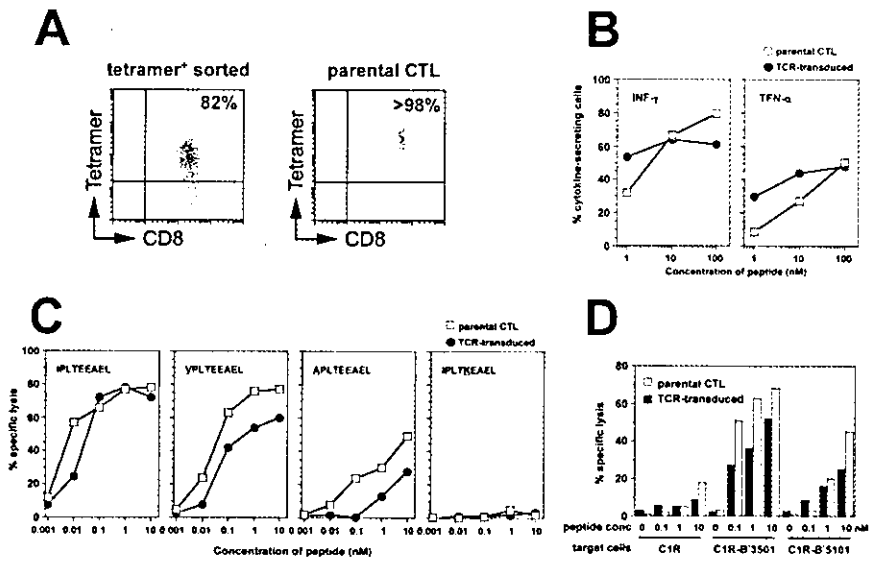

Fig. 3. Ligand specificity and functional avidity of TCR-transduced T cells compared with those of parental CTL 589. (A) The CD8$^+$ tetramer$^+$ subset of the TCR-transduced cells were sorted and cultured with autologous feeder cells pulsed with the Pol peptide. The resultant tetramer-sorted TCR-transduced cells and the parental CTL 589 were stained with anti-CD8-PerCP and tetramer-allophycocyanin and analyzed by flow cytometry. The frequency of the CD8$^+$ tetramer$^+$ subset within the total cell population is shown in the each dot plot. MFI values of tetramer-sorted TCR-transduced cells and the parental CTL 589 for the tetramer were 225 and 411, respectively. (B) Cytokine secretion activity of the tetramer-sorted TCR-transduced cells and the parental CTL 589 were analyzed in response to C1R-B*3501 cells pulsed with various concentrations of Pol peptide at an E/T ratio of 1:1. Percent cytokine-producing cells in response to C1R-B*3501 cells without pulsing the peptide was always <0.2%. Data shown are the means of duplicate assays. (C) Cytotoxic activity of the tetramer-sorted TCR-transduced cells and of the parental CTL 589 was analyzed in response to C1R-B*3501 cells pulsed with various concentrations of Pol peptide or a series of mutant peptides at an E/T ratio of 2:1. The sequences of the peptides tested are shown in each graph. The specific lysis activity of both cells in response to C1R-B*3501 cells without pulsing the peptide was always <5%. Data shown are the means of duplicate assays. (D) Cytotoxic activity of the tetramer-sorted TCR-transduced cells and of the parental CTL 589 was analyzed in response to C1R cells or transfectants expressing HLA-B*3501 or HLA-B*5101 pulsed with various concentrations of Pol peptide. The E/T ratio was 2:1. Data shown are the means of duplicate assays.

cells had promiscuous HLA restriction like their parental CTL 589. Moreover, there were virtually no signs of allogeneic responses in the TCR-transduced T cells toward target cells expressing these HLA molecules (Fig. 3D), despite the fact that the recipient T cells were derived from an individual who carries *HLA-B**3501 but not *HLA-B**5101.

These results indicate that the genetic transfer of HIV-specific TCR resulted in redirection of the antigen specificity of recipient T cells toward the HIV antigen, with activity of cytotoxicity and cytokine secretion (Fig. 3B, C) as well as specificity to peptides and HLA restriction elements (Fig. 3C, D) that were all comparable to those of the parental CTL clone. However, the tetramer binding level of the TCR-transduced T cells was slightly lower than that of the parental CTL clone (Fig. 3A).

2.4 Relationships between levels of tetramer binding and functional avidity of the TCR-transduced T cells

Ectopic expression of α and β chains of TCR in mature T cells will lead to the heterologous pairing of either protein with endogenous α and β chains of TCR. These 'hybrid' TCR would also result in decreased surface expression of the desired pair of TCR (*i.e.* ectopic chains) and may potentially lead to reduced avidity of the TCR-transduced T cells. In fact, TCR-transduced T cells obtained after sorting tetramer$^+$ fractions showed decreased tetramer binding activity compared to the parental CTL 589 (Fig. 3A). To examine this issue, we analyzed the relationships between HLA tetramer binding activity and functional capacity of the TCR-transduced T cells, since the tetramer binding activity of the TCR-transduced T cells could be dependent on the surface expression of the desired pair of TCR.

We first examined the effect of the expression level of transgenes on the surface expression of the desired TCR chains on the TCR-transduced T cells. The TCR-transduced T cells were gated in terms of tetramer$^+$ and tetramer$^-$ phenotypes, and each fraction was then analyzed for its expression levels of GFP and CD24 antigen (Fig. 4A). The tetramer$^+$ subset showed about twofold higher MFI values for both GFP and CD24 than the tetramer$^-$ subset (Fig. 4A), indicating that the difference in the levels of transgene expression contributed at some extent to the different tetramer binding activities of the TCR-transduced T cells. However, the tetramer$^+$ and tetramer$^-$ subsets showed markedly

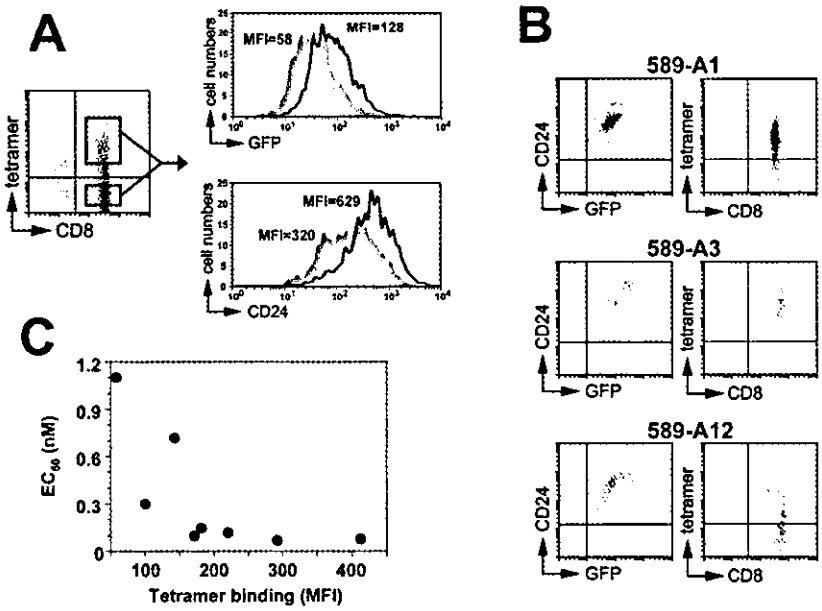

Fig. 4. Functional avidity versus tetramer binding activity of TCR-transduced cells. (A) The tetramer$^+$ (solid lines) and tetramer$^-$ (shaded areas) subsets of TCR-transduced, antigen-stimulated cells were gated and analyzed for their expression levels of GFP and CD24. The MFI values of each subset are indicated in the histograms. (B, C) The single-cell-sorted TCR-transduced cells were analyzed for their levels of tetramer binding and transgene expressions by flow cytometry. Representative dot plots are shown (B). Relationships between the tetramer binding levels and EC_{50} values of the TCR-transduced clones were analyzed (C). The EC_{50} values were determined from the cytolytic activities of these cells toward C1R-B*3501 cells pulsed with various concentrations of the Pol peptide at an E/T ratio of 2:1. The data are summarized in Table 1.

overlapped patterns in terms of both GFP and CD24 expression (Fig. 4A), suggesting that other factors, including heterodimer formation with endogenous TCR chains, influence the different surface expression of the desired TCR chains on the individual TCR-transduced T cells.

Next, the $CD8^+$ GFP^+ $CD24^+$ subset of transduced cells, including both $tetramer^+$ and $tetramer^-$ fractions, was single-cell sorted and cultured for 12 days in the presence of autologous feeder cells pulsed with the Pol peptide. Among the cells that had been positive for growth, eight clones were analyzed for their expression levels of CD8, GFP, and CD24 as well as for their levels of tetramer binding (Table 1, Fig. 4B). Tetramer binding by these cells resulted in MFI values ranging from approximately 50 to 400 (Table 1).

We then analyzed their cytotoxic activities toward C1R-B*3501 cells pulsed with various concentrations of the Pol peptide and determined their functional avidities as the 50% effective concentration of the Pol peptide (EC_{50}). The EC_{50} values of the TCR-transduced clones were also distributed widely, ranging from 0.07 to 1.1 nM (Table 1). A set of clones with the higher tetramer binding activity (MFI>200) showed relatively constant EC_{50} values (average EC_{50} of 0.09±0.026 nM), and these values were comparable to the value for the parental CTL 589 (EC_{50} of 0.07 nM) (Fig. 4C). In contrast, another set of clones with the lower tetramer binding activity (MFI<200) showed low functional avidity (average EC_{50} of 0.47±0.43 nM) and their EC_{50} values varied, mostly depending on their tetramer binding activity (Fig. 4C). These results are in good agreement with the data showing that the tetramer-sorted TCR-transduced T cells and the parental CTL 589 had comparable functional avidity (Fig. 3B, C) and yet had different tetramer binding activity (Fig. 3A).

Thus we concluded that although both the level of transgene expression and the extent of 'hybrid' TCR formation can differentially affect the surface expression levels of desired TCR chains in the individual TCR-transduced T cells, the fraction of TCR-transduced T cells with the surface expression of desired TCR chains above a certain threshold (*i.e.* MFI>200 for the tetramer under the assay condition tested in this study) had virtually identical functional avidities toward peptide-pulsed target cells.

2.5 Antiviral replication activity of TCR-transduced CD8 T cells

We further examined the ability of TCR-transduced cells to inhibit HIV-1 replication *in vitro*. CD4-expressing 221-B*3501 cells were infected with HIV-1 HXB2D and co-cultured with the TCR-transduced clones for 6 days. We determined the amounts of p24 Gag antigen production in the culture supernatant by an enzyme-linked immunosorbent assay (Fig. 5A), and the frequency of $p24^+$ cells, by flow cytometric analysis (Fig. 5B). In both assays, all of the TCR-transduced clones showed substantial suppression activity toward HIV-1 replication, whereas virtually no suppression activity was found in the case of mock-transduced cells (Fig. 5). It is of interest that all of the tested clones showed similar inhibitory activity against HIV replication (Fig. 5), although the functional avidity of these clones toward peptide-pulsed target cells showed a broad distribution, with EC_{50} values ranging

Table 1. Summary of functional and phenotypic characterization of mock- or TCR-transduced cells

	EC_{50} (nM)	MFI			
		Tetramer	CD8	GFP	CD24
A1	0.10	172	453	143	769
A3	0.08	412	584	286	1,405
A6	0.72	143	434	55.5	1,353
A11	0.15	182	311	220	161
A12	1.10	57.5	529	106	633
A14	0.07	292	453	288	809
A16	0.12	221	422	650	1,239
A23	0.30	100	331	50.7	1,101
Mock	>100	3.8	303	3.3	2.7

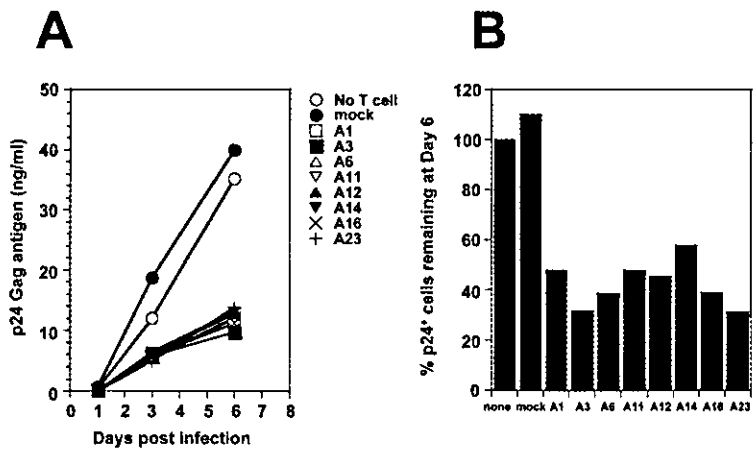

Fig. 5. Inhibitory potency of TCR-transduced clones toward HIV-1 replication *in vitro*. (A) The 221-CD4-B*3501 cells (2×10^4 cells/well) infected with HIV-1 HXB2D were co-cultured or not with the mock- or TCR-transduced clones at an E/T ratio of 0.5:1. On days 1, 3, and 6 post-infection, a portion of the culture supernatant was collected and the amounts of p24 antigen produced by the HIV-infected 221 cells were then determined. (B) The HIV-infected 221 cells were collected on day 6 and intracellularly stained with FITC-conjugated mAb specific for the p24 Gag antigen for flow cytometry. The relative frequency of $p24^+$ cells at day 6 in the co-cultures with the indicated TCR-transduced T cells is shown. The data are given as the means of duplicate assays in one experiment. These results are representative of those of two additional independent experiments.

from 0.07 to 1.1 nM (Table 1). Particularly, clone A12 showed potent suppression activity for HIV replication (Fig. 5) despite its modest functional avidity and tetramer binding activity (Table 1). This finding appears to be consistent with the recently reported study demonstrating that epitope specificity of CTL, rather than functional avidity of CTL, is a key factor in the ability of CTL to control HIV replication, and that the process of epitope presentation on HIV-infected cells greatly influences CTL efficiency *in vivo* [29].

It should be noted that we used an Epstein-Barr virus-transformed B cell line as an HIV-infected target cell. Since kinetics of HIV replication is generally variable dependent on cell types as well as HIV strains, it may be possible that inhibition potency of HIV-specific CD8 T cells toward HIV replication is also variable, dependent on host cell types both *in vitro* and *in vivo*. In this regard, suppression activity of HIV-specific CD8 T cells toward HIV replication is intriguing as assessed using primary human CD4 T cells and macrophages as HIV-infected target cells.

The introduction of a chimeric TCR containing human CD4 or HIV-specific immunoglobulin sequences linked to the signaling domain of the TCR ζ chain (universal TCR) into CD8 T cells for targeting of HIV-infected cells has been reported [30–32]. In comparison with chimeric receptor approaches, the transfer of native TCR may have distinct advantages. In a chimeric receptor approach, high-affinity receptor-ligand interaction will most likely result in incomplete T cell activation owing to omission of immunoreceptor tyrosine-based activation motifs normally present in the CD3 complex. More importantly, it is unclear whether or not chimeric receptor-ligand engagement results in functional maturation of transduced T cells to a fully differentiated effector phenotype, which character of CD8 T cells has been shown to be important to control HIV replication *in vivo* [16].

In summary, the data presented in this study suggest that the reconstitution of HIV-specific immunoreactive T cells engineered by genetic transfer of the native form of αβ TCR into primary CD8 T cells is a viable strategy to suppress HIV replication. This approach represents a potential alternative to other types of immunotherapy for HIV infection, although further studies to elucidate the phenotypic and functional properties of such engineered HIV-specific CD8 T cells *in vivo* are required.

3 Materials and methods

3.1 T cell culture

The Jurkat variant that lacked expression of either α or β TCR was kindly provided by Bent Rubin (Unite de Physiopathologie Cellulaire et Moleculaire, CNRS, France) and maintained in RPMI 1640 and 10% fetal bovine serum. CTL clone 589 cells were generated before [33] from an HIV-infected patient (*HLA-A*2402/A*2601*, *HLA-B*3501/B*5101*) following stimulation of peripheral lymphocytes with an HLA-B*3501-restricted and HIV Pol-derived epitope peptide (HIV $Pol_{448-456}$; IPLTEEAEL). CTL clones were maintained in

RPMI 1640 and 10% human serum supplemented with 200 U/ml rIL-2, and were stimulated weekly with irradiated C1R-B*3501 cells or autologous PBMC pulsed with 100 nM Pol peptide as needed.

3.2 Construction of retroviral vectors and gene transfer

The genes encoding full-length α and β TCR of CTL 589 (DDBJ accession numbers AB164620 and AB164621, respectively) were subcloned into the pGC-based retroviral vector pGCDNsap(MSCV) [28]. The genes encoding a mouse heat-stable antigen (CD24) or GFP were also incorporated into the constructs along with an IRES following the α or β TCR gene (Fig. 1A) to facilitate monitoring the expression of α or β TCR genes, respectively, in the transduced cells. Amphotropic retrovirus containing each of these constructs was then prepared, essentially as described previously [28].

Human primary CD8 T cells were isolated from PBMC of an HIV-negative healthy donor (*HLA-A*0201/A*2402, HLA-B*3501/B*4002*) using anti-CD8 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). The resultant CD8 T cells (>90% of the cells were $CD8^+$) were activated by plate-coated anti-CD3 mAb (OKT3) for 3 days, plated on recombinant fibronectin-coated plates (Takara Shuzo, Otsu, Japan), and incubated with the retrovirus supernatant containing the TCR 589 α-IRES-CD24 gene for 72 h, during which interval the cells were exposed to fresh retrovirus supernatant every 12 h. Transduced CD8 T cells expressing CD24 antigens were isolated by use of PE-labeled anti-CD24 mAb (PharMingen, San Diego, CA) and anti-PE magnetic beads (Miltenyi Biotech). The isolated cells were subsequently transduced by the construct containing TCR 589 β-IRES-GFP, as above. The fraction of transduced CD8 T cells that were positive for GFP, CD24, and CD8 molecules was obtained by sorting with a FacsVantage (BD Biosciences, San Jose, CA). The sorted T cells were propagated by stimulation with OKT3 mAb and subsequently by irradiated autologous PBMC pulsed with 100 nM Pol peptide. These T cells were maintained in RPMI 1640 and 10% human autologous serum supplemented with 200 U/ml rIL-2.

3.3 HLA-tetramer analysis

The HLA-B*3501-tetramer complex with allophycocyanin-conjugated avidin (Molecular Probes, Inc., Eugene, OR) was prepared as previously described [26]. T cells were first stained by the tetramer for 15 min at 37°C, and then stained for 20 min at 4°C with other antibodies, such as anti-CD8 mAb and anti-mouse CD24 mAb conjugated to peridinin chlorophyll protein (PerCP) and PE (BD Biosciences), respectively. They were then washed twice with a washing buffer (2% newborn bovine serum in phosphate-buffered saline) and fixed in a 1% paraformaldehyde solution. The resultant cells were analyzed by flow cytometry.

3.4 Cytotoxicity assay

The cytotoxic activity of the CTL clone 589 and the TCR-transduced T cells was determined by a standard ^{51}Cr-release assay as described [26]. 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 for 1 h and incubated with the effector T cells for an additional 4 h at 37°C. For virus-infected target cells, C1R-B*3501 cells or 221-B*3501 cells expressing human CD4 antigen (2×10^3 cells/well) were infected with HIV-1 HXB2D or HIV-1 LAI. These viruses have the same epitope sequence as was used for the synthetic Pol peptide. The cells were subsequently labeled with ^{51}Cr and incubated with the effector T cells for 6 h at 37°C. Note that >80% cells were positive for intracellular p24 Gag antigen when HIV-infected cells were used for CTL assays.

3.5 Cytokine secretion assay

CTL clone and TCR-transduced T cells were co-cultured with 221-CD4-B*3501 cells pulsed with various concentrations of the Pol peptide for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the cultures were continued for an additional 4 h. Cells were permeabilized and stained with anti-IFN-γ and anti-TNF-α mAb conjugated to PE and allophycocyanin, respectively (PharMingen), as previously described [34]. Frequencies of IFN-γ^+ and TNF-α^+ $CD8^+$ cells were determined by flow cytometry.

3.6 Antiviral replication assay

The 221-CD4-B*3501 cells were infected with HIV-1 HXB2D at 10 $TCID_{50}$/ml for 6 h at 37°C and washed with culture medium. The resultant infected cells were plated in a 96-well U-bottom plate at 2×10^4 cells/well, and then the mock- or TCR-transduced $CD8^+$ T cells were added to each well at an E/T ratio of 0.5:1. The culture medium contained RPMI 1640 and 10% human serum supplemented with 200 U/ml rIL-2. On days 1, 3, and 6 post-infection, a portion of the culture supernatant was collected and the amounts of p24 antigen produced by HIV-infected 221 cells were determined by use of an enzyme-linked immunosorbent assay (ZeptoMetrix Corporation, New York, NY). In addition, on day 6, cells were collected, intracellularly stained with FITC-conjugated mAb specific for p24 Gag antigen (KC57; Coulter Immunology, Hialeah, FL), and analyzed by flow cytometry.

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