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ARTICLE

# T Lymphocytes Transduced with a Lentiviral Vector Expressing F12-*vif* Are Protected from HIV-1 Infection in an APOBEC3G-Independent Manner

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**The viral infectivity factor (Vif) is an essential component of the HIV-1 infectious cycle. Vif counteracts the action of the cytidine deaminase APOBEC3G (AP3G), which confers nonimmune antiviral defense against HIV-1 to T lymphocytes. Disabling or interfering with the function of Vif could represent an alternative therapeutic approach to AIDS. We have expressed a natural mutant of Vif, F12-Vif, in a VSV-G-pseudotyped lentiviral vector under the Tat-inducible control of the HIV-1 LTR. Conditional expression of F12-Vif prevents replication and spreading of both CXCR4 and CCR5 strains of HIV-1 in human primary T lymphocyte and T cell lines. T cells transduced with F12-Vif release few HIV-1 virions and with reduced infectivity. Several lines of evidence indicate that HIV-1 interference requires the presence of both wild-type and F12-Vif proteins, suggesting a dominant-negative feature of the F12-Vif mutant. Surprisingly, however, the F12-Vif-mediated inhibition does not depend on the reestablishment of the AP3G function.**

**Key Words: gene therapy, lentiviral vectors (LV), HIV/AIDS, Vif, APOBEC3G**

## INTRODUCTION

Anti-HIV-1 gene therapy aims at providing AIDS patients with genetically modified, HIV-1-resistant T cells competent to restore immune functions and to control the HIV-1-induced pathogenesis. Hematopoietic stem cells (HSCs), T cell precursors, or T lymphocytes can be genetically modified with sequences encoding ribozymes, decoys, antisense and small interfering RNA molecules directed against viral and cellular genes, or proteins such as intrakines, toxins, single-chain antibodies, and dominant-negative mutants of HIV-1 gene products [1,2]. Most of the preclinical and clinical studies carried out so far have been based on the use of retroviral vectors derived from the Moloney murine leukemia virus (MLV) to transduce HSCs or T cells. However, MLV-derived vectors have shown major limitations for clinical applications, such as poor efficiency in transducing nondividing cells and propensity to induce neoplasia by insertional activation of oncogenes [3]. Conversely, HIV-1-derived lentiviral vectors transduce human HSCs at high efficiency without compromising their self-renewing, repopulating, and differentiating capacity [4,5]. The first clinical application of a second-generation lentiviral vector for anti-HIV gene therapy, recently authorized in the United States, is based upon preclinical studies demonstrating the feasibility and efficacy of an *env* antisense to control HIV-1 replication in primary T lymphocytes from HIV-1-infected patients [6,7].

Among the possible targets of anti-HIV gene therapy, there is the product of the *vif* gene. Vif is one of the four accessory proteins of HIV-1, expressed at a late phase during virus replication in a Rev-dependent manner [8]. Vif is required for high viral infectivity in nonpermissive cells, which include the natural targets of HIV-1 (T cells and macrophages) and some T cell lines (i.e., CEM, H9, and HUT 78), whereas it is dispensable in a few permissive cell lines (i.e., SupT1, CEMss, 293T, C8166, and HeLa) [9]. A major function of Vif is to counteract the action of the cellular inhibitor APOBEC3G (AP3G), whose expression is restricted to nonpermissive cells [10]. AP3G, in the absence of Vif, causes massive G-to-A hypermutation in proviral DNA. Deamination generates highly mutated and unstable cDNA that strongly affects virus viability. Vif overcomes the action of AP3G by inducing



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its degradation in the 26S proteasome and exclusion from virions. Lately, other members of the APOBEC family, such as APOBEC3F and APOBEC3B, have been shown to deaminate HIV-1 provirus similar to AP3G [11–13].

Disabling or interfering with the function of Vif could represent an alternative anti-HIV-1 therapeutic approach. We report the anti-HIV-1 activity of a natural mutant of Vif (F12-Vif) carrying 14 unique amino acid substitutions ([14] and Fig. 1B), originally identified in the F12 HIV-1 variant, a nonproducer HIV-1 provirus cloned from HUT 78 cells infected with a primary HIV-1 isolate [15]. The F12 HIV provirus is capable of blocking superinfection by HIV-1 [16,17], and this activity has been previously transferred to T cells in the framework of a retroviral vector [18]. We have developed a lentiviral vector expressing F12-*vif* under the control of the HIV-1 LTR, which acts as an inducible promoter in the presence of the viral transactivator Tat and is therefore transcriptionally activated preferentially in HIV-1-infected cells. Here, we show that conditional expression of F12-Vif efficiently prevents replication and spreading of both CXCR4 (X4) and CCR5 (R5) HIV-1 strains in transduced primary T lymphocytes and T cell lines. F12-Vif inhibits the production and reduces the infectivity of HIV-1 virions released by transduced permissive and nonpermissive T cells by interfering in a transdominant fashion with the wild-type Vif, but without restoring AP3G function.

## RESULTS

### Lentiviral Vectors for Conditional Expression of Vif Proteins

We inserted the F12-*vif* open reading frame into the HIV-1-based pHR2 lentiviral vector [19] under the control of the wild-type, Tat-inducible HIV-1 LTR. The Rev-responsive element (RRE), essential for an efficient production of Vif, was included downstream of the packaging signal. The vector (F12-VifPΔN) (Fig. 1A) contains a phosphoglycerokinase (PGK) promoter-driven internal expression cassette for the constitutive expression of the ΔLNGFR surface marker [20]. Control vectors were PΔN, which carries only the PGK-ΔLNGFR cassette, and WT-VifPΔN, in which the WT-*vif* (Fig. 1A) has replaced the F12-*vif* gene.

We pseudotyped the vectors with the vesicular stomatitis envelope glycoprotein (VSV-G) in a second-generation packaging system and used them to transduce T cell lines (CEM A3.01, SupT1, and CEMss) and T lymphocytes derived from healthy human donors. When necessary, we enriched transduced cells by immune selection with an anti-NGFR antibody at >92% purity, as confirmed by FACS analysis of ΔLNGFR expression. Southern blot analysis of genomic DNA derived from transduced cells revealed only bands of the expected size, indicating unrearranged genomic integration of the vectors (data not shown).

We analyzed expression of the F12-*vif* transgene at both RNA and protein level in permissive transduced SupT1 cells mock infected and infected with Δ*vif* HIV-1 for a week by Northern and Western blot, respectively (Figs. 1C and 1D). The 1-kb ΔLNGFR internal transcript is constitutively expressed in both mock- and HIV-infected cells, while the LTR-driven, unspliced, and singly spliced transcripts are induced only in HIV-infected cells (Fig. 1C). Identical results were obtained in transduced CEM A3.01 cells infected with WT HIV-1 (data not shown). Both F12-Vif and WT-Vif proteins are detectable at weak, basal levels in mock-infected SupT1 cells and significantly induced 7 days after infection (Fig. 1D). We normalized protein content by probing the filter with an anti-human actin Ab, whereas we assayed productive infection of the cell culture with an anti-HIV-1 serum (Fig. 1D) and by measuring the reverse transcriptase (RT) activity in the culture supernatant (data not shown).

### F12-Vif Inhibits Replication of Both X4 and R5 HIV-1 Strains in Primary T Lymphocytes and CEM A3.01

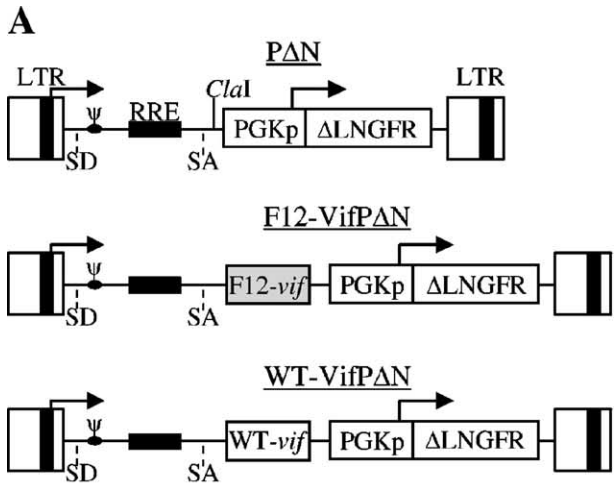
We initially assessed the F12-Vif antiviral activity in T lymphocytes derived from buffy coats of healthy normal donors. We infected mock-transduced and vector-transduced cells with the X4 HIV-$1_{IIIB}$ strain at the m.o.i. of 0.1 and monitored RT activity for 21 days. In contrast to mock-transduced and WT-Vif-expressing cells, T lymphocytes carrying the integrated F12-*vif* gene were protected from HIV-1 infection throughout the experiment (Fig. 2A).

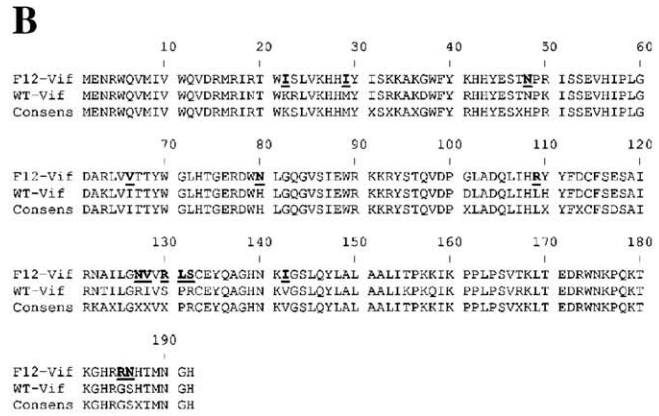
To test the inhibitory activity of F12-Vif against an R5 HIV-1 strain, we challenged the transduced T lymphocytes with the HIV-$1_{AD8}$ molecular clone at the m.o.i. of 0.1 and analyzed them for viral production for 28 days. As shown in Fig. 2B, cultures of T cells expressing F12-Vif control viral replication, whereas mock-transduced or PΔN-transduced cell cultures show a normal viral release.

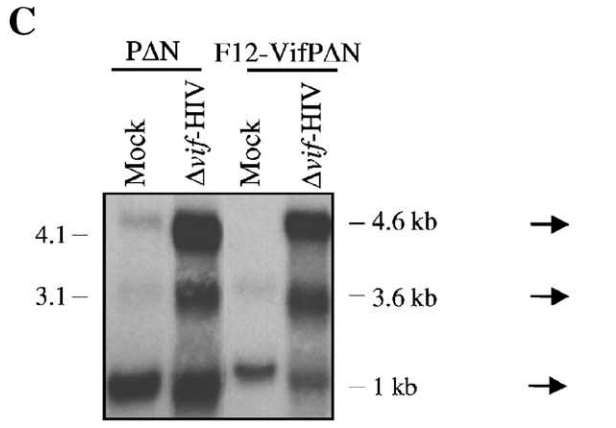
To provide a quantitative estimate of the inhibition generated by F12-Vif, we measured the concentration of p24Ag in mock-transduced and vector-transduced CEM A3.01 cells infected with the molecular clone HIV-$1_{NL4-3}$ at the m.o.i. of 0.01 (Fig. 2C). At the peak of infection of the control cells (days 11 and 17), previously evaluated by RT activity, p24 Ag was undetectable in F12-VifPΔN-transduced cells, whereas it was at least 5 orders of magnitude higher in mock-, PΔN-, and WT-VifPΔN-transduced cells (day 17: $2.3 \times 10^5$, $1.3 \times 10^5$, $1.8 \times 10^5$ pg/ml, respectively). Overall, these data indicate that the expression of F12-Vif specifically reduces acute infection of HIV-1 and that the antiviral activity of the mutant is independent of the HIV-1 coreceptor usage.

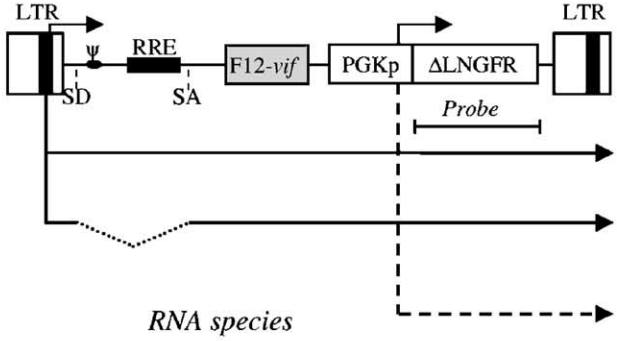
### F12-Vif Inhibits HIV-1 Replication in an AP3G-independent Manner

To gain insight into the mechanism of F12-Vif protective function, we evaluated whether the mutant could restore the endogenous AP3G antiviral activity in a dominant-negative fashion. To verify this hypothesis,



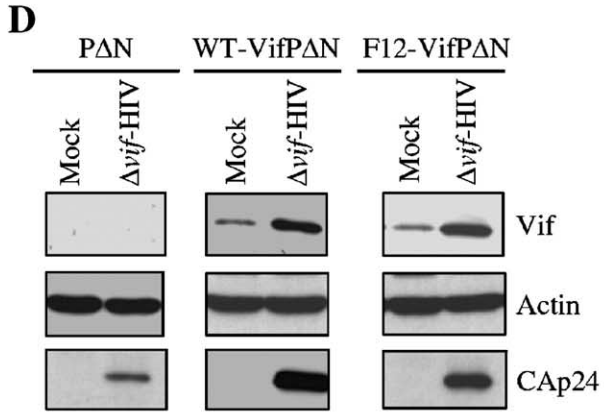




**FIG. 1.** Structure and HIV-1-dependent expression of the lentiviral vectors. (A) Schematic representation of the HIV-1-based PΔN, WT-VifPΔN, and F12-VifPΔN lentiviral vectors in their proviral forms. Wild-type HIV-1 long terminal repeats (LTR), packaging signal (ψ), splice donor and acceptor sites (SD and SA), Rev-responsive element (RRE), and *Cla*I restriction sites are specified. Arrows indicate transcription start sites. (B) Amino acid sequence of F12-Vif compared to the WT-Vif (HIV-$1_{NL4-3}$) and a consensus sequence generated from five HIV-1 strains (HXB2 Accession No. K03455, BRU Accession No. K02013, SF2 Accession No. K02007, PV22 Accession No. K02083, MN Accession No. M17449). The aa substitutions unique to F12-Vif are in bold and underlined letters. (C) Northern blot analysis of total RNA of transduced SupT1 cells, mock infected (Mock) or infected for 7 days with a *vif*-deficient virus at m.o.i. of 0.1 (Δ*vif*-HIV). The expected viral RNA transcripts are schematically represented on the right. (D) Western blot analysis of WCE obtained from transduced SupT1 cells mock infected or infected for 7 days with the Δ*vif*-HIV-1 at the m.o.i. of 0.1. The membrane was sequentially probed with an anti-Vif antiserum (Vif), an anti-human actin Ab (actin), and a patient-derived anti-HIV-1 serum (CAp24).

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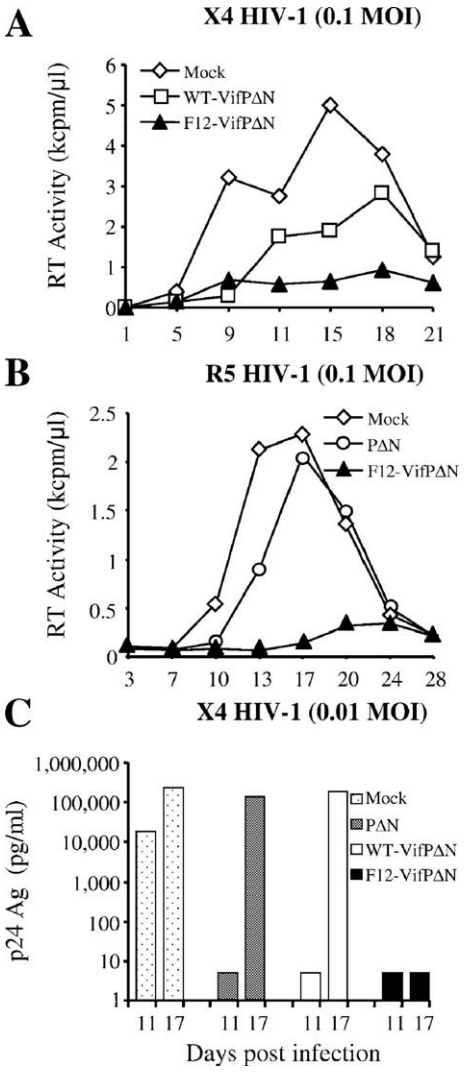


**FIG. 2.** F12-Vif inhibits the replication of both X4 and R5 HIV-1 strains in T lymphocytes and CEM A3.01 cells. (A and B) Peripheral blood lymphocytes were isolated from healthy donors, mock transduced or transduced with the different lentiviral vectors, and then infected with (A) the X4 strain HIV-$1_{IIIB}$ or (B) the R5 molecular clone HIV-$1_{AD8}$ at the m.o.i. of 0.1. Supernatants were collected every 4 days, and assessed for RT activity. (C) CEM A3.01 cells mock transduced or transduced with the lentiviral vectors were infected with the X4 molecular clone HIV-$1_{NL4-3}$ at the m.o.i. of 0.01. Supernatants corresponding to the days of maximal viral production by RT activity measurement were analyzed for p24Ag production by ELISA.

we performed Western blot analysis of protein derived from 293T cells cotransfected with a fixed amount of the hemagglutinin antigen (HA)-tagged AP3G (AP3G:-HA) and WT-Vif plasmids and variable amounts of the F12-Vif plasmid (Fig. 3A). Contrary to our expectation, we found that F12-Vif does not prevent the Vif-mediated degradation of AP3G in every tested ratio. Indeed, the disappearance of AP3G correlates directly with the increasing doses of F12-Vif (Fig. 3A), indicating that F12-Vif contributes to AP3G degradation. To demonstrate directly that F12-Vif by itself induces the enzyme degradation, we examined whole-cell extract (WCE) derived from 293T cells cotransfected with a constant amount of AP3G:HA plasmid and increasing amounts of either WT-Vif or F12-Vif plasmids. As shown in Fig. 3B, F12-Vif alone eliminates AP3G in a manner comparable to that of the WT-Vif.

Next, we looked at the protein composition of viral particles produced from CEM A3.01 cells mock trans-

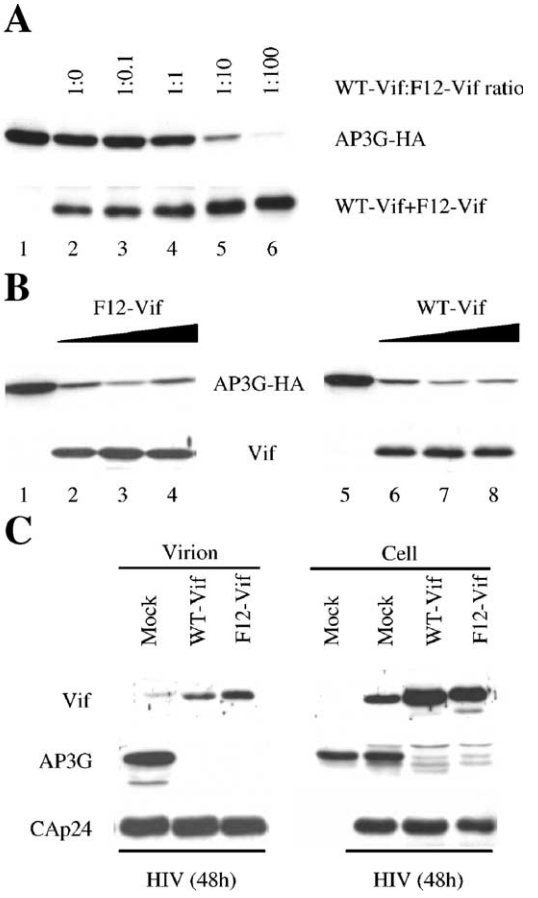


**FIG. 3.** F12-Vif induces AP3G degradation in a manner identical to and in synergy with WT-Vif. (A) Western blot analysis of whole-cell extract (WCE) obtained from 293T cells cotransfected with a fixed amount of AP3G:HA (30 ng/8 $\times$ $10^5$ cells) and WT-Vif-expressing vectors (10 ng/8 $\times$ $10^5$ cells) and various amounts of F12-Vif-expressing vector at the indicated ratios. The membrane was sequentially probed with the anti-HA monoclonal Ab and the anti-Vif serum (top and bottom, respectively). (B) Western blot analysis of 293T cells cotransfected with a fixed amount of AP3G:HA vector (30 ng/8 $\times$ $10^5$ cells) and increasing amounts of either WT-Vif- or F12-Vif-expressing vectors (400, 600, and 800 ng). The membrane was sequentially probed with the anti-HA monoclonal Ab and anti-Vif serum. (C) Western blot analysis of proteins derived from isolated virions produced by CEM A3.01 cells expressing either WT-Vif or F12-Vif and infected with a VSV-G-pseudotyped Δ*env*-HIV molecular clone. WCEs from the mock-infected and virion-producing infected cells were run in parallel. The membrane was sequentially probed with the anti-AP3G, recognizing the endogenous protein; the anti-Vif, and a patient-derived anti-HIV-1 serum.

duced and transduced with WT-Vif- or F12-Vif-expressing vectors after a single-cycle infection. To obtain a high percentage of infected cells and thus a high number of virions, we used the envelope-deficient HIV-1 molecular clone R9Δ*env* pseudotyped with VSV-G (VSV-G/R9Δ*env*HIV-1). After 48 h, we analyzed proteins derived from cell-free virions and infected cells for Vifs and AP3G content (Fig. 3C). Of note, although the cellular level of F12-Vif and WT-Vif is comparable between the two cell types (right), we found in this experiment a 3-fold higher (values of densitometric evaluation of five independent blots are from 2.5- to 8-fold) level of Vif proteins in the viral particles released from the F12-Vif- compared to the WT-Vif-expressing cells. In agreement with our previous observations, both cell types show undetectable levels of intravirion AP3G compared to the mock-transduced cells, indicating that both Vifs produced *in trans* from the vectors are extremely potent in eliminating AP3G. In contrast, in mock-transduced cells the AP3G level is higher, likely because its complete degradation requires a productive rather than a single-cycle infection or because only a fraction of the cells within the population is infected. As shown by Sheely et al. [10] AP3G levels are reduced in infected cells only after 8–10 days.

### F12-Vif, Like WT-Vif, Inhibits Viral Replication of Wild-Type, but Not Δ*vif*-HIV-1, in Permissive CEMss Cells

To demonstrate further that F12-Vif inhibits HIV-1 replication in an AP3G-independent manner, we infected the permissive CEMss mock-transduced and vector-transduced cells with the X4 HIV-$1_{NL4–3}$ molecular clone at the m.o.i. of 0.1. In line with the previous results, we found that F12-Vif expression reduces HIV-1 replication (Fig. 4A). However, WT-Vif also decreases the production of HIV-1 in these cells. Furthermore, to establish whether the mere expression of F12-Vif or WT-Vif produced *in trans* was sufficient to inhibit HIV-1 replication also in the absence of WT-Vif derived by the challenging virus, we infected the cells with Δ*vif* HIV-1. As shown in Fig. 4B, the *vif*-deficient virus efficiently replicates in all cell types, suggesting that WT-Vif of the infectious virus is required for HIV-1 inhibition.

### F12-Vif, Like WT-Vif, Rescues Viral Replication of Δ*vif* HIV-1 in Nonpermissive Cells

To investigate further whether F12-Vif inhibitory activity was dependent on the presence of WT-Vif, we infected the nonpermissive CEM A3.01 mock- and vector-transduced cells with Δ*vif* HIV-1 at the m.o.i. of 0.01 (Fig. 5A). As expected, Δ*vif* HIV-1 did not replicate in mock- and PΔN-transduced cells, while it efficiently grew in cells in which WT-Vif was supplied *in trans*. Remarkably, Δ*vif* HIV-1 was released also from cells expressing F12-Vif, indicating that the mutant, in the absence of the WT counterpart, mimics its function (Fig. 5A). We also

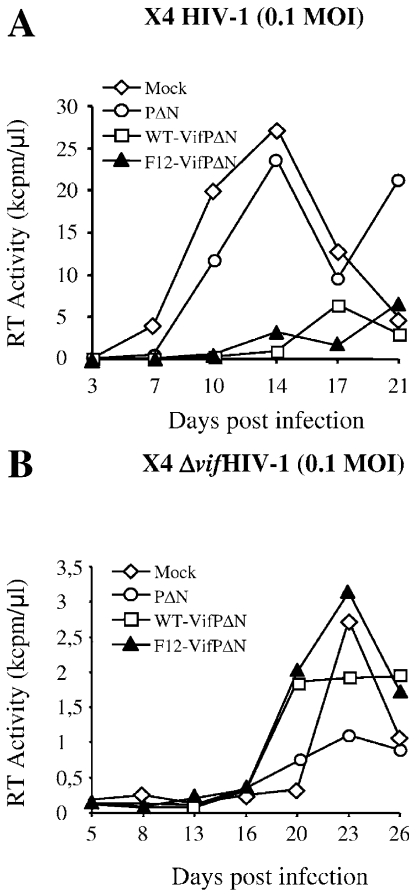


**FIG. 4.** F12-Vif and WT-Vif similarly inhibit viral replication of wild-type, but not Δ*vif* HIV-1, in permissive CEMss cells. (A and B) CEMss cells mock transduced or transduced with the lentiviral vectors were infected with either (A) the X4 molecular clone HIV-$1_{NL4–3}$ or (B) its Δ*vif* derivative at the m.o.i. of 0.01.

infected transduced cells with the wild-type virus $HIV_{NL4–3}$ to confirm the F12-Vif-mediated viral interference within the experiment (Fig. 5B).

To test whether F12-*vif* might behave as a "rescue gene" in case it encounters Δ*vif* HIV-1 quasispecies silently harbored in a patient, we collected the virus emerging from F12-Vif- and WT-Vif-expressing cells and assessed their infectivity in nonpermissive (CEM A3.01) and permissive cells (CEMss) (Fig. 5C). Of interest, neither virus replicated in CEM A3.01 cells, whereas, as expected, both grew in CEMss cells, ruling out the possibility that F12-*vif* could be detrimental rather than beneficial in a potential gene therapy approach.

### F12-Vif-expressing Cells Release Fewer Viral Particles Compared to Control Cells after a Single Round of Infection

To determine whether F12-Vif-expressing cells restrain HIV-1 production by limiting viral release, we measured the level of p24Ag in culture supernatants after 24 h,

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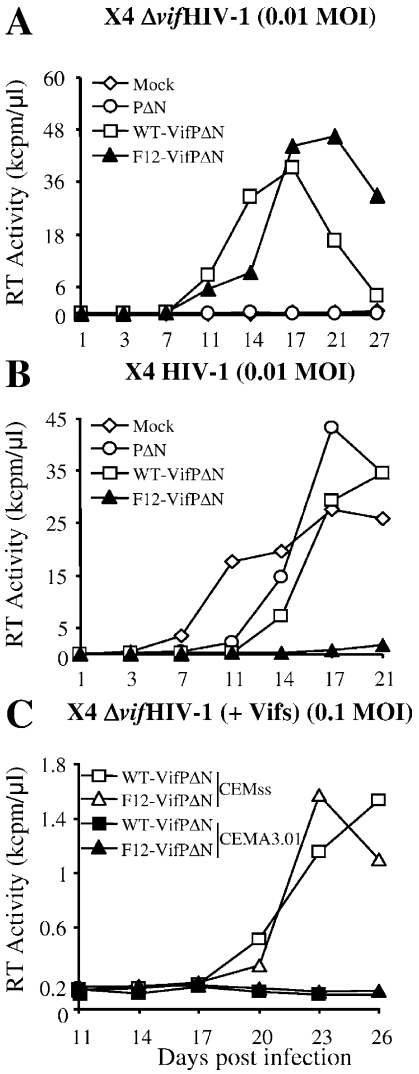


**FIG. 5.** F12-Vif and WT-Vif equally rescue viral replication of Δ*vif* HIV-1 in nonpermissive CEM A3.01 cells. (A and B) CEM A3.01 cells mock transduced or transduced with the different lentiviral vectors were infected with the X4 molecular clones (A) Δ*vif*-HIV and (B) HIV-$1_{NL4-3}$ at the m.o.i. of 0.01. Supernatants were collected every 4 days and tested for RT activity. (C) Day 17 supernatants of the WT-Vif- and F12-Vif-expressing cells from the experiment of (A) were used to infect permissive CEMss and nonpermissive CEM A3.01 cells at the m.o.i. of 0.1.

which corresponds to a single-cycle infection period. We adsorbed $HIV_{NL4-3}$ to CEM A3.01 mock transduced and transduced with the lentiviral vectors at the m.o.i. of 1 for 5 to 16 h and then removed it by trypsin treatment. We estimated the fold of increment of viral production by measuring the p24Ag levels after removal of the excess input virus (time 0) and 24 h afterward. As shown in Fig. 6, the F12-Vif-expressing cells release 60% less HIV-1, whereas the control cells PΔN and WT-VifPΔN show equal or more viral production compared to the mock-transduced cells, respectively. These results demonstrate that F12-Vif considerably diminishes the production of viral particles from HIV-1-infected cells.

### HIV-1 Emerging From F12-Vif-Expressing Cells is Noninfectious

Next, we questioned the infectivity of the small, but detectable, amount of virus produced from F12-*vif*-carrying cells. Since cocultivation of virus-producing and target cells, even in the absence of cell-to-cell contact, increases viral infectivity, we performed HIV-1 infection in transwell plates (Fig. 7). We infected mock-transduced and vector-transduced T lymphocytes with $HIV_{NL4-3}$ and seeded them in the upper chamber of a transwell plate that contained activated untransduced T lymphocytes in the lower chamber. After 16–24 h—the time sufficient to produce enough virus to infect the cells underneath—we removed the upper chamber. We collected supernatants from the lower chamber immediately after removal of the upper chamber (day 1) and every 3 days and tested them for p24Ag production by ELISA. Fig. 7 shows that, although the absolute levels of p24Ag were comparable at day 1 among the transduced cells (day 1: PΔN 21 pg/ml, WT-VifPΔN 28 pg/ml, and F12-VifPΔN 19 pg/ml), the virus produced from F12-Vif-expressing cells did not

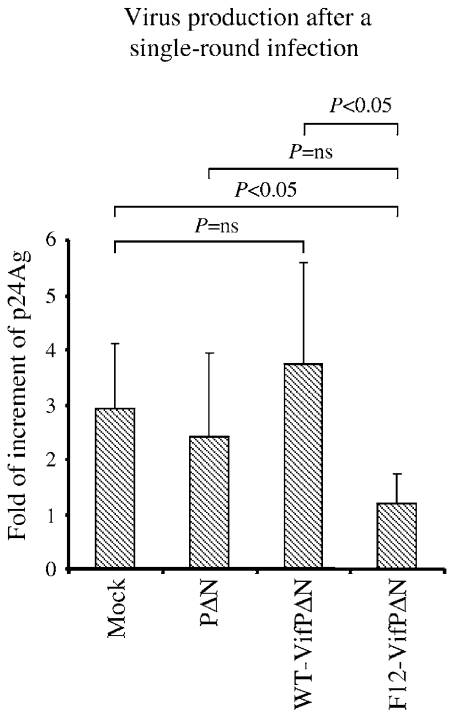


**FIG. 6.** HIV-1 virus production after a single-round infection is strongly decreased in F12-Vif-expressing cells. CEM A3.01 cells mock transduced or transduced with the lentiviral vectors were infected with the X4 HIV-$1_{NL4-3}$ for a single cycle. The fold of increase of viral production was calculated by measuring the level of p24Ag of the virus released after 24 h and that of the input virus. Values represent the means of four different experiments.

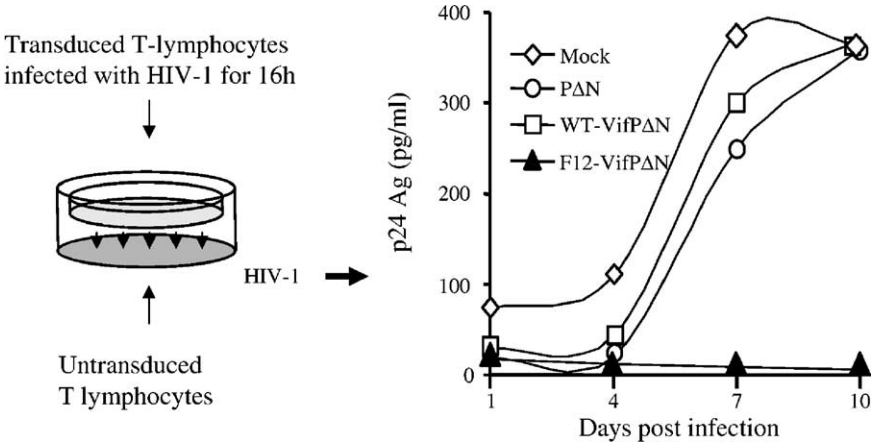


**FIG. 7.** HIV-1 emerging from F12-Vif-expressing cells is noninfectious. T lymphocytes mock transduced or transduced with the lentiviral vectors were infected with X4 HIV-$1_{NL4-3}$ at the m.o.i. of 1 for 5–16 h. Infected cells were seeded in the upper chamber of a transwell plate, whereas untransduced lymphocytes were plated in the lower chamber (left). After 16 h, the upper chamber was removed, and supernatants from the lower chamber were collected every 3 days and tested for p24Ag production by ELISA.

trigger a productive infection. Overall, these findings indicate that the reduction of viral production and infectivity combined is responsible for the inhibitory activity of F12-Vif.

## Discussion

Many years of preclinical investigation have shown that the HIV-1 life cycle can be interfered with at many levels in human T cells and proved the concept of anti-HIV gene therapy [1,2]. However, early clinical trials with T lymphocytes transduced with retroviral vectors expressing transdominant mutants of viral proteins or anti-HIV-1 ribozymes were disappointing [21,22], due mainly to low gene transfer efficiency, insufficient engraftment, and short *in vivo* persistence of the genetically modified T cells. Transplantation of HSCs expressing appropriately regulated gene products in their T cell and macrophage progeny should theoretically overcome these problems and allow the establishment and long-term persistence of an HIV-resistant immune repertoire in infected patients. For patients failing conventional treatment, autologous transplantation of genetically modified HSCs could represent a reasonable therapeutic alternative.

In this paper, we describe the development of an HIV-1-based lentiviral vector (F12-VifPΔN) for the conditional expression of F12-Vif, a natural mutant of Vif originally discovered in the F12 nonproducer variant of HIV-1 [14,15,23]. We show that expression of F12-Vif is highly effective in inhibiting HIV-1 replication in primary T cell cultures and T cell lines infected *in vitro* with either X4 or R5 HIV-1 strains. F12-Vif inhibits the production and reduces the infectivity of HIV-1 by transdominant inhibition of the WT-Vif function, without restoring the activity of AP3G.

An important feature of the F12-VifPΔN lentiviral vector is that the F12-*vif* transgene is under the transcriptional control of a Tat-dependent, wild-type HIV-1 LTR and the posttranscriptional control of the Rev/RRE system. Synthesis of F12-Vif is therefore activated only in HIV-1-infected cells. Avoiding unnecessary expression of a foreign antigen in transduced HSCs and their progeny is a crucial factor in a gene therapy approach to AIDS. Immunogenicity of constitutively expressed transgene products was reported to cause T-cell-mediated rejection of genetically modified cells and is among the causes of the limited persistence of transduced T cells observed in previous clinical trials [24,25]. By itself, Vif is one of the less immunogenic among the HIV-1 proteins [26]. F12-Vif is not expected to differ significantly from WT-Vif under this respect, at least on the basis of both BIMAS (http://bimas.cit.nih.gov/) and RANKPEP (http://www.mifoundation.org/Tools/rankpep.html) algorithms for predicting HLA-I-binding peptides (data not shown). Low immunogenicity and Tat-dependent expression of F12-Vif should avoid, or at least minimize, immune response against uninfected F12-Vif-expressing cells *in vivo*. On the other hand, the wild-type HIV-1 LTR guarantees very high expression levels of the transgene in HIV-1-infected cells, a crucial requirement for the activity of a transdominant mutant protein.

Production of Vif occurs late in the normal HIV-1 life cycle, while F12-Vif is already abundantly accumulated in transduced T cells by the time WT-Vif is synthesized by the incoming HIV-1. Early expression of Vif has no antiviral activity per se in permissive cells since these cells transduced by a lentiviral vector expressing WT-Vif do not become resistant to HIV-1. The only effect observed is a delay in the kinetics of infection. This is manifest also in cells transduced by the empty PΔN vector and is an already known effect of lentiviral vectors, which slightly reduce infectivity and spreading of HIV-1 by competing for encapsidation [27] or for limiting substrates required for reverse transcription and packaging [28]. Early expression of Vif could instead explain the HIV-1 inhibition observed in permissive cells (CEMss) transduced with a vector expressing either

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F12-Vif or WT-Vif. As reported by Akari et al. [29] high levels of Vif interfere with viral infectivity.

Analysis of the mechanism of action of F12-Vif shows that the reduction of viral infectivity in nonpermissive cells does not depend on the restoration of the AP3G function, but on a different, transdominant mechanism that requires the presence of WT-Vif. Interestingly, the activity of F12-Vif is indistinguishable from that of WT-Vif in inducing the intracellular degradation of AP3G and reducing its incorporation into the virions. In the absence of WT-Vif, F12-Vif rescues the replication of a Δ*vif* HIV-1 mutant in nonpermissive cells, indicating its functional equivalence with its WT counterpart. Furthermore, WT-Vif-expressing cells, which normally support HIV-1 replication, are devoid of AP3G, similar to F12-Vif-expressing cells. Therefore, the antiviral activity of F12-Vif is independent of AP3G function. These results are not entirely surprising, since none of the 14 unique mutations of F12-Vif (Fig. 1B and [14,17]) affects the SLQ(Y/F)LAΦΦΦΦ (where Φ is any hydrophobic amino acid) sequence that is required, together with an N-terminus domain, for AP3G degradation [30,31].

F12-Vif is likely to affect assembly and/or budding of new virions because they are released by transduced cells in reduced quantity and appear to be noninfectious when tested on normal, untransduced T lymphocytes. In this regard, Vif is known to have a role in viral budding, through its interaction with the HP-68-Gag complex in the late phases of capsid assembly [32]. Finally, we show that F12-Vif is incorporated with higher efficiency than WT-Vif into HIV-1 virions.

Multimerization through the proline-rich region spanning amino acids 151–164 was previously shown to be necessary for Vif function [33]. F12-Vif carries one unique amino acid substitution within this region ($V^{142} \rightarrow I$) [14,17]. We speculate that formation of F12-Vif/WT-Vif heterodimers/multimers may somehow impair, and ultimately affect, viral assembly, budding, and/or infectivity in nonpermissive cells. Interestingly, homodimers/multimers of F12-Vif are apparently compatible with a normal viral cycle, and it is only the combination of F12-Vif and WT-Vif that reduces viral infectivity. We are currently investigating whether the increased incorporation of F12-Vif into virions and heterodimer formation may play a role in reducing viral infectivity.

It is interesting to note that a statistically significant correlation between one of the unique F12-Vif mutations, $R^{132} \rightarrow S$, and low viral load has been observed within a cohort of long-term nonprogressor HIV-1-infected individuals [34]. This suggests that $S^{132}$ may be at least one of the mutations responsible for the low infectivity of HIV-1 virions produced in the presence of F12-Vif.

In conclusion, this study shows that T cells transduced with F12-VifPΔN interfere with HIV-1 infection by a novel mechanism, that is, by producing noninfectious viral particles. Most of the genes used so far as potential anti-HIV-1 therapeutics were designed with the goal of interfering with the HIV-1 life cycle at the level of transcription, RNA processing, or packaging and, in only one case, with viral entry [35]. None of the genes tested so far has shown 100% efficiency in reducing viral replication and spreading. Although F12-Vif expression per se would not be sufficient to eradicate HIV-1 infection, it would be an ideal function to combine with genes preventing HIV-1 entry in a single vector that would at the same time reduce viral infection and disable any escaping virion. The only drawback of using F12-Vif is its potential ability to rescue the replication of a *vif*-deficient HIV-1 quasispecies silently harbored in the patient's T cells. However, this would be a risk only in the case of a T-cell-based gene therapy, since the possibility of transducing a HSC harboring a *vif*-deficient provirus is extremely low.

## MATERIALS AND METHODS

***Cells.*** CEM A3.01 [36], CEMss, and SupT1 T lymphoblastoid cells were grown in RPMI 1640 supplemented with 10% FCS (EuroClone Ltd., UK) and a combination of penicillin–streptomycin and glutamine (PSG). 293T cells were propagated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and PSG. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy human donors by centrifugation on a Ficoll–Hypaque (Lymphoprep, Nycomed Pharma AS, Norway) gradient following standardized procedures. Fresh and thawed cryopreserved cells were stimulated for 3 days with soluble anti-human CD3 (30 ng/ml) (Orthoclone OKT3, Janssen-Cilag, UK) and recombinant human IL-2 (rhIL-2, 50 U/ml) (Chiron, Emeryville, CA, USA), washed twice with PBS, and maintained in IMDM supplemented with 10% FCS, PGS, and rhIL-2.

***Plasmids.*** The lentiviral vector PΔN was generated by cloning the PGK-ΔLNGFR cassette [20], encoding a truncated form of the low-affinity nerve growth factor receptor (ΔLNGFR) under the control of the PGK promoter, into the *Cla*I/*Sac*II sites of the lentiviral vector pHR2 [19]. The F12-VifPΔN and WT-VifPΔN vectors were obtained by inserting a PCR-amplified, 611-bp F12-*vif* or wild-type *vif* sequence, respectively, into the *Cla*I site of the PΔN vector. The wild-type HIV-$1_{NL4-3}$ (Accession No. M19921) and the F12 HIV-1 (Accession No. Z11530) DNAs were used as *vif* templates for PCR amplification using the following primers: for, 5′ -GCAAAGA**ATCGAT**-GGGATTATGGAAAACAG-3′ ; rev, 5′ -CTCCTCTA**ATCGAT**GCTAGTGTC-CATTCATTG-3′ (*Cla*I sequence in bold). The pCEM15:HA [37] expression vector was a kind gift from M. Malim (King's College, London, UK).

***Production of pseudotyped lentiviral vectors and viruses.*** Pseudotyped lentiviral vector stocks were produced by transient cotransfection of 293T cells with the transfer vector, a second-generation minimal packaging construct pCMVΔR8.74, and the pMD.G plasmid encoding the VSV-G [38] at the ratio of 1.5:1:0.5 by Fugene6 (Roche Diagnostics Corp., Indianapolis, IN, USA). VSV-G-pseudotyped HIV-1 was obtained by cotransfection of 293T cells with R9ΔEnv HIV-1 (a kind gift from D. Trono, University of Geneva, Switzerland) and pMD.G plasmids at 1:1 ratio. Supernatants were harvested 48 h after transfection, cleared by low-speed centrifugation (10 min at 480*g*), and filtered through a 0.45-μm pore-size filter.

***Transduction and ΔLNGFR immune selection.*** Cells were transduced with the described lentiviral vectors by spinoculation. Briefly, supernatants were incubated with target cells at the m.o.i. of 5 and centrifuged

at 1240*g* for 1 h in the presence of Polybrene (8 μg/ml). Transduction efficiency was monitored at least 1 week after spinoculation by flow cytometry analysis of ΔLNGFR expression (FACScan, Becton–Dickinson, Mountain View, CA, USA) using the anti-human p75-LNGFR monoclonal antibody 20.4 (ATCC, Rockville, MD, USA). Immune selection of ΔLNGFR$^+$ cells was obtained by magnetic cell sorting using MiniMACS microbeads (Miltenyi Biotec, Inc., Sunnyvale, CA, USA) according to the manufacturer's instructions. Preactivated T lymphocytes were transduced by a slightly modified protocol (Polybrene concentration 0.8 μg/ml and two cycles of spinoculation separated by an overnight resting phase in complete medium). Primary T cells were reactivated by anti-CD3 stimulation in the presence of feeder cells (6000-rad-irradiated heterologous PBMC) at 1:1 ratio, before infection with HIV-1.

***Northern blot analysis.*** Total RNA was extracted using the Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions, run on 0.8% agarose–formaldehyde gels, transferred onto nylon membrane (Hybond–N; Amersham, Little Chalfont, UK) by capillary transfer, probed with $10^6$ dpm/ml of a $^{32}$P-labeled 1-kb ΔLNGFR fragment in PerfectHyb PLUS hybridization buffer (Sigma Chemical Corp., St. Louis, MO, USA), and exposed to X-ray films at −70°C.

***Western blot analysis.*** WCEs were prepared as previously described [39], and viral proteins derived from isolated cell-free virions were obtained by centrifugation of the supernatants at 15,700*g* for 90 min at 4°C in a microcentrifuge. Pellets were resuspended in PBS containing 0.5% NP-40 and immediately boiled in loading buffer. Proteins were size-fractionated by SDS–PAGE and electroblotted to Hybond ECL nitrocellulose membranes (Amersham). Membranes were blocked in 5% low-fat dry milk and then incubated with the appropriate primary Ab. HIV-$1_{HXB2}$ Vif rabbit antiserum [40] was obtained from Dana Gabuzda through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD, USA) and used at 1:1000 dilution. The rabbit antiserum against human actin and the mouse monoclonal anti-HA (clone HA-7) were purchased from Sigma and used at 1:250 and 1:10,000 dilution, respectively. The rabbit anti-serum against human AP3G was a generous gift from W. C. Greene (Gladstone Institute of Virology and Immunology, San Francisco, CA, USA) [41] and used at 1:4000 dilution. A serum obtained from an AIDS patient was used at 1:2000 dilution. Ab binding was visualized using the enhanced chemiluminescence system ECL (Amersham).

***HIV-1 infection.*** Cells were acutely infected with the following HIV-1 strains: the laboratory adapted X4 HIV-$1_{IIIB/LAI}$ (Advanced Biotechnologies, Columbia, MD, USA), the molecular clones X4 HIV-$1_{NL4-3}$ and its *vif*-deficient (Δ*vif*) derivative [42], and the molecular clone R5 HIV-$1_{AD8}$ (Advanced Biotechnologies). Viruses (m.o.i. ranging from 0.01 to 1) were adsorbed to the cells for 2–5 h at 37°C and then washed out twice with PBS. Cells were eventually resuspended in complete medium and seeded at 0.5–1 × $10^6$/ml in triplicate in 96-well plates. Culture supernatants were harvested every 4 days and stored at −80°C until tested for $Mg^{2+}$-dependent RT activity or by p24 ELISA (Coulter Corp., Westbrook, ME, USA) following standard procedures. The experiment described for Fig. 7 was performed as follows: HIV-$1_{NL4-3}$ (m.o.i. of 0.5) was adsorbed for 5 h at 37°C on mock-transduced and transduced cells. The excess virus was then removed by gentle trypsin treatment (PBS containing 0.25% trypsin for 5 min at 37°C). After extensive washes, infected cells (5 × $10^5$) were seeded in the upper chamber of a 24-transwell plate (Corning Costar, Rochester, NY, USA), whereas in the lower chamber 5 × $10^5$ untransduced cells were plated. After 16 h, the upper chamber was removed and supernatants were collected every 3 days for 10 days and tested for p24Ag production by ELISA.

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