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

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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-Vα12

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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

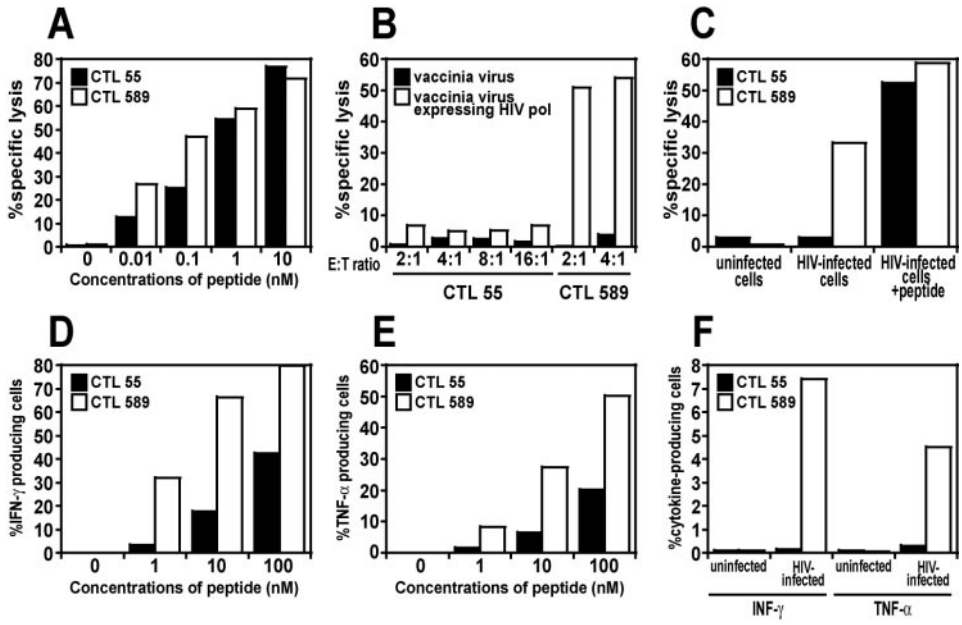


FIGURE 1. Analysis of effector functions of the CTL clones. *A* and *B*, Cytotoxic activity of CTL 55 and 589 toward C1R-B*3501 cells either pulsed with the indicated concentrations of the Pol peptide (*A*) or infected with vaccinia virus expressing HIV-1 GagPol polyproteins at the indicated E:T cell 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.

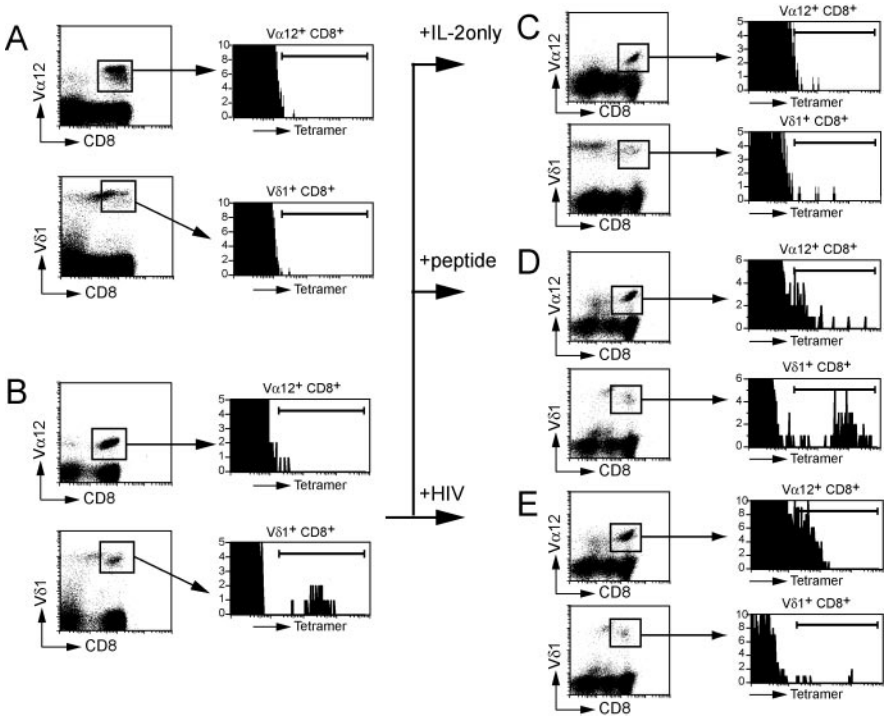


FIGURE 2. Ex vivo analysis of the tetramer$^+$ CD8 T cells in the peripheral lymphocytes in response to Ag stimulation. Cryopreserved PBMC of a representative HIV-negative donor (5×10^6; *A*) and the HIV-infected patient (1×10^6; *B*) were stained with the tetramer and anti-CD8 and anti-TCR Abs. The 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.

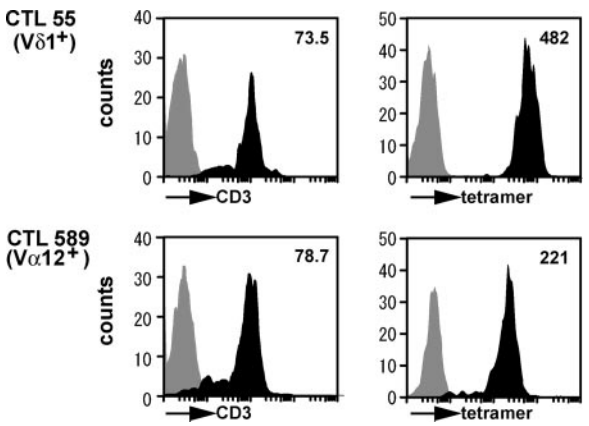


FIGURE 3. Flow cytometric analysis of CTL clones. Two CTL clones, CTL 55 and 589, selected from the Vδ1$^+$ and Vα12$^+$ CD8 T cell subsets, respectively, were stained (■) or not stained (▨) with anti-CD3 mAb or the tetramer at 4°C, and analyzed by flow cytometry. MFI values are shown in the *upper right*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

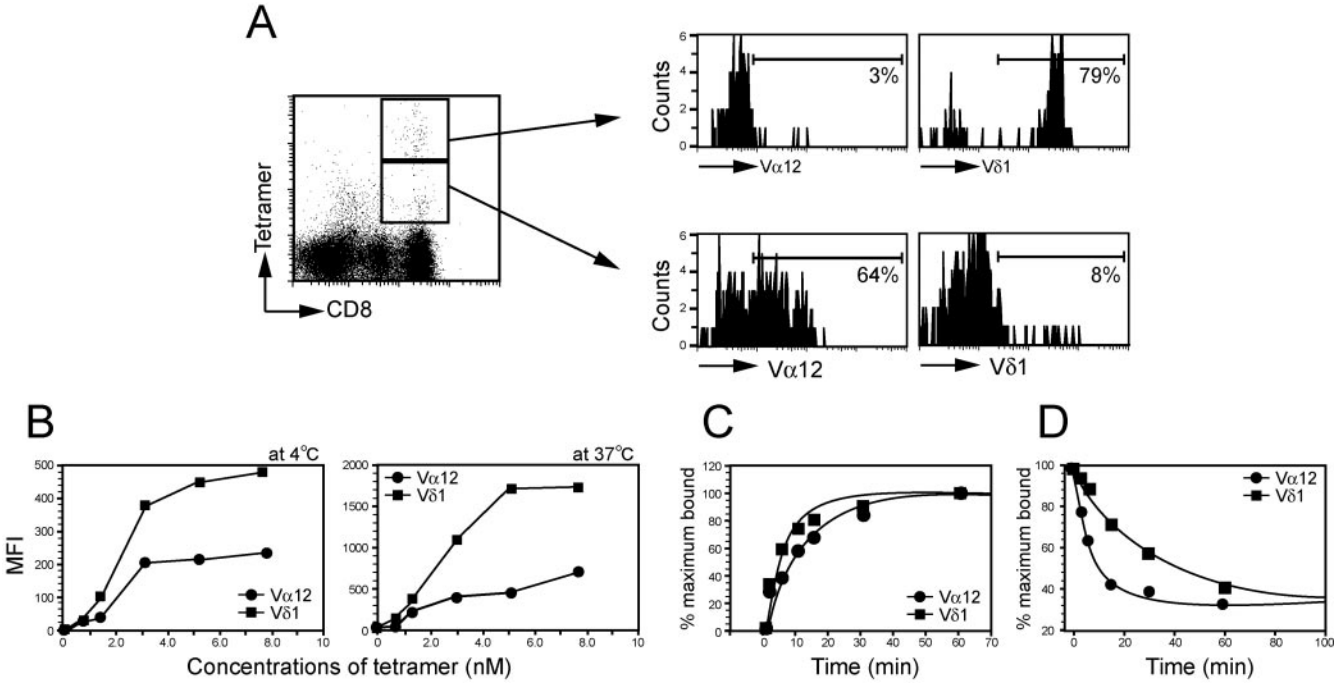


FIGURE 4. Flow cytometric analysis of kinetic interaction between the tetramer and CD8 T cell subsets. *A*, TCR analysis of tetramer^{high} and tetramer^{low} CD8 T cell subsets in Pol peptide-stimulated peripheral lymphocytes of the HIV-infected patient. *B*, Tetramer binding activities of V$\delta1^+$ 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.

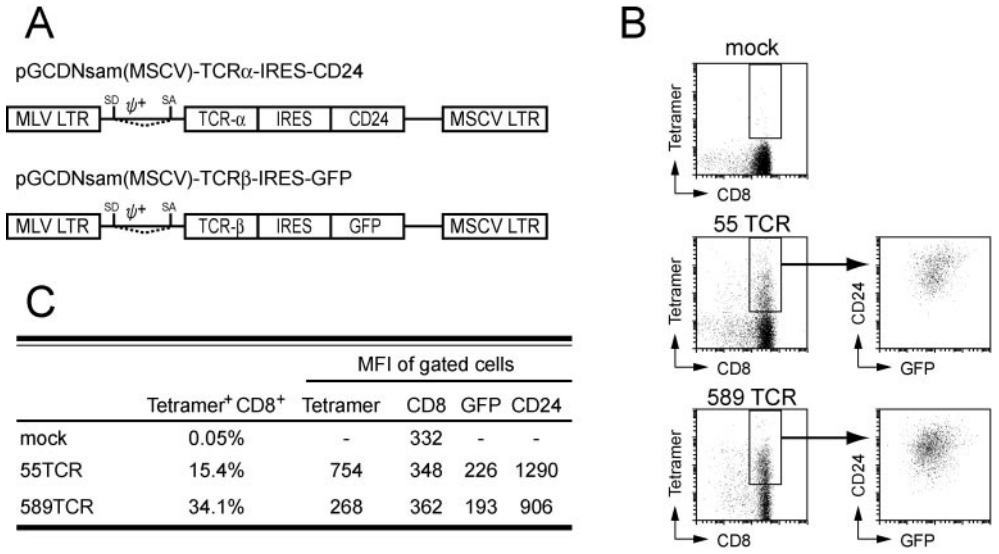
the V$\delta1^+$ and V$\alpha12^+$tetramer$^+$CD8 T cells were solely due to the difference in the TCR.

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.

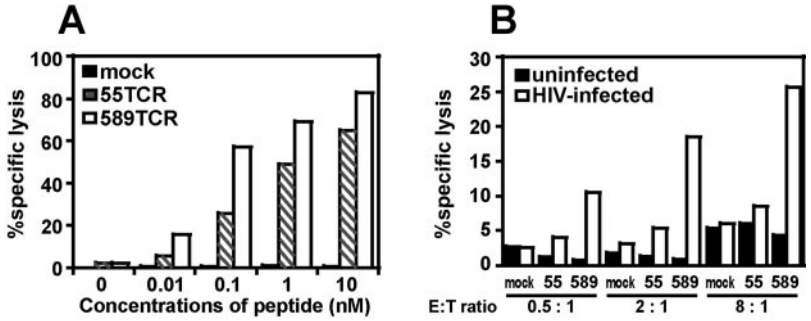


FIGURE 6. Cytotoxic activity of the 55 and 589 TCR-transduced CD8 T cells. The CD8$^+$tetramer$^+$ subsets of the 55 and 589 TCR-transduced cells were sorted (see Fig. 5) and analyzed for their cytotoxic activity toward C1R-B*3501 cells pulsed with the indicated concentrations of the Pol peptide at an E:T cell ratio of 1:1 (*A*). The same set of TCR-transduced cells was assessed for their cytotoxic activity toward C1R-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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References

1. McMichael, A. J., and S. L. Rowland-Jones. 2001. Cellular immune responses to HIV. *Nature 410:980*.
2. Lieberman, J., P. Shankar, N. Manjunath, and J. Andersson. 2001. Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. *Blood 98:1667*.
3. van Baarle, D., S. Kostense, M. H. van Oers, D. Hamann, and F. Miedema. 2002. Failing immune control as a result of impaired $CD8^+$ T-cell maturation: CD27 might provide a clue. *Trends Immunol. 23:586*.
4. Yang, O. O. 2003. Will we be able to "spot" an effective HIV-1 vaccine? *Trends Immunol. 24:67*.
5. Hanke, T. 2003. Development of prophylactic AIDS vaccines: the current state of affairs. *Curr. Opin. Mol. Ther. 5:25*.
6. Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, et al. 2000. HIV-specific $CD8^+$ T cells produce antiviral cytokines but are impaired in cytolytic function. *J. Exp. Med. 192:63*.
7. Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, et al. 2002. HIV-specific $CD8^+$ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat. Immunol. 3:1061*.
8. Spencer, J., and T. Braciale. 2000. Incomplete $CD8^+$ T lymphocyte differentiation as a mechanism for subdominant cytotoxic T lymphocyte responses to a viral antigen. *J. Exp. Med. 191:1687*.
9. Franco, A., D. Tilly, I. Gramaglia, M. Croft, L. Cipolla, M. Meldal, and H. Grey. 2000. Epitope affinity for MHC class I determines helper requirement for CTL priming. *Nat. Immunol. 1:145*.
10. Fuller, C., and V. Braciale. 1998. Selective induction of $CD8^+$ cytotoxic T lymphocyte effector function by staphylococcus enterotoxin B. *J. Immunol. 161:5179*.
11. Kersh, E. N., G. J. Kersh, and P. M. Allen. 1999. Partially phosphorylated T cell receptor ζ molecules can inhibit T cell activation. *J. Exp. Med. 190:1627*.
12. Alam, S. M., P. J. Travers, J. L. Wung, W. Nasholds, S. Redpath, S. C. Jameson, and N. R. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature 381:616*.
13. Savage, P. A., J. J. Boniface, and M. M. Davis. 1999. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity 10:485*.
14. Holler, P. D., and D. M. Kranz. 2003. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity 18:255*.
15. al-Ramadi, B. K., M. T. Jelonek, L. F. Boyd, D. H. Margulies, and A. L. Bothwell. 1995. Lack of strict correlation of functional sensitization with the apparent affinity of MHC/peptide complexes for the TCR. *J. Immunol. 155:662*.
16. Alam, S. M., G. M. Davies, C. M. Lin, T. Zal, W. Nasholds, S. C. Jameson, K. A. Hogquist, N. R. Gascoigne, and P. J. Travers. 1999. Qualitative and quantitative differences in T cell receptor binding of agonist and antagonist ligands. *Immunity 10:227*.
17. Baker, B. M., S. J. Gagnon, W. E. Biddison, and D. C. Wiley. 2000. Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling. *Immunity 13:475*.
18. Kalergis, A. M., N. Boucheron, M. A. Doucey, E. Palmieri, E. C. Goyarts, Z. Vegh, I. F. Luescher, and S. G. Nathenson. 2001. Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. *Nat. Immunol. 2:229*.
19. Savage, P. A., and M. M. Davis. 2001. A kinetic window constricts the T cell receptor repertoire in the thymus. *Immunity 14:243*.
20. Tomiyama, H., K. Miwa, H. Shiga, Y. I. Moore, S. Oka, A. Iwamoto, Y. Kaneko, and M. Takiguchi. 1997. Evidence of presentation of multiple HIV-1 cytotoxic T lymphocyte epitopes by HLA-B*3501 molecules that are associated with the accelerated progression of AIDS. *J. Immunol. 158:5026*.
21. Ferrini, S., I. Prigione, C. Bottino, E. Ciccone, G. Tambussi, S. Mammoliti, L. Moretta, and A. Moretta. 1989. Monoclonal antibodies which react with the T cell receptor $\gamma\delta$ recognize different subsets of $CD3^+WT31^-$ T lymphocytes. *Eur. J. Immunol. 19:57*.
22. Ueno, T., H. Tomiyama, and M. Takiguchi. 2002. Single T cell receptor-mediated recognition of an identical HIV-derived peptide presented by multiple HLA class I molecules. *J. Immunol. 169:4961*.
23. Ueno, T., H. Tomiyama, M. Fujiwara, S. Oka, and M. Takiguchi. 2003. HLA class I-restricted recognition of an HIV-derived epitope peptide by a human T cell receptor α chain having a Vδ1 variable segment. *Eur. J. Immunol. 33:2910*.
24. Kaneko, S., M. Onodera, Y. Fujiki, T. Nagasawa, and H. Nakauchi. 2001. Simplified retroviral vector GCsap with murine stem cell virus long terminal repeat allows high and continued expression of enhanced green fluorescent protein by human hematopoietic progenitors engrafted in nonobese diabetic/severe combined immunodeficient mice. *Hum. Gene Ther. 12:35*.
25. Willcox, B. E., G. F. Gao, J. R. Wyer, J. E. Ladbury, J. I. Bell, B. K. Jakobsen, and P. A. van der Merwe. 1999. TCR binding to peptide-MHC stabilizes a flexible recognition interface. *Immunity 10:357*.
26. Whelan, J. A., P. R. Dunbar, D. A. Price, M. A. Purbhoo, F. Lechner, G. S. Ogg, G. Griffiths, R. E. Phillips, V. Cerundolo, and A. K. Sewell. 1999. Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent. *J. Immunol. 163:4342*.
27. Crawford, F., H. Kozono, J. White, P. Marrack, and J. Kappler. 1998. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity 8:675*.
28. Goulder, P. J., Y. Tang, C. Brander, M. R. Betts, M. Altfeld, K. Annamalai, A. Trocha, S. He, E. S. Rosenberg, G. Ogg, et al. 2000. Functionally inert HIV-specific cytotoxic T lymphocytes do not play a major role in chronically infected adults and children. *J. Exp. Med. 192:1819*.
29. Kelleher, A. D., and S. L. Rowland-Jones. 2000. Functions of tetramer-stained HIV-specific $CD4^+$ and $CD8^+$ T cells. *Curr. Opin. Immunol. 12:370*.
30. Kostense, S., K. Vandenberghe, J. Joling, D. Van Baarle, N. Nanlohy, E. Manting, and F. Miedema. 2002. Persistent numbers of $tetramer^+$ $CD8^+$ T cells, but loss of interferon-γ^+ HIV-specific T cells during progression to AIDS. *Blood 99:2505*.
31. Demotte, N., D. Colau, S. Ottaviani, D. Godelaine, A. Van Pel, T. Boon, and P. van der Bruggen. 2002. A reversible functional defect of $CD8^+$ T lymphocytes involving loss of tetramer labeling. *Eur. J. Immunol. 32:1688*.
32. Echchakir, H., G. Dorothee, I. Vergnon, J. Menez, S. Chouaib, and F. Mami-Chouaib. 2002. Cytotoxic T lymphocytes directed against a tumor-specific mutated antigen display similar HLA tetramer binding but distinct functional avidity and tissue distribution. *Proc. Natl. Acad. Sci. USA 99:9358*.
33. Lim, D. G., K. B. Bourcier, G. J. Freeman, and D. A. Hafler. 2000. Examination of $CD8^+$ T cell function in humans using MHC class I tetramers: similar cytotoxicity but variable proliferation and cytokine production among different clonal $CD8^+$ T cells specific to a single viral epitope. *J. Immunol. 165:6214*.
34. McKeithan, T. W. 1995. Kinetic proofreading in T-cell receptor signal transduction. *Proc. Natl. Acad. Sci. USA 92:5042*.
35. Davis, M., J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by $\alpha\beta$ T cell receptors. *Annu. Rev. Immunol. 16:523*.
36. Valitutti, S., and A. Lanzavecchia. 1997. Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. *Immunol. Today 18:299*.
37. Holler, P. D., L. K. Chlewicki, and D. M. Kranz. 2003. TCRs with high affinity for foreign pMHC show self-reactivity. *Nat. Immunol. 4:55*.
38. Keir, M. E., M. G. Rosenberg, J. K. Sandberg, K. A. Jordan, A. Wiznia, D. F. Nixon, C. A. Stoddart, and J. M. McCune. 2002. Generation of $CD3^+CD8^{low}$ thymocytes in the HIV type 1-infected thymus. *J. Immunol. 169:2788.*
39. Keir, M. E., C. A. Stoddart, V. Linquist-Stepps, M. E. Moreno, and J. M. McCune. 2002. IFN-α secretion by type 2 predendritic cells up-regulates MHC class I in the HIV-1-infected thymus. *J. Immunol. 168:325*.