Published in final edited form as:
J Gene Med. 2006 January ; 8(1): 18–28. doi:10.1002/jgm.825.

HIV-1-based defective lentiviral vectors efficiently transduce human monocytes-derived macrophages and suppress replication of wild-type HIV-1

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Abstract

Background—Human monocytes play an important role in mediating human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system (CNS), and monocytes-derived macrophages (MDM) represent a major viral reservoir within the brain and other target organs. Current gene transduction of MDM is hindered by a limited efficiency. In this study we established a lentiviral vector-based technique for improved gene transfer into human MDM cultures *in vitro* and demonstrated significant protection of transduced MDM from super-infection with wild-type HIV-1.

Methods—HIV-1-based lentiviral vector stocks were prepared in 293T cells by the established calcium phosphate transfection method. Human monocytes were isolated from donors' blood by Ficoll-Paque separation and cultured *in vitro*. To establish an effective technique for vector-mediated gene transfer, primary cultures of human MDM were transduced at varying multiplicities of infection (MOI) and at a range of time points following initial isolation of cells (time-in-culture). Transduced cells were then examined for transgene (green fluorescent protein (GFP)) expression by fluorescent microscopy and reverse transcription polymerase chain reaction (RT-PCR). These cultures were then exposed to wild-type HIV-1, and viral replication was quantitated by p24 assay; production of neurotoxic effector molecules by the transduced MDM was also examined, using indicator neurons.

Results—We have demonstrated that primary human MDM could be efficiently transduced (>50%) with concentrated HIV-1-based defective lentiviral vectors (DLV). Furthermore, DLV-mediated gene transduction was stable, and the transduced cells exhibited no apparent difference from normal MDM in terms of their morphology, viability and neurotoxin secretion. Challenge of DLV-transduced MDM cultures with HIV-$1_{Ba\text{-}L}$ revealed a 4- to 5-fold reduction in viral replication, as measured by p24 antigen production. This effect was associated with the mobilization of the GFP-expressing DLV construct by the wild-type virus.

Conclusions—These data demonstrate the inhibition of HIV-1 replication in primary MDM, by a DLV vector that lacks any anti-HIV-1 transgene. These findings lay the initial groundwork for future studies on the ability of DLV-modified monocytes to introduce anti-HIV-1 genes into the CNS.

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Lentiviral vector-mediated gene delivery to the CNS by monocytes/macrophages is a promising, emerging strategy for treating neuro-AIDS.

Keywords

defective lentiviral vector (DLV); monocots-derived macrophages (MDM); blood-derived monocytes (BDM); HIV-1; transduction efficiency; vector mobilization (VM); green fluorescent protein (GFP)

Introduction

HIV-1-based vectors are attractive gene delivery tools because of their ability to efficiently transduce target cells independent of their cell division status at both dividing and non-dividing phases, and their capacity to establish long-lasting transgene expression due to chromosomal integration of the proviral DNA. Among the cell types which are susceptible to HIV-1-based vectors are hematopoietic stem cells [1–7] and monocytes/macrophages [8–13] which represent important targets for human gene therapy [14–16].

Monocytes arise in the bone marrow from myeloid stem cells and are released into the blood. Within a short time, they migrate into various tissues and mature into macrophages. These mononuclear cells also have the ability to cross the blood-brain barrier (BBB) and to enter the central nervous system (CNS), where they act as an important target and reservoir of HIV-1 [17–21]. HIV-1-infection of the brain can lead to HIV dementia, a primary disorder of the CNS, which affects nearly 25% of untreated, HIV-1-infected individuals [22]. Highly active antiretroviral therapy (HAART) has failed to eliminate this aspect of HIV-1-associated disease, and in fact the prevalence of HIV dementia has increased in the post-HAART era, possibly because of HAART's positive effects on overall patient survival time [23–25]. It has further been suggested that HAART may be altering the presentation and/or pathogenesis of HIV dementia, resulting in a more slowly progressive, chronic form of the disease [23–25]. These observations highlight the need for new, adjunct therapeutic approaches aimed specifically at ameliorating or preventing HIV dementia.

One exciting approach is to genetically modify MDM, so as to render them resistant to HIV-1 infection. An additional merit of this approach is that blood-derived monocytes (BDM) are able to traverse the BBB, and to differentiate into stable, long-lived, CNS-resident macrophages; this suggests that BDM may represent a potential vehicle to introduce anti-HIV-1 or neuroprotective genes into the brain. Partly because of this, there is a growing interest in the development of efficient methods for gene transfer into primary human monocyte-derived macrophages (MDM) and for manipulation of gene expression in these cells. Several groups have attempted to transduce MDM recently and different viral vectors have been tested for their effectiveness at transducing monocytes and macrophages [8,26–29]. However, only limited success has been obtained to date. One of the major obstacles in delivering therapeutic genes into human MDM is the poor transduction efficiency under most currently available gene-transfer conditions [8,26,28–31].

We have previously described the construction of defective lentiviral vectors (DLV) based on a modified HIV-1 genome [13,32]. We have used these vectors to develop an optimized protocol for the efficient transduction of primary human MDM. We explored the effect of a range of different parameters on the efficiency of DLV-mediated gene transfer into these cells, including cell density, time-in-culture, and the multiplicity of infection (MOI) of the vector. By doing so, we developed an optimized protocol, which allowed us to transduce approximately 50% of our target cell population. These transduced cells showed stable, long-term expression of the green fluorescent protein (GFP) and exhibited no apparent difference

from untransduced normal MDM in terms of cell morphology and growth kinetics. When challenged with an infectious wild-type HIV-1_{Ba-L} strain, the DLV-transduced cultures showed a significant ability to inhibit HIV-1 replication, as evidenced by a roughly 5-fold reduction in p24 levels in the transduced MDM compared to untransduced normal MDM. These results represent the initial report of inhibition of wild-type HIV-1 replication in human MDM by a DLV vector that lacks any anti-HIV-1 transgene, suggesting the possibility that DLV-modified MDM may represent a potentially useful approach for anti-HIV-1 therapy.

Materials and methods

Vector production and concentration

HIV-1-based defective lentiviral vectors (DLV) encoding selected *cis*-acting elements were produced by transient transfection of human embryonic kidney 293T cells with a packaging construct, a VSV-G envelope construct and a transfer construct containing the reporter gene GFP (Figure 1) [13,33]. In brief, 24 h prior to transfection, 293T cells were seeded in 75 cm^2 tissue culture (TC) flasks at a density of about 6.5×10^6 cells/flask with 14 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and incubated at 37 °C with 5% CO_2. Cell growth medium was replaced with 14 ml fresh medium 2 h prior to transfection. For transfection, a mixture of 6.25 μg packaging plasmid, 6.25 μg transfer plasmid and 2.50 μg VSV-G encoding envelope plasmid was added dropwise to each 75 cm^2 TC flask, and then chloroquine was added to a final concentration of 25 μM. Eight hours post-transfection, the medium was aspirated from these flasks and 8 ml/flask of fresh DMEM containing 2% FBS was added. Vector produced in the transfected cultures was harvested at days 1,2, and 3 post-transfection and stored at −70 °C. Titration of vector was performed using a human T cell line, CEM (AIDS Research and Reference Reagent Program, NIH), by a 10-fold serial dilution method and vector titer was determined at day 3 post-inoculation by counting the number of GFP-positive cells at the endpoint of vector dilution using a fluorescence microscope.

To obtain highly concentrated DLV stocks, vectors stored at −70 °C from different batches of viral transfection were thawed and pooled. Following low-speed centrifugation (1800 *g*) for 30 min at 4°C, recovered supernatant was passed through a 0.45 μm filter (Nalgene, USA). Filtrate containing the vector was then ultra-centrifuged at 113 000 *g* for 3 h at 4°C using a Beckman SW28 rotor. Vector pellet was resuspended in a small amount (1% original volume) of RPMI-1640 containing no FBS. Vector aliquots (0.1–0.2 ml/tube) were stored at −70 °C for future use.

In vitro isolation and cultivation of monocytes

Blood was drawn from consented healthy donors into BD Vacutainer™ ACD (Beckton Dickinson), and peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation through Ficoll-Paque™ PLUS (Amershan Biosciences AB, Sweden). The purified PBMC were then resuspended in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated human serum (Gemini Bio-Products, CA, USA), 20% heat-inactivated FBS (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 0.292 mg/ml L-glutamine (Sigma). The purified PBMC were then seeded into 25 cm^2 TC flasks (Corning) at a range of different densities (0.3 to 3.0×10^7 cells) and incubated at 37 °C with 5% CO_2 to allow the attachment of freshly isolated monocytes. Following 3 h incubation, non-adherent cells were removed by aspiration and the remaining adherent cells were washed extensively with Dulbecco's phosphate-buffered saline (DPBS) (Sigma), prior to the addition of 5 ml RPMI-1640 growth medium per flask, and subsequent cultivation at 37 °C. Cell growth and monolayer formation were observed daily using a phase-contrast inverted microscope (Olympus).

To verify the purity of the attached cells, these monolayer cultures were stained with human CD14 monoclonal antibody conjugated with R-phycoerytherin (Caltag Laboratories, CA, USA). Briefly, the antibody was diluted 50–100 times with DPBS and MDM cultures at incubation days 1, 4, 7 and 11 were stained with the diluted antibody. After 1–2 h incubation, stained cells were rinsed twice with DPBS, and then examined under an inverted fluorescence microscope (Olympus IX70). Mouse isotype IgG2a conjugated with R-PE was also included in this study as a negative control.

Transduction of primary human MDM

Before DLV transduction, cell growth medium was removed from flasks and MDM cultures were washed gently with DPBS three times to remove all cellular debris and residual serum. Transduction was initiated by adding 0.5 ml of DLV stock to each 25 cm^2 TC flask, in the presence of 8 μg/ml polybrene (Sigma). Following a 2-h adsorption with gentle shaking every 15–20 min, transduced cells were rinsed twice with DPBS to remove residual vectors and then 5 ml of complete RPMI-1640 growth medium were added to each flask. The cultures were subsequently evaluated for the expression of the transgene (GFP) by visual inspection using an inverted phase-contrast fluorescence microscope. Efficiency of DLV-mediated transduction of primary MDM was determined at day 3 post-transduction by calculating the percentage of GFP-positive cells within the transduced cell population.

This transduction protocol was used to test and evaluate freshly isolated monocytes (day 0) and primary MDM cultivated for different time periods *in vitro* (days 3, 5, 7, 9 and 13) for their sensitivity to DLV infection at an MOI of 10. In some experiments, the efficiency of DLV-mediated transduction was also examined using a range of different concentrations of vector MOI (1, 10, 50 and 100), and the effect of multiple rounds of vector transduction was also determined.

Neurotoxin production by DLV-transduced MDM

For analysis of the *in vitro* neurotoxicity of MDM-derived supernatants, we followed our previously described methods [34,35]. In brief, cell culture conditioned medium was collected from DLV-transduced MDM at days 0, 5 and 10 days post-infection and stored at −70 °C until analysis. Cell culture supernatants were also collected from untransduced normal MDM and HIV-$1_{Ba\text{-}L}$-infected MDM cultures as controls. To determine neurotoxin production, conditioned medium collected from DLV-infected MDM and control MDM cultures was diluted at 1:10 and then applied to primary neuronal cells prepared from the cerebella of postnatal day 8 Sprague-Dawley rats as previously described [36–38]. Following 24-h incubation, cells were washed and fixed with 4% paraformaldehyde in 0.1 M PBS, and then subjected to an *in situ* terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end-labeling (TUNEL) assay (Oncor, Gaithersburg, MD, USA) as described previously [35]. Determination of neurotoxin production by transduced MDM was performed by measuring the percentage of apoptotic neurons from 12 or more microscopic fields of TUNEL-stained cells.

Wild-type HIV-1 infection of DLV-transduced MDM

HIV-$1_{Ba\text{-}L}$, obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, was propagated once in primary MDM in order to generate a cell-free virus stock, which was then used to infect primary MDM. For this experiment, a DLV-transduced cell population containing approximately 45 ± 3% GFP-positive cells, and a mock-transduced control population, was challenged with wild-type HIV-$1_{Ba\text{-}L}$ (40 ng of p24 antigen) at day 5 post-transduction. Following 1 h adsorption at 37 °C, the virus was aspirated from the flasks and the cells were washed twice with DPBS (5 ml/wash), prior to the addition of growth medium. Culture supernatants were collected every 3 days for a total period of 4 weeks, and HIV-1

production in these infected cultures was determined by measuring the level of HIV-1 p24 antigen in these supernatants.

Statistical analysis

Both one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (software 'Prism') and *t*-test were employed for statistical analysis in this study. The difference between two samples was considered to be significant if the *p* value was less than 0.05.

Results

Primary cultures of human MDM

To optimize an *in vitro* method for consistent preparations of primary cultures of human MDM for vector-based genetic modification, we evaluated the effect of initial cell-seeding density and cell attachment time on cell growth and longevity *in vitro*. We found that an 80–90% confluent cell monolayer was usually formed within 3 days of seeding $1.5 \pm 0.5 \times 10^7$ PBMC into a 25 cm^2 TC flask. Staining of the adherent cells with a mouse anti-human CD14 monoclonal antibody showed that 94–98% of the cells were CD14-positive (Figure 2), indicating the purity of prepared monocyte cultures.

Transduction of primary MDM

We tested DLV for their ability to transduce cultures of primary human monocytes that had been maintained *in vitro* for different time periods (0, 3, 5, 7, 9 and 13 days). Freshly isolated monocytes (day 0) were found to be susceptible to DLV transduction at an MOI of 10, but at a very low efficiency (1.9 ± 0.4%; Figure 3). However, the cells became progressively more susceptible to DLV-mediated transduction during their first week in culture. DLV-directed transduction efficiency of MDM increased to over 4% at day 3 and reached approximately 17% at day 5 ($p < 0.001$). When day 7 cultures were used, over 27% of the cell population was successfully transduced at the vector MOI of 10. However, as indicated in Figure 1, the transduction efficiency did not significantly change after day 7 ($p > 0.05$). These results suggest that DLV-mediated gene transfer into primary MDM may be influenced by cellular differentiation or by other factors associated with *in vitro* maintenance of primary monocytes [8,39].

We also evaluated the impact of vector concentration on transduction efficiency of primary MDM. For this experiment, primary MDM cultures on day 7 *in vitro* (DIV-7) were exposed to 1, 10, 50, and 100 MOI of the DLV. We demonstrated that the DLV-mediated transduction efficiency was directly correlated with the vector MOI that was used (Figure 4), although not in a linear fashion (i.e., gene transfer efficiency was elevated only about 2.5-fold, when going from an initial MOI of 1.0 to an MOI of 100). Nonetheless, we were able to attain a high efficiency of the gene transfer at the highest MOI (52% of cells in the culture being GFP-positive at this MOI), indicating a statistically significant increase of DLV-mediated gene transduction ($p < 0.001$).

To further enhance DLV-mediated gene transfer into primary MDM, we tested and established a supertransduction protocol. We hypothesized that initial efficiency of DLV-mediated transduction of human MDM would be significantly elevated by repeated vector transduction, i.e., by re-transducing a previously transduced MDM population with DLV. As summarized in Table 1, initial transduction efficiency could be substantially increased by multiple rounds of subsequent transduction.

Both transduced and super-transduced primary MDM were analyzed for their morphology and growth kinetics compared to untransduced control cells; we found that these transduced cells exhibited no apparent difference from normal MDM. Consistent with this, we observed that the expression of the GFP reporter gene in transduced MDM remained stable for >7 weeks in culture by phase-contrast microscopy (Figure 5A) and by reverse transcription polymerase chain reaction (RT-PCR) (Figure 5B).

To evaluate whether vector transduction might result in cellular activation and release of potentially neurotoxic effector molecules, we also examined the *in vitro* neurotoxicity of supernatants collected from normal non-transduced MDM and MDM cultures infected with DLV or wild-type HIV-$1_{Ba\text{-}L}$. This comparative analysis revealed that culture supernatants from DLV-transduced MDM elicited identical, baseline, levels of toxicity in indicator rat neurons, as compared to supernatants from normal untransduced MDM (Figure 6). In contrast, supernatants from HIV-1-infected MDM elicited significant levels of neuronal apoptosis, as expected [40].

DLV-transduced cultures resist challenge with infectious HIV-1

To determine if DLV-mediated transduction of MDM could alter cellular susceptibility to infection with full-length HIV-1, we performed a challenge experiment in which we exposed both transduced and untransduced MDM to HIV-$1_{Ba\text{-}L}$. As shown in Figure 7, HIV-$1_{Ba\text{-}L}$ replication was detected in the control MDM shortly after viral inoculation (day 3) and gradually reached peak levels by day 21 post-infection. On the other hand, HIV-$1_{Ba\text{-}L}$ replication in DLV-transduced MDM was significantly inhibited. As shown in Figure 7A, initial detection of viral replication was delayed and peak levels of virus production were suppressed by 4- to 5-fold, as compared to the non-transduced cultures. These results suggest that the HIV-1-based lentiviral vector conferred a considerable degree of protection against wild-type HIV-1 challenge in primary human MDM. In agreement with this, an HIV-1-induced cytopathic effect (CPE) in untransduced MDM was evident by the presence of abnormally large cells, multinucleated cells, and debris resulting from late stages of cell death. However, we observed only very modest levels of viral-induced CPE in the DLV-transduced cultures, as compared to control cultures (Figure 7B).

The DHIV-CTE vector used in our experiments contains an intact *vpu* gene that is located in an intron. This could, in theory, be expressed upon HIV-1 infection of DLV-transduced target cells and might interfere with wild-type virus replication due to effects on CD4 receptor expression, or for other reasons. With this in mind, we modified DHIV-CTE by restriction enzyme-mediated ablation of the *vpu* gene to generate DHIV-CTEΔvpu. Infection with DHIV-CTEΔvpu displayed similar transduction efficiency as the parental DHIV-CTE (data not shown). We then performed a challenge experiment, in which DHIV-CTEΔvpu-transduced MDM cultures containing 44 ± 2% GFP-positive cells were exposed to wild-type HIV-$1_{Ba\text{-}L}$ under the same conditions as used in the experiment shown in Figure 7. The results showed a substantial reduction (67 ± 3%) in HIV-$1_{Ba\text{-}L}$ replication as compared with untransduced control MDM by measuring p24 antigen production at days 18–24 post-infection (*t*-test, $p < 0.01$). The extent of the anti-HIV-1 effect was very slightly lower than that in the DHIV-CTE-transduced cultures (76 ± 2% viral reduction; Figure 7A), but the two data sets did not differ to a statistically significant degree (*t*-test, $p > 0.05$). These findings suggest that the HIV *vpu* gene product may contribute to the DLV-mediated inhibition of wild-type HIV-1 replication at a very limited level in our experimental system.

Finally, to evaluate DLV mobilization, we examined the percentage of GFP-positive cells in the virus-challenged cell cultures described above. This analysis revealed that the percentage of GFP-positive cells increased from an initial level of 44–45% at the time of infection, to a level of 55–57% at day 30 following infectious virus challenge, in both the DHIV-CTE- and

DHIV-CTEΔvpu-transduced MDM cell cultures. This change could be attributed to (a) vector mobilization which might have occurred within the culture system and/or (b) preferential killing of untransduced (GFP-negative) cells. Our experimental results do not provide enough information to allow us to infer whether (a) or (b) was more prominent. To examine the former possibility, culture supernatants were collected from the DHIV-CTE-transduced MDM at 15 days post infection with HIV-1_{-Ba-L}. After performing low-speed centrifugation (1800 *g*, 30 min) and membrane filtration (0.45 μm filter), the resulting cell-free supernatants were then used to infect freshly prepared monocytes at day 7. This resulted in the detection of some GFP-positive cells as early as post-infection day 2 (0.5 ± 0.1%) which reached 4.9 ± 0.17% within the infected MDM population at day 6, consistent with the mobilization of the vector by HIV-1_{Ba-L} (no mobilization was detected in cultures that were not exposed to HIV-1_{Ba-L}, nor was any mobilization detected in supernatants from untransduced MDM that were exposed to HIV-1_{Ba-L}; Figure 8). Importantly, these newly infected monocyte cultures remained GFP-positive over an extended time period (12 days) and showed no alteration in the number of GFP-positive cells during the extent of the experiment, indicating that pseudotransduction or transcription from unintegrated virus was not responsible for the detection of GFP.

Discussion

In this paper, we describe a reproducible and simple method for the highly efficient transduction of primary human monocytes and MDM. This method results in transgene expression among >50% of the transduced cell population, and therefore represents a major improvement over previously published methods [8]. It may be possible to further improve the efficiency of gene transfer by incorporating the cPPT element into our vector backbone, as described by Neil and coworkers [8].

Our results demonstrate that the efficient transduction of primary human monocytes with HIV-1-based vectors is dependent, in part, on the *in vitro* cultivation of these cells for a period of approximately 7 days (at least under our cell culture conditions). This is in good agreement with results from other investigators, and reinforces the notion that freshly isolated human monocytes are somewhat resistant to HIV-1 infection [8], for reasons which may relate to a block in nuclear import of the viral pre-integration complex [8], and/or deficiencies in cellular cofactors that are required for efficient viral reverse transcription [39].

Importantly, DLV-mediated transduction of primary monocytes did not result in changes in cell growth properties or in the production of neurotoxic effector molecules – suggesting that it had only a minimal impact on cellular activation. At the same time, transgene expression in transduced MDM was very stable *in vitro*. Collectively, these findings bode well for the potential future use of lentivirus-transduced monocytes in an *in vivo* setting (e.g., to act as 'Trojan horses' to deliver transgenes into the CNS, for long-term expression within the CNS).

Because human MDM are major targets and reservoirs of HIV-1 within the CNS [17,20], we wished to determine whether DLV-transduced MDM might be partially or completely resistant to infection by wild-type HIV-1. We therefore examined the susceptibility of untransduced MDM and matched, transduced MDM to infection by HIV-1_{Ba-L}. We found that the replication of HIV-1 in the transduced MDM was significantly inhibited when compared to untransduced normal MDM. These data demonstrate a DLV-mediated inhibitory effect on HIV-1 infection in primary culture of human MDM (using vectors which lack any designated anti-HIV-1 insert sequence), which extend our previous findings in dividing cells [32].

DLV-mediated interference of HIV-1 replication is a recently described phenomenon that has been demonstrated in transduced primary lymphocytes and CD4+ T cell lines by several other investigators [32,41–47]. There are several potential mechanisms that may account for this

antiviral effect: (a) TAR and RRE decoy effects of the vectors at both the transcriptional and post-transcriptional levels [45]; (b) competition of the vectors for substrates necessary for reverse transcription and RNA encapsidation [44,46]; and (c) co-packaging and/or dimerization of wild-type and DLV genomes, resulting in the generation of defective virus particles [48]. Precedents for some or all of these possibilities exist in the literature, and include the fact that co-packaging of wild-type HIV genome along with a modified HIV genome and subsequent inhibition of HIV-1 replication has been documented using a Moloney retrovirus vector [49]. The present study suggests that these mechanisms of HIV-1 suppression hold true not only in T cell lines or primary CD4+ T cells, but also in primary human MDM.

Previous studies have shown that defective HIV-1-based vectors can be mobilized by wild-type HIV-1, and subsequently trafficked to previously untransduced cells within a culture [32,41,44–46]. This phenomenon remains relatively poorly explored, but its existence suggests that it may be possible to design a gene therapy strategy in which one might be able to protect a large number of virus-susceptible cells by transducing a much smaller initial subset of cells (either *ex vivo* or *in vivo*). In our experiments, we demonstrated that DLV mobilization can occur in transduced human monocytes, although the efficiency of vector mobilization appeared to be modest. We are currently examining whether it may be possible to increase the efficiency of mobilization.

It should be noted that the defective lentiviral vector (DHIV-CTE) used in this study contains an intact HIV-1 *tat* gene. It has been documented in the literature that the presence or absence of Tat has little or no effect on vector production or on the efficiency of vector transduction [44]. However, deletion of the *tat* gene from our vector would prevent detection of the GFP reporter gene, which is under the transcriptional control of the Tat-dependent HIV-1 long terminal repeat (LTR) [44]. Thus, we believe that the presence of Tat in the vector does not detract from the main conclusions of the present work, particularly since Tat has been described to enhance HIV-1 replication and cell killing, rather than protecting cells from viral infection [50–52]. Future studies will examine Tat-defective constructs using different reporter gene cassettes.

A second viral gene, *vpu*, was also included in the DHIV-CTE vector. To assess the potential role of *vpu* in vector-mediated gene transduction and inhibition of wild-type virus replication, DHIV-CTE was modified by ablation of the *vpu* gene, thereby generating DHIV-CTEΔvpu. Vpu was found to be dispensable for vector production and transduction of MDM, as previously reported by An *et al.* [44]. However, challenge experiments with infectious HIV-1 revealed that DHIV-CTEΔvpu-transduced MDM were very slightly less resistant to wild-type virus infection than DHIV-CTE-transduced cells. This suggests that *vpu* may contribute to the observed inhibition of wild-type HIV-1 at very limited level by our DLV constructs. Possible reasons might include the fact that Vpu is involved in CD4 receptor degradation [53–56].

Overall, the mechanistic basis for the inhibitory effect of our DLV vectors on the replication of wild-type HIV-1 remains uncertain; possible reasons may include competition for necessary intracellular cofactors and/or preferential replication or packaging of the DLV genomes. Future experiments will be needed to address this. Such studies will also allow for the optimization of DLV-based approaches to the inhibition of HIV-1 replication, which is presently considerably less efficient than other antiviral approaches such as siRNA or dominant negative mutants [12,57].

In conclusion, we have developed an optimized strategy which allows for highly efficient *ex vivo* transduction of primary human monocyte-derived macrophages with defective HIV-1-based lentivirus vectors pseudotyped with the VSV-G glycoprotein. Using this method, we obtained stable long-term gene expression in cultured human MDM. Furthermore, we have

demonstrated that DLV-transduced primary MDM cultures are at least partially refractory to infection by wild-type HIV-1. These findings will form the basis for future *in vivo* studies, in which we intend to explore the ability of DLV-transduced MDM to cross the BBB and enter the CNS, thereby acting as a novel delivery system for CNS gene therapy.

Acknowledgments

This study was supported by grants from the National Institutes of Health (S11NS043499, G12RR003061, P01MH064570 and R01AI049057) and by the Hawaii Community Foundation.

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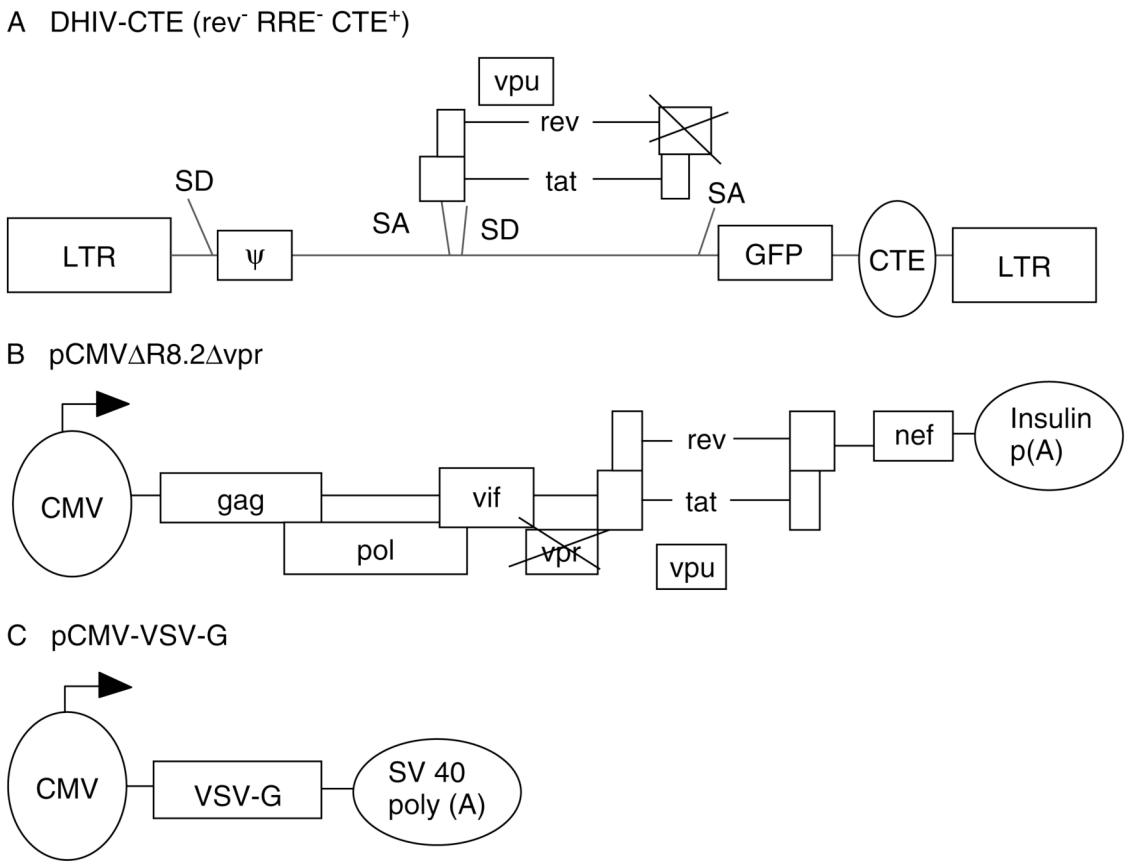

Figure 1.

Generation and design of defective lentiviral vector (DLV). (A) HIV-1-based transfer construct lacking structural genes (*gag/pol* and *env*) and most accessory genes (*vif*, *vpr*, *nef* and *rev*) (32). (B) pCMVΔR8.2Δvpr is the lentiviral packaging construct [42]. (C) The envelope construct, pCMV-VSV-G, encodes the vesicular stomatitis virus glycoprotein (G) for pseudo-typing [1]

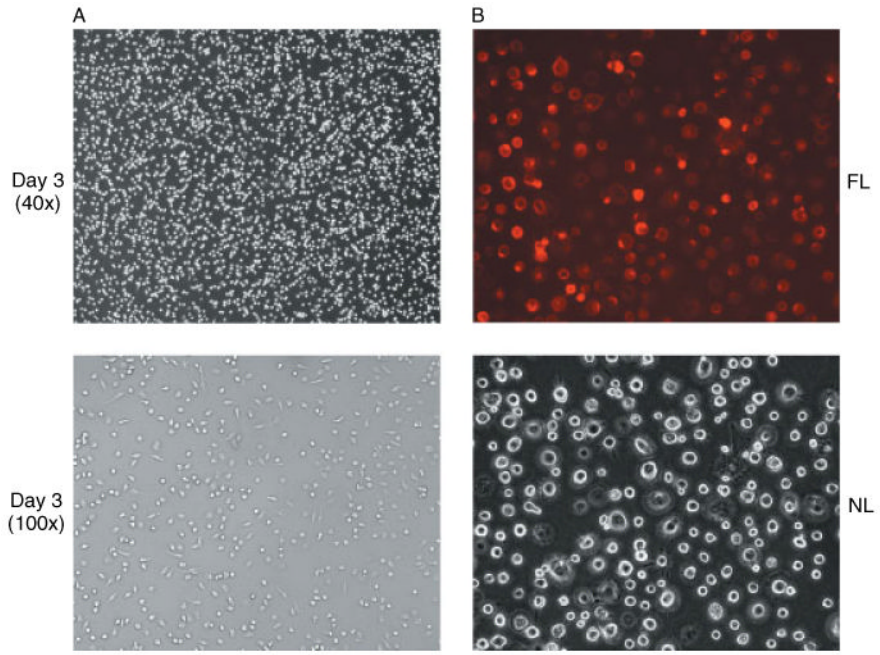

Figure 2.
Photomicrographs of primary cultures of human MDM. (A) Day 3 cultures of human MDM showing the formation of cell monolayers under phase-contrast microscopy. (B) Staining of primary cultures of human MDM at day 10 with mouse anti-human CD14 monoclonal antibody conjugated with R-phycoerytherin showing the same field under either normal light (NL) phase-contrast microscopy or fluorescent light (FL) microscopy (magnification 200×)

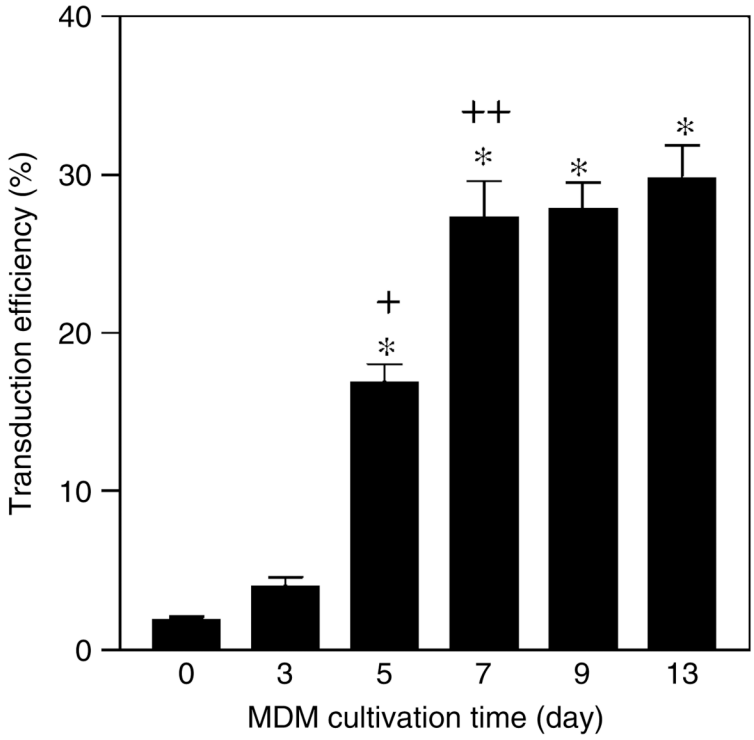

Figure 3.

Effect of time-in-culture on the susceptibility of primary human monocytes to DLV transduction. Adherent monocytes were grown in RPMI supplemented with 10% human serum and 20% FBS and transduced with DLV (MOI = 10) at the indicated times. Transduction efficiency was determined by calculating the percentage of GFP+ cells within the culture. Transduction efficiency increased significantly from day 3 to day 5 ($^{+}p < 0.001$) and from day 5 to day 7 ($^{++}p < 0.01$), but there was no significant increase after day 7 ($^{*}p > 0.05$) using one-way ANOVA followed by Bonferroni's multiple comparison test

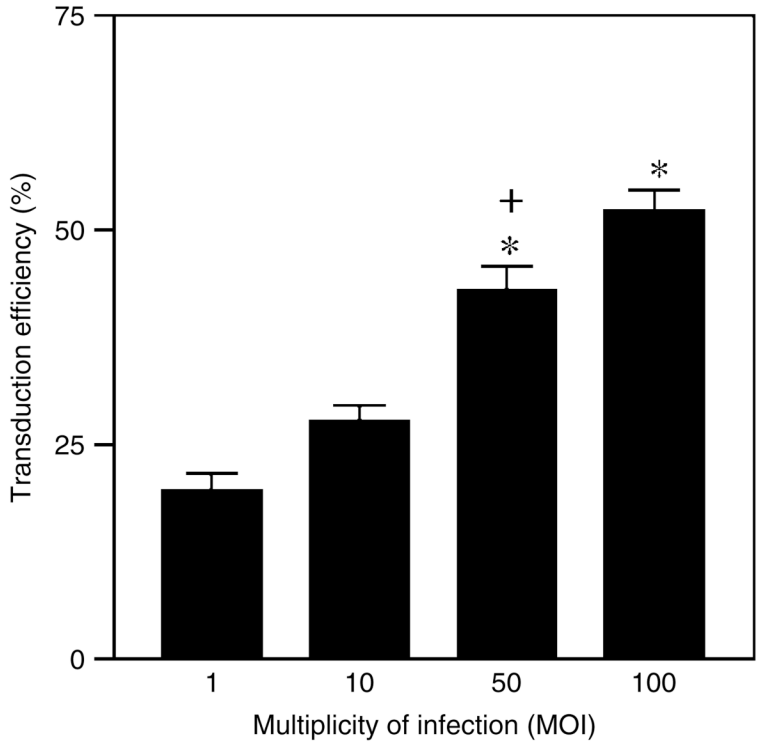

Figure 4.

Effect of different vector MOI on transduction efficiency in human monocyte cultures. MDM cultures (day 7 *in vitro*) were seeded at a common density and then transduced with the indicated vector MOI. The percentage GFP+ cells within the culture was then determined at day 5 post-transduction. Data are given as the mean of multiple samples from at least three independent experiments for each cell culture time point. Transduction efficiency increased significantly when a MOI of 50 or 100 was used as compared to MOI of 1 (*$p < 0.005$) and 10 ($^{+}p < 0.01$) by one-way ANOVA followed by Bonferroni's multiple comparison test

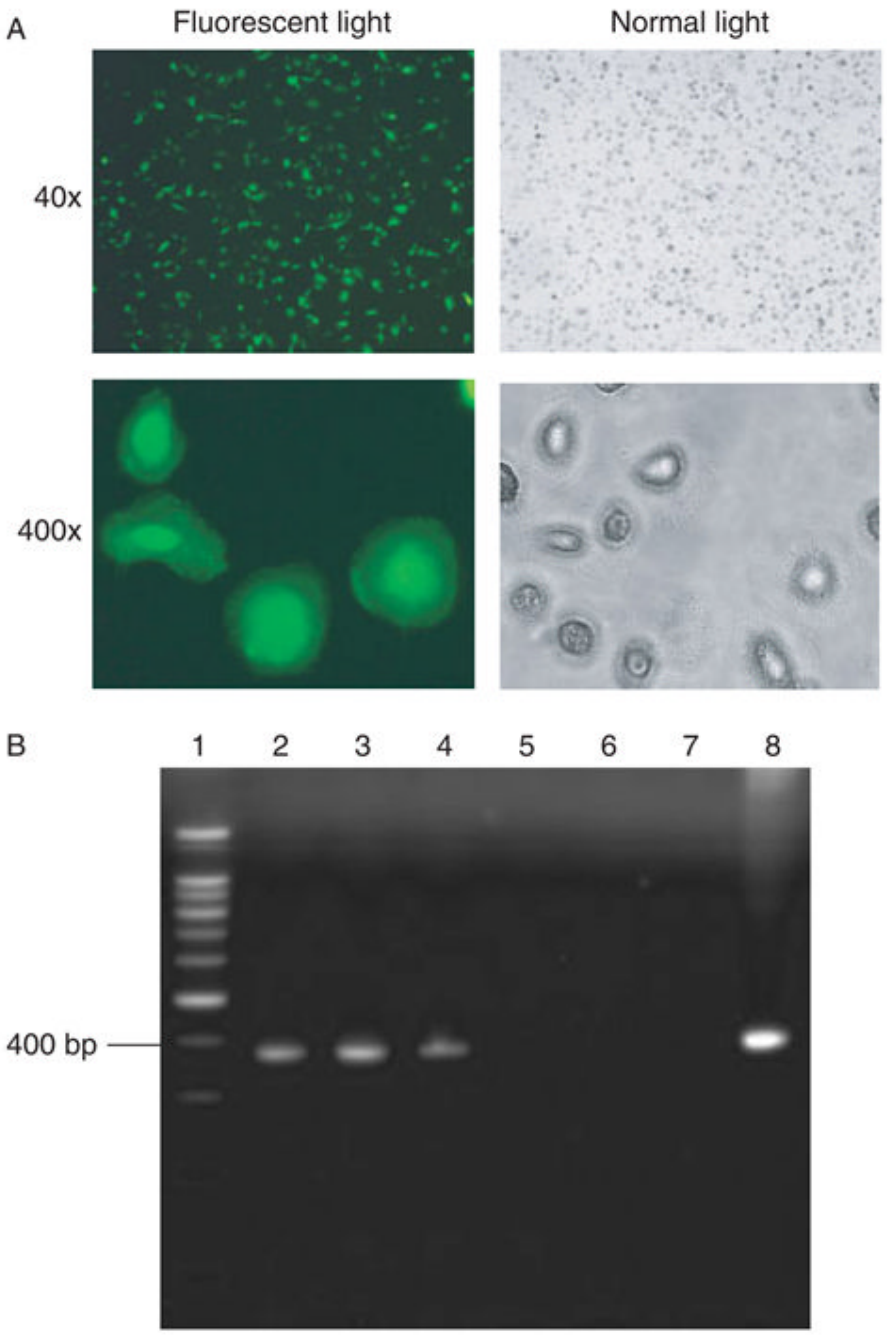

Figure 5.

Stable gene expression in human MDM. (A) Primary cultures of human MDM were transduced with the defective lentiviral vector (MOI = 50) at day 7 *in vitro*, and GFP expression was then examined 46 days later (one of four representative experiments is shown). (B). RT-PCR detection of transgene expression in DLV-transduced human MDM. Total RNA was isolated from both DLV-transduced and normal MDM and RT-PCR amplification was conducted with the use of GFP-specific primers flanking a 373-bp fragment. The PCR products were subjected to 2% agarose gel electrophoresis. Lanes: 1 = 100 bp DNA ladder; 2 = DLV-transduced MDM at day 5; 3 = DLV-transduced MDM at day 39; 4 = DLV-transduced MDM infected with

HIV-$1_{Ba\text{-}L}$ at day 25; 5 = untransduced normal MDM at day 25; 6 = negative control (water) for RT; 7 = negative control (water) for PCR; and 8 = positive control, pDLV

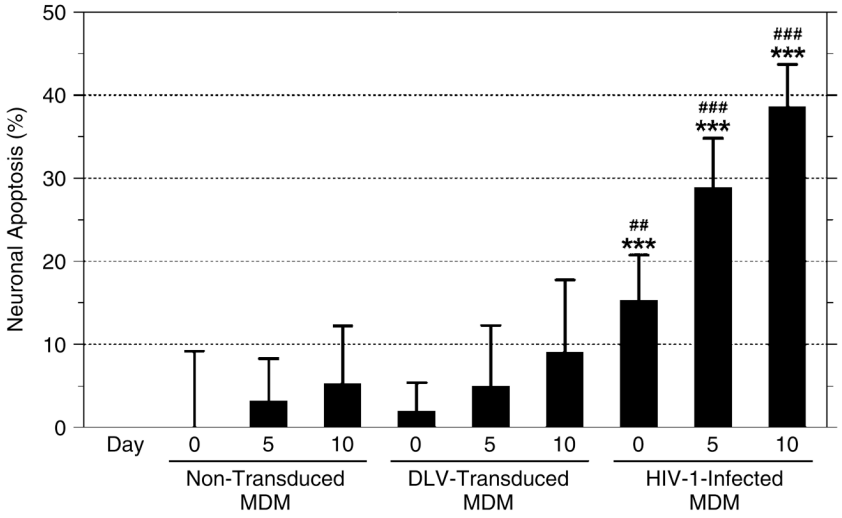

Figure 6.

Macrophage conditioned medium (MCM) collected at various days post-infection (0, 5 and 10 days) from non-transduced, DLV-transduced, and HIV-1-infected MDM was added (at a dilution 1 : 10) into cultures of rodent cerebellar granule neurons for 24 h. At the end of this incubation period, neuronal apoptosis was evaluated by TUNEL assay. The data are presented as mean values (% apoptotic cells) ± the standard error of the mean. All values shown represent treatment-specific values (i.e., the basal level of apoptosis in untreated cells has been subtracted from these data). Statistical significance was determined by one-way ANOVA followed by Bonferroni's multiple comparison test. ***, ##, and ### denote statistical significance $p < 0.001$ as compared to non-transduced MCM on the corresponding day, $p < 0.01$ and $p < 0.001$ as compared to DLV-transduced MCM on the corresponding day, respectively

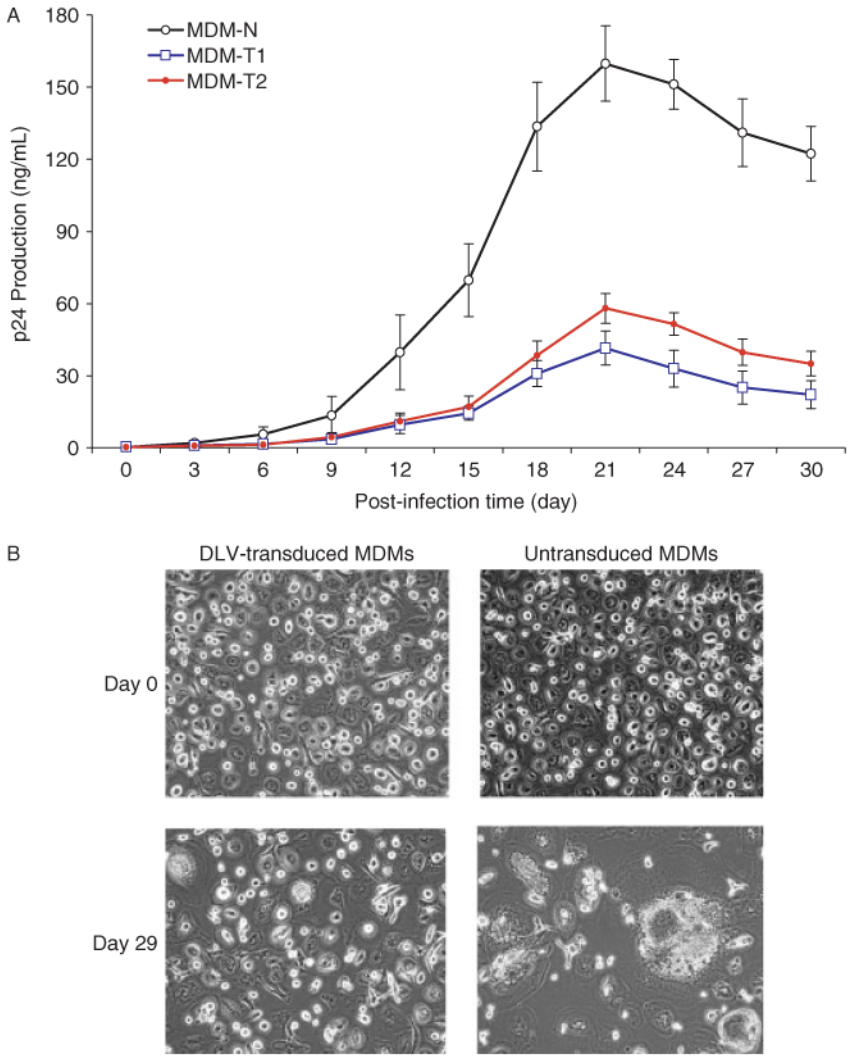

Figure 7.

Lentiviral vector inhibits HIV-1 replication in primary monocyte cultures. (A) A comparison of the kinetics of HIV-1_{Ba-L} replications shown, in both normal (non-transduced; MDM-N) and vector-transduced (MDM-T1 = DHIV-CTE transduced and MDM-T2 = DHIV-CTEΔvpu transduced) MDM. The data show a significant inhibition of HIV-1 replication in both the vector-transduced MDM cultures as compared to untransduced MDM (*t*-test, $p < 0.01$), but no statistical difference between MDM-T1 and MDM-T2 ($p > 0.05$). (B) DLV vector transduction suppresses HIV-1 cytopathicity in MDM cultures. Normal (non-transduced) and DHIV-CTE-transduced cultures of primary MDM were exposed to HIV-1_{Ba-L}, and examined under light microscopy at days 0 and 29 following virus infection (100× magnification). It can be readily appreciated that the DLV strongly suppressed HIV-1-mediated cytopathic effects, resulting in a reduction in the number of giant cells in the culture

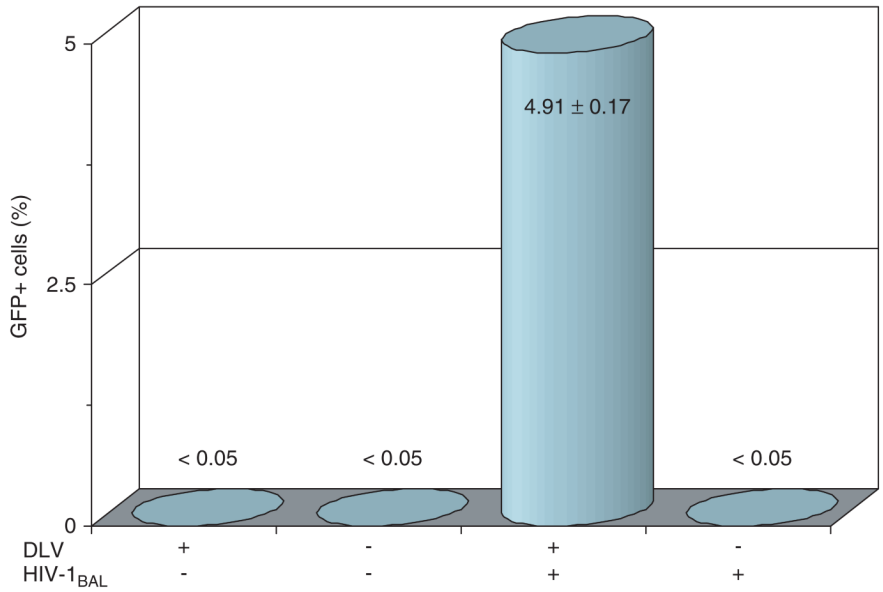

Figure 8.

DLV mobilization by wild-type HIV-1 infection. Mobilization of the GFP-expressing DLV vector was assessed by collecting cell-free culture supernatants from the indicated cell populations. These culture supernatants were then added to fresh MDM and the percentage of GFP-positive cells was enumerated 3 days later. Results shown represent mean values from three independent experiments (± the standard deviation). The data clearly show that DLV mobilization was induced in transduced MDM when they were subsequently infected with HIV-$1_{Ba\text{-}L}$, but not in transduced but uninfected cells, or in untransduced MDM

Table 1

Effect of DLV-mediated supertransduction on gene transfer efficiency in monocyte-derived macrophages

Primary transduction			Second transduction			Third transduction		
MOI	**Efficiency**	**Mean ± SD**	**MOI**	**Efficiency**	**Mean ± SD**	**MOI**	**Efficiency**	**Mean ± SD**
10	30.67% (46/150)		10	43.00% (56/130)		10	46.23% (49/106)	
	24.85% (42/169)	27.83 ± 2.91%		33.50% (57/170)	38.10 ± 4.76%		53.66% (66/123)	50.26 ± 3.75
	27.96% (52/186)			37.08% (66/178)			50.89% (57/112)	
50	43.41% (73/150)			54.87% (62/113)				
	38.10% (56/147)	43.02 ± 4.74%	10	48.80% (61/125)	52.67 ± 3.36%	ND	ND	
	47.56% (78/164)			54.35% (50/92)				

MOI = multiplicity of infection, SD = standard deviation