4-day intervals and viral production was monitored by HIV of p24 Gag antigen ELISA kit (RETRO TEC; ZeptoMetrix, Buffalo, NY).

Real-time RT-PCR (qRT-PCR) analysis of HIV-1 infection

HIV-1–infected cells were collected and total DNA was prepared 3, 8 and 12 hours after infection. For the detection and quantification of individual forms of HIV-1 DNA, oligonucleotide primer and probe sequences were designed specifically for the *TaqMan* assay as described elsewhere.[19] All probes (Biosearch Technologies, Novato, CA.) were 5′-labeled with the fluorophore FAM as the reporter dye, and 3′-labeled with Black Hole Quencher-1 (BHQ-1) as the quencher dye. The qRT-PCR analysis was performed on an Mx3000P (Stratagene) and the amount of HIV-1–specific DNA per cell was normalized to β-globin gene.

Kinetics of virus production in MDMs and reporter analysis

MDMs (2×10^5/well) were cultured in 48-well tissue-culture plates and infected either with wild-type HIV-1_{NF462} or HIV-$1_{NF462\Delta Nef}$. MDMs were infected with lentivirus at a multiplicity of infection (MOI) of 2 or 10 and washed extensively. The next day, cells were exposed to HIV-1 (5 ng/well) for 2 hours. Cell supernatants were harvested at 3- or 4-day intervals, and viral production was monitored by p24 antigen ELISA.

The cell-culture supernatants at 10 days after HIV infection were examined for infectivity, and 10 days after HIV infection, cell supernatants were collected (termed HIV-1/Lenti cont and HIV-1/Lenti shNef366). CEMx174 CCR5/LTR-EGFP cells were infected with HIV-1/Lenti cont or HIV-1/Lenti shNef366, and the number of HIV-1–infected EGFP$^+$ T cells was determined by fluorescence-activated cell sorter (FACS).

Detection of chemokines

For the detection of chemokine production in MDMs, the cytometric bead array (CBA) kit (BD Bioscience, San Jose, CA) was used, which measured 4 chemokines (IL-8, MIP-1α, MIP-1β, MCP-1) simultaneously.

Restimulation assay of lentivirus-transduced U1 cells

Latent HIV-1–infected U1 cells were transduced with Lenti cont or Lenti shNef366 at an MOI of 1. Two weeks later, EGFP$^+$ cells were sorted and stimulated with 1 ng/mL recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). Culture supernatants were collected at days 2 and 5, and the level of p24 antigen was measured by ELISA.

Results

siRNA suppresses *nef* mRNA and Nef protein expression

In the HIV-1 genome, *nef* is located at the 3′ end of the viral genome, partially overlapping the 3′ LTR (Figure 1A). Jacque and colleagues demonstrated previously that siRNA targeting of the 5′ region of *nef* (nucleotides 164-185) suppressed HIV replication.[20] Therefore, we selected 3 distinct regions of the HIV-1_{NL432} *nef* sequence using a Web-based program for designing DNA-directed RNAi systems, focusing on the Nef coding region overlapping the 3′ LTR. These were designated as Nef338, 366, and 479 based on the position of the first nucleotide of the siRNA. From initial screening experiments, we found that Nef366 was the most effective target site (data not shown).

The type 1 interferon response is an innate defense mechanism in eukaryote cells against viral infection. It has been shown that some types of siRNA induce type 1 interferon, which in turn mediates the gene-specific effect of RNAi.[21-23] The stealth siRNA system was developed to avoid the interferon response to siRNA in cells (Invitrogen manual). We prepared synthetic stealth siRNAs, designated Nef355st and Nef366st, and a control siRNA designated EGFPst, to determine the effect of RNAi using sequences based on Nef366 (the U3-overlapping region of the Nef-coding region). Neff355st was synthesized based on a Web-based computer program for generating stealth siRNA (Invitrogen), whereas Nef366st represents a slightly modified version of the stealth target site (6-nucleotide difference), so that it conformed to the target sequence as described. These stealth Nef siRNA sequences differed by only 5 nucleotides (Figure 1A).

We established a stable Nef-expressing HeLa-CD4 clonal cell line, designated as HeLa-CD4-Nef. HeLa-CD4-Nef cells were transfected either with 2.5 μM EGFPst or *nef* stealth siRNAs (Nef355st or Nef366st), and harvested 48 hours after transfection. Total RNA was extracted and the level of *nef* mRNA was measured by qRT-PCR. We observed that transfection with Nef366st reduced *nef* mRNA expression more than 90% (Figure 1B), whereas Nef355st suppressed the level of *nef* mRNA approximately 80%, compared with EGFPst controls. When cell lysates of the transfected cells were analyzed by Western blot, we found that both Nef366st and Nef355st suppressed Nef protein levels to below the detection limit of the assay (Figure 1C). Taken together, these results clearly showed that Nef366 is an efficient target sequence for the inhibition of *nef* gene expression by siRNA.

shRNA suppresses *nef* mRNA and Nef protein expression

To assess the effect of endogenous expression of Nef366 siRNA, we constructed expression vectors that encoded shRNAs corresponding to Nef366, or *lacZ* as a control, driven by the human U6 polymerase III promoter, designated as pENTR/shNef366 and pENTR/shLacZ, respectively (Figure 2A). HeLa-CD4-Nef cells were transfected with either pENTR/shNef366 or pENTR/shLacZ by FuGene6 reagent (Roche) and cells were harvested 72 hours after transfection. Total RNA was extracted and analyzed by qRT-PCR. We observed that the level of *nef* mRNA was suppressed by approximately 80% in cells transfected with pENTR/shNef366 (Figure 2B). Western blot analysis confirmed that pENTR/shNef366 strongly suppressed Nef protein levels as well (data not shown). These results indicated that promoter-driven endogenous

Figure 2. RNAi by transfection with shRNA expression vectors. (A) Schematic of the expression vectors (pENTR/shRNA) encoding shRNAs of Nef366 or *lacZ*, designated as pENTR/shNef366 and pENTR/shLacZ, respectively, in which expression is driven by the human U6 polymerase III promoter. (B) Nef-expressing HeLa-CD4 cells were transfected either with 1.0 μg pENTR/shNef366 or pENTR/shLacZ and cells were harvested 72 hours after transfection. Total RNA was extracted and analyzed by qRT-PCR. The data represent the average ± SD of 3 independent experiments.

expression of shNef366 was able to mediate RNAi of *nef* in HeLa-CD4-Nef cells.

Inhibition of HIV-1 replication in U937 cells by lentivirus-based shRNA expression

The transfection efficiency of the entry vectors used in suspension cells was quite low, and the objective here is to introduce siRNAs into primary macrophages. Therefore we constructed HIV-1–based lentivirus vectors expressing Nef366 shRNA or shRNA targeting lacZ as a control (Lenti shNef366 and Lenti control) using Gateway technology. The structure of the lentivirus vector used in the following studies is illustrated in Figure 3A.

To test whether Nef366 shRNA was able to efficiently block HIV-1 replication, we infected U937 cells with Lenti shNef366 or Lenti control, both of which encoded GFP driven by the EF-1α promoter (EGFP), at an MOI of 1. Two weeks after infection, nearly 30% of the cells stably expressed EGFP (Figure 3B upper panel). We sorted the EGFP$^+$ cells by fluorescence-activated cell sorter (FACSaria; BD Biosciences), after which the purity of the Lenti control– and Lenti shNef366–transfected, EGFP$^+$ cells was 97.2% and 99.7%, respectively (Figure 3B lower panel; U937/Lenti cont and U937/Lenti shNef366). The purified cell populations were then infected with 2 inoculation doses of HIV-1 (Figure 3C upper and lower panels; p24: 20 ng and 100 ng, respectively). The culture supernatants were collected at 3- or 4-day intervals, and the level of p24 antigen was measured by ELISA. We observed that at both inoculation doses HIV-1 replication in U937 cells was inhibited by Lenti shNef366, especially at the peak of HIV-1 production. The reverse transcriptase activity was also measured in parallel, and the result was consistent with that of p24 ELISA (data not shown). The inhibition of HIV-1 replication was sustained at least for 1 week, following which HIV-1 production gradually decreased in all cell populations, presumably because of the cytopathic effect of HIV-1 infection.

To further evaluate the effect of RNAi on the early steps of HIV-1 infection, we prepared cell lysates at different time points after inoculation (3, 8, and 12 hours after infection) and analyzed the level of reverse transcription activity by measuring the amount of different forms of proviral DNA (HIV-1 *2LTR* and *U5-Gag*) by the qRT-PCR. The copy number of these proviral DNA forms decreased in U937/Lenti shNef366 cells, relative to that seen in U937/Lenti control cells at all time points. The amount of these DNA forms normalized to β-globin gene at 12 hours after HIV-1 infection is depicted in Figure 3D. The copy number of *2LTR* and *U5-Gag* was 16.9% and 13.4% of control, respectively. These results suggested that the inhibition of HIV-1 replication occurred early after virus entry, presumably during uncoating or reverse transcription, not integration.

A type 1 interferon response has been shown to be induced by synthetic siRNAs via protein kinase R- (PKR) or toll-like receptor 7 (TLR 7)–mediated signaling pathways.[21-23] To eliminate the possibility that we were generating an interferon response following shRNA expression in our system, we analyzed the level of 2′ 5′-oligoadenylate synthetase mRNA expression in Lenti shNef366–infected U937 cells by qRT-PCR. We detected no such message (data not shown), indicating that the interferon response plays a

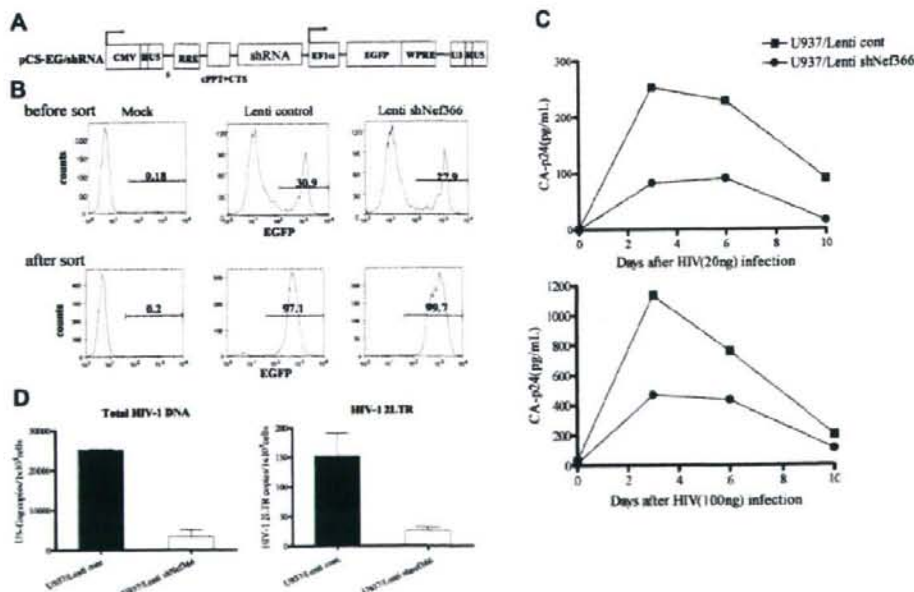

Figure 3. Inhibition of HIV-1 replication in U937 cells by lentivirus-mediated shRNA. (A) The structure of the shRNA lentiviral expression vector. The HIV-1–based lentivirus vector for expressing shRNA was constructed using Gateway technology. pCS-EG/shRNA consisted of U6-shRNA upstream of an EF1α promoter–driven EGFP expression cassette, which allowed simultaneous expression of shRNA and EGFP. (B) U937 cells were infected with lentivirus expressing either shNef366 (Lenti shNef366) or shLacZ (Lenti control) at an MOI of 1. After 2 hours of infection, cells were washed and maintained in culture. Cells expressing EGFP were analyzed by FACS, and EGFP$^+$ cells were collected. EGFP$^+$ cells were analyzed by FACSaria 1 week later (designated as U937/Lenti control and U937/Lenti shNef366). (C) U937/Lenti cont or U937/Lenti shNef366 cells (1×10^5/well) were infected with HIV-1_{NL432}, and the culture supernatants of these cells were collected at 3- or 4-day intervals after infection. The level of p24 antigen in the culture supernatants was measured by ELISA. (D) HIV-1–infected cells were collected and total DNA was prepared 12 hours after infection. Total HIV-1 and 2LTR DNA was analyzed by qRT-PCR. The amount of HIV-1–specific DNA per cell was normalized to β-globin gene expression. The data represent the average ± SD of 3 independent experiments.

minimal role, if any, in the observed inhibitory effect on HIV replication by Lenti shNef366.

Lentivirus-based nef shRNA inhibits HIV-1 replication and affects chemokine production in MDMs

Swingler and coworkers reported that HIV-1 Nef expression in macrophages mediated lymphocyte chemotaxis and activation through the induction of MIP-1α and MIP-1β expresion.[8] To determine the effect of Nef expression during HIV-1 infection in MDMs, we infected MDMs with wild-type HIV-1_{NF462} or the corresponding *nef* gene-deletion mutant, HIV-1_{NF462}dNef, and assessed the kinetics of virus replication by p24-specific ELISA. Representative results from 2 donors are shown in Figure 4A. We consistently observed that the level of HIV-1_{NF462} replication was 2- to 6-fold higher than that of HIV-1_{NF462}dNef in MDMs. These results were consistent with those reported by Swingler et al.[9] Although no apparent T-cell damage was observed during cultivation for 3 weeks following HIV-1 infection, the amount of virus production gradually decreased. We analyzed chemokine production in MDMs infected with HIV-1 wild-type and *nef*-deleted HIV-1 at days 10, 14, and 17 after infection. The level of chemokine production in uninfected MDMs varied depending on the donor, but both donors produced a high level of IL-8 and monocyte chemotactic protein-1 (MCP-1), and a low level of MIP-1α and MIP-1β (data not shown). HIV infection per se, independent of the presence or absence of Nef, did not affect this trend, in that the levels of these chemokines, with the exception of MIP-1β, were only slightly affected by HIV infection. Notably, virus replication resulted in an increased production of MIP-1β, which peaked at 14 days after infection, in parallel with the peak of viral replication. Figure 4B shows the results of the analysis of the levels of MIP-1β and MIP-1α in the 2 donors. HIV-1 infection induced a 2-fold increase in the level of MIP-1β compared with mock-infected MDMs. In contrast, infection with Nef-deleted HIV-1 caused a reduction in the level of MIP-1β in the MDMs from both donors, indicating that Nef is responsible for the up-regulation of MIP-1β, but does not affect MIP-1α, MCP-1, or IL-8 production.

To examine whether shRNAs against the U3-overlapping region of *nef* were able to block HIV-1 replication in MDMs, we infected MDMs with Lenti control or Lenti shNef366, at an MOI of 10 or 2 (Figure 5A left and right panels, respectively). After 2 hours of incubation, cells were extensively washed and cultivated overnight, and the following day, they were infected with HIV-1_{NF462}. Culture supernatants were collected every 3 or 4 days and the level of p24 antigen was measured by ELISA. Of note, despite the extensive washing after lentivirus infection, the level of p24 was quite high up to 7 days after HIV-1 infection. We detected a second peak of virus production, which we interpreted as true HIV-1 replication in MDMs transduced with lentiviral vectors expressing shRNAs. In addition, presumably because of the toxic effect of infection by lentivirus pseudotyped with VSV, the level of p24 antigen was lower than that in MDMs infected with HIV-1 virus. Nevertheless, we observed a similar level of inhibition of HIV-1 replication in MDMs by Lenti shNef366 at 2 different doses of infection (Figure 5A), and the inhibition was maintained for at least 3 weeks after HIV-1 infection.

Macrophages can mediate efficient infection of lymphocytes *in trans*,[9,24] suggesting that macrophages serve as a major reservoir and vehicle for HIV-1 dissemination. We were interested in whether the progeny virus produced from MDMs harboring Nef366 shRNA maintained their ability to infect T cells. Supernatants from MDM cells transduced with Lenti control or Lenti shNef366 were collected 10 days after HIV infection, and the level of p24 antigen was measured and used to quantitate the amount of HIV present. These sources of HIV were designated as HIV/Lenti cont or HIV/Lenti shNef366. Using CEMx174 CCR5/LTR-EGFP cells as indicator cells, we estimated the infectivity of HIV/Lenti cont or HIV/Lenti shNef366 by analyzing the number of $EGFP^+$ T cells following infection (Figure 5B). Compared with HIV-1/Lenti cont, HIV-1/Lenti shNef366 had a significant loss of infectivity in $CCR5^+$ T cells. Our results suggested that Lenti shNef366 has the potential to protect HIV-1 dissemination to T cells by HIV-1–infected MDMs

We also examined the level of chemokine production following HIV infection of MDMs transduced with shRNA lentivirus vectors. Although the basal level of MIP-1α and MIP-1β production was slightly increased following lentivirus infection, the level of MIP-1β decreased in Lenti shNef366 cells compared with Lenti control (Figure 5C). The levels of MCP-1 and IL-8 were either unaffected or somewhat restored by Lenti shNef366 (data not shown).

Lentivirus-based nef shRNA protects progression from latent HIV-1 infection to productive infection

Latent HIV-1 infection can be established following provirus integration into the host genome.[25-27] A small number of infected cells re-enter the resting stage, harboring an integrated copy of the HIV-1 genome. These latent HIV-infected cells represent a barrier to successful virus eradication because subsequent cytokine or

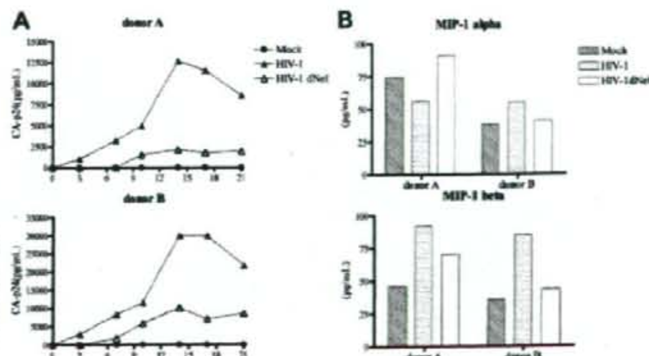

Figure 4. The effect of Nef expression during HIV-1 infection in MDMs. (A) MDMs (2×10^5/well) of 2 donors were infected either with wild-type HIV-1_{NF462} or HIV-$1_{NF462dNef}$. The supernatants of these wells were harvested at 3- or 4-day intervals after infection, and viral production was monitored by sequential quantitation of p24 by ELISA. (B) The CBA kit was used to measure the level of chemokines (MIP-1α and MIP-1β) in cell supernatants 14 days after HIV infection.

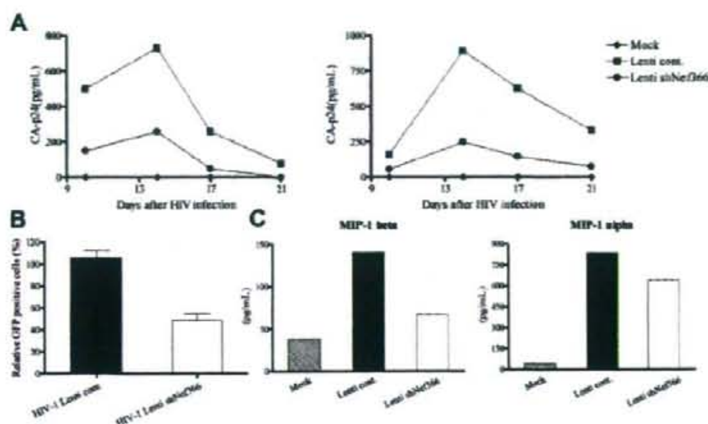

Figure 5. Lentivirus-expressed nef shRNA inhibits HIV-1 replication and affects chemokine production in MDMs. (A) MDMs were transduced with Lenti cont or Lenti shNef366 at an MOI of 2. At 2 hours after infection, cells were washed twice, then cultured for another 24 hours, at which point the cells were infected with HIV-1_{NF462}. The culture supernatants were collected at 3- or 4-day intervals after HIV infection, and the level of p24 antigen was measured by ELISA. (B) MDMs transduced either with Lenti control or Lenti shNef366 were infected with HIV-1 and supernatants were collected 10 days after infection and designated as HIV-1 Lenti cont and HIV-1 Lenti shNef366, respectively. CEMx174 CCR5/LTR-EGFP cells were infected either with HIV-1 Lenti cont or HIV-1 Lenti shNef366 and GFP^+, HIV-1–infected T cells were analyzed by FACS 48 hours later. The data represent the average ± SD of 3 independent experiments. (C) The culture supernatants of MDMs transduced with lentivirus vectors were collected 14 days after infection and the levels of the chemokines MIP-1α and MIP-1β were measured.

other stimuli can reactivate viral gene expression, and reinitiate HIV-1 replication.[28-31] We were interested in whether Lenti shNef366 was able to regulate the progression of latent HIV-1 infection to productive infection in U1 cells.[17] U1 cells are U937 cells in which a latent HIV-infection has been established, and HIV-1 replication can be induced in these cells on appropriate activation. We transduced U1 cells with Lenti control or Lenti shNef366 at an MOI of 1. After 2 hours of infection, cells were extensively washed and maintained in culture. Two weeks after transduction, the cells were sorted by FACSaria, and the $EGFP^+$ cell population was stimulated with 1 ng/mL recombinant GM-CSF. Culture supernatants were collected at different time points (days 2 and 5) and the level of p24 antigen was measured by ELISA. As shown in Figure 6, the levels of p24 antigen were dramatically decreased in U1 cells harboring Lenti shNef366 at all time points examined.

Discussion

In this study, we constructed an shRNA expression system that targeted HIV *nef* gene sequences that overlap the 3′ LTR U3 (Nef366) and showed that Nef366 shRNA had a strong inhibitory effect on *nef* gene expression in Nef-expressing HeLa-CD4 cells. Furthermore, expression of shNef366 in monocytic cell lines strongly inhibited the replication of HIV-1 at an early stage of HIV infection. The rational for using shNef366 to target HIV *nef* was several-fold. Because the U3 region is required during reverse transcription for first template transfer and integration of the viral genome into the host genome, siRNA targeting of the U3 region may induce not only specific degradation of *nef* mRNA, but also inhibit HIV-1 reverse transcription. Furthermore, although others have observed escape mutations in RNAi experiments targeting *nef* or *tat*,[32,33] the nef/U3 sequence we targeted is highly conserved as discussed in the paragraph after the next one. If a mutation were to occur in the U3 region, it would affect the overall transcription efficiency of HIV-1 after integration because the U3 region of the HIV-1 LTR contains the transcription initiation or promoter/enhancer sites that are essential for efficient HIV transcription. Of note, the strategy used Jacque et al[20] using siRNA targeting of the 5′ region of *nef* turned out to induce an escape mutant.[33] Although we did not extensively test for the emergence of escape mutants, targeting the 3′ LTR U3-overlapping region of *nef* (Nef366) represented a potentially potent strategy for controlling HIV-1 replication.

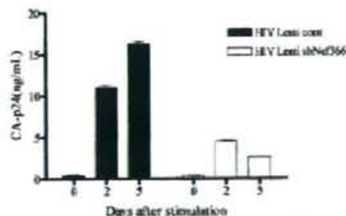

Figure 6. The effect of Lenti shNef366 on latent HIV-1 infection. Latent HIV-1–infected U1 cells were transduced with Lenti control or Lenti shNef366 at an MOI of 1. Two weeks after infection, $EGFP^+$ cells were sorted by FACSaria, and $EGFP^+$ cells were stimulated with 1 ng/mL recombinant GM-CSF. Cell-culture supernatants were collected 0, 2, and 5 days after stimulation, and the level of p24 antigen was measured by ELISA. The data represent the averages ± SDs of 3 independent experiments.

Macrophages are one of the major target cell populations in the early phase of HIV-1 infection, when R5 viruses predominate.[34] HIV-1 replication in macrophages is usually slow and less cytopathic compared with that in activated T cells, allowing the virus to survive long after infection. Thus, macrophages serve as one of the reservoirs for HIV in an infected individual.[35] Therefore, therapeutic strategies that target macrophages are promising approaches to the control of persistent HIV-1 infection in vivo. Taking advantage of the lentivirus expression system, which is an efficient way to introduce a desired gene into primary cells, we were able to show that expression of Nef366 shRNAs in primary MDMs inhibited HIV-1 replication in these cells.

In this context, several groups have demonstrated that RNAi, mediated by the introduction of HIV-specific siRNA duplexes, can inhibit viral replication in T cells, although the effect was transient.[20,36-38] Das et al were able to show a stable inhibitory effect on viral replication using a murine retrovirus vector expressing Nef-specific siRNAs in T-cell lines. However, the block in virus replication was not absolute and escape mutants emerged.[33] These previous results prompted us to develop a novel strategy of RNAi-mediated inhibition of HIV infection that did not induce a type 1 interferon and had a stable, long-term effect. We chose to

transduce Nef366 shRNA into low or nondividing primary macrophages, as opposed to actively proliferating T cells, using a lentivirus expression vector, and were able to demonstrate RNAi effect during macrophage cultivation for 3 weeks. Using an alignment of 200 HIV-1 sequences obtained by BLAST search analysis, only one base mismatch in the Nef366 region was detected in a subtype A virus (GenBank no. AB098332 and no. AB098333, HIV-1 UG029). Further study will be required to determine whether this subtype A virus is resistant to shRNA Nef366. Because Nef/LTR is in a completely conserved region, at least among subtype B viruses, this region might have quite an important function for HIV-1 replication. We speculate that if escape mutants were to emerge in the presence of lentiviral-shRNA Nef366, the compensatory mutation would occur outside of this region.

Importantly, using this system, we were also able to demonstrate a decrease in the infectivity of HIV-1 produced from infected MDMs. This attenuation effect is potentially significant because it implies that lentivirus-mediated RNAi may also reduce transmissibility of HIV-1 overall. However, in light of the significant problem of viral escape during chronic HIV infection, it may become necessary to combine multiple sites of siRNAs targeting the *nef-U3* region in the future.

Control of the latent phase of HIV infection is a key issue for effective therapeutic intervention. We demonstrated here that Lenti shNef366 was able to suppress the reactivation of HIV from latently infected cells. The expression of integrated HIV-1 in latently infected cells is controlled at the level of transcription by cellular factors and the viral transactivator Tat, both of which act through the HIV-1 LTR.[39] Transcription of integrated viral RNA is initiated at the R region of the 5′ LTR. The fact that shNef366, which targeted the U3-overlapping region of Nef, was effective in latently infected cells, suggests that shNef366 can directly target cleavage of *nef* mRNAs or total viral RNAs at the 3′ end. Therefore, our lentivirus-based shRNA expression system appears to be able to control both early and latent HIV-1 infection.

MIP-1α and MIP-1β are ligands of the HIV-1 coreceptor, CCR5. Through interaction with the CCR5 receptor, they promote the maturation of Th1 cells.[40,41] Swingler et al reported that MIP-1α and MIP-1β were induced by Nef in macrophages during HIV infection and that culture supernatants derived from Nef-expressing macrophages induced both chemotaxis and activation of resting T lymphocytes, enabling productive HIV-1 infection of those T cells.[8] These and other results have led to a model of HIV infection in which expression of Nef in HIV-infected MDMs enhances the secretion of MIP-1β, which recruits mainly $CCR5^+$ Th1 cells, resulting in the expansion of R5 tropic HIV-1 during macrophage–T-cell interactions. Our results were partially consistent with this model because the degradation of *nef* mRNA expression resulted in the decreased MIP-1β production. Of note, the production of MIP-1α in our system appeared to be unaffected by Nef expression but was induced by lentivirus infection. Because the production of MIP-1α in HIV-infected MDMs was similar to that in uninfected MDMs, it seems likely that MIP-1α production was enhanced by a non–HIV-specific component of the lentivirus expression system, perhaps VSV-G protein. Although the levels of MCP-1 and IL-8 varied depending on the donor and were independent of Nef expression, we cannot rule out the possibility that other unknown chemokines are induced by Nef. Any such dysregulated chemokine production by Nef expression in macrophages might provide an appropriate environment for HIV to establish an efficient infection and dissemination.

In summary, we demonstrated the feasibility of using lentiviral expression vectors to express shRNAs against the U3-overlapping region of *nef* in primary MDMs, as a type of intracellular immunization and potential gene therapy approach against HIV-1. Future development of an AIDS vaccine based on the specific inhibition of viral gene expression combined with existing therapeutic strategies may provide keys to help eradicate HIV.

Acknowledgments

We thank Masayuki Ishige and Rieko Iwaki for their excellent technical assistance.

References

1. Kestler HW 3rd, Ringler DJ, Mori K, et al. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell. 1991;65:651-662.
2. Agopian K, Wei BL, Garcia JV, Gabuzda D. A hydrophobic binding surface on the human immunodeficiency virus type 1 Nef core is critical for association with p21-activated kinase 2. J Virol. 2006;80:3050-3061.
3. Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. Immunity. 2002;16: 493-497.
4. Geyer M, Fackler OT, Peterlin BM. Structure–function relationships in HIV-1 Nef. EMBO Rep. 2001;2:580-585.
5. Steffens CM, Hope TJ. Recent advances in the understanding of HIV accessory protein function. AIDS. 2001;15(suppl 5):S21-S26.
6. Na YS, Yoon K, Nam JG, et al. Nef from a primary isolate of human immunodeficiency virus type 1 lacking the EE(155) region shows decreased ability to down-regulate CD4. J Gen Virol. 2004;85: 1451-1461.
7. Deacon NJ, Tsykin A, Solomon A, et al. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science. 1995;270:988-991.
8. Swingler S, Mann A, Jacque J, et al. HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nat Med. 1999;5:997-103.
9. Swingler S, Brichacek B, Jacque JM, Ulich C, Zhou J, Stevenson M. HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection. Nature. 2003;424:213-219.
10. Hannon GJ. RNA interference. Nature. 2002;418: 244-251.
11. Coburn GA, Cullen BR. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J Virol. 2002;76: 9225-9231.
12. Lee MT, Coburn GA, McClure MO, Cullen BR. Inhibition of human immunodeficiency virus type 1 replication in primary macrophages by using Tat- or CCR5-specific small interfering RNAs expressed from a lentivirus vector. J Virol. 2003;77: 11964-11972.
13. Novina CD, Murray MF, Dykxhoorn DM, et al. siRNA-directed inhibition of HIV-1 infection. Nat Med. 2002;8:681-686.
14. Qin XF, An DS, Chen IS, Baltimore D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci U S A. 2003; 100:183-188.
15. Song E, Lee SK, Dykxhoorn DM, et al. Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. J Virol. 2003;77:7174-7181.
16. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. J Virol. 1998;72:8150-8157.
17. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. Science. 1987;238:800-802.
18. Tsunetsugu-Yokota Y, Kato T, Yasuda S, et al. Transcriptional regulation of HIV-1 LTR during antigen-dependent activation of primary T cells by dendritic cells. J Leukoc Biol. 2000;67:432-440.
19. Yamamoto N, Tanaka C, Wu Y, et al. Analysis of human immunodeficiency virus type 1 integration by using a specific, sensitive and quantitative assay based on real-time polymerase chain reaction. Virus Genes. 2006;32:105-113.
20. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. Nature. 2002;418:435-438.
21. Hornung V, Guenthner-Biller M, Bourquin C, et al. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med. 2005;11:263-270.
22. Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL,

Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet. 2003;34:263-264.

23. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol. 2003;5:834-839.

24. Carr JM, Hocking H, Li P, Burrell CJ. Rapid and efficient cell-to-cell transmission of human immunodeficiency virus infection from monocyte-derived macrophages to peripheral blood lymphocytes. Virology. 1999;265:319-329.

25. Garcia-Blanco MA, Cullen BR. Molecular basis of latency in pathogenic human viruses. Science. 1991;254:815-820.

26. McCune JM. Viral latency in HIV disease. Cell. 1995;82:183-188.

27. Finzi D, Siliciano RF. Viral dynamics in HIV-1 infection. Cell. 1998;93:665-671.

28. Chun TW, Stuyver L, Mizell SB, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci U S A. 1997;94:13193-13197.

29. Finzi D, Hermankova M, Pierson T, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. 1997;278:1295-1300.

30. Wong JK, Hezareh M, Gunthard HF, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. 1997;278:1291-1295.

31. Chun TW, Engel D, Mizell SB, Ehler LA, Fauci AS. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. J Exp Med. 1998;188:83-91.

32. Boden D, Pusch O, Lee F, Tucker L, Ramratnam B. Human immunodeficiency virus type 1 escape from RNA interference. J Virol. 2003;77:11531-11535.

33. Das AT, Brummelkamp TR, Westerhout EM, et al. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virol. 2004;78:2601-2605.

34. Moore JP, Kitchen SG, Pugach P, Zack JA. The CCR5 and CXCR4 coreceptors—central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses. 2004;20:111-126.

35. Aquaro S, Calio R, Balzarini J, Bellocchi MC, Garaci E, Perno CF. Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir. Antiviral Res. 2002;55:209-225.

36. Capodici J, Kariko K, Weissman D. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J Immunol. 2002;169:5196-5201.

37. Dave RS, Pomerantz RJ. Antiviral effects of human immunodeficiency virus type 1-specific small interfering RNAs against targets conserved in select neurotropic viral strains. J Virol. 2004;78:13687-13696.

38. Stevenson M. Dissecting HIV-1 through RNA interference. Nat Rev Immunol. 2003;3:851-858.

39. Cullen BR. HIV-1 auxiliary proteins: making connections in a dying cell. Cell. 1998;93:685-692.

40. Loetscher P, Uguccioni M, Bordoli L, et al. CCR5 is characteristic of Th1 lymphocytes. Nature. 1998;391:344-345.

41. Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. Nat Immunol. 2001;2:102-107.