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Intrabody-mediated knockout of the high-affinity IL-2 receptor in primary human T cells using a bicistronic lentivirus vector

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A bicistronic human immunodeficiency virus type 1 (HIV-1)-based vector is described in which the expression of a selectable marker and a second gene of interest are forcibly coupled by means of an internal ribosome entry site. The vector provides high-level expression of the coselected gene in approximately 90% of transduced cells and has been used to express an endoplasmic reticulum-targeted single-chain antibody (intrabody) directed against a subunit of the interleukin-2 receptor, IL-2Rα. In the established T cell line Kit225 and also in primary human T cells stably transduced with the intrabody vector, the cell surface expression of IL-2Rα could be reduced to a low or undetectable level. Responsiveness to IL-2 was reduced 10-fold in the IL-2Rα-negative cells, consistent with a lack of high-affinity IL-2 receptors. Pseudotyping of the HIV-1 core with the vesicular stomatitis virus G protein improved particle stability by two- to three-fold and enhanced vector entry into established T cell lines up to 230-fold. Vector entry into primary human T cells was most efficient when the amphotropic murine leukemia virus envelope was used. The forced, high-expression capability of the bicistronic vector, together with the capacity of HIV-1 vectors to infect nondividing cells, make this an attractive tool for the genetic manipulation of primary cell types.

Keywords: *lentivirus vector; IRES; VSV-G protein; single-chain antibody; IL-2Rα*

Introduction

Retroviral vectors based on Moloney murine leukemia virus (MoMLV) have been widely used *in vitro* and are the method of choice for *ex vivo* gene delivery in a majority of human gene therapy trials because of their broad target cell range, efficient gene transfer and stable, single copy gene insertion into the host cell genome.[1,2] A significant limitation of MLV-based vectors, however, is the requirement for mitotic division of the target cell, which appears to reflect the need for breakdown of the nuclear membrane in order for the MLV preintegration complex to access the host cell chromatin and integrate.[3,4] The preintegration complex of lentiviruses, a family of retroviruses which includes the human immunodeficiency virus type 1 (HIV-1), have been shown to possess nuclear targeting signals which allow these viruses to infect nondividing cells including macrophages.[5–7] The karyophilic properties of the HIV-1 preintegration complex have been attributed both to the matrix protein and to the vpr protein, which mediate the active transport of HIV-1 preintegration complexes across the nuclear membrane.[8–10] The capacity of HIV-1 vectors to transduce nondividing cells stably has been demonstrated *in vitro*[11] and also *in vivo* with transduction of terminally differentiated neurons as well as astroglial cells following injection of a β-gal expressing HIV-1 vector into the brains of adult mice.[12]

A second feature of HIV-1-based vectors is the ability to manipulate the target cell range by substituting the HIV-1 envelope glycoprotein, gp160, with envelope proteins from other viruses. Use of the native HIV-1 envelope allows targeted delivery of vectors to CD4/coreceptor-positive cells, a property that may be desirable when delivering gene-based therapeutics aimed at inhibiting HIV-1 replication. HIV-1 particles have been successfully pseudotyped with envelope glycoproteins from other retroviruses including A-MLV, SRV3 and HTLV-I which each confer an extended host range.[13–15] HIV-1 particles have also been pseudotyped with envelope proteins from unrelated viruses such as herpes simplex virus and the G protein of vesicular stomatitic virus (VSV).[12,16–18] The robust association between VSV-G protein and the virion core allows virus particles to be concentrated without loss of infectivity and has enabled the production of HIV-1 vector stocks with titers exceeding 10^9/ml.[11,17–19] HIV-1 vectors have therefore been developed to a point of potential clinical utility and offer considerable potential as an *in vivo* tool for the manipulation of both dividing and nondividing cells.

A frequent requirement of gene transfer vectors is the ability to express more than one gene, for example the gene of interest and a selectable marker. Retroviral vectors have previously been described which are capable of expressing two genes by alternate splicing of the vector RNA or by the use of an internal promoter.[1] A problem frequently encountered when using an internal promoter is that of promoter interference, whereby transcription of

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the nonselected gene is suppressed by a poorly understood epigenetic mechanism.[20,21] Splicing vectors have also proved problematic and their use has been limited by the difficulty of achieving the desired balance of spliced and unspliced vector RNAs.[22,23] More recently, bicistronic mRNAs incorporating an internal ribosome entry site (IRES) have been found to provide a more balanced expression of two genes in retroviral and other vector systems.[24–29] In this article we describe a bicistronic HIV-1-based vector, in which the expression of a selectable gene and a second gene are forcibly coupled by means of an IRES. This strategy resulted in efficient expression of the coselected gene in a high proportion of transduced cells. Furthermore, we exploit the powerful Tat/TAR transactivation system of HIV-1[30,59,60] to boost gene expression to an extremely high level.

Results

Optimization of conditions for HIV-1 vector transduction into PBMC

Choice of envelope gene: Previous studies have demonstrated the capability of HIV-1 particles pseudotyped with A-MLV, SRV3, HTLV-I or VSV envelope glycoproteins to infect CD4-negative cell lines of human and nonhuman origin such as HOS, Hela and NIH3T3.[13–19] To establish which viral envelopes most efficiently mediate entry into primary human T cells, a HIV-1 vector encoding chloramphenicol acetyltransferase (CAT) was pseudotyped with a panel of viral envelopes and used to infect PHA-activated PBMC. Vector entry into two CD4 expressing T cell lines, Jurkat and SupT1, was examined in parallel. The results are summarized in Figure 1a. The entry of HIV particles bearing the native gp160 from the HIV-1 molecular clone HXB2 was roughly equivalent in the three cell types examined. The entry of A-MLV and VSV-G-pseudotyped particles varied significantly depending upon the cell type. In SupT1 cells, vector entry was enhanced 3.6- and 230-fold by substitution of the native gp160 with the A-MLV envelope or VSV-G protein, respectively. A 50-fold enhancement was seen in Jurkat cells using the VSV-G protein. Interestingly, the Jurkat cells were refractory to infection by A-MLV-pseudotyped particles. Vector entry into activated PBMCs was enhanced 1.76-fold and 5.1-fold by use of the VSV and A-MLV envelope proteins, respectively.

Envelope glycoproteins from visna/maedi virus and human foamy virus did not permit entry at a detectable level into activated PBMCs either by cell-free infection or by cocultivation. It is unclear at present whether this is due to an insufficient expression level of the envelope glycoproteins in the packaging cell, to poor incorporation of the glycoproteins into virion particles or to the lack of appropriate receptors on human PBMCs. Among the nonretroviral envelopes tested, the fusion (F) and hemagglutinin (HN) proteins of NDV and the E glycoproteins of Sindbis virus also failed to yield particles capable of infecting PBMCs.

Infection kinetics and greater efficiency of cell-to-cell infection: To determine the time required for cell-free virus binding and entry, PBMC were exposed to a fixed amount (50 000 RT units) of HXB2, A-MLV or VSV-G-coated virus for periods ranging from 30 min to 24 h. In all cases, the transduction level increased linearly up to 8 h and rose a further 1.5- to two-fold over the next 18 h (Figure 1b). Addition of the polycation polybrene had little or no effect on the infectivity HIV(HXB2) particles but did enhance the entry of HIV(A-MLV) particles two- to three-fold at a concentration of 8 μg/ml (data not shown). Polybrene was not routinely used, however, as significant toxicity was observed at this concentration. Cocultivation of the producer and target cells enhanced the efficiency of HIV(HXB2) transduction by 30-fold and gave a more modest three- to four-fold increase in HIV(A-MLV)-mediated transfer (data not shown).

Particle stability: To examine the effect of storage on particle infectivity, virus harvested from COS-1 cells 60 h after transfection was stored at 4°C, 18°C or 37°C for up to 48 h before infection. At time zero, freshly harvested virus was used to inoculate PBMCs which had been activated 3 days earlier. The results in Figure 1c show that HIV(A-MLV), HIV(HXB2) and HIV(VSV-G) particles decayed with half lives of approximately 8, 12 and 24 h, respectively. The experiment was repeated three times with similar results. In each case, the rate of decay was not strongly influenced by the storage temperature. Between 80 and 100% of the infectivity was retained if virus stocks were frozen in aliquots at –70°C (data not shown).

Requirement for T cell activation: To examine the effect of activation state on the efficiency of vector transduction, 1×10^6 non-activated or PHA-activated PBMCs were infected with 10 000 RT units of a frozen HIV-1 (A-MLV) vector stock. Activated cells were infected 1, 3, 5, 7 and 9 days after PHA treatment. Vector stocks were stored at –70°C and thawed immediately before use. As shown in Figure 1d, transduction levels were maximal on day 5, but did not show a sharp peak. Owing to the greater expansion potential of cells transduced shortly after PHA stimulation, infection on day 3 was found to yield the greatest absolute number of transduced cells. As expected, non-activated cells were transduced at low levels relative to activated cells. Several groups have previously reported a block in the infection of quiescent T cells which has been attributed to slow or incomplete reverse transcription of the incoming viral genome[31–34] as well as a failure of the reverse transcribed DNA to integrate in non-activated T cells.[35,36]

Evaluation of a bicistronic HIV-1 vector

A bicistronic vector was designed in which the encephalomyocarditis virus IRES is inserted between a selectable gene and the gene of interest, allowing cap-independent translation of the downstream gene (Figure 2a).[37] The bicistronic vector has been used to express an endoplasmic reticulum (ER)-targeted single-chain antibody, or intrabody (sFvTac), directed against the α subunit of the high-affinity interleukin-2 receptor (IL-2Rα). We previously showed that the expression of the sFvTac gene in the human T cell line Jurkat led to a phenotypic knockout of IL-2Rα due to intrabody-mediated retention of the receptor in a pre-Golgi compartment.[38] Kit225, a growth factor-dependent human T cell line that expresses high levels of IL-2Rα (about 200 000 receptors per cell), was used to test the efficacy and expression characteristics of

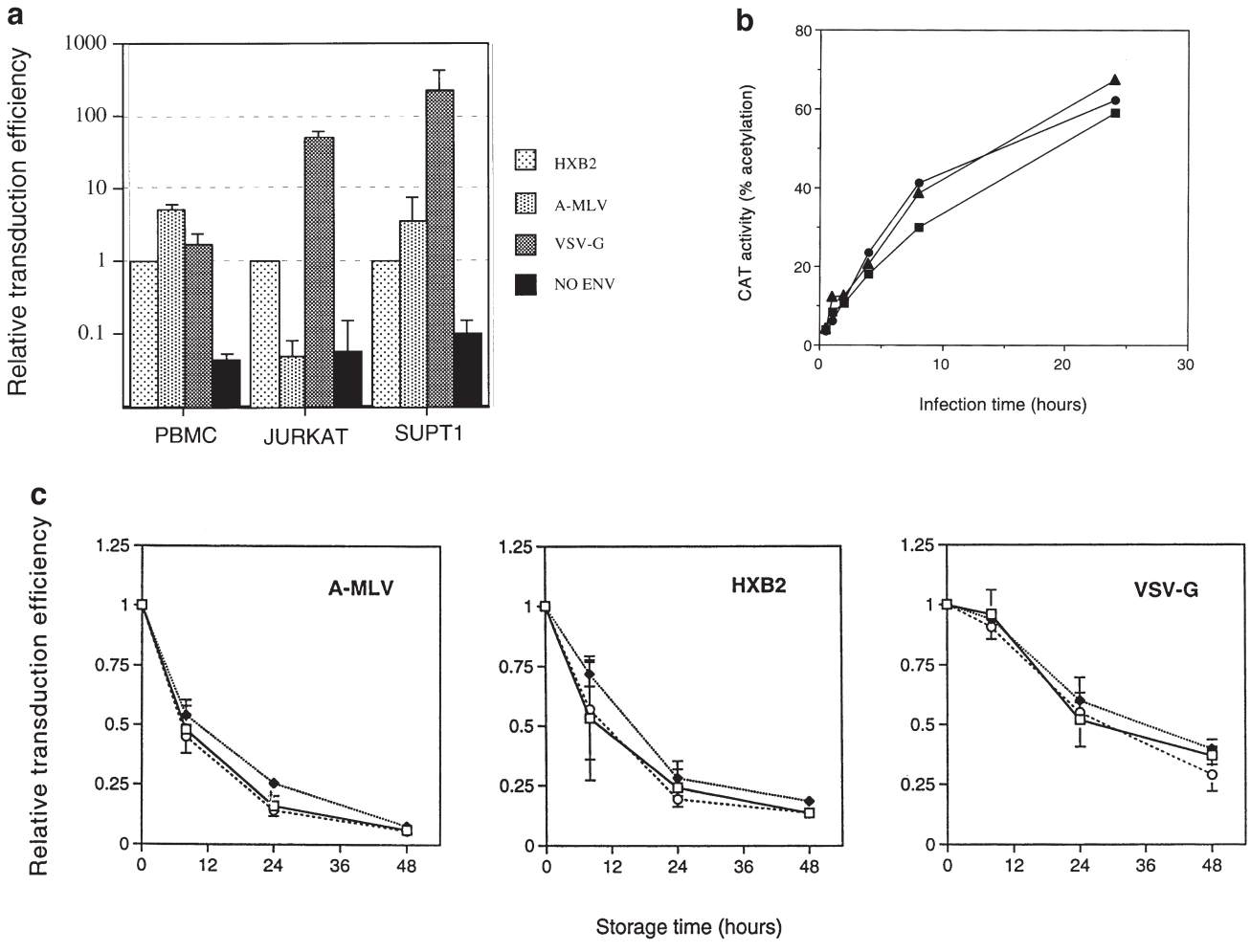

Figure 1 *(a) Transduction efficiencies of wild-type and pseudotyped HIV-1 CAT vector. CAT activities measured 72 h after infection are expressed relative to the level obtained using the wild-type (HXB2) envelope. Standard deviations are shown. (b) Influence of infection time on the level of vector transduction. PHA-activated PBMCs were exposed to HIV (HXB2) (▲), HIV (A-MLV) (●) and HIV (VSV) (■) vector stocks for the indicated period of time and harvested 3 days later. CAT activity is expressed as the percentage of ^{14}C-chloramphenicol to be acetylated in a 1 h assay using 20 μg (HXB2, VSV) or 4 μg (A-MLV) of cell extract. (c) Effect of storage on particle infectivity. HIV (HXB2), HIV (VSV) and HIV (A-MLV) particles were stored for up to 48 h at 4°C (◆), 18°C (□) or 37°C (○) before infection of PHA-activated PBMCs. Cells were harvested 72 h after infection for measurement of CAT activity. Bars represent one standard deviation. (d) Influence of activation state and day of infection on vector transduction efficiency. PBMCs were infected with 10 000 RT units of a frozen HIV (A-MLV) vector stock either before or 1, 3, 5, 7 and 9 days after PHA activation. Cultures were harvested 72 h after infection and 15 μg of each extract was used to measure CAT activity.*

HVTac, a HIV-1-based vector in which sFvTac and puromycin acetyltransferase (puro) genes are expressed from a bicistronic mRNA. HIV(A-MLV) particles encapsidating the HVTac vector, which contains an internal SL3–3 promoter driving expression of the puro-IRES-sFvTac cassette, were produced in COS-1 cells and used to infect Kit225 cells that were subsequently maintained in 100 U/ml IL-7. A bulk population of transduced cells was obtained by puromycin selection and analyzed for cell surface IL-2Rα expression by flow cytometry. As shown in Figure 3, greater than 90% of the puromycin-resistant Kit225 cells showed little or no cell surface expression of IL-2Rα, suggesting that virtually all transduced cells expressed the sFvTac intrabody. Similar results were obtained with the human T cell lines C8166–45 and HUT102 (data not shown). Phenotypic knockout of IL-2Rα was also achieved with a vector lacking the internal SL3–3 promoter, in which the bicistronic puro/sFvTac mRNA is produced by splicing of the full-length vector transcript (data not shown). These results underscore the

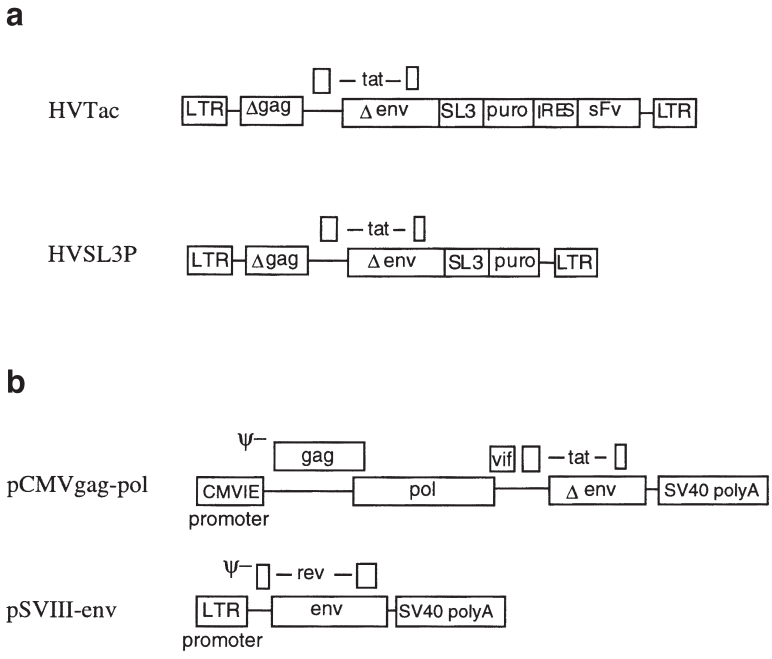

Figure 2 *Genetic organization of HIV-1-based retroviral vectors (a) and helper virus constructs (b) used for vector encapsidation.*

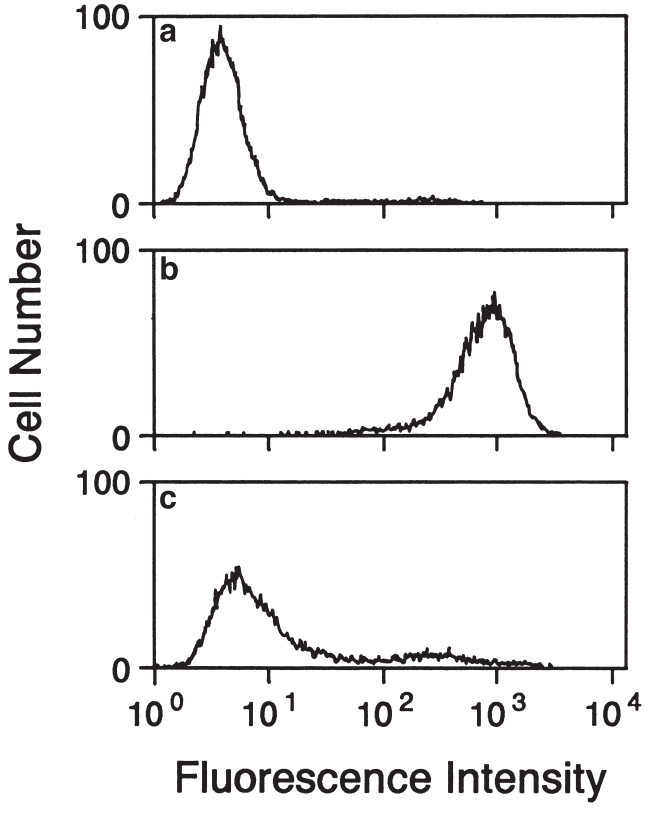

Figure 3 *IL-2Rα expression on Kit 225 cells transduced with the HVTac vector. The cells were stained with anti-Tac (b and c) or an irrelevant antibody (a). (a and c) HVTac-transduced cells, bulk population; (b) parent Kit 225 cells.*

efficiency of the forced expression system as well as the ability of ER-targeted antibodies to constitutively down-regulate a highly abundant cell surface receptor.

Stable transduction of primary human T cells by HIV-1 vectors

The vectors HVTac and HVSL3P were encapsidated by HIV(HXB2) or HIV(A-MLV) particles and introduced into PHA-activated PBMCs by cell-free transduction. Puromycin selection was applied 24 h later and the cultures were maintained in 100 U/ml IL-7. Large numbers of stably transduced T cells were obtained by re-stimulating the cultures on day 14 as described in Materials and methods. No viable cells were seen in nontransduced cultures after re-stimulation. In contrast, cultures transduced with HVTac or the control vector expanded 10- to 20-fold upon re-stimulation, yielding $>10^7$ puromycin-resistant cells within 7 days. The CD4/CD8 phenotype of these cells was analyzed by flow cytometry (Figure 4). Cells transduced using the HXB2 envelope were >98% CD4 positive and illustrate the potential of the HIV vector system for targeting genes to the MHC class II-restricted T cell subset. When the A-MLV envelope was used, both CD4 and CD8 cells were represented in the transduced population (Figure 4). The inverted CD4/CD8 ratio in the transduced population compared with the ratios normally found in peripheral blood of healthy donors may reflect more efficient transduction of the CD8 population or greater expansion of the CD8 cells under these culture conditions.

Constitutive down-regulation of IL-2Rα in peripheral blood T cells transduced with the HVTac vector

The cell surface expression of IL-2Rα in the transduced populations was analyzed by flow cytometry. The bulk PBMC population transduced with HVTac showed a marked reduction in IL-2Rα expression. Only 33.4% of

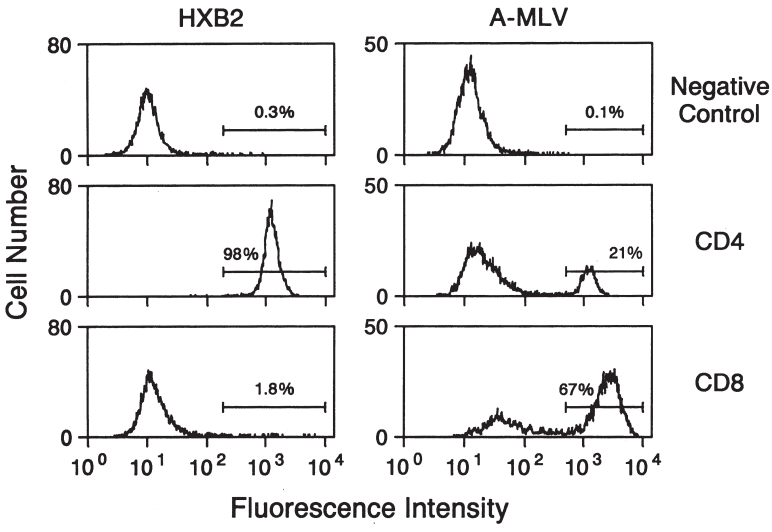

Figure 4 *CD4 and CD8 expression on stably transduced PBMCs. CD4 and CD8 expression was detected by indirect immunofluorescent staining with Leu3a and Leu2a, respectively. Control cells were stained with the secondary antibody alone. The envelope used for vector transfer is indicated at the top of the Figure.*

these cells stained positive for IL-2Rα (Figure 5a, center left panel) whereas 88.0% were positive in the control population transduced with the HVSL3P vector (Figure 5a, lower left panel). Furthermore, the mean fluorescence intensity of the HVTac-transduced cells was low (8.76) relative to that of the control population (52.9). Single-cell clones were generated from the HVTac-transduced population by subcloning in the presence of IL-7 and analyzed for IL-2Rα expression by FACS analysis. Of the 10 clones analyzed, five (50%) did not express IL-2Rα at a detectable level (Figure 5a).

Immunoprecipitation studies confirmed a low level of mature IL-2Rα chains in the bulk HVTac-transduced cells (Figure 5b). Newly synthesized IL-2Rα is detected in the ER as a 40 kDa high-mannose glycoprotein (p40) which, on passage through the Golgi compartment, is converted into the mature 55 kDa form (p55) that is transported to the cell surface.[38] Immunoprecipitation of metabolically labeled IL-2Rα revealed normal levels of p55 in the HVSL3P-transduced PBMC but a virtual absence of p55 in the HVTac-transduced cells (Figure 5b, lanes 1 and 2, respectively). In contrast to our findings in established T cell lines,[38,39] the intracellular retention of IL-2Rα in PBMCs was not associated with accumulation of the p40 precursor. As IL-2Rα mRNA synthesis is stimulated by IL-2 receptor signaling in primary T cells, the absence of p40 may reflect reduced IL-2Rα gene transcription in the sFvTac expressing cells, which lack high-affinity IL-2 receptors. Alternatively, the complexes formed between p40 and sFvTac in the ER may be degraded more rapidly in primary T cells than in T cell lines.

Reduced IL-2 responsiveness in HVTac-transduced T cell clones

A thymidine incorporation assay was used to compare the IL-2 responsiveness of an IL-2Rα-negative and an IL-2Rα-positive clone (clones 2 and 5, see Figure 5a). The IL-2Rα negative clone 2 did not respond to low doses of IL-2 (Figure 5c). Some proliferation was seen at IL-2 concentrations of 10 and 100 U/ml but when compared with the IL-2Rα-positive clone 5, approximately 10 times more IL-2 was required to achieve an equivalent proliferative response. These data demonstrate functional as well as phenotypic evidence for the absence of high-affinity IL-2 receptors in the sFvTac-transduced cells. Responsiveness to high concentrations of IL-2 is expected in the IL-2Rα-negative clone, as these cells continue to express the intermediate-affinity IL-2 receptor, composed of the IL-2Rβ and γ_c chains.[40]

Discussion

Interest in lentivirus vectors has recently been spurred by the recognition that these viruses are able to infect many noncycling cells including macrophages, neurons and astroglial cells,[12] contact-arrested human skin fibroblasts and human peripheral blood $CD34^+$ cells[11] as well as by technical innovations which have allowed the production of high-titer vector stocks.[17–19] In this article we demonstrate the stable transduction of primary human T cells by HIV-1-based vectors. HIV-1 vector entry into PHA-activated PBMCs was improved five-fold by substituting the native HIV-1 envelope glycoprotein with that of A-MLV. Pseudotyping with VSV-G protein dramatically enhanced HIV-1 vector entry into Jurkat and SupT cell lines but was found to have a minimal (less than two-fold) effect on vector entry into PBMCs.

The vector described here incorporates several features designed to provide reliable, high-level expression of intrabodies or other proteins in primary human T cells. First, expression of the intrabody gene was coupled to the expression of a selectable marker gene by means of an IRES. Second, we incorporated the HIV-1 *tat* gene into the vector to take advantage of the powerful Tat/TAR transactivation system which renders the HIV-1 LTR one of the most powerful promoters available for human T cells. Third, the *rev* gene, which is required for the nuclear export of full-length and incompletely spliced viral RNAs, is disrupted in the vector so that only terminally spliced mRNAs encoding Tat and/or sFvTac will be produced in the target cell (Figure 6) (see Ref. 41 for an overview of Rev function). As the incorporation of an

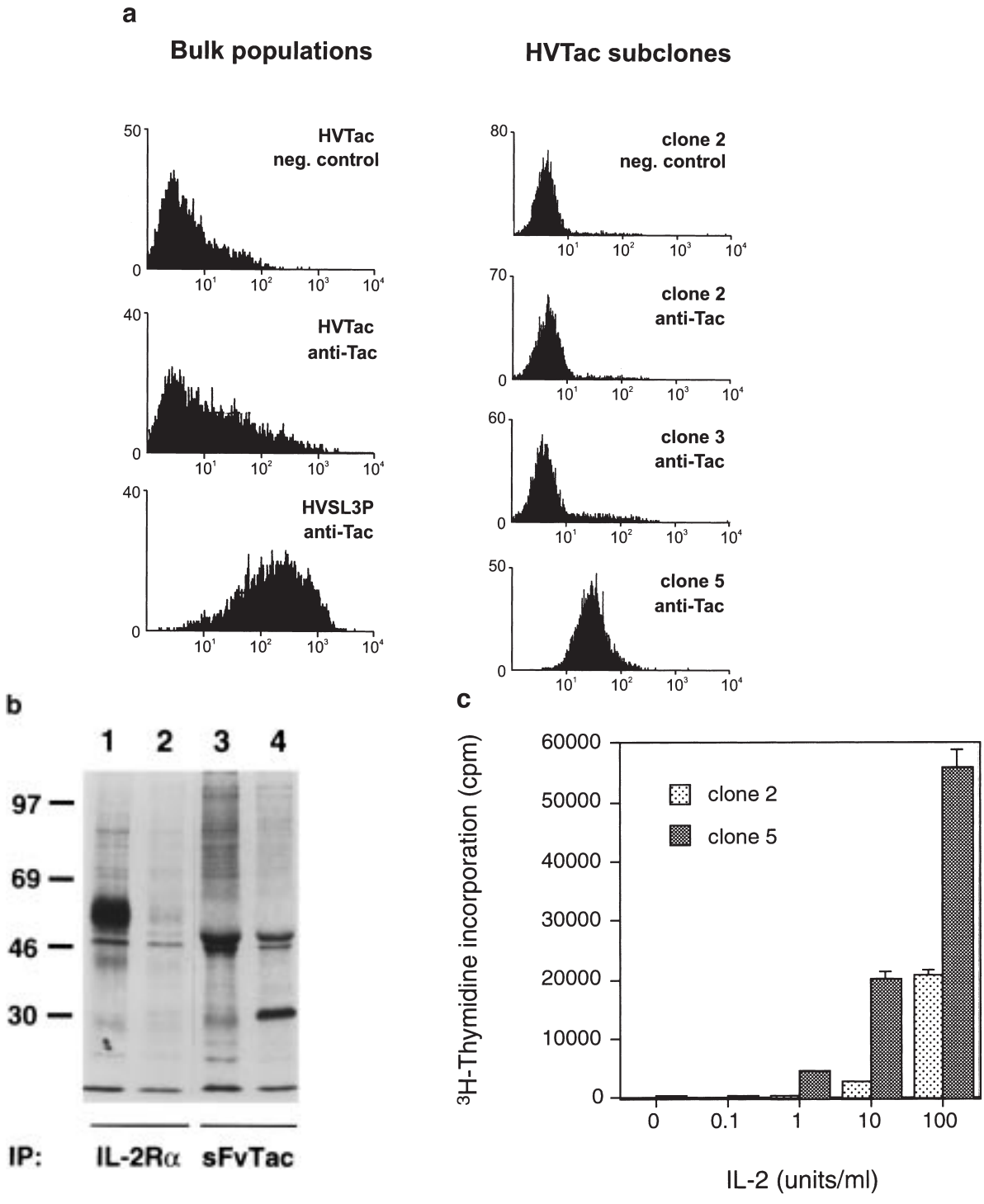

Figure 5 *(a) IL-2Rα expression on PHA-activated peripheral blood mononuclear cells transduced with HVTac or a control vector, HVSL3P. The cells were stained as indicated with anti-Tac mAb or with the secondary antibody alone (negative control). (b) Immunoprecipitation of IL-2Rα and sFvTac from bulk populations of transduced PBMCs. IL-2Rα was precipitated with mAb 7G7/B6 (lanes 1 and 2) and sFvTac with rabbit anti-mouse IgG (lanes 3 and 4). The transducing vector was HVSL3P (lanes 1 and 3) or HVTac (lanes 2 and 4). (c) IL-2 induced proliferation in peripheral blood T cell clones which are positive (clone 5) or negative (clone 2) for IL-2Rα expression. Bars represent standard error of the mean.*

intron in the 5′ untranslated region has been demonstrated to improve the efficiency of gene expression in many mammalian expression systems,[42–45] the extensive vector RNA splicing that takes place in the absence of Rev may itself contribute to efficient expression of the sFvTac gene. Lastly, the 3′ location of the IRES-sFvTac cassette ensures that every vector transcript, whether spliced or unspliced, can be translated to produce the sFvTac intrabody (Figure 6). As an MLV vector encoding *neo* and sFvTac genes from a bicistronic mRNA had no effect on the expression level of IL-2Rα in Kit225 and C8166–45 cells (Figure 7), we conclude that the high-

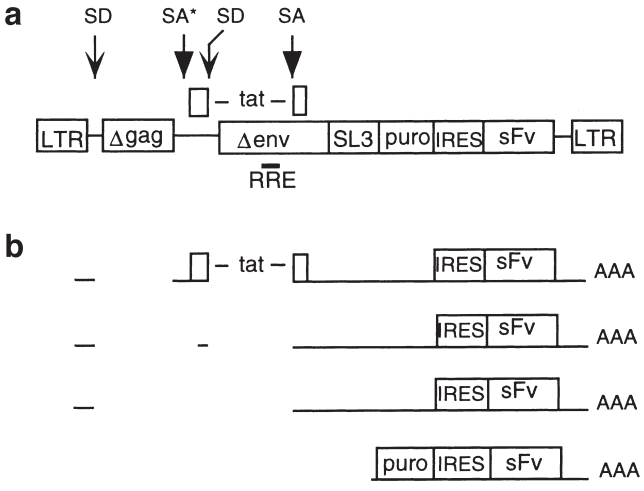

Figure 6 *Structure (a) and predicted transcripts (b) of the HVTac vector with their translation products. Splice donor (SD) and splice acceptor (SA) sites are shown. SA* denotes a cluster of four splice acceptor sites. Only terminally spliced transcripts are shown in (b). Other single and multiply spliced transcripts are possible.*[56–58]

expression capability of the HIV-1 bicistronic vector was a critical factor in achieving the complete elimination of IL-2Rα from the surface of Kit 225 cells.

Our objectives in this study were first, to develop a system for stable, high-level gene expression in primary human T cells and second, to determine whether intracellular antibodies can be used to alter the phenotype or function of primary T cells. The bicistronic HIV-1-based vector provided a highly effective means of intrabody gene expression, which has allowed the phenotypic knockout of the high affinity IL-2 receptor in primary T cells. One obvious application of these cells is in the investigation of activation-induced cell death (AID), a phenomenon in which signaling by the high-affinity IL-2 receptor is thought to play a key role.[46,47] The vector described here is most suitable for use with replicating cells because the selection step relies on outgrowth of a puromycin-resistant cell population. With minor modifications, the vector could be adapted for use with nonmitotic cells. Current studies to replace the *puro* gene with a fluorescent or cell surface marker will allow the rapid enrichment of transduced cells by cell sorting or immunoselection methods that do not rely on cell division. In the therapeutic setting, it may also be desirable to replace the Tat-driven expression system with an alternative promoter, eliminating the requirement for *tat* gene expression.

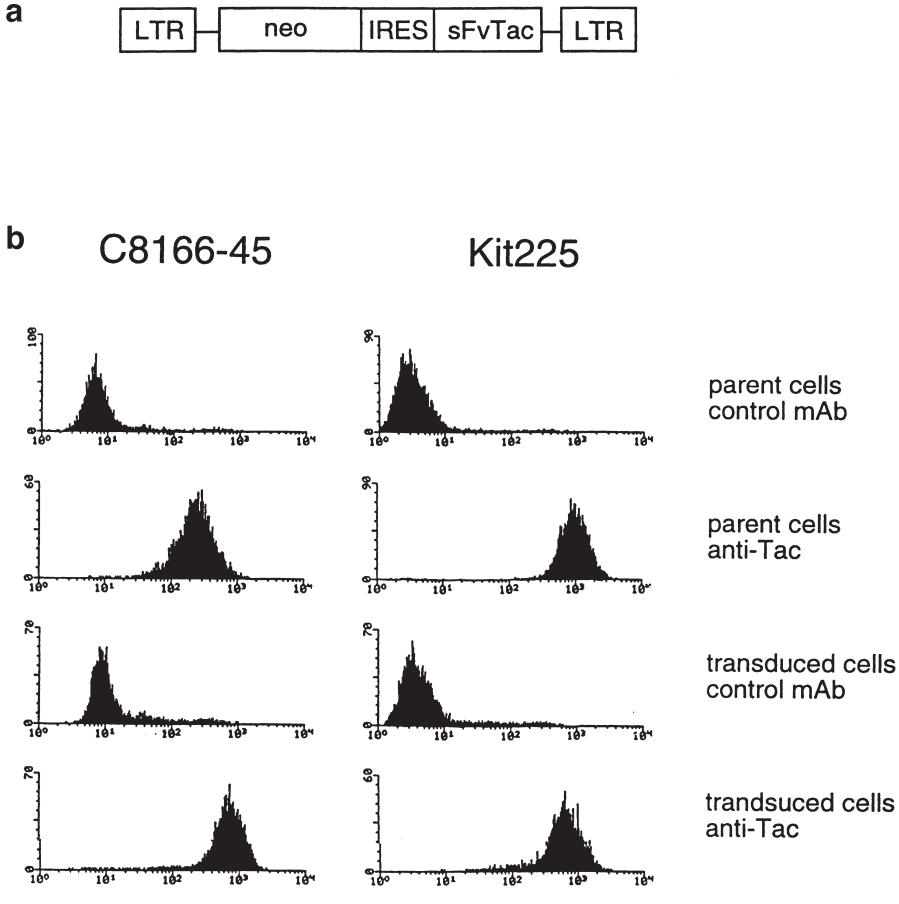

Figure 7 *(a) Structure of a bicistronic MLV-based vector, LNITac. (b) Flow cytometric analysis of IL-2Rα expression on parental and LNITac-transduced C8166–45 and Kit225 cells. Bulk populations were stained as indicated with anti-Tac or an isotype-matched control mAb.*

Materials and methods

Cell culture

COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Kit225 cells[48] were maintained in RPMI-1640 with 10% FBS and 100 U/ml recombinant human IL-7. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by Ficoll–Paque density centrifugation and stimulated with 1 μg/ml phytohemagglutinin (PHA-P, from Murex Diagnostics, Norcross, GA, USA). IL-2, in the form of 5% T-Stim (Becton Dickinson, Bedford, MA, USA) or 100 U/ml recombinant human IL-7 R&D Systems, Minneapolis, MN, USA) was added 2 days later and maintained continuously thereafter. For bulk re-stimulation, PBMC were cultured in 96-well plates at 10^4 cells per well with 1 μg/ml PHA, γ-irradiated (50 Gy) allogeneic PBMC as feeder cells (10^5 per well) and either 5% T-Stim or 100 U/ml IL-7.

Construction of bicistronic vectors

The vectors used here are based on the HIV-1 proviral clone HXB2 and depicted in Figure 2. Three deletions (2096–5743), (7041–7621) and (8475–9015) remove part or all of the *gag, pol, env, rev, vif* and *nef* open reading frames. The two exons of *tat* are intact, as are the splice donor and acceptor sites required for *tat* mRNA splicing. The Rev-responsive element (RRE) is retained to maximize production and packaging of the full-length vector RNA in the packaging cell, where Rev is supplied in *trans*. In the vector HVTac, a cassette composed of a puromycin acetyltransferase gene (puro) driven by the MLV SL3–3 promoter, an internal ribosome entry site (IRES) and a second gene (sFvTac), was inserted at position 8475, immediately downstream of the *nef* splice acceptor site. The encephalomyocarditis virus (EMCV) IRES was obtained from pCITE 2a (Novagen, Madison, WI, USA) and the sFvTac gene inserted at an *Nco*I site such that the initiator codon falls precisely at the natural EMCV polyprotein start site. sFvTac is a single-chain antibody derivative of the anti-Tac monoclonal antibody, which binds to the extracellular domain of IL-2Rα. The version of sFvTac used here has a C-terminal ER retention signal (amino acids K-D-E-L) and was designated sFvTacKDEL in a previous publication.[38] The vector HVSL3P was used as a control in some experiments.

Envelope expression plasmids

HIV-1 *env* and *rev* genes were expressed using pSVIII-env[49] A *Kpn*I–*Stu*I-deleted version of this plasmid, which expresses *rev* but not *env*, was used throughout the study as a negative control. pL/env, a gift of Dr B Cullen, encodes the visna/maedi virus L and *env* genes.[50] pA-MLV-env and pTMO, encoding the amphotropic MLV and Mason Pfizer monkey virus envelope genes were provided by Dr D Littman and Dr E Hunter, respectively.[51,52] Newcastle disease virus (strain AV) HN and F protein expression vectors were provided by Dr T Morrison and the Sindbis virus E gene expressor plasmid was a gift of Dr U Olshevski. pHCMV-G expressing the VSV G protein was provided by Dr T Friedmann.[19] A vector expressing the human foamy virus *env* gene was constructed by subcloning an *Xho*I to *Eco*RI fragment of the HFV genome[53] provided by Dr R Flugel into pRC/CMV (Invitrogen, Carlsbad, CA, USA).

Vector encapsidation and transduction

For optimization of conditions for PBMC infection, we used an env-deleted provirus carrying a CAT reporter gene in the *nef* position.[54] The defective genome is able to undergo a single round of infection when an envelope glycoprotein is provided in *trans*. Experiments were repeated three or more times.

Cell-free infection: For cell-free vector transfer, the CAT vector and an envelope expressor plasmid were transiently co-expressed in COS-1 cells using the DEAE dextran technique. Culture supernatants harvested 48–72 h later were normalized for reverse transcriptase activity and used to infect approximately 1×10^6 PBMC which had been stimulated 3 days earlier with PHA. CAT activity was measured in the PBMC culture 72 h after infection.

Cocultivation: For cocultivation, COS-1 cells transfected 48 h earlier were treated with 100 μg/ml mitomycin C for 30 min at 37°C. After washing three times with serum-free medium, the cells were cocultivated with 2×10^6 PHA-activated PBMC for 8–16 h. PBMC were washed, maintained in IL-2 and harvested no sooner than 12 days after infection for measurement of CAT activity. Where necessary, residual COS-1 cells were eliminated by re-stimulation of the PBMC with PHA and feeder cells. The re-stimulated cells were harvested 6 days later for measurement of CAT activity.

Selectable vectors: In the absence of an efficient stable packaging cell line for HIV-1 vectors, vector particles were generated by transfecting COS-1 cells with the vector and two or more plasmids encoding the structural and regulatory virus proteins required for HIV-1 particle formation (Figure 2b). *Gag, pol, vif* and *tat* genes were expressed from pCMVgag–pol and HIV-1 *env* and *rev* functions from pSVIIIenv. In experiments using A-MLV env, a separate *rev* expressor plasmid was included. Each of the plasmids contains an SV40 origin of replication allowing high-level expression in COS-1 cells. COS-1 cells were transfected with the vector and packaging plasmids at a ratio of 5:2:1:0.5 (gag–pol: vector: env: rev) using the DEAE dextran technique. Virus containing supernatants were harvested 48 and 72 h later, passed through a 0.45 μm filter and used immediately to infect 2×10^6 target cells.

Stable vector transfer into primary T lymphocytes

Ficoll-separated PBMCs stimulated 3 days earlier with 1 μg/ml PHA were infected with 2–5 ml of the vector stock (equivalent to a reverse transcriptase activity of 50–100 000 c.p.m.). The infection was repeated with fresh virus 24 h later and puromycin was applied 24 h after the second transduction at 0.5 μg/ml. Transduced PBMCs were fed at 3–4 day intervals with medium containing IL-7 (100 U/ml) and puromycin. Those cells still viable on day 14 were re-stimulated with PHA, feeder cells and IL-7, as described above. Seven to 14 days later, the emergent puromycin-resistant cell population was analyzed for cell surface phenotype, intrabody expression etc. Single-cell subclones were generated from the PBMC culture by limiting dilution as described.[55] Briefly, 14 days after the previous stimulation, puromycin-resistant cells were seeded at one cell per well of a V-bottom 96-

well plate in the presence of PHA, feeder cells and 100 U/ml IL-7. Wells were fed at 3–4 day intervals with medium containing IL-7. Positive wells were maintained by restimulation at 14-day intervals.

FACS analysis

Cell surface IL-2Rα, CD4 and CD8 were detected by staining with anti-Tac (kindly provided by Dr TA Waldmann, NCI, Bethesda, MD, USA), Leu3a and Leu2a monoclonal antibodies (Becton Dickinson) followed by FITC-conjugated goat anti-mouse IgG.

Immunoprecipitation

Metabolic labeling and immunoprecipitations were performed as described.[38] Briefly, 1×10^7 cells were metabolically labeled for 12 h with ^{35}S-cysteine and then lysed in RIPA buffer (0.15 m NaCl, 0.05 m Tris.HCl pH 7.2, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate). IL-2Rα and sFvTac were immunoprecipitated sequentially from the lysate using mAb 7G7/B6 and polyclonal rabbit anti-mouse IgG (Sigma, St Louis, MO, USA), respectively. Samples were analyzed by 10% SDS-PAGE and autoradiography.

Proliferation assays

Seven to 10 days after PHA stimulation, cells were washed three times to remove traces of IL-2, then plated in triplicate at 1×10^5 cells per well of a 96-well round bottom plate. IL-2 was added at doses ranging from 0 to 100 U/ml. Forty-eight hours later, wells were pulsed with 1 μCi titrated thymidine. Cells were harvested 18 h later and thymidine incorporation was measured by liquid scintillation counting.

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