Lentivirus vectors expressing short hairpin RNAs against the U3-overlapping region of HIV nef inhibit HIV replication and infectivity in primary macrophages


Although successful attempts to inhibit HIV-1 replication in T cells using RNAi have been reported, the effect of HIV-specific RNAi on macrophages is not well known. Macrophages are key targets for anti–HIV-1 therapy because they are able to survive long after the initial infection with HIV and can spread the virus to T cells. In this study, we identified a putative RNAI target of HIV, consisting of the portion of the nef gene overlapping the U3 region (Nef366), and generated a lentivirus-based short hairpin RNA (shRNA) expression vector (Lenti shNef366). We show that Lenti shNef366 inhibits (1) HIV-1 replication in a monocyctic cell line and in primary monocyte-derived macrophages (MDMs), (2) reactivation of latent HIV-1 infection, and (3) the production of secondary HIV-1 from MDMs harboring a genomic copy of Nef366. Moreover, we found that the up-regulated production of macrophage inflammatory protein 1β (MIP-1β), but not MIP-1α, in MDMs by Nef expression was considerably suppressed by Lenti shNef366, which suggests that HIV-1 dissemination to T cells through its interaction with HIV-1–infected MDMs can also be controlled by Lenti shNef366. Thus, lentivirus-mediated shRNA expression targeting the U3-overlapping region of HIV nef represents a feasible approach to genetic vaccine therapy for HIV-1.

Introduction

HIV Nef, which is uniquely conserved among HIV-1, HIV-2, and SIV, is essential for viral replication in vivo. Nef is located at the 3′ end of the viral genome, partially overlapping the 3′ long terminal repeat (LTR). The nef gene is one of the earliest expressed genes during HIV-1 replication and is transcribed at particularly high levels, often accounting for up to 80% of HIV-1–specific RNA in the early stages of viral replication. The Nef protein is multifunctional, having been shown to be involved in the down-regulation of CD4 receptor molecules, cell apoptosis, and signal transduction. From studies of HIV-infected individuals, accumulating evidence indicates that Nef plays an important, albeit currently not clearly understood, role in the pathogenesis of AIDS.

Recent investigations have shown that Nef has evolved macrophage-specific functions, such as the recruitment of T cells to sites of infection. Macrophages expressing Nef secrete a high level of macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, thus recruiting peripheral T cells to lymph nodes. More recently it was shown that Nef regulates the release of paracrine factors from macrophages; at least 2 proteins have been identified, which enhance lymphocyte susceptibility to HIV-1 infection in the absence of cell-cycle progression. These results provide ample evidence that Nef functions as a virulence factor that contributes to the manifestation of the clinical symptoms of immunodeficiency. Thus, any therapeutic intervention aimed at either completely blocking or at least partially reducing the expression of nef during HIV infection would likely enhance the ability of the immune system to fight HIV infection.

Sequence-specific degradation of viral mRNA by the process of RNAi is a mechanism for selectively inhibiting the synthesis of viral proteins that are critical for HIV-1 replication. RNAi therapy is based on an existing mechanism of gene regulation that is ubiquitous in plants and animals, in which targeted mRNAs are degraded in a sequence-specific manner. Quite recently, several groups reported the use of RNAi to successfully inhibit HIV-1 replication.

To study the effect of stable expression of short hairpin RNA (shRNA) against the U3-overlapping region of HIV-1 nef on virus replication and Nef-mediated cytokine regulation in primary macrophages, we established a lentivirus vector system expressing HIV-specific shRNAs. We show that HIV replication in primary macrophages was considerably suppressed following transfection of shRNAs targeting the U3-overlapping region of genomic HIV nef. Moreover, RNAi was able to control CC-chemokine

From the Department of Immunology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo; Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN Tsukuba Institute, Tsukuba; Department of Molecular Virology, Bio-Response, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo; AIDS Research Center, National Institute of Infectious Diseases, Shinjuku, Tokyo; and Division of Cellular and Molecular Biology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, Shirokane-dai, Minato-ku, Tokyo, Japan.


Supported by a grant from the Ministry of Health, Labor, and Welfare of Japan and from the Japan Health Sciences Foundation.

T.Y. and Y.T.-Y. performed laboratory experiments, data management, and the biostatistical analysis; T.Y., Naoki Y., J.-i.I., and Y.T.-Y. were responsible for the general design of the study; H.M. and Norio Y. were responsible for the design of the specific parts on lentivirus vectors and quantitative PCR analysis, respectively; T.Y., Y.T.-Y., and J.-i.I. were involved in the interpretation of the results and general outline of the paper; and T.Y. and Y.T.-Y. wrote the article.

Reprints: Yasuko Tsunetsugu-Yokota, Department of Immunology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan; e-mail: yyokota@nih.go.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2006 by The American Society of Hematology

BLOOD, 15 NOVEMBER 2006 • VOLUME 108, NUMBER 10

3305
production associated with Nef expression in HIV-1–infected macrophages. Thus, lentivirus-vector–based RNAi of the U3-overlapping region of HIV-1 nef might have potential usefulness as a genetic vaccine against HIV-1 infection.

Materials and methods

Construction of plasmids

To express gene-specific shRNAs under the human U6-RNA promoter, sense and antisense oligonucleotides 47 bp in length were ligated into pENTR/U6 (Invitrogen, Carlsbad, CA). The sequences of the oligonucleotides were as follows: lacZ, sense oligonucleotide, 5'-cagcgatttggtgtgtag-3', and antisense oligonucleotide, 5'-ttatactcactaacctgctatggttgtaagatggtaggtggttaagg-3'; Nef666 (nucleotides 366-385 of the HIV-1NL4.3 nef ORF overlapping the 3' LTR); sense oligonucleotide, 5'-ccagcgatttggtgtagttgtgtaagatggtaggtggttggagttttgggcaattc-3', and antisense oligonucleotide, 5'-aaacaggtaggacacattcatcctctctctctctttggttaggtggtgtaagatggtaggtggttggagttttgggcaattc-3'. The resulting entry vectors were termed pENTR/shLacZ and pENTR/shNef666, respectively.

A Gateway-compatible (Invitrogen) HIV-1–based vector, pCS-RfA, containing elongation factor 1α promoter (EF-1α)–driven green fluorescent protein (EGFP) (pCS-RfA-EGF), was used to construct the lentivirus vectors, pCS-EGF/shLacZ and pCS-EGF/shNef666, according to the manufacturer’s instructions (Invitrogen).

Cell culture and transfection

The human cell line 293T and human monocytic cell lines U937 and U117 were maintained in Dulbecco modified Eagle medium (DMEM) and RPMI 1640 medium (Gibco, Grand Island, NY), respectively, supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 μg/mL). To establish CCR5+ CEMx174 cells expressing EGFP driven by HIV-LTR, CEMx174 cells were transfected with pEF-BOSbst-HuCCR5 and pHIV-1 LTR-EGFPpuro (kind gifts from M. Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan) and CEMx174 CCR5/LTR-EGFP cells were established.

HeLa-CD4 cells (obtained from the National Institutes of Health AIDS Reagent Program) were transfected with pEF-Nef bst, and Nef-expressing HeLa-CD4 cells were established (HeLa-CD4-Nef).

RNAi target site selection

A Web-based program for designing siRNA targets (Promega, Madison, WI), BLOCK-iT RNAi Target Designer (Invitrogen), and the National Center for Biotechnology Information Web site were used for the selection of siRNA and shRNA sequences, and for BLAST searches. Stealth siRNAs were synthesized (Figure 1) and HeLa-CD4-Nef cells were transfected with 2.5 μL Stealth siRNA complexed to 2.5 μL Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Total RNA was extracted and analyzed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) using specific LUX primers (Invitrogen) and the SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen). The sequences of the qRT-PCR primers were as follows: nef forward, labeled at its 3' terminus with a reporter fluorophore 6-carboxyfluorescein (FAM), 5'-cagcgatttggtgtagttgtgtaagatggtaggtggttggagttttgggcaattc-3'; nef reverse, 5'-ttatactcactaacctgctatggttgtaagatggtaggtggttggagttttgggcaattc-3'; ef-1α forward labeled at its 3' terminus with a reporter fluorophore 6-carboxyfluorescein-4', 5'-dichloro-2', 7'-dimethoxyfluorescein (JOE), 5'-aaacaggtaggacacattcatcctctctctctctttggttaggtggtgtaagatggtaggtggttggagttttgggcaattc-3'. The reactions were performed using an MX3000P (Stratagene, La Jolla, CA).

For Western blot analysis, cell lysates were prepared, subjected to 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with anti-Nef monoclonal antibody (mAb; F3, a kind gift from Dr Y. Fuji, Graduate School of Pharmaceutical Science, Nagoya City University, Nagoya, Japan). The blot was reacted with biotinylated goat anti–mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA), then with streptavidin–POD (Roche, Indianapolis, IN), then cells were washed 5 times. Culture supernatants were subjected to 12.5% SDS-PAGE and immunoblotted with anti-Nef mAb.

Proteins were visualized by the SuperSignal Western Dura Extended Duration Substrate (Pierce, Rockford, IL) using an LAS3000 analyzer (Fuji Film, Tokyo, Japan).

Preparation of lentivirus vector

The lentivirus shRNA expression vectors were produced by transient transfection of 293T cells with a self-inactivating (SIN) vector construct, VSVG-G- and Rev-expressing plasmid pCMV-VSV-G-RSV-Rev, and the packaging construct pCAG-HIVgp using the calcium phosphate precipitation method. The lentiviral vector was concentrated by ultracentrifugation and the final solution was assayed for p24 antigen by an in-house enzyme-linked immunosorbent assay (ELISA). The infectivity was determined by using 293T cells based on the EGFP expression.

Preparation of HIV-1 virus stocks

To prepare HIV-1, COS-7 cells were transfected with either pNL432, pNf462 (a kind gift from A. Adachi, Tokushima University, Tokushima, Japan), or pNf462dNef, in which the nef gene was deleted by digestion with XhoI and KpnI, as described previously.

Primary MDM culture

From peripheral blood mononuclear cells (PBMCs) of healthy, HIV-1–donors, CD14+ monocytes were enriched using a magnetic-activated cell sorter (MACS; Miltenyi Biotec, Cologne, Germany) as described. Monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, 5% human AB plasma, and 10 ng/mL macrophage colony-stimulating factor (M-CSF) for 1 week to allow differentiation into monocyte-derived macrophages (MDMs).

Kinetics of virus production in stable shRNA-expressing U937 cells

Stable shRNA-expressing cells were infected with HIV-1NL4.3 for 2 hours, then cells were washed 5 times. Culture supernatants were harvested at 3-
Latent HIV-1–infected U1 cells were transduced with Lenti cont or Lenti

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. All probes (Biosearch Technologies, Novato, Calif.) 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 × 10⁵/well) were cultured in 48-well tissue-culture plates and infected either with wild-type HIV-1 NL432 or HIV-1/Lenti shNef. 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

Detection of chemokine production in MDMs, the cytokine bead array (CBA) kit (BD Bioscience, San Jose, Calif.) 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 cultured 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. 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. 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). Nef355st 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 transduced 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). 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

From www.bloodjournal.org by guest on October 2, 2017. For personal use only.
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 Lentivirus 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 (FACsSaria; 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 role in the inhibition of HIV-1 replication.
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 expression. To determine the effect of Nef expression during HIV-1 infection in MDMs, we infected MDMs with wild-type HIV-1NF462 or the corresponding nef gene-deletion mutant, HIV-1NF462dNef, 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-1NF462 replication was 2- to 6-fold higher than that of HIV-1NF462dNef in MDMs. These results were consistent with those reported by Swingler et al. 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 Lentivirus shRNA expressing 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 Lentivirus shNef366 cells compared with Lentivirus control (Figure 5C). The levels of MCP-1 and IL-8 were either unaffected or somewhat restored by Lentivirus shNef366 (data not shown).

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

Lentivirus 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. 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
strongly inhibited the replication of HIV-1 at an early stage of HIV infection. Furthermore, expression of shNef366 in monocytic cell lines had a strong inhibitory effect on nef expression and showed that Nef366 shRNA had a strong inhibitory effect on HIV replication, 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 monocytes 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 al20 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.

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.35 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 NefLTR 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 Lentiviral 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. 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. Swinger 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. 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 siRNAs 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


Lentivirus vectors expressing short hairpin RNAs against the U3-overlapping region of HIV nef inhibit HIV replication and infectivity in primary macrophages