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Pseudotyping of Moloney leukemia virus-based retroviral vectors with simian immunodeficiency virus envelope leads to targeted infection of human CD4+ lymphoid cells

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In view of our recent findings that a truncated form of the envelope (Env) glycoprotein of human immunodeficiency virus type 1 (HIV-1) was efficiently incorporated into MoMLV particles, we studied the generation of Moloney murine leukemia virus (MoMLV)/simian immunodeficiency virus (SIV) pseudotypes. Unlike HIV-1, both the wild-type SIV Env and a truncated form, which lacks most of the cytoplasmic domain of the transmembrane glycoprotein, were incorporated into MoMLV particles and generated infectious retroviral vectors which could transduce CD4+ sMAGI macaque cells. The infection depended on target cell CD4 expression, and was neutralized by both soluble CD4 and sera from SIV-infected macaques. We also observed pseudotype-mediated gene transfer of a green fluorescent protein marker into the CD4+ CEMX174 and C8166 lymphoid cell lines. More importantly, primary human lymphocytes were also successfully transduced ex vivo by MoMLV/SIV pseudotypes, albeit at lower efficiency, and gene transfer was specifically restricted to the CD4+ subset. These findings demonstrate that MoMLV/SIV pseudotypes can be used to transduce cells which are susceptible to SIV infection, and thus might be advantageously employed in animal models for direct in vivo delivery of gene therapy-based approaches.

Keywords: *pseudotype; SIV, retroviral vector; lymphocytes; flow cytometry*

Introduction

MoMLV-based retroviral vectors are the main vehicles employed to date in gene therapy clinical trials, especially those aiming at stable correction of genetic defects. While direct *in vivo* gene delivery represents the ultimate goal in this field, target cells are generally manipulated genetically *ex vivo*; this is due to several technical and safety constraints, as well as the broad distribution of the Pit-2 receptor of the amphotropic MoMLV (A-MLV) on human tissues,[1] which would probably lead to the undesirable transduction of many cell types following *in vivo* administration of the vector. In addition, some cell types, including hepatocytes and bone marrow cells, are inefficiently infected by A-MLV-based vectors, and this constitutes a limitation for gene therapy approaches to diseases affecting these cell types.[2] Thus, engineering of viral tropism is actively studied, because it could lead to significant improvements in the field.

It was recently demonstrated that MoMLV particles can be efficiently pseudotyped by other envelope proteins, including vesicular stomatitis virus G (VSV-G) glycoprotein,[3] gibbon ape leukemia virus (GaLV),[4] and human foamy virus (HFV) envelope protein.[5] Moreover, we recently observed efficient pseudotyping of MoMLV particles with a largely truncated form of human immunodeficiency virus type 1 (HIV-1) Env.[6] The inclusion of VSV-G, GaLV and HFV Envs would modify, and in some cases broaden, the tropism of the vector, and might be useful for the *ex vivo* transduction of cell types usually resistant to MoMLV vectors carrying the A-MLV Env. On the other hand, the incorporation of HIV-1 Env into vector particles restricts the virus' tropism to cells expressing the CD4 marker and indicates the possible *in vivo* utilization of such vectors to deliver therapeutic genes specifically to CD4+ cells.

In view of the potential applications in humans, we focused on the development of an animal model to preliminarily assay CD4-targeted vectors *in vivo*. As a first step in this direction, we established simian immunodeficiency virus (SIV) *env* expressing plasmids, and tested the generation of MoMLV/SIV pseudotypes *in vitro*. SIV is a simian lentivirus closely related to HIV-1 that utilizes the CD4 molecule as a principal receptor on target cells, and causes an acquired immunodeficiency syndrome-like disease in monkeys.[7] We report here that MLV/SIV pseudotypes can be efficiently generated with both wild-type and a truncated form of SIVmac239 Env, and show that this approach is feasible for the *ex vivo* transduction of primary human lymphocytes. This system might be

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further developed to test direct *in vivo* gene transfer to CD4-expressing cells.

Results

Establishment and characterization of SIVmac239 env expression plasmids

We generated two SIV *env* subgenomic constructs, as detailed in Materials and methods, expressing either wild-type (pHCMV-SIV*env*) or a truncated SIV Env (pHCMV-SIVΔCT*env*), which lacks most of the cytoplasmic domain of the transmembrane protein (Figure 1). To minimize possible instability of truncated Env, this construct was generated by mutating codon 734 of SIV *env* gene, which also naturally undergoes mutations when SIV is passaged in human cell lines.[8] According to our findings in the HIV-1 system,[6] mutant Env was expected to be incorporated into heterologous particles more efficiently than wild-type Env. In preliminary experiments, we compared the *in vitro* activity of the two SIV Env expressors. When 293T cells were transfected with pHCMV-SIV*env*, we observed the expression of precursor gp160, as well as gp130 and gp41 cleavage products (Figure 2, lane 2). As expected, transfection of pHCMV-SIVΔCT*env* resulted in the expression of a smaller precursor protein, which migrated between gp160 and gp130, as well as gp130 and gp28 cleavage products (Figure 2, lane 3). Gp28 was an expected cleavage product, which results from the truncation of the cytoplasmic tail of gp41, as observed recently by Johnston *et al*.[9] HCMV vector lacking *env* sequences was used as a negative control in the transfection experiments, and did not yield any specific band in radioimmunoprecipitation studies (Figure 2, lane 1). Overall, both *env* expression plasmids yielded comparably high levels of Env precursor glycoproteins, which were correctly processed into the expected mature products in transfected 293T cells.

Generation of infectious virus by pseudotyping of MoMLV particles with both wild-type and truncated SIV Env

To determine whether SIV envelope proteins could be functionally incorporated into virions, we tested the

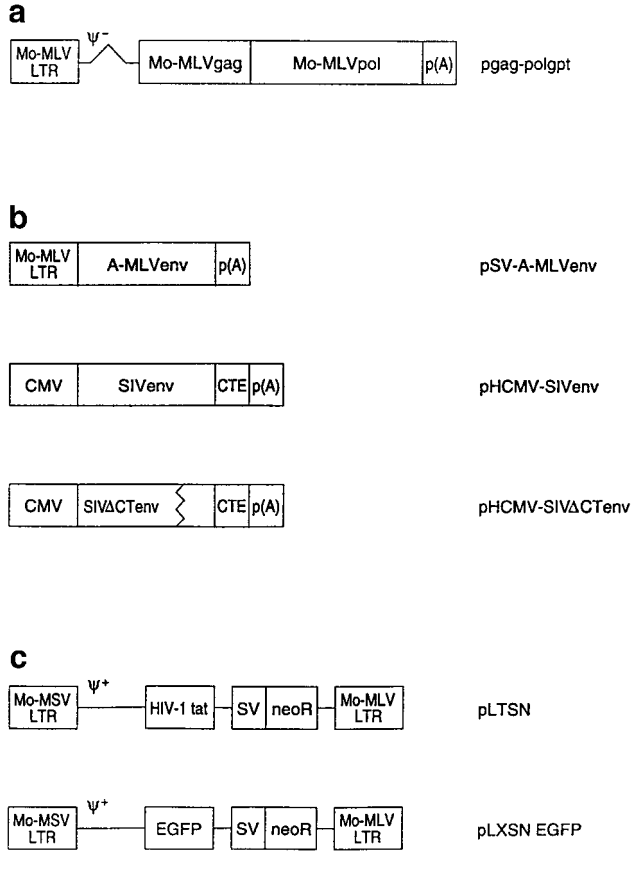

Figure 1 *Schematic representation of the constructs used to produce MoMLV-based vectors by transient transfection. (a) MoMLV Gag-Pol expression plasmid. (b) A-MLV and SIV Env expression plasmids. (c) Tat and EGFP transfer vectors. The MoMLV packaging signal (ψ) is deleted in pgag-polgpt but is present in both pLTSN and pLXSNEGFP. CMV, cytomegalovirus promoter; Mo-MSV, Moloney murine sarcoma virus; CTE, SRV-1 constitutive transport element; p(A), polyadenylation signal; SV, simian virus 40 promoter; neoR, neomycin phosphotransferase gene.*

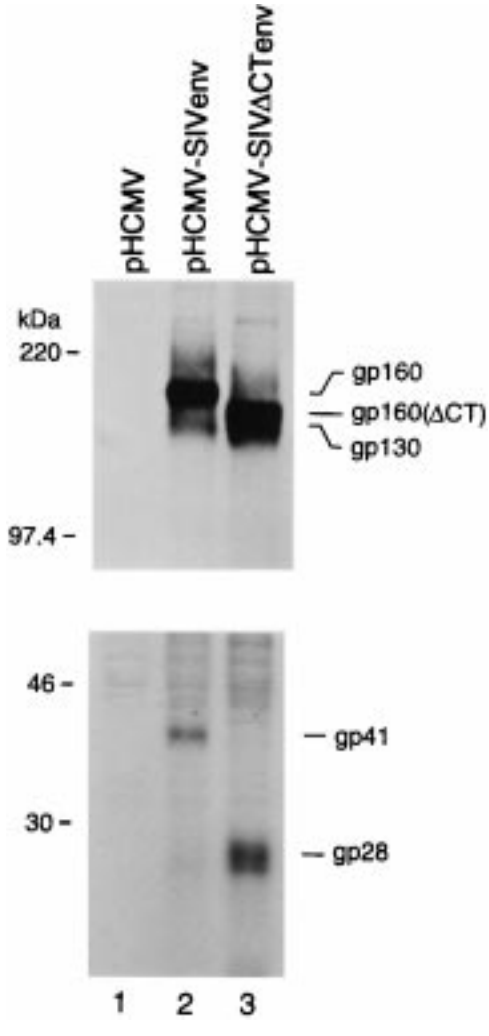

Figure 2 *Synthesis and processing of SIVmac239 Env proteins in transfected cells. 293T cells were transfected with either the wild-type (pHCMV-SIVenv) or the truncated (pHCMV-SIVΔCTenv) SIV Env expression constructs. Forty-eight hours later 293T cells were radiolabeled with ^{35}S-methionine and ^{35}S-cysteine for 7 h, lysed and analyzed by SDS-PAGE after radioimmunoprecipitation with serum from SIV^{+} macaques. The positions of the different SIV Env proteins are indicated on the right and the molecular weight marker on the left. pHCMV was used as negative control.*

infectivity of pseudotyped MoMLV particles. Pseudotyped viral particles were transiently generated by cotransfection of 293T cells with the constructs described in Figure 1, as detailed in Materials and methods; a packageable transcript was provided by cotransfection of pLTSN, which expresses HIV-1 *tat*. As target cells we used the sMAGI cell line, a macaque mammary tumor cell line which expresses human CD4 and harbors a β-galactosidase (β-gal) gene under the transcriptional control of HIV-1 LTR sequences, including the Tat responsive element (TAR).[10] In this setting, infectious pseudotyped viral particles were expected to transduce the *tat* gene, and thus induce the synthesis of β-gal in the target cells.

The use of both A-MLV Env and the C-terminally truncated SIV Env, respectively encoded by the SV-A-MLV-*env* and the HCMV-SIVΔCT*env* constructs, resulted in the appearance of blue foci following β-gal staining, indicating successful vector transduction (Figure 3a and c, respectively). The A-MLV Env, which served as a positive control, yielded titers in the order of 10^4–10^5 transducing units/ml, while the truncated SIV Env generated 10- to 100-fold less infectious viral particles (Table 1). Interestingly, the wild-type SIV Env, encoded by the HCMV-SIV*env* construct, could also efficiently pseudotype MoMLV particles, and yielded titers similar to those obtained with the truncated SIV *env* construct (Figure 3b and Table 1). This result strikingly differs from what we observed in the case of HIV-1, where efficient gene transduction could be obtained only when the truncated form of the Env complex was used.[6] Positive cells were not detected when no *env* expression construct was used for transfection of 293T cells (Figure 3d and Table 1).

Remarkably, no gene transfer into the human HeLa-CD4 (MAGI) cell line with MoMLV/SIV pseudotypes

Table 1 Infectivity of MoMLV particles pseudotyped with A-MLV or SIV Env proteins[a]

Expt No.	*Virus titer (No. of blue foci/ml)*			
	A-MLV env	*SIV WT env*	*SIV ΔCT env*	*env⁻*
1	4×10^5	2×10^3	2×10^3	0
2	ND	2×10^3	2×10^3	0
3	3.5×10^4	1×10^3	1.9×10^3	0
4	3.7×10^4	1.2×10^3	2.1×10^3	0
5	2.7×10^5	0.5×10^3	1×10^3	0
6	2.1×10^5	2.5×10^3	3.2×10^3	0

[a]On CMMT-CD4-LTR-β-gal indicator cells. Pseudotyped virions were produced in 293T cells by cotransfection of p*gag-pol*gpt, pLTSN and SV-A-MLV-*env* (A-MLVenv), pHCMV-SIV*env* (SIV WT env), pHCMV-SIV·ΔCT*env* (SIV ΔCT env), or HCMV (env⁻). WT, wild-type; ND, not determined.

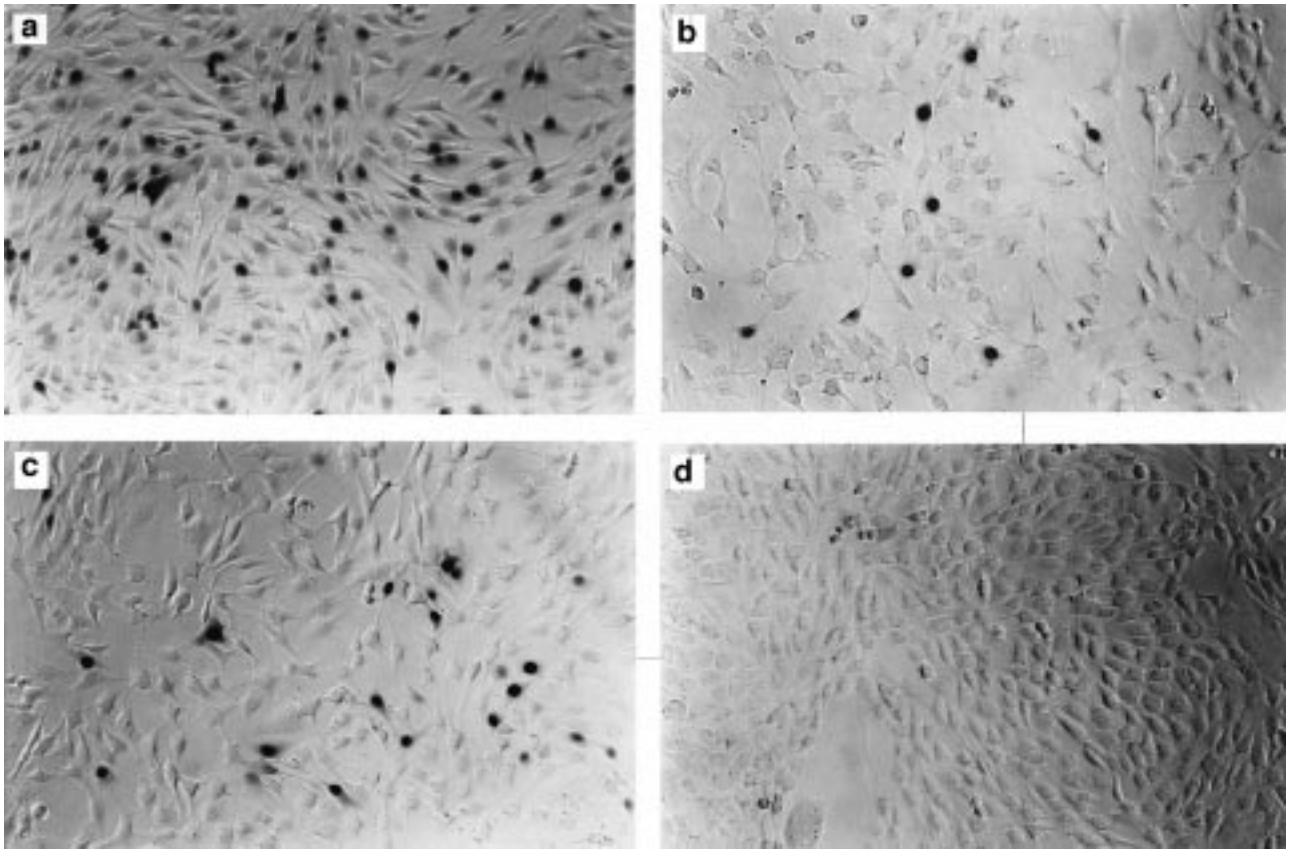

Figure 3 *Transduction of sMAGI cells by pseudotyped vectors. Thirty-six hours after infection with 0.5 ml retroviral vector-containing supernatants, target cells were fixed and underwent β-gal staining. Both the use of SIVenvWT (b), and SIVenvΔCT (c) resulted in the appearance of foci of blue-stained cells, indicating successful vector transduction. The A-MLVenv (a) and the env⁻ (d) served, respectively, as positive and negative controls. One representative experiment is shown.*

was observed (data not shown). This particular cell line expresses human CD4 and can be infected by T-tropic strains of HIV-1 but not SIV;[11] it is reported to be negative for CCR5 expression and positive for CXCR-4 or another protein that functions as a T-tropic HIV-1 coreceptor.[12] On the other hand, MAGI cells were efficiently infected by the A-MLV vector (data not shown), as well as by the MoMLV/HIV-1 Env pseudotype.[6] This finding agrees with Marcon *et al*,[13] who observed that cotransfection of CD4 and CCR5 expression plasmids is necessary to infect HeLA cells with SIVmac239 isolates, and also suggests that coreceptor usage is conserved when the SIVmac239 Env is carried on heterologous particles.

MoMLV/SIV pseudotype relies on gp130–CD4 interaction for transduction of target cells

To verify that the transduction of MoMLV particles pseudotyped with SIV Env was mediated by the SIV envelope, we attempted to inhibit target cell infection with anti-gp130 antibodies. Pretreatment of MoMLV particles pseudotyped by either wild-type or truncated SIV Env with pooled serum from three SIV-infected macaques reduced the virus titer measured on sMAGI cells in a dose-dependent manner (Figure 4a), whereas a control serum from uninfected macaques did not affect the virus titer of the MoMLV/SIV pseudotypes (Figure 4a). On the other hand, under identical conditions, the serum from the SIV$^+$ macaques had no significant effect on the infectivity of particles pseudotyped by A-MLV Env (data not shown), thus demonstrating that the neutralization was specific for pseudotypes bearing SIV Env.

To verify further that the entry of MoMLV particles pseudotyped with wild-type SIV Env was dependent on the interaction between gp130 and CD4, pseudotypes were preincubated with soluble CD4 (sCD4) before the infection of target cells. Pretreatment of MoMLV particles pseudotyped by either wild-type or truncated SIV Env led to an 80–85% loss of infectivity. Under identical conditions, sCD4 had no significant effect on the infectivity of particles pseudotyped by A-MLV Env (Figure 4b).

Transduction of CD4$^+$ lymphoid cell lines

We next tested whether MoMLV/SIV pseudotypes could also transduce CD4$^+$ lymphoid cell lines which are susceptible to infection by wild-type SIV. To this end, we transfected 293T cells with the *gag-pol* expressor along with the different *env* expression plasmids. As a template for genomic RNA, we transfected pLXSNEGFP, which carries an enhanced form of the green fluorescent protein (EGFP) driven by the MoMLV LTR (Figure 1). Green fluorescent proteins are useful markers for gene transfer procedures, because the vector-transduced target cells can be easily identified and counted cytofluorimetrically.[14–16] We transduced CEMX174, a CD4$^+$ cell line suitable for infection by different SIV strains, including SIVmac239, either by cocultivation with transfected cells or by addition of vector-containing supernatants; findings are summarized in Table 2 and Figure 5. We observed that transduction by cocultivation was a generally more efficient procedure, yielding up to 13.1% transduced CEMX174 cells with the amphotropic vector (Table 2 and Figure 5, A-MLVenv). We also observed that both SIV pseudotypes efficiently infected this cell line; the pseudotype with wild-type Env transduced 7.2% target cells (Table 2 and Figure 5, SIVenv), and the one carrying the truncated SIV Env could infect 4.3% CEMx174 cells (Table 2 and Figure 5, SIVΔCTenv). Following cocultivation on transfected 293T cells in the absence of Env, only background values were found; these low levels were associated with cells whose level of EGFP

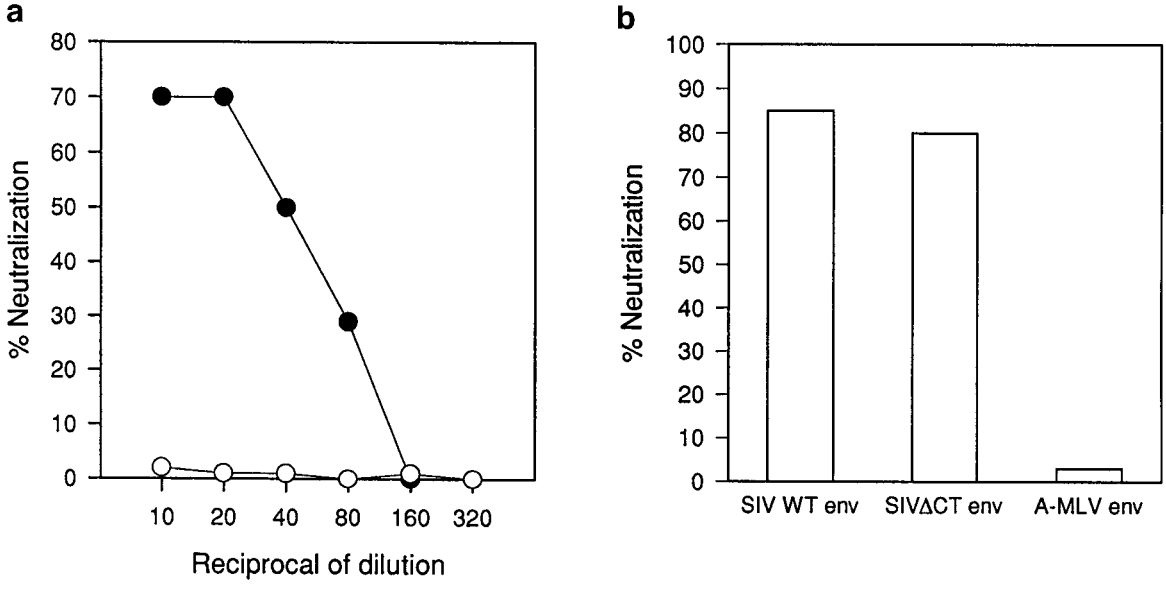

Figure 4 *Neutralization of SIV envelope-specific infectivity. MoMLV particles pseudotyped with wild-type SIV Env were generated by transient transfection of 293T cells. (a) Cell-free supernatants (1 ml) were incubated with a pooled heat-inactivated serum from three SIV infected macaques (filled circles), or with control serum from uninfected macaques (open circles), at the dilutions reported on the x axis, for 1 h at 37°C before the addition to sMAGI cells. Thirty-six hours after transduction, the titer was determined as described in Materials and methods, and the percentage of neutralization is indicated on the y axis. In experiments with sCD4 (b), the indicated vector-containing supernatants were incubated with 100 μg/ml sCD4 for 1.5 h at 37°C before transduction of sMAGI cells, according to the protocol described in Materials and methods. The experiments were carried out twice with similar levels of relative inhibition of the infectivity of the MoMLV/SIV pseudotyped retroviral vectors: one representative experiment is shown.*

Table 2 Transduction of CD4+ lymphoid cell lines with retroviral vectors pseudotyped with different envelope proteins

Cell line	*Experiment No.*	*% EGFP positive cells*				*Method*
		A-MLV env	*SIV WT env*	*SIV ΔCT env*	*env⁻*	
CEMX174	1	2.8	1.3	0.6	0	S
	2	6.9	2.6	0.6	0	S
	3	13.1	7.2	4.3	0.2	C
	4	12.1	7.9	2.3	0.3	C
C8166	1	3.7	2.1	1.1	0	S
	2	6.3	5.4	2.7	0.3	C

Target cells (CEMX174, C8166; 2×10^5 cells per well) were infected with 1 ml supernatant (S) of transfected 293T cells or by cocultivation, where indicated (C). Pseudotyped virions were produced by cotransfection of p*gag-pol*gpt, pLTSN, and SV-A-MLV-*env* (A-MLVenv), pHCMV-SIV*env* (SIV WT env), pHCMV-SIVΔCT*env* (SIV ΔCT env), or HCMV (env⁻). Forty-eight hours after infection, the cells were labeled with anti-CD4PE and analyzed by FACS. The percentage of vector-transduced cells in individual experiments is reported. WT, wild-type.

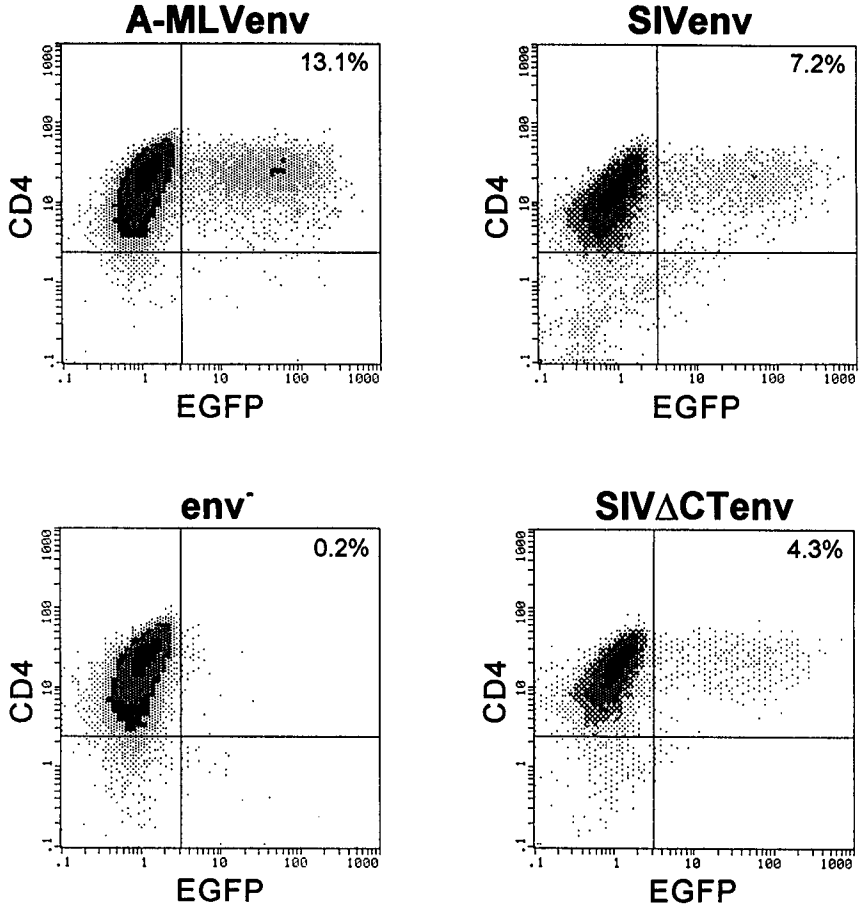

Figure 5 *Transduction of CD4+ lymphoid cells by different retroviral vectors carrying EGFP as a marker gene. CEMX174 cells were infected with different pseudotyped MoMLV particles generated by transient transfection of 293T cells. Forty-eight hours after transduction cells were labeled with anti-CD4PE and the percentage of EGFP-expressing cells was quantified by FACS analysis. Vector-transduced CD4+ cells are shown in the upper right panel of each diagram. The individual env constructs used for pseudotyping and the mean percentage of EGFP-positive cells for each construct are indicated.*

expression was close to the threshold of acceptability (Table 2 and Figure 5, env⁻). Similar findings were obtained following transduction of the C8166 cell line, although the percentage of vector-transduced cells was lower, compared with CEMX174 cells (Table 2). Interestingly, the pseudotype generated by wild-type SIV Env transduced both human CD4+ cell lines tested slightly better than the one carrying the truncated Env.

In two experiments, infected CEMX174 cells were grown up to 3 weeks in G418 medium in order to enrich

for vector-transduced cells. Indeed, we could successfully derive EGFP$^+$-G418 resistant cultures from both A-MLV Env and SIV Env pseudotyped MoMLV particles, while no culture was obtained from the env$^-$ control (data not shown). These data confirmed the results of the short-term experiments and demonstrated the stable transduction of target cells by the MoMLV/SIV pseudotypes.

Transduction of human primary lymphocytes

To evaluate whether the MoMLV/SIV pseudotype might be useful for targeted transduction of primary CD4$^+$ T lymphocytes, we cocultivated PHA-activated peripheral blood mononuclear cells (PBMC) from healthy donors with transiently transfected 293T cells; EGFP was detected on the CD4 and CD8 subsets by FACS analysis. Gene transfer in lymphocytes was overall more difficult than in the CD4$^+$ lymphoid cell lines. In six independent experiments with different donors the standard A-MLV vector could transduce up to 5% of the CD3$^+$ cells (range 1–5%). As expected, both CD4$^+$ and CD8$^+$ subsets were infected, at levels proportional to their percentages in the transduced population. In a representative experiment shown in Figure 6, 3.4% CD3$^+$ lymphocytes were transduced by the A-MLV vector; 2.3% belonged to the CD4$^+$ and 1.1% to the CD8$^+$ subsets, which accounted, respectively, for 61.8 and 38.2% of the total CD3$^+$ population. Transduction of the cells by SIV wild-type Env pseudotype also resulted in gene transfer in the CD3$^+$ cells, but at a lower efficiency (0.7%), which probably reflected the lower amounts of virus produced in the cultures. Remarkably, transduced lymphocytes belonged almost exclusively to the CD4$^+$ subset (0.6%) (Figure 6), and only background (0.1%) values were found among the CD3$^+$CD8$^+$ cells, similar to those obtained following cocultivation of lymphocytes with the env$^-$ control (Figure 6). In conclusion, these experiments indicate a restricted tropism of the pseudotyped vector, and show that it might be employed *ex vivo* for selective gene transfer into human CD4$^+$ lymphocytes.

Discussion

One of the major goals of present day gene transfer technologies is to restrict the tropism of retroviral vectors, in order to direct gene transfer into defined tissues or cell types. Clearly, this would be a critical step towards the development of vectors which may eventually be used *in vivo* for a number of therapeutic interventions.[17] To this end, an obvious modification of the existing vectors concerns the envelope proteins they display. In this respect, the generation of chimeric molecules, displaying a single-chain antibody fragment fused to the natural envelope proteins, constitutes an elegant approach to cell targeting. Indeed, this strategy was successful in targeting human

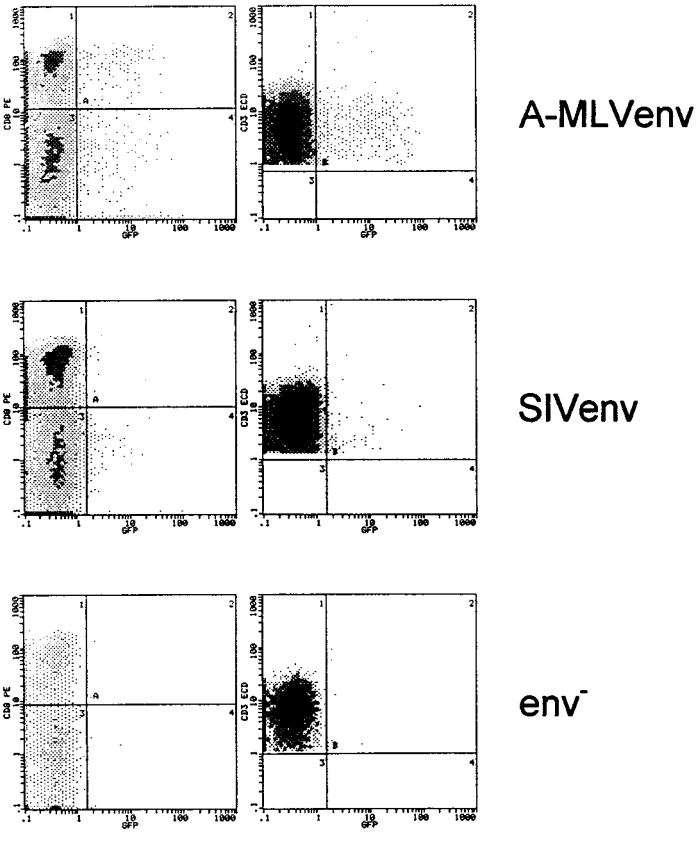

Figure 6 *Transduction of human primary T lymphocytes with different retroviral vectors carrying EGFP as a reporter gene. PHA-activated human PBMC were infected by cocultivation on transfected 293T cells releasing different pseudotyped MoMLV particles. Forty-eight hours after transduction, PBMC were labeled with both anti-CD3 and anti-CD8 and the percentage of EGFP-expressing lymphoid cells was quantified by FACS analysis. FACS diagrams on the right show CD3$^+$EGFP$^+$ cells (upper right quadrant of each panel). Figures on the left indicate vector-transduced CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ cells, which are shown, respectively, in the lower and upper right panels of each diagram. The individual env constructs used for pseudotyping are indicated.*

cells via the major histocompatibility complex class I molecules.[18] On the other hand, this and similar studies have also shown that the very low titers of vector currently obtained represent a major technical limitation.[19]

The possibility of mounting the Env of another virus, which naturally shows restricted tropism, on Env devoid MLV particles represents an alternative approach to this problem. Pseudotyping in general is a successful procedure as indicated by VSV-G protein MoMLV pseudotypes, whose infectious titers are remarkable.[3,20] Such a vector might be very useful for the transduction of cells which are resistant to infection with amphotropic vectors; on the other hand, *in vivo* applications in the near future are not likely, given the even broader tropism acquired through VSV pseudotyping.

In view of these considerations, we addressed the feasibility of exploiting the naturally occurring tropism of HIV-1 and SIV for $CD4^+$ cells to generate tissue-specific retroviral vectors. We previously described successful pseudotyping of MoMLV particles with HIV-1 Env. Here we describe the construction and CD4-specific targeting of MoMLV/SIV vectors. The generation of pseudotypes with SIV is very attractive due to the availability of a well-known animal model for testing the *in vivo* delivery of the vector, in association with either marker or antiviral therapeutic genes.

Interestingly, we observed that while pseudotypes mounting wild-type HIV-1 Env were inefficiently generated, the same was not true for wild-type SIV Env. On the contrary, SIV*env* and SIVΔCT*env* expression constructs generated pseudotypes with similar titers. This finding is not readily explained because, according to the results with HIV-1 pseudotypes, the long cytoplasmic tail of SIV Env was expected to be just barely mounted on MLV particles, due to difficult accomodation into heterologous particles.[6] At present, we can only speculate that the difference might depend on the secondary structure of SIV Env, which, unlike HIV-1 Env, might be more compatible with the MoMLV core.

We addressed the specificity of the MoMLV/SIV pseudotypes for $CD4^+$ cells by complementary approaches. First, the requirement of a functional gp130 for infectivity was shown by neutralization experiments with anti-gp130 antibody-containing serum. Second, the usage of CD4 as a receptor by the pseudotypes was indicated by inhibition of infection with sCD4. Third, we observed that murine $CD4^-$ NIH-3T3 cells, which are optimally transduced by standard MoMLV-based vectors, were not infected by the MoMLV/SIV pseudotypes, although the vector complemented by the A-MLV Env, used as a positive control, could infect up to 90% of NIH-3T3 cells (data not shown). This finding indirectly reinforced the evidence that SIV Env pseudotyped MoMLV particles require the gp130/CD4 interaction for the transduction of target cells.

Another interesting point is coreceptor usage by these pseudotypes. SIVmac239 utilizes either CCR-5 or a newly described CXCR4-like molecule as coreceptor but not fusin.[13,21] The wild-type SIV does not infect HeLa-CD4 cells, unless they are transduced with a CCR-5 expression vector, while it efficiently infects sMAGI cells.[12] According to our findings, this property also seems conserved when SIV Env is pseudotyped with a heterologous core. Direct proof of coreceptor usage by the MoMLV/SIV pseudotypes would be provided by the transduction of CCR-5 into HeLa-CD4 cells and the demonstration of successful infection by the vector.

These vectors might have applications both *in vitro* and *in vivo*, particularly if higher infectious titers can be obtained. Transduction of primary lymphocytes, which also represent an important target for *ex vivo* gene transfer, occurred at very low levels; on the other hand, transduction was restricted to the $CD4^+$ expressing subset. Any clinically relevant application, therefore, must await further improvements in vector production technology. Finally, the *in vivo* administration of these vectors, which can be envisaged in animal models to confirm their tropism for $CD4^+$ cells, will also require the definition of a convenient administration route, besides the titer improvement. Since MoMLV-based vectors only transduce proliferating cells, it might be necessary to deliver the vector to lymphoid compartments where $CD4^+$ cell proliferation readily occurs, such as the thymus, in order to increase the efficiency of gene transfer. Our ongoing studies will hopefully disclose the potential *in vivo* applications of these vectors.

Materials and methods

Plasmids

The MoMLV Gag-Pol expression construct p*gag-pol*gpt harbors the MoMLV *gag* and *pol* genes under the control of the MoMLV long terminal repeat (LTR) and a SV40 polyadenylation signal.[22] The construct lacks ψ packaging sequences as a consequence of a 134-bp deletion between the MoMLV LTR and *gag* gene. The amphotropic MoMLV Env expression construct SV-A-MLV*env* has the AMLV *env* gene inserted between MoMLV LTR sequences and a SV40 polyadenylation signal.[23] The SIV Env expression plasmid pHCMV-SIV*env* carries the SIVmac239 *env* gene downstream of human cytomegalovirus early promoter/enhancer sequences (HCMV). This construct expresses SIV Env exploiting a Rev-independent pathway of Env expression which relies on a constitutive transport element (CTE) derived from the simian retrovirus 1 (SRV-1) genome.[24] Details of plasmid construction are described elsewhere.[25] This construct has also been used to prepare pHCMV-SIVΔCT*env*, a variant of pHCMV-SIV*env* with a premature termination codon (TGA) in place of codon 734 of the SIV *env* gene, obtained by site-directed mutagenesis. This results in the truncation of the transmembrane protein from wild-type 354 amino acids (gp41) to 207 (gp28), as already reported by others.[9]

Finally, the Tat-transducing construct pLTSN is a derivative of the retroviral vector pLXSN, which contains HIV-1 *tat* under the control of Moloney murine sarcoma virus (MoMSV) LTR sequences.[26] The pLXSN EGFP construct was used to transduce lymphoid cells. This construct is a derivative of pLXSN carrying the gene for an enhanced green fluorescent protein (EGFP) driven by the MoMSV LTR.[14] An HCMV-driven β-gal expression plasmid, named pCMVβ-gal, was used in some experiments to check for transfection efficiency.

Cell culture and transfections

293T human kidney cells were obtained from ATCC, and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Gibco-

BRL, Gaithersburg, MD, USA) and 1% l-glutamine. The simian sMAGI cell line (macaque mammary tumor CMMT cells expressing human CD4 and the HIV-1 LTR fused to the β-gal reporter gene), and the human HeLa-CD4-LTR-β-gal cell line (MAGI) were cultured in complete DMEM plus 0.2 mg of G418 (Gibco-BRL) per milliliter, and 0.1 mg hygromycin per milliliter (Boehringer Mannheim, Mannheim, Germany).[10,11] Lymphoid cell lines CEMX174 and C8166 were grown in RPMI 1640 supplemented with 10% FCS and 1% l-glutamine. Finally, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque (Pharmacia-LKB, Uppsala, Sweden) gradient centrifugation as described,[27] and cultured for 48 h in RPMI 1640 supplemented with 10% FCS and 1% l-glutamine, in the presence of phytohemagglutinin (PHA-P; Difco, Detroit, MI, USA), before retroviral vector transductions. The day before transfection, 1.5×10^6 293T cells were seeded in 25 cm^2 tissue culture flasks. The cultures were transfected with plasmid DNA using a calcium phosphate precipitation technique.[28]

Radioimmunoprecipitation analysis (RIPA)

Forty-eight hours after transfection, 293T cells were metabolically labeled for 7 h with a mixture of ^{35}S-methionine and ^{35}S-cysteine (Promix; Amersham, Little Chalfont, UK; 14 000 μCi/ml, prepared in methionine and cysteine-deficient DMEM), and lysed in RIPA buffer (140 mm NaCl/8 mm Na_2HPO_4/2 mm NaH_2PO_4/1% Nonidet P-40/0.5% sodium deoxycholate/0.05% SDS), as described.[6] To control for transfection efficiency among the different samples, pCMVβ-gal was cotransfected, and cell lysate β-gal activity was measured in a β-counter (Packard, Grove Hills, IL, USA). SIV proteins were then immunoprecipitated overnight at 4°C from the cell lysates using a pooled serum from SIV-infected macaques and protein A-Sepharose (Sigma, St Louis, MO, USA). Bound proteins were released by boiling in 2-mercaptoethanol-containing buffer, and separated by SDS-PAGE through 10% polyacrylamide gels. To reduce non-specific binding to cellular proteins, the sera were combined with the protein A-Sepharose, and pre-adsorbed for 2 h with an unlabeled cell lysate from the nontransfected cells. Immunoprecipitation of the transfected cell lysates using sera from SIV-uninfected animals did not yield any specific signal (data not shown).

Transduction of CD4$^+$ cells

Pseudotyped virions were generated by transfection of 293T cells with 3 μg of the MoMLV Gag-Pol expression construct p*gag-pol*gpt, along with 6 μg of either pLTSN or pLXSN EGFP as transducing vector, and 0.1 to 3 μg of the different Env expression constructs. Fresh medium was added to the cultures 12–18 h before the supernatant was collected and passaged though 0.45-μm pore-size filters. To assess the ability of the pseudotyped virions to transduce CD4$^+$ cells, serial dilutions of the filtered supernatants were layered over either CMMT-CD4-LTR-β-gal or HeLa-CD4-LTR-β-gal cells, which had been seeded into 12-well culture plates the day before infection at 3×10^4 cells per well. Protamine sulphate (8 μg/ml) (Sigma, St Louis, MO, USA) was added to the wells, and the cells were kept in a total volume of 1 ml. After 12 h at 37°C, 3 ml of medium were added to dilute the protamine sulphate; 36 h later, the cells were washed, fixed and stained as previously described,[11] and blue cells expressing Tat were counted using a light microscope. Titers were expressed as the number of blue foci per milliliter of added supernatant.

Transduction of CEMX174 and C8166 cell lines was performed by using either cell-free supernatant or cocultivation techniques. In the first case, 1 ml of retroviral vector containing supernatant was incubated at 37°C with 2×10^5 target cells for 6–9 h in the presence of protamine sulfate (8 μg/ml) with occasional stirring. Lymphoid cells were then pelleted, resuspended in fresh medium and grown for an additional 48 h before FACS analysis. For the cocultivation experiments, 10^6 cells in 1 ml RPMI 1640 medium were seeded on transfected 293T cells releasing the different retroviral vectors, and cocultivated for 9 h at 37°C. Suspension cells were then gently removed, transferred to 12-well plates containing 2 ml RPMI 1640 supplemented with 10% FCS and 1% l-glutamine, and cultivated for an additional 48 h before EGFP detection. In some experiments, transduced CEMX174 cells were cultured for 2–3 weeks in G418-containing medium (Gibco-BRL, 500 μg/ml active compound), to enrich for vector-tranduced cells.

Freshly isolated human lymphocytes were transduced by cocultivation techniques, as detailed above except that, following cocultivation for 9 h, the cells were resuspended in complete RPMI supplemented with 100 U/ml recombinant IL2 (rIL2; EuroCetus, Milan, Italy), and cultured for an additional 48 h before EGFP detection. In a set of experiments, transduction of PBMC by retroviral vector-containing supernatants was carried out as described for lymphoid cell lines, but resulted in poor efficiency of gene transfer, compared with cocultivation and was not further pursued.

Neutralization assays

Neutralization assays with sera from SIV-infected monkeys were performed following a modification of a previously described protocol.[29] As a neutralizing serum a pooled heat-inactivated serum from three SIV-infected macaques was used, which contained anti-gp130 Env antibodies, as shown by prior evaluation by Western blotting (not shown). As control serum, a heat-inactivated serum from an uninfected macaque was used. Briefly, 100 μl of diluted serum (six two-fold dilutions, starting with the 1:10 dilution) and 100 μl of virus-containing supernatant were mixed. Following 1 h incubation at 37°C, 0.8 ml complete DMEM was added and the supernatant was applied to sMAGI cells, in the presence of protamine sulphate (8 μg/ml); 36 h after transduction, the number of blue foci was evaluated by β-gal staining.

Neutralization with sCD4 (SmithKline Beecham, King of Prussia, PA, USA) was performed as reported.[6,30] Briefly, 100 μl retroviral vector-containing supernatant were incubated for 1.5 h at 37°C in the presence of 100 μg/ml sCD4. The supernatant was then applied to sMAGI cells. Transduction and titer calculations were carried out as described above.

Cytofluorographic analysis

Vector-transduced lymphoid cells were analyzed on an Elite cytofluorometer (Coulter, Hialeah, FL, USA). Forty-eight hours after infection, cells were pelleted, washed and fixed. CEMX174 and C8166 cells were labeled with anti-CD4-PE monoclonal antibody (mAb; Dako, Glostrup, Denmark). PBMC were labeled with both anti-

CD3ECD (Coulter) and anti-CD8PE (Dako) mAbs. Two- and three-color immunofluorescence was carried out as reported,[31] and analyzed using the PRISM parameter of the Elite cytofluorometer; the negative control setting for each mAb was determined by using labeled Ig of the corresponding isotype.

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