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**RESEARCH ARTICLE**

# Efficient lentiviral transduction of Herpesvirus saimiri immortalized T cells as a model for gene therapy in primary immunodeficiencies

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*Infection of human T lymphocytes with the* Herpesvirus saimiri *(HVS) yields immortalized T-cell lines (HVS-T) which retain all the phenotypical and functional characteristics of their parental cells. This represents a new experimental model for studying genetic disorders of T lymphocytes. In spite of the efforts of many laboratories, no satisfactory way has been found so far to modify HVS-T cells genetically. We have analyzed the capacity of oncoretroviral (MLV)- and lentiviral (HIV-1)-based vectors pseudotyped with vesicular stomatitis virus glycoprotein (VSVg) to transduce HVS-T cells. HIV-1-derived vectors efficiently transduced HVS-T cell lines, reaching up to 85% of cells expressing the transgene in a single round of infection. MLV-based vectors, on the other hand, were unable to transduce more than 1% of any of the HVS-T cell lines analyzed. Lentiviral-driven gene expression was maintained constant and stable in HVS-T cells for a minimum of 48 days. We also observed that although the lentiviral transduction efficiency achieved on HVS-T cells is lower than that obtained with tumor or primary endothelial cells, it is nevertheless similar to that found with activated primary T cells.*

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## Introduction

The isolation and characterization of genetic functions involved in the biology of healthy and pathological human T lymphocytes demand suitable experimental systems. Thus, the molecular and functional analysis of T lymphocytes requires the adaptation of primary cells to long-term *in vitro* culture. This has been achieved either by generating antigen-specific T-cell clones or by infecting T lymphocytes with the HTLV-I retrovirus. Nevertheless, diverse factors have limited the extensive application of these two models. Primary T-cell clones show a stable phenotype and retain all the characteristics of their parental cells,[1] but they have a limited lifespan and *in vitro* culture requires cyclic stimulation with the specific antigen as well as the addition of exogenous IL-2. HTLV-I infected cells, on the other hand, are initially IL-2 dependent and functionally competent,[2] although after some weeks in culture a downregulation of relevant cellular proteins takes place, giving rise to IL-2 independent, giant multinucleated cells that no longer retain T-cell characteristics.[3,4]

More recently, it has been reported that certain strains of *Herpesvirus saimiri* (HVS) immortalize human T lymphocytes (HVS-T), thus yielding long-term cell lines with stable surface markers and functional phenotypes.[5,6] This strategy has provided a suitable, convenient experimental approach to examine T-cell function. HVS is non-pathogenic in its natural host, the squirrel monkey, but provokes extremely aggressive lymphomas and lymphocytic leukemias in other primates,[7] where it enters the cell and remains as nonintegrated viral episomes.[5,6] Although HVS encodes two antiapoptotic proteins,[8,9] all evidence suggests that it is not interference with apoptotic programs that promotes HVS immortalization of T cells but rather interactions between viral proteins and normal T-cell signaling pathways.[10]

HVS-T cells are potentially important in the study of both primary and acquired immunodeficiencies. When studying primary immunodeficiencies, HVS-T cell lines are particularly useful due to the scarcity of patients and the low numbers of primary cells usually available. Our group[11] and others[12,13] have shown the suitability of HVS immortalization for characterizing T-cell defects that are directly linked to mutated genes. Therefore, HVS-T cells from primary immunodeficient patients are ideal targets to assess functional reconstitution after the introduction of therapeutic genes.

In spite of numerous efforts by many laboratories, all attempts to introduce foreign genes into HVS-T cell lines by transfection or transduction with oncoretroviral vectors have met with little success. This has impeded the general use of HVS-T cells in experimental gene-therapy models. Lentiviral vectors have been

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described recently as the most powerful of all integrative vector systems.[14] They are stable, easy to concentrate and have broad tropism. Virtually all the cells analyzed, including nondividing ones, have been efficiently transduced.[15] We report here on our successful transduction with lentiviral vectors of several HVS-T cell lines from primary immunodeficient patients as well as healthy individuals. In addition, we also observed that although the efficiency achieved is lower than that obtained with tumor or primary endothelial cells, it is similar to that found with activated primary T cells.

## Materials and methods

### Cell lines and culture media

The generation and characterization of HVS-T cells: GOR-1/HVS; GOR-7/HVS (derived from two patients with the Wiskott–Aldrich syndrome) and ITA/HVS has been described in detail.[11] The HVS-T cell line ITA/HVS was derived from a healthy female subject, mother of a patient with a severe combined autosomal immunodeficiency from whom the SPA/HVS cell line was derived. CA/HVS cells were obtained from an unrelated normal individual. All HVS-T cell lines were cultured in a 1:1 mixture of RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium) and Panserin 401 medium (PAN Biotech, Aidenbach, Germany), supplemented with 10% fetal calf serum, glutamine, penicillin–streptomycin (all from Bio-Whittaker), and 50 UI/ml recombinant human IL-2 (rIL-2; obtained through the AIDS Research and Reference Program, National Institutes of Health, Rockville, MD, USA). 293 T cells (kidney epithelial cells), JEG-3 cells (extravillous trophoblast coriocarcinoma cells) and RKO cells (colon adenocarcinoma cells) were grown in Dulbecco's modified Eagle's medium (DMEM High Glucose; Gibco), supplemented with 10% FCS, glutamine and antibiotics as above; and HUVEC cells (endothelial human primary cells; PROMOCELL, Heidelberg, Germany) were grown in endothelial cell-growth medium (PROMOCELL). Jurkat T cells were cultured in RPMI 1640, supplemented as above without the addition of rIL-2.

### Plasmids

HIV packaging (pCMVΔR8.91) and VSV-G (pMD.G) plasmids were kindly provided by Dr D Trono and are described elsewhere.[14,33] The packaging plasmid pCMVΔR8.91 encodes *gag*, *pol*, *tat* and *rev* genes. The pMD.G plasmid encodes the vesicular stomatitis virus (VSV) G protein. The lentiviral vector plasmid HRSIN-CSGW[34] contains a spleen focus-forming virus (SFFV) LTR, which drives an eGFP expression cassette. The HRSIN-CSGW vector also contains the Woodchuck post-transcriptional regulatory element (WPRE) and the central polypurine tract (cPPT) to enhance viral titer and efficiency within nondividing cells. The MLV vectors are based on the pHIT system[35] (pHIT60 plasmid encoding MLV *gag-pol*, and the pCNCG plasmid containing the vector genome encoding a CMV-driven eGFP) and were kindly provided by Oxford Biomedica (Oxford, UK). Plasmid pLCMV, encoding the envelope glycoprotein from the LCMV virus, was kindly provided by Dr D Sanders (Purdue University, West Lafayette, IN, USA).

### Vector production

Transfection of packaging cells was carried out by lipofection. Briefly, 293 T cells ($6 \times 10^6$) were plated over a 10-cm tissue culture grade Petri dish (Sarstedt, Newton, NC, USA) the day before transfection to ensure exponential growth and 80% confluence. Vector plasmids, together with packaging and envelope plasmids (27 μg total DNA; plasmid proportions of 3:2:1, respectively) were resuspended in 1.5 ml OPTI-MEM medium (Gibco) and mixed at room temperature for 20 min with 60 μl Lipofectamine 2000 (Invitrogen, Calsbad, CA, USA) before being diluted in 1.5 ml OPTI-MEM. The plasmid–lipofectamine mixture was added to prewashed cells and then incubated for 6–8 h. The producer cells were then washed and further cultured for 48 h in 10 ml OPTI-MEM medium. The viral supernatants were collected and filtered through a 0.45 μm filter (Nalgene, Rochester, NY, USA), alliquoted and immediately frozen at −80°C.

### Cell transduction and vector titration

Exponentially growing target cells were washed in PBS, and $2 \times 10^5$ cells were seeded per well in 500 μl of their appropriate medium in 24-well plates. PBTLs were isolated and stimulated with OKT3 in the presence of exogenous IL-2 as described elsewhere[36] 3 days before transduction. Supernatants containing retroviral vectors were added to the culture and incubated overnight. After 72 h or other time if indicated, the cells were collected, washed, fixed in 0.2% paraformaldehyde and analyzed in a FACScan Flow Cytometer (Becton-Dickinson, San Jose, CA, USA). The percentage of transduced cells was determined on the basis of fluorescence increase due to the expression of eGFP. Viral titers (transduction units/ml) were calculated on the basis of the percentage of $GFP^+$ cells detected in the linear range of a serial dilution of the supernatant. MOI is defined as the number of viral vectors/cell, and was estimated in a highly permissive cell line such as 293 T.

## Results

### Lentiviral vectors are efficient tools for stably transducing HVS-T cells

We produced oncoretroviral (murine leukemia virus (MLV)-based) and lentiviral (HIV-1-based) vectors by the cotransfection of 293 T cells with three plasmids: pCNCG, pHIT60 and pMD. G for MLV-based vectors and pHRSIN-CSGW, pCMVΔR8.9 and pMD.G for HIV-1-based vectors (see Materials and methods). Both vectors encode the enhanced-green fluorescent protein (eGFP) gene and are pseudotyped by the same envelope (VSVg). In either case the transduction of target cells results in an increase in fluorescence, which is easily measured by flow cytometry. Cells deriving from hematopoietic and nonhematopoietic tumors (Jurkat; RKO; JEG-3), primary tissues (HUVEC) and HVS-T cells from both immunodeficient patients (GOR-1/HVS; GOR-7/HVS; SPA/HVS) and healthy individuals (ITA/HVS; CA/HVS) were transduced with a single round of both lentiviral and oncoretroviral vectors.

Oncoretroviral vectors failed to transduce HVS-T cells efficiently, achieving not more than 1% cell transduction with any HVS-T cell analyzed, even at an multiplicity of infection (MOI) of 40 (Figure 1a, dark bars, and Figure

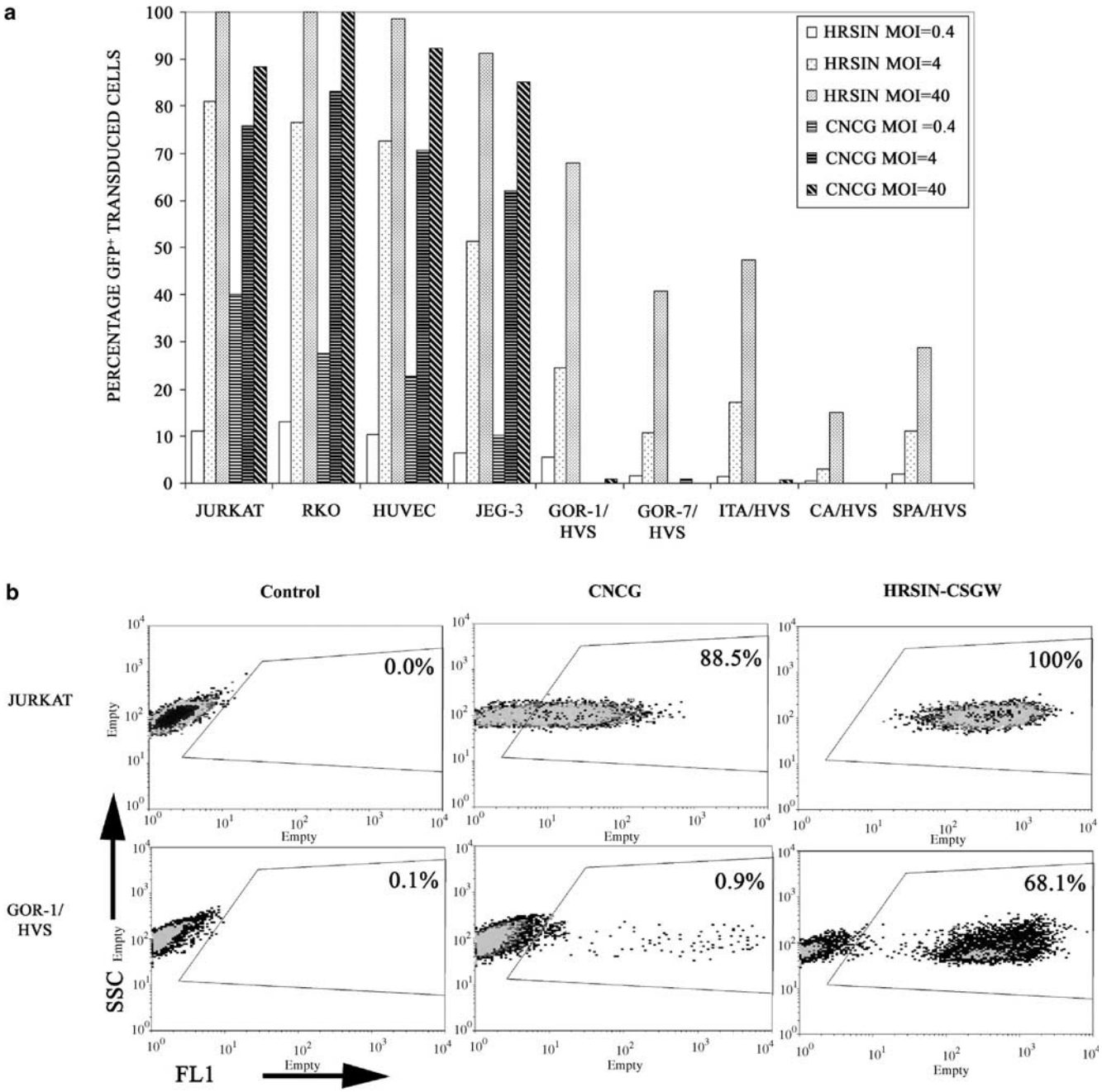


***Figure 1*** *Lentiviral vectors but not oncoretroviral vectors can efficiently transduce HVS-T cells. (a) Transduction efficiency of lentiviral vectors HRSIN-CSGW (light bars) and oncoretroviral vector CNCG (dark bars) was determined in primary (HUVEC) and tumor cell lines (JEG-3, RKO, Jurkat) together with HVS-T cell lines derived from healthy individuals (ITA/HVS, CA/HVS) and immunodeficient patients (GOR-1/HVS, GOR-7/HVS, SPA/HVS). The data represent percentages of eGFP$^+$ cells detected by flow cytometry 7 days after one single round of transduction. One representative experiment out of three is shown. (b) Flow cytometry plots of transduced cells. Typical flow cytometry dot-plot showing T-leukemia cells Jurkat (top row) and the GOR-1/HVS (bottom row) transduced at an MOI of 40 with either the oncoretroviral vector CNCG (middle column) or the lentiviral vector HRSIN-CSGW (right-hand column).*

1b, lower-middle plot). All the HVS-T cell lines, however, were efficiently transduced by lentiviral vectors, at levels ranging from 15 to 65% fluorescent cells at an MOI of 40 and from 3 to 25% at an MOI of 4 after one single round of infection (Figure 1a, light bars, and Figure 1b, bottom-right plot). Both oncoretroviral and lentiviral vectors transduced tumor and primary cells with comparably high efficiency (Figure 1a, dark and light bars).

We assessed the gene-expression stability of the HRSIN-CSGW vector in two HVS-T cell lines (ITA/HVS and GOR-1/HVS) and the Jurkat T-cell leukemia line after transduction at high MOI. This is important since transgene detection in target cells may be due to pseudotransduction[16] (especially at high MOIs) or expression from nonintegrated vectors.[17,18] Additionally, the expression of the transgene by transduced cells can be blocked by gene silencing.[19] The expression of eGFP protein was determined on days 7, 28 and 48 after a single round of cell transduction on day one. Figure 2 shows that the high percentage of transduced cells

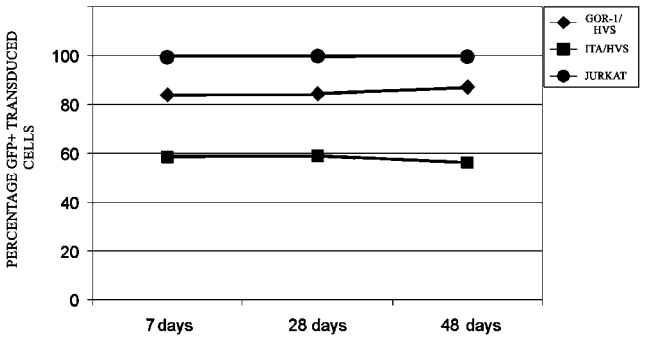


***Figure 2*** *Lentiviral transgene expression on HVS-T cells is stable. Protein expression stability on HVS-T cells (GOR-1/HVS and ITA/HVS) and Jurkat cells was determined by measuring eGFP expression by flow cytometry over time. The different cell lines were transduced on day one with a single round of the HRSIN-CSGW lentiviral vector at an MOI of 400, maintained in culture for 48 days and analyzed at the indicated times.*

obtained (60; 82 and 100% for ITA/HVS, GOR-1/HVS and Jurkat cell lines, respectively) remained stable over a period of 48 days. Furthermore, the eGFP gene-expression levels determined by cell-fluorescence intensity remained unchanged over time in all the three cell lines analyzed (data not shown).

### *HVS-T cells are restricted to retroviral-vector transduction*

On the basis of the data shown in Figure 1, we may also conclude that oncoretroviral vectors are 100–700 times less efficient for HVS-T cells than for non-HVS-T cells, whereas this difference is only 2–7 times in the case of lentiviral vectors. This suggests that HVS-T cells are strongly restricted to MLV-based vectors and only partially resistant to HIV-1-based vectors. We were keen to determine whether HIV-1-based vectors were partially restricted in HVS-T cells. Thus, we transduced GOR-1/HVS and ITA/HVS T cells with lentiviral vectors at MOIs ranging from 0.02 to 400, using Jurkat cells as lineage-matched controls. Figure 3 shows that the percentage of transduced cells reached a plateau at an MOI of 100 in the two HVS-T cells analyzed (GOR-1/HVS and ITA/HVS; solid squares and triangles), indicating that a subpopulation of these cells was strongly restricted to lentiviral transduction. The percentage of cell transduction with the most permissive HVS-T cell line, GOR-1/HVS, was never higher than 80%, even at MOIs of 400 (Figure 3, solid squares). On the other hand, we routinely achieved 90% cell transduction in Jurkat cells with an MOI of 10 and 100% transduced cells with an MOI of 40 (solid diamonds).

### *Partial restriction of lentiviral vectors to transduce HVS-T cells is not overcome using a different envelope*

Since receptor usage is the most highly limiting factor for a virus to infect different cell types,[20,21] we investigated whether by using a different envelope we could overcome the partial restriction of lentiviral vectors to transduce HVS-T cells and therefore increase transduction efficiency. Jurkat cells as well as the HVS-T cells were transduced with the HRSIN-CSGW lentiviral plasmid pseudotyped with either the VSVg or lymphocytic chorimeningitis virus (LCMV) envelopes at increasing MOIs ranging from 0.1 to 10. In Figure 4, we show that

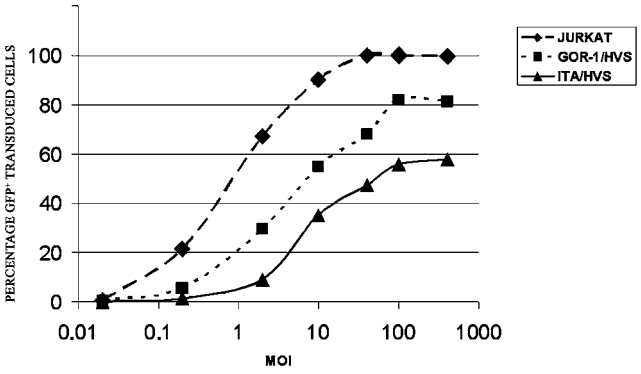


***Figure 3*** *HVS-T cells are restricted to lentiviral vector transduction. Jurkat leukemia T cells and HVS-T cell lines GOR-1/HVS and ITA/HVS were transduced with the HRSIN-CSGW lentiviral vectors at increasing MOIs up to 400. The eGFP gene expression was assessed by flow cytometry 7 days after cell transduction. One typical experiment out of three is shown.*

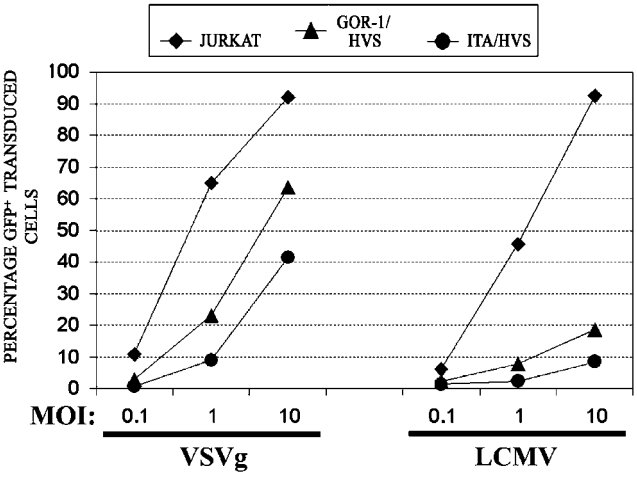


***Figure 4*** *LCMV pseudotyping does not overcome lentiviral vector restriction of HVS-T cell transduction. Jurkat tumor T cells (solid diamonds) and HVS-T cell lines GOR-1/HVS (solid triangles) and ITA/HVS (solid circles) were transduced with the HRSIN-CSGW lentiviral vector pseudotyped with either VSVg (left-hand side) or LCMV (right-hand side) glycoproteins at the indicated MOIs. Percentage of eGFP$^+$-transduced cells was determined by flow cytometry after 1 week.*

lentiviral vectors pseudotyped with the LCMV envelope remain restricted to HVS-T cell transduction. Furthermore, whereas Jurkat cells are transduced with similar efficiency with both envelopes (Figure 4, solid diamonds), HVS-T cells are transduced significantly more successfully when the HRSIN-CSGW lentiviral vector is pseudotyped with the VSVg envelope (Figure 4, solid triangles and circles).

### *Equivalent permissiveness of HVS-T cells and activated primary T cells to lentiviral transduction*

The low permissiveness of HVS-T cells to lentiviral vector transduction could be due to a behavior mirroring that of blood primary T cells. Therefore, OKT3-activated peripheral blood T lymphocytes (PBTLs) were transduced with lentiviral vectors at increasing MOIs (1, 10 and 100) concomitantly with the HVS-T cells GOR-1/HVS, ITA/HVS and CA/HVS as well as Jurkat cells (Figure 5). Again, nearly 100% of the Jurkat cells were transduced at low MOI (10) (Figure 5, left bars), whereas

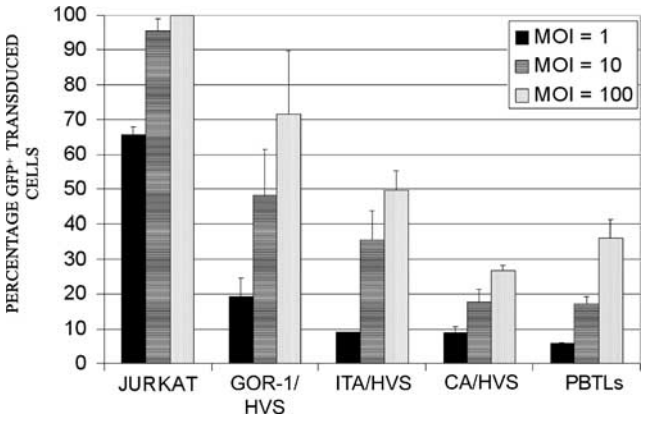


***Figure 5*** *HVS-T cell lentiviral transduction efficiency is similar to that of OKT-3-activated peripheral T lymphocytes. Jurkat and HVS-T cells GOR-1/HVS, ITA/HVS and CA/HVS were transduced at the indicated MOIs in parallel with human peripheral blood T lymphocytes (PBTLs) activated with OKT-3 3 days before transduction. Percentages of cells expressing eGFP were determined by flow cytometry 7 days after transduction.*

the percentage of transduction of HVS-T cells ranged from 30 to 70% at an MOI of 100 (Figure 5, central bars), which is similar to the values obtained with stimulated peripheral T lymphocytes (Figure 5, right bars).

## Discussion

The genetic bases of many T-cell immunodeficiencies have been identified over the last few years. Gene reconstitution of defective progenitor cells is a promising therapeutic perspective for these patients, but unfortunately the clinical implementation of gene therapy protocols has been set back due to a lack of suitable *in vitro* experimental systems for testing new gene delivery vectors. There is an urgent need, therefore, to find a way around the scarcity of primary immunodeficient patients and the limited number of circulating T lymphocytes that can be obtained from them.

HVS-T cell lines are a promising model for addressing the functional rescue of primary immunodeficient cells after gene reconstitution, but the initial enthusiasm stirred by the discovery of this process of cell immortalization was tempered by repeated failures in introducing foreign genes into these cells. Electroporation or lipofection of drug-selectable vectors, as well as cell transduction with MLV-oncoretroviral vectors failed to stably express the transgene (our data).

To investigate new vectors, which might deliver genes stably to HVS-T cells, we selected two different retroviral vector systems based equally on their high efficiency, broad tropism and stability of expression. The CNCG vector is based on the oncoretrovirus MLV and the HRSIN-CSGW vector on the lentivirus HIV-1. HIV-1-based vectors in particular have demonstrated their high efficiency in almost any cell line, including those resistant to MLV-based transduction.[15] Moreover, since wild-type HIV-1 strains can infect HVS-T cells[22,23] we hypothesized that vectors deriving from HIV-1 should transduce these cells. We show here that HIV-1-based vectors can efficiently and stably transduce HVS-T cells. We easily reached up to 80% transduction efficiency after only one round of exposure to lentiviral vectors in all the HVS-T cells analyzed. MLV-based vectors, on the other hand, were quite inefficient and never transduced more than 1% of the cells. We demonstrated that eGFP detection in HVS-T cell lines transduced with HRSIN-CSGW represents vector transduction (and not pseudotransduction[16] or expression from nonintegrated vectors[17,18]) since the percentage of eGFP$^+$ cells remained stable for at least 48 days as shown in Figure 2. This also supports that gene silencing[19] is not occurring in lentiviral-transduced HVS-T cells.

The fact that we never obtained more than 60–80% transduction of HVS-T cells, even at an MOI of 400, indicates not only that HVS-T cells are less easily transduced than non-HVS cell lines but also that 40–20% of HVS-T cells are highly restricted against transduction by lentiviral vectors. The differences in susceptibility to lentiviral transduction among different HVS-T cell lines are difficult to interpret but may be due to donor variability.

Since receptor usage is the most highly limiting factor for a virus to infect different cell types,[20,21] we investigated whether by using a different envelope we could overcome the partial restriction and increase transduction efficiency in HVS-T cells. We chose LCMV envelope because (i) it is efficiently incorporated into HIV-1 vectors,[24,25] (ii) LCMV and VSVg bind different cell receptors[26,27] and therefore vectors pseudotyped with LCMV and VSVg use different entry mechanisms and (iii) LCMV but not VSVg may allow the stable production of broad-host-range retroviral vectors, which can be concentrated by ultracentrifugation.[24,25] We found that not only do LCMV-pseudotyped lentiviral vectors remain restricted to HVS-T cell transduction but also that HVS-T cell lines are transduced better with the VSVg pseudotypes. Since the receptor for VSVg is a membrane phospholipid[26] and for LCMV it is α-dystroglycan,[27] the restriction observed must occur at some stage after entry.

Therefore, the low permissiveness of HVS-T cells may be due either to intrinsic cellular factors present in T cells[28,29] or to the possible expression of HVS latent genes that might interfere with lentiviral vector transduction. In our case, viral interferences are unlikely since HVS cells are permissive to HIV-1 infection[30,31] and the expression of the HVS latent gene stpC enhances HIV-1 replication.[32] If HVS immortalization does not block HIV replication but rather enhances it, T-cell factors must be primarily involved in explaining the relatively low permissiveness of HIV-1 vectors in HVS-T cells. In fact, T lymphocytes have several mechanisms that are capable of inhibiting HIV-1 replication[28,29] and so it is likely that the restriction observed results from a behavior mirroring primary T cells. This would explain why the lentiviral vector acts equally well or even more efficiently on HVS-T cells than it does on primary T cells (Figure 5).

In summary, we have demonstrated that HVS-T cells from healthy individuals as well as immunodeficient patients can be stably transduced by lentiviral vectors and that they are just as susceptible as primary T cells. These results support the idea that HVS-T cells are an adequate model for gene therapy.

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