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RESEARCH ARTICLE

T-cell engineering by a chimeric T-cell receptor with antibody-type specificity for the HIV-1 gp120

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Immune-based approaches of cell therapy against viral pathogens such as the human immunodeficiency virus type 1 (HIV-1) could be of primary importance for the control of this viral infection. Here, we designed a chimeric cell surface receptor (105TCR) to provide primary human T-lymphocytes with antibody-type specificity for the HIV-1 envelope glycoprotein. This receptor includes the single chain Fv domain of the neutralizing anti-gp120 human monoclonal antibody F105, CD8α hinge and the transmembrane and the cytoplasmic domains of TCRζ. Our results show that 105TCR is expressed at the cellular surface and is capable of recognizing the HIV-1 envelope glycoprotein inducing highly efficient effector T-cell responses, including extracellular signal-regulated kinase phosphorylation and cytokine secretion. Moreover, human primary CD8+ T-lymphocytes transduced by oncoretroviral and lentiviral vectors containing the 105TCR gene are able to mediate in vitro-specific cytolysis of envelope-expressing cells and HIV-1-infected CD4+ T-lymphocytes. These findings suggest that 105TCR is particularly suited for in vivo efficacy studies.
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Introduction

The CD8+ cellular cytotoxic response specifically directed toward human immunodeficiency virus type 1 (HIV-1)-infected cells plays a critical role in the control of HIV-1 infection and is likely to be a major component of the host immune response both in acute and chronic infection.[1–4] Genetic approches offer a potential means to enhance immune recognition and elimination of infected cells. Our group has previously proposed a new strategy of adoptive immunotherapy based on *ex vivo* transfer of autologous CD4 T-cell lines, resistant to HIV-1, which recognize specific antigen of HIV-1 and opportunistic infectious agents.[5–7] However, the HLA-restricted nature of viral antigen recognition by the T-cell receptor limits the application of adoptive immunotherapy strategies to MHC-matched individuals. To overcome this problem, one promising strategy is to engineer T-lymphocytes genetically to express artificial TCRs that direct cytotoxicity toward viral antigens.[8–10] Artificial receptors typically comprise a viral antigen-specific recognition element derived from a single-chain antibody variable fragment (scFv).[11,12] When used to reprogram T-cell specificity, such fusion receptors allow MHC-independent recognition of antigen.[8,10,13] ScFv-based TCRs are engineered to contain a signaling domain that delivers an activation stimulus.[14] Several scFv-based chimeric TCR (cTCR) have been developed that contain the TCRζ cytoplasmatic domain. This domain is capable of inducing a potent activation signal in the absence of the remaining components of TCR-CD3 complex, thus activating cytolytic functions.[15] It has been demonstrated that chimeric receptors containing the TCRζ chain and the CD8α hinge are not associated with endogenous subunits of the TCR complex.[16–18] Therefore, generation of unpredictable and deleterious immune specificity is not likely to occur.

Roberts *et al*[19] have developed two classes of HIV-specific chimeric receptors, in which the antigen binding domain is comprised of either CD4 or a single chain specific for the envelope glycoprotein gp41, characterized by the ability of activating a number of T-cell effector functions in response to target cells. *In vitro* studies and clinical trials have confirmed the feasibility and safety of adoptive therapy with CD4+[20,21] or CD4+ and CD8+[22,23] T cells modified with cTCR containing the human CD4 molecule as the extracellular domain. In turn, these engineered T cells were endowed with antiviral activity. Indeed, a sustained decrease of replication-competent virus cultured from blood, as well as of HIV-DNA isolated from rectal mucosa, was achieved. These data are encouraging and provide evidence that the cTCR strategy is potentially useful as an immunotherapeutic approach *in vivo*.

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Therefore, we were prompted to develop and characterize a novel MHC-unrestricted anti-HIV-1 cTCR capable of redirecting the antigen specificity of primary T-cell populations against the HIV-1 envelope glycoprotein gp120. To this end, we designed the 105TCR chimeric molecule, composed of the ζ chain subunit of the CD3 T-cell receptor as the cytoplasmic and transmembrane domains, fused to the hinge region of CD8α. The extracellular domain consisted of an scFv region, derived from the human monoclonal antibody F105 specific for a gp120 epitope mapping within the CD4 binding site. The human F105 antibody has been shown to have neutralizing activity towards T-tropic HIV-1 isolates.[24] Here, we demonstrate the expression of 105TCR on T-lymphocytes and the ability of 105TCR to induce T-cell activation and T-cell-mediated cytolysis following contact with gp120-expressing cells and HIV-1-infected CD4+ T-lymphocytes.

Results

Design and expression of 105TCR in human lymphoblastoid cell line

The 105TCR we developed is schematically represented in Figure 1a. The chimeric receptor is characterized, in its extracellular domain, by the presence of the 105scFv specific for the HIV-1_{env} glycoprotein gp120. In turn, the 105scFv is derived from the human monoclonal antibody F105, which reacts with a conformationally defined epitope on the HIV-1 gp120 exposed on the free virions and located within or topographically near the CD4

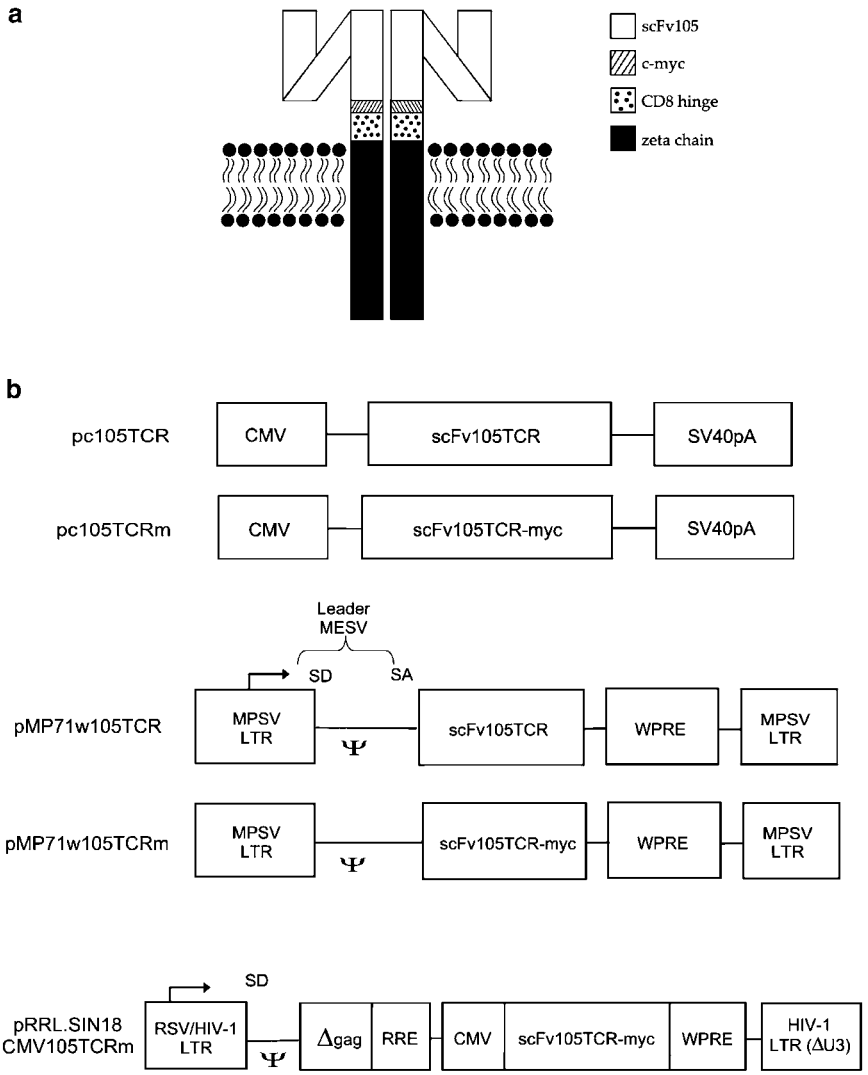

Figure 1 *Structure of the 105TCR receptor and schematic representation of the constructs. (a) The gp120-specific TCR receptor (scFv105TCR) encompasses an scFv, derived from the 105 hybridoma, consisting of the VH and VL fragments connected by a linker peptide. A CD8α hinge links the scFv to the transmembrane and intracellular region domains of the human ζ chain. An SP is present at the N-terminus for targeting the chimeric molecule to the cell surface. A c-myc (m) tag has been included in the extracellular domain to allow monitoring of 105TCR expression. (b) The gp120-specific receptor was expressed using the eukaryotic expressor plasmid pCDNA 3.1 (pc105TCR and pc105TCRm), the MP71 oncoretroviral vector (MP71w105TCR and MP71w105TCRm) and the lentiviral vector RRL.SIN18 (RRL.SIN18CMV105TCRm). Only the relevant parts of the constructs are indicated. MPSV: myeloproliferative sarcoma virus long terminal repeat (LTR) promoter; SD: splice donor site; SA: splice acceptor site; Ψ: packaging signal; MESV leader: 5′-untranslated leader of murine embryonic stem cell virus; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; RSV: Rous sarcoma virus; HIV-1 LTR: R and U5 regions from the HIV-1 LTR; ΔU3 LTR: HIV-1 LTR containing a deletion within the U3 region.*

binding site.[24,25] The F105 antibody is a neutralizing antibody that binds to the envelope glycoprotein of multiple T-tropic HIV-1 strains.[24,25] The scFv105 is coupled to the CD8α hinge and to the transmembrane and intracellular signaling domains of the CD3ζ chain of the TCR. In addition, a signal peptide (SP) was introduced upstream of the single-chain sequence for targeting 105TCR to the cellular membrane. A *c-myc* tag was also included within the extracellular domain of some constructs to monitor the 105TCR's expression. 105TCR was inserted into the pCDNA 3.1 eukaryotic expression plasmid (pc105TCR and pc105TCRm) under the transcriptional control of the human cytomegalovirus (CMV) immediate-early promoter to produce the cTCR (Figure 1b). To assess the expression of 105TCR, the lymphoblastoid Jurkat T-cell line was transfected with the pc105TCRm plasmid and processed for Western blot analysis using an anti-human *c-myc* tag monoclonal antibody. As reported in Figure 2a, a distinct band of molecular mass of 58 kDa, corresponding to the 105TCR protein, was revealed. This band did not appear in mock-transfected cells. Surface expression of 105TCR in transfected cells was determined by immunofluorescence analyses with confocal microscopy of paraformaldehyde fixed cells using an anti-human *c-myc* tag antibody (Figure 2b). Our results indicate that lymphoblastoid T cells express the 105TCR protein not only at the cytoplasmatic level but also at the membrane surface. Expression of 105TCR could not be detected on T cells transfected with the negative control.

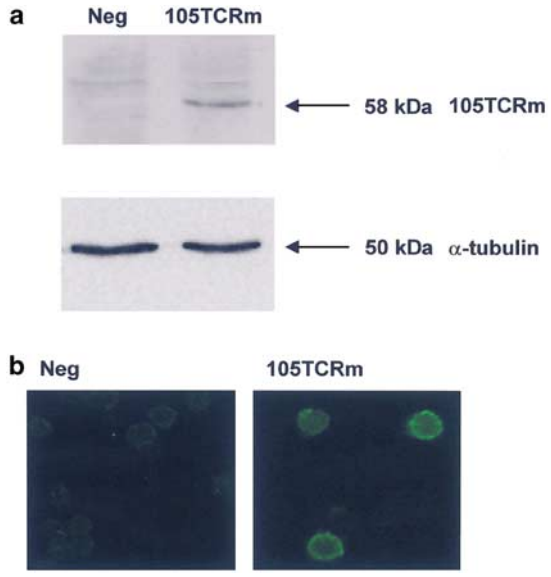

Figure 2 *cTCR expression and cellular membrane localization in transfected T-cell lines. (a) Jurkat cells were transfected with either the pc105TCR or the pc105TCRm plasmid and lysed 72 h later; the cellular extracts were separated in 10% acrylamide gels and transferred to PVDF membrane. Upper panel: the proteins were incubated with 2 μg/ml of anti-c-myc tag monoclonal antibody. Lower panel: the proteins were incubated with anti-α-tubulin monoclonal antibody (1:2000) to normalize for gel loading and membrane transfer. Arrows indicate the position of 105TCR (58 kDa) and α-tubulin (50 kDa). (b) Transfected Jurkat cells were stained by indirect immunofluorescence with 0.2 μg/μl of anti-c-myc tag monoclonal antibody followed by incubation with FITC-anti-mouse antibody and analyzed by confocal microscopy.*

105TCR elicits extracellular signal-regulated kinase (ERK) phosphorylation and IL-2 production in T cells expressing the chimeric receptor upon engagement with envelope expressing transfected cells

It has been reported that activation of ERK, a mitogen-activated protein (MAP) kinase, is required for stimulation of T cells.[26] ERK consists of two isotypes ERK1 (42 kDa) and ERK2 (44 kDa), both of which are activated by phosphorylation following TCR engagement.[27] To assess the ability of 105TCR to activate the signaling pathway, lymphoblastoid CD4+ cells transfected with the pc105TCR plasmid were cocultivated with COS-1 cells transfected with the HIV-1 envelope expressor plasmid. ERK activity was investigated by immunoblotting using a polyclonal antibody that recognizes both the phosphorylated active forms of ERK (Figure 3). In response to interaction with gp120 expressed on the cellular membrane, 105TCR led to phosphorylation of both ERK isotypes (ERK 42 kDa and ERK 44 kDa) in

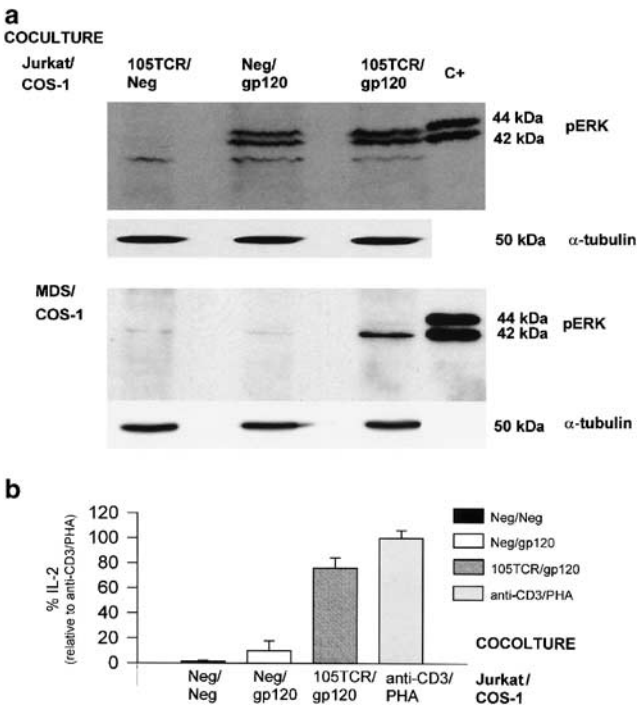

Figure 3 *Functional analysis of transfectants. (a) Lymphoblastoid T-cell lines expressing high (Jurkat, upper panel) or low (MDS, lower panel) levels of the CD4 molecule were transfected with either the pc105TCR plasmid or the pCDNA 3.1 control plasmid, and cocultivated for 5 min with gp120-expressing COS-1 cells. As a negative control, COS-1 cells were transfected with the pCDNA 3.1 plasmid. 72 h later, 1×10^6 T cells were lysed and immunoblotted with the anti-active form of ERK antibody diluted 1:3000. A cellular extract of NGF-stimulated PC12 cells was used as a positive control. The proteins were incubated with anti-α-tubulin monoclonal antibody (1:2000) to normalize for gel loading and membrane transfer. The positions of ERKs (44 and 42 kDa) and α-tubulin (50 kDa) are indicated. (b) Jurkat cells expressing 105TCR were evaluated for their ability to secrete IL-2 in response to incubation in the presence of PHA with COS-1 cells transfected with the envelope glycoprotein expressing plasmid pSVIIIenv or with the control plasmid. In parallel, Jurkat cells were stimulated with anti-CD3 (10 μg/ml) and PHA (1 μg/ml). The amount of IL-2 released from Jurkat cells after cocultivation with COS-1 cells for 24 h was quantified in the culture supernatants by ELISA. The bars indicate the relative amount of IL-2 protein normalized to an IL-2 value of 100 obtained after stimulation of Jurkat cells with anti-CD3 and PHA. One of three representative experiments is shown.*

CD4+ Jurkat cells (Figure 3a, upper panel). However, strong ERK activation was also observed when gp120-positive COS-1 cells were cocultivated with 105TCR-negative T cells. Given that the lymphoblastoid Jurkat cell line expresses high level of CD4 receptor (90%, data not shown), ERK activation in this cell line is likely derived from CD4–gp120 interaction. To investigate whether TCR is functionally active on its own in driving signal transduction upon antigen recognition, the MDS lymphoblastoid T-cell line, expressing low level of the CD4 molecule (<20%, data not shown), was transfected with the pc105TCR plasmid and used in the coculture experiments. As reported in Figure 3a (lower panel), ERK phosphorylation was detectable only when the MDS 105TCR transfectants were cocultivated with gp120-expressing COS-1 cells. In this regard, the predominant ERK isoform expressed in MDS cells was found to be the 42 kDa protein corresponding to ERK2. These data confirm that binding of 105TCR to gp120 expressed on the cellular surface mediates T cells activation.

We then tested the ability of 105TCR-transfected T-lymphocytes to secrete IL-2 in response to recognition of gp120 expressed on the cellular surface of COS-1 cells. As shown in Figure 3b, 105TCR can activate Jurkat cells to release IL-2 upon interaction with membrane-bound gp120. Specifically, the IL-2 response obtained from Jurkat cells upon 105TCR stimulation by gp120-positive COS-1 cells is similar to that observed in Jurkat cells after activation of the native T-cell receptor with the anti-CD3 monoclonal antibody and PHA stimulation. Jurkat cells transfected with negative control plasmid did not respond to stimulation by the gp120-positive COS-1 cells.

Consistent with the above results, these data strongly suggest that 105TCR mediates the full activation of T cells leading to cytokine production.

Binding of 105TCR to soluble gp120

One problem associated with the use of cTCR is that soluble antigen can inhibit the receptor's activity. During HIV-1 infection, gp120 is frequently shed into serum of patients as a soluble protein.[28,29] Therefore, this soluble gp120 could be recognized by 105TCR present on the T-cell surface leading to cell activation. Should such an activation occur *in vivo*, it could trigger CD8+ T-cell-mediated activity in the absence of target cells. To investigate binding of soluble antigen, COS-1 cells expressing 105TCR were incubated with a high concentration of soluble biotinylated gp120 (3 μg). Cells were then labeled with streptavidin–phycoerythrin (PE) and analyzed by fluorescence-activated cell sorting (FACS). As shown in Figure 4a, no significant binding of 105TCR to soluble gp120 was detectable (2.5% positive cells). Conversely, a higher proportion (10%) of COS-1 cells expressing the CD4 molecule bound soluble gp120. Since the 105TCR and CD4 molecules were similarly expressed at the cellular surface (45 and 44.7%, of positive cells, respectively) (Figure 4b), differences in 105TCR and CD4 expression are unlikely to account for the observed differences in gp120 binding. The F105 and CD4 affinity for monomeric gp120 are in a similar nanomolar range (W Marasco, personal communication); therefore, one explanation for the observed difference in binding of 105TCR and CD4 to soluble gp120 could be that the affinity and/or the overall binding capacity of the single-chain construct within the 105TCR might differ from that of the complete IgG.

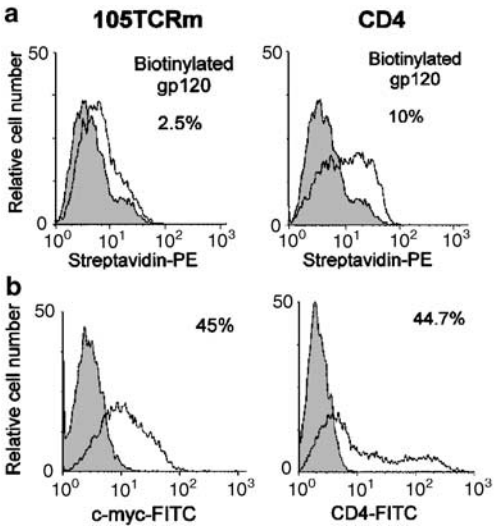

Figure 4 *Binding of 105TCR to soluble HIV-1 gp120. (a) COS-1 cells were transfected with the pc105TCRm plasmid (left) or the pCDNA 3.1CD4 plasmid as a positive control (right). After 72 h, the cells were labeled with biotinylated gp120, incubated with streptavidin–PE and analyzed by flow cytometry. Dark histograms refer to COS-1 cells transfected with the pCDNA 3.1 plasmid (negative control). (b) As a positive control of 105TCR and CD4 expression on the cellular surface, COS-1 cells transfected with the pc105TCRm or the pCDNA 3.1CD4 plasmid were stained with anti-human c-myc tag or anti-CD4 monoclonal antibody, respectively, and analyzed by flow cytometry.*

Thus, these results show that soluble gp120 is poorly recognized by 105TCR expressed on the lymphocyte cell surface.

105TCR expression in transduced primary human CD8+ T-lymphocytes

Based on the results obtained with the eukaryotic expressor plasmid, we next developed retroviral vectors containing 105TCR in order to transduce primary T-lymphocytes. To this end, last-generation retroviral vectors were adopted. As an oncoretroviral vector the MP71 vector was used (Figure 1b), which allows expression of 105TCR under the control of the murine proliferative sarcoma virus (MPSV) LTR promoter–enhancer sequences and of an improved 5′-untranslated sequence derived from the murine embryonic stem cell virus (MESV).[30–32] As a lentiviral vector the RRL.SIN18 vector was used, in which the expression of 105TCR is regulated by the human CMV internal promoter.[33] Transfection of the oncoretroviral vectors MP71w105TCR and MP71w105TCRm into the PG13 GALV packaging cell line led to production of GALV-pseudotyped virions.[34] Transducing lentiviral particles pseudotyped with the VSV-G envelope glycoprotein were prepared by transient transfection of 293T cells.[35] Recombinant oncoretroviral and lentiviral particles were titered on 293 and 293T cell lines, respectively, and tested in parallel for their ability to infect human CD8+ cells purified by positive selection from the PBMCs of normal donors (see Materials and methods). As a first approach, a transgene containing the coding region for the enhanced green fluorescent protein (EGFP) was used to assess percentage

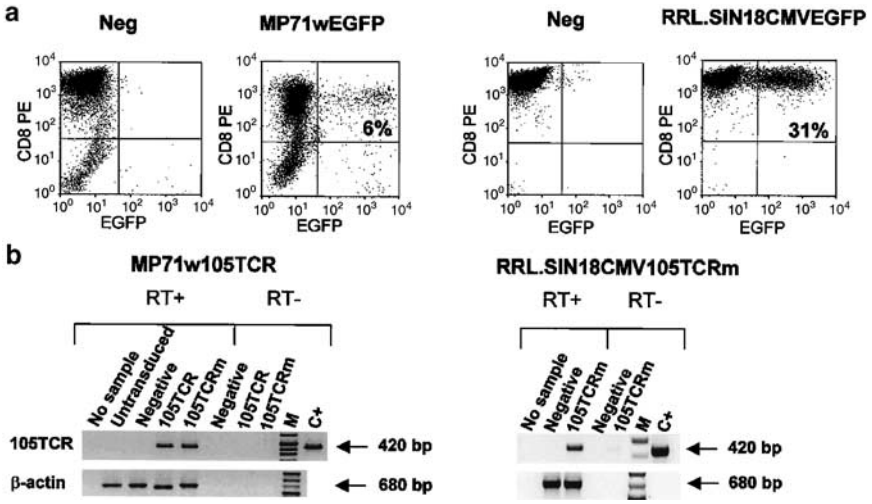

Figure 5 *Expression of 105TCR in primary human CD8+ T-lymphocytes. (a) To determine the percentage transduction of CD8+ cells, samples were transduced with either the MP71w oncoretroviral or the RRL.SIN18 lentiviral vector containing the EGFP (MP71wEGFP and RRL.SIN18CMVEGFP, respectively). On day 8, gene transfer efficiency was assessed by flow cytometry. CD8+ cells were stained with anti-CD8 monoclonal antibody and then with anti-mouse antibody PE conjugated and analyzed for EGFP and PE emission. The percentage of EGFP+/CD8+ T cells is reported in the upper right panels. (b) Transduced human CD8+ T cells express high levels of 105TCR mRNA. To investigate expression of 105TCR, RT-PCR analysis was performed in the presence (RT+) and absence (RT–) of reverse transcriptase. RNA was extracted from the CD8+ cells transduced with the 105TCR-expressing retroviral vectors (oncoretroviral vectors MP71w105TCR and MP71w105TCRm, left; lentiviral vector RRL.SIN18CMV105TCRm, right) on day 3 after transduction. To normalize for gel loading, the β-actin mRNA expression was determined (lower panels). Amplified products were analyzed on 1% agarose gels. Arrows indicate the position of 105TCR and β-actin amplification products and the relative size in base pairs (420 and 680 bp, respectively). No sample: PCR-negative control; untransduced: control untransduced CD8+ T cells; negative: CD8+ T cells transduced with the pCDNA 3.1 plasmid; 105TCR: CD8+ T cells transduced with the 105TCR-expressing oncoretroviral MP71w vector; 105TCRm: CD8+ T cells transduced with the 105TCRm-expressing oncoretroviral MP71w (left) or lentiviral RRL.SIN18 (right) vector; M: size markers; c+: the pc105TCR plasmid served as PCR positive control.*

of transgenic cells by flow cytometry. EGFP replaced the scFv105TCR within the vectors' framework. As shown in Figure 5a (left panel), the EGFP expression yield was 6% with the MP71 vector, in agreement with data obtained by Uckert *et al*[36] under similar experimental conditions. When the RRL.SIN18 lentiviral vector was used, the EGFP expression achieved was significantly higher (35%, Figure 5a, right panel) and stable long-term expression of the transgene, up to 5 months post-transduction, was observed (data not shown). Differences in viral titers as well as in viral tropism of oncoretroviral and lentiviral vector particles were likely to be responsible for the observed differences in gene transduction efficiency in T-lymphocytes.

We next examined the expression of 105TCR mediated by retroviral vectors in transduced CD8+ T cells by RT-PCR using specific primers. In the experiment shown (Figure 5b), we analyzed the mRNAs extracted from CD8+ T cells transduced with the oncoretroviral (left panel) and lentiviral (right panel) vectors. The mRNA extracted from mock-transduced CD8+ T cells and from CD8+ cells transduced with the pCDNA 3.1 plasmid served as the controls. A clear mRNA product was amplified from CD8+ T cells transduced with both retroviral vectors, demonstrating specific transcription of the 105TCR gene.

105TCR-modified primary CD8+ T lymphocytes specifically lyse envelope expressing cells and HIV-1-infected cells

The ability of retrovirally transduced CD8+ T-lymphocytes to mediate specific lysis of HIV-1 env-expressing lymphoblastoid T target cells was evaluated in standard

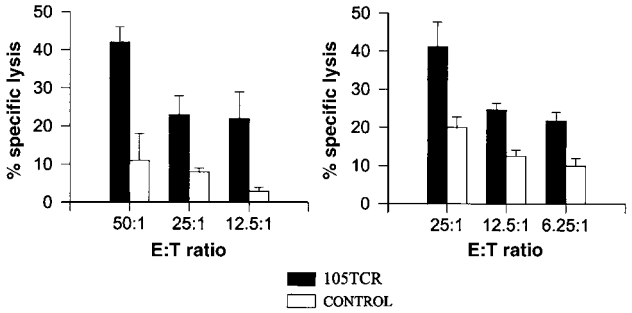

Figure 6 *Lysis of gp120-expressing T cells by 105TCR-transduced human CD8+ T-lymphocytes. Lymphoblastoid T-cell lines (MDS and Jurkat, left and right panel, respectively) were transfected with either the envelope glycoprotein expressing plasmid or the control plasmid, and used as target cells (T) in standard ^{51}Cr release assay. Effector cells (E) were human CD8+ cells transduced with either the oncoretroviral MP71w105TCR vector (left panel) or the lentiviral RRL.SIN18CMV105TCRm vector (right panel). As a control, effector cells were transduced with the pCDNA 3.1 plasmid. The cytolytic function of the transduced T cells was evaluated in a 4 h ^{51}Cr release at the indicated ratios. No specific lysis was observed using control target cells. The spontaneous lysis was $<10\%$ in all assays. Results are expressed as specific ^{51}Cr release $\pm$ s.d. (%) for triplicate samples. Results from one representative experiment out of three are shown.*

4 h ^{51}Cr release assays at a range of effector to target (E:T) ratios. Although the levels of cytolysis showed some experimental variation, the relative activity of 105TCR was consistent in all the experiments. A representative assay is shown in Figure 6. Primary CD8+ T cells expressing the 105TCR receptor exhibited specific lysis of env-positive MDS and Jurkat lymphoblastoid target cells, while control primary CD8+ T cells, transduced with the pCDNA 3.1 plasmid, displayed slight cytolytic

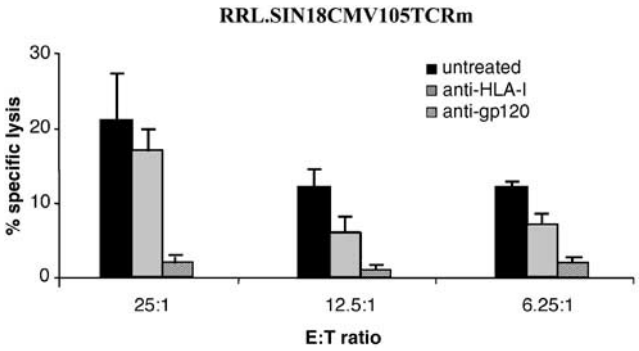

Figure 7 *105TCR-mediated MHC class I-unrestricted cytolysis of envelope-expressing cells by transduced CD8+ cells. A ^{51}Cr-labeled Jurkat lymphoblastoid cell line transfected with the envelope glycoprotein-expressing plasmid was preincubated for 60 min with monoclonal antibody recognizing monomorphic determinants of MHC class or the gp120 glycoprotein, then incubated for 4 h with CD8+ T cells transduced with the lentiviral vector expressing the 105TCR. Results are expressed as net specific lysis ± s.d. (%) for triplicate samples. Lysis of envelope-positive target cells by control CD8+ T cells were 20, 14 and 12%. Results from one representative experiment out of three performed are shown.*

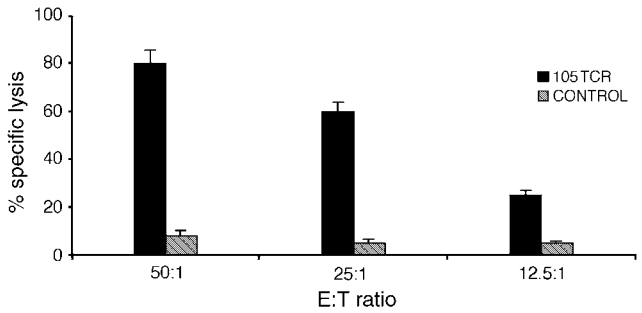

Figure 8 *Lysis of HIV-1-infected CD4+ T cells by 105TCR-transduced human CD8+ T-lymphocytes. Purified CD4+ T-lymphocytes were infected with HIV-1 IIIB and used as target cells (T) in standard ^{51}Cr release assay. Effector cells (E) were control human CD8+ cells transduced with the pCDNA 3.1 plasmid or human CD8+ cells transduced with the lentiviral RRL.SIN18CMV105TCRm vector. No specific lysis was observed using uninfected target cells. The spontaneous lysis was <10% in all assays. Results are expressed as specific ^{51}Cr release ± s.d. (%) for triplicate samples. Results from one representative experiment out of three are shown.*

activity against env-positive target cells. CD8+ T cells expressing 105TCR or CD8+ control T cells were unable to lyse the env-negative expressing target cell lines (data not shown).

To determine whether an MHC-independent engagement of 105TCR was indeed responsible for the observed cytolysis, the cytolytic assay was performed in the presence of an antibody specific for the HLA class I. As reported in Figure 7, preincubation with HLA class I blocking antibody only slightly affected the lysis of env-positive target cells ($P = 1.09$), indicating an MHC-unrestricted recognition by 105TCR. The specificity of the interaction between 105TCR and the gp120-expressing target cells was confirmed by preincubation of envelope-positive target cells with an anti-gp120 monoclonal antibody, resulting in complete inhibition of lysis by 105TCR-transduced cells ($P = 0.016$). The antigen specificity of this response was further demonstrated by the lack of abrogation of T-cell lysis on addition of an IgG isotype control monoclonal antibody (data not shown). Altogether, these results provide evidence that 105TCR specifically engages gp120 in an MHC-independent antigen recognition process.

To assess the capacity of CD8+ 105TCR-transduced cells to lyse HIV-1-infected cells, purified CD4+ T-lymphocytes were infected with HIV-1 IIIB and used as target in cytotoxicity assays. As shown in Figure 8, CD8+ 105TCR-transduced lymphocytes specifically killed infected cells, while control CD8+ cells did not. Uninfected CD4+ lymphocytes were not recognized by 105TCR-expressing or control CD8+ lymphocytes (data not shown).

Discussion

Cellular immunotherapy adopting autologous or host-compatible antigen-specific T-lymphocytes has shown significant potential in the treatment of malignant and infectious diseases including HIV-1 infection.[17,37,38] Adoptive transfer of HIV-1-specific *ex vivo*-expanded CD8+ T cells was capable of augmenting the antiviral response. As an alternative to the use of conventional T-lymphocytes, chimeric receptor-modified T-lymphocytes has recently shown promise in clinical studies.[22] In this regard, an ideal approach of adoptive immunotherapy of AIDS should be primarily aimed at producing modified T cells capable of viral antigen recognition in an MHC-unrestricted manner, since it has been shown that HIV-1 can downregulate expression of MHC class I molecules.[39,40] Secondly, this approach should take into account the need for a generalized response against varieties of epitopes. Thirdly, modified T cells should be able to recognize different cell types that support viral replication. Selection of T cells engineered to express cTCR and their use as anti-HIV-1 effector cytotoxic cells should accomplish these requirements.

In this study, we describe an scFv-based chimeric T-cell receptor (105TCR) capable of recognizing the HIV-1 envelope glycoprotein gp120 present on the surface of target cells and of inducing effector T-cell responses including kinase activation and cytokine secretion. We show that retrovirally transduced human primary T cells expressing 105TCR are able to mediate specific cytolytic function towards envelope-expressing cells and HIV-1-infected CD4+ T-lymphocytes.

According to the results of experiments in which MAP kinases are activated following TCR engagement,[26,41] our data show that 105TCR is capable of transmitting intracellular signaling leading to ERK phosphorylation. Furthermore, we demonstrated that the expression of 105TCR enables T-lymphocytes to secrete IL-2 in response to gp120 recognition. These results are in agreement with those reported by Roberts and other groups using cTCR containing, as an extracellular domain, the human CD4 molecule or scFvs specific for either gp120 or gp41 HIV-1 envelope subunits.[19,42–45] Moreover, our data confirm that the presence of the ζ chain as an intracellular domain is sufficient to stimulate T cells.[14,46] Costimulatory function is likewise crucial for adequate T-cell activation. Indeed, modified

chimeric receptors containing different signaling moieties have been recently proposed. Maher *et al*[47] developed scFv-based receptors, recognizing a prostate-specific membrane antigen, and comprising both TCRζ and CD28 sequences. This combination has been shown to amplify human T-cell responses; thus, for an *in vivo* application, smaller doses of engineered T cell may be sufficient to allow for T-cell expansion following infusion. Although we have not yet utilized hybrid constructs containing both receptor and costimulatory molecules, our data with 105TCR are encouraging to pursue further development in this direction.

Some disadvantages of TCR engineered T cells with respect to native T cells that recognize the foreign peptide in the context of an MHC class molecule include binding of circulating antigen shed from the viral envelope. This may lead to blockage of target recognition and/or inappropriate activation.[44,48] Indeed, cTCR-expressing T cells can be inhibited by soluble antigens in several models.[49] It is known that soluble gp120 can be found in the serum of HIV-infected patients.[28,29] We were therefore concerned that T cells with envelope specificity could be relatively ineffective under conditions similar to those present *in vivo*. However, this was not the case with our TCR-transduced cells since they poorly bound soluble gp120. The likely explanation for this phenomenon may reside in the larger antigen recognition pocket of an scFv as compared to that of a native TCR. This structural feature would permit specific recognition of a conformational epitope formed in the context of a lipid viral membrane. Our scFv is in fact derived from the F105 monoclonal antibody, an antibody capable of virus neutralizing activity.[24]

Since retrovirus-mediated gene transfer into peripheral blood T cells is crucial for efficient expression of the cTCR, we introduced 105TCR into both oncoretroviral and lentiviral vectors, for efficient transduction of target cells.[32,35] Our data show that retrovirally transduced human CD8+ T cells expressed high levels of 105TCR and that transduced CD8+ cells were able to lyse HIV-1-envelope-expressing cells specifically in an MHC-unrestricted manner. Moreover, TCR-transduced CD8+ cells killed with high-efficiency HIV-1-infected CD4+ T-lymphocytes.

The ability of 105TCR to recognize cells independently of antigen processing could be advantageous in bypassing both the natural selection of viral escape mutants and the potential downregulation of cell surface class I molecules. In addition, the use of cTCRs would circumvent the alteration of viral antigen processing and presentation. Indeed, endogenous processing of viral proteins *in vivo* can be more complex than *in vitro* studies with synthetic peptides would predict.[50] Another important property for engineered CD8+ T cells is their ability to clear many different HIV-1-infected cell types, such as monocytes and dendritic cells, which are both important sites of viral replication *in vivo*, as shown recently.[51]

In summary, our results establish the principle that a conserved site of gp120 can be the target for a cell-mediated response using T cells engineered to express an scFv TCR endowed with broad-spectrum viral neutralizing activity. In this regard, a neutralizing cTCR could represent a novel therapeutic system for the immunotherapy of AIDS. While the safety and feasibility of chimeric receptor-based T-cell therapy have already been proved in human clinical trials,[22] efforts should now focus on the refinement on further development of 105TCR for its therapeutic *in vivo* applications.

Materials and methods

Cell lines and transfections

COS-1 (green monkey transformed fibroblast cell line, ATCC CRL 1650), PG13 (retrovirus packaging cell line containing the gibbone ape leukemia virus, GALV, envelope, ATCC CRL-10686), 293 (human embryonic kidney cell line, ATCC CRL-1573) and Jurkat (CD4+ human T lymphoblastoid cell line, ATCC TIB-152) were supplied by American Type Culture Collection (ATCC, Manassas, VA, USA). The 293T cell line (293 stably transduced with the simian virus 40 T antigen) was provided by Dr D Baltimore. All the above except the Jurkat cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, San Diego, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St Louis, Missouri, USA). The CD4+ human T lymphoblastoid MDS cell line was supplied by Dr G Acs.[52] Jurkat and MDS cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Transfections of COS-1 and PG13 cells were performed with lipofectamine plus (Invitrogen) following the manufacturer's instructions, while transfections of 293T cells were conducted using the calcium phosphate method.[53]

Transfections of T cells were carried out by the electroporation method as described previously.[54]

Development of chimeric receptor in expression constructs

The single-chain antibody chimeric immune receptor (scFv105TCR or 105TCR) used in these studies comprise the sFv105-B domain linked to the signal transducing domain (residues 28–165; numbering according to Weissman *et al*[55]) of the TCR/CD3 ζ subunit. The CD8α hinge domain (residues 105–165; numbering according to Zomoyska *et al*[56]) is localized in the membrane proximal region of the extracellular domain in order to provide accessibility for ligand binding. The sFv105-B domain is derived from the human F105 monoclonal antibody previously characterized,[57,58] which recognizes determinants within the CD4 recognition site of the HIV-1 envelope glycoprotein gp120. To obtain the scFv105TCR, the genetic fragments of the SP, CD8α hinge and CD3ζ chain regions were isolated by RT-PCR reactions from the mRNA of healthy donor PBMCs. The RT-PCR reactions were performed with primers 5′-CCCAAGCTTGCCTCCC AGCCTCTTTCTGA-3′ and 5′-GGAATTCCTG TGCCT CTGT AATCGGCAACT-3′ containing *Hind*III/*Eco*RI restriction sites for the SP region; primers 5′-CTCGAGAG CAACTCCATCATGTACTTC-3′ and 5′-GGTACCGGCGA AGTCCAGCCCCCTCGTGTGCAC-3′ containing *Xho*I/*Kpn*I restriction sites for the CD8 region; and primers 5′-GGTACC CCCAAACTCTGCTACCTGCTGG-3′ and 5′-GCTCTAGATTAGCGAG GGGGCAGGGCCT-3′ contain-

ing *Kpn*I/*Xba*I restriction sites for the ζ chain region (the underline indicates the restriction enzyme sites). Finally, a fragment of DNA coding for the anti-gp120 scFv was amplified by PCR from the pSFv105-B domain using primers 5′-CGCGAATTCATGGAACATCTGTG-3′; 5′-TCGGCTCGAGACGCGTTGGTGCAGC-3′ containing *EcoR*I/*Xho*I sites. The scFv105TCR was cloned into the pCDNA 3.1 plasmid (Invitrogen) for expression. To this end, the PCR products were firstly subcloned into the pCD2.1 plasmid (Invitrogen) with the TA cloning method; these fragments were obtained after digestion with the restriction enzymes described above and ligated into the pCDNA 3.1 plasmid restricted with *Xho*I/*Xba*I and *Hind*III/*Xho*I, yielding the pc105TCR plasmid. The pc105TCR plasmid containing a *c-myc* tag (pc105TCRm) was obtained by different cloning steps. A fragment of DNA encompassing the CD8 region was amplified from the pc105TCR plasmid using primer 5′-CCGCTCGAGGAA CAAAAACTTATTTCTGAAGAAGATCTGAGCAACTCC ATCATGTACTTCAGC-3′, containing *Xho*I restriction site, the *c-myc* sequence (EQKLISEEDL) and primer 5′-GG GGGTACCGGCGAAGTCCAG-3′, containing *Kpn*I restriction site. CD8 overlapping sequences were positioned at both the 5′- and 3′-ends of the primers. The PCR product was digested with *Xho*I/*Kpn*I and ligated to the pc105TCR plasmid previously digested with the same enzymes to generate the pc105TCRm plasmid.

The oncoretroviral vectors MP71w105TCR and MP71w105TCRm containing the scFv105TCR and scFv105TCRm, respectively, were obtained by using the MP71wEGFP vector kindly provided by Dr C Baum.[59,60] This construct combines the promoter/enhancer sequences of the MPSV LTR and improved 5′-untranslated sequences derived from the MESV. The DNA fragment encompassing the scFv105TCR and scFv105TCRm was amplified from the pc105TCR and pc105TCRm plasmids, respectively, using oligonucleotide primers 5′-TTGGCGGCCGCCATG GAGTGGAAGGCG-3′, containing the *Not*I restriction site, and 5′-TTAGCGAGGGGG CAGGGCCTGCATGTG-3′. The PCR product was digested with *Not*I and cloned into the oncoretroviral vector MP71wEGFP previously digested with *BspE*I, filled in with Klenow polymerase and then digested with *Not*I to eliminate the EGFP sequence. The lentiviral vector RRL.SIN18CMV105TCRm containing the scFv105TCRm was obtained by using the pRRL.SIN18CMVEGFP vector kindly provided by Dr L Naldini.[35] The DNA fragment containing the scFv105TCRm was amplified from the pc105TCRm plasmid using oligonucleotide primers 5′-TTGGATATCCCGCCATGGAGTGGAAGGCG-3′ and 5′-CAGT CGTCGACTTAGCGAGGGGGCA-3′, containing *EcoR*V and *Sal*I restriction sites, respectively. The PCR product was digested with *EcoR*V and *Sal*I, and then cloned into the pRRL.SIN18CMVEGFP lentiviral vector previously *BamH*I digested, filled in with Klenow polymerase and digested with *Sal*I to eliminate the EGFP sequence.

All the constructs were confirmed by direct sequencing. Sequencing reactions were performed using ABI PRISM™ Dye terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA, USA). DNA sequences were determined using an ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Culture and transduction of human primary T cells

CD8+ and CD4+ cells were isolated from buffy coats from healthy donors by magnetic Dynal beads coated with anti-CD8 antibody (Dynal, Oslo, Norway) or anti-CD4 antibody (Dynal). The purity of the CD8+ and CD4+ cells population ranged from 95 to 100% as estimated by FACS (Becton Dickinson, San Jose, CA, USA) analysis using mouse monoclonal antibody against the human CD8 antigen (Becton Dickinson) or the human CD4 antigen (Becton Dickinson) and a PE-conjugated anti-mouse antibody as secondary reagent (Jackson ImmunoResearch laboratories, Inc., West Grove, PA, USA).

Before transduction, the CD8+ enriched target cell population (1.5×10^6 cells/ml) was prestimulated in RPMI1640 (Gibco), supplemented with PHA (1 μg/ml) (Murex Dartford, Kent, UK), PMA (50 ng/ml) (Sigma) and IL-2 (100 U/ml) (BD Bioscience, Bedford, MA, USA).

To generate oncoretrovirus-containing supernatants, PG13 packaging cells (4×10^5) were plated into a 60 mm tissue culture dish and after 24 h mock transfected or transfected with 3 μg of either the MP71w105TCR or the MP71wEGFP oncoretroviral vector. The medium was changed at 48 h post-tranfection, and GALV-pseudotyped retroviral supernatants were harvested and filtered 6 h later. As a control, the pCDNA 3.1 plasmid replaced the oncoretroviral vector.

The titer of EGFP-transducing oncoretrovirus was determined by infecting 293 cells with serial dilutions of virus supernatants in 24-well plates. After 48 h, the percentage of EGFP-transduced cells was determined by flow cytometry. Titers were $2–3 \times 10^6$ GFU/ml, where GFU stands for GFP green colony-forming units.

Transduction of CD8+ cells was performed over 2 consecutive days by replacing half of the T-cell culture medium with GALV-pseudotyped supernatants supplemented with IL-2 (100 U/ml) and protamine sulfate (4 μg/ml, Sigma). Cells were then centrifuged at 2500 r.p.m. for 120 min at 25°C before incubation at 37°C. To improve the transduction efficiency, the plates were coated with a recombinant fibronectin fragment CH296 (Takahara, Otsu, Japan) before seeding the cells. At day 8 after transduction, cells were analyzed for transduction efficiency by monitoring EGFP expression or assayed for cytolytic activity.

Pseudotyped HIV-1-derived vectors were generated by transient transfection of 293T.[33] The 293T cells were plated at 80% confluence on a 75 cm^2 flask and transfected by calcium phosphate DNA precipitation with 6.5 μg of the pMDLgRRE plasmid, 3 μg of the pRSV-REV plasmid, 3.5 μg of the pMD2.VSV-G plasmid and 8 μg of the pRRL.SIN18CMV105TCR or of the pCDNA 3.1 plasmid. The medium was replaced with fresh culture medium 24 h later. As a control, the pCDNA 3.1 plasmid was used in place of the lentiviral vector. Viral supernatant was harvested 48 h after transfection, filtered through 0.45 μm filters, aliquoted and stored at −80°C. Viral vector titers were determined by transduction of 293T cells, seeded in six-well plates at a density of 3×10^5 cells using serial dilutions of vector supernatants, in the presence of polybrene (8 μg/μl). FACS analysis was performed 5 days later: titers ranged from 0.5 to 5×10^7 GFU/ml. For transduction of CD8+ cells, 10^5 activated CD8+ T-lymphocytes were inoculated with the lentiviral particles in a total volume of 2 ml RPMI 1640 10% FBS supplemented with 5 μg/ml polybrene and

centrifuged at 1000 *g* for 1.5 h at 25°C. After inoculation, T-lymphocytes were washed in PBS and cultured in RPMI1640 10% FBS supplemented with IL-2 (100 U/ml) at 37°C for 5 days.

Western blotting analysis

Jurkat cells (4×10^6) were mock transfected or transfected with the pc105TCRm plasmid, washed 72 h later in PBS and lysed on ice in 100 µl of lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% IGEPAL CA 360 (Sigma), 0.5% sodium deoxycholate, 1% SDS; 1 × protease inhibitor mix (Roche Molecular Systems, New Jersey, USA) and then centrifuged for 5 min at 10 000 *g* to remove cell debris. The proteins were resuspended in a volume of 2 × SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT), boiled for 5 min, electrophoresed on a 10% SDS-PAGE gel and electrotransferred onto a PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blocked with 2% nonfat milk (Sigma) in PBS for 2 h at room temperature and washed three times in PBS–0.05% Tween 20 (PBS/T). The membranes were then probed with a primary mouse anti-human *c-myc* tag (1:1000 diluted) (Roche) in 2% nonfat milk for 1 h at room temperature. After three washes in PBS/T, secondary horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham) in 2% nonfat milk was applied for 1 h at room temperature, followed by five additional washes in PBS/T. The blots were developed using a chemiluminescent kit (Pierce, Rockford, IL, USA) as directed by the manufacturer, exposed to an X-ray film (Kodak, Paris, France) and analyzed using the Versa Doc Imaging System (Bio-Rad, Hercules, CA, USA). To test for equal protein loading, the same blots were incubated with mouse anti-human α-tubulin (1:2000) (Sigma).

Immunocytofluorescence and flow cytometric analyses

To assess for cTCR surface expression, Jurkat cells (5×10^6) were transfected with 10 µg of the pc105TCRm plasmid by electroporation.[54] After 72 h, cells were fixed in 2% paraformaldehyde and incubated with unconjugated mouse anti-human *c-myc* tag antibody (1:10 diluted, Roche Molecular Biochemicals, Indianapolis, IN, USA). Bound antibodies were detected with an anti-mouse antibody fluorescein isothiocyanate (FITC)-conjugated (Santa Cruz, CA, USA). The cells were then excited with a krypton-argon laser and visualized using a LEICA DM IRBE confocal microscopy (Leica, Solms, Germany).

CD8+ cells population was controlled using an unconjugated mouse anti-human CD8 antibody (Becton Dickinson), which was detected with a PE-anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA). Flow cytometric analysis was performed on a FacScan cytometer (Becton Dickinson). Data were analyzed using WinMDI 2.8 software.

ERK phosphorylation

Lymphoblastoid Jurkat or MDS T-cell lines (5×10^6) transfected with 10 µg of the pc105TCR plasmid were evaluated for their ability to induce MAP kinase activity, in particular, ERK phosphorylation. Activated ERK was assessed by Western blotting using rabbit polyclonal antibody that recognizes the dually phosphorylated active forms of ERK (Promega, Madison, WI, USA). Lymphoblastoid cells were incubated with COS-1 target cells transfected with the pSVIII*env* plasmid.[61] This plasmid expresses the HIV-1 envelope glycoprotein gp120 (HXBc2 molecular clone) under the transcriptional control of the HIV-1 LTR. COS-1 and lymphoblastoid T cells were incubated at a 1:1 ratio in six-well plates for 30 min on ice. After the incubation, T cells were washed in PBS and resuspended in 100 µl of PBS containing 1 µg of goat IgG fraction of mouse immunoglobulins (Cappel, Aurora, OH, USA). Following 5 min of incubation at 37°C, the cells were washed in PBS, resuspended in 1 × SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT), passed a few times through a 0.33 mm needle and then processed for Western blotting.

IL-2 release

Jurkat cell lines expressing the scFv105TCR were evaluated for their ability to secrete IL-2 in response to incubation with the COS-1 cell line displaying or not displaying the HIV-1 envelope gp120. Jurkat cells were incubated with COS-1 gp120-negative or -positive target cells at a ratio of 1:1 in six-well plates for 24 h in the presence of 1 µg/ml PHA. In parallel, Jurkat cells were stimulated via the native T-cell receptor with 1 µg/100 µl of anti-CD3 monoclonal antibody (Becton Dickinson) for 30 min at 4°C before adding 1 µg/ml PHA. After 24 h, supernatants were removed and assayed for IL-2 concentration by solid-phase ELISA (R&D Systems, Minneapolis, MN, USA). The detection limit of the IL-2 ELISA was 7 pg/ml.

Binding of soluble gp120

COS-1 cells were transfected with the pc105TCRm plasmid and incubated in PBS/0.1% BSA to a final volume of 100 µl with 1 µg of biotinylated recombinant HIV envelope gp120 (Intracell, Seattle WA, USA) for 30 min at 4°C. The cells were then washed three times and incubated under the same conditions with 2 µl of streptavidin–PE (Becton Dickinson). The cells were washed as above and the level of binding was assessed by FACS analysis. As a negative control, the samples were incubated with the secondary reagent alone, while as a positive control cells were transfected with a plasmid expressing the human CD4 molecule under the transcriptional control of the CMV promoter (pCDNA 3.1CD4), kindly provided by Dr J Sodroski.

Analysis of gene expression by RT-PCR

Total RNA was extracted from CD8+ cells pellets using a commercial Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. To eliminate residual contaminating DNA, 1 U of RNase free DNase I (Roche) was added to each sample; after 30 min of incubation at 37°C, DNase I was heat inactivated at 94°C for 3 min, followed by quick cooling on ice. First strand cDNA was made from 5 µg of total RNA in a 50 µl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5.5 mM $MgCl_2$, 10 mM random hexanucleotide primers, 1 mM of each dNTP, 0.3 U RNase inhibitor and 1.5 U Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase (RT) (Applied Biosystem). A negative RT sample control (no Mo-MuLV RT) was included to ensure that the RNA was free of viral DNA. RNA samples were incubated in the reaction mixture for 10 min at 25°C, 60 min at 48°C

and then at 95°C for 5 min. A measure of 10 μl of the reverse-transcribed mixture was amplified with primers specific to the TCR chimeric sequence (forward primer 5′-TTGGCGGCCGCCATGGAGTGGAAGGCG-3′ and reverse primer 5′-TTAGCGAGGGGGCAGGGCCTGCAT GTG-3′). As a positive control for each RNA preparation and RT-PCR, the β-actin cellular sequence was also amplified simultaneously, in separated tubes, using forward (5′-TGACGGGGTCACCCACACTGTGCCCA TCT-3′) and reverse (5′-CGAAGCATTGCG GTGGAC-GATGGAGC-3′) specific primers. PCR was performed in a final volume of 50 μl containing 25 pmol of each primer, 250 μM of each dNTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 1 U of Amplitaq DNA polymerase (Applied Biosystem). In all, 40 amplification cycles were performed after an initial denaturation step of 1 min at 94°C. Each cycle was carried out at 94°C for 30 s, 68°C for 2 min, 68°C for 10 min, followed by a final cycle at 68°C for 10 min, prior to cooling to 4°C. A measure of 15 μl of each amplified sample was subjected to agarose gels electrophoresis, visualized under a UV lamp after staining with ethidium bromide and then analyzed using GEL Document 1000 (Bio-Rad, Hercules, CA, USA).

Virus stocks and infection of human primary T cells with HIV-1

HIV-1 IIIB stocks were obtained from the supernatant of freshly infected Jurkat cells and stored at −80°C. The viral titer (50% tissue culture infectious dose ($TCID_{50}$)/ml) was determined by titer determination on C8166 cells as described previously.[62]

Before infection, the CD4+ enriched target cells population (1.5×10^6 cells/ml) was prestimulated in RPMI 1640 (Gibco), supplemented with PHA (1 μg/ml) (Murex Dartford) and IL-2 (100 U/ml) (BD Bioscience). For infection, 1×10^6/ml activated CD4+ T-lymphocytes were incubated with HIV-1 IIIB at a multiplicity of infection of 5×10^5 $TCID_{50}$/cell for 6 h at 37°C. After infection, the T-lymphocytes were washed in PBS, seeded in 24-well plates at a density of 1×10^6 cells/ml, cultured in RPMI 1640 10% FBS supplemented with IL-2 (100 U/ml) at 37°C for 18 h and then tested in cytotoxicity assays.

Cytotoxic T-cell assay

Cytotoxicity of retroviral vector-transduced human primary CD8+ T cells was evaluated by a standard ^{51}Cr release method. Jurkat and MDS lymphoblastoid T-cell lines transfected with the HIV-1 gp120-expressing plasmid pSVIIIenv and purified CD4+ T-lymphocytes infected with HIV-1 IIIB were used as targets. The target cells were labeled with 50 μCi of [^{51}Cr]isoquinoline (Medi Physics Inc., Arlington, IL, USA) for 1 h at 37°C, extensively washed and seeded at a density of 0.5×10^4 in round-bottomed 96-well microplates in 100 μl of complete RPMI-1640. Transduced CD8+ effector (E) cells were suspended in 100 μl of AIM-V medium (Invitrogen) and added to target cells at different E:T ratios. The plates were incubated at 37°C for 4 h, and 100 μl of the supernatant of each sample were harvested for γ-counting in order to assess ^{51}Cr release. Calculations were carried out in triplicate and standard deviations were determined. All of the experiments were repeated at least three times. Specific lysis was calculated as follows:

$$\text{Specific lysis \%} = \frac{\text{Observed release (c.p.m.)} - \text{spontaneous release (c.p.m.)}}{\text{Total release (c.p.m.)} - \text{spontaneous release (c.p.m.)}} \times 100$$

where c.p.m. is the count/min released by targets incubated with effector cells. Spontaneous release was determined from wells to which 100 μl of complete medium were added instead of effector cells. Total releasable radioactivity was measured after treating the target with 100 μl of 1% Triton X-100.

For the blocking experiments, the anti-human HLA-class I monoclonal antibody (Cymbus Biotechnology, Ltd, UK) or the anti-gp120 monoclonal antibody (ABI, Columbia, MD, USA) was added to the ^{51}Cr-loaded target cells and incubated for 1 h before the cytotoxic assay.

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