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Inducible-costimulator-mediated suppression of human immunodeficiency virus type 1 replication in CD4+ T lymphocytes

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Abstract

We investigated the effects of signaling through CD28 family molecules on human immunodeficiency virus type 1 (HIV-1) replication in vitro. A monoclonal antibody (mAb) specific for inducible costimulator (ICOS) suppressed both X4 and R5 HIV-1 replication in CD4+ peripheral blood mononuclear cells (PBMC). This suppression was not attributable to reduced cell growth or viability. CD28 mAb showed variable effects and also suppressed HIV-1 replication when immobilized. Replication of pseudotype viruses with HIV-1-but not with vesicular stomatitis virus G-envelope was efficiently suppressed in CD4+ PBMC treated with ICOS or CD28 mAbs. However, CD4, CXCR4, and CCR5 expression on the surface was not down-regulated. Moreover, HIV-1 replication in CD4+ PBMC was suppressed by a soluble form of human B7-H2, a ligand of ICOS, but was enhanced by soluble B7-1, a ligand for CD28. These findings suggest that natural or artificial ligands for ICOS potentially suppress HIV-1 replication mainly at the entry stages.

Keywords: HIV-1 suppression; Immune regulation; Costimulatory molecule; ICOS; CD28; T cell activation

Introduction

HIV-1 proliferates extensively and creates viremia in the acute phase of HIV-1 infection. Following the acute phase, the peripheral HIV-1 load declines to low levels that are continued during the asymptomatic phase of several years (Ho et al., 1989). Even during the asymptomatic period, viral replication is maintained at a low level (Pantaleo et al., 1993; Piatak et al., 1993). HIV-1 production in vivo is performed by both continuous de novo infections to uninfected short-lived cells, and viral persistence in long-life cells (Ho et al., 1995; Perelson et al., 1996; Wei et al., 1995). Individuals who receive long-term highly active antiretroviral therapy retain replication-competent HIV-1 predominantly in the memory T cell fractions, suggesting that these cells may be the main reservoir of persistently infecting HIV-1 (Finzi et al., 1997; Wong et al., 1997).

The mechanisms regulating HIV-1 replication in vivo are not fully understood. HIV-1-specific cytotoxic T cells (CTL) are thought to kill HIV-1-infected cells, although Nef-mediated down-regulation of MHC-I may limit CTL function (Walker et al., 1987). CD8+ cell-mediated unidentified soluble and cellular molecules may also contribute to the suppression of viral replication (Kannagi et al., 1990; Walker et al., 1986). Neutralizing antibodies might have some effect on limiting the spread of HIV-1. Besides these host defense mechanisms, the status of T cell activation may also affect HIV-1 replication by altering the expression of HIV-1 receptors and cytokines, as well as the status of intracellular molecules responsible for nuclear transport or transcription.

T cell activation is regulated by signals through a T cell receptor/CD3 complex and various costimulatory molecules that interact with their ligands on antigen-presenting cells (APC) (Lenschow et al., 1996). The first well-characterized costimulatory molecule was CD28 (Hara et al., 1985), which mediates positive signals in T cells following interactions with B7-1/CD80 or B7-2/CD86 (Linsley et al., 1990). Inhibition of CD28 receptors prevented CD3-mediated HIV-1 replication, indicating that CD28 signals are crucial in HIV-1 replication (Diegel et al., 1993). In contrast, CD28 may also transmit signals inhibiting HIV-1 replication when ligated (Levine et al., 1996).

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Several other members of the CD28 family molecules have been identified including cytotoxic T lymphocyte antigen 4 (CTLA-4) (Brunet et al., 1987), inducible costimulator (ICOS)/activation-inducible lymphocyte immunomediatory molecule (AILIM)/H4 (Buonfiglio et al., 2000; Hutloff et al., 1999; Tamatani et al., 2000), and programmed death gene 1 (PD-1) (Nishimura and Honjo, 2001). CTLA-4 is an inducible molecule homologous to CD28 and also binds B7-1/CD80 and B7-2/CD86, but transmits negative signals to T cells (Azuma et al., 1993; Krummel and Allison, 1995; Walunas et al., 1994). ICOS transmits positive signals following interactions with the ligand B7-H2/B7 homologous protein (B7h)/B7-related protein 1 (B7RP-1) (Swallow et al., 1999; Yoshinaga et al., 1999). Stimulation of CD28 results in activation of IL-2, while stimulation of ICOS induces the production of other cytokines such as IL-4, IL-5, IL-10, IL-13, IFN-γ, TNF-α, and GM-CSF (Beier et al., 2000; Coyle et al., 2000; Hutloff et al., 1999). PD-1 binds PD-L1 (B7-H1) and PD-L2 (B7-DC) (Dong et al., 1999; Freeman et al., 2000; Latchman et al., 2001; Tseng et al., 2001), and inhibits TCR-mediated proliferation of T cells and cytokine production. CD28 is constitutively expressed by T cells, while other molecules are induced upon activation (Sharpe and Freeman, 2002).

B7 family molecules are expressed on monocytes, dendritic cells, and B cells, and sometimes on T cells, epithelial cells, and endothelial cells (Frauwirth and Thompson, 2002; Sharpe and Freeman, 2002). Although B7-1/B7-2 are mainly expressed on lymphoid organs, B7h, PD-L1, and PD-L2 are also found on nonlymphoid organs such as the heart, lung, and kidney (Latchman et al., 2001; Lenschow et al., 1996; Sharpe and Freeman, 2002; Swallow et al., 1999). Thus, interactions between molecules from the CD28-B7 family may play an important role not only in priming T cells, but also in regulating activated T cells in both lymphoid and nonlymphoid organs in vivo.

In the present study, we investigate effects of signals through CD28 family molecules on HIV-1 replication, and showed that ICOS monoclonal antibody (mAb) mediate suppression of HIV-1 replication in PHA-stimulated CD4+ T cells in vitro mainly at the early stages of HIV-1 replication. Furthermore, a soluble form of B7-H2 (B7h) also mediated signals suppressing HIV-1 replication, suggesting that natural or artificial ligands for ICOS might be applicable as a biological strategy to control HIV-1 infection.

Results

The effects of mouse monoclonal antibodies (mAbs) for the CD28 family molecules on HIV-1 replication

To examine the effect of activation signals through the CD28 family molecules on HIV-1 replication, phytohemagglutinin (PHA)-stimulated CD4+ peripheral blood mononuclear cells (PBMC) were infected by the X4 strain HIV-1 NL4-3, and cultured in the presence of mAbs for various CD28 family molecules at concentrations ranging from 0.5 to 10 μg/ml. HIV-1 replication was monitored by measuring the levels of HIV-1 p24 in the culture supernatant after 4 days of culture. The results of four experiments using PBMC isolated from different donors are summarized in Table 1. Although there was individual variability, SA12, a mAb to ICOS, significantly suppressed HIV-1 replication. The effects of the anti-CD28 mAb TN228 varied widely, enhancing viral replication in some samples, while suppressing it in others. MAbs to CTLA-4 and PD-1 did not markedly affect HIV-1 replication. Flow cytometric analysis indicated that PHA-stimulated CD4+ PBMC expressed significant levels of CD28 and ICOS, but low or undetectable levels of PD-1 and CTLA-4 (Fig. 1). We therefore investigated the effects of ICOS and CD28 signals on HIV-1 replication thereafter.

Table 1
Effects of mAbs to various CD28 family molecules on HIV-1 replication

	Concentration of mAbs (μg/ml)	HIV-1 p24 production (pg/ml) in CD4+ PBMC culture in the presence of					
		Medium	TN228 anti-CD28	MIH8 anti-CTLA-4	SA12 anti-ICOS	MIH4 anti-PD-1	Mouse IgG1
Exp. 1	0	130.1 ± 6.1					
	10		147.1 ± 32.0	158.0 ± 27.0	100.0 ± 12.1	147.3 ± 79.3	135.0 ± 16.1
Exp. 2	0	152.8 ± 9.7					
	1		123.5 ± 11.2	119.0 ± 12.9*	129.1 ± 9.6	134.8 ± 20.4	136.0 ± 9.6
	2.5		75.9 ± 17.5*	149.6 ± 13.7	58.3 ± 5.5*	136.4 ± 6.2	140.5 ± 8.9
Exp. 3	0	77.2 ± 13.8					
	10		133.0 ± 8.6*	70.2 ± 20.0	39.6 ± 7.4*	79.4 ± 8.3	82.0 ± 11.0
Exp. 4	0	168.8 ± 20.6					
	0.5		183.7 ± 14.0	138.4 ± 14.5	78.5 ± 4.4*	146.2 ± 14.9	151.5 ± 38.2
	5		199.2 ± 59.0	149.7 ± 10.5	49.9 ± 17.3*	155.1 ± 72.3	141.2 ± 34.6

PHA-stimulated CD4+ PBMC cells were infected with the X4 HIV-1 strain NL4-3 for 2 h and cultured either without or with the indicated mAbs at the indicated concentration in 96-well round-bottomed plates. HIV-1 p24 in the supernatant was measured by ELISA after 4 days. The results of four independent experiments using PBMC from different donors are shown. The values indicate the mean ± SD from triplicate determinations.
* $P < 0.05$.

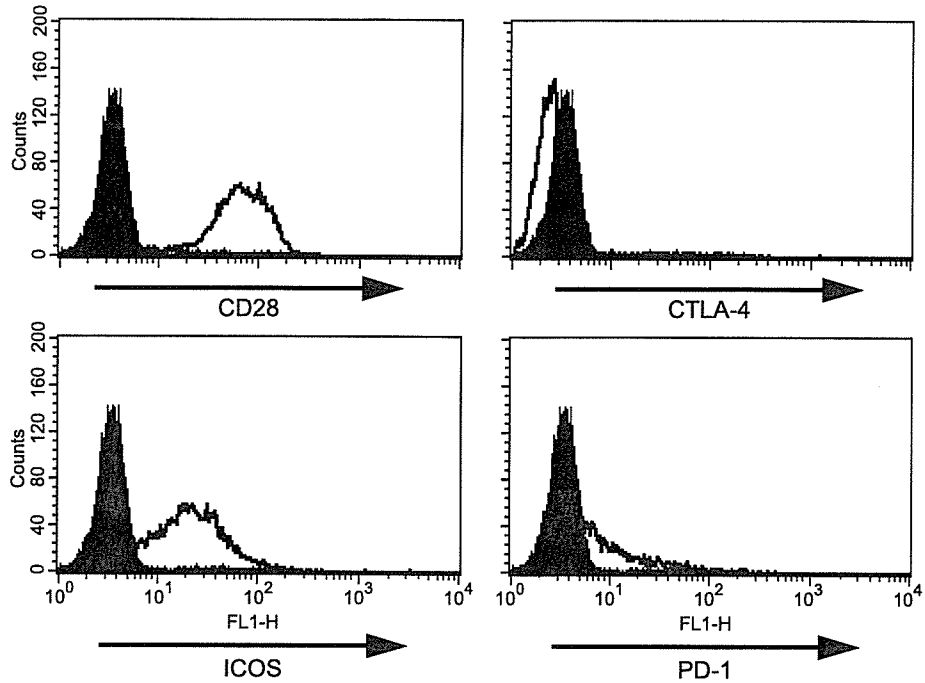

Fig. 1. Cell surface expression of CD28 family molecules on PHA-stimulated CD4 + PBMC. PHA-stimulated CD4+ PBMC cultured in the presence of IL-2 for 6 days were stained with mAbs to CD28, CTLA-4, ICOS, or PD-1 (open histogram), and subsequently stained with FITC-labeled second antibody. Closed histogram indicates cells stained with second antibody alone.

Inhibition of X4 and R5 HIV-1 replication by immobilized ICOS and CD28 mAbs

It has previously been shown that immobilized CD28 mAbs suppress HIV-1 replication in PBMC (Levine et al., 1996). We then assessed the effects of immobilized ICOS and CD28 mAbs on X4 and R5 HIV-1 replication. As shown in Fig. 2, immobilized CD28 and ICOS mAbs significantly inhibited replication of both X4 HIV-1 NL4-3 and R5 HIV-1 JR-CSF in CD4+ PBMC. The inhibitory effect of immobilized ICOS and CD28 mAbs on HIV-1 replication was observed in repetitive experiments. Thus, ligation of ICOS or CD28 mediated efficient inhibitory signals in HIV-1 replication in CD4+ T cells regardless of X4 or R5 HIV-1 strains.

Immobilized ICOS and CD28 mAbs do not suppress cell proliferation or viability

We next examined whether ICOS- or CD28-mediated inhibition of viral replication is associated with reduced proliferation or viability of CD4+ T cells. Fig. 3A shows HIV-1 replication in PHA-stimulated CD4+ T cells cultured in either the presence of immobilized ICOS or CD28 mAbs at concentrations ranging from 0.0125 to 5 μg/ml for 4 days. HIV-1 replication was dose-dependently suppressed by immobilized ICOS or CD28 mAbs, except at the highest concentration of CD28 mAb, which showed some irregularity. Simultaneously prepared uninfected CD4+ T cells from the same donor underwent a [^{3}H]thymidine uptake assay after 4 days of culture in the presence of similar concentrations of immobilized mAbs. As shown in Fig. 3B, in contrast to HIV-1 replication, proliferation of CD4+ T cells was positively correlated with the presence of either immobilized ICOS or CD28 mAbs in a dose-dependent manner. As a control, immobilized mouse IgG1 did not affect either HIV-1 replication or cell proliferation. To assess whether cell death occurred, we daily monitored the levels of lactate dehydrogenase (LDH) released into the supernatant from CD4+ T cells cultured with immobilized mAbs. As shown in Fig. 3C, the amount of LDH did not differ among these CD4+ T cell cultures during 3 days of culture. These results indicated that the inhibition of HIV-1 replication by immobilized ICOS or CD28 mAbs was not due to inhibition of cell proliferation or viability.

ICOS and CD28 mediate inhibition of the early stages of a HIV-1 replication cycle

To determine at which steps of the life cycle of HIV-1 the antiviral effect of ICOS or CD28 mAbs occurred, we monitored HIV-1 cDNA synthesis after infection. PHA-stimulated CD4+ T cells infected with HIV-1 NL4-3 for 2 h were cultured with immobilized ICOS or CD28 mAbs, and 10^6 cells in each well were harvested for DNA analysis 2, 24, and 48 h after infection (Fig. 4). The reverse transcripts were amplified by PCR using specific primer pairs for HIV-1 LTR R and gag regions (R/gag)(Masuda et al., 1995; Zack et al., 1990). Twenty-four hours after infection, the amount of viral cDNA (R/gag) was considerably less in

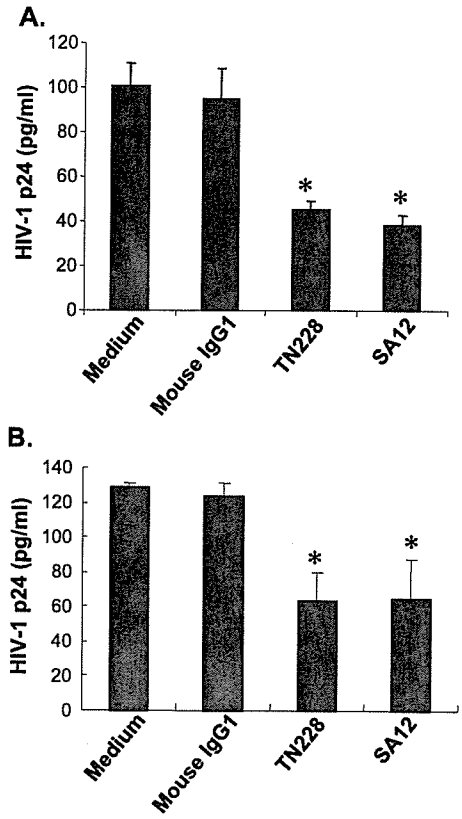

Fig. 2. Immobilized mAbs for ICOS (SA12) and CD28 (TN228) inhibited HIV-1 replication. CD4+ PBMC cultured for 4–6 days after PHA stimulation were infected with X4 HIV-1 NL4-3 (A) or R5 HIV-1 JR CSF (B) for 2 h, and cultured in medium alone or in the plates precoated with 5 μg/ml antibodies indicated. After 4 days, HIV-1 p24 levels in the supernatant were measured by ELISA. The results indicate the mean ± SD from triplicate determinations. Similar results were obtained in an independent experiment. $*P < 0.05$.

ICOS mAb-treated CD4+ T cells compared with the control cells not treated with mAbs. Although to a lesser degree than ICOS mAb, CD28 mAb also inhibited cDNA synthesis of HIV-1. Similar inhibition of viral cDNA synthesis by these mAbs was also observed at 14 h following HIV-1 infection in an independent experiment (data not shown). These results suggest that immobilized ICOS and CD28 mAbs prevent HIV-1 infection during the steps before or at reverse transcription in the HIV-1 life cycle.

The effects of immobilized ICOS and CD28 mAbs on pseudotype HIV-1 expression

We next investigated whether ICOS or CD28 mAbs could suppress the entry steps of HIV-1 replication by using pseudotype HIV-1 (Masuda et al., 1995; Planelles et al., 1995) consisting of an envelope-defective HIV-1 expressing

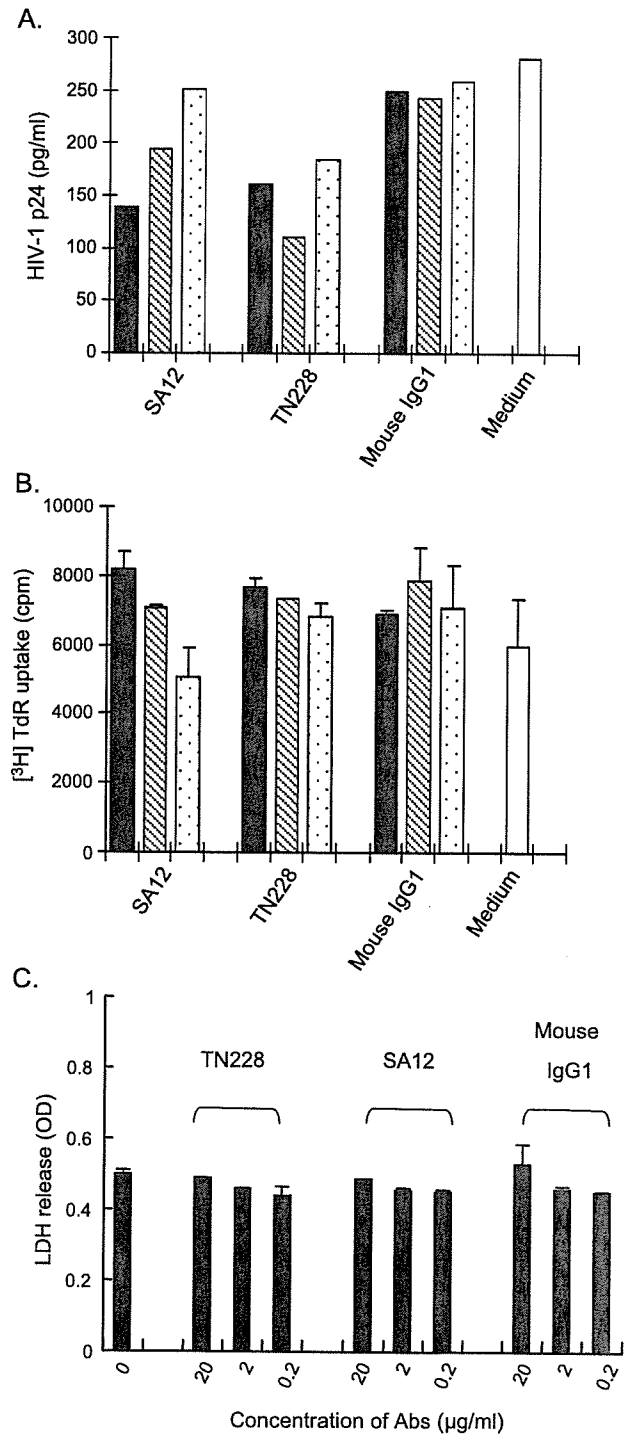

Fig. 3. Stimulation of ICOS or CD28 suppressed HIV-1 replication but not the proliferation or viability of CD4+ T cells. (A) PHA-stimulated purified CD4+ T cells infected with HIV-1 NL4-3 were cultured on plates precoated with antibodies at the indicated concentration of 5 μg/ml (■), 0.25 μg/ml (▨), and 0.0125 μg/ml (⊡), or in the medium alone (□) for 4 days, and HIV-1 P24 levels in the supernatant was measured by ELISA. (B) Uninfected PHA-stimulated purified CD4+ T cells from the same donor as A were simultaneously cultured in plates similarly precoated with antibodies for 4 days, and the incorporation of [^{3}H]thymidine (TdR) into the cells during the last 16 h was measured as described in Materials and methods. The results indicate the mean ± SD from triplicate determinations. (C) Cell death of PHA-stimulated purified CD4+ T cells incubated on plates precoated with antibodies at the indicated concentrations was determined daily from the level of LDH in the supernatant over 3 days. The results are shown for 24 h after the initiation of culture. Similar results were obtained in the second and third days.

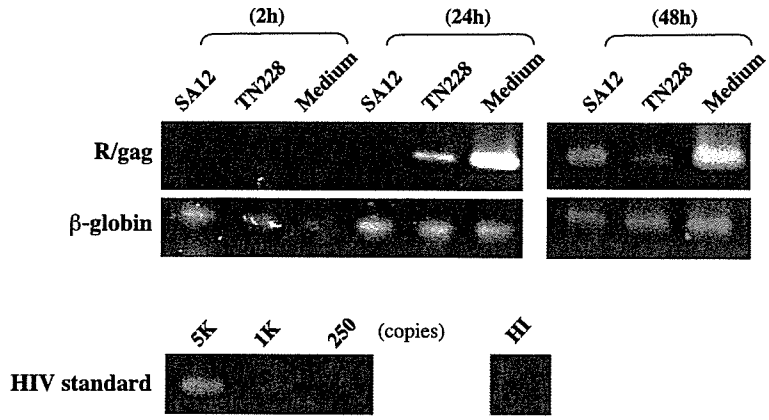

Fig. 4. Analysis of HIV-1 DNA synthesis in NL4-3-infected CD4+ PBMC treated with immobilized ICOS mAb. CD4+ PBMC cultured for 4 days after PHA stimulation were infected with DNase-treated HIV-1 NL4-3 for 2 h and cultured in 12-well flat-bottomed plates (10^6 cells/well) precoated with ICOS or CD28 mAbs (5 μg/ml) or medium alone. Total DNA was extracted from the cells harvested at the indicated periods after infection and underwent PCR analysis with the primer pairs for HIV-1 R/gag region. For HIV-1 DNA standards, 250–5000 copies of linearized HIV-1 JR-CSF DNA were amplified in parallel. Amplified products were resolved on a 2% agarose gel and visualized by Syber-Green staining (FMC Bioproduct). HIV-1 treated at 65 °C for 30 min before inoculation was used as a heat-inactivated control (HI).

luciferase and either HIV-1 LAI envelope (Poon et al., 1998) or VSV-G envelope (Yee et al., 1994). The pseudotype viruses with HIV-1 envelope enter into cells by envelope-mediated membrane fusion, whereas the pseudotype viruses with VSV-G envelope use endocytotic mechanisms. Fig. 5 shows luciferase activities of PHA-stimulated CD4+ PBMC that were infected with pseudotype HIV-1 and cultured in the presence of immobilized ICOS or CD28 mAbs. Luciferase expression in the CD4+ PBMC infected with VSV-G envelope pseudotype virus was increased by CD28 mAbs and unchanged by ICOS mAbs (Fig. 5A). By contrast, expression of HIV-1 envelope pseudotype virus was significantly inhibited by both ICOS and CD28 mAbs (Fig. 5B). These results indicate that immobilized ICOS and CD28 mAbs mainly affected the entry stages of HIV-1 replication.

ICOS or CD28 stimulation did not reduce HIV-1 receptor expression on CD4+ T cells

We next assessed whether pretreatment with immobilized ICOS or CD28 mAbs reduces the expression of HIV-1-specific receptors on CD4+ T cells. Following 24 h of preincubation with immobilized ICOS or CD28 mAbs, the expression of CD4, CXCR4, and CCR5 on PHA-stimulated CD4+ cells was analyzed by flow cytometry. The purity of the CD4+ T cells was approximately 93%. The results are shown in Fig. 6. The expression of CCR5 was significantly upregulated in cultures pretreated with CD28 or ICOS mAbs. In these cultures, increased CCR5 expression persisted during the first 4 days after stimulation at least. In contrast, the levels of CD4 and CXCR4 expression were not altered by these mAbs. Thus, ICOS- or CD28-mediated suppression of both X4 and R5 HIV-1 replication could not be attributed to a reduction in HIV-1-specific receptor expression on the cell surface.

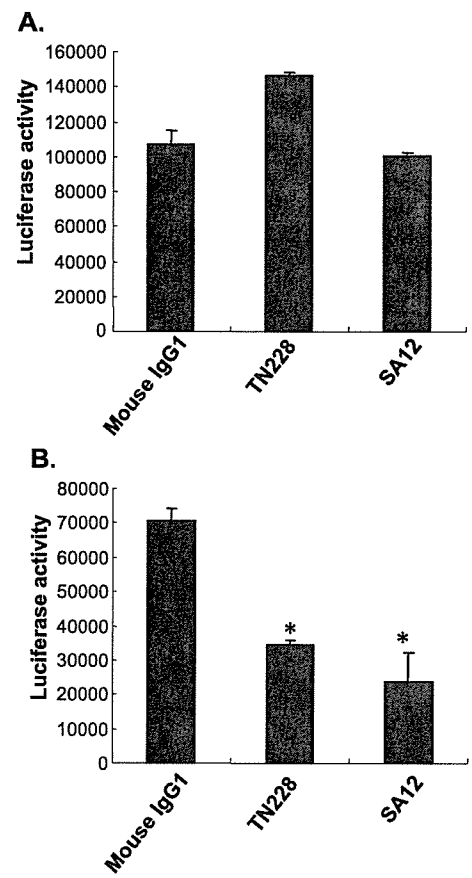

Fig. 5. Effects of ICOS or CD28 stimulation on pseudotype HIV-1 gene expression. CD4+ PBMC cultured for 4 days after PHA stimulation were incubated overnight in plates coated with immobilized ICOS or CD28 mAbs or control mouse IgG (1 μg/ml) (10^6/well in 24-well plates), then infected with luciferase-expressing pseudotype HIV-1 possessing a VSV-G envelope (A) or a HIV-1 LAI envelope (B) for 2 h. The cells were further cultured for 48 h on plates coated with the same antibodies as above, and luciferase activity was measured. The results show the mean ± SD from duplicate determinations. *$P < 0.05$.

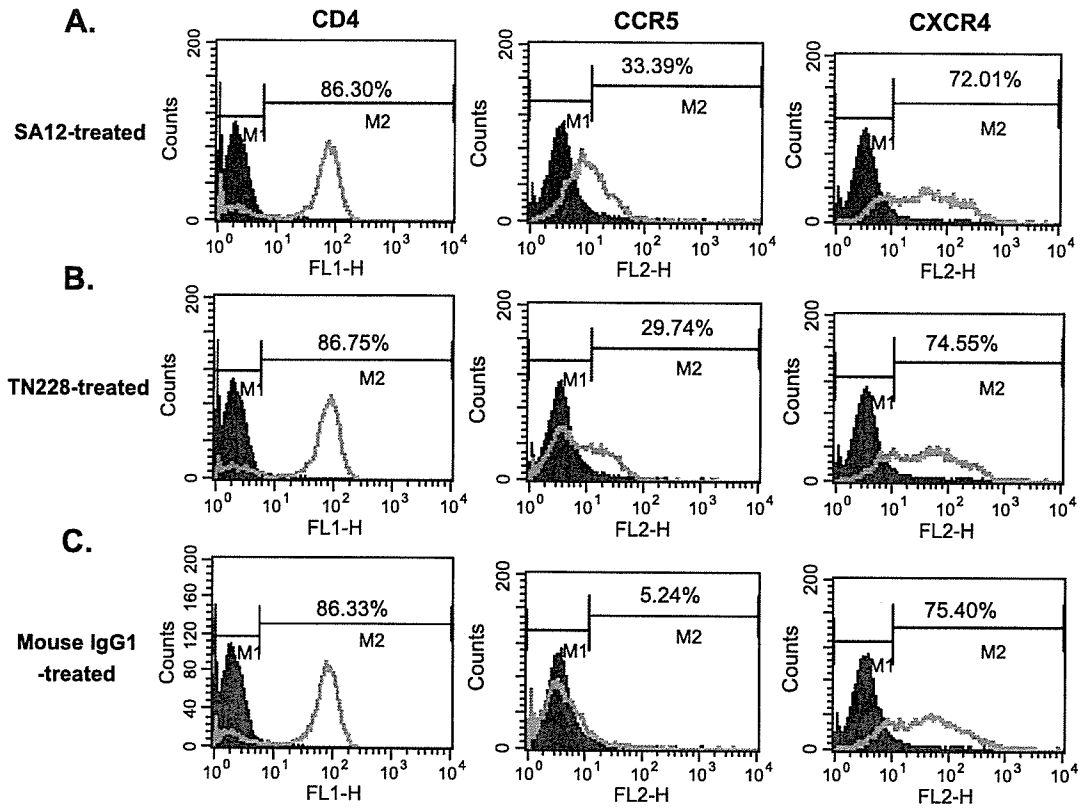

Fig. 6. Effects of ICOS or CD28 stimulation on HIV-1 receptor expression by CD4+ T cells. PHA-stimulated CD4+ PBMC were treated either with immobilized mAbs to ICOS (A) or CD28 (B) or control mouse IgG1 (C) (5 μg/ml) in 24-well flat-bottomed plates, and the expression of CXCR4 and CCR5 receptors was examined by FACS (open histogram) after 24 h of culture. Closed histogram indicates control cells stained with the second antibody alone. Similar results were obtained in an independent experiment cultured for 3 days.

Effect of ICOS or CD28 stimulation on cytokine production

We next examined whether ICOS and CD28 mAbs altered the production of cytokines and chemokines that might affect HIV-1 replication. In most of the experiments above, we added ICOS or CD28 stimulation to CD4+ PBMC that had been cultured for several days after PHA stimulation. In this condition, the cells stimulated with PHA alone produced detectable levels of IL-5, IFN-γ, MIP-1α, and RANTES, and very low levels of IL-10 (Table 2). Among them, IL-5 production was further enhanced by CD28-stimulation, but other cytokines were not significantly altered by ICOS or CD28 mAbs.

We also examined cytokine production by CD4+ PBMC stimulated with ICOS or CD28 mAbs immediately after PHA stimulation. In this condition, the cells with PHA stimulation alone produced significant levels of IL-5, IL-10, TNFα, IFNγ, RANTES, and MIP-1α. Stimulation of ICOS and CD28 enhanced IL-5 and IL-10, but reduced TNFα and IFNγ production. RANTES and MIP-1α were not significantly affected by ICOS and CD28 mAbs. SDF-1 was not detectable in any culture tested.

We also confirmed that the expression of pseudotype virus with HIV-1 envelope was significantly inhibited in the cells that were stimulated with ICOS or CD28 mAbs immediately after PHA stimulation, (Fig. 7A), whereas similar treatment did not affect cell proliferation (Fig. 7B).

Table 2
Effect of ICOS and CD28 stimulation on cytokine production (pg/ml) from PHA-stimulated CD4+ PBMC

Cytokines	Early stimulation with			Late stimulation with		
	Mouse IgG1	SA12	TN228	Mouse IgG1	SA12	TN228
IL-4	N.D.	4.4	2.3	N.D.	N.D.	1.3
IL-5	621.3	1785.1	1514.9	679.6	773.6	1053.4
IL-10	171.6	274.7	246.8	8.9	11.2	13
TNF-α	1599.2	1075.7	942.4	1.8	2.4	2.1
IFN-γ	13814.5	2913.4	3858.3	338.5	356.6	419
MIP-1α	1512.7	1629.9	1554.5	379.1	356.7	394
RANTES	3524.4	3565.9	3177.8	426.7	400	429.6
SDF-1α	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

CD4+ PBMC were placed into plates that were precoated with mouse IgG1, or SA12 (anti-ICOS) or TN228 (anti-CD28) mAbs at a concentration of 5 μg/ml, immediately (early stimulation) or 4 days (late stimulation) after 30 min of PHA stimulation, and cultured in the RPMI medium containing IL-2. Cytokines and chemokines in the supernatants were measured by using either Cytometric Bead Array Kit (IL-4, IL-5, IL-10, TNF-α) or ELISA (IFN-γ, MIP-1α, RANTES, SDF-1α) 3 days after mAb stimulations.

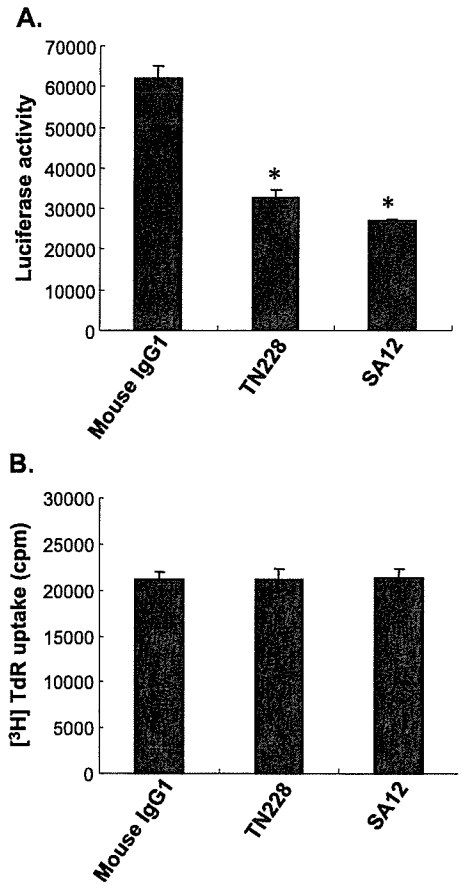

Fig. 7. Effects of ICOS or CD28 stimulation immediately after PHA stimulation. CD4+ PBMC were stimulated with PHA for 30 min, washed, and cultured on plates precoated with immobilized ICOS or CD28 mAbs or control mouse IgG (1 μg/ml) in the presence of IL-2. HIV-1 expression (A) and proliferation (B) of these cells were examined. (A) Two days after stimulation, the cells were infected with pseudotype virus with a HIV-1 envelope, and further cultured for 48 h in the mAb-coated wells, and then luciferase activity was measured. *$P < 0.05$. (B) The proliferation of the cells was measured by the [^{3}H]thymidine incorporation method after a 3-day culture without infection. The results show the mean ± SD from duplicate (A) or quadruplicate (B) determinations.

Leflunomide reversed the inhibition of HIV-1 replication mediated by immobilized ICOS or CD28 mAbs

The intracellular transduction pathway underlying ICOS signaling is not fully understood. Consequently, we investigated the effects of inhibitors for NF-κB (Leflunomide), MEK (PD98059), and p38 MAP kinase (SB202190) on ICOS- or CD28-induced inhibition of HIV-1 replication. As shown in Fig. 8, Leflunomide significantly attenuated CD28-induced inhibition of HIV-1 replication in CD4+ T cells. Although Leflunomide alone suppressed HIV-1 replication, the coexistence of immobilized CD28 mAb resulted in overwhelming viral replication. A low level of enhancement in HIV-1 replication was also observed in ICOS mAb-treated cells in the presence of Leflunomide, although it was not statistically significant. In the presence of PD98059, CD28 but not ICOS mAb significantly enhanced HIV-1 replication. SB202190 marginally altered the effects of ICOS- or CD28-mAbs. These results suggested that CD28 and ICOS in a lesser degree mediated positive signals in HIV replication besides inhibitory signals, and that the inhibitory signals were suppressed by inhibiting NF-κB and/or MEK pathways.

Natural ligands for ICOS-inhibited HIV-1 replication

Finally, we assessed whether the natural ligands of ICOS or CD28 affect HIV-1 replication. Recombinant chimeric proteins of various B7-CD28 family molecules and the Fc region of human IgG were added to CD4+ T cell cultures

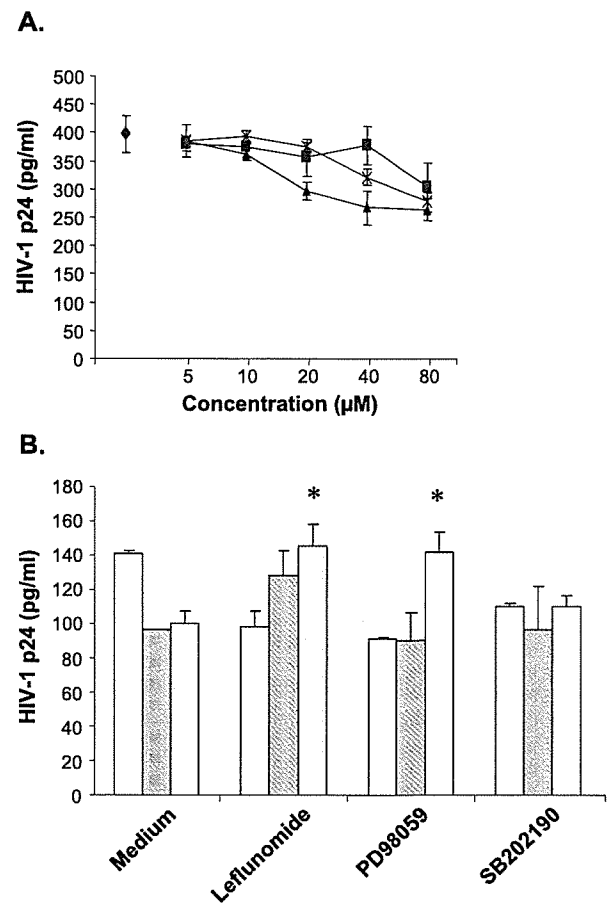

Fig. 8. The effects of Leflunomide on ICOS- or CD28-induced inhibition of HIV-1 replication. (A) PHA-stimulated CD4+ PBMC infected with HIV-1 NL4-3 were cultured in the absence (◆) or presence of various concentrations of Leflunomide (NF-κB-inhibitor) (■), PD98059 (MEK-inhibitor) (▲), or SB202190 (p38 MAP kinase inhibitor) (×), for 4 days, and HIV-1 p24 levels in the supernatant were measured. (B) PHA-stimulated CD4+ PBMC infected with HIV-1 NL4-3 were cultured either without (□) or with immobilized ICOS (▨) or CD28 (▨) mAbs (5 μg/ml) in 48-well flat-bottomed plates in either the absence or presence of 20 μM Leflunomide, PD98059, or 10 μM of SB202190. HIV-1 p24 levels in the supernatant were measured by ELISA 4 days after infection. The results indicate the mean ± SD from triplicate determinations. Similar results were obtained in an independent experiment. *$P < 0.05$.

Table 3
Effects of various soluble B7-CD28 family molecules on HIV-1 replication

Reagents	HIV-1 p24 (pg/ml) in the supernatant of HIV-1-infected CD4+ PBMC culture	
	Exp. 1	Exp. 2
Medium	77.2 ± 13.8	168.8 ± 20.6
Mouse IgG1	82.0 ± 11.0	N.T.
CD28/Fc	65.3 ± 9.1	139.8 ± 4.1
CTLA-4/Fc	41.1 ± 12.0*	84.9 ± 17.0*
ICOS/Fc	72.5 ± 8.0	N.T.
B7-1(CD80)/Fc	96.9 ± 13.9	N.T.
B7-H2(B7h)/Fc	43.7 ± 5.9*	95.9 ± 13.4*
CD28/Fc + B7-H2/Fc	N.T.	79.5 ± 15.6*
CTLA-4/Fc + B7-H2/Fc	N.T.	71.0 ± 5.0*

PHA-stimulated CD4+ PBMC were infected with HIV-1 NL4-3, and cultured in the medium containing IL-2 in the presence of 10 μg/ml of the indicated recombinant chimeric proteins of various B7-CD28 family molecules and the human Fc region of IgG. HIV-1 p24 in the supernatant was measured by ELISA after 4 days. The values indicate the mean ± SD from triplicate determinations.
N.T., not tested.
*$P < 0.05$.

following infection with HIV-1 NL4-3. The results are shown in Table 3. B7-H2 (B7h)/Fc and CTLA-4/Fc significantly suppressed HIV-1 replication. CD28/Fc produced low levels of suppression, but the effects of ICOS/Fc were minimal. B7-1 (CD80)/Fc enhanced viral replication. A mixture of B7-H2 (B7h)/Fc and either CD28/Fc or CTLA-4/Fc resulted in maximal suppression. These results indicate that B7-H2 (B7h), a natural ligand of ICOS, suppressed HIV-1 replication in a similar manner to ICOS-mAbs, whereas B7-1, a natural ligand of CD28 and CTLA-4, enhanced HIV-1 replication.

Discussion

In the present study, we showed that ICOS transmitted signals suppressing HIV-1 replication in CD4+ T cells in vitro. ICOS-mAb suppressed HIV-1 replication when the mAb was either added directly to the cell culture or was immobilized. A soluble form of B7-H2 (B7h), a natural ligand for ICOS, also suppressed HIV-1 replication. Immobilized CD28-mAb suppressed HIV-1 replication in CD4+ cells as previously reported (Levine et al., 1996), while direct application of CD28 mAb or its natural ligand B7-1 mostly enhanced viral replication consistently with other reports, indicating that CD28 costimulation supports HIV-1 replication (Diegel et al., 1993).

Immobilized ICOS or CD28 mAbs significantly inhibited replication of both X4 and R5 wild-type HIV-1 (Fig. 2). In addition, these mAbs inhibited the expression of pseudotype HIV-1 possessing HIV-1 envelope but not VSV-G envelope (Fig. 5). Replication of pseudotype HIV-1 with VSV-G-envelope was even enhanced especially when the cells were pretreated with CD28 mAb. Moreover, immobilized ICOS or CD28 mAbs significantly inhibited DNA synthesis during the initial cycle of viral replication in the cells infected with wild-type HIV-1 (Fig. 4). These observations indicated that the entry stages of the viral life cycle were mainly affected by these mAbs. However, CD4 and CXCR4 expression on the cell surface was not affected by stimulation with ICOS and CD28 mAbs, and CCR5 levels were even enhanced by treatment with these mAbs (Fig. 6). Thus, the suppression of HIV-1 replication by these mAbs was not attributable to the down-regulation of HIV-1 receptors or coreceptors.

The cytokines and chemokines potentially modify HIV-1 replication. Stimulation of CD28 induces IL-2, while stimulation of ICOS results in the production of other cytokines (Beier et al., 2000; Coyle et al., 2000; Hutloff et al., 1999). We measured several cytokines and chemokines in two different culture conditions. When the cells were treated with ICOS or CD28 mAbs immediately after PHA stimulation, ICOS and CD28 stimulation enhanced IL-5 and IL-10, but suppressed TNFα and IFNγ production. When the cells were treated with mAbs 4 days after PHA stimulation, the levels of cytokine production were generally low, and alteration of cytokine levels by ICOS and CD28 mAbs was limited. Because HIV-1 expression was suppressed by these mAbs in both culture conditions as well (Figs. 5B and 7A), alteration of cytokines observed here was unlikely to be a major reason for the viral suppression. The precise mechanisms underlying the suppression of HIV-1 replication by ICOS and CD28 mAbs remains to be determined.

The signaling pathways downstream of CD28 and ICOS are not fully understood. Stimulation of the T cell receptor in concert with CD28 induces activation of JNK, P38 MAPK, AP-1, and interleukin-2 (Avraham et al., 1998; Faris et al., 1996; Hehner et al., 2000), which involves activation of Vav and Rac-1 (Raab et al., 2001; Salojin et al., 1999). Activation of NF-κB also occurs following CD28 costimulation, which results from IκB degradation (Harhaj and Sun, 1998; Kane et al., 2002). Recent reports have shown that MAPKKK might activate the IKK complex, and lead to degradation of IκB following CD28 costimulation (Tuosto et al., 2000). In the present study, ICOS- and CD28-mediated HIV-1 suppression was reversed by the NF-κB inhibitor Leflunomide, which prevents degradation of IκB (Manna and Aggarwal, 1999). Because NF-κB also positively regulates HIV-1 replication itself, Leflunomide alone suppressed HIV-1 production. Nevertheless, the levels of HIV-1 replication in the presence of Leflunomide recovered by stimulation with CD28 mAb to the control levels and also with ICOS mAb to a lesser extent (Fig. 8). Leflunomide has been shown to inhibit protein tyrosine kinase and MAP kinase pathways involving MEK (Brazelton and Morris, 1996; Manna and Aggarwal, 1999). In the presence of PD98059, the MEK inhibitor, CD28, but not ICOS mAb, enhanced HIV-1 replication (Fig. 8). These results implies that the CD28-mediated positive effects on HIV-1 replication overcame its inhibitory effects in the presence of Leflunomide or PD98059, indicating that the CD28-medi-

ated signals suppressing HIV-1 replication might involve the NF-κB and MEK pathways. ICOS also showed a weak positive effect on HIV-1 replication in the presence of Leflunomide, indicating that its inhibitory effects might involve partly common pathways to those of CD28.

Among the chimeric Fc-fusion proteins with various B7-CD28 family molecules tested, CTLA-4/Fc and B7-H2 (B7h)/Fc significantly inhibited HIV-1 replication in CD4+ PBMC (Table 2). CTLA-4/Fc, known to efficiently block interactions between B7-1 (CD80)/B7-2 (CD86) and CD28, suppressed replication of HIV-1. A similar suppression of HIV-1 replication by CTLA-4/Fc has been previously reported (Diegel et al., 1993; Smithgall et al., 1995). This occurs probably because CTLA-4/Fc blocked the positive regulation through B7-1 (CD80)/B7-2 (CD86) that is expressed in a small proportion of PBMC. Although immobilized CD28-mAb mediated HIV-1 suppression in vitro, natural ligands of CD28 may preferentially mediate positive signals for HIV-1 replication. This notion is partly supported by previous reports showing that stimulation of CD28 with cross-linking mAb, but not with natural ligand B7-1, could activate ERK2 and p21ras (Nunes et al., 1994).

Because B7-H2 (B7h) is a natural ligand of ICOS, it was not unexpected that B7-H2 (B7h)/Fc suppressed HIV-1, as B7-H2 (B7h)/Fc binds ICOS and potentially activates signals downstream to ICOS on CD4+ T cells. This is consistent with our current findings that ICOS mAbs mediated suppression of HIV-1. The simple addition of ICOS/Fc, which is capable of binding B7-H2 (B7h), to the PBMC culture did not alter HIV-1 replication, suggesting that the levels of natural ligands of ICOS present in the culture is insufficient to suppress HIV-1. Nevertheless, suppression of HIV-1 replication by B7-H2 (B7h)/Fc indicates that natural or artificial ligands of ICOS potentially elicit suppressive effects on HIV-1 replication.

B7-H2 (B7h) is more broadly expressed than B7-1 (CD80)/B7-2 (CD86) in both lymphoid and a variety of nonlymphoid tissues (Swallow et al., 1999), implying that ICOS-expressing cells could be regulated by various peripheral cells. A recent clinical study on HIV-1 infection showed that ICOS expression peaked during the early stages of primary infection, dropped to control levels in the asymptomatic period, and subsequently increased as AIDS developed (Lucia et al., 2000). They also showed that HIV-1 gp120 induced transient ICOS expression in naïve T cells. Our results suggest that ICOS stimulation may render uninfected T cells resistant to HIV-1 replication, and less efficiently suppress HIV-1 expression in persistently infected cells. ICOS-mediated suppression of HIV-1 replication may play some roles in regulating HIV-1 in vivo.

In conclusion, stimulation of ICOS by mAb or its ligands mediated suppression of the early steps of HIV-1 replication in CD4+ PBMC. These findings contribute to our understanding of HIV-1 regulation in vivo, and the development of strategies to control HIV-1.

Materials and methods

Cells

PBMC from healthy individuals were isolated by Ficoll-Hypaque density centrifugation. CD4+ T cell fractions were negatively purified from PBMC by magnetic cell sorting following incubation with a cocktail of mAbs for CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγδ, and Glycophorin A, using MACS Cell Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry showed that these fractions contained 93% CD4+ cells. Unfractionated PBMC or CD4+ T cells were stimulated with 0.05% PHA P (Becton Dickinson, MD) for 30 min, washed, and then cultured in RPMI 1640 (GIBCO BRL, Long Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 U/ml recombinant human interleukin-2 (IL-2) (Shionogi, Osaka, Japan) and 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37 °C in a 5% CO_2 incubator at a concentration of 1×10^6 cells/ml for 4–6 days. Cells were then used for HIV-1 infection. In some experiments, Dynabeads (Dynal A.S., Oslo, Norway) coated with anti-CD8 mAbs were used to enrich CD4+ PBMC. 293T cells were maintained in Dulbecco's Modification of Eagle's Medium (ICN Biomedicals Inc., OH) supplemented with 10% FCS.

Flow cytometric analysis

The cells were incubated with mAbs antigens or control mouse immunoglobulin, washed, and subsequently stained with FITC-conjugated goat anti-mouse IgG + IgM mAbs (Immunotech, Marseilles, France) for 30 min at room temperature. The cells were washed and analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA).

Antibodies and reagents

The mAbs to human CD28 family molecules used were TN228, anti-CD28 (Lanier et al., 1995), MIH8, anti-CTLA-4, SA12, anti-ICOS (Sakamoto et al., 2001), and MIH4, anti-PD-1 (Youngnak et al., 2003), all of which were prepared as purified mouse IgG1. MIH8 anti-CTLA4 was generated by immunizing DBA/2 mice with human CTLA-4-transfected P815 cells (Oki et al., 1999). SA12 was kindly provided by Japan Tobacco Inc. (Osaka, Japan). Fluorescein isothiocyanate (FITC)-conjugated CD4, phycoerythrin (PE)-conjugated CXCR4 and CCR5, and their isotype-matched control antibodies were purchased from BD Bioscience (San Diego, CA). Human CD28/Fc, CTLA-4/Fc, B7-1/Fc, and B7-H2/Fc, which were chimeric recombinant proteins fused to the Fc region of human IgG, were purchased from R & D Systems, Inc. (Minneapolis, MN). Human ICOS/Fc was provided by Japan Tobacco Inc. A NF-kB inhibitor Leflunomide, MEK (MAPKK) inhibitor PD98059 (Sigma-

Aldrich, St. Louis, MO), and p38 MAP kinase inhibitor SB202190 (Calbiochem, San Diego, CA) were also used.

Proliferation assay

Cell proliferation was measured using the [^{3}H]thymidine uptake method. PHA-stimulated CD4+ T cells cultured for 4–6 days in a medium containing IL-2 were incubated in 96-well flat-bottom plates at a concentration of 10^5 cells/200μl in either the presence or absence of mAbs for 4 days. [^{3}H]thymidine (37 kBq) was added into each well during the last 16 h. Cells were then harvested on a glass filter, and the incorporation of [^{3}H]thymidine into the cells was monitored with a MicroBeta scintillation counting system (Pharmacia Biotech, Cambridge, UK).

Cytotoxicity test

PHA-stimulated CD4+ T cells cultured in IL-2-containing medium for 4–6 days were incubated in 96-well flat bottom plates at a concentration of 10^5 cells/well in either the presence or absence of mAbs, and LDH released into the supernatant was monitored every 24 h over 3 days using a Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI).

Virus preparation and infection

X4 HIV 1 strain NL4-3 (Adachi et al., 1986) and R5 HIV-1 strain JR-CSF (Koyanagi et al., 1987), kindly provided by Dr. Y. Koyanagi (Tohoku University), were propagated in PBMC and the supernatant was used as virus source. For HIV-1 infection, PHA-stimulated unfractionated or CD4+ PBMC were cultured in a medium containing IL-2 for 4–6 days, incubated with HIV-1-containing supernatant at a concentration of 4×10^6/ml for 2 h at 37 °C, washed, and then cultured in a fresh medium containing IL-2. HIV-1 pseudotype virus was prepared as described elsewhere (Masuda et al., 1995; Planelles et al., 1995). Briefly, using Lipofectamine (GIBCO BRL), 10^6 of 293T cells were transfected with 1 μg each of pNL4-3lucΔenv vector and pLET-LAI (Poon et al., 1998) or pHCMVG (Yee et al., 1994), all of which were kindly provided by Dr. Irvin S.Y. Chen, University of California. pNL4-3 lucΔenv is an envelope defective pNL4-3 vector containing the luciferase gene inserted at the *Nef* site (Masuda et al., 1995). pLET-LAI encodes envelope proteins of HIV-1 LAI strain (Poon et al., 1998), and pHCMVG encodes a vesicular stomatitis virus envelope (Yee et al., 1994). Culture supernatant (5 ml) of the transfected 293T cells was harvested 48 h posttransfection, filtered through a 0.45-μm filter, and used as pseudotype HIV-1. For infection, PHA-stimulated CD4+ T cells were incubated for 3 h with a pseudotype HIV-1-containing supernatant that had been treated with DNase I (140 u/ml, Worthington, NJ) in the presence of 10 mM $MgCl_2$ at 37 °C for 1 h to avoid DNA contamination before infection. The cells were then washed and cultured in a IL-2-containing medium at a concentration of 5×10^5 cells/ml in 24- or 12-well plates for 48 h in either the presence or absence of mAbs. Virus replication was determined from luciferase activity.

Treatment of HIV-1-infected cells with mAbs

MAbs at concentrations ranging between 0.025 and 10 μg/ml were either added directly to the culture or precoated in the wells before culture. For precoating, mAbs diluted in PBS was incubated overnight in 96-well or 48-well flat-bottomed plates at 4 °C. The wells were washed with PBS immediately before the culture of HIV-1-infected cells.

Analysis of HIV-1 cDNA synthesis

HIV-1-infected cells were harvested after various incubation periods, washed, and 10^6 cells were disrupted in a 150 μl urea lysis buffer (4.7 M urea, 1.3% sodium dodecyl sulfate, 0.23 M NaCl, 0.67 mM EDTA [pH 8.0], 6.7 mM Tris–HCl [pH 8.0]) and underwent phenol–chloroform extraction and ethanol precipitation. Precipitated DNA was then resuspended in 30 μl water. A 0.5 μg DNA pellet of each sample underwent PCR using primer pairs (Zack et al., 1990) amplifying a DNA fragment between HIV-1 LTR U3 and HIV gag (M667/M661) for 35 cycles at 95 °C for 1 min, 65 °C for 2 min, and 72 °C for 2 min as previously described (Tsurutani et al., 2000; Zack et al., 1990). For HIV-1 DNA standards, 250–5000 copies of linearized HIV-1 JR-CSF DNA were amplified in parallel. Amplified products were resolved on a 2% agarose gel and stained with SYBER-Green (FMC Bioproduct, Rocklan, MN).

Enzyme-linked immunosorbent assay (ELISA)

HIV-1 p24, human RANTES, IFN-γ, SDF-1α, and MIP-1α in cell-free supernatants were measured by HIV-1 p24 Antigen ELISA kit (ZeptoMetrix, NY), and human RANTES, IFN-γ, SDF-1α, and MIP-1α immunoassay kit (R & D Systems), respectively. Absorbances were measured at 450 nm using microplate reader (Bio-Rad, Hercules, CA) and analyzed with Microplate Manager III software.

Measurement of cytokines

Human IL-4, IL-5, IL-10, and TNFα in the culture supernatants were measured by the Cytometric Bead Array Kit (BD Biosciences, Pharmingen, CA) in accordance with the manufacturer's instructions.

Luciferase assay

For luciferase analysis, cells were lysed by 100 μl of 1× luciferase lysis buffer (Promega) 24 h postinfection, and

luciferase expression was measured with a luciferase assay system (Lumat, EG&G Berthold, Germany).

Statistical analysis

Differences among HIV-1 p24 values of individual sample groups vs. controls were evaluated by Dunnett's *t* test using SPSS Base 11.0J (SPSS Inc., Chicago, IL). *P* values less than 0.05 were considered to be statistically significant.

Acknowledgments

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Original article

Expression of small hairpin RNA by lentivirus-based vector confers efficient and stable gene-suppression of HIV-1 on human cells including primary non-dividing cells

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Abstract

RNA interference (RNAi) is a sequence-specific RNA degradation process mediated by short double-stranded RNAs termed small interfering RNAs. Here, we describe the lentivirus-based vector small interfering RNA system expressing. As a pilot study, we generated constructs expressing small hairpin RNA (shRNA) specific for luciferase gene (shLuc) or green fluorescence protein (shGFP) under the control of human H1 RNA polymerase III promoter. The effect of the shRNA was evaluated against HIV-1 infection in a single-round or multiple-round infectious system using an HIV-1 molecular clone carrying the *luc* or *GFP* gene. In the single-round infectious system, cells transduced with shLuc by lentiviral vector significantly reduced (~90% reduction) viral gene expression after challenge infection at a multiplicity of infection of 10. These transduced cells continued to resist against at least four sequentially repeated challenge infections. Importantly, this efficient antiviral activity persisted over 35 days in culture. In a multiple-round infectious system using a replication-competent HIV-1 molecular clone carrying the *GFP* gene, we also observed that a lentiviral vector expressing shGFP could inhibit HIV-1 replication for at least 1 week. The profound effect of lentiviral shRNA was also observed in human primary monocyte-derived macrophages. Thus, shRNA introduced through the lentiviral vector can be useful for efficient and stable gene suppression in human cells including primary non-dividing cells. Moreover, quantitative analysis of viral cDNA synthesis on challenge infection showed that viral genomic RNAs packaged in incoming virus core might not be targeted by shLuc. Instead, the degradation of transcripts from integrated proviral DNAs might be a major cause of the profound reduction in HIV-1 gene expression by shRNA in our system.

Keywords: Small hairpin RNAs (shRNA); Lentivirus vector; Monocyte-derived macrophages (MDMs); HIV-1; H1 promoter

1. Introduction

RNA interference (RNAi) is a sequence-specific RNA degradation process conserved in eukaryotes [1,2]. Short (21–25 nucleotides) double-stranded RNA (dsRNA) processed by an RNase III-related nuclease (Dicer)[3], termed small interfering RNAs (siRNAs), can mediate sequence-specific RNA degradation through a dsRNA-induced silencing complex (RISC) [4–6]. Synthetic duplexes of small RNAs mimicking siRNA can specifically silence genes without activating non-specific suppression by dsRNA-dependent protein kinase [7–9], becoming a powerful tool for the analysis of gene functions. In addition, synthetic siRNAs as possible therapeutic agents against specific pathogens such as HIV-1 have recently been reported [9–12].

Meanwhile, RNA polymerase III-based small hairpin RNA (shRNA) expression vector systems have been established to induce RNAi in mammalian cells [13–17]. Although these vectors provide certain advantages over chemically synthesized siRNAs, some disadvantages still remain, including transient shRNA expression and low transfection efficiency, especially in primary non-dividing cells. To overcome these limitations, shRNA delivery systems using retroviral vectors [18–20], adenoviral vector [21] and, more recently, lentiviral vector [22,23] have been reported. We

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describe here a lentivirus-mediated shRNA delivery system. As a pilot study, we generated lentiviral vector [24] expressing shRNA specific for luciferase (*luc*) gene [6,7] or green fluorescence protein (GFP) [14] under the control of human H1 RNA polymerase III promoter [13]. We show that the introduction of shRNA through lentiviral vectors efficiently and specifically reduces viral gene expression of HIV-1 carrying the *luc* gene in 293T cells for more than 35 days in culture. A similar specific and profound reduction in viral gene expression was also reproduced in human monocyte-derived macrophages. Thus, shRNA introduced through the lentivirus gene-delivery system can confer an efficient and stable antiviral state on human cells, including primary non-dividing cells. In addition, Stevenson and coworkers recently reported that siRNAs direct the specific degradation of genomic HIV-1 RNA, thereby preventing the subsequent synthesis of viral cDNA [9]. In contrast to this reported result, quantitative analysis of viral cDNA synthesis revealed that shRNAs from our RNA pol III-expression system did not target HIV-1 genomic RNA at steps prior to reverse transcription, suggesting that our shRNA might target mainly viral transcripts from integrated proviral DNA.

2. Materials and methods

2.1. Constructs

To generate pGEM-H1, the H1-RNA promoter was amplified by PCR using genomic DNA derived from 293T cells as a template, with oligonucleotides H1-F (5′CCAT GGAATTCGAACGCTGACGTCAT3′) and H1-R (5′GGAAGATCTGTGGTCTCATACA GAACTTATAAGATTC3′). The PCR product was inserted into pGEM-T Easy (Promega) by TA-cloning. pGEM-H1-shLuc was constructed by ligating an annealing product of sense oligonucleotides (5′GATCCCCGTACGCGGAATACTTCGATTCAAGAGATCGAAGTAT TCCGCGTACGTTTTTGGAAAG3′) and antisense oligonucleotides (5′TCGACTTTCCAA AAACGTACGCGGAATACTTCGATCTCTTGAATCGAAGTATTCCGCGTACGGG3′) with the 3.2-kb *Bgl*II–*Sal*I fragment from pGEM-H1. To generate pGEM-H1-shGFP, pGEM-H1 was digested with *Bgl*II–*Sal*I. The 3.2-kb *Bgl*II–*Sal*I fragment from pGEM-H1 was ligated with an annealing product of sense oligonucleotides (5′GATCCCGAAGAAGTCGTGCTGCTTCAAGAGAGAAGCAGCACGACTTCTTCTTTTTGGAAAG3′) and antisense oligonucleotides (5′TCGACTTTCCAAAAAGAAGAAGTCGTGCTGCTTCTCTCTTGAAGAAGCAGCACGACTTCTTCGG3′). pGEM-H1-shVif was constructed such that the 3.2-kb *Bgl*II–*Sal*I fragment from pGEM-H1 was inserted into an annealing product of sense oligonucleotides (5′GATCCCAGCACACAAGTAGACCCTGTTCAAGAGACAGGGTCTACTTGTGTGCTTTTTTGGAAAG3′) and antisense oligonucleotides (5′TCGACTTTC CAAAAAAGCACACAAGTAGACCCTGTCTCTTGAACAGGGTCTACTTGTGTGCTG G3′). To construct pCS-H1, the 0.2-kb *Eco*RI–*Spe*I fragment from pGEM-H1 was cloned into the *Eco*RI–*Spe*I sites of pT7Blue (Novagen). The resultant plasmid was digested by *Eco*RI and *Sal*I. The DNA fragment that encoded H1 RNA promoter sequence was ligated into the 7.9-kb *Eco*RI–*Xho*I fragment of pCS-CDF-CG-PRE vector, in which the woodchuck hepatitis virus post-transcriptional regulatory element and the central DNA flap sequence were inserted into original self-inactivating lentivirus vector (pCS-CG) [24] (details of cloning are available on request). pCS-H1-shLuc, pCS-H1-shGFP or pCS-H1-shVif were constructed by inserting the 0.3-kb *Eco*RI–*Sal*I fragment from pGEM-H1-siLuc, pGEM-H1-shGFP or pGEM-H1-shVif into the 7.9-kb *Eco*RI–*Xho*I fragment of pCS-CDF-CG-PRE, respectively.

2.2. Cells

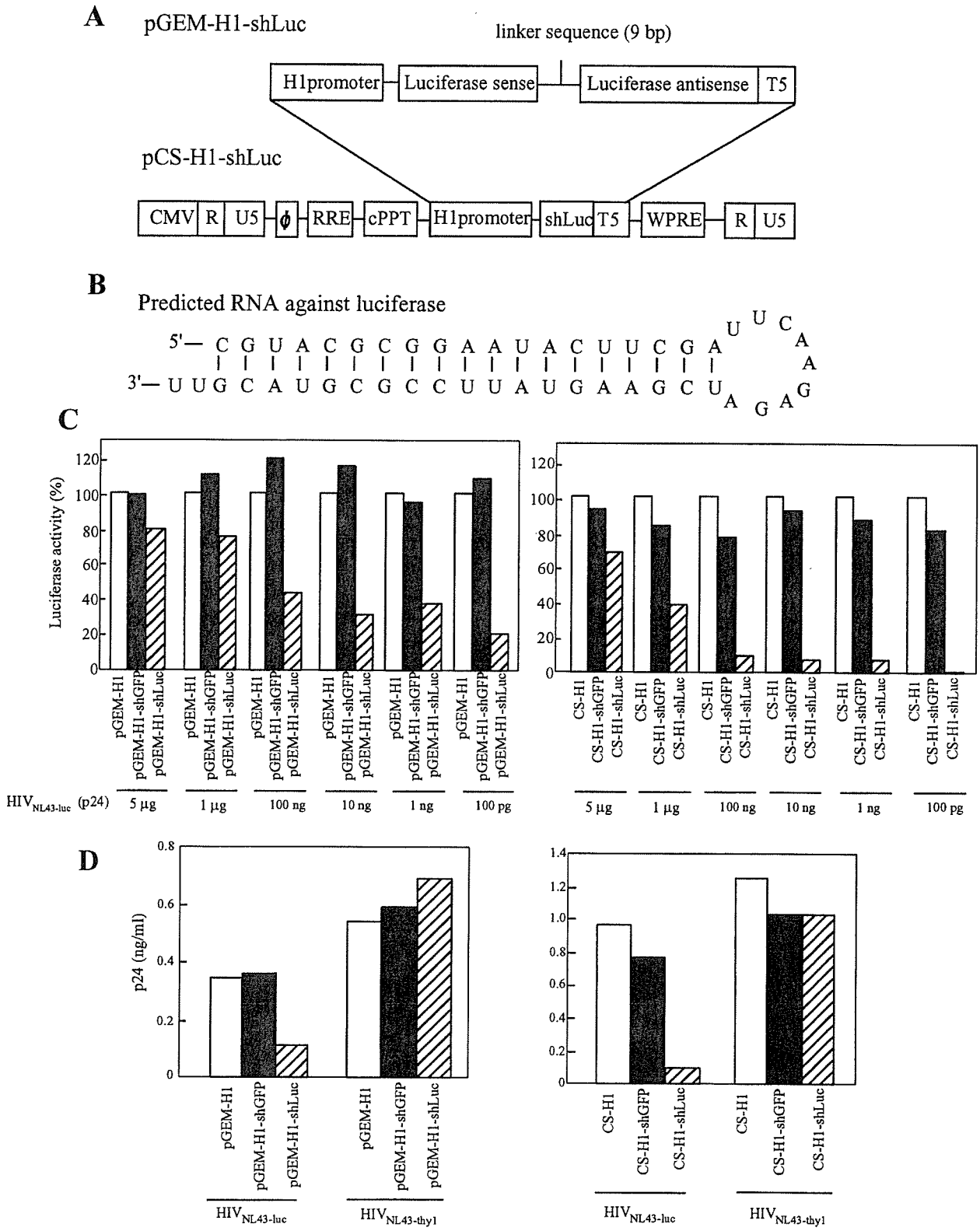
293T cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS). Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by the Ficoll centrifugation method. PBMCs were resuspended at 10^6 cells per ml in RPMI-1640 medium containing 10% FCS and plated. After 2 h of adhesion at 37 °C, adherent cells were collected. To allow differentiation of macrophages, monocytes were cultured for 7 days in RPMI-1640 medium supplemented with 10% human AB serum.

2.3. Virus preparation

The pseudotyped virus was generated by co-transfection of 293T cells with 1 μg of the pNLlucΔenv and 1 μg of the amphotropic Moloney murine leukemia virus envelope expression vector (pJD-1) or VSV-G (pMD.G). The culture supernatants were harvested at 48 h post-transfection and filtered through 0.45-μm pore-size filters. The replication-competent HIV-1 carrying GFP was similarly generated by transfection of 293T cells with pNL-EGFP vector [25] (kind offer from Dr. Y.C. Sung). The VSV-G pseudotyped lentiviral vectors were produced by transient transfection of 293T cells (5×10^6) plated in 100-mm dishes. A vector construct (17 μg), VSV-G-expressing construct pMD.G (5 μg), rev-expressing construct pRSV-Rev (5 μg) and the packaging construct pMDLg/p.RRE (12 μg) [26] were cotransfected into 293T cells using the calcium phosphate precipitation method. Supernatants were harvested and filtered through 0.45-μm pore-size filters. The lentiviral vector was concentrated ~40-fold by low centrifugation at 6000 × *g* for 16 h and resuspended in 2 ml of RPMI-1640 medium.

2.4. Transfection

293T cells (1×10^5 cells) were transfected with 1 μg of pGEM-H1 or pGEM-H1-shLuc or pGEM-H1-shGFP by Lipofectamine (Invitrogen) according to the manufacturer's instructions. The chemically synthesized siRNAs (QIAGEN)


A
pGEM-H1-shLuc
linker sequence (9 bp)
H1promoter
Luciferase sense
Luciferase antisense
T5
pCS-H1-shLuc
CMV
R
U5
ϕ
RRE
cPPT
H1promoter
shLuc
T5
WPRE
R
U5
B
Predicted RNA against luciferase
5'— C G U A C G C G G A A U A C U U C G A U U C A A
3'— U U G C A U G C G C C U U A U G A A G C U A G A G
C
Luciferase activity (%)
pGEM-H1
pGEM-H1-shGFP
pGEM-H1-shLuc
CS-H1
CS-H1-shGFP
CS-H1-shLuc
HIVNL43-luc (p24)
5 μg
1 μg
100 ng
10 ng
1 ng
100 pg
D
p24 (ng/ml)
HIVNL43-luc
HIVNL43-thy1

used for the siLuc are 5′-CGUACGCGGAAUACUU-CGAdTdT-3′ (sense) and 5′-UCGAAGUAUUCCGCG-UACG dTdT-3′ (antisense), and for the siGEP, are 5′-GCAAGCUGACCCUGAAGUUCAUdTdT-3′ (sense) and 5′-GAACUUCAGGGUCAGCUUGCCGdTdT-3′ (antisense). 293T cells were transfected with 100 pmol of each synthetic siRNA in the presence of oligofectamine (Invitrogen) according to the manufacturer's protocol.

2.5. HIV-1 infection

293T cells or human primary monocyte-derived macrophages (MDMs) were infected with 1 ml pseudotype virus. After 48 h, cells were washed with PBS and suspended in cell lysis buffer. The level of HIV-1 p24 antigen in each cell lysate was determined using the enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol.

2.6. Analysis of HIV-1 DNA

Cells were infected with 1 ml DNaseI-treated virus. Total DNA was extracted at 2 days post-infection from the cells by using the urea-lysis method. Briefly, cells were lysed with 0.3 ml of urea lysis buffer (7 M urea, 2% SDS, 1 mM EDTA, 10 mM Tris–HCl pH 8.0, 0.35 M NaCl). Total DNA was purified from the cell lysates by phenol–chloroform extraction followed by ethanol precipitation. Analysis of HIV-1 DNA was performed by quantitative PCR with HIV-1 specific primers (R/U5: 5′-GGCTAACTAGGGAACCCAC-TG-3′ and 5′-CTGCTAGAGATTTTCCACACTGAC-3′, R/gag: 5′-GGCTAACTAGGGAACCCACT G-3′ and 5′-GA-CGCTCTCGCACCCATCTC-3′ or vif-F/vif-R: 5′-GAG-ATATAGCACAC AAGTAGACC-3′ and 5′-GCTAGTGC-CAAGTACTGTGAGAT-3′) by *Taq* DNA polymerase (Invitrogen). The thermal program was 1 min hot start at 94 °C, followed by 30 cycles of denaturizing at 94 °C for 1 min, annealing at 65 °C for 2 min and extension at 72 °C for 2 min. PCR products were separated on 2% agarose gels and stained with SYBER Green. Quantitative analysis was carried out with the LightCycler quick real-time PCR system 330 (Roche Diagnostics).

3. Results

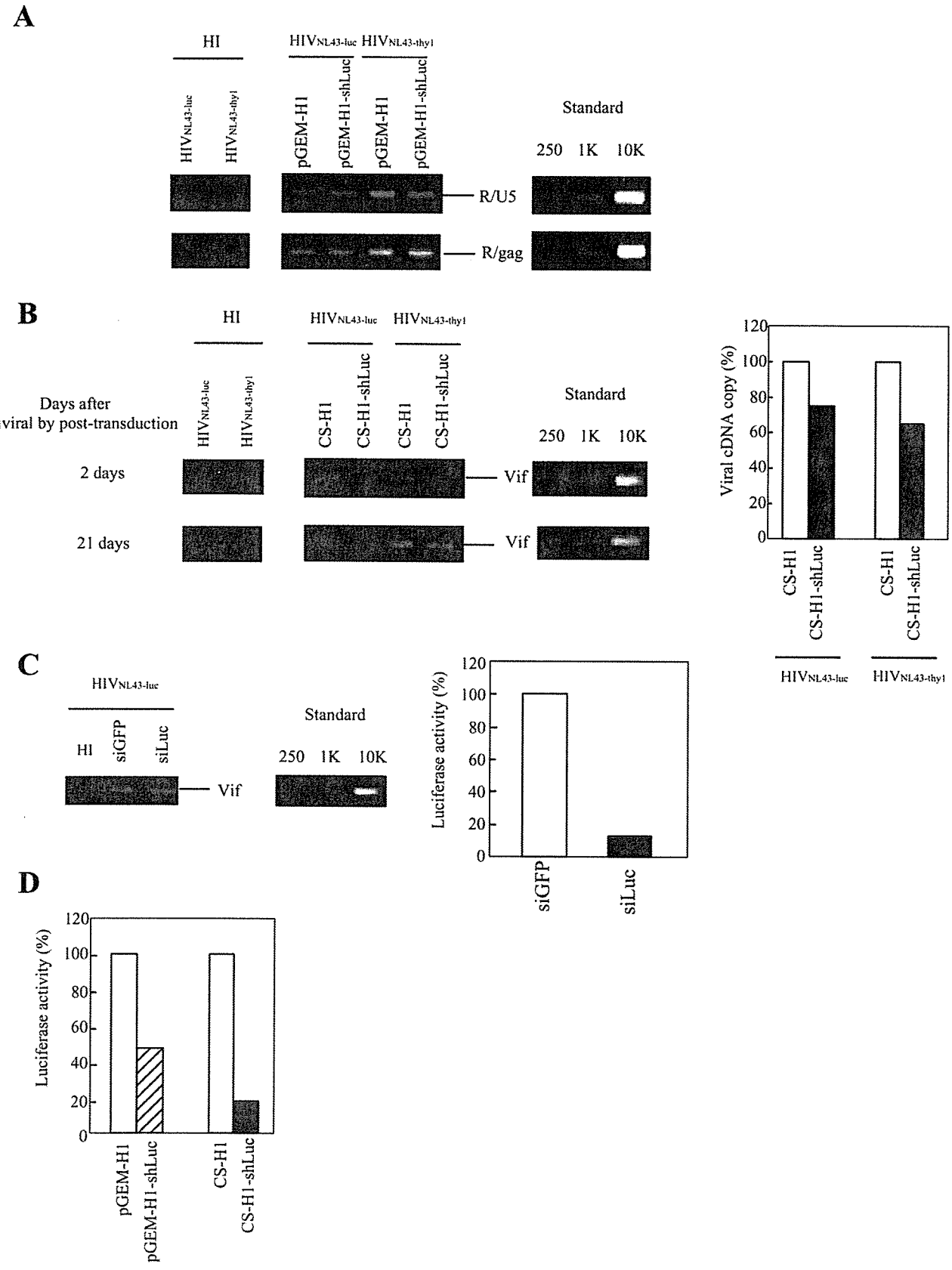
3.1. Construction of shRNA expression vector against luciferase

Based on the previous report by Agami and coworkers [13], we generated plasmid-based (pGEM-H1) and self-inactivating lentivirus-based [24] (pCS-H1) vectors to express shRNA under the control of human H1 RNA polymerase III (H1 pol III) promoter (Fig. 1A). As a pilot study, we inserted the shRNA sequence targeted for the firefly luciferase gene (*luc*) downstream of the H1 promoter with five thymidines (T5) at the 3′ end as a termination signal. The predicted stem-loop structure of the transcripts consists of a 19-nucleotide (nt) sequence derived from the Luc transcript, separated by a loop of 9 nt from the reverse complement of the same 19-nt sequences with two 3′ overhanging uridines (Fig. 1B).

3.2. Inhibition of HIV-1 gene expression by shRNA expression vector

To evaluate the effect of the shRNA against the firefly luc (shLuc) expressed from plasmid-based (pGEM-H1-shLuc) or lentivirus-based (pCS-H1-shLuc) vectors, we used a single-round infection assay using an env-defective HIV-1 molecular clone [27] carrying the firefly luc gene (pNLlucΔenv) [28] pseudotyped with Moloney MuLV envelope or G protein of vesicular stomatitis virus ($HIV_{NL43\text{-}luc}$ pseudotype). This single-round infection assay was previously shown to be useful for quantitative monitoring of each event during a single round of HIV replication, including cDNA synthesis from viral genomic RNA and protein expressions from proviral DNA that is stably integrated into host chromosomal DNA [28]. We introduced the shLuc into 293T (human embryonic kidney) cells by transfection of pGEM-H1-shLuc plasmid or by infection with the VSV-G pseudotyped lentivirus (CS-H1-shLuc) generated with a third-generation packaging system in which all accessory (*vif, vpr, vpu, nef* and *tat*) genes have been eliminated [26]. At 48 h post-shLuc introduction, cells were challenged by infection with $HIV\text{-}1_{NL43\text{-}luc}$ pseudotypes at various virus concentrations. One nanogram of p24 protein per 10^5 cells corre-

Fig. 1. Construction of an shRNA expression vector against luciferase. (A) The genome structure to express shRNA for the luciferase gene, in which the luciferase target sequence (19 nt) in sense and antisense orientations with a 9-bp linker sequence is driven by the H1 RNA promoter (pGEM-shLuc). The shRNA transcript terminates with five thymidines (T5) as a termination signal. A self-inactivating (SIN) lentiviral vector [24] was used to express the shRNA (pCS-H1-shLuc). (B) The predicted secondary structure of shRNA designed to target the luciferase gene. (C) Inhibition of HIV-1 gene expression by the shRNA expression vector. We introduced shRNA into 293T cells by transfection with 0.5 μg of pGEM-H1 (empty control), pGEM-H1-shGFP (irrelevant shRNA) or pGEM-H1-shLuc (left) or by infection with a lentivirus packaging CS-H1 (empty control), CS-H1-shGFP (irrelevant shRNA) or CS-H1-shLuc (right). At 48 h after shRNA introduction, the cells were infected by $HIV_{NL43\text{-}luc}$ pseudotypes (VSV-G) at various p24 concentrations (100 pg–5 μg). The level of viral gene expression was evaluated by measuring the luciferase activity 48 h later. (D) The level of viral gene expression in 293T cells transfected with pGEM-H1, pGEM-H1-shGFP or pGEM-H1-shLuc was also evaluated by measuring the level of p24, using the ELISA at 48 h post-challenge infection by $HIV_{NL43\text{-}luc}$ pseudotypes (~20 ng p24). In the 293T cells transduced with lentiviral vector (CS-H1, CS-H1-shGFP or CS-H1-shLuc), challenge infection by $HIV_{NL43\text{-}luc}$ or $HIV_{NL43\text{-}thy}$ pseudotypes was done at 7 days post-transduction to avoid cross-detection of p24 carried over by lentivirus particles. The level of p24 in the lentiviral-transduced cells at 48 h after challenge infection is shown.


A
HI
HIVNL43-luc
HIVNL43-thy1
HIVNL43-luc
HIVNL43-thy1
pGEM-H1
pGEM-H1-shLuc
pGEM-H1
pGEM-H1-shLuc
Standard
250 1K 10K
R/U5
R/gag
B
HI
HIVNL43-luc
HIVNL43-thy1
Days after lentiviral by post-transduction
HIVNL43-luc
HIVNL43-thy1
CS-H1
CS-H1-shLuc
CS-H1
CS-H1-shLuc
Standard
250 1K 10K
2 days
21 days
Vif
Vif
Viral cDNA copy (%)
120
100
80
60
40
20
0
CS-H1
CS-H1-shLuc
CS-H1
CS-H1-shLuc
HIVNL43-luc
HIVNL43-thy1
C
HIVNL43-luc
HI
siGFP
siLuc
Standard
250 1K 10K
Vif
Luciferase activity (%)
120
100
80
60
40
20
0
siGFP
siLuc
D
Luciferase activity (%)
120
100
80
60
40
20
0
pGEM-H1
pGEM-H1-shLuc
CS-H1
CS-H1-shLuc

sponds to a multiplicity of infection (MOI) of approximately 0.1. Virus gene expressions were determined at 48 h post-challenge infection by measuring the luciferase activity (Fig. 1C) and the p24 level (Fig. 1D) in the cell lysates. To control for non-specific effects of the shLuc, empty vector lacking the shRNA (pGEM-H1) or vector expressing an irrelevant shRNA against the *GFP* gene (pGEM-H1-shGFP) was also examined in parallel. Challenge infection of HIV-$1_{NL43\text{-}luc}$ at low input of p24 (less than 10 ng of p24 corresponding to an MOI of 1.0), pGEM-H1-shLuc exhibited an 80% reduction of the luciferase activity compared with the level by control vectors pGEM-H1 or pGEM-H1-shGFP (Fig. 1C left). However, at high concentrations of challenged HIV-$1_{NL43\text{-}luc}$ (1 or 5 μg of p24 corresponding to an MOI of 100 or 500, respectively), the inhibitory effect by pGEM-H1-shLuc was reduced to as low as ~20% reduction compared with the level by control vectors. On the other hand, a more profound reduction in HIV-$1_{NL43\text{-}luc}$ gene expression was observed when shLuc was introduced in 293T cells through the lentiviral vector system (Fig. 1C right). Compared with lentiviral vector expressing empty (CS-H1) or irrelevant shRNA (CS-H1-shGFP), lentiviral vector expressing shLuc (CS-H1-shLuc) reduced more than 90% of the HIV-$1_{NL43\text{-}luc}$ gene expression when the transduced 293 T cells (CS-H1-shLuc) were infected with as high as 100 pg p24 of virus, corresponding to an MOI of 0.01. Significant (~60%) reduction was still noticed when the transduced 293T cells were infected with 1 μg input of p24, which corresponds to an MOI of 100. To confirm the specific effect of the shLuc to the luc sequence, we examined the pGEM-H1-shLuc and CS-H1-shLuc against challenge infection of $HIV_{NL43\text{-}thy}$, which is identical to $HIV_{NL43\text{-}luc}$ except that it carries the mouse thy-1.2 gene instead of the luc gene [29]. These effects of shLuc are specific to the luc gene, since no obvious effect was observed when cells were challenged by infection with $HIV_{NL43\text{-}thy}$ pseudotype (Fig. 1D).

3.3. Effect of shRNA expression on viral cDNA synthesis

Following binding and entry of HIV-1 into the target cells, viral cDNA is synthesized in the cytoplasm using genomic viral RNA originally packaged in the incoming virus particle as a template. There have been recent reports that siRNAs direct the specific degradation of genomic HIV-1 RNA, thereby preventing the subsequent synthesis of viral cDNA [9,10,30]. We addressed our attention to the effects of shLuc on viral cDNA synthesis after infection. Total DNA was harvested from cells 48 h after challenge infection and subjected to quantitative PCR analysis to monitor viral cDNA synthesis. We used HIV-1-specific primer pairs M667/AA55 for the early viral DNA species (R/U5) or M667/M661 for the formation of complete or nearly complete viral DNA (R/gag) [31]. No significant reduction in the levels of early (R/U5) and late (R/gag) stage of viral cDNA intermediates was observed when shLuc was introduced through plasmid-based expression (pGEM-H1-shLuc) (Fig. 2A). A similar experiment was done to evaluate the effect of shLuc introduced by the lentiviral system (CS-H1-shLuc). Since pCS-H1-shLuc has homologous sequences to the R/U5 and R/gag of HIV-1, we used the primer pair for the *vif* gene (vifF/vifR) to detect the viral cDNAs of challenged HIV-1 specifically. CS-H1-shLuc reduced the level of the vifF/vifR to around 75% of the control level when virus ($HIV_{NL43\text{-}luc}$ pseudotype) was challenged at 2 days after lentiviral transduction (Fig. 2B). This slight reduction in viral cDNA synthesis might not be luc gene specific, since similar reduction was observed when $HIV_{NL43\text{-}thy}$ pseudotype was challenged. This reduction in viral cDNA synthesis was not observed when HIV was challenged at 21 days after lentiviral transduction (Fig. 2B). Since the efficient antiviral status persisted as long as 35 days after lentivirus transduction (Fig. 3A), these results suggested that viral genomic RNAs, templates for the viral cDNA synthesis by viral reverse transcriptase, might not be efficiently targeted by the siRNA in our shRNA expression system. Recent research has indicated that viral genomic RNA originally packaged in viral particles can also be targeted by chemically synthesized siRNA during its entry into cells [9,10,30]. Then, we evaluated whether chemically synthesized siRNAs could attack the HIV-1 genomic RNA. Chemically synthesized siRNA designed to target the same sequence of luc gene (siLuc) indeed suppressed viral gene expression efficiently (~90% reduction) compared with the effect by irrelevant siRNA designed to target *GFP* gene (Fig. 2C right). However, we could not observe significant reduction in the viral cDNA synthesis by the chemically synthesized siRNAs (Fig. 2C left). To specify the effect of the

Fig. 2. Effect of shRNA expression on viral cDNA synthesis. (A) 293T cells were transfected with 2 μg of pGEM-H1-shLuc or pGEM-H1. DNaseI-treated $HIV_{NL43\text{-}luc}$ or $HIV_{NL43\text{-}thy}$ pseudotypes were inoculated into cells at 48 h post-transfection. At 2 days after challenge infection, total DNA was extracted from the infected cells and subjected to quantitative PCR analysis by using primers for R/U5 or R/gag. For the HIV-1 DNA standard, linearized pNL43lucΔenv was amplified in parallel. Virus treated at 65 °C for 30 min prior to inoculation was used as heat-inactivated control (HI). (B) 293T cells were transduced with lentiviral vector CS-H1 or CS-H1-shLuc. At day 2 or 21 after transduction, cells were infected with DNaseI-treated $HIV_{NL43\text{-}luc}$ or $HIV_{NL43\text{-}thy}$ (~10 ng of p24). Viral cDNA synthesized from $HIV_{NL43\text{-}luc}$ or $HIV_{NL43\text{-}thy}$ pseudotype was analyzed by quantitative PCR using primers for vif-F and vif-R (vif). The level of viral cDNA (*vif*) was calculated with the LightCycler quick real-time PCR system 330 (Roche Diagnostics). The level of viral cDNA in the same amount of cellular DNA (adjusted by β-globin gene) was indicated by normalizing to the level in the control cells (CS-H1) as 100% (right). (C) 293T cells were transfected with 100 pmol of chemically synthesized siRNA (siGFP or siLuc). At 20 h after transfection, 293T cells were infected with $HIV_{NL43\text{-}luc}$ pseudotype (~10 ng of p24). At 24 h after challenge infection, total DNA from the 293T cells was subjected to quantitative PCR using primers for vif to monitor the level of viral cDNA synthesis (left). At the same time, viral gene expression in the 293T cells was examined by measuring the luciferase activity (right). (D) 293T cells were first infected with $HIV_{NL43\text{-}luc}$. Then, at 24 h after infection, infected cells were transfected with 2 μg of pGEM-H1-shLuc or pGEM-H1, or transduced with lentiviral vector CS-H1 or CS-H1-shLuc. Two days later, the level of viral protein production was evaluated by measuring the luciferase activity.