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

Takamasa Ueno[1], Mamoru Fujiwara[1], Hiroko Tomiyama[1], Masafumi Onodera[2] and Masafumi Takiguchi[1]

[1] Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[2] Major of Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaragi, Japan

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.

Key words: TCR / CD8 T cell / HIV/AIDS / Gene therapy

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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

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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].

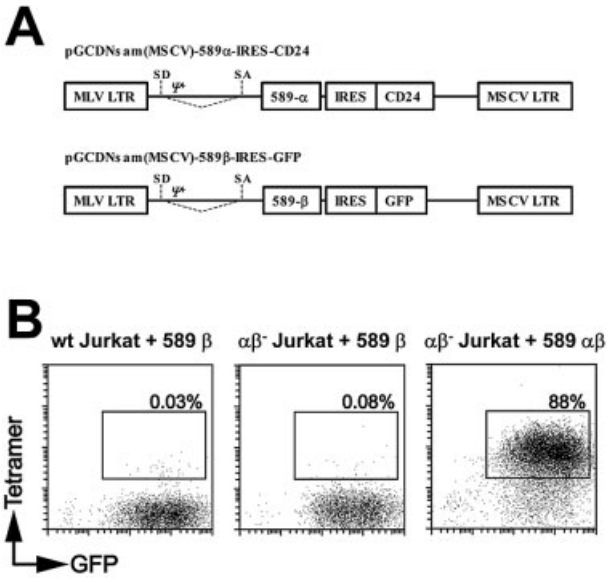

Fig. 1. Creation of functional retroviral vectors for TCR transduction. (A) Linear representation of retroviral vector constructs for transduction of αβ TCR 589 genes. SD and SA, splicing donor and acceptor, respectively; Ψ^+, packaging signal. (B) Wild-type (wt) and TCR-negative variant ($\alpha\beta^-$) 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.

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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.

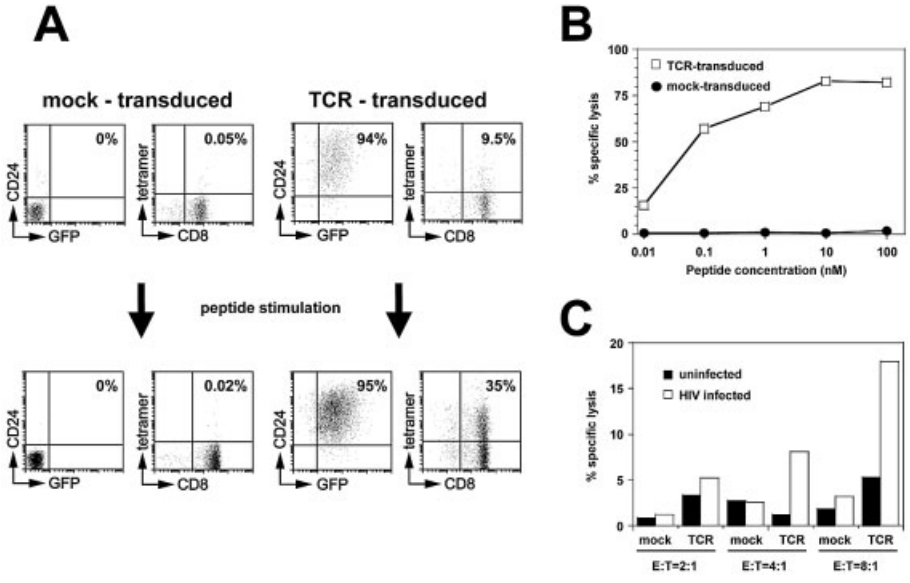

Fig. 2. Flow-cytometric and functional analysis of TCR-transduced primary CD8 T cells. (A) Primary human CD8 T cells that had been sorted into the GFP$^+$ CD24$^+$ fraction after transduction with TCR-encoded vectors were stained with anti-CD24-PE, anti-CD8-PerCP, and tetramer-allophycocyanin, and analyzed 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.

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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

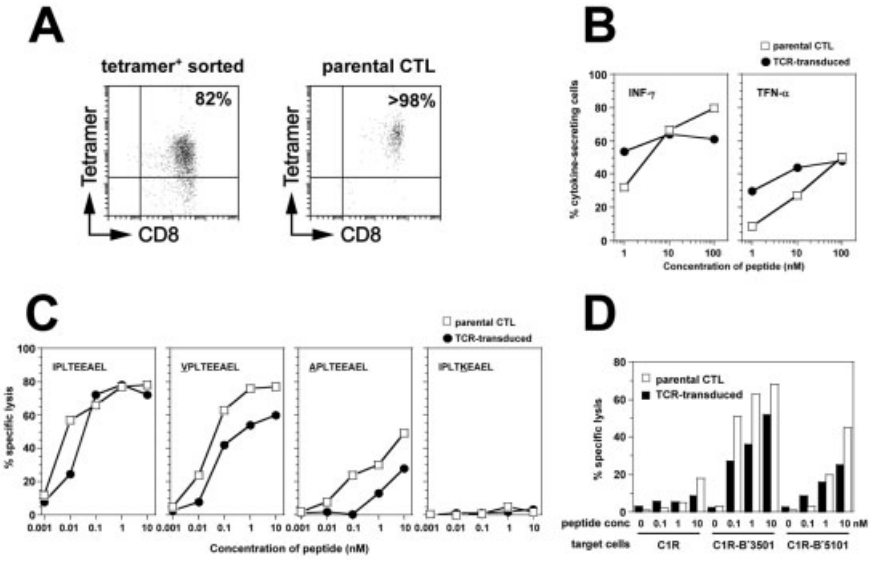

Fig. 3. Ligand specificity and functional avidity of TCR-transduced T cells compared with those of parental CTL 589. (A) The CD8$^+$ tetramer$^+$ subset of the TCR-transduced cells were sorted and cultured with autologous feeder cells pulsed with the Pol peptide. The resultant tetramer-sorted TCR-transduced 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.

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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

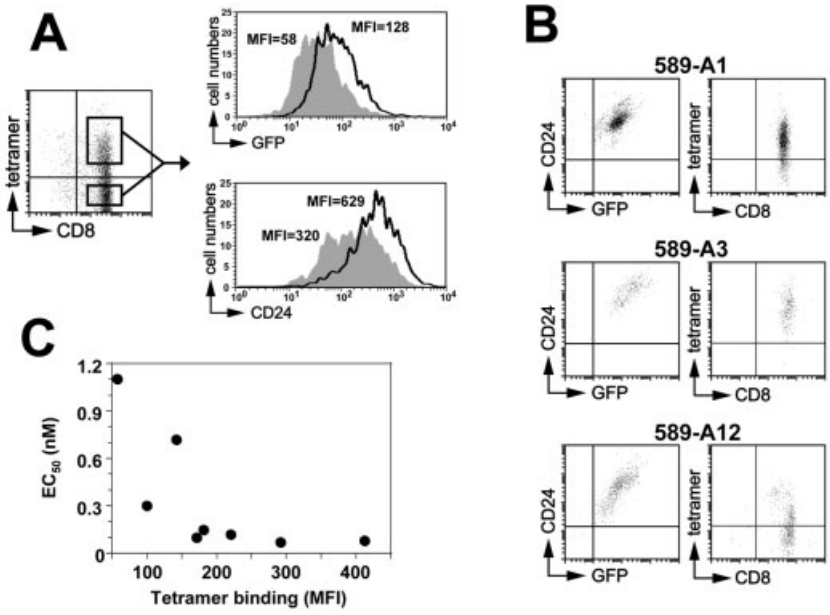

Fig. 4. Functional avidity versus tetramer binding activity of TCR-transduced cells. (A) The tetramer$^+$ (solid lines) and tetramer$^-$ (shaded areas) subsets of TCR-transduced, antigen-stimulated cells were gated and analyzed for their expression levels of GFP and CD24. The MFI values of each subset 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.

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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

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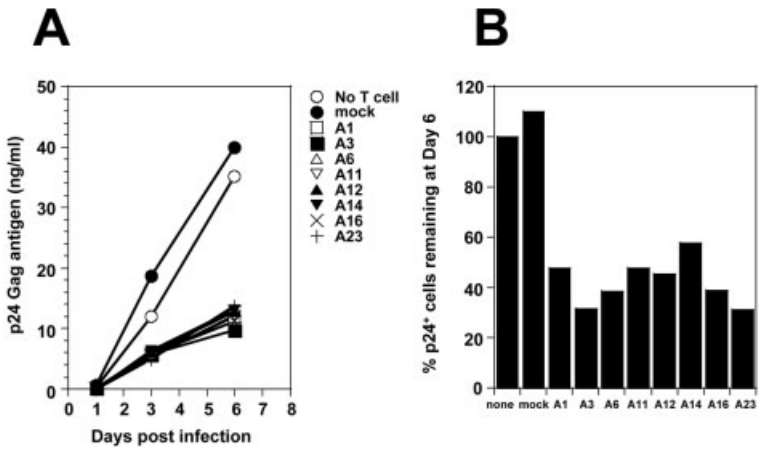

Fig. 5. Inhibitory potency of TCR-transduced clones toward HIV-1 replication *in vitro*. (A) The 221-CD4-B*3501 cells (2×10^4 cells/well) infected with HIV-1 HXB2D were co-cultured or not with the mock- or TCR-transduced clones at an E/T ratio of 0.5:1. On days 1, 3, and 6 post-infection, a portion of 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 $\alpha\beta$ 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

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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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Correspondence: Takamasa Ueno, Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto, 860-0811, Japan
Fax: +81-96-373-6532
e-mail: uenot@kaiju.medic.kumamoto-u.ac.jp

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