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.

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References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59 (2), 284–291.

Avraham, A., Jung, S., Samuels, Y., Seger, R., Ben-Neriah, Y., 1998. Co-stimulation-dependent activation of a JNK-kinase in T lymphocytes. Eur. J. Immunol. 28 (8), 2320–2330.

Azuma, M., Ito, D., Yagita, H., Okumura, K., Phillips, J.H., Lanier, L.L., Somoza, C., 1993. B70 antigen is a second ligand for CTLA-4 and CD28. Nature 366 (6450), 76–79.

Beier, K.C., Hutloff, A., Dittrich, A.M., Heuck, C., Rauch, A., Buchner, K., Ludewig, B., Ochs, H.D., Mages, H.W., Kroczek, R.A., 2000. Induction, binding specificity and function of human ICOS. Eur. J. Immunol. 30 (12), 3707–3717.

Brazelton, T.R., Morris, R.E., 1996. Molecular mechanisms of action of new xenobiotic immunosuppressive drugs: tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. Curr. Opin. Immunol. 8 (5), 710–720.

Brunet, J.F., Denizot, F., Luciani, M.F., Roux-Dosseto, M., Suzan, M., Mattei, M.G., Golstein, P., 1987. A new member of the immunoglobulin superfamily—CTLA-4. Nature 328 (6127), 267–270.

Buonfiglio, D., Bragardo, M., Redoglia, V., Vaschetto, R., Bottarel, F., Bonissoni, S., Bensi, T., Mezzatesta, C., Janeway Jr., C.A., Dianzani, U., 2000. The T cell activation molecule H4 and the CD28-like molecule ICOS are identical. Eur. J. Immunol. 30 (12), 3463–3467.

Coyle, A.J., Lehar, S., Lloyd, C., Tian, J., Delaney, T., Manning, S., Nguyen, T., Burwell, T., Schneider, H., Gonzalo, J.A., Gosselin, M., Owen, L.R., Rudd, C.E., Gutierrez-Ramos, J.C., 2000. The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. Immunity 13 (1), 95–105.

Diegel, M.L., Moran, P.A., Gilliland, L.K., Damle, N.K., Hayden, M.S., Zarling, J.M., Ledbetter, J.A., 1993. Regulation of HIV production by blood mononuclear cells from HIV-infected donors: II. HIV-1 production depends on T cell–monocyte interaction. AIDS Res. Hum. Retroviruses 9 (5), 465–473.

Dong, H., Zhu, G., Tamada, K., Chen, L., 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat. Med. 5 (12), 1365–1369.

Faris, M., Kokot, N., Lee, L., Nel, A.E., 1996. Regulation of interleukin-2 transcription by inducible stable expression of dominant negative and dominant active mitogen-activated protein kinase kinase kinase in jurkat T cells. Evidence for the importance of Ras in a pathway that is controlled by dual receptor stimulation. J. Biol. Chem. 271 (44), 27366–27373.

Finzi, D., Hermankova, M., Pierson, T., Carruth, L.M., Buck, C., Chaisson, R.E., Quinn, T.C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D.D., Richman, D.D., Siliciano, R.F., 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science 278 (5341), 1295–1300.

Frauwirth, K.A., Thompson, C.B., 2002. Activation and inhibition of lymphocytes by costimulation. J. Clin. Invest. 109 (3), 295–299.

Freeman, G.J., Long, A.J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L.J., Malenkovich, N., Okazaki, T., Byrne, M.C., Horton, H.F., Fouser, L., Carter, L., Ling, V., Bowman, M.R., Carreno, B.M., Collins, M., Wood, C.R., Honjo, T., 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp. Med. 192 (7), 1027–1034.

Hara, T., Fu, S.M., Hansen, J.A., 1985. Human T cell activation. II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44 kilodalton polypeptide (9.3 antigen). J. Exp. Med. 161 (6), 1513–1524.

Harhaj, E.W., Sun, S.C., 1998. IkappaB kinases serve as a target of CD28 signaling. J. Biol. Chem. 273 (39), 25185–25190.

Hehner, S.P., Hofmann, T.G., Dienz, O., Droge, W., Schmitz, M.L., 2000. Tyrosine-phosphorylated Vav1 as a point of integration for T-cell receptor- and CD28-mediated activation of JNK, p38, and interleukin-2 transcription. J. Biol. Chem. 275 (24), 18160–18171.

Ho, D.D., Moudgil, T., Alam, M., 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. N. Engl. J. Med. 321 (24), 1621–1625.

Ho, D.D., Neumann, A.U., Perelson, A.S., Chen, W., Leonard, J.M., Markowitz, M., 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373 (6510), 123–126.

Hutloff, A., Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., Kroczek, R.A., 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 397 (6716), 263–266.

Kane, L.P., Lin, J., Weiss, A., 2002. It's all Rel-ative: NF-kappaB and CD28 costimulation of T-cell activation. Trends Immunol. 23 (8), 413–420.

Kannagi, M., Masuda, T., Hattori, T., Kanoh, T., Nasu, K., Yamamoto, N., Harada, S., 1990. Interference with human immunodeficiency virus (HIV) replication by CD8+ T cells in peripheral blood leukocytes of asymptomatic HIV carriers in vitro. J. Virol. 64 (7), 3399–3406.

Koyanagi, Y., Miles, S., Mitsuyasu, R.T., Merrill, J.E., Vinters, H.V., Chen, I.S., 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science 236 (4803), 819–822.

Krummel, M.F., Allison, J.P., 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J. Exp. Med. 182 (2), 459–465.

Lanier, L.L., O'Fallon, S., Somoza, C., Phillips, J.H., Linsley, P.S., Okumura, K., Ito, D., Azuma, M., 1995. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J. Immunol. 154 (1), 97–105.

Latchman, Y., Wood, C.R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I., Iwai, Y., Long, A.J., Brown, J.A., Nunes, R., Greenfield, E.A., Bourque, K., Boussiotis, V.A., Carter, L.L., Carreno, B.M., Malenkovich, N., Nishimura, H., Okazaki, T., Honjo, T., Sharpe, A.H., Freeman, G.J., 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat. Immunol. 2 (3), 261–268.

Lenschow, D.J., Walunas, T.L., Bluestone, J.A., 1996. CD28/B7 system of T cell costimulation. Annu. Rev. Immunol. 14, 233–258.

Levine, B.L., Mosca, J.D., Riley, J.L., Carroll, R.G., Vahey, M.T., Jagodzinski, L.L., Wagner, K.F., Mayers, D.L., Burke, D.S., Weislow, O.S., St Louis, D.C., June, C.H., 1996. Antiviral effect and ex vivo CD4+ T cell proliferation in HIV-positive patients as a result of CD28 costimulation. Science 272 (5270), 1939–1943.

Linsley, P.S., Clark, E.A., Ledbetter, J.A., 1990. T-cell antigen CD28 medi-

ates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc. Natl. Acad. Sci. U. S. A. 87 (13), 5031–5035.

Lucia, M.B., Buonfiglio, D., Bottarel, F., Bensi, T., Rutella, S., Rumi, C., Ortona, L., Janeway Jr., C.A., Cauda, R., Dianzani, U., 2000. Expression of the novel T cell activation molecule hpH4 in HIV-infected patients: correlation with disease status. AIDS Res. Hum. Retroviruses 16 (6), 549–557.

Manna, S.K., Aggarwal, B.B., 1999. Immunosuppressive leflunomide metabolite (A77 1726) blocks TNF-dependent nuclear factor-kappa B activation and gene expression. J. Immunol. 162 (4), 2095–2102.

Masuda, T., Planelles, V., Krogstad, P., Chen, I.S., 1995. Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 att site: unusual phenotype of mutants in the zinc finger-like domain. J. Virol. 69 (11), 6687–6696.

Nishimura, H., Honjo, T., 2001. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. Trends Immunol. 22 (5), 265–268.

Nunes, J.A., Collette, Y., Truneh, A., Olive, D., Cantrell, D.A., 1994. The role of p21ras in CD28 signal transduction: triggering of CD28 with antibodies, but not the ligand B7-1, activates p21ras. J. Exp. Med. 180 (3), 1067–1076.

Oki, S., Kohsaka, T., Azuma, M., 1999. Augmentation of CTLA-4 expression by wortmannin: involvement of lysosomal sorting properties of CTLA-4. Int. Immunol. 11 (9), 1563–1571.

Pantaleo, G., Graziosi, C., Demarest, J.F., Butini, L., Montroni, M., Fox, C.H., Orenstein, J.M., Kotler, D.P., Fauci, A.S., 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362 (6418), 355–358.

Perelson, A.S., Neumann, A.U., Markowitz, M., Leonard, J.M., Ho, D.D., 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell lifespan, and viral generation time. Science 271 (5255), 1582–1586.

Piatak Jr., M., Saag, M.S., Yang, L.C., Clark, S.J., Kappes, J.C., Luk, K.C., Hahn, B.H., Shaw, G.M., Lifson, J.D., 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science 259 (5102), 1749–1754.

Planelles, V., Haislip, A., Withers-Ward, E.S., Stewart, S.A., Xie, Y., Shah, N.P., Chen, I.S., 1995. A new reporter system for detection of retroviral infection. Gene Ther. 2 (6), 369–376.

Poon, B., Grovit-Ferbas, K., Stewart, S.A., Chen, I.S., 1998. Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents. Science 281 (5374), 266–269.

Raab, M., Pfister, S., Rudd, C.E., 2001. CD28 signaling via VAV/SLP 76 adaptors: regulation of cytokine transcription independent of TCR ligation. Immunity 15 (6), 921–933.

Sakamoto, S., Tezuka, K., Tsuji, T., Hori, N., Tamatani, T., 2001. AILIM/ICOS: its expression and functional analysis with monoclonal antibodies. Hybrid. Hybridomics 20 (5–6), 293–303.

Salojin, K.V., Zhang, J., Delovitch, T.L., 1999. TCR and CD28 are coupled via ZAP-70 to the activation of the Vav/Rac-1-/PAK-1/p38 MAPK signaling pathway. J. Immunol. 163 (2), 844–853.

Sharpe, A.H., Freeman, G.J., 2002. The B7-CD28 superfamily. Nat. Rev. Immunol. 2 (2), 116–126.

Smithgall, M.D., Wong, J.G., Linsley, P.S., Haffar, O.K., 1995. Costimulation of CD4+ T cells via CD28 modulates human immunodeficiency virus type 1 infection and replication in vitro. AIDS Res. Hum. Retroviruses 11 (8), 885–892.

Swallow, M.M., Wallin, J.J., Sha, W.C., 1999. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity 11 (4), 423–432.

Tamatani, T., Tezuka, K., Hanzawa-Higuchi, N., 2000. AILIM/ICOS: a novel lymphocyte adhesion molecule. Int. Immunol. 12 (1), 51–55.

Tseng, S.Y., Otsuji, M., Gorski, K., Huang, X., Slansky, J.E., Pai, S.I., Shalabi, A., Shin, T., Pardoll, D.M., Tsuchiya, H., 2001. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. J. Exp. Med. 193 (7), 839–846.

Tsurutani, N., Kubo, M., Maeda, Y., Ohashi, T., Yamamoto, N., Kannagi, M., Masuda, T., 2000. Identification of critical amino acid residues in human immunodeficiency virus type 1 IN required for efficient proviral DNA formation at steps prior to integration in dividing and nondividing cells. J. Virol. 74 (10), 4795–4806.

Tuosto, L., Costanzo, A., Guido, F., Marinari, B., Vossio, S., Moretti, F., Levrero, M., Piccolella, E., 2000. Mitogen-activated kinase kinase kinase 1 regulates T cell receptor- and CD28-mediated signaling events which lead to NF-kappaB activation. Eur. J. Immunol. 30 (9), 2445–2454.

Walker, C.M., Moody, D.J., Stites, D.P., Levy, J.A., 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science 234 (4783), 1563–1566.

Walker, B.D., Chakrabarti, S., Moss, B., Paradis, T.J., Flynn, T., Durno, A.G., Blumberg, R.S., Kaplan, J.C., Hirsch, M.S., Schooley, R.T., 1987. HIV-specific cytotoxic T lymphocytes in seropositive individuals. Nature 328 (6128), 345–348.

Walunas, T.L., Lenschow, D.J., Bakker, C.Y., Linsley, P.S., Freeman, G.J., Green, J.M., Thompson, C.B., Bluestone, J.A., 1994. CTLA-4 can function as a negative regulator of T cell activation. Immunity 1 (5), 405–413.

Wei, X., Ghosh, S.K., Taylor, M.E., Johnson, V.A., Emini, E.A., Deutsch, P., Lifson, J.D., Bonhoeffer, S., Nowak, M.A., Hahn, B.H., et al., 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373 (6510), 117–122.

Wong, J.K., Hezareh, M., Gunthard, H.F., Havlir, D.V., Ignacio, C.C., Spina, C.A., Richman, D.D., 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278 (5341), 1291–1295.

Yee, J.K., Friedmann, T., Burns, J.C., 1994. Generation of high-titer pseudotyped retroviral vectors with very broad host range. Methods Cell Biol. 43 (Pt. A), 99–112.

Yoshinaga, S.K., Whoriskey, J.S., Khare, S.D., Sarmiento, U., Guo, J., Horan, T., Shih, G., Zhang, M., Coccia, M.A., Kohno, T., Tafuri-Bladt, A., Brankow, D., Campbell, P., Chang, D., Chiu, L., Dai, T., Duncan, G., Elliott, G.S., Hui, A., McCabe, S.M., Scully, S., Shahinian, A., Shaklee, C.L., Van, G., Mak, T.W., et al., 1999. T-cell co-stimulation through B7RP-1 and ICOS. Nature 402 (6763), 827–832.

Youngnak, P., Kozono, Y., Kozono, H., Iwai, H., Otsuki, N., Jin, H., Omura, K., Yagita, H., Pardoll, D.M., Chen, L., Azuma, M., 2003. Differential binding properties of B7-H1 and B7-DC to programmed death-1. Biochem. Biophys. Res. Commun. 307 (3), 672–677.

Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A., Chen, I.S., 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 61 (2), 213–222.

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Inhibition of human immunodeficiency virus type 1 replication by Z-100, an immunomodulator extracted from human-type tubercle bacilli, in macrophages

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Z-100 is an arabinomannan extracted from *Mycobacterium tuberculosis* that has various immunomodulatory activities, such as the induction of interleukin 12, interferon gamma (IFN-γ) and β-chemokines. The effects of Z-100 on human immunodeficiency virus type 1 (HIV-1) replication in human monocyte-derived macrophages (MDMs) are investigated in this paper. In MDMs, Z-100 markedly suppressed the replication of not only macrophage-tropic (M-tropic) HIV-1 strain (HIV-1_{JR-CSF}), but also HIV-1 pseudotypes that possessed amphotropic Moloney murine leukemia virus or vesicular stomatitis virus G envelopes. Z-100 was found to inhibit HIV-1 expression, even when added 24 h after infection. In addition, it substantially inhibited the expression of the pNL43lucΔenv vector (in which the *env* gene is defective and the *nef* gene is replaced with the firefly luciferase gene) when this vector was transfected directly into MDMs. These findings suggest that Z-100 inhibits virus replication, mainly at HIV-1 transcription. However, Z-100 also downregulated expression of the cell surface receptors CD4 and CCR5 in MDMs, suggesting some inhibitory effect on HIV-1 entry. Further experiments revealed that Z-100 induced IFN-β production in these cells, resulting in induction of the 16-kDa CCAAT/enhancer binding protein (C/EBP) β transcription factor that represses HIV-1 long terminal repeat transcription. These effects were alleviated by SB 203580, a specific inhibitor of p38 mitogen-activated protein kinases (MAPK), indicating that the p38 MAPK signalling pathway was involved in Z-100-induced repression of HIV-1 replication in MDMs. These findings suggest that Z-100 might be a useful immunomodulator for control of HIV-1 infection.

INTRODUCTION

Monocytes/macrophages are presumed to be the initial target of human immunodeficiency virus type 1 (HIV-1) in mucosa, and these cells might serve as virus reservoirs during all stages of HIV-1 infection (Connor & Ho, 1994; Mann *et al.*, 1990; van't Wout *et al.*, 1994). HIV-1 entry into monocytes/macrophages is mediated by binding of HIV-1 envelope glycoproteins to the cell-surface receptor CD4 and a coreceptor (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996; Hill *et al.*, 1997), and most primary HIV-1 isolates are macrophage-tropic (M-tropic), utilizing the CCR5 coreceptor. Individuals with the CCR5 Δ32 mutation (Stephens *et al.*, 1998) have partial resistance to HIV-1 infection and this indicates clearly that suppression of virus entry into monocytes/macrophages could be a strategy for limiting primary HIV-1 infection.

HIV-1 replication in monocytes/macrophages is also influenced by their differentiation status. HIV-1 replication can be detected in tissue macrophages present in the lung, skin and mucosa *in vivo*. HIV-1 replicates well in monocyte-derived macrophages (MDMs) *in vitro*, but poorly in monocytes (Kalter *et al.*, 1991; Pauza *et al.*, 1988; Smith *et al.*, 1994). However, conversely, in the presence of *Mycobacterium tuberculosis*, HIV-1 replication is inhibited in macrophages, but enhanced in monocytes (Mancino *et al.*, 1997; Weiden *et al.*, 2000). This observation suggests that permissiveness of monocytes/macrophages for HIV-1 infection depends on their state of activation and differentiation. Zybarth *et al.* (1999) have demonstrated that lipopolysaccharide (LPS), a potent stimulator of macrophages, inhibits HIV-1 replication in MDMs. Similarly, HIV-1 replication is reduced in dendritic cells where maturation has been driven by CD40 ligand binding

(Bakri *et al.*, 2001). As LPS also induces dendritic cell maturation (Kaisho *et al.*, 2001), macrophages and dendritic cells might share an LPS-responsive signalling pathway. LPS is known to bind to the cell-surface receptor CD14 and to transmit intracellular signals through Toll-like receptor (TLR) 4 (da Silva Correia *et al.*, 2001). These immunomodulators, in inducing macrophage activation or dendritic-cell maturation, might induce resistance to HIV-1 infection. Interestingly, murabutide, a synthetic derivative of peptidoglycan muramyl dipeptide that is undergoing HIV-1 clinical trials, inhibits HIV-1 replication by activating macrophages (De La Tribonniere *et al.*, 2003; Vidal *et al.*, 2001).

Z-100, a mixture of an arabinomannan and other small-sized components (Kobatake *et al.*, 1981), is used clinically in Japan in patients with leukopenia caused by radiation therapy. This compound is purified from an extract of human-type *M. tuberculosis* strain Aoyama B. Z-100 consists mainly of three fractions, A, B and C, when separated by high performance gel filtration chromatography. Fraction A contains 67·6 % sugars, mainly arabinomannan; fraction B contains 83·5 % sugars, mainly mannan; and fraction C contains 41·1 % sugars, mainly mannan and glucan, with large amounts of amino-acid-containing peptidoglycan.

The immunomodulatory activities of Z-100 have been described previously. These include anti-tumour (Sasaki *et al.*, 1993; Suzuki *et al.*, 1986) and anti-metastatic (Emori *et al.*, 1996; Kobayashi *et al.*, 1997) activities against several syngeneic tumours in experimental animals, protective effects against opportunistic infections in immuno-suppressed hosts (Kawamura *et al.*, 1990; Sasaki *et al.*, 1997) and cytokine-inducing activities, such as interleukin (IL) 12 (Kobayashi *et al.*, 1995) and interferon (IFN)-γ (Hayashi *et al.*, 1981) production *in vivo* and *in vitro*. The anti-tumour activities of Z-100 might be due to suppression of tumour-associated Th2-type cytokines, for example IL4 and IL10 (Oka *et al.*, 1999). Our group have demonstrated previously that Z-100 restored the balance of Th1/Th2 cell responses in tumour-bearing mice by upregulating IL12 and downregulating IL10 production by macrophages (Oka *et al.*, 2003).

In addition to its anti-tumour activity, Z-100 decreased the severity of murine AIDS (caused by LP-BM5 murine leukemia virus in C57BL/6 mice) by repressing Th2-type responses (Sasaki *et al.*, 2001). Recently, we found that Z-100 could induce β-chemokines, for example macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES, which are natural ligands for CCR5 in human MDMs. Furthermore, Z-100 induces MIP-1α in a phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophage cell line by activating the p38 mitogen-activated protein kinase (MAPK) signalling pathway (K. Yoshinaga, personal communication). These findings encouraged us to investigate the anti-HIV-1 effects of Z-100.

In this paper, we demonstrate that Z-100 suppresses HIV-1 replication in acutely infected MDMs. Although Z-100 induced β-chemokine production by MDMs, we found that, in the main, Z-100 suppressed late stages of HIV-1 replication, presumably at the level of virus transcription. Further analysis revealed that this suppression required IFN-β induction in MDMs, mediated through activation of the p38 MAPK signalling pathway. These findings suggest that Z-100 might be a useful immunomodulator for control of HIV-1 infection.

METHODS

Cell culture. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy seronegative donors by Ficoll–Hypaque density-gradient centrifugation (Ficoll-Paque PLUS; Amersham Bioscience). Monocytes were purified from fresh PBMCs by magnetic cell sorting (MACS) using a monocyte isolation kit (Miltenyi Biotec). Monocytes were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 5 % heat-inactivated human AB blood group serum (Sigma), 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 5 ng granulocyte/macrophage colony-stimulating factor (GM-CSF) ml^{-1} (Stemcell Technologies) in order to generate MDMs. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma) and stimulated to differentiate into macrophages by the addition of PMA (5 nM; Sigma) for 24 h prior to infection. Monkey kidney fibroblast line COS7 or human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10 % FBS.

Virus preparation and infection. M-tropic HIV-$1_{JR\text{-}CSF}$ strain (Koyanagi *et al.*, 1987) (kindly provided by Y. Koyanagi, Tohoku University) was grown in phytohaemagglutinin (PHA; Difco Laboratories)-stimulated PBMCs cultured in RPMI 1640 medium supplemented with 10 % FBS and 10 IU recombinant IL2 (Shionogi) ml^{-1} for 5–7 days. The supernatants were filtered and stored at −80 °C until use. To infect MDMs *in vitro*, culture supernatant that contained HIV-$1_{JR\text{-}CSF}$ (1 ng p24 ml^{-1}) was added to each well and incubated for 24–48 h at 37 °C. After this time, virus was removed by washing the cells twice and fresh medium was added. Cultures were incubated for 7 days with fresh medium in the presence or absence of Z-100 at a range of concentrations and at 37 °C in humidified 5 % CO_2. Virus replication was assessed by the level of virus p24 antigen in the supernatants. Pseudotype viruses were generated by co-transfection of the monkey kidney fibroblast line COS7 or human embryonic kidney 293T cells with the pNL43lucΔenv vector (in which the *env* gene is defective and the *nef* gene is replaced with the firefly luciferase gene) and either an M-tropic HIV-1 envelope expression vector (pJR-FL) (kindly provided by Dr Yoshio Koyanagi, Tohoku University), an amphotropic Moloney murine leukemia virus (MuLV) envelope expression vector (pJD-1) or a vesicular stomatitis virus (VSV)-G envelope protein (pHCMVG) (Masuda *et al.*, 1995; Yee *et al.*, 1999) (kindly provided by Dr Irvin S. Y. Chen, University of California), using Lipofectamine (Gibco BRL). Culture supernatants (4·5 ml) from the transfected cells were harvested at 48 h post-transfection, filtered through 0·45-μm-pore filters and used as virus preparations. Viral gene expression from each plasmid vector was confirmed by luciferase activity. The cells were lysed at 48 h post-transfection with 1 ml 1× luciferase lysis buffer (Promega) and 1 μl of each cell lysate was assayed for luciferase. MDMs ($2·5–5 \times 10^5$) were infected with 0·5–1 ml of these viruses in the presence of 10 μg polybrene (Sigma) ml^{-1} at 37 °C for 6 h. Viruses were then removed and the cells were cultured with fresh medium in the presence or absence of Z-100 at 37 °C in humidified 5 % CO_2. Virus replication in infected

MDMs was assessed by luciferase activity. In some experiments, Z-100 was added to MDMs either 24 h before infection or simultaneously with infection and then left in the culture medium. MAPK inhibitors were added to the MDM cultures 30 min before Z-100 treatment.

Transfection of viral DNA. MDMs (5×10^5) were transfected with the pNL43lucΔenv vector using JetPEI (PolyPlus transfection). Following 24 h incubation, MDMs were washed, overlaid with fresh medium and incubated at 37 °C in humidified 5 % CO_2. Cells were lysed 96 h post-transfection with 100 µl 1× luciferase lysis buffer and the luciferase activity was assessed.

Reagents and antibodies. Z-100, consisting of a mixture of polysaccharides including arabinose, mannose, glucose and other small-sized components, was prepared from hot-water extracts of *M. tuberculosis* strain Aoyama B, purified by deproteinization and dialysis, and was supplied by Zeria Pharmaceutical Co. Ltd (Tokyo, Japan). The Z-100 stock solution was of clinical-use grade and confirmed as containing 2 mg D-arabinose ml^{-1} and being free from endotoxins, and was kept at 4 °C and diluted just before use. The inhibitors SB 203580 and Ro 318220 were purchased from Calbiochem. PD 98059 and PDTC (ammonium pyrrolidine dithiocarbamate) inhibitors were purchased from Sigma. SP 600125 inhibitor was purchased from Tocris Cookson Inc. Anti-CCAAT/enhancer binding protein (C/EBP) β (C-19) and anti-rabbit horseradish peroxidase (HRP) antibodies were purchased from Santa Cruz Biotechnology. Anti-human IFN-β neutralizing antibody and isotype-matched control antibody (normal goat IgG) were purchased from R&D Systems. Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and anti-CCR5 monoclonal antibodies (mAbs) and their isotype-matched control immunoglobulins (Igs) were purchased from BD Biosciences.

Flow cytometry analysis. MDMs (5×10^5) were incubated for 15 min at room temperature with FITC-conjugated anti-CD4 or FITC-conjugated anti-CCR5 mAbs or with isotype-matched control Igs to assess surface receptor expression. Cells were washed twice, resuspended, fixed in 1 % paraformaldehyde and analysed with a FACSCalibur flow cytometer (Becton Dickinson). Live cells were gated according to forward- and side-scatter characteristics and the percentage of positive cells and the mean fluorescence intensity (MFI) were recorded.

ELISA. The level of HIV-1 p24 antigen in the culture supernatants was measured by ELISA using a RETRO-TEK HIV-1 p24 Antigen ELISA kit (ZeptoMetrix). The level of MIP-1α, MIP-1β and RANTES in MDM culture supernatants was determined using ELISA kits purchased from R&D Systems. An ELISA kit specific for human IFN-β detection (PBL Biomedical Laboratories) was also used.

Luciferase activity. Infected cells were harvested at 4 days post-infection for luciferase analysis. The cell pellet from individual wells was washed twice with PBS and lysed with 150 µl 1× luciferase lysis buffer. Cell lysate (10 µl) was analysed using a luminometer (Lumat LB 9507; EG&G Berthold). The luciferase activity was indicated as U μl^{-1}.

Preparation of nuclear extracts and Western blot analysis. MDMs (4×10^6) or PMA-differentiated THP-1 cells (4×10^6) were stimulated with 100 µg Z-100 ml^{-1} for 96 h at 37 °C. Nuclear extracts were then prepared using a Nuclear Extract kit (Active Motif) according to the manufacturer's instructions. The protein concentration of the extracts was determined by Bradford protein assay (Bio-Rad Laboratories). Nuclear protein (60 µg) was separated by SDS-PAGE on a 10–20 % gel (SuperSep; Wako) and transferred onto a nitrocellulose membrane (ATTO). The membrane was incubated with anti-C/EBPβ antibodies followed by anti-rabbit HRP antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (ECL Western blotting detection reagents; Amersham Biosciences) and quantified using a Science Lab 98 Image Gauge (Fuji Photo Film).

Statistical analyses. The paired *t*-test was used to compare the means of the two groups. For the one-way analysis of variance (ANOVA) followed by multiple comparisons tests, Dunnett's two-tailed test was used. In both tests, P values $<0{\cdot}05$ were considered statistically significant.

RESULTS

Z-100 inhibits wild-type HIV-1 replication in acutely infected MDMs

A range of Z-100 concentrations (0·1–100 µg ml^{-1}) was added to the replication-competent HIV-$1_{JR\text{-}CSF}$-infected MDM culture in order to evaluate its HIV-suppressive activity. Fig. 1(a) shows the level of virus p24 antigen in untreated or Z-100-treated MDM culture supernatant, assayed by ELISA at 7 days post-infection. The addition of Z-100 at 0·1, 1, 10 or 100 µg ml^{-1} resulted in 31·8, 55·7, 79·0 or 93·5 % inhibition of virus replication, respectively; that is, in a dose-dependent manner ($P<0{\cdot}01$). Z-100 at a concentration of 100 µg ml^{-1} inhibited HIV-1 replication markedly; however, Z-100 added at <10 µg ml^{-1} had varied effects in blood donors. Therefore, a concentration of 100 µg ml^{-1} was used in further experiments to ensure maximal inhibition.

The effect of adding Z-100 at different time points following HIV-$1_{JR\text{-}CSF}$ infection of MDMs was evaluated. Z-100 (100 µg ml^{-1}) was added to MDMs either 24 h before infection (−24 h), simultaneously with infection (0 h) or following the infection period (+48 h) and the results are shown in Fig. 1(b). HIV-1 replication was suppressed markedly (>97·9 %) when Z-100 was added either −24 or 0 h from infection. Virus replication was suppressed significantly (78·1 %, $P<0{\cdot}01$) even when Z-100 was added at +48 h post-infection, indicating that Z-100 suppressed HIV-1 replication even after completion of the initial virus infection.

Effects of Z-100 on β-chemokine production and cell surface HIV-1 receptors

β-Chemokines, natural ligands of CCR5, are known to block M-tropic HIV-1 entry competitively (Cocchi *et al.*, 1995; Verani *et al.*, 1997). High levels of β-chemokines (8·1 ng MIP-1α ml^{-1}, 11·0 ng MIP-1β ml^{-1} and 2·5 ng RANTES ml^{-1}) were detected in MDM culture supernatants treated with Z-100 at 100 µg ml^{-1} for 24 h. Therefore, we evaluated whether these β-chemokines are responsible for the HIV-suppressive activity of Z-100 by adding neutralizing antibodies against MIP-1α, MIP-1β and RANTES into Z-100-treated, HIV-1-infected MDM cultures. We found that these neutralizing antibodies had no effect on the Z-100-induced HIV suppression (data not shown).

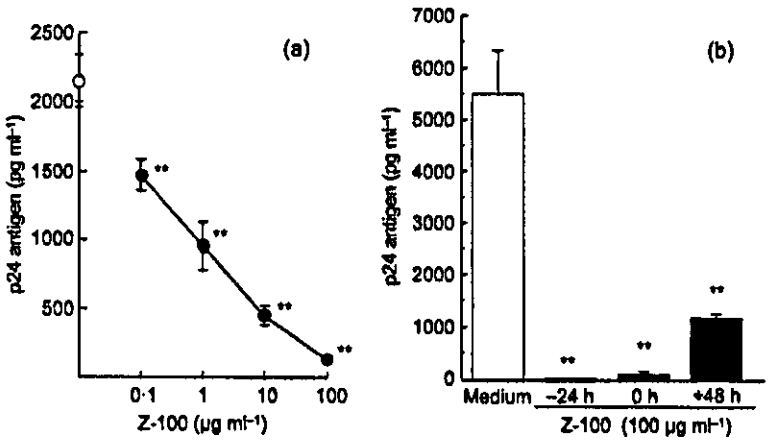

Fig. 1. Z-100 inhibits M-tropic HIV-1 replication in acutely infected MDMs. (a) Dose-dependent inhibition of HIV-1 replication by Z-100. MDMs were infected with HIV-$1_{JR\text{-}CSF}$ for 24 h, washed and maintained in the absence (○) or presence (●) of Z-100 at 0·1–100 μg ml^{-1}. (b) Effect of Z-100 (100 μg ml^{-1}) on HIV-1 replication in MDMs infected with HIV-$1_{JR\text{-}CSF}$ for 48 h when added 24 h before infection (−24 h), simultaneously with infection (0 h) or following infection (+48 h). For the MDM samples pre-treated with Z-100, the same concentration of Z-100 was re-added after washing at 48 h and then left in the culture thereafter. Virus p24 antigen levels were measured in culture supernatants by ELISA 7 days post-infection. Values represent means ± SEM of four replicates. **, $P<0·01$ compared to medium control (Dunnett's test).

The influence of Z-100 on CD4 and CCR5 receptor expression in MDMs was examined further by flow cytometry (Fig. 2). Both CD4 and CCR5 receptor expression levels were reduced slightly in MDMs cultured with Z-100 for 24 h (Fig. 2). This result explains partly why Z-100 suppressed HIV-1-replication in MDMs more effectively when added before rather than after HIV-1 infection.

Z-100 inhibits single-round virus replication of pseudotype HIV-1 in MDMs at late stages

Single-round infection assay was used to determine the stages of HIV-1 suppression by Z-100. In this assay, we used an *env*-defective HIV-1 clone carrying the luciferase gene (pNLlucΔenv) pseudotyped with an envelope from either M-tropic HIV-$1_{JR\text{-}FL}$, amphotropic MuLV or VSV-G. MDMs, pre-treated with Z-100 for 24 h, were exposed to these viruses and maintained for 4 days in the absence or presence of Z-100. Virus expression was evaluated by luciferase activity expressed in MDMs (Fig. 3a). Z-100 suppressed virus expression of M-tropic HIV-$1_{JR\text{-}FL}$ (88·1 %), amphotropic MuLV (81·9 %) and VSV-G (67·1 %) significantly. As MuLV and VSV-G pseudotype viruses do not require β-chemokine receptors for infection, this result indicates that Z-100 suppressed HIV-1 replication at the post-entry stages.

The addition of Z-100 to MDM cultures at different time points following amphotropic MuLV or VSV-G pseudotype HIV-1 infection confirmed this result. As shown in Fig. 3(b), pseudotype HIV-1 expression was inhibited significantly in any culture to which Z-100 was added −24 h, or 6 h and 24 h post-infection. It is noteworthy that Z-100 suppressed virus replication even when added 24 h after infection. These findings suggest that Z-100 inhibits virus replication mainly at late stages of virus replication, presumably at the level of virus transcription, because proviral DNA integration must have been completed by 24 h post-infection (Masuda *et al.*, 1995).

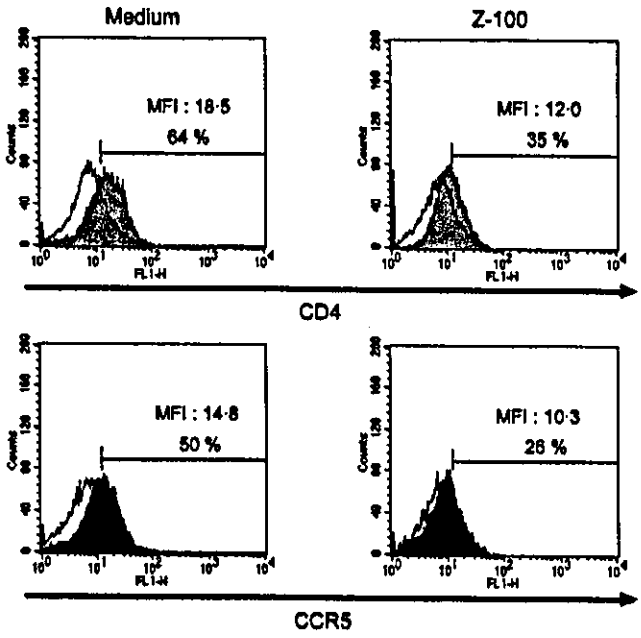

Fig. 2. Downregulation by Z-100 of CD4 and CCR5 membrane expression in MDMs. MDMs were cultured in the absence or presence of Z-100 (100 μg ml^{-1}) for 24 h and the expression of CD4 or CCR5 (filled histograms) and isotype-matched control (open histograms) in these cells was analysed using a flow cytometer. The percentage of positive cells and the MFI for CD4 and CCR5 receptors are indicated.

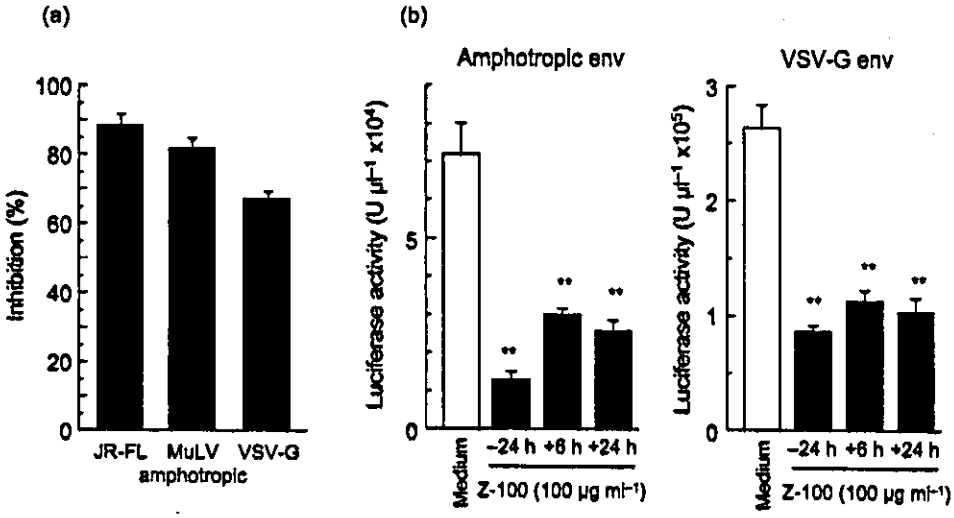

Fig. 3. Z-100 inhibits single-round virus replication of pseudotype HIV-1 in MDMs. (a) Suppressive activity of Z-100 on the replication of pseudotype pNL43lucΔenv-based viruses with either M-tropic env (pJR-FL), amphotropic MuLV env (pJD-1) or VSV-G (pHCMVG) in MDMs. MDMs pre-treated with Z-100 (100 μg ml^{-1}) for 24 h were exposed for 6 h to the pseudotype viruses indicated and maintained in the absence or presence of Z-100 at 100 μg ml^{-1}. Luciferase activity in MDMs was assayed with a luminometer following 4 days incubation. Values represent means ± SEM of percentage inhibition of medium control for each virus. (b) Effect of Z-100 (100 μg ml^{-1}) on expression of amphotropic MuLV or VSV-G pseudotype virus in MDMs when added 24 h before infection (−24 h), following infection (+6 h) or 24 h after infection (+24 h). For the MDM samples treated with Z-100 before infection, Z-100 was re-added to same concentration at 6 h following cell washing. Values represent means ± SEM of three or four replicates. **, $P<0{\cdot}01$ compared to medium control (Dunnett's test).

Suppression of virus transcription by Z-100 requires p38 MAPK activation

The p38 MAPK protein is an important mediator of stress- or inflammatory cytokine-induced gene expression (Ono & Han, 2000). In particular, p38 kinase is known to play a key role in LPS-induced signal transduction pathways that lead to cytokine synthesis (Lee & Young, 1996). p38 MAPK activation has been suggested recently as a major mechanism for LPS-mediated HIV-suppressive activity in macrophages (Zybarth *et al.*, 1999). The effect of a specific p38 MAPK inhibitor, SB 203580, on Z-100 inhibition of virus replication was evaluated in order to determine whether Z-100-induced, HIV-1 suppression involves a mechanism similar to that of LPS (Fig. 4a). M-tropic HIV-$1_{JR\text{-}CSF}$ replication was suppressed by 91·4% in the presence of Z-100 compared to without Z-100. When SB 203580 (1 μM) was added to HIV-1-infected MDM cultures 30 min before Z-100 treatment, HIV-1 suppression by Z-100 was reduced significantly, to 27·7%. A similar dose-dependent alleviation of Z-100-mediated HIV-1 suppression was observed when amphotropic MuLV pseudotype HIV-1 was used on MDMs or PMA-differentiated THP-1 cells (Fig. 4b). The addition of SB 203580 alone to infected MDM cultures did not alter virus replication markedly. These results suggest that Z-100 HIV-suppressive activity requires p38 MAPK activation, analogous to that with LPS.

Various inhibitors were used to assess whether other MAPK signalling cascades were involved in Z-100-mediated HIV suppression. These included SP 600125, a selective and reversible inhibitor of c-*jun* N-terminal kinase (JNK), PD 98059, a selective inhibitor of MAP kinase kinase-1 (MKK1), Ro 318220, a potent inhibitor of mitogen- and stress-activated protein kinase-1 (MSK1) and MAPK-activated protein kinase-1 (MAPKAP-K1) activity, and PDTC, a specific inhibitor of NF-κB. As shown as Table 1, SB 203580 abolished Z-100-mediated HIV suppression. In addition, PDTC restored virus replication in Z-100-treated MDM cultures (Table 1); however, it was not clear whether there was involvement of NF-κB pathways in Z-100-induced HIV-1 suppression, because PDTC alone markedly enhanced virus replication in MDM cultures without Z-100. We therefore investigated the role of MAPK signalling pathways.

Effects of Z-100 in HIV-1 transcription

The results shown in Fig. 3 suggested that Z-100 inhibits virus replication at post-entry stages of virus replication. Therefore we assessed the inhibitory effect of Z-100 on virus transcription by transfecting MDMs directly with *env*-defective HIV-1 vector expressing luciferase (pNL43lucΔenv). As shown in Fig. 5, luciferase activity in MDMs cultured for 4 days post-transfection with Z-100 was markedly lower than in the control culture without Z-100 ($P<0{\cdot}01$; Fig. 5). Moreover, pre-treatment of MDMs with SB 203580 at 1 μM significantly alleviated Z-100 suppression ($P<0{\cdot}05$). These results indicate that Z-100 suppressed virus transcription and that this suppression required p38 MAPK activation.

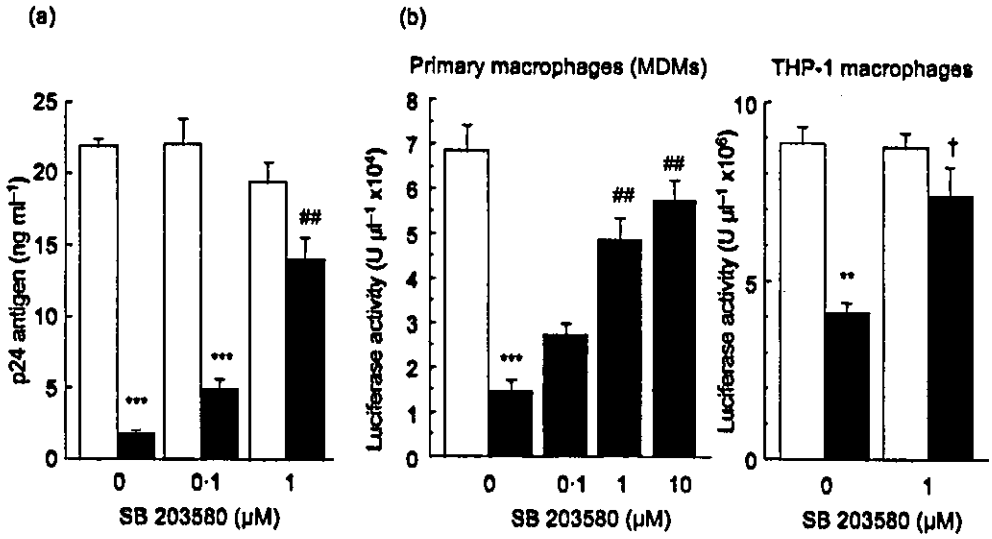

Fig. 4. Inhibitory effect of a p38 MAPK inhibitor on the HIV-suppressive activity of Z-100. (a) MDMs were infected with HIV-$1_{JR\text{-}CSF}$ for 48 h, washed and maintained in the absence (open bars) or presence (filled bars) of Z-100 at 100 µg ml^{-1}. A specific p38 MAPK inhibitor, SB203580, was added to the culture at 0·1 or 1 µM 30 min before Z-100 treatment. Virus p24 antigen levels in culture supernatants were detected by ELISA 7 days post-infection. (b) MDMs or PMA-differentiated THP-1 macrophages were infected with pseudotype virus (amphotropic MuLV) for 6 h, washed and maintained in the absence (open bars) or presence (filled bars) of Z-100 at 100 µg ml^{-1}. SB203580 was added to these cells at 0·1–10 µM 30 min before Z-100 treatment. Luciferase activity of these macrophages was assayed with a luminometer at 4 days post-infection. Values represent means ± SEM of three or four replicates. Statistically significant differences are indicated as: *** and **, $P<0·001$ and $P<0·01$, respectively, compared to medium control (*t*-test); ##, $P<0·01$ compared to the control of Z-100 alone (Dunnett's test); †, $P<0·05$ compared to the control of Z-100 alone (*t*-test).

Z-100 enhances the induction of inhibitory 16-kDa C/EBPβ isoform in macrophages

HIV-1 transcription is controlled by cellular and virus factors that bind the HIV-1 long terminal repeat (LTR) (Gaynor, 1992). Recent reports have demonstrated that the C/EBP family of transcription factors is essential for HIV-1 replication in macrophages, but not in CD4$^+$ T cells (Henderson & Calame, 1997). C/EBPβ (also called NF-IL6) and intact HIV-1 LTR binding sites are required for virus transcription; however, the dominant-negative 16-kDa isoform of C/EBPβ inhibits HIV-1 replication and LTR promoter function (Descombes & Schibler, 1991; Henderson *et al.*, 1995, 1996).

Nuclear protein extracted from PMA-differentiated THP-1 cells incubated with or without Z-100 for 4 days was analysed to evaluate if Z-100 could induce the inhibitory isoform. As shown in Fig. 6, the 16-kDa inhibitory C/EBPβ isoform increased 5·7-fold in the presence of Z-100, whereas expression of the 37-kDa stimulatory C/EBPβ isoform was only increased slightly by Z-100 treatment (Fig. 6a) in these cells. This resulted in an increase in the ratio of inhibitory C/EBPβ isoform to stimulatory isoform of 89 % following Z-100 addition. Pre-treatment of THP-1 macrophages with SB 203580 increased the stimulatory isoform and decreased the inhibitory isoform in Z-100-treated THP-1 macrophages (Fig. 6a), resulting in a decrease in the ratio of inhibitory to stimulatory isoforms to 51 %. Induction of the inhibitory C/EBPβ isoform by Z-100 and its suppression by SB 203580 was observed in primary macrophages (Fig. 6b). These results are consistent with those in Fig. 4(b) that show Z-100-mediated HIV suppression in MDMs was alleviated greatly by SB 203580 pre-treatment (10 µM). These findings suggested that Z-100 inhibits HIV-1 transcription by repressing LTR by induction of the inhibitory 16-kDa C/EBPβ isoform, for which p38 MAP kinase activation was required.

Z-100 inhibits HIV-1 replication by induction of the inhibitory C/EBPβ isoform by IFN-β release from MDMs

Honda *et al.* (1998) have reported that *M. tuberculosis* infection induces an IFN-β response in macrophages and that IFN-β induces the expression of an inhibitory 16-kDa C/EBPβ transcription factor. Consequentially, we examined IFN-β involvement in induction of the inhibitory 16-kDa C/EBPβ isoform in Z-100-treated MDMs. As shown in Fig. 7, the addition of IFN-β-neutralizing antibodies (1 ng ml^{-1}) to Z-100-treated MDMs suppressed the enhanced inhibitory C/EBPβ isoform levels to that of the control (without Z-100). The role of IFN-β in Z-100-induced HIV-1 suppression was examined using MuLV pseudotype HIV-1. As shown in Fig. 7(b), virus expression, suppressed by Z-100, was elevated by adding IFN-β

Table 1. Inhibitory effect of MAPK or NF-κB inhibitors on HIV-suppressive activity of Z-100 in MDMs

Six hours after amphotropic MuLV pseudotype HIV-1 infection, MDMs were washed and maintained in the absence or presence of Z-100 at 100 μg ml^{-1}. Inhibitors were added to MDMs at the concentrations indicated 30 min before Z-100 treatment. Luciferase activity in MDMs was assayed with a luminometer at 4 days post-infection. Values represent means ± SEM of four replicates. Statistically significant differences are indicated by: ***, $P<0.001$ compared to medium control (*t*-test); ## and #, $P<0.01$ and $P<0.05$, respectively, compared to Z-100 without inhibitors (Dunnett's test).

Inhibitor	Concentration (μM)	Luciferase activity (U μl^{-1} x 10^4)	
		Medium	Z-100
None	–	143·4±19·7	33·3±11·4***
SB 203580	0·1	187·2±3·4	83·8±6·0##
	1	175·6±2·8	113·6±3·1##
	10	158·0±11·2	106·5±3·8##
SP 600125	0·01	135·7±27·2	48·2±4·7
	0·1	138·9±7·7	38·7±3·3
	1	117·5±16·7	46·7±1·3
PD 98059	0·1	139·8±5·9	35·3±10·2
	1	54·6±12·7	15·3±4·1
	10	133·7±2·3	38·4±3·7
Ro 318220	0·01	125·4±24·2	52·3±1·8
	0·1	149·0±12·3	51·3±2·1
	1	39·4±0·8	19·0±2·2
PDTC	1	149·0±16·7	40·8±6·1
	10	205·8±5·5	72·9±4·1#
	100	284·9±20·3	104·9±6·2##

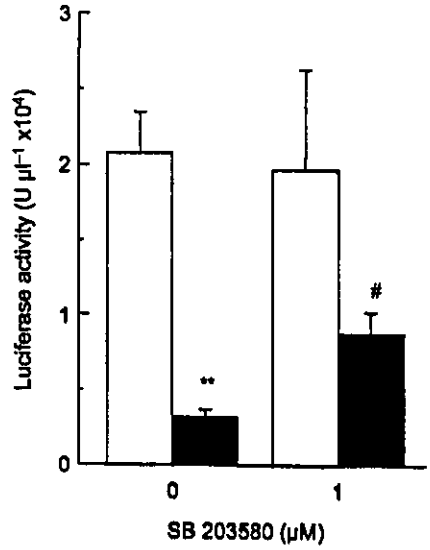

Fig. 5. Z-100 inhibits transcription of HIV-1 in MDMs. MDMs were transfected with pNL43lucΔenv for 24 h using JetPEI, washed and incubated with fresh medium, without (open bar) or with (filled bar) Z-100 at 100 μg ml^{-1}. SB203580 (1 μM) was added to cultures 30 min before Z-100 treatment. The luciferase activity in MDMs was assayed with a luminometer at 4 days post-transfection. Values represent means ± SEM of four replicates. **, $P<0.01$ compared to medium control (*t*-test); #, $P<0.05$ compared to Z-100 alone sample (*t*-test).

neutralizing antibodies ($P<0.01$, Fig. 7b). These results suggest that MDM-produced IFN-β was required for Z-100-mediated HIV suppression and induction of the inhibitory C/EBPβ isoform.

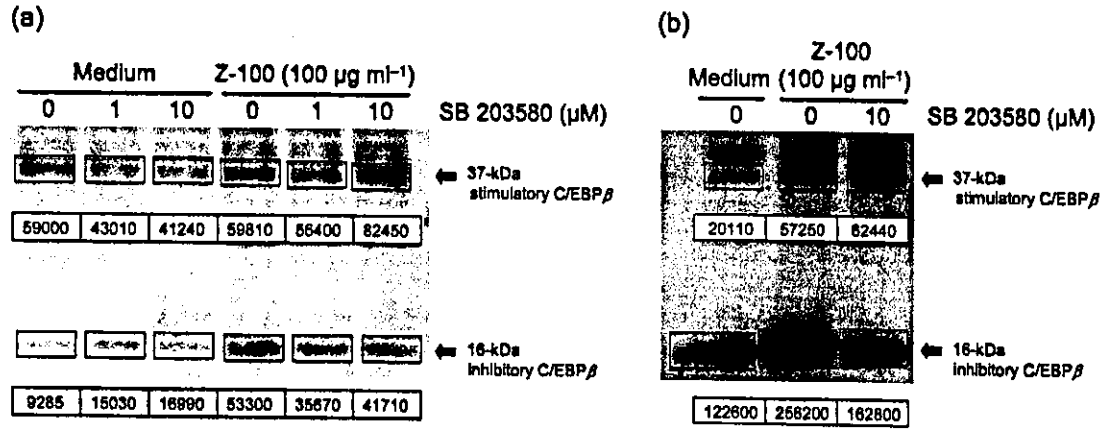

Fig. 6. Induction of inhibitory 16 kDa C/EBPβ isoform in Z-100-treated macrophages and its suppression by a p38 MAPK inhibitor. PMA-differentiated THP-1 macrophages (a) or MDMs (b) (4 x 10^6) were incubated with Z-100 at 100 μg ml^{-1} in the absence or presence of SB203580 (1 or 10 μM). After incubation for 4 days, nuclear proteins were extracted and subjected to Western blot analysis. Membrane-bound nuclear proteins were incubated with anti-C/EBPβ antibodies and visualized by ECL. Two isoforms of C/EBPβ, 37-kDa stimulatory C/EBPβ and 16-kDa inhibitory C/EBPβ, are indicated by arrows. The value below each band represents its density quantified using a Science Lab 98 Image Gauge.

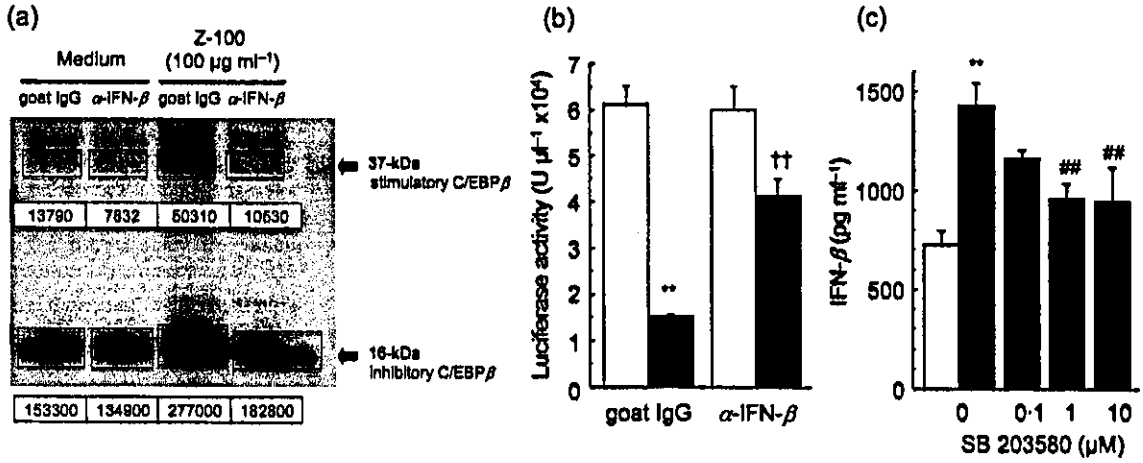

Fig. 7. Z-100 inhibits HIV-1 replication by the induction of inhibitory C/EBPβ through the release of IFN-β from MDMs. (a) Z-100-mediated induction of inhibitory 16-kDa C/EBPβ was suppressed by neutralizing antibodies to IFN-β (α-IFN-β). MDMs (4×10^6) were incubated with Z-100 (100 μg ml^{-1}) and anti-IFN-β (1 ng ml^{-1}) or isotype-matched control goat IgG (1 ng ml^{-1}) for 4 days and nuclear proteins extracted from MDMs were subjected to Western blot analysis, probed with anti-C/EBPβ antibodies. Immunoreactive bands visualized by ECL were quantified using a Science Lab 98 Image Gauge. (b) Anti-IFN-β neutralizing antibodies alleviated the HIV-suppressive activity of Z-100. MDMs infected with amphotropic MuLV pseudotype HIV-1 infection for 6 h, were washed and incubated with medium (open bars) or Z-100 (100 μg ml^{-1}) (filled bars) and anti-IFN-β neutralizing antibodies (1 ng ml^{-1}) or isotype-matched control goat IgG (1 ng ml^{-1}). Luciferase activity in MDMs at 4 days post-infection was assayed with a luminometer. Values represent means ± SEM of five replicates. **, $P<0.01$ compared to control culture of goat IgG alone (*t*-test); ††, $P<0.01$ compared to control culture with Z-100 and goat IgG (*t*-test). (c) Enhancement of IFN-β release from MDMs by Z-100 was inhibited by SB 203580. MDMs infected with amphotropic MuLV pseudotype HIV-1 for 6 h were washed and incubated with medium (open bar) Z-100 (100 μg ml^{-1}) (filled bars) in the absence or presence of SB203580 (0·1–10 μM), 30 min before Z-100 treatment. The cells were incubated for 4 days and then the IFN-β level in the culture supernatants was measured by ELISA. Values represent means ± SEM of four replicates. **, $P<0.01$ compared to medium control (*t*-test); ##, $P<0.01$ compared to control culture in the presence of Z-100 (Dunnett's test).

Finally, we assessed whether IFN-β was produced by Z-100-treated MDMs. IFN-β levels were measured in culture supernatants from the pseudotype HIV-1-infected MDMs samples in Fig. 4(b). Z-100-treated MDMs produced about twice the amount of IFN-β compared to the untreated control ($P<0.01$; Fig. 7c). In addition, Z-100-induced IFN-β production was inhibited significantly by the addition of SB 203580 ($P<0.01$). These results indicate strongly that Z-100 induces MDMs to produce IFN-β via activation of p38 MAPK, leading to HIV-1 suppression by induction of the inhibitory C/EBPβ isoform.

DISCUSSION

In this study, we demonstrated that Z-100, an arabinomannan extracted from *M. tuberculosis*, inhibits HIV-1 replication in macrophages. Z-100 significantly inhibited replication in MDMs of pseudotype HIV-1 that possess either an amphotropic MuLV or a VSV-G envelope (Fig. 3a). These results indicate clearly that Z-100 suppressed post-entry stages of HIV-1 replication, because these pseudotype viruses do not require β-chemokine receptors for infection. Z-100 suppressed expression of pseudotype HIV-1 significantly, even when added at 24 h post-infection (Fig. 3b), suggesting that Z-100 inhibits virus replication at post-integration stages. Moreover, Z-100 substantially inhibited expression of the pNL43lucΔenv vector, transfected directly into MDMs (Fig. 5). These findings indicate strongly that Z-100 inhibits transcription of HIV-1.

Although Z-100 induced β-chemokines such as MIP-1α, MIP-1β and RANTES, the addition of their neutralizing antibodies had no effect on the HIV-suppressive activity of Z-100 (data not shown), corresponding with previous reports indicating that β-chemokines (Moriuchi *et al.*, 1996) or pre-treatment of these (Kelly *et al.*, 1998) cannot block the M-tropic HIV-1 infection of MDMs. However, Z-100 downregulated HIV-1 receptors (CD4 and CCR5) in MDMs (Fig. 2), suggesting that Z-100 might also inhibit M-tropic virus entry to a small degree. This result explains partially why expression of M-tropic-enveloped, but not VSV-enveloped, pseudotype HIV-1 was inhibited slightly more in Z-100-pretreated MDMs than in cells treated with Z-100 post-infection (Fig. 1b). The mechanism of CD4 and CCR5 receptor downregulation is unclear, but Z-100-induced β-chemokines might contribute to downregulation, at least for CCR5.

It has been reported that LPS inhibits HIV-1 replication in macrophages and that p38 MAPK is involved in this suppression, probably through a signalling pathway downstream of CD14 and TLR4 (Zybarth *et al.*, 1999). As Z-100 HIV-suppressive activity was repressed significantly by SB 203580, a specific inhibitor of p38 MAPK (Fig. 4), Z-100 might suppress HIV-1 replication by a mechanism similar to that of LPS. In our preliminary studies, Z-100 induced MIP-1α production through activation of p38 MAPK (data not shown). Recent findings have demonstrated that LPS binds mainly TLR4 and activates MDMs through MyD88-dependent and -independent signalling pathways (Kaisho *et al.*, 2001; Kawai *et al.*, 1999). Although the major receptor for Z-100 is unclear, potentially its major component, arabinomannan, and the small amount of peptidoglycan found would bind the mannose receptor and TLR2/TLR6, respectively, and mediate various signalling pathways including the MAPK cascade. In this study, Z-100-mediated HIV-1-suppression was alleviated by a p38 MAPK inhibitor, but not by inhibitors of JNK or MKK1.

The p38 MAPK has been reported to consist of four known members of the p38 family, p38α, p38β, p38γ and p38δ, all of which have different functions (Ono & Han, 2000). In macrophages, p38α and p38δ are abundant, but p38β is undetected (Hale *et al.*, 1999). The pyridinyl imidazole p38 inhibitors, SB 203580 and SB 202190, nearly are equipotent for p38α and p38β, but do not inhibit p38γ or p38δ (Kumar *et al.*, 1997; Wang *et al.*, 1997). In this study, Z-100-mediated, HIV suppression was alleviated by SB 203580 (Fig. 4 and Table 1), indicating that Z-100 might inhibit HIV-1 replication by activation of the p38α isoform, but not p38δ. Ro 318220, a potent inhibitor of MSK1/MAPKAP-K1 located downstream of MAPK, could not alleviate Z-100-mediated HIV suppression (Table 1). As p38α and p38β, but not p38γ or p38δ, are known to phosphorylate MAPKAP-K2 (Keesler *et al.*, 1998; Kumar *et al.*, 1997; Wang *et al.*, 1997), HIV-suppressive activity by Z-100 might be mediated through the p38α MAPK–MAPKAP-K2 pathway.

In monocytes/macrophages, viral gene expression using the HIV-1 LTR is regulated by its interactions with C/EBPβ, which is also known to be an important regulator of inflammation among the C/EBP transcription factor family (Henderson *et al.*, 1995; Natsuka *et al.*, 1992). Three C/EBP-binding sites in the negative regulatory element (NRE) of the HIV-1 LTR (Tesmer *et al.*, 1993) have been identified as DNA elements responsible for LPS-mediated HIV-1 suppression in macrophages (Bernstein *et al.*, 1991). The stimulatory 37-kDa C/EBPβ isoform promotes transcription, whereas an inhibitory 16-kDa isoform acts in a dominant-negative manner, competing for C/EBP sites when expressed even at levels lower than that of the stimulatory isoform (Descombes & Schibler, 1991). In Z-100-treated macrophages, the inhibitory 16-kDa C/EBPβ isoform was induced more strongly than the stimulatory 37-kDa C/EBPβ isoform (Fig. 6), suggesting that Z-100 might repress HIV-1 LTR activity by altering the balance between the C/EBPβ isoforms towards suppression. In addition, SB 203580 inhibited Z-100-induction of the inhibitory isoform, indicating that p38 MAPK-dependent, Z-100-mediated HIV-1 suppression was through 16-kDa C/EBPβ induction by Z-100.

Interferons are known to inhibit HIV-1 replication in macrophages at various stages, including early stages (Meylan *et al.*, 1993) and transcription (Tissot & Mechti, 1995). In the present study, we found that Z-100 induced IFN-β production from MDMs. Moreover, neutralizing antibodies for IFN-β inhibited Z-100-mediated, HIV suppression and induction of inhibitory C/EBPβ (Fig. 7a, b). These findings indicate that Z-100-mediated suppression of HIV-1 transcription is dependent partly on IFN-β induction. In addition, IFN-β production by Z-100 was inhibited by a p38 MAPK inhibitor, SB 203580 (Fig. 7c), indicating that Z-100 induces IFN-β through activation of the p38 MAPK pathway. These findings are consistent with reports demonstrating that *M. tuberculosis* infection, or LPS, induced the 16-kDa inhibitory C/EBPβ isoform through IFN-β induction and, coincidentally, repressed HIV-1 LTR transcription in THP-1 macrophages (Honda *et al.*, 1998). The precise mechanisms of how IFN-β induces 16-kDa C/EBPβ remain to be clarified.

In response to Z-100, TLR2 and the mannose receptor potentially mediate signals to induce IFN-β. The transcription enhancer of the IFN-β promoter contains four positive regulatory domains (PRDI to PRDIV), which activate IFN-β expression cooperatively in response to virus infection (Maniatis *et al.*, 1998). The transcription factors that bind to these elements include NF-κB, which binds to PRDII, IRF3, which binds to adjacent PRDIII and PRDI (PRDIII-I), and the heterodimeric transcription factor (ATF)-2-c-Jun, which binds to PRDIV. Although the mechanisms for IFN-β induction in Z-100-treated MDMs are not clear, p38 MAPK might contribute to activation of NF-κB and ATF-2 transcription factors, because these transcription factors are located downstream from p38 and bind to PRDII and PRDIV, respectively, in the IFN-β enhancer.

In conclusion, Z-100 suppressed the replication of HIV-1 in macrophages, mainly at virus transcription and partially at virus entry. This suggests that Z-100 might be a useful immunomodulator for control of HIV-1 infection.

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REFERENCES

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. & Berger, E. A. (1996). CC CKR5: a RANTES,

MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955–1958.

Bakri, Y., Schiffer, C., Zennou, V., Charneau, P., Kahn, E., Benjouad, A., Gluckman, J. C. & Canque, B. (2001). The maturation of dendritic cells results in postintegration inhibition of HIV-1 replication. *J Immunol* **166**, 3780–3788.

Bernstein, M. S., Tong-Starksen, S. E. & Locksley, R. M. (1991). Activation of human monocyte-derived macrophages with lipopolysaccharide decreases human immunodeficiency virus replication in vitro at the level of gene expression. *J Clin Invest* **88**, 540–545.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C. & Lusso, P. (1995). Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by $CD8^+$ T cells. *Science* **270**, 1811–1815.

Connor, R. I. & Ho, D. D. (1994). Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J Virol* **68**, 4400–4408.

da Silva Correia, J., Soldau, K., Christen, U., Tobias, P. S. & Ulevitch, R. J. (2001). Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. *J Biol Chem* **276**, 21129–21135.

De La Tribonniere, X., Mouton, Y., Vidal, V. & 7 other authors (2003). A phase I study of a six-week cycle of immunotherapy with Murabutide in HIV-1 patients naïve to antiretrovirals. *Med Sci Monit* **9**, PI61–PI68.

Deng, H., Liu, R., Ellmeier, W. & 12 other authors (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661–666.

Descombes, P. & Schibler, U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67**, 569–579.

Dragic, T., Litwin, V., Allaway, G. P. & 8 other authors (1996). HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667–673.

Emori, Y., Sasaki, H., Hayashi, Y. & Nomoto, K. (1996). Effect of Z-100, an immunomodulator extracted from human type tubercle bacilli, on the pulmonary metastases of Lewis lung carcinoma in attempt to regulate suppressor T cells and suppressor factor, IL-4. *Biotherapy* **9**, 249–256.

Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877.

Gaynor, R. (1992). Cellular transcription factors involved in the regulation of HIV-1 gene expression. *AIDS* **6**, 347–363.

Hale, K. K., Trollinger, D., Rihanek, M. & Manthey, C. L. (1999). Differential expression and activation of p38 mitogen-activated protein kinase α, β, γ, and δ in inflammatory cell lineages. *J Immunol* **162**, 4246–4252.

Hayashi, Y., Ebina, T., Suzuki, F. & Ishida, N. (1981). Interferon-inducing activity of an immunotherapeutic anticancer agent, SSM, prepared from *Mycobacterium tuberculosis* strain Aoyama B. *Microbiol Immunol* **25**, 305–316.

Henderson, A. J. & Calame, K. L. (1997). CCAAT/enhancer binding protein (C/EBP) sites are required for HIV-1 replication in primary macrophages but not $CD4^+$ T cells. *Proc Natl Acad Sci U S A* **94**, 8714–8719.

Henderson, A. J., Zou, X. & Calame, K. L. (1995). C/EBP proteins activate transcription from the human immunodeficiency virus type 1 long terminal repeat in macrophages/monocytes. *J Virol* **69**, 5337–5344.

Henderson, A. J., Connor, R. I. & Calame, K. L. (1996). C/EBP activators are required for HIV-1 replication and proviral induction in monocytic cell lines. *Immunity* **5**, 91–101.

Hill, C. M., Deng, H., Unutmaz, D., Kewalramani, V. N., Bastiani, L., Gorny, M. K., Zolla-Pazner, S. & Littman, D. R. (1997). Envelope glycoproteins from human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus can use human CCR5 as a coreceptor for viral entry and make direct CD4-dependent interactions with this chemokine receptor. *J Virol* **71**, 6296–6304.

Honda, Y., Rogers, L., Nakata, K., Zhao, B-Y., Pine, R., Nakai, Y., Kurosu, K., Rom, W. N. & Weiden, M. (1998). Type I interferon induces inhibitory 16-kD CCAAT/enhancer binding protein (C/EBP)β, repressing the HIV-1 long terminal repeat in macrophages: pulmonary tuberculosis alters C/EBP expression, enhancing HIV-1 replication. *J Exp Med* **188**, 1255–1265.

Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K. & Akira, S. (2001). Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol* **166**, 5688–5694.

Kalter, D. C., Nakamura, M., Turpin, J. A., Baca, L. M., Hoover, D. L., Dieffenbach, C., Ralph, P., Gendelman, H. E. & Meltzer, M. S. (1991). Enhanced HIV replication in macrophage colony-stimulating factor-treated monocytes. *J Immunol* **146**, 298–306.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K. & Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**, 115–122.

Kawamura, I., Mitsuyama, M. & Nomoto, K. (1990). Enhanced protection of cyclophosphamide-treated mice against infection with *Pseudomonas aeruginosa* after treatment with Z-100, a polysaccharide-rich extract from *Mycobacterium tuberculosis* Aoyama B. *Immunopharmacol Immunotoxicol* **12**, 331–343.

Keesler, G. A., Bray, J., Hunt, J. & 8 other authors (1998). Purification and activation of recombinant p38 isoforms α, β, γ, and δ. *Protein Expr Purif* **14**, 221–228.

Kelly, M. D., Naif, H. M., Adams, S. L., Cunningham, A. L. & Lloyd, A. R. (1998). Dichotomous effects of β-chemokines on HIV replication in monocytes and monocyte-derived macrophages. *J Immunol* **160**, 3091–3095.

Kobatake, H., Suekane, T., Murakami, Y., Niwa, S., Okahira, A. & Kushida, H. (1981). Studies on hot water extract of *Mycobacterium tuberculosis*. I. Structural analyses of polysaccharides. *Yakugaku Zasshi* **101**, 713–722.

Kobayashi, M., Nakajima, N., Pollard, R. B. & Suzuki, F. (1995). Tumor immunotherapeutic lipid-arabinomannan extracted from *Mycobacterium tuberculosis* (Z-100) induces the production of IL-12. In *Abstracts of the 86th Annual Meeting of the American Association for Cancer Research*, Toronto, p. 467.

Kobayashi, M., Pollard, R. B. & Suzuki, F. (1997). Inhibition of pulmonary metastasis by Z-100, an immunomodulatory lipid-arabinomannan extracted from *Mycobacterium tuberculosis*, in mice inoculated with B16 melanoma. *Anticancer Drugs* **8**, 156–163.

Koyanagi, Y., Miles, S., Mitsuyasu, R. T., Merrill, J. E., Vinters, H. V. & Chen, I. S. (1987). Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. *Science* **236**, 819–822.

Kumar, S., McDonnell, P. C., Gum, R. J., Hand, A. T., Lee, J. C. & Young, P. R. (1997). Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun* **235**, 533–538.

Lee, J. C. & Young, P. R. (1996). Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J Leukoc Biol* **59**, 152–157.

Mancino, G., Placido, R., Bach, S., Mariani, F., Montesano, C., Ercoli, L., Zembala, M. & Colizzi, V. (1997). Infection of human

monocytes with *Mycobacterium tuberculosis* enhances human immunodeficiency virus type 1 replication and transmission to T cells. *J Infect Dis* **175**, 1531–1535.

Maniatis, T., Falvo, J. V., Kim, T. H., Kim, T. K., Lin, C. H., Parekh, B. S. & Wathelet, M. G. (1998). Structure and function of the interferon-β enhanceosome. *Cold Spring Harb Symp Quant Biol* **63**, 609–620.

Mann, D. L., Gartner, S., Le Sane, F., Buchow, H. & Popovic, M. (1990). HIV-1 transmission and function of virus-infected monocytes/macrophages. *J Immunol* **144**, 2152–2158.

Masuda, T., Planelles, V., Krogstad, P. & Chen, I. S. Y. (1995). Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 *att* site: unusual phenotype of mutants in the zinc finger-like domain. *J Virol* **69**, 6687–6696.

Meylan, P. R. A., Guatelli, J. C., Munis, J. R., Richman, D. D. & Kornbluth, R. S. (1993). Mechanisms for the inhibition of HIV replication by interferons-α, -β, and -γ, in primary human macrophages. *Virology* **193**, 138–148.

Moriuchi, H., Moriuchi, M., Combadiere, C., Murphy, P. M. & Fauci, A. S. (1996). $CD8^+$ T-cell-derived soluble factor(s), but not β-chemokines RANTES, MIP-1α, and MIP-1β, suppress HIV-1 replication in monocyte/macrophages. *Proc Natl Acad Sci U S A* **93**, 15341–15345.

Natsuka, S., Akira, S., Nishio, Y., Hashimoto, S., Sugita, T., Isshiki, H. & Kishimoto, T. (1992). Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. *Blood* **79**, 460–466.

Oka, H., Emori, Y., Ohya, O., Kobayashi, N., Sasaki, H., Tanaka, Y., Hayashi, Y. & Nomoto, K. (1999). An immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis*, Z-100, restores the balance of Th1/Th2 cell responses in tumor bearing mice. *Immunol Lett* **70**, 109–117.

Oka, H., Shiraishi, Y., Sasaki, H., Yoshinaga, K., Emori, Y. & Takei, M. (2003). Antimetastatic effect of an immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis* strain Aoyama B, Z-100, through the production of interleukin-12. *Biol Pharm Bull* **26**, 1336–1341.

Ono, K. & Han, J. (2000). The p38 signal transduction pathway: activation and function. *Cell Signal* **12**, 1–13.

Pauza, C. D., Galindo, J. & Richman, D. D. (1988). Human immunodeficiency virus infection of monoblastoid cells: cellular differentiation determines the pattern of virus replication. *J Virol* **62**, 3558–3564.

Sasaki, H., Schmitt, D., Hayashi, Y., Pollard, R. B. & Suzuki, F. (1993). Antitumor mechanisms of Z-100, an immunomodulatory arabinomannan extracted from *Mycobacterium tuberculosis*: the importance of lymphocytes infiltrated into tumor sites. *Nat Immun* **12**, 104–112.

Sasaki, H., Kobayashi, M., Emori, Y., Ohya, O., Hayashi, Y. & Nomoto, K. (1997). Z-100, a polysaccharide-rich preparation extracted from the human type *Mycobacterium tuberculosis*, improves the resistance of Meth-A tumor-bearing mice to endogenous septic infection. *Biotherapy* **10**, 139–143.

Sasaki, H., Kobayashi, M., Pollard, R. B. & Suzuki, F. (2001). Effects of Z-100, a *Mycobacterium-tuberculosis*-derived arabinomannan, on the LP-BM5 murine leukemia virus infection in mice. *Pathobiology* **69**, 96–103.

Smith, P. D., Fox, C. H., Masur, H., Winter, H. S. & Alling, D. W. (1994). Quantitative analysis of mononuclear cells expressing human immunodeficiency virus type 1 RNA in esophageal mucosa. *J Exp Med* **180**, 1541–1546.

Stephens, J. C., Reich, D. E., Goldstein, D. B. & 36 other authors (1998). Dating the origin of the *CCR5*-Δ*32* AIDS-resistance allele by the coalescence of haplotypes. *Am J Hum Genet* **62**, 1507–1515.

Suzuki, F., Brutkiewicz, R. R. & Pollard, R. B. (1986). Importance of Lyt 1^+ T-cells in the antitumor activity of an immunomodulator, SSM, extracted from human-type Tubercle bacilli. *J Natl Cancer Inst* **77**, 441–447.

Tesmer, V. M., Rajadhyaksha, A., Babin, J. & Bina, M. (1993). NF-IL6-mediated transcriptional activation of the long terminal repeat of the human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **90**, 7298–7302.

Tissot, C. & Mechti, N. (1995). Molecular cloning of a new interferon-induced factor that represses human immunodeficiency virus type 1 long terminal repeat expression. *J Biol Chem* **270**, 14891–14898.

van't Wout, A. B., Kootstra, N. A., Mulder-Kampinga, G. A. & 7 other authors (1994). Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J Clin Invest* **94**, 2060–2067.

Verani, A., Scarlatti, G., Comar, M., Tresoldi, E., Polo, S., Giacca, M., Lusso, P., Siccardi, A. G. & Vercelli, D. (1997). C–C chemokines released by lipopolysaccharide (LPS)-stimulated human macrophages suppress HIV-1 infection in both macrophages and T cells. *J Exp Med* **185**, 805–816.

Vidal, V. F., Castéran, N., Riendeau, C. J., Kornfeld, H., Darcissac, E. C., Capron, A. & Bahr, G. M. (2001). Macrophage stimulation with Murabutide, an HIV-suppressive muramyl peptide derivative, selectively activates extracellular signal-regulated kinases 1 and 2, C/EBPβ and STAT1: role of CD14 and Toll-like receptors 2 and 4. *Eur J Immunol* **31**, 1962–1971.

Wang, X. S., Diener, K., Manthey, C. L. & 10 other authors (1997). Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J Biol Chem* **272**, 23668–23674.

Welden, M., Tanaka, N., Qiao, Y. & 14 other authors (2000). Differentiation of monocytes to macrophages switches the *Mycobacterium tuberculosis* effect on HIV-1 replication from stimulation to inhibition: modulation of interferon response and CCAAT/enhancer binding protein β expression. *J Immunol* **165**, 2028–2039.

Yee, J. K., Friedmann, T. & Burns, J. C. (1994). Generation of high-titer pseudotyped retroviral vectors with very broad host range. *Methods Cell Biol* **43**, 99–112.

Zybarth, G., Reiling, N., Schmidtmayerova, H., Sherry, B. & Bukrinski, M. (1999). Activation-induced resistance of human macrophages to HIV-1 infection in vitro. *J Immunol* **162**, 400–406.

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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

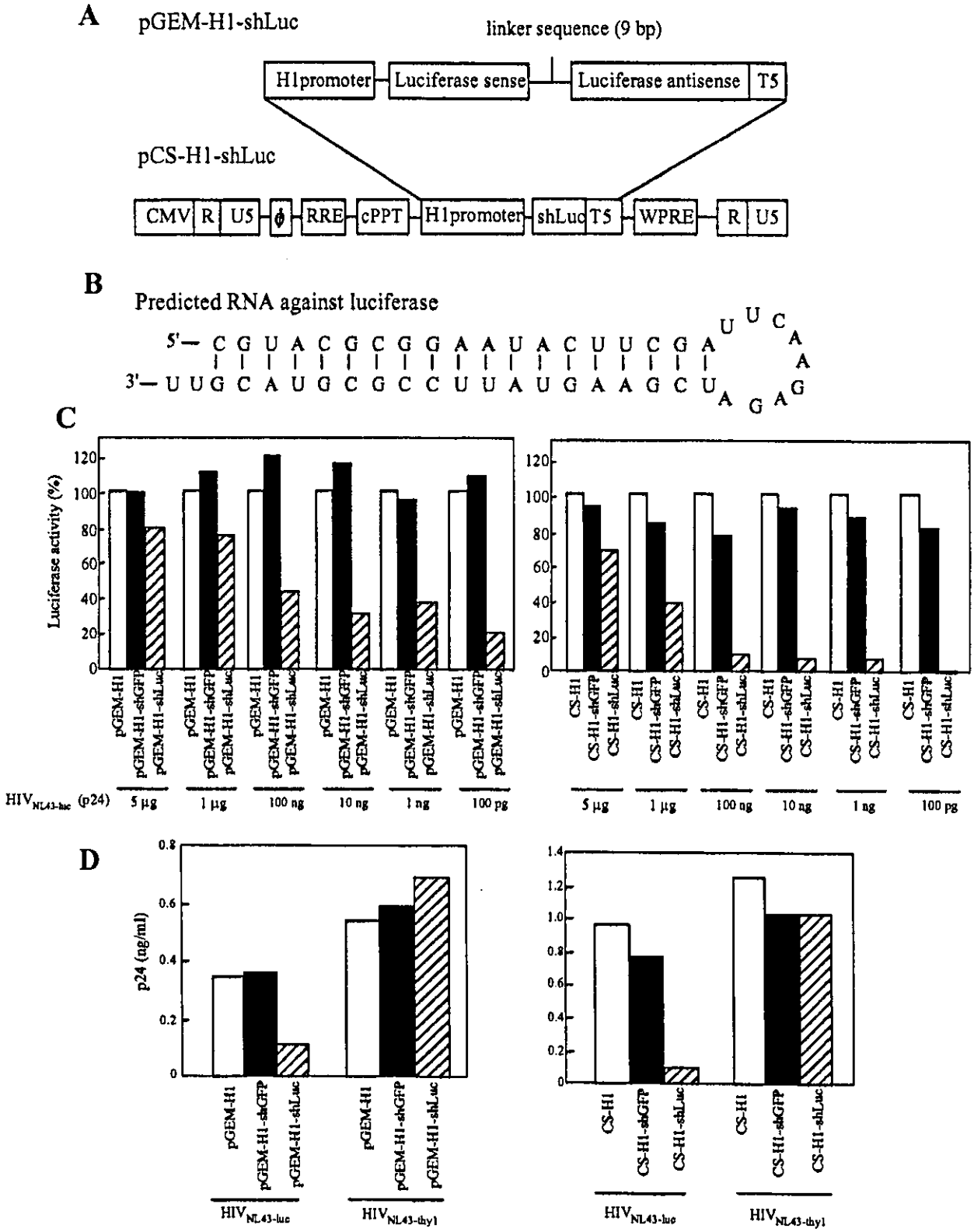
293T cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS). Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by the Ficoll centrifugation method. PBMCs were resuspended at 10^6 cells per ml in RPMI-1640 medium containing 10% 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
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
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
HIV NL43-luc (p24)
5 μg
1 μg
100 ng
10 ng
1 ng
100 pg
D
p24 (ng/ml)
HIV NL43-luc
HIV NL43-thy1

used for the siLuc are 5′-CGUACGCGGAAUACUUCGAdTdT-3′ (sense) and 5′-UCGAAGUAUUCCGCGUACG 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′-GGCTAACTAGGGAACCCACTG-3′ and 5′-CTGCTAGAGATTTTCCACACTGAC-3′, R/gag: 5′-GGCTAACTAGGGAACCCACT G-3′ and 5′-GACGCTCTCGCACCCATCTC-3′ or vif-F/vif-R: 5′-GAGATATAGCACAC AAGTAGACC-3′ and 5′-GCTAGTGCCAAGTACTGTGAGAT-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

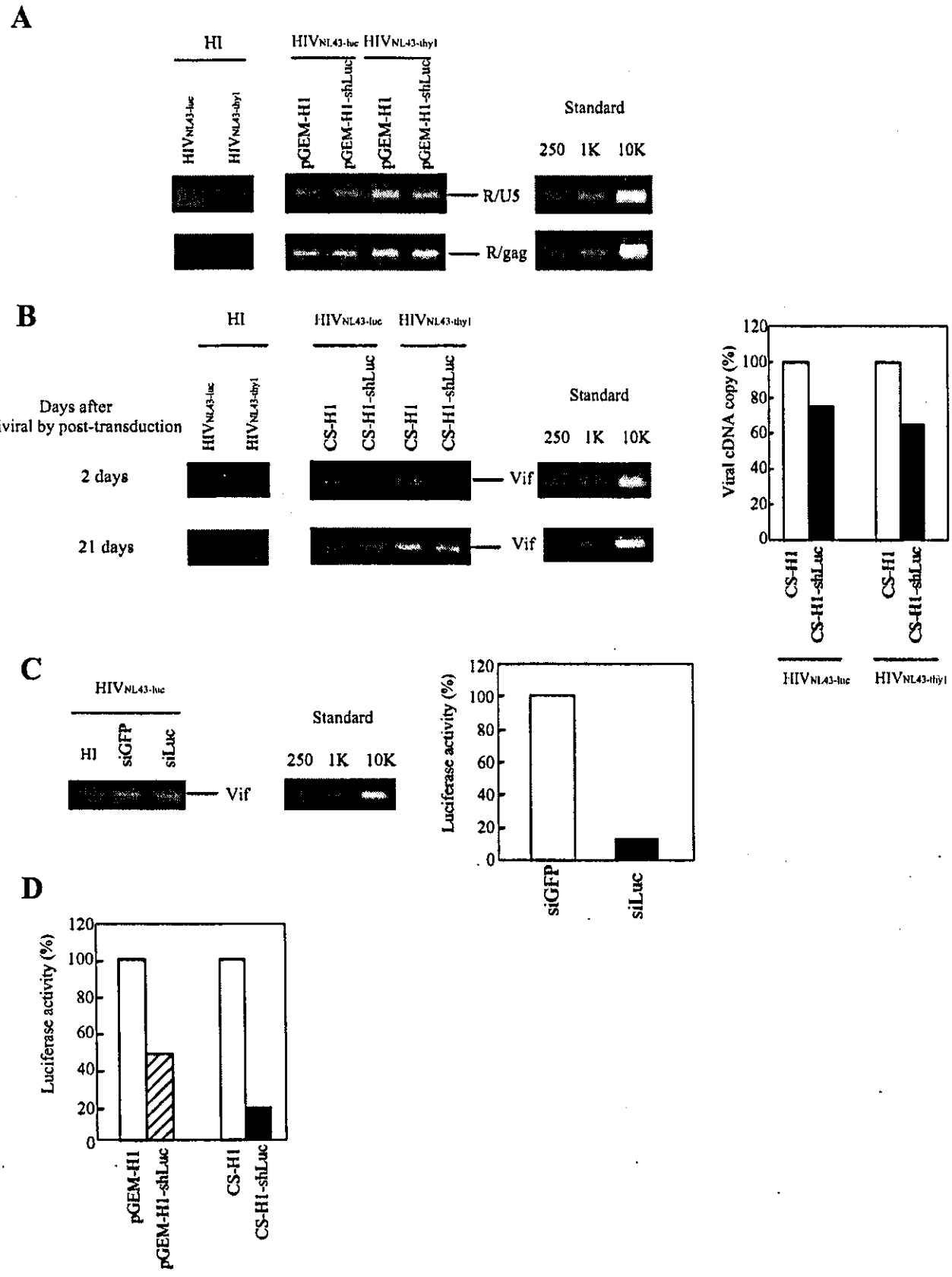
3.1. Construction of shRNA expression vector against luciferase

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