HLA class I-restricted recognition of an HIV-derived epitope peptide by a human T cell receptor α chain having a Vδ1 variable segment

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A subset of human peripheral αβ T cells have been shown to express TCR α chains containing Vδ1 segments, although what antigens the Vδ1$^+$ αβ T cells recognize via these TCR is not known yet. We eventually established a human CD8 T cell clone that expressed αβ TCR and Vδ1 antigens. Corroboratively, a unique in-frame Vδ1.1JαCα transcript was found in the clone. The clone showed cytotoxic activity and IFN-γ production towards cells expressing HLA-B*3501 pulsed with an HIV Pol-derived epitope peptide (IPLTEEAEL). By flow-cytometric analysis *ex vivo* using an HLA-B*3501 tetramer, a fraction of Vδ1$^+$CD8$^+$tetramer$^+$ cells was found in peripheral lymphocytes of an HIV-infected patient, indicating the existence of HLA-restricted and HIV-specific Vδ1$^+$ CD8 T cells *in vivo*. Moreover, retrovirus-mediated transfer of the TCR-encoding genes into TCR-negative hybridoma cells showed that the transduced cells were stained by the tetramer and were activated in response to the Pol peptide, further confirming the ligand specificity of the TCR. Together, these results clearly demonstrate that Vδ1$^+$ αβ TCR are restricted to engaging peptide antigens in the context of classical MHC class I molecules, highlighting the difference in the ligand specificity between Vδ1$^+$ αβ TCR and Vδ1$^+$ γδ TCR.

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1 Introduction

The mammalian immune system includes three types of cells, B cells, αβ T cells, and γδ T cells that use somatic gene rearrangement to produce genes encoding their defining surface receptors. Among them, αβ T cells are restricted to engaging antigen in the context of a major histocompatibility complex (MHC) molecule, whereas γδ T cells recognize small molecules and intact proteins without the requirement for antigen processing [1]. Most γδ T cells bear a tissue-specific, restricted set of Vγ and Vδ domains. For example, human γδ T cells bearing Vδ1-encoded TCR account for the vast majority of γδ T cells in tissues such as the spleen and intestine [2]. Although the antigens recognized by the Vδ1$^+$ γδ T cells remain enigmatic, recent studies have demonstrated that a subset of Vδ1$^+$ γδ T cells can recognize stress-inducible MHC class I-like MIC molecules expressed by epithelial cells via specific TCR interactions [3]. In addition, the major tissue population of γδ T cells expressing Vδ1 TCR have been shown to recognize CD1c, one member of a family of nonpolymorphic CD1 molecules expressed on the surface of dendritic cells and B cells [4].

TCR genes are assembled from separate variable (V), diversity (D), and joining (J) elements by a site-specific recombination mechanism termed V(D)J recombination. The TCR α and δ genes form a single complex locus, spanning approximately one mega base on chromosome 14 in humans. Despite the δ locus being embedded in the α locus in the chromosome, the α and δ genes encode proteins participating exclusively in the αβ or γδ TCR complexes, which in turn are expressed in the two separate lineages of αβ and γδ T cells, respectively (see reference [5] for reviews). It has been reported, however, that some Vδ genes are rearranged either to Jα or to Jδ genes and can therefore be used in the synthesis of TCR-α or δ chains, which are expressed in the αβ and γδ T cells, respectively. For example, a subset of human peripheral T cells expressing Vδ1 antigens on their surface were found in both αβ and γδ T cell lineages as

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revealed by multi-color flow cytometry [6]. Sequencing analysis of TCR genes revealed the existence of Vδ1JαCα transcripts encoded by a unique Vδ1Jα rearrangement in the Vδ1-expressing αβ T cells [7]. However, what antigen or ligand the Vδ1-expressing αβ T cells recognize via the TCR has not been directly investigated yet. In addition, functional properties of such Vδ1-expressing αβ T cells in the periphery have yet to be examined.

In the present study, we clearly demonstrated that a Vδ1⁺ αβ T cell clone established from an HIV-infected patient could kill cells pulsed with an HIV Pol-derived epitope peptide presented by syngeneic HLA-B*3501. In addition, the finding that the Vδ1-bearing αβ TCR could recognize an engaging peptide as antigen in the context of a classical HLA class I molecule highlights the difference in the ligand specificity between Vδ1-expressing αβ and γδ TCR.

2 Results and discussions

2.1 Generation of a T cell clone and its functional characterization

An HIV Pol peptide (HIV $Pol_{448–456}$; IPLTEEAEL) has been shown to be a T cell epitope endogenously processed and presented by HLA-B*3501 [8]. We established CD8 T cell clones from an HIV-infected patient (carrying HLA-B*3501) following stimulation of peripheral lymphocytes from the patient with the Pol peptide. Among them, the T cell clone, designated as CTL 55, showed substantial cytotoxic activity toward T2 cells expressing HLA-B*3501 pulsed with the Pol peptide even at 0.1 nM concentration (Fig. 1A), which clone showed potency comparable to that of some other T cell clones with the same antigen specificity [8]. CTL 55 also showed significant IFN-γ production in response to the peptide-pulsed.221 cells expressing HLA-B*3501 as revealed by intracellular flow cytometry (Fig. 1B). These functional assays indicated that CTL 55 could recognize the Pol peptide presented by HLA-B*3501.

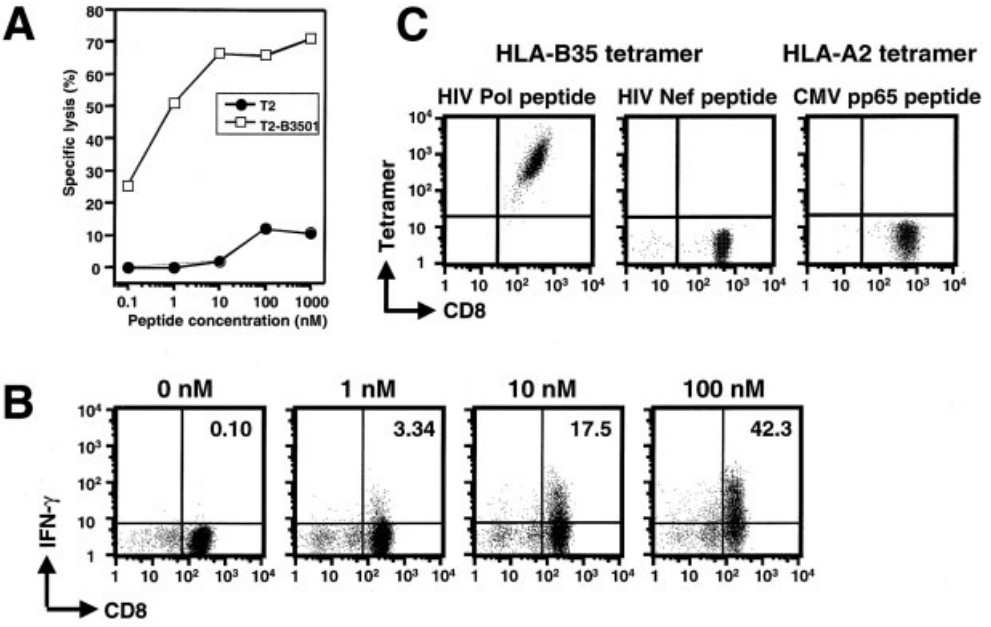

Fig. 1. Functional analysis of CTL 55 in response to the Pol peptide. (A) Cytotoxic activity of CTL 55 was examined by a standard Cr^{51}-release assay against T2 cells or a transfectant expressing HLA-B*3501 pulsed with the indicated concentrations of Pol peptide at effector to target ratio of 2:1. (B) IFN-γ production activity of CTL 55 was examined by an intracellular flow cytometric assay against.221 transfectants expressing HLA-B*3501 pulsed with the indicated concentrations of the Pol peptide at effector to target ratio of 1:1. Per cent $CD8^+IFN\text{-}\gamma^+$ cells is shown in each panel. (C) CTL 55 were stained with the HLA-B*3501 tetramer in complex with the HIV Pol peptide (IPLTEEAEL) or the HIV Nef peptide (RPQVPLRPMTY), or the HLA-A*0201 tetramer in complex with the human cytomegalovirus pp65 peptide (NLVPMVATV). Cells were then stained with the anti-CD8 mAb and analyzed by flow cytometry.

We next prepared an HLA-B*3501 tetramer in complex with the Pol peptide, and tested the ability of CTL 55 to bind the tetramer. As clearly shown in Fig. 1C, virtually all fractions of CTL 55 (>98%) were stained by the HLA-B35 tetramer in complex with the Pol peptide, whereas no fractions of CTL 55 (<0.1%) bound the HLA-B35 tetramer in complex with an HIV Nef peptide (RPQVPLRPMTY). Nor was stained CTL 55 by the HLA-A2 tetramer in complex with a human cytomegalovirus peptide (pp65, NLVPMVATV) as shown in Fig. 1C. These data confirmed the HLA-B35-restricted and HIV Pol epitope-specific antigen recognition property of CTL 55.

2.2 Analysis of TCR-encoding genes of CTL 55

Next we analyzed V-region segments of the TCR on CTL 55 by cloning the TCR-encoding genes by using the rapid amplification of 5'-cDNA ends protocol. When we used an anti-sense primer specific for Cα transcripts, the genes amplified by the PCR contained a Vδ1 gene segment followed by a JαCα-encoded gene segment (Table 1 and Fig. 2). This result confirmed the previous findings by Miossec et al. [6, 7] that a small subset (<0.5%) of human peripheral T cells expressed Vδ1-encoded variable segments on αβ T cells. Using a Cβ-specific antisense primer, we isolated TCR-β cDNA containing a Vβ13.3-encoded gene segment (Table 1), suggesting that the Vδ1.1-bearing TCR-α chain was paired with TCR-β chain on the surface of CTL 55.

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        10         20         30         40         50         60
atgctgttctccagcctgctgtgtgtatttgtggccttcagctactctggatcaagtgtg
M  L  F  S  S  L  L  C  V  F  V  A  F  S  Y  S  G  S  S  V

        70         80         90        100        110        120
gcccagaaggttactcaagcccagtcatcagtatccatgccagtgaggaaagcagtcacc
A  Q  K  V  T  Q  A  Q  S  S  V  S  M  P  V  R  K  A  V  T

       130        140        150        160        170        180
ctgaactgcctgtatgaaacaagttggtggtcatattatatttttttggtacaagcaactt
L  N  C  L  Y  E  T  S  W  W  S  Y  Y  I  F  W  Y  K  Q  L

       190        200        210        220        230        240
cccagcaaagagatgattttccttattcgccagggttctgatgaacagaatgcaaaaagt
P  S  K  E  M  I  F  L  I  R  Q  G  S  D  E  Q  N  A  K  S

       250        260        270        280        290        300
ggtcgctattctgtcaacttcaagaaagcagcgaaatccgtcgccttaaccatttcagcc
G  R  Y  S  V  N  F  K  K  A  A  K  S  V  A  L  T  I  S  A

       310        320        330        340        350        360
ttacagctagaagattcagcaaagtacttttgtgctcttggggaaggggggagcccagaag
L  Q  L  E  D  S  A  K  Y  F  C  A  L  G  E  G  G  A  Q  K

       370        380        390        400        410
ctggtatttggccaaggaaccaggctgactatcaacccaaatatccagaat
L  V  F  G  Q  G  T  R  L  T  I  N  P  N  I  Q  N
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Fig. 2. Nucleotide and amino acid sequence of TCR-α chain of CTL 55. TCR-α transcript of CTL 55 encoded by Vδ1.1JαCα gene segment is shown (see also Table 1).

Table 1. TCR-encoding genes isolated from CTL 55[a)]

	V gene	J segment	CDR3 sequence
Vδ1.1	TRDV1*01	TRAJ54*01	CALGEGGAQKLVF
Vβ13.3	TRBV6-1*01	TRBJ2-7*01	CASRTGGTLIEQYF

a) Alignment of variable and joining regions of the TCR genes were analyzed by IMGT, the international ImMunoGeneTics database (home page at http://imgt.cines.fr/). Complementary determining region 3 (CDR3) sequences for α and β TCR are shown.

2.3 Flow-cytometric analysis of CTL 55 and peripheral lymphocytes

Phenotypic analysis showed CTL 55 were $CD3^+$ (clone: UCHT1) lymphocytes that were positive for αβ TCR (clone: BMA031) and negative for γδ TCR (clone: B1.1, Fig. 3). In addition, mAb A13 [9], specific for an epitope encoded by Vδ1 gene segments transcribed along with either JαCα or JδCδ [6] reacted with CTL 55 (Fig. 3A). These results indicate that CTL 55 expressed αβ TCR complexes including a Vδ1 variable segment on their surface. In contrast, another Vδ1-specific mAb (clone: δTCS1) did not react with CTL 55 (data not shown), consistent with the previous observations that the δTCS1 mAb recognized the epitope spanning Vδ1 and Jδ1 domains [10].

We then examined the presence of the antigen-specific $V\delta1^+$ T lymphocytes in the periphery *in vivo*. Peripheral lymphocytes of an HIV-infected patient were directly stained by the HLA-B35 tetramer in complex with the Pol peptide, anti-CD8 mAb and anti-Vδ1 mAb, and were analyzed by flow cytometry. As shown in the Fig. 3B, a subset of $CD8^+$ $tetramer^+$ cells (0.09% in total $CD8^+$ cells) was gated and the surface expression of the Vδ1 antigen was determined. More than 70% cells in the $CD8^+$ $tetramer^+$ subset were positive for the Vδ1 antigen (Fig. 3B). In contrast, virtually no fractions of $CD8^+$ $tetramer^+$ cells (less than 0.02% in total $CD8^+$ cells) were found in peripheral lymphocytes of HIV-negative donors (Fig. 3C). These data clearly indicated the existence of the HLA-B35-restricted and HIV Pol-specific $V\delta1^+$ T cells in the peripheral lymphocytes of the HIV-infected patient.

It is conceivable that $V\delta1^+$ αβ T cells constitute a dominant repertoire of HIV Pol-specific HLA-B35-restricted T cells among HIV-infected patients carrying HLA-B35, and that the frequency of HIV Pol-specific $V\delta1^+$ T cells is correlated with disease progression, anti-retroviral treatment, or CTL escape. In this regard, we have been testing this hypothesis by examining the frequency of HIV Pol-specific $V\delta1^+$ T cells of the same patient at several

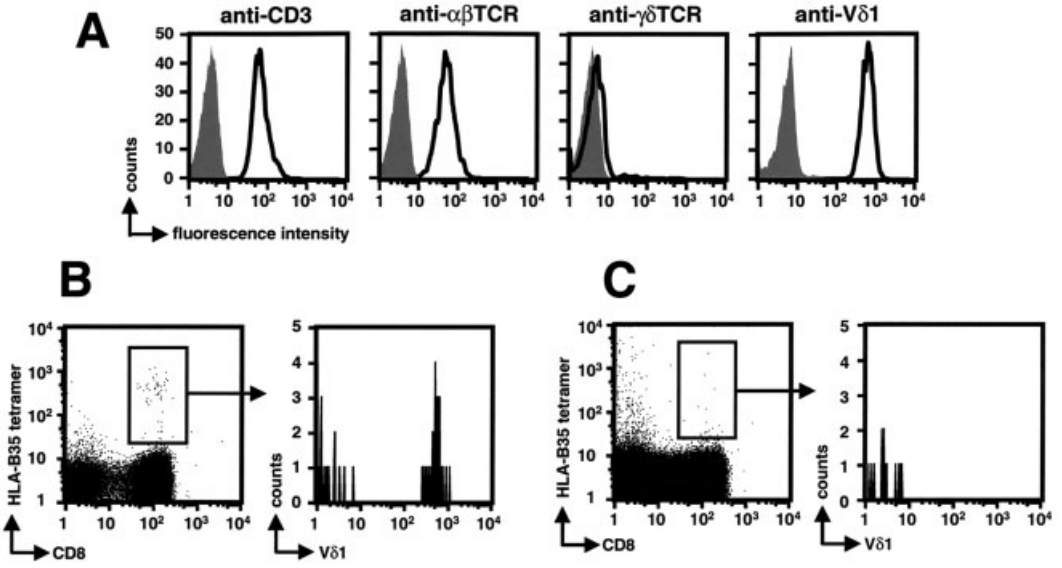

Fig. 3. Flow-cytometric analysis of antigen-specific Vδ1⁺ T lymphocytes. (A) CTL 55 was stained (solid lines) or not (shaded areas) with mAb and analyzed by flow cytometry. The mAb tested were anti-human CD3 (clone UCHT1), anti-human αβ TCR (clone BMA031), anti-human γδ TCR (clone B1.1), and anti-human Vδ1 TCR (clone A13). (B) HIV-infected patient's peripheral lymphocytes (1×10^6) were stained by the HLA-B35 tetramer, anti-CD8 mAb, and anti-Vδ1 mAb, and were analyzed by flow cytometry. A subset of CD8⁺ tetramer⁺ cells were gated (left panel) and the Vδ1 antigen expressed on the surface of gated cells was analyzed (right panel). (C) Peripheral lymphocytes (4×10^6) of an HIV-negative donor carrying HLA-B*3501 were stained and analyzed as above. The data show one representative of four different HIV-negative donors.

time points during the course of HIV infection: the frequency of Vδ1⁺ T cells in the CD8⁺ tetramer⁺ subset appeared to vary between 5 to 80% (T. Ueno, unpublished data). Further studies focusing on the association between the frequency of HIV-specific Vδ1⁺ αβ T cells and the disease progression to AIDS as well as functional properties of HIV-specific Vδ1⁺ αβ T cells toward HIV-infected cells *in vivo* are intriguing.

2.4 Reconstruction of the TCR complex on TCR-negative T cell hybridoma

Despite the allelic exclusion system operating on TCR genes, as many as 30 and 1% of human peripheral αβ T cells have been reported to coexpress two different α or β chains on their surface, respectively [11–13]. In addition, γδ T cells expressing two different γ or δ chains have also been reported [14–16]. We therefore cannot simply exclude the possibility that different TCR chains expressed on the surface of CTL 55 played a role in apparent antigen specificity, although only a unique set of α and β transcripts was identified in CTL 55.

Thus, in order to address the ligand specificity of the Vδ1.1/Vβ13.3 TCR, we used the retrovirus-mediated system for transduction of both TCR genes into TG40/CD8 cells [17], a human CD8α-expressing mouse T cell hybridoma cell line deficient in αβ TCR expression. The TCR-transduced TG40/CD8 cells showed the surface expression of CD3ε and Vδ1 antigens (Fig. 4A), indicating that the Vδ1.1/Vβ13.3 TCR had been successfully reconstituted on the surface of the transduced TG40/CD8 cells.

We then tested the TCR-transduced cells for their ability to recognize the Pol peptide presented by HLA-B*3501. The Vδ1.1/Vβ13.3 TCR-transduced cells showed specific reactivity with the tetramer, whereas mock-transduced cells did not (Fig. 4A). Furthermore, the TCR-transduced cells, but not the mock-transduced ones, secreted IL-2 in response to the HLA-B*3501-expressing cells pulsed with even a very low 10 nM concentration of the Pol peptide (Fig. 4B). These results provide direct evidence that the Vδ1.1/Vβ13.3 TCR was solely responsible for the peptide-specific response in the context of HLA-B*3501.

In summary, employing functional and phenotypic analysis including a tetramer staining technology toward the human T cell clone and human peripheral lymphocytes as well as the TCR-transduced cell, we provided evidence here that the Vδ1-bearing αβ TCR recognized a peptide presented by HLA class I molecules, as do other

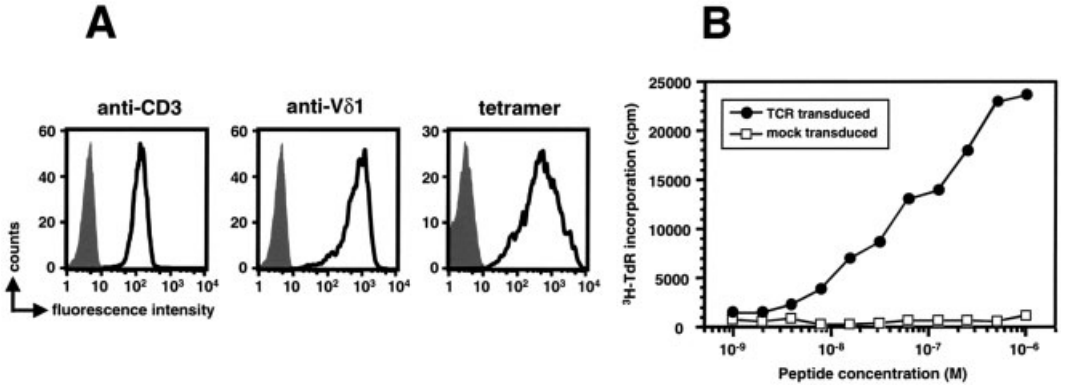

Fig. 4. Phenotypic and functional analysis of the Vδ1.1/Vβ13.3 TCR in TG40/CD8 cells following transduction with TCR-encoding genes. (A) TG40/CD8 cells transduced with the TCR-encoding genes were stained with anti-CD3ε mAb, anti-Vδ1 mAb, or HLA-B35 tetramer and were analyzed by flow cytometry (solid lines). Mock-transduced cells stained by the same reagents are shown as shaded areas. (B) The TCR- or mock-transduced TG40/CD8 cells (2×10^4) were incubated with C1R transfectants expressing HLA-B*3501 (1×10^4) in the absence or the presence of the indicated concentrations of the Pol peptide. The amounts of IL-2 secreted by TG40/CD8 cells were determined by assaying the proliferation activity of CTLL-2 cells.

Vα-bearing αβ TCR. The findings suggest that the V gene, whether comes from the α or δ locus, once assembled with JαCα as a result of somatic gene arrangements becomes a part of αβ TCR by pairing with a TCR-β chain, and thus plays a role in recognizing peptide antigens in the context of classical MHC class I molecules.

3 Materials and methods

3.1 Cell lines and antibodies

C1R, T2, and.221 transfectants expressing HLA-B*3501 (C1R-B*3501, T2-B*3501, and.221-B*3501, respectively) were previously generated [8, 18]. The TCR-negative mouse T cell hybridoma TG40 (kindly provided by T, Saito, Chiba University, Chiba, Japan) transduced with a human CD8α gene (TG40/CD8) was previously generated [17].

The following antibodies were used: phycoerythrin (PE)-conjugated anti-human IFN-γ mAb, anti-mouse CD3ε mAb (2C11), anti-human CD3 mAb (clone UCHT1), and anti-human γδ TCR mAb (clone B1.1) from PharMingen (San Diego, CA); fluorescein-5-isothiocyanate (FITC)-conjugated anti-human CD8 mAb and PE-conjugated anti-mouse immunoglobulins from DAKO Corporation (Glostrup, Denmark); and PE-conjugated anti-human TCR Pan α/β mAb (clone BMA031) from Immunotech (Marseilles Cedex, France). Anti-Vδ1 mAb A13 [9] was kindly provided by L. Moretta (Istituto di Istologia ed Embriologia Generale, Genova, Italy).

3.2 Preparation of tetrameric peptide-MHC complex

A tetrameric complex of peptide, β2-microglobulin (β2M), and HLA-B*3501 was prepared as described [17]. Briefly, an ectodomain of HLA-B35 (15 mg) and β2M (100 mg) produced in *Escherichia coli* as inclusion bodies were first solubilized in denaturing buffer containing 8 M urea and then refolded in refolding buffer (100 mM Tris-HCl, pH 8.0, containing 400 mM arginine, 2 mM ethylenediaminetetraacetic acid, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.1 mM phenylmethylsulfonyl fluoride) in the presence of 5 mg of a chemically synthesized peptide (Sawady Technologies, Inc., Tokyo) for 48 h at 4°C. The resultant 45-kDa ternary complex was purified by size-exclusion and anion-exchange chromatographies. Purified complexes were enzymatically biotinylated at a birA recognition sequence located at the C terminus of the heavy chain, and were mixed with PE- or allophycocyanin-conjugated avidin (Molecular Probes, Inc., Eugene, OR) at a molar ratio of 4:1 to give the HLA-tetramer complex.

3.3 Generation of antigen-specific CTL clone

CTL clones were established from an HIV-infected patient (HLA-A*2402/A*2601, HLA-B*3501/B*5101) following stimulation of peripheral lymphocytes with an HIV Pol-derived synthetic epitope peptide (HIV $Pol_{448–456}$; H-IPLTEEAEL-OH) as described [8]. Briefly, a bulk CTL culture was seeded at a density of 0.8 cells/well in U-bottom 96-well microtiter plate with a cloning mixture (5×10^5 irradiated allogeneic peripheral lymphocytes from a healthy donor and 5×10^4 irradiated T2-B*3501 cells prepulsed with a 1 μM concentration of the Pol peptide in RPMI1640 supplemented with 10% fetal calf

serum and 100 U/ml human rIL-2). Two weeks later, cells positive for growth were tested for cytolytic activity by the ^{51}Cr-release assay described below. Positive clones were maintained in RPMI1640 and 10% fetal calf serum supplemented with 100 units/ml rIL-2 and were stimulated weekly with irradiated T2-B*3501 cells pulsed with the Pol peptide.

3.4 Cytotoxic assay

The cytotoxic activity of the CTL clone was determined by using a standard ^{51}Cr-release assay as described [8]. T2 cells, a human lymphoblastoid line deficient in peptide transport, or transfectants expressing HLA-B*3501 were first incubated with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Pharmacia) in saline for 1 h at 37°C, and then washed three times with culture medium. The ^{51}Cr-labeled T2 cells (2×10^3 cells/well) were plated in a 96-well round-bottom microtiter plate in the absence or presence of various concentrations of the peptide. After 1 h, CTL clones (2×10^3 cells/well) were added and the plate was incubated for an additional 4 h at 37°C. To determine maximum and spontaneous ^{51}Cr release, we also added 5% Triton X-100 or culture medium alone, respectively, to different wells. A portion of the culture supernatant (100 μl) was then removed from each well and analyzed by a γ counter. The percent specific lysis was calculated as described [8].

3.5 IFN-γ secretion assay

The IFN-γ production activity of the CTL clone was determined by an intracellular flow cytometry as described [19]. Briefly, CTL clones were stimulated by .221-B*3501 cells pulsed with various concentrations of the peptide for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h. Cells were collected and stained by anti-CD8 mAb. After having been treated with paraformaldehyde solution, cells were permeabilized in a permeabilization buffer (0.1% saponin and 20% newborn bovine serum in phosphate-buffered saline) at 4°C for 10 min and stained with anti-IFN-γ mAb. After thorough washing with the permeabilization buffer, the cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

3.6 Flow-cytometric analysis *ex vivo*

Cryopreserved peripheral lymphocytes of an individual (HLA-A*2402/2601, HLA-B*3501/5101) with chronic HIV-1 infection (1×10^6 cells) or of HIV-negative individuals (4×10^6 cells) were stained first by the tetramer at 37°C for 15 min. Cells were subsequently stained by anti-Vδ1 (A13), anti-mouse Ig-PE, and anti-CD8-PerCP antibodies at 4°C for 15 min. Cells were then washed twice with a washing buffer (10% newborn bovine serum in phosphate-buffered saline), and analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

3.7 Cloning and sequencing analysis of TCR-encoding genes

Total RNA was prepared from the T cell clone (approximately 1×10^5 cells) by using a total RNA isolation kit (QIAGEN GmbH, Hilden, Germany). cDNA clones encoding α and β TCR were obtained by reverse transcription using a SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA), isolated total RNA (50 ng), and a primer specific for Cα (5'-actggattagagtctctcagctggtaca-3') or Cβ (5'-ttgggtgtgggagatctctgcttctgatg-3'), respectively. cDNA was then amplified by polymerase chain reaction (PCR) using a highly specific Taq DNA polymerase (AccuPrime SuperMix I; Invitrogen Corp., Carlsbad, CA) and was cloned into a plasmid. The DNA sequence of the α and β TCR-encoding genes was determined by using a Genetic Analyzer 310 (PE Biosystems). Alignment of V and J regions of the TCR genes was analyzed by use of the International ImMunoGeneTics database (http://imgt.cnusc.fr:8104) created by M.-P Lefranc [20].

3.8 Construction of retroviral vectors and gene transfer

Retrovirus-mediated gene transfer was used to reconstruct αβ TCR complexes on TG40/CD8 cells as previously described [17]. Briefly, the genes encoding full-length α and β TCR were subcloned into the retroviral vector pMX [21], which was kindly provided by T. Kitamura (Tokyo University, Tokyo, Japan). The ecotropic virus packaging cell line Plat-E (also a gift from T. Kitamura, Tokyo University, Tokyo, Japan) was first transfected with the resulting constructs by using the transfection reagent Lipofectamine 2000 (Invitrogen Corp.). Two days later, the culture supernatant containing recombinant virus was collected and then incubated with TG40/CD8 cells in the presence of 10 μg/ml polybrene for 6 h. Transduced TG40/CD8 cells showing bright staining by anti-mouse CD3ε mAb were selected by fluorescence-activated cell sorting and cloned for use in further functional assays.

3.9 Interleukin-2 assay for cellular activation

C1R transfectants expressing HLA-B*3501 (10^4 cells/well) were preincubated in a 96-well microtiter plate for 30 min at 37°C in the absence or presence of various concentrations of the Pol peptide. The mock- or TCR-transduced TG40/CD8 cells (2×10^4 cells/well) were added to the culture medium containing RPMI1640 and 10% fetal calf serum in a total volume of 200 μl. A portion of the culture supernatant (100 μl) was removed from each well after a 48-h incubation at 37°C, transferred to another culture plate, and frozen at –20°C. The amount of IL-2 produced by the TG40/CD8 cells was determined by analyzing the proliferative activity of the IL-2 indicator cell line CTLL-2. CTLL-2 cells (2×10^4/well) were incubated with the prepared culture supernatants for 18 h at 37°C. Then 0.5 μCi of [^{3}H]thymidine (TdR; Amersham Pharmacia) was added, and the cells were subsequently

incubated for an additional 6 h before being harvested onto glass fiber filters. After thorough washing of the filters with distilled water, the amount of [^{3}H]TdR incorporated by CTLL-2 cells that was retained on the filters was determined with a scintillation counter (MicroBeta Trilux; Warrac Oy, Turku, Finland).

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