46. Naumann M, Scheidereit C. Activation of NF-κB in vivo is regulated by multiple phosphorylations. EMBO J 1994; 13:4597–4607.

47. Zhong H, Voll RE, Ghosh S. Phosphorylation of NF-κB p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell 1998; 1:661–671.

48. Jiang X, Takahashi N, Matsui N, Tetsuka T, Okamoto T. The NF-κB activation in the lymphotoxin beta receptor signaling depends on the phosphorylation of p65 at serine 536. J Biol Chem 2003; 278:919–926.

49. Jiang X, Takahashi N, Ando K, Otsuka T, Tetsuka T, Okamoto T. NF-κB p65 transacvation domain is involved in the NF-κB-inducing kinase (NIK) pathway. Biochem Biophys Res Commun 2003; 301:583–590.

50. Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. IκB kinases phosphorylate NF-κB p65 subunit on serine 536 in the transactivation domain. J Biol Chem 1999; 274:30353–30356.

51. Cao Y, Bonizzi G, Seagroves TN, Greten FR, Johnson R, Schmidt EV, Karin M. IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell 2001; 107:763–775.

52. Ghosh S, Karin M. Missing pieces in the NF-κB puzzle. Cell 2002; 109:S81–S96.

53. Wang D, Baldwin AS Jr. Activation of nuclear factor-κB-dependent transcription by tumor necrosis factor-α is mediated through phosphorylation of RelA/p65 on serine 529. J Biol Chem 1998; 273:29411–29416.

54. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr, Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. Mol Cell Biol 2000; 20:1626–1638.

55. Neumann M, Grieshammer T, Chuvpilo S, Kneitz B, Lohoff M, Schimpl A, Franza BR Jr, Serfling E. RelA/p65 is a molecular target for the immunosuppressive action of protein kinase A. EMBO J 1995; 14:1991–2004.

56. Ollivier V, Parry GC, Cobb RR, de Prost D, Mackman N. Elevated cyclic AMP inhibits NF-κB-mediated transcription

in human monocytic cells and endothelial cells. J Biol Chem 1996; 271:20828–20835.

57. Takahashi N, Tetsuka T, Uranishi H, Okamoto T. Inhibition of NF-κB transcriptional activity by protein kinase A. Eur J Biochem 2002; 269:1–7.

58. Uranishi H, Tetsuka T, Yamashita M, Asamitsu K, Shimizu M, Itoh M, Okamoto T. Involvement of the pro-oncoprotein TLS (translocated in liposarcoma) in nuclear factor-κB p65-mediated transcription as a coactivator. J Biol Chem 2001; 276: 13395–13401.

59. Tetsuka T, Uranishi H, Imai H, Ono T, Sonta S, Takahashi N, Asamitsu K, Okamoto T. Inhibition of nuclear factor-κB-mediated transcription by association with the amino-terminal enhancer of split, a Groucho-related protein lacking WD40 repeats. J Biol Chem 2000; 275:4383–4390.

60. Ashburner BP, Westerheide SD, Baldwin AS Jr. The p65 (RelA) subunit of NF-κB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol Cell Biol 2001; 21:7065–7077.

61. Anest V, Hanson JL, Cogswell PC, Steinbrecher KA, Strahl BD, Baldwin AS. A nucleosomal function for IκB kinase-α in NF-κB-dependent gene expression. Nature 2003; 423:659–663.

62. Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB. Histone H3 phosphorylation by IKK-a is critical for cytokine-induced gene expression. Nature 2003; 423:655–659.

63. Imbert V, et al. Tyrosine phosphorylation of IκBα activates NF-κB without proteolytic degradation of IκBα. Cell 1996; 86:787–798.

64. Livolsi A, Busuttil V, Imbert V, Abraham RT, Peyron JF. Tyrosine phosphorylation-dependent activation of NF-κB. Requirement for p56 LCK and ZAP-70 protein tyrosine kinase. Eur J Biochem 2001; 268:1508–1515.

65. Ozaki M, et al. Inhibition of the Rac1 GTPase protects against nonlethal ischemia/reperfusion-induced necrosis and apoptosis in vivo. FASEB J 2000; 14:418–429..

66. Sulciner DJ, Irani K, Yu ZX, Ferrans VJ, Goldschmidt-Clermont P, Finkel T. Rac1 regulates a cytokine-stimulated,

redox-dependent pathway necessary for NF-κB activation. Mol Cell Biol 1996; 16:7115–7121.

67. Sundaresan M, Yu ZX, Ferrans VJ, Sulciner DJ, Gutkind JS, Irani K, Goldschmidt-Clermont PJ, Finkel T. Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. Biochem J 1996; 318(Pt 2):379–382.

68. Tozawa K, Sakurada S, Kohri K, Okamoto T. Effects of anti-nuclear factor κB reagents in blocking adhesion of human cancer cells to vascular endothelial cells. Cancer Res 1995; 55: 4162–4167.

69. Roederer M, Staal FJ, Raju PA, Ela SW, Herzenberg LA. Cytokine-stimulated human immunodeficiency virus replication is inhibited by *N*-acetyl-L-cysteine. Proc Natl Acad Sci USA 1990; 87:4884–4888.

70. Merin JP, Matsuyama M, Kira T, Baba M, Okamoto T. Alpha-lipoic acid blocks HIV-1 LTR-dependent expression of hygromycin resistance in THP-1 stable transformants. FEBS Lett 1996; 394:9–13.

71. Schmidt KN, Traenckner EB, Meier B, Baeuerle PA. Induction of oxidative stress by okadaic acid is required for activation of transcription factor NF-κB. J Biol Chem 1995; 270:27136–27142.

72. Suzuki YJ, Mizuno M, Packer L. Signal transduction for nuclear factor-κB activation. Proposed location of antioxidant-inhibitable step. J Immunol 1994; 153:5008–5015.

73. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K. Evidence that reactive oxygen species do not mediate NF-κB activation. EMBO J 2003; 22: 3356–3366.

74. Okamoto T, Ogiwara H, Hayashi T, Mitsui A, Kawabe T, Yodoi J. Human thioredoxin/adult T cell leukemia-derived factor activates the enhancer binding protein of human immunodeficiency virus type 1 by thiol redox control mechanism. Int Immunol 1992; 4:811–819.

75. Hayashi T, Ueno Y, Okamoto T. Oxidoreductive regulation of nuclear factor κB. Involvement of a cellular reducing catalyst thioredoxin. J Biol Chem 1993; 268:11380–11388.

76. Matthews JR, Wakasugi N, Virelizier JL, Yodoi J, Hay RT. Thioredoxin regulates the DNA binding activity of NF-κB by reduction of a disulphide bond involving cysteine 62. Nucleic Acids Res 1992; 20:3821–3830.

77. Yoshida S, Katoh T, Tetsuka T, Uno K, Matsui N, Okamoto T. Involvement of thioredoxin in rheumatoid arthritis: its costimulatory roles in the TNF-α-induced production of IL-6 and IL-8 from cultured synovial fibroblasts. J Immunol 1999; 163:351–358.

78. Tagaya Y, Maeda Y, Mitsui A, Kondo N, Matsui H, Hamuro J, Brown N, Arai K, Yokota T, Wakasugi H, Yodoi J. ATL-derived factor (ADF), an IL-2 receptor/Tac inducer homologous to thioredoxin; possible involvement of dithiol-reduction in the IL-2 receptor induction. EMBO J 1989; 8:757–764.

79. Toledano MB, Leonard WJ. Modulation of transcription factor NF-κB binding activity by oxidation–reduction in vitro. Proc Natl Acad Sci USA 1991; 88:4328–4332.

80. Ghosh G, van Duyne G, Ghosh S, Sigler PB. Structure of NF-κB p50 homodimer bound to a κB site. Nature 1995; 373: 303–310.

81. Muller CW, Rey FA, Sodeoka M, Verdine GL, Harrison SC. Structure of the NF-κB p50 homodimer bound to DNA. Nature 1995; 373:311–317.

82. Qin J, Clore GM, Kennedy WM, Huth JR, Gronenborn AM. Solution structure of human thioredoxin in a mixed disulfide intermediate complex with its target peptide from the transcription factor NFκB. Structure 1995; 3:289–297.

83. Sakurada S, Kato T, Okamoto T. Induction of cytokines and ICAM-1 by proinflammatory cytokines in primary rheumatoid synovial fibroblasts and inhibition by *N*-acetyl-L-cysteine and aspirin. Int Immunol 1996; 8:1483–1493.

84. Taniguchi Y, Taniguchi-Ueda Y, Mori K, Yodoi J. A novel promoter sequence is involved in the oxidative stress-induced expression of the adult T-cell leukemia-derived factor (ADF)/human thioredoxin (Trx) gene. Nucleic Acids Res 1996; 24:2746–2752.

85. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thiore-

doxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 1998; 17:2596–2606.

86. Gotoh Y, Cooper JA. Reactive oxygen species- and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-α signal transduction. J Biol Chem 1998; 273:17477–17482.

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Glycoglycerolipid from the Membranes of *Acholeplasma laidlawii* Binds to Human Immunodeficiency Virus-1 (HIV-1) and Accelerates Its Entry into Cells

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Abstract. We have reported previously that glycoglycerolipids derived from the membranes of *Acholeplasma laidlawii*, 3-*O*-[2′-*O*-(α-D-glucopyranosyl)-6′-*O*-acyl-α-glucopyranosyl]-1,2-di-*O*-acyl-*sn*-glycerols (GAGDGs) bind to human cell lines. In addition, the GAGDGs were found to augment HIV-1 infection in human cell lines. Here we show that GAGDG binds to HIV-1 and facilitates the entry of HIV-1 into cells. The binding ability of GAGDG to HIV-1 was blocked by anti-GAGDG serum. Binding assay with synthetic GAGDGs and related compounds showed that the presence of branching form of acyl chains at the C14 or C16 position, glucose, and the acyl chain binding to the glucose were critical for efficient binding. GAGDG efficiently augmented the entry of HIV-1 into cells in a single-cycle replication assay. These results indicate that GAGDG of *A. laidlawii* membranes participates in the facilitation of HIV-1 infection.

HIV is well known to be the etiological agent of AIDS. However, the progression of AIDS is highly variable in individuals. Several factors, such as viral strains or host factors, have been attributed to such variations. Infectious agents, including various viruses, parasites, and bacteria, are considered as cofactors in the progression of AIDS [2]. Mycoplasmas are wall-less, parasitic, Gram-positive bacteria and the smallest organisms capable of self-replication [27]. *Mycoplasma fermentans* (*M. incognitus*) and *M. penetrans* were isolated from the tissues and urine of patients with AIDS [14, 15, 17]. And these mycoplasmas were shown to enhance the cytopathic effect of HIV-1 infection [13, 16]. In addition, mycoplasmas and acholeplasmas were reported to enhance HIV-1 replication in vitro [4, 5]. Thus, mycoplasmas might be reasonable candidates for cofactors in the progression of AIDS, but the precise chemical components responsible for enhancing HIV-1 replication are unknown. Possible mycoplasma components involved in enhancement of HIV infection include glycoglycerolipids. Indeed, our colleague has found that the glycoglycerolipid containing two glucose and three acyl chains, 3-*O*-[2′-*O*-α-D-glucopyranosyl]-6′-*O*-acyl-α-D-glucopyranosyl]-1,2-di-*O*-acyl-*sn*-glycerol (GAGDG) isolated from the membranes of *A. laidlawii* enhances the amount of p24 in culture supernatants of HIV-1 infected cells, although GAGDG failed to enhance the production of proinflammatory cytokines and LTR promoter activity [9]. The findings suggest that GAGDG might act mostly on viral entry and not on viral production. Moreover, GAGDG was reported to bind to lymphoid cells [23], but the mechanisms by which GAGDG enhances HIV replication remain to be defined.

In this study, we examined the interaction of GAGDG with HIV infection. We found that GAGDG coated on cover glasses was able to bind to HIV-1 and to facilitate the entry of HIV-1 into cells with a single-cycle replication assay. Furthermore, we examined the chemical structures of GAGDG essential for binding ability. Branching form of acyl chains with C14 or C16 were required for the ability. Both glucose and the acyl chains binding to glucose were also highly critical.

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Materials and Methods

Cells. A human T-cell line MOLT-4 cells were maintained in exponential growth in RPMI1640 medium containing 10% fetal calf serum (FCS, Mitsubishi Chemical, Tokyo, Japan), 2 mM L-glutamine, 100 μg mL^{-1} penicillin G, and 100 μg mL^{-1} streptomycin. To exclude the possibility of mycoplasma infections in the cell cultures, these cell suspensions were inoculated onto Hayflick's mycoplasma agar medium once a week.

Virus. HIV-1 IIIB was obtained from Y. Koyanagi [3]. Replication-incompetent luciferase reporter viruses were generated by cotransfecting 293T cells with pNL-Luc-E^-R^- [6] and plasmids encoding HIV-1 IIIB T-tropic Env protein or HIV-1 SF162 M-tropic Env protein or murine leukemia (MuLV) amphotropic (A-MLV) Env protein. pNL-Luc-E^-R^- reporter plasmid, which encodes full-length HIV-1 NL43 proviral DNA with a frameshift in *env* and expresses luciferase in place of *nef*, was obtained through the NIH AIDS Research and Reference Reagent Program. For expression of HIV-1 IIIB and SF162 Envs, 3.4 kb *Eco*RI-*Eco*RV fragment containing the *env* gene of HIV-1 IIIB and SF162 was subcloned into the β-actin-based expression vector pCAGGS and designated as pGGS-IIIB-env and pGGS-SF162-env, respectively. pSV-A-MLV-env was obtained from N. Landau [20]. Culture supernatants were harvested at 2 days post-transfection and were analyzed for their HIV-1 p24 content by a Retro Tek HIV-1 p24 antigen ELISA (Zepto Metrix, Buffalo, NY).

Glycoglycerolipids. GAGDGs with various acyl chains and 3-*O*-[2'-*O*-α-D-glucopyranosyl-α-D-glucopyranosyl]-1,2-di-*O*-acyl-*sn*-glycerol (GGDG), 3-*O*-(6'-*O*-acyl-α-D-glucopyranosyl)-1,2-di-*O*-acyl-*sn*-glycerol (AGDG), and 3-*O*-α-D-glucopyranosyl-1,2-di-*O*-acyl-*sn*-glycerol (GDG) were synthesized as previously described [11, 23–26].

HIV-1 binding assay. Fifty μL of GAGDG and its derivatives dissolved in chloroform were spread on individual 10-mm-diameter cover glasses (Matsunami, Osaka, Japan) to make thin films of GAGDGs as previously described [23]. The cover glasses coated with GAGDGs were incubated with 50 ng mL^{-1} of HIV-1 in 24-well cell culture plate for 24 h at 37°C. The cover glasses were washed two times with PBS, and bound viruses were quantitated by using the Retro Tek HIV-1 p24 antigen ELISA.

Antiserum and blocking assay. One mg mL^{-1} GAGDG containing palmitoyl (normalC16) as acyl chains was mixed with an equal volume of Freund's complete adjuvant (DIFCO, Detroit, MI) and was injected into New Zealand White rabbits. The animals were boosted with the same amount of antigen in Freund's incomplete adjuvant (DIFCO) every 4 weeks. Antiserum collected at 12 weeks was tested for reactivity with GAGDG by enzyme-linked immunosorbent assay (ELISA). The antiserum was diluted 1:10 with PBS and incubated with cover glasses coated with GAGDG for 1 h. The cover glasses were washed twice with PBS, and then the binding ability was measured as described above.

Luciferase reporter virus and HIV-1 entry assay. Luciferase reporter virus pseudotyped by T-tropic HIV-1 IIIB (HIV-Luc) was produced as mentioned above. Fifty μg of GAGDG in chloroform-methanol (2:1, by vol.) was dried in a round-bottom glass tube and suspended in 1 mL PBS by sonication for 1 min at output 5 (Sonifier cell disruptor 200, Branson, Danbury, CT). MOLT-4 cells (5×10^5 cell) in 1 mL of RPMI 1640 were incubated with 1 or 3 ng mL^{-1} luciferase reporter virus for 48 h in the presence of 1 μg mL^{-1} suspension of GAGDG. Luciferase activity was measured by using a Luciferase Assay Kit (Promega, Madison, WI). The activities (relative light unit) were normalized by protein concentrations of whole cells.

Results

Binding ability of GAGDG to HIV-1. We previously reported that *A. laidlawii* membranes are able to enhance HIV-1 replication [4] and that GAGDGs isolated from the membranes bind to lymphoid cells [23], which prompted us to assume that GAGDG could mediate the infection of HIV-1 to cells. To test this hypothesis, we initially examined the binding ability of GAGDG to HIV-1. Since GAGDG containing three isomyristoyl (isoC14) as acyl chains exhibited a high binding ability to lymphoid cells [23], we used GAGDG-isoC14 for binding assay. HIV-1 IIIB was incubated with cover glasses coated with GAGDG-isoC14, and the amount of HIV captured by the coverglasses was measured. As shown in Fig. 1a, HIV-1 could bind to the cover glasses coated with GAGDG in a dose-dependent manner. When 1.0 μg GAGDG-isoC14 was coated, the levels of captured HIV-1 IIIB reached a plateau and were approximately eightfold higher than those of uncoated ones. In contrast, 1.0 μg of synthetic analogue of GAGDG, containing three myristoyl (normalC14) as acyl chains failed to capture HIV-1, even though 10 μg of GAGDG normalC14 slightly captured HIV-1 ($p < 0.05$). To further determine the binding ability of GAGDG to various HIV-1 strains, we used HIV-1 viruses pseudotyped with HIV-1 IIIB T-tropic envelopes, SF162 M-tropic envelopes, and murine leukemia virus amphotropic envelopes (A-MLV). When these viruses were incubated with cover glasses coated with GAGDG-isoC14, the levels of captured viruses were significantly higher than that of the control (Fig. 1b).

Structures required for binding ability. To examine the structure of GAGDG required for binding ability, we used various derivatives of GAGDG (Fig. 2a). We initially examined the effect of diverse acyl chains on the binding ability to HIV-1. HIV-1 IIIB was incubated with a cover glass coated with GAGDG containing isobutyroyl (isoC4), isocaproyl (isoC8), isomyristoyl (isoC14), myristoyl (normalC14), isopalmitoyl (isoC16), or palmitoyl (normalC16) as acyl chains. HIV-1 was captured efficiently on the cover glasses coated with GAGDG-isoC16 and -isoC14, and less efficiently with -normalC14. Nevertheless, GAGDG-isoC4, -isoC8, and -normalC16 failed to show efficient ability to capture HIV-1 (Fig. 2b). To further elucidate the role of glucose, AGDG, one of the glucoses, was deleted from GAGDG, was coated on cover glasses followed by incubation with HIV-1. The cover glasses coated with AGDG failed to capture HIV-1. Subsequently, GGDG, devoid of the acyl

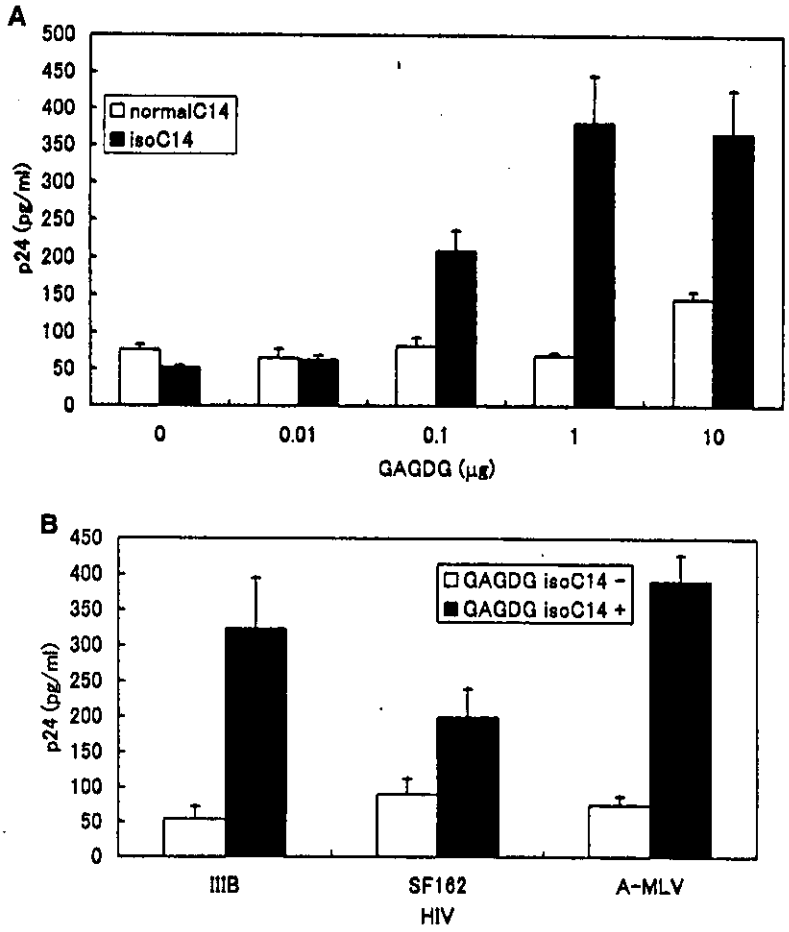

Fig. 1. Binding ability of GAGDG to HIV-1. T-tropic HIV-1 IIIB (A) and pseudotyped strain of HIV-1 IIIB, M-tropic SF162, and amphotropic A-MLV (B) were incubated with cover glasses coated with GAGDG-isoC14. The amount of HIV-1 captured on cover glass was measured by p24 ELISA. All values represent the means and SD of three assays.

chain binding to glucose at the C-6′ position, on cover glasses was incubated with HIV-1. The cover glasses also failed to capture HIV-1. Finally, GDG, in which the combination of the glucose and the acyl chain was deleted, as expected, lost the ability to capture HIV-1 (Fig. 2c).

Blocking of binding ability by anti-GAGDG serum. To confirm that the binding of HIV-1 to the GAGDG coated on cover glasses is GAGDG-specific, we performed a blocking assay using a rabbit antiserum against GAGDG-normalC16. This antiserum was found to cross-react with GAGDG-isoC16 by an enzyme-linked immunosorbent assay (Fig. 3a). The cover glasses coated with GAGDG-isoC16 were preincubated with the antiserum or pre-immune serum and then incubated with HIV-1 IIIB. As shown in Fig. 3b, the treatment of anti-GAGDG-normalC16 serum completely reduced the HIV-1 binding down to the levels of control without treatments, whereas pre-immune serum failed to do it.

Enhancement of HIV-1 entry by GAGDG. To examine whether the GAGDG bound to HIV would enhance the infection of HIV-1 to cells, the level of HIV-1 entry into cells was measured by using a luciferase reporter virus. An env from T-tropic HIV-1 IIIB was used to produce pseudotyped virus with an Env-defective provirus engineered to express a luciferase reporter gene. Incubation of cells with the pseudotyped virus resulted in a single round of infection and subsequent expression of the luciferase after viral integration. Determination of the luciferase activity provides a quantitative measure of viral entry [6]. As shown in Fig. 4a, MOLT-4 cells were efficiently infected with HIV-1 IIIB pseudotyped virus at a concentration of 3 ng mL^{-1} in the absence of GAGDG, although the presence of GAGDG seemed to augment the viral infection. Therefore, the pseudotyped virus was diluted up to a concentration of 1.0 ng mL^{-1}, in which the luciferase activity was not detected in the absence of GAGDG. When GAGDG was added under these conditions, the luciferase activity was augmented approximately sevenfold. Similarly, we examined whether other GAGDGs could induce HIV-1 infection. However, GAGDG-isoC4, -isoC8, and -normalC14 failed to augment the luciferase activity (Fig. 4b). These results indi-

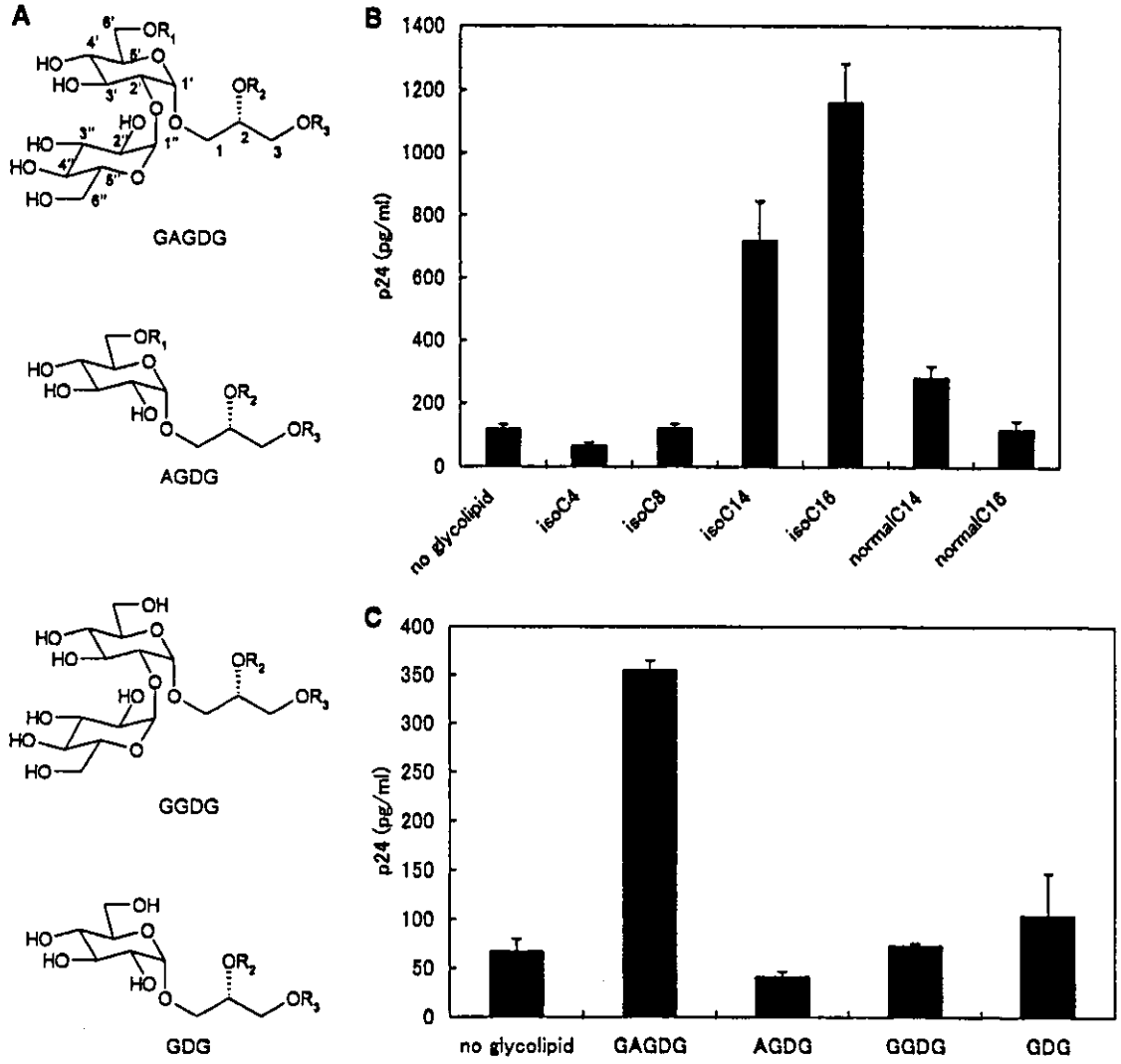

Fig. 2. Chemical structures and binding activity. (A) Chemical structures of GAGDG, AGDG, GGDG, and GDG. (B) Effect of different acyl chains on GAGDG binding to HIV-1. One μg of GAGDG-isoC4, -isoC8, -isoC14, -normalC14, -isoC16, and -normalC16 was coated on cover glass and incubated with HIV-1 IIIB. The amount of HIV-1 captured on cover glass was measured by p24 ELISA. All values represent the means and SD of three assays. (C) Effect of glucose and acyl chain on GAGDG binding to HIV-1. GAGDG-isoC14, AGDG-isoC14, GGDG-isoC14, and GDG-isoC14 were coated on cover glasses and incubated with HIV-1 IIIB. The amount of HIV-1 captured on cover glass was measured by p24 ELISA. All values represent the means and SD of three assays.

cate that GAGDG-isoC14 would significantly facilitate the infection of HIV-1 IIIB to MOLT-4 cells.

Discussion

In this study, we demonstrated that GAGDGs had the ability to capture HIV-1 and enhance HIV-1 infection. In our previous report, GAGDGs were shown to possess the ability to bind to lymphoid cells [23]. These findings suggest that GAGDGs may bind to both lymphoid cells and HIV-1 virus, leading to the augmentation of HIV-1 infection. AGDG, a glucose-deleted glycolipid, caused reduction of the ability to capture HIV-1, indicating that glucose is critical for capturing HIV-1. GGDG, in which an acyl chain binding to glucose was deleted, also caused the reduction of the ability. The result suggests that at least one acyl chain at the C-6′ position of glucose may play an important role for binding ability, although the roles of acyl chains at two other positions have not yet been examined. Both GAGDG-isoC14 and -isoC16 showed efficient ability to capture HIV-1, whereas the ability of GAGDG-isoC4, -isoC8, -normalC14, and -normalC16 was significantly reduced (Fig. 2b). These results indicate that the branching forms of acyl chains with C14 or C16 are necessary for efficiently capturing HIV-1.

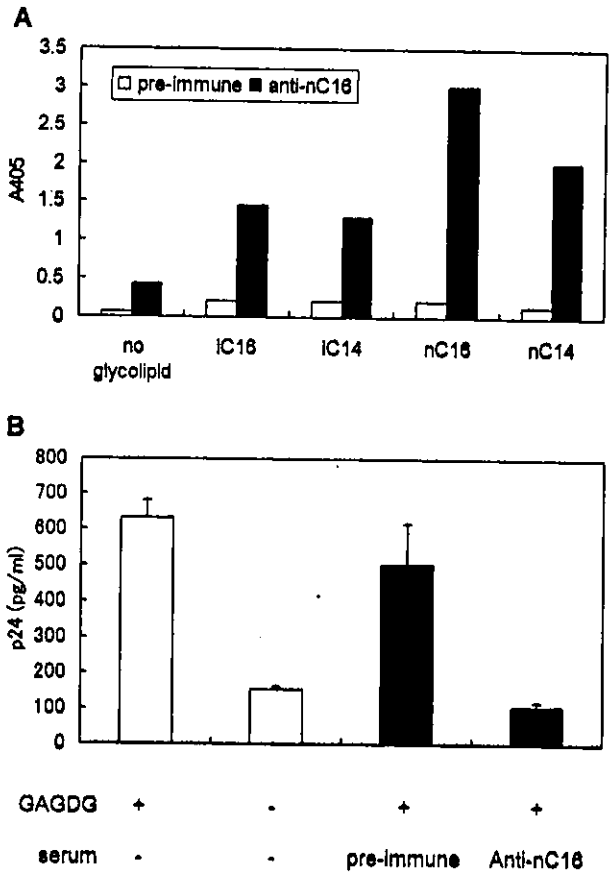

Fig. 3. Antiserum blocks GAGDG-isoC16 binding to HIV-1. (A) 1.0 μg of various GAGDGs was coated on cover glass and incubated with antiserum against GAGDG-normalC16 followed by incubation with HRP-conjugated anti-rabbit IgG. The affinity was measured by ELISA. (B) GAGDG-isoC16 was coated on cover glass and incubated with antiserum against GAGDG-normalC16 followed by incubation with HIV-1 IIIB. The amount of HIV-1 captured on cover glass was measured by p24 ELISA. All values represent the means and SD of three assays.

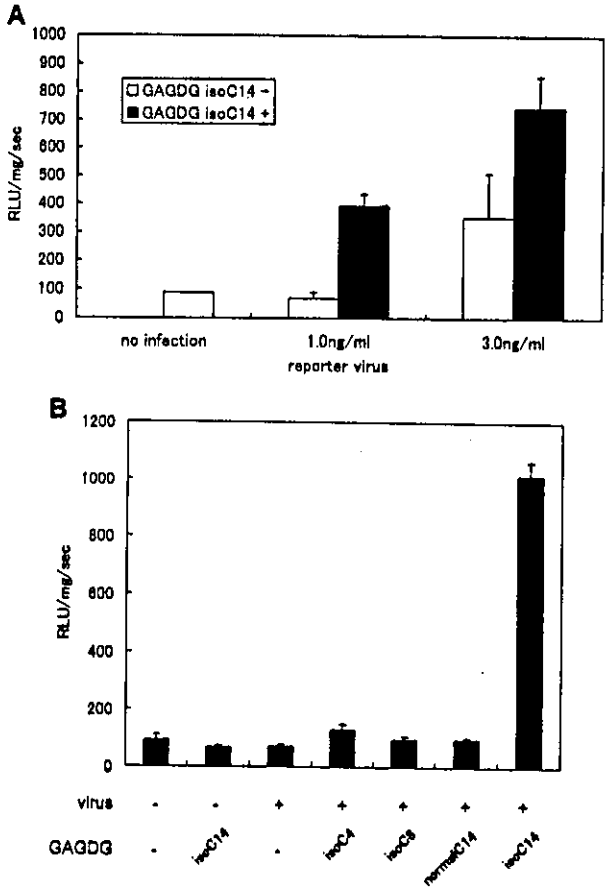

Fig. 4. Enhancement of HIV-1 infection by GAGDG. (A) 1 or 3 ng mL^{-1} HIV-1 IIIB pseudotyped luciferase reporter viruses was incubated with MOLT-4 cells in the presence of GAGDG-isoC14. (B) 1 ng mL^{-1} HIV-1 IIIB pseudotyped luciferase reporter virus was incubated with MOLT-4 cells in the presence of various GAGDGs. After 48 h incubation, luciferase activity was measured. All values represent the means and SD of three assays.

To examine the effect of GAGDG-isoC14 on HIV-1 IIIB infection, MOLT-4 cells were incubated with HIV-1 IIIB pseudotyped virus in the presence of GAGDG-isoC14. As shown in Fig. 4, the GAGDG-isoC14 could significantly augment HIV-1 IIIB pseudotyped virus infection, whereas GAGDG-isoC4, -isoC8, and -normalC14 failed to cause the HIV-1 infection. We also found that, in the presence of GAGDG, neither CXCR4-positive, CD4-negative HeLa cells, nor CXCR4-negative, CD4-positive THP-1 cells were infected with HIV-1 IIIB (data not shown). These findings suggest that, even in the presence of GAGDG, HIV-1 infection may be dependent on both CD4 and CXCR4 molecules on the cell surface and that GAGDG does not serve as a receptor or co-receptor for HIV-1 infection. Early reports showed that galactosylceramide, glycolipid of human cells, enhances HIV-1 infection by binding to envelope glycoprotein gp120 [7, 10, 18, 21], and the binding ability was inhibited by antibodies to gp120 [1]. In contrast, we found that in our experimental systems, the polyclonal antibody against gp120 did not block the binding between GAGDG and HIV-1 (data not shown), and that HIV-1 containing murine leukemia amphotropic (A-MLV) Env protein instead of HIV-1 Env protein could bind to GAGDG (Fig. 1b). The results indicate that the gp120 may not participate in the binding of GAGDG to HIV-1 and that GAGDG may enhance HIV-1 infection in different mechanisms from that of galactosylceramide. In our previous report, GAGDG-isoC14 was shown to have a higher binding ability to lymphoid cells than GAGDG-normalC14 [23], consistent with a hierarchy of the enhancement of HIV-1 entry by GAGDGs (Fig. 4). These findings suggest that GAGDG-isoC14

may capture both HIV-1 and lymphoid cells in a similar mechanism. In addition, a recent report showed that HIV-1 buds selectively from glycolipid-enriched membrane, lipid rafts and consequently possesses various proteins, including CD59 and Thy-1, derived from host cells [19]. The findings indicate that in vivo GAGDG may use these proteins as ligands to bind to both HIV-1 and lymphoid cells.

GAGDGs are found not only in the cell membranes of mycoplasmas but also in those of Gram-positive and Gram-negative bacteria and their L-forms [12]. But mycoplasmas are completely wall-less, and GAGDG is thought to be on the cell surface, indicating that GAGDG may interact directly with both host cells and HIV-1. It remains unclear how GAGDG functions *in vivo*, but several reports suggest that the fusion of mycoplasma with eukaryotic cells may result in the delivery of mycoplasma components into host cells [8, 22]. These findings suggest that GAGDG, which is delivered to host cells, may bind to HIV-1, resulting in the enhancement of HIV-1 infection. Alternatively, GAGDG on the surface of mycoplasma may bind to HIV-1, and then the binding complexes reach cells for viral entry.

Collectively, our data provide evidence that GAGDG of *A. laidlawii* membranes would augment the entry of HIV-1 into cells. Mycoplasma infection could be one of the cofactors, which might account for the progression of AIDS.

Literature Cited

1. Bhat S, Spitalnik SL, Gonzalez-Scarano F, Silberberg DH (1991) Galactosyl ceramide or a derivative is an essential component of the neural receptor for human immunodeficiency virus type 1 envelope glycoprotein gp120. Proc Natl Acad Sci USA 88:7131–7134
2. Blanchard A, Montagnier L, Gougeon ML (1997) Influence of microbial infections on the progression of HIV disease. Trends Microbiol 5:326–331
3. Cann AJ, Koyanagi Y, Chen IS (1988) High efficiency transfection of primary human lymphocytes and studies of gene expression. Oncogene 3:123–128
4. Chowdhury IH, Munakata T, Koyanagi Y, Kobayashi S, Arai S, Yamamoto N (1990) Mycoplasma can enhance HIV replication in vitro: A possible cofactor responsible for the progression of AIDS. Biochem Biophys Res Commun 170:1365–1370
5. Chowdhury MI, Munakata T, Koyanagi Y, Arai S, Yamamoto N (1994) Mycoplasma stimulates HIV-1 expression from acutely- and dormantly-infected promonocyte/monoblastoid cell lines. Arch Virol 139:431–438
6. Connor RI, Chen BK, Choe S, Landau NR (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206:935–944
7. Fantini J, Cook DG, Nathanson N, Spitalnik SL, Gonzalez-Scarano F (1993) Infection of colonic epithelial cell lines by type 1 human immunodeficiency virus is associated with cell surface expression of galactosylceramide, a potential alternative gp120 receptor. Proc Natl Acad Sci USA 90:2700–2704
8. Franzoso G, Dimitrov DS, Blumenthal R, Barile MF, Rottem S (1992) Fusion of Mycoplasma fermentans strain incognitus with T-lymphocytes. FEBS Lett 303:251–254
9. Fujimoto N (2002) Enhancement of HIV-1 replication by glycoglycerolipid from membrane of Acholeplasma laidlawii PG8. J Kurume Med Assoc 65:186–194
10. Hug P, Lin HM, Korte T, Xiao X, Dimitrov DS, Wang JM, Puri A, Blumenthal R (2000) Glycosphingolipids promote entry of a broad range of human immunodeficiency virus type 1 isolates into cell lines expressing CD4, CXCR4, and/or CCR5. J Virol 74:6377–6385
11. Imai H, Oishi T, kikuchi T, Hirama M (2000) Concise synthesis of 3-*O*-(2-*O*-α-D-glucopyranosyl-6-*O*-acyl-α-D-glucopyranosyl)-1,2-di-*O*-acyl-*sn*-glycerols. Tetrahedron 56:8451–8459
12. Kates M (1990) Glyco-, phosphoglyco-, and sulfoglycoglycerolipids of bacteria. In: Kates M (ed) Handbook of lipid research. New York: Plenum Press, pp 1–123
13. Lemaitre M, Henin Y, Destouesse F, Ferrieux C, Montagnier L, Blanchard A (1992) Role of mycoplasma infection in the cytopathic effect induced by human immunodeficiency virus type 1 in infected cell lines. Infect Immun 60:742–748
14. Lo SC, Dawson MS, Wong DM, Newton PB, 3rd, Sonoda MA, Engler WF, Wang RY, Shih JW, Alter HJ, Wear DJ (1989) Identification of Mycoplasma incognitus infection in patients with AIDS: An immunohistochemical, in situ hybridization and ultrastructural study. Am J Trop Med Hyg 41:601–616
15. Lo SC, Hayes MM, Wang RY, Pierce PF, Kotani H, Shih JW (1991a) Newly discovered mycoplasma isolated from patients infected with HIV. Lancet 338:1415–1418
16. Lo SC, Tsai S, Benish JR, Shih JW, Wear DJ, Wong DM (1991b) Enhancement of HIV-1 cytocidal effects in CD4+ lymphocytes by the AIDS-associated mycoplasma. Science 251:1074–1076
17. Lo SC, Hayes MM, Tully JG, Wang RY, Kotani H, Pierce PF, Rose DL, Shih JW (1992) Mycoplasma penetrans sp. nov., from the urogenital tract of patients with AIDS. Int J Syst Bacteriol 42:357–364
18. Long D, Berson JF, Cook DG, Doms RW (1994) Characterization of human immunodeficiency virus type 1 gp120 binding to liposomes containing galactosylceramide. J Virol 68:5890–5898
19. Nguyen DH, Hildreth JE (2000) Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. J Virol 74:3264–3272
20. Page KA, Landau NR, Littman DR (1990) Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J Virol 64:5270–5276
21. Puri A, Hug P, Jernigan K, Barchi J, Kim HY, Hamilton J, Wiels J, Murray GJ, Brady RO, Blumenthal R (1998) The neutral glycosphingolipid globotriaosylceramide promotes fusion mediated by a CD4-dependent CXCR4-utilizing HIV type 1 envelope glycoprotein. Proc Natl Acad Sci USA 95:14435–14440
22. Rottem S, Cirillo VP, Kruyff Bd, Shinitzky M, Razin S (1973) Cholesterol in mycoplasma membranes. Correlation of enzymic and transport activities with physical state of lipids in membranes of Mycoplasma mycoides var. capri adapted to grow with low cholesterol concentrations. Biochim Biophys Acta 323:509–519
23. Toujima S, Kuwano K, Zhang Y, Fujimoto N, Hirama M, Oishi T, Fukuda S, Nagumo Y, Imai H, Kikuchi T, Arai S (2000) Binding of glycoglycerolipid derived from membranes of *Acholeplasma laidlawii* PG8 and synthetic analogues to lymphoid cells. Microbiology 146:2317–2323
24. van Boeckel C, Avan Boom JH (1985a) Synthesis of phosphatidyl-α-glucosyl glycerol containing a dioleoyl phosphatidyl moiety.

Application of the tetraisopropyldisiloxane-1,3-diyl (tips) protecting group in sugar chemistry, part III. Tetrahedron 41:4545–4555

25. van Boeckel CA, van Boom JH (1985b) Synthesis of phosphatidyl-α-glucosyl glycerol containing a dioleoyl phosphatidyl moiety. Application of the tetraisopropyldisiloxane-1,3-diyl (tips) protecting group in sugar chemistry, part IV. Tetrahedron 41:4557–4565
26. van Boeckel CA, van Boom JH (1985c) Synthesis of phosphatidyl-α-glucosyl glycerol containing a dioleoyl phosphatidyl moiety. Application of the tetraisopropyldisiloxane-1,3-diyl (tips) protecting group in sugar chemistry, part V. Tetrahedron 41:4567–4575
27. Weisburg WG, Tully JG, Rose DL, Petzel JP, Oyaizu H, Yang D, Mandelco L, Sechrest J, Lawrence TG, Van Etten J, Maniloff J, Woese CR (1989) A phylogenetic analysis of the mycoplasmas: Basis for their classification. J Bacteriol 171:6455–6467

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

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Abstract

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

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

Introduction

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

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

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

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

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

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

Results

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

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

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

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

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

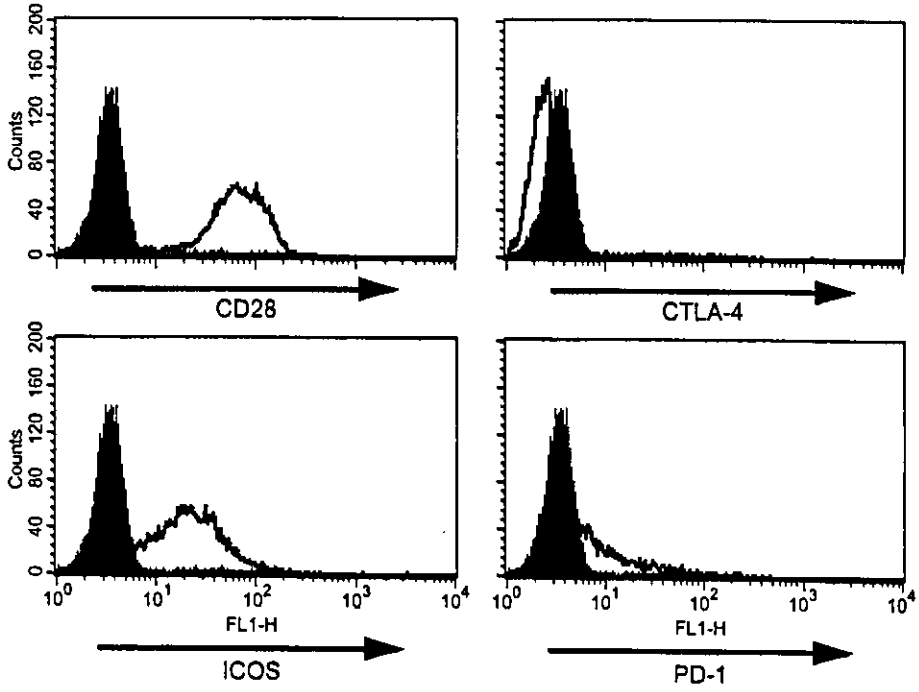

Fig. 1. Cell surface expression of CD28 family molecules on PHA-stimulated CD4 + PBMC. PHA-stimulated CD4+ PBMC cultured in the presence of IL-2 for 6 days were stained with mAbs to CD28, CTLA-4, ICOS, or PD-1 (open histogram), and subsequently stained with FITC-labeled second antibody. Closed histogram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

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

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

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

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

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

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

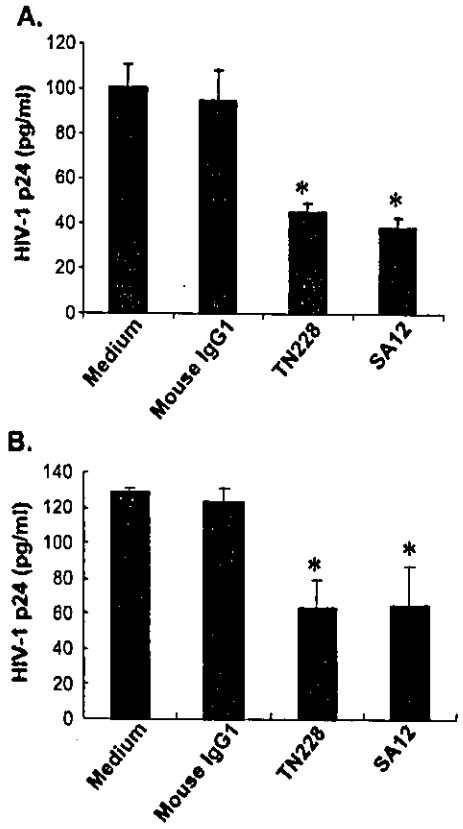

Fig. 2. Immobilized mAbs for ICOS (SA12) and CD28 (TN228) inhibited HIV-1 replication. CD4+ PBMC cultured for 4–6 days after PHA stimulation were infected with X4 HIV-1 NL4-3 (A) or R5 HIV-1 JR CSF (B) for 2 h, and cultured in medium alone or in the plates precoated with 5 μg/ml antibodies indicated.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

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

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

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

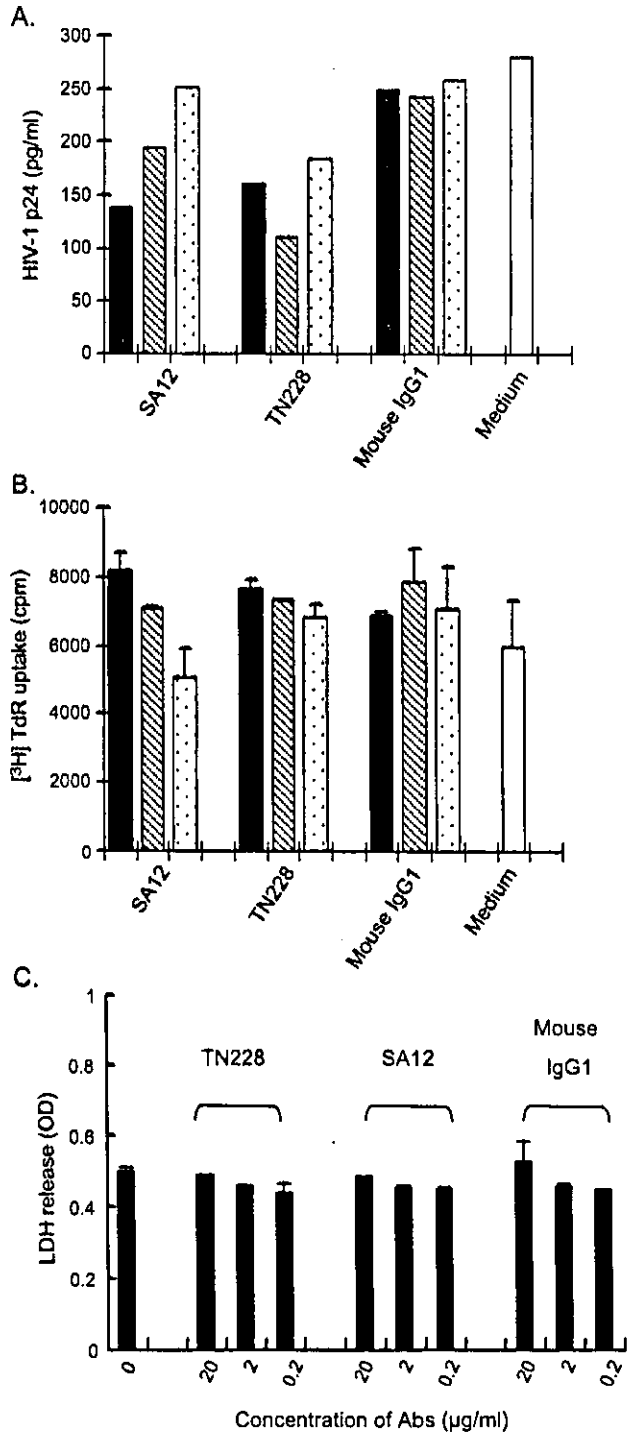

Fig. 3. Stimulation of ICOS or CD28 suppressed HIV-1 replication but not the proliferation or viability of CD4+ T cells. (A) PHA-stimulated purified CD4+ T cells infected with HIV-1 NL4-3 were cultured on plates precoated with antibodies at the indicated concentration of 5 μg/ml (■), 0.25 μg/ml (▨),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

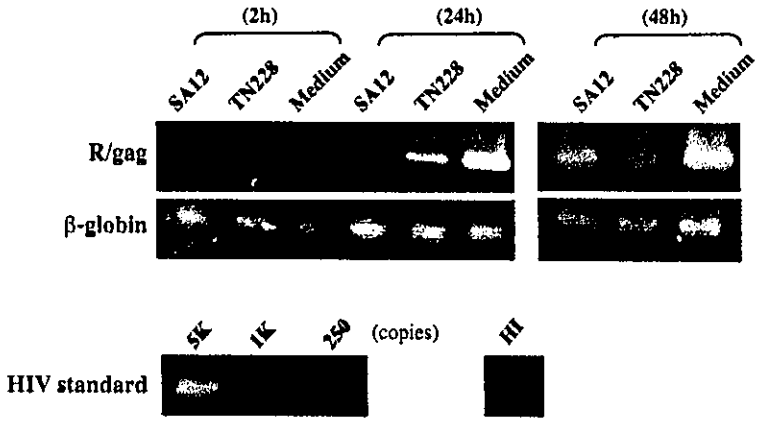

Fig. 4. Analysis of HIV-1 DNA synthesis in NL4-3-infected CD4+ PBMC treated with immobilized ICOS mAb. CD4+ PBMC cultured for 4 days after PHA stimulation were infected with DNase-treated HIV-1 NL4-3 for 2 h and cultured in 12-well flat-bottomed plates (10^6 cells/well) precoated with ICOS or CD28 mAbs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

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

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

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

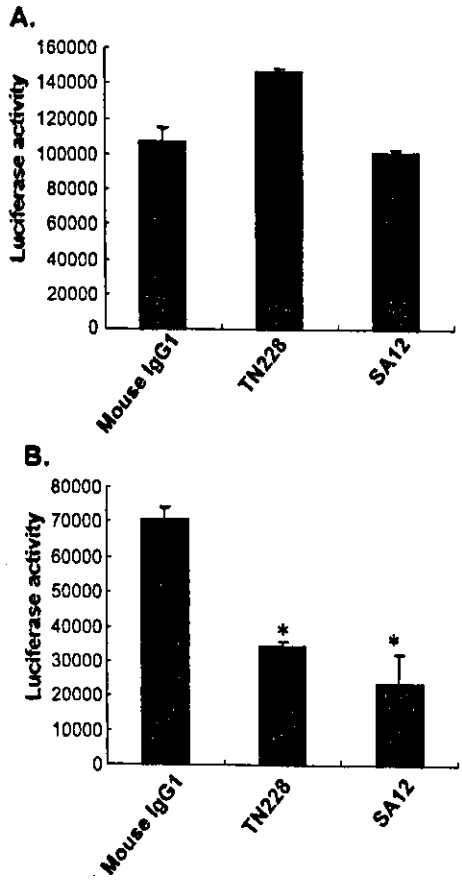

Fig. 5. Effects of ICOS or CD28 stimulation on pseudotype HIV-1 gene expression. CD4+ PBMC cultured for 4 days after PHA stimulation were incubated overnight in plates coated with immobilized ICOS or CD28 mAbs or control mouse IgG (1 μg/ml) (10^6/well in 24-well plates), then infected with luciferase-expressing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

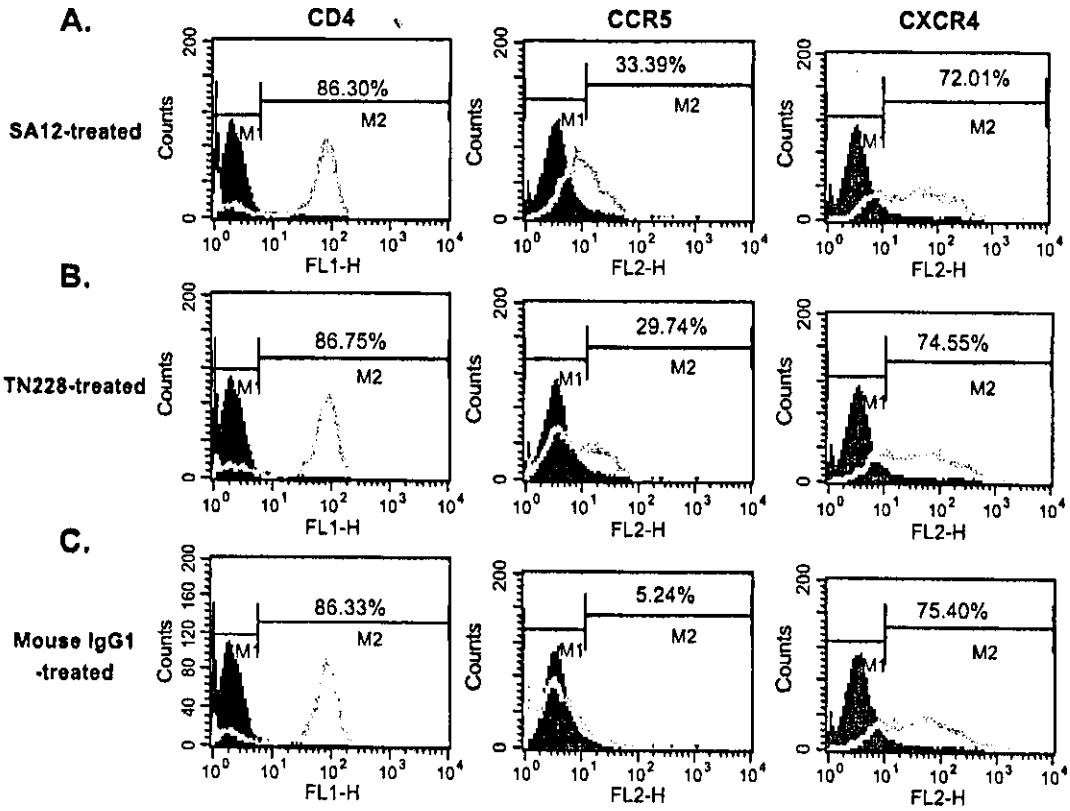

Fig. 6. Effects of ICOS or CD28 stimulation on HIV-1 receptor expression by CD4+ T cells. PHA-stimulated CD4+ PBMC were treated either with immobilized mAbs to ICOS (A) or CD28 (B) or control mouse IgG1 (C) (5 μg/ml) in 24-well flat-bottomed plates, and the expression of CXCR4 and CCR5 receptors was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

Effect of ICOS or CD28 stimulation on cytokine production

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

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

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

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

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

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

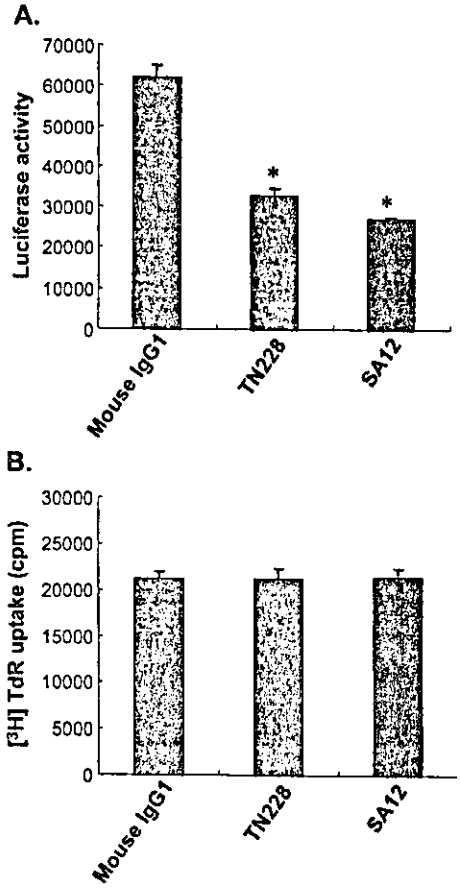

Fig. 7. Effects of ICOS or CD28 stimulation immediately after PHA stimulation. CD4+ PBMC were stimulated with PHA for 30 min, washed, and cultured on plates precoated with immobilized ICOS or CD28 mAbs or control mouse IgG (1 μg/ml) in the presence of IL-2. HIV-1 expression (A) and proliferation (B)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

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

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

Natural ligands for ICOS-inhibited HIV-1 replication

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

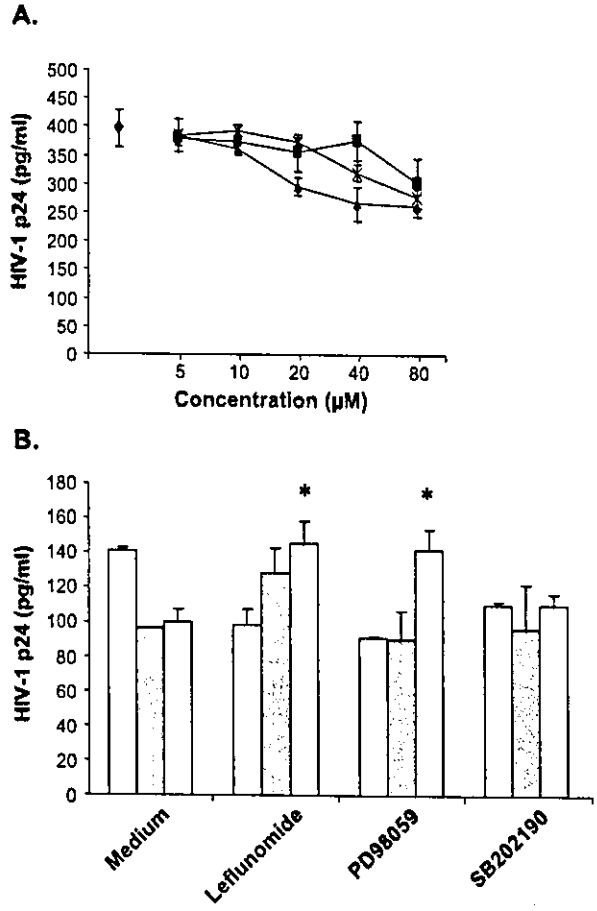

Fig. 8. The effects of Leflunomide on ICOS- or CD28-induced inhibition of HIV-1 replication. (A) PHA-stimulated CD4+ PBMC infected with HIV-1 NL4-3 were cultured in the absence (♦) or presence of various concentrations of Leflunomide (NF-κB-inhibitor) (■), PD98059 (MEK-inhibitor) (▲), or SB202190 (p38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

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

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

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

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

Discussion

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

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

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

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