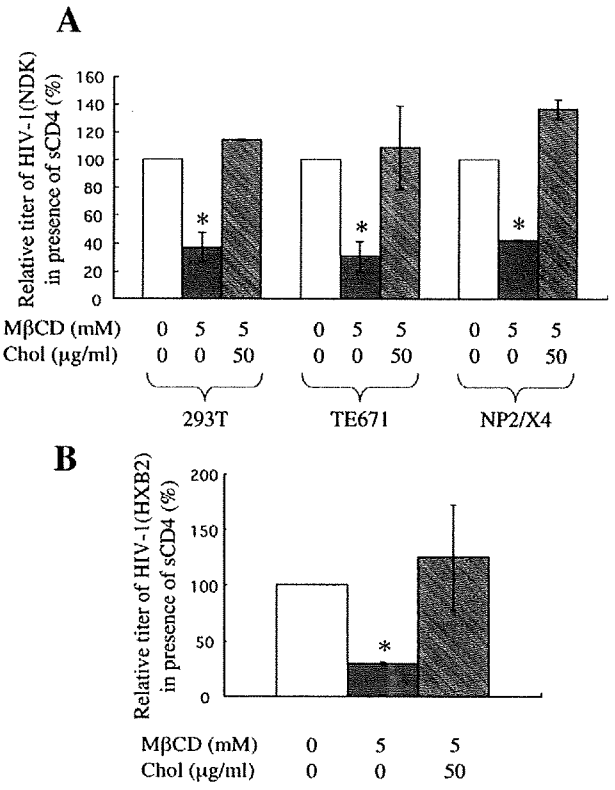

Fig. 7. Effect of MβCD on soluble CD4-induced CD4-dependent HIV-1 infection. (A) The HIV-1 vector having the NDK Env protein was inoculated into indicated cells in presence of soluble CD4 (sCD4) (20 μg/ml). (B) The HIV-1 vector having the HXB2 Env protein was inoculated into treated TE671 cells in presence of sCD4. Relative transduction titers to those in untreated cells are indicated. These experiments were independently repeated three times. Asterisks indicate statistically significant differences compared to their controls.

kindly obtained from Dr. U.Hazan (Dumonceaux et al., 1998). The CD4-independent HIV-1 Env (8X strain) expression plasmid was kindly provided from Dr. R. Doms (Hoffman et al., 1999). The CD4-dependent HIV-1 (HXB2 strain) Env expression plasmid was kindly obtained from Dr. Y. Yokomaku. The VSV-G expression plasmid (pHEF-VSVG) was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAIS, NIH, USA (Chang et al., 1999).

Cells

Human glioma NP2 (Soda et al., 1999), human rhabdosarcoma TE671, human cervical cancer HeLa, and human embryonal kidney (HEK) 293 T cell were cultured in Dulbecco's modified Eagle's medium (Sigma) at 37 °C in 5% CO_2. The culture media were supplemented with 8% fetal bovine serum (Biofluids). The original NP2 cells do not express endogenous CXCR4 and CD4 proteins, whereas NP2/X4 and NP2/CD4/X4 cells were processed to express exogenous CXCR4 alone and both of CD4 and CXCR4, respectively (Soda et al., 1999). TE671/CD4 and 293T/CD4 cells were constructed as follows. HEK 293T cells were transfected with murine leukemia virus (MLV) *gag–pol* (3 μg) (TAKARA), CD4-encoding retroviral vector (3 μg), and VSV-G expression plasmid (3 μg) (Chang et al., 1999) by the TransIT LT1 regent (30 μl) (Mirus). The cells were washed 24 h after transfection, and cultured for 24 h in fresh medium. Culture supernatant of the transfected cells was inoculated into 293 T or TE671 cells. The inoculated cells were selected by puromycin (10 μg/ml). The puromycin-resistant cell pool was utilized in this study. TE671 and 293 T cells over-expressing CXCR4 were constructed by transduction of these cells with CXCR4-encoding MLV vector as described above.

Transduction assay

To obtain HIV-1 vector particles containing Env protein, human 293T cells were transfected with a packaging construct of HIV-1 (R8.91) (Naldini et al., 1996), LacZ-containing HIV-1 vector (Iwakuma et al., 1999), and the appropriate Env expression plasmids (3 μg each) by the Trans IT-LT1 reagent. The transfected cells were washed with medium 24 h after transfection, and continued to be cultured in fresh medium for 24 h . Target cells were inoculated with the culture supernatants of the transfected cells in presence or absence of soluble CD4 (20 μg/ml) (obtained from AIDS Research and Reference Reagent program, NIH). The inoculated cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Wako) 2 days after inoculation. Blue cells were counted to estimate transduction titer. Undiluted CD4-independent and -dependent vectors induced usually around 5×10^2 and 5×10^4 blue cells. Therefore, in this study, CD4-dependent vectors were diluted 100 times with medium.

Cholesterol depletion

The cells were treated with methyl-β-cyclodextrin (MβCD) (Sigma) in FBS-free medium, or with 5 mM MβCD and 50 μg/ml cholesterol (Wako) for 30–120 min at 37 °C. As control, the cells were exposed to FBS-free medium alone. After incubation, the cells were washed with phosphate buffer saline (PBS) before being used to remove MβCD and cholesterol.

Filipin staining

The treated cells were fixed with 1% p-formaldehyde in PBS for 10 min at room temperature. The fixed cells were washed and stained with filipin (0.1 g/ml) (Sigma) in PBS for 2 h at room temperature. After washing with PBS, the cells were collected by scraper and fluorescence strength at 525 nm of the cells was analyzed by a flow cytometry (Becton Dickinson). Fluorescence strength of filipin at 525 nm is reduced by its binding to cholesterol (Severs and Robenek, 1983; Castanho et al., 1992).

FACS

To analyze cell surface expression of CXCR4, suspended cells were treated with a rat anti-CXCR4 antibody (A80) for 1 h at 4 °C. The CXCR4 antibody (A80) recognizes the third extracellular loop of CXCR4 (Tanaka et al., 2001). The cells were washed with PBS 3 times, and then treated with a FITC-conjugated anti-rat IgG antibody (Sigma). The stained cells were applied to a flow cytometry (Coulter).

Assay of HIV-1 Env-mediated cell fusion

HEK 293T cells were transfected with the HIV Env expression plasmid. The plasmid additionally encodes the tat protein. HEK 293T cells were transfected with a tat expression plasmid as a control. Target cells were transfected with the LTR-LacZ plasmid, and then were treated with MβCD. These cells were mixed 48 h after transfection, and cultured for 24 h . LacZ activities of the cell lysates prepared from the mixed cultures were measured by the high sensitive β-gal activity measurement kit (Stratagene).

Fractionation of raft membrane microdomains

HA-tagged CXCR4 and CD4 expressing cells were washed and lysed on ice by 0.1% Triton X-100. The cell lysates were centrifuged and its supernatant was defined as soluble fraction. Equal volume of sample buffer was added to its precipitates, and it was defined as insoluble fraction. These fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%), and were transferred onto a PVDF membrane (Millipore). Western immunoblotting was performed

using a mouse anti-HA monoclonal (Convance) or anti-CD4 antibody (Santa Cruz Biotechnology). A horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Red) was used as secondary antibody. Antibody-binding proteins were visualized using ECL Western blotting detection regents (Amersham Phaemacia Biotech).

Immunofluorescence microscopy

CXCR4 expressing cells were cultured on four-well culture slides (Miles) for 24 h . Cells were incubated with the rat anti-CXCR4 antibody (A80) for 1 h at 4 °C, followed by AlexaFlour 555-conjugated CT-B and FITC-conjugated anti-rat IgG for 1 h at 4 °C. Cells were observed using a confocal fluorescence microscope (Leica).

Statistical analysis

Differences between groups of data were deter,imed by Student's *t*-test. Statistical significance was set as $P<0.01$ for all tests.

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The Novel CXCR4 Antagonist KRH-3955 Is an Orally Bioavailable and Extremely Potent Inhibitor of Human Immunodeficiency Virus Type 1 Infection: Comparative Studies with AMD3100▽

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The previously reported CXCR4 antagonist KRH-1636 was a potent and selective inhibitor of CXCR4-using (X4) human immunodeficiency virus type 1 (HIV-1) but could not be further developed as an anti-HIV-1 agent because of its poor oral bioavailability. Newly developed KRH-3955 is a KRH-1636 derivative that is bioavailable when administered orally with much more potent anti-HIV-1 activity than AMD3100 and KRH-1636. The compound very potently inhibits the replication of X4 HIV-1, including clinical isolates in activated peripheral blood mononuclear cells from different donors. It is also active against recombinant X4 HIV-1 containing resistance mutations in reverse transcriptase and protease and envelope with enfuvirtide resistance mutations. KRH-3955 inhibits both SDF-1α binding to CXCR4 and Ca^{2+} signaling through the receptor. KRH-3955 inhibits the binding of anti-CXCR4 monoclonal antibodies that recognize the first, second, or third extracellular loop of CXCR4. The compound shows an oral bioavailability of 25.6% in rats, and its oral administration blocks X4 HIV-1 replication in the human peripheral blood lymphocyte-severe combined immunodeficiency mouse system. Thus, KRH-3955 is a new promising agent for HIV-1 infection and AIDS.

The chemokine receptors CXCR4 and CCR5 serve as major coreceptors of human immunodeficiency virus type 1 (HIV-1), along with CD4 as a primary receptor for virus entry (2, 15, 18, 19). SDF-1α, which is a ligand for CXCR4, blocks the infection of CXCR4-utilizing X4 HIV-1 strains (7, 34). On the other hand, ligands for CCR5 such as RANTES inhibit CCR5-utilizing R5 HIV-1 (10). These findings made chemokines, chemokine derivatives, or small-molecule inhibitors of chemokine receptors attractive candidates as a new class of anti-HIV-1 agents. Many CCR5 antagonists have been developed as anti-HIV-1 drugs. These include TAK-779 (Takeda Pharmaceutical Company) (5), TAK-652 (6), TAK-220 (45), SCH-C (Schering-Plough) (43), SCH-D (vicriviroc) (42), GW873140 (aplaviroc; Ono Pharmaceutical/Glaxo Smith Kline) (28), and UK-427,857 (maraviroc; Pfizer Inc.) (17). Of these, maraviroc was approved by the U.S. FDA in 2007 for the treatment of R5 HIV-1 in treatment-experienced adult patients, combined with other antiretroviral treatment. Several classes of CXCR4 antagonists have also been reported. The bicyclam AMD3100 showed antivirus activity against many X4 and some R5X4 HIV strains in peripheral blood mononuclear cells (PBMCs) but not against R5 strains (16, 40). The pharmacokinetics and antiviral activity of this compound were also evaluated in humans (21, 22). T22, [Tyr-5,12, Lys-7]polyphemusin II, which is an 18-mer peptide derived from horseshoe crab blood cells, was reported to specifically inhibit X4 HIV-1 strains (30). Studies on the pharmacophore of T140 (a derivative of T22) led to the identification of cyclic pentapeptides (46).

In 2003, we reported that KRH-1636 is a potent and selective CXCR4 antagonist and inhibitor of X4 HIV-1 (23). Although the compound was absorbed efficiently from the rat duodenum, it has poor oral bioavailability. Continuous efforts to find more potent CXCR4 antagonists that are bioavailable when administered orally allowed us to develop KRH-3955 by a combination of chemical modification of the lead compound and biological assays. In this report, we describe the results of a preclinical evaluation of KRH-3955, including its in vitro anti-HIV-1 activity, its in vivo efficacy in the human peripheral blood lymphocyte (hu-PBL)-severe combined immunodeficiency (SCID) mouse model, and its pharmacokinetics in rats in comparison with those of AMD3100.

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MATERIALS AND METHODS

Compounds. The synthesis and purification of KRH-3955, *N,N*-dipropyl-*N'*-[4-({[(1*H*-imidazol-2-yl)methyl][(1-methyl-1*H*-imidazol-2-yl)methyl]amino}methyl)benzyl]-*N'*-methylbutane-1,4-diamine tri-(2*R*,3*R*)-tartrate, were carried out by Kureha Corporation. The chemical structure of KRH-3955 is shown in Fig. 1. The CXCR4 antagonist AMD3100 and zidovudine (AZT) were obtained from Sigma. Saquinavir was obtained

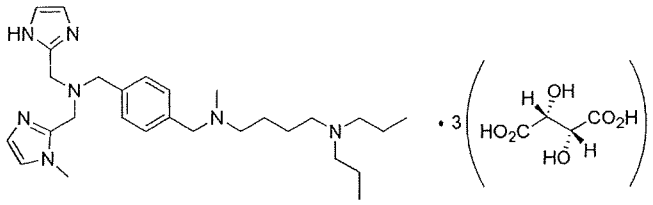

FIG. 1. Chemical structure of KRH-3955.

from the NIH AIDS Research and Reference Reagent Program, NIAID, Bethesda, MD. AMD070 and SCH-D were synthesized at Kureha Corporation.

Cells. Molt-4 no. 8 cells (24) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and antibiotics (50 ng/ml penicillin, 50 ng/ml streptomycin, and 100 ng/ml neomycin; Invitrogen), which is referred to as RPMI medium. Chemokine receptor-expressing human embryonic kidney 293 (HEK293) cells (ATCC CRL-1573) and Chinese hamster ovary (CHO) cells (ATCC CCL-61) were maintained in minimal essential medium or F-12 (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (50 ng/ml penicillin, 50 ng/ml streptomycin, and 100 ng/ml neomycin). PBMCs from HIV-1-seronegative healthy donors were isolated by Ficoll-Hypaque density gradient (Lymphosepal; IBL, Gunma, Japan) centrifugation (31) and grown in RPMI medium supplemented with recombinant human interleukin-2 (rhIL-2; Roche, Mannheim, Germany) at 50 U/ml.

Viruses. Viral stocks of HIV-1_{NL4-3}, HIV-1_{JR-CSF}, and HIV-$1_{89.6}$ were each produced in the 293T cell line by transfection with HIV-1 molecular clone plasmids pNL4-3 (1), pYK-JRCSF (25), and p89.6 (11), respectively, by the calcium phosphate method. The 50% tissue culture infective dose was determined by an end-point assay with PBMC cultures activated with immobilized anti-CD3 monoclonal antibody (MAb) (33, 51). Subtype B HIV-1 primary isolates 92HT593, 92HT599 (N. Hasley), and 91US005 (B. Hahn) and AZT-resistant HIV-1 (A018) (D. D. Richman) (26) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. These clinical isolates were propagated in the activated PBMCs prepared as described above.

Anti-HIV-1 assays. Human PBMCs activated with immobilized anti-CD3 MAb (OKT-3; ATCC, Manassas, VA) in RPMI medium for 3 days were infected with various HIV-1 strains, including primary clinical isolates, at a multiplicity of infection of 0.001. After 3 h of adsorption, the cells were washed and cultured in RPMI medium supplemented with rhIL-2 (50 U/ml) in the presence or absence of the test compounds. Amounts of HIV-1 capsid (p24) antigen produced in the culture supernatants were measured by an enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY) 7 to 10 days after infection. The cytotoxicities of the compounds were tested on the basis of the viability and proliferation of the activated PBMCs, as determined with Cell Proliferation Kit II (XTT) from Roche (36).

Susceptibility of multidrug-resistant HIV-1 to CXCR4 antagonists was also measured by using recombinant viruses in a single replication cycle assay (9, 49). HIV-1 resistance test vectors (RTVs) contain the entire protease (PR) coding region and the reverse transcriptase (RT) coding region, from amino acid 1 to amino acid 305, amplified from patient plasma and a luciferase expression cassette inserted in the *env* region. The RTVs in this study contain patient-derived PR and RT sequences that possess mutations associated with resistance to PR, RT, or both PR and RT. Env-pseudotyped viruses were produced by cotransfecting 293 cells with RTV plasmids and expression vectors encoding the Env protein of well-characterized X4-tropic laboratory strain HXB2, NL4-3, or NL4-3 containing the Q40H enfuvirtide (T20) resistance mutation introduced by site-direct mutagenesis. The virus stocks were harvested 2 days after transfection and used to infect U87 $CD4^+$ cells (kind gifted from N. Landau, NYU School of Medicine) expressing CXCR4 in 96-well plates, with serial dilutions of CXCR4 antagonists. Target cells were lysed, and luciferase activity was measured to assess virus replication in the presence and absence of inhibitors. Drug concentrations required to inhibit virus replication by 50% (IC_{50}) were calculated.

Immunofluorescence. Molt-4 cells or CXCR4-expressing HEK293 cells were treated with various concentrations of KRH-3955 or AMD3100 in RPMI medium or phosphate-buffered saline containing 1% bovine serum albumin and 0.05% NaN_3 (fluorescence-activated cell sorting [FACS] buffer). In washing experiments, cells were washed with RPMI medium or FACS buffer. The cells were Fc blocked with 2 mg/ml normal human immunoglobulin G (IgG) in FACS buffer and then stained directly with mouse MAbs 12G5-phycoerythrin (PE) and 44717-PE (R&D Systems, Inc., Minneapolis, MN) or rat MAb A145-fluorescein isothiocyanate (FITC) and indirectly with MAb A80. The A145 and A80 MAbs were produced in ascitic fluid of BALB/c nude mice, and IgG fractions were obtained from ascitic fluid by gel filtration chromatography with Superdex G200 (Amersham Pharmacia). Goat anti-rat IgG (heavy and light chains) labeled with FITC was purchased from American Corlex (47). After washing, the cells were analyzed on a FACScalibur (BD Biosciences, San Jose, CA) flow cytometer with CellQuest software (BD Biosciences).

DNA construction and transfection. Chemokine receptor-expressing CHO cells were generated as reported previously (23). Human CXCR4 cDNA was cloned into the pcDNA3.1 vector. Mutations were introduced by using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing and transfected into 293 cells by using the Lipofectamine reagent (Invitrogen) (48). Stable transfectants were selected in the presence of 400 μg/ml G418 (Invitrogen). The COOH-terminal intracellular domain of CXCR4 (residues 308 to 352) was deleted in all mutants and the wild type. This deletion has no influence on HIV-1 infection or on SDF-1α binding and signaling but abolishes ligand-induced endocytosis (3).

Ligand-binding assays. Chemokine receptor-expressing CHO cells (5×10^6/0.2 ml per well) were cultured in a 24-well microtiter plate. After 24 h of incubation at 37°C, the culture medium was replaced with binding buffer (RPMI medium supplemented with 0.1% bovine serum albumin). Binding reactions were performed on ice in the presence of ^{125}I-labeled chemokines (final concentration of 100 pmol/liter; PeproTech Inc., Rocky Hill, NJ) and various concentrations of test compounds. After washing away of unbound ligand, cell-associated radioactivity was counted with a scintillation counter as described previously (23).

CXCR4-mediated Ca^{2+} signaling. Fura2-acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan)-loaded CXCR4-expressing CHO cells were incubated in the absence or presence of various concentrations of KRH-3955 or AMD3100. Changes in intracellular Ca^{2+} levels in response to SDF-1α (1 μg/ml) were determined by using a fluorescence spectrophotometer as described previously (30).

Detection of KRH-3955 in blood after oral administration. The plasma concentration-time profile of R-176211 (distilled water was used as a vehicle), the free form of KRH-3955, was examined after a single oral administration of KRH-3955 at a dose of 10 mg/kg or intravenous administration at a dose of 10 mg/kg to male Sprague-Dawley rats (CLEA, Kanagawa, Japan). R-176211 in plasma was measured by liquid chromatography-tandem mass spectrometry. Pharmacokinetic parameters were calculated by using WinNonlin Professional (ver. 3.1; Pharsight Co.).

Infection of hu-PBL–SCID mice. Two groups of C.B-17 SCID mice (CLEA, Kanagawa, Japan) were administered a single dose of either KRH-3955 or tartrate (2% glucose solution was used as the vehicle) as a control orally (p.o.) and fed for 2 weeks. These mice were then engrafted with human PBMCs (1×10^7 cells/animal intraperitoneally [i.p.]) and after 1 day were infected i.p. with 1,000 infective units of X4 HIV-1_{NL4-3}. IL-4 (2 μg per animal) was administrated i.p. on days 0 and 1 after PBMC engraftment to enhance X4 HIV-1 infection. After 7 days, human lymphocytes were collected from the peritoneal cavities and spleens of the infected mice and cultured in vitro for 4 days in RPMI medium supplemented with 20 U/ml rhIL-2. HIV-1 infection was monitored by measuring p24 levels in the culture supernatant. We used a selected donor whose PBMCs could be engrafted at an efficiency of >80% in C.B-17 SCID mice. Usually, 5×10^5 to 10×10^5 human $CD4^+$ T cells can be recovered from each hu-PBL–SCID mouse. Mice with no or low recovery of human $CD4^+$ T cells at the time of analysis were omitted. For ex vivo cultures, we used a quarter of the cells recovered from a mouse. The protocols for the care and use of the hu-PBL–SCID mice were approved by the Committee on Animal Research of the University of the Ryukyus before initiation of the present study.

RESULTS

Anti-HIV-1 activities of KRH-3955 in activated PBMCs. The inhibitory activity of KRH-3955 against X4 HIV-1 (NL4-3), R5X4 HIV-1 (89.6), and R5 HIV-1 (JR-CSF) was examined in activated human PBMCs from two different donors. KRH-3955 inhibited the replication of both X4 and R5X4 HIV-1 in activated PBMCs with 50% effective concentrations (EC_{50}) of 0.3 to 1.0 nM but did not affect R5 HIV-1 replication, even at concentration of up to 200 nM (Table 1). In contrast, the CCR5 antagonist SCH-D (vicriviroc) inhibited R5 HIV-1 rep-

TABLE 1. Anti-HIV-1 activity of KRH-3955 in activated PBMCs[a]

Virus	Donor	EC_{50} (nM)[b]					
		KRH-3955	AMD3100	AMD070	SCH-D	AZT	SQV
NL4-3	A	1.1	41	35	>1,000	11	9.0
X4	B	0.33	15	15	>1,000	8.0	29
89.6	A	0.38	44	55	>1,000	7.4	9.9
R5X4	B	ND[c]	ND	ND	ND	ND	ND
JR-CSF	A	>200	>200	>200	0.37	0.96	2.6
R5	B	>200	>200	>200	1.2	6.2	8.0
A018H (X4) (pre-AZT)	C	1.4	38	ND	ND	1.9	ND
A018G (X4) (post-AZT)	C	1.3	32	ND	ND	87,000	ND

[a] PBMCs from two different donors were used in each assay. Anti-HIV-1 activity was determined by measuring the p24 antigen level in culture supernatants.
[b] Assays were carried out in triplicate wells. The average of two to four experiments is shown.
[c] ND, not determined.

lication but inhibited neither X4 nor R5X4 HIV-1 replication (Table 1). The anti-HIV activity of KRH-3955 against the 89.6 virus from donor B was not determined because the virus did not replicate enough for calculation of the anti-HIV activity of KRH-3955 and other drugs. Notably, the anti-HIV-1 activity of KRH-3955 was much higher than that of AMD3100, a well-known X4 HIV-1 inhibitor, or AMD070, the other X4 inhibitor that is bioavailable when administered orally. KRH-3955 also inhibited the replication of clinical isolates of X4 HIV-1 (92HT599) and R5X4 HIV-1 (92HT593) with EC_{50} ranging from 4.0 to 4.2 nM (data not shown). Although both KRH-3955 and AMD3100 were effective against at least some R5X4 HIV-1 strains in activated PBMCs, neither KRH-3955 nor AMD3100 inhibited the infection of CD4/CCR5 cells by R5 or R5X4 HIV-1, even at a concentration of 1,660 nM (data not shown). Importantly, the 50% cytotoxic concentration of KRH-3955 in activated PBMCs (donor A) was 57 μM, giving a high therapeutic index (51,818) in the case of NL4-3 infection, which was higher than that of AZT (8,000 in the case of donor A). These results indicate that the compound is a selective inhibitor of HIV-1 that can utilize CXCR4 as a coreceptor. Since a CXCR4 antagonist should be used in combination with a CCR5 antagonist in a clinical setting, we next examined whether the combined use of both antagonists efficiently blocks mixed infection with X4 and R5 HIV-1. Combination of KRH-3955 and SCH-D at 4 plus 4 nM and 20 plus 20 nM blocked the replication of 50:50 mixtures of NL4-3 and JR-CSF by 91 and 96%, respectively (data not shown). Thus, KRH-3955 is a highly potent and selective inhibitor of X4 HIV-1.

Anti-HIV-1 activities of KRH-3955 in activated PBMCs from different donors. It has been observed that the anti-HIV-1 activity of compounds in PBMCs varies from donor to donor. Therefore, the anti-HIV-1 activity of KRH-3955 against X4 HIV-1 was examined in activated PBMCs from eight different donors. The levels of p24 antigen in NL4-3-infected cultures ranged from 17 to 120 ng/ml (Table 2). KRH-3955 inhibited the replication of NL4-3 with EC_{50} ranging from 0.23 to 1.3 nM and with EC_{90} ranging from 2.7 to 3.5 nM (Table 2), demonstrating that the anti-HIV-1 activity of KRH-3955 was independent of the PBMC donor.

Anti-HIV-1 activities of KRH-3955 against drug-resistant HIV-1 strains. To further assess the efficacy of KRH-3955, we used a single-cycle assay to evaluate the activity of KRH-3955 against a panel of recombinant viruses that express an X4-tropic envelope protein (HXB2) but contain PR and RT sequences containing a wide variety of mutations associated with resistance to PR inhibitors (PIs), nucleoside RT inhibitors (NRTIs), and non-NRTIs (NNRTIs). This assessment was also performed with recombinant viruses that express an X4-tropic envelope protein (NL4-3) that contains the Q40H mutation and displays resistance to T20 (an entry inhibitor). The results of these experiments demonstrate that both KRH-3955 and AMD3100 inhibited the infection of CD4/CXCR4 cells by these recombinant drug-resistant viruses, including viruses resistant to PIs, NRTIs, or NNRTIs; multidrug-resistant viruses; and T20-resistant viruses (Table 3). We also observed that KRH-3955 inhibited the replication of A018G, a highly AZT-resistant strain, in activated PBMCs with an EC_{50} of 1.3 nM (Table 1).

KRH-3955 selectively inhibits ligand binding to CXCR4. To investigate whether KRH-3955 specifically blocks ligand binding to CXCR4, the inhibitory effect of the compound on chemokine binding to CHO cells expressing CXCR4, CXCR1, CCR2b, CCR3, CCR4, or CCR5 was determined. KRH-3955 efficiently inhibited SDF-1α binding to CXCR4 in a dose-dependent manner (Fig. 2 and 3b), and the IC_{50} for SDF-1α binding was 0.61 nM, which is similar to its EC_{50} against HIV-1. Similar results were obtained when we used a Molt-4 T cell line as the CXCR4-expressing target cell (Fig. 3a). Interestingly, the inhibitory activity of AMD3100 against SDF-1α binding was much weaker than its anti-HIV-1 activity (Fig. 3), suggesting that the binding sites of these two compounds are different. In contrast, the compound did not affect the binding

TABLE 2. Anti-HIV-1 activity of KRH-3955 against NL4-3 infection of PBMCs from eight different donors

Donor	p24 level (ng/ml)	EC_{50} (nM)	EC_{90} (nM)
1	31	1.30	3.2
2	25	1.20	3.2
3	17	1.20	3.3
4	40	0.70	2.9
5	120	0.77	2.9
6	58	1.50	3.5
7	49	0.23	2.7
8	53	1.00	3.0
Mean ± SD	49 ± 32	0.99 ± 0.40	3.1 ± 0.30

TABLE 3. KRH-3955 susceptibilities of drug-resistant viruses[a]

Virus[b]	IC_{50} (nM)[c]	
	KRH-3955	AMD3100
NL4-3	0.50	4.6
HXB2	0.60	6.2
NRTI-Res (HXB2-env)	0.60	9.0
NNRTI-Res (HXB2-env)	0.80	7.0
PI-Res (HXB2-env)	0.70	9.2
MDR (HXB2-env)	0.70	5.3
T20-Res (NL4-3-env)	0.40	2.3

[a] Susceptibility of drug-resistant HIV-1 was measured by using a single-cycle recombinant virus assay (see Materials and Methods).
[b] The pseudoviruses containing X4-tropic envelope (HXB2 or NL4-3) and patient-derived PR and RT sequences containing mutations associated with resistance to PR (PI-Res), RT (NRTI-Res or NNRTI-Res), or both (MDR) (the mutations are not shown). T20-Res contains a site-directed mutation (Q40H) in the NL4-3 envelope.
[c] IC50, 50% inhibitory concentration of CXCR4 antagonists.

of ^{125}I-labeled SDF-1α, ^{125}I-labeled RANTES, ^{125}I-labeled MCP-1, ^{125}I-labeled TARC, ^{125}I-labeled RANTES, or ^{125}I-labeled IL-8 to CXCR4, CCR1, CCR2b, CCR4, CCR5, or CXCR1, respectively (Fig. 2). Thus, KRH-3955 selectively blocks the binding of SDF-1α to CXCR4.

KRH-3955 exhibits inhibition of Ca^{2+} signaling through CXCR4. We next examined whether KRH-3955 acts as an agonist or antagonist of CXCR4 by using CXCR4-expressing CHO cells. The addition of KRH-3955 inhibited the SDF-1α-induced increase in the intracellular Ca^{2+} concentration in a dose-dependent manner, whereas 100 nM AMD3100 did not affect Ca^{2+} mobilization (Fig. 4). KRH-3955 itself did not affect Ca^{2+} mobilization at up to 1 μM (data not shown). We performed the Ca^{2+} mobilization assay with human PBMCs but could not detect an SDF-1α-induced Ca^{2+} signal mainly due to low expression of CXCR4 (data not shown). Thus, KRH-3955 inhibits Ca^{2+} signaling through CXCR4.

Effect of KRH-3955 on anti-CXCR4 antibody binding to CXCR4-expressing cells. To localize the binding site(s) of KRH-3955, the effects of KRH-3955 and AMD3100 on the binding of four types of anti-CXCR4 MAbs were first examined. We used MAbs A145, 12G5, 44717, and A80, which are specific for the N terminus, extracellular loop 1 (ECL1) and ECL2, ECL2, and ECL3, respectively. Neither KRH-3955 nor AMD3100 inhibited A145 binding to CXCR4-expressing Molt-4 cells (Fig. 5). Both compounds inhibited the binding of MAbs 12G5, 44717, and A80 to Molt-4 cells in a dose-depen-

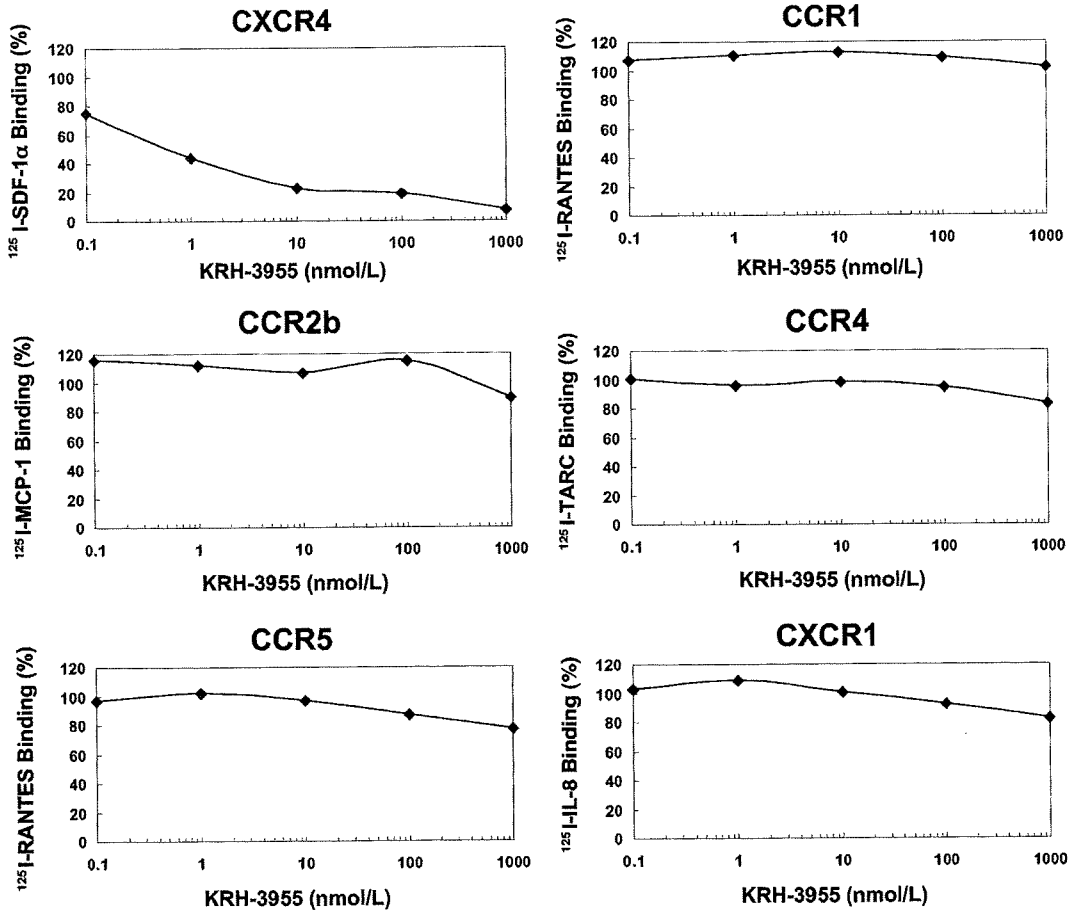

FIG. 2. Inhibitory effects of KRH-3955 on chemokine binding to CXCR4-, CCR1-, CCR2b-, CCR4-, CCR5-, or CXCR1-expressing CHO cells. Chemokine receptor-expressing CHO cells were incubated with various concentrations of KRH-3955 in binding buffer containing ^{125}I-labeled chemokine. Binding reactions were performed on ice and were terminated by washing out the unbound ligand. Cell-associated radioactivity was measured with a scintillation counter. Percent binding was calculated as 100 × [(binding with inhibitor − nonspecific binding)/(binding without inhibitor − nonspecific binding)]. The data represent the means in duplicate wells in a single experiment.

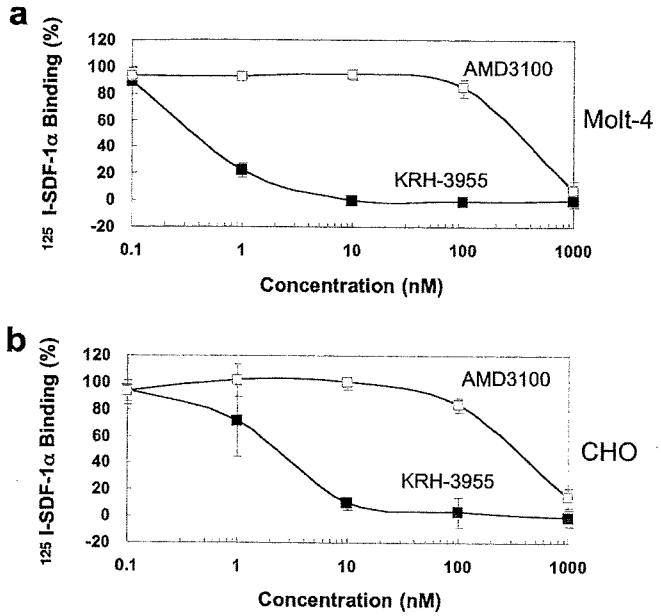

FIG. 3. Concentration-dependent inhibition by KRH-3955 of SDF-1α binding to (a) Molt-4 and (b) CXCR4-expressing CHO cells. CXCR4-expressing CHO cells were incubated with various concentrations of KRH-3955 (■) or AMD3100 (□) in binding buffer containing ^{125}I-labeled SDF-1α. Binding reactions were performed, and percent binding was calculated as described in the legend to Fig. 2. The data represent the means ± standard deviations of three independent experiments.

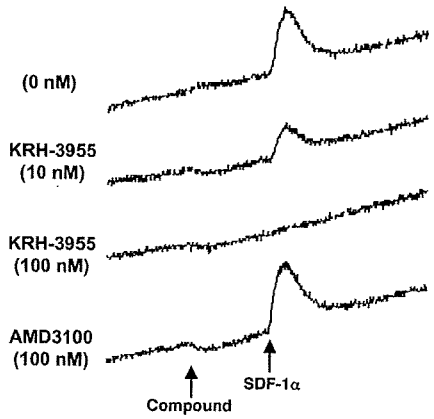

FIG. 4. Inhibitory effects of KRH-3955 on SDF-1α-induced Ca^{2+} mobilization in CXCR4-expressing CHO cells. Fura-2-acetoxymethyl ester-loaded CXCR4-expressing CHO cells were incubated in the presence or absence of various concentrations of KRH-3955 or AMD3100. Changes in intracellular Ca^{2+} levels in response to SDF-1α (1 μg/ml) were determined with a fluorescence spectrophotometer. The data show representative data for two independent experiments.

dent manner. The inhibitory activity of KRH-3955 is similar to its anti-HIV-1 activity, whereas the inhibitory activity of AMD3100 is much weaker than its anti-HIV-1 activity. Similar data were obtained when activated human PBMCs were used as target cells (data not shown). KRH-3955 itself did not induce internalization of CXCR4 at concentrations of up to 1 μM (data not shown), as KRH-1636 did (23). These results suggest that the binding sites of KRH-3955 are located in a region composed of all three ECLs of CXCR4.

Long-lasting inhibitory effects of KRH-3955 on the binding of MAb 12G5. The inhibitory effect of KRH-3955 on the binding of MAb 12G5 was examined with or without washing of the compound from the cells. Molt-4 cells were treated with 10 nM KRH-3955 or 1,000 nM AMD3100 for 15 min. With or without washing, the cells were stained with MAb 12G5-PE and the amount of bound antibody was analyzed by flow cytometry. KRH-3955 strongly inhibited MAb 12G5 binding to Molt-4 cells irrespectively of washing (Fig. 6a). In contrast, AMD3100 efficiently inhibited MAb 12G5 binding without washing away of the compound but lost its inhibitory activity after washing away of the compound (Fig. 6a). The long-lasting inhibitory effect of KRH-3955 on the binding of MAb 12G5 was further tested. Molt-4 cells were preincubated with or without KRH-3955 at 10 nM. The compound was washed away, and the cells were further incubated at 37°C in compound-free growth medium. At 0, 3, and 6 h after compound removal, the cells were stained with MAb 12G5-PE and analyzed by flow cytometry. Even at 6 h after washing away of the compound, KRH-3955 inhibited MAb 12G5 binding by approximately 40% (Fig. 6b). These results suggest that KRH-3955 has a strong binding affinity for CXCR4 and a slow dissociation rate, although competition assays with the two molecules (KRH-3955 versus MAb 12G5 with radioactive, nonradioactive, or different labeling) are necessary to provide definitive conclusions.

Inhibition of MAb 12G5 binding to CXCR4 mutants by KRH-3955. The effects of different CXCR4 mutations on the inhibitory activity of KRH-3955 against MAb 12G5 binding to CXCR4 were examined. HEK293-CXCR4 transfectants were preincubated with various concentrations of KRH-3955 and AMD3100, after which the compound was washed away. The binding of PE-conjugated MAb 12G5 was measured by flow cytometry. As reported previously, AMD3100 substantially lost its blocking activity against MAb 12G5 binding for D171A (TM4), D262A (TM6), and E288A/L290A (TM7) mutants, as shown by previous reports (Table 4) (20, 37, 38). In contrast, the blocking activity of KRH-3955 against MAb 12G5 binding was not affected by the above mutations. In contrast, the H281A (ECL3) mutant displayed decreased inhibition of MAb 12G5 binding by KRH-3955 (Table 4). These data further support the hypothesis that the CXCR4 interaction sites of KRH-3955 are different from those of AMD3100.

Pharmacokinetic studies of KRH-3955 in rats. In pharmacokinetics studies, KRH-3955 was orally or intravenously administrated to Sprague-Dawley rats at a dose of 10 mg/kg. The plasma concentration of R-176211, the free form of KRH-3955, was monitored by liquid chromatography-tandem mass spectrometry. In these studies, KRH-3955 was found to be well absorbed and the absolute oral bioavailability in rats was calculated to be 25.6% based on the area under the plasma concentration-time curve (Table 5). However, KRH-3955 also showed a long elimination half-life after single-dose administration to rats, suggesting long-term accumulation of the compound in tissues (Table 5). KRH-3955 was found to be stable in human hepatic microsomes, and no significant inhibition of CYP450 liver enzymes by this compound was observed (data

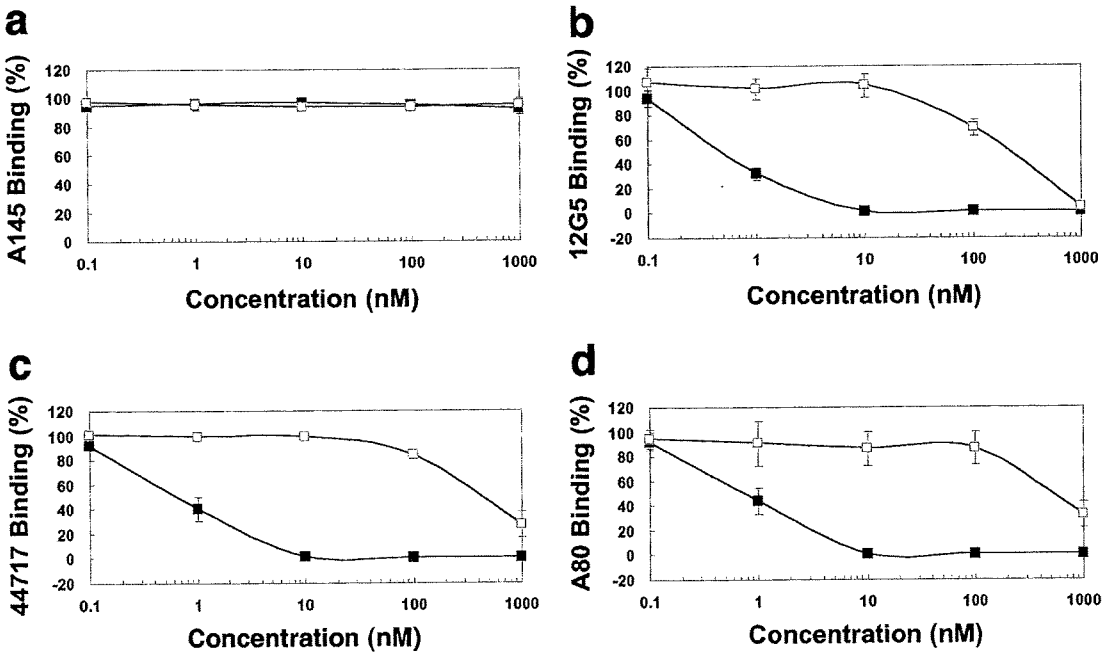

FIG. 5. Effect of KRH-3955 on the binding of four different MAbs to the CXCR4 receptor. Molt-4 cells were incubated with various concentrations of KRH-3955 (■) or AMD3100 (□). The cells were stained directly with MAbs 12G5 (recognizes ECL1 and ECL2 of CXCR4)-PE, 44717 (recognizes ECL2 of CXCR4)-PE, and A145 (recognizes the N terminus of CXCR4)-FITC or indirectly with MAb A80 (recognizes ECL3 of CXCR4). The mean fluorescence of the stained cells was analyzed with a FACScalibur flow cytometer. Percent binding was calculated with the equation described in the legend to Fig. 2. The data represent the means ± standard deviations of three independent experiments.

not shown). Thus, orally administered KRH-3955 is bioavailable in rats.

KRH-3955 efficiently suppresses X4 HIV-1 infection in hu-PBL–SCID mice. We then examined whether KRH-3955 can interfere with X4 HIV-1 infection in vivo by using hu-PBL–SCID mice. Mice were administrated a single dose (10 mg/kg) of either KRH-3955 or tartrate (as a control) p.o. and fed for 2 weeks. These mice were then engrafted with human PBMCs, and after 1 day, these "humanized" mice were infected with infectious X4 HIV-1 (NL4-3). After 7 days, human lymphocytes harvested from the peritoneal cavities and spleens of the infected mice were cultured for 4 days in vitro in the presence of rhIL-2 in order to determine the level of HIV-1 infection by the p24 enzyme-linked immunosorbent assay. The maximum concentration of KRH-3955 in blood after p.o. administration was estimated to be 100 nM (data now shown). Under these conditions, four of five mock-treated mice were infected whereas only one of five mice treated with KRH-3955 was infected (Table 6). The one infected mouse in the KRH-3955-treated group (no. 5)

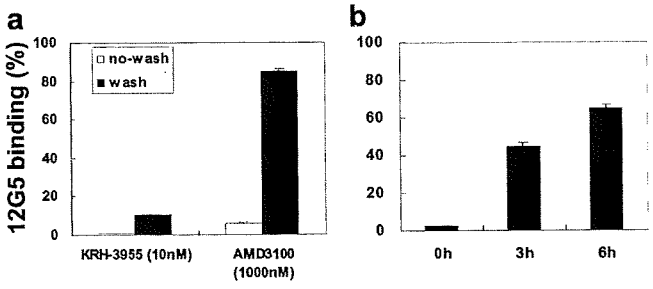

FIG. 6. Long-lasting inhibitory effects of KRH-3955 on the binding of MAb 12G5. (a) Molt-4 cells were treated with 10 nM KRH-3955 or 1,000 nM AMD3100 for 15 min. With (■) or without (□) washing, the cells were staining with MAb 12G5-PE and analyzed by flow cytometry. (b) Long-lasting inhibitory effect of KRH-3955 on the binding of MAb 12G5. Molt-4 cells were preincubated with or without KRH-3955 at 10 nM. The compound was washed away, and the cells were further incubated at 37°C in compound-free RPMI medium. At 0, 3, and 6 h after removal of the compound, the cells were staining with MAb 12G5-PE and analyzed by flow cytometry. The data represent the means of triplicate wells in a single experiment.

TABLE 4. Affinity of KRH-3955 and AMD3100 for wild-type CXCR4 and various mutant forms of CXCR4[a]

CXCR4 (location)	KRH-3955		AMD3100	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Wild type	2.8 ± 0.5	8.2 ± 0.4	289.1 ± 25.5	971.1 ± 31.2
V99A (ECL1)	1.5 ± 0.2	7.4 ± 0.2	258.5 ± 25.9	>1,000
V112A (TM3)	2.2 ± 0.2	>10	196.6 ± 28.5	821.3 ± 15.4
H113A (TM3)	0.8 ± 0.3	6.3 ± 0.2	296.4 ± 112.2	>1,000
D171A (TM4)	3.2 ± 0.1	>10	>1,000	>1,000
D181A (ECL2)	0.5 ± 0.1	5.1 ± 0.3	143.7 ± 29.3	795.6 ± 79.9
H203A (TM5)	0.5 ± 0.1	5.3 ± 0.1	259.0 ± 11.5	860.6 ± 22.4
D262A (TM6)	1.6 ± 0.3	8.1 ± 0.5	>1,000	>1,000
E275A (ECL3)	1.0 ± 0.2	6.4 ± 0.1	235.6 ± 30.2	930.2 ± 26.1
E277A (ECL3)	3.1 ± 0.1	8.7 ± 0.1	469.5 ± 19.2	>1,000
V280A (ECL3)	1.0 ± 0.2	6.1 ± 0.1	175.3 ± 10.3	821.2 ± 47.3
H281A (ECL3)	14.1 ± 5.2	248.3 ± 74.9	72.7 ± 42.9	572.2 ± 118.1
W283A (ECL3)	1.3 ± 0.2	6.9 ± 0.2	300.2 ± 10.5	>1,000
I284A (TM7)	1.2 ± 0.2	6.8 ± 0.5	265.8 ± 20.8	>1,000
E288A/L290A (TM7)	1.6 ± 0.1	7.7 ± 0.3	>1,000	>1,000

[a] The data shown, which represent means ± SDs (n = 3) of nanomolar concentrations, were obtained from competition binding on HEK293 cells expressing the wild-type or mutant CXCR4 receptors with MAb 12G5.

TABLE 5. Pharmacokinetic parameters of KRH-3955 after single oral administration in rats[a]

Parameter	Value when given i.v. or p.o. at 10 mg/kg
Bioavailability (%)[b]	25.6
I.v. half-life (h)	99.0 ± 13.1
I.v. CL (liters/h/kg)[c]	3.9 ± 0.07
V_1 (ss) (liters/kg)[d]	374.0 ± 14
P.o. C_{max} (ng/ml)[e]	86.3 ± 23.6
T_{max} (h)[f]	2.3 ± 1.53
P.o. AUC_{0-336} (ng · h/ml)[g]	325.0 ± 38

[a] The data shown are means ± SDs (n = 3).
[b] Bioavailability = $(AUC_{oral}/AUC_{i.v.}) \times (dose_{i.v.}/dose_{oral}) \times 100$.
[c] CL, clearance.
[d] V_1 (ss), volume of distribution in central compartment at steady state.
[e] C_{max}, maximum concentration of drug in serum.
[f] T_{max}, time to maximum concentration of drug in serum.
[g] AUC_{0-336}, area under the plasma concentration-time curve from time zero to 336 h.

TABLE 6. Inhibition of infection of hu-PBL–SCID mice with X4 HIV-1 by KRH-3955[a]

Group and mouse no.	p24 produced (pg/ml)
Control	
1	747
2	10,263
3	<5
4	5,821
5	1,902
KRH-3955	
6	<5
7	<5
8	<5
9	<5
10	36

[a] Two groups of C.B-17 SCID mice (n = 5) were administrated a single dose of either KRH-3955 or tartrate (as a control) p.o. and fed for 2 weeks. These mice were then engrafted with human PBMCs (1×10^7 per animal i.p.), and after 1 day, these "humanized" mice were infected with 1,000 infective units of X4 HIV-1_{NL4-3}. IL-4 (2 mg per animal) was administrated i.p. on days 0 and 1 after PBMC engraftment to enhance X4 HIV-1 infection. After 7 days, human lymphocytes were harvested from the infected mice and cultured in vitro for 4 days in medium containing 20 U/ml IL-2. HIV-1 infection was monitored by measuring p24 levels. Means from duplicate determinations are shown. <5, below detection level.

showed low levels of p24 production. These results indicate that single-dose p.o. administration of KRH-3955 was very effective in protecting against X4 HIV-1 infection in an in vivo mouse model.

DISCUSSION

In this study, we clearly demonstrate that KRH-3955, a KRH-1636 derivative that is bioavailable when administered orally, is a potent inhibitor of HIV-1 infection both in vitro and in vivo. KRH-3955 selectively inhibited X4 HIV-1 strains, including clinical isolates, as we have previously shown with KRH-1636. Furthermore, KRH-3955 is approximately 40 times more potent than KRH-1636 in its anti-HIV-1 activity in activated PBMCs (Table 1). The anti-HIV-1 activity of KRH-3955 was independent of the PBMC donor (Table 2). KRH-3955 also inhibited the infectivity of recombinant viruses resistant to NRTIs, NNRTIs, PIs, and T20 (Table 3). Pharmacokinetic studies of KRH-3955 indicated that the compound is bioavailable in rats when administered orally (Table 5). In addition, oral administration of the compound efficiently inhibited the replication of X4 HIV-1 in the hu-PBL–SCID mouse model (Table 6). Although we could show that KRH-3955 is a potent inhibitor of subtype B HIV-1 isolates, we need to examine the efficacy of this compound against non-subtype B HIV-1 isolates because of the global nature of the HIV/AIDS epidemic and the regional diversity of HIV-1 subtypes.

R5 HIV-1 is isolated predominantly during the acute and asymptomatic stage (12) and is also believed to be important for virus transmission between individuals. In contrast, X4 HIV-1 strains emerge in approximately 50% of infected individuals and their emergence is associated with a rapid $CD4^+$ T-cell decline and disease progression (35, 50). One recent report also indicated that detection of X4 HIV-1 at baseline independently predicted disease progression (13), although it is still not known whether the emergence of X4 HIV-1 is a cause or outcome of disease progression. These findings strongly support the need for highly potent CXCR4 inhibitors that are bioavailable when administered orally such as KRH-3955.

Inhibition of ligand binding to chemokine receptors by KRH-3955 was specific for CXCR4 (Fig. 2), as we observed previously for KRH-1636. This specific inhibition of SDF-1α binding to CXCR4 by KRH-3955 is absolutely necessary for developing an anti-HIV agent to avoid immune dysregulation by nonspecific inhibition of binding by other chemokines. It is of note that the inhibitory activity of the compound against SDF-1α binding is similar to that against HIV-1 infection, which is different from that of control compound AMD3100. Where on the CXCR4 molecule is the binding site(s) of KRH-3955? Experiments to examine the effect of KRH-3955 on the binding of several anti-CXCR4 MAbs suggest that the binding sites of KRH-3955 are located in all three ECLs of CXCR4 (Fig. 5). To further define the binding site(s) of KRH-3955, we examined the effects of CXCR4 point mutations on the inhibitory activity of KRH-3955 against MAb 12G5 binding to the receptor. AMD3100 was used as a control. The inhibitory activity of AMD3100 against MAb 12G5 binding to the receptor was greatly reduced by the mutations D171A (TM4), D262A (TM6), and E288A/L290A (TM7), as reported previously (Table 4) (20, 37, 38). Of note, these mutations also affect SDF-1α binding and/or CXCR4 coreceptor activity (8). Unexpectedly, none of these three mutations affected the inhibition of MAb 12G5 binding by KRH-3955 (Table 4). Only the H281A (ECL3) mutant showed decreased inhibition of MAb 12G5 binding by KRH-3955 (Table 4). Interestingly, the same mutant modestly increased the blocking activity of AMD3100 against MAb 12G5 binding. In addition, the H281A mutation markedly impaired inhibition of MAb 12G5 binding by AMD3465, one of the prototype monocyclams (37). Further experiments with different CXCR4 mutants are necessary to identify the exact site(s) on CXCR4 targeted by this compound.

Pharmacological tests of KRH-3955 were performed with rats, and the compound was found to be bioavailable when administered orally (Table 5), which is favorable for anti-HIV drugs. However, the compound also indicated a long half-life after single-dose administration to rats, suggesting long-term accumulation of the compound in tissues, which can be either advantageous

in terms of inhibiting HIV-1 infection in hu-PBL–SCID mice (Table 6) or disadvantageous in terms of toxicity. Further studies are ongoing to determine the safety and pharmacokinetics of the compound in other animals such as dogs and monkeys. To evaluate the in vivo efficacy of KRH-3955, we used the hu-PBL–SCID mouse model and showed that oral administration of the compound strongly protected against X4 HIV-1 infection in this model system (Table 6). To achieve substantial replication of X4 HIV-1 in this system, recombinant IL-4 was added after human PBMC engraftment as described previously (23). Notably, KRH-3955 was administered only once 2 weeks before PBMC engraftment and was effective enough to block X4 HIV-1 infection, suggesting that the compound can be used as a preexposure prophylaxis agent to prevent HIV infection. This long-lasting antiviral effect of KRH-3955 can be partly explained by the strong affinity of the compound for CXCR4 (Fig. 6) and long-term accumulation of the compound in tissues.

In terms of safety of anti-HIV drugs, CCR5 antagonists are considered to be relatively safe because of the lack of obvious health problems in individuals homozygous for the CCR5 delta32 allele (27, 39). Indeed, maraviroc, a CCR5 antagonist, was approved by the U.S. FDA in 2007. In contrast, CXCR4 antagonists, which inhibit SDF-1α–CXCR4 interactions, may cause severe adverse effects because knocking out either the SDF-1α or the CXCR4 gene in mice causes marked defects such as abnormal hematopoiesis and cardiogenesis, in addition to vascularization of the gastrointestinal tract (32, 44, 52). However, no severe side effects have been reported for either AMD3100, a well-characterized CXCR4 antagonist, or AMD070, an oral CXCR4 antagonist, in human volunteers and/or HIV-infected patients. Milder side effects, including gastrointestinal symptoms and paresthesias, were common at higher doses of AMD3100. These results indicate the feasibility of using CXCR4 antagonists as anti-HIV-1 drugs in a clinical setting (21, 22, 41).

Besides the physiological roles mentioned above, the CXCR4–SDF-1 axis is also involved in various diseases such as cancer metastasis, leukemia cell progression, rheumatoid arthritis, and pulmonary fibrosis. CXCR4 antagonists such as AMD3100 and T140 have demonstrated activity in treating such CXCR4-mediated diseases (14, 46). In addition, AMD3100 is considered to be a stem cell mobilizer for transplantation in patients with cancers such as non-Hodgkin's lymphoma. Recently, AMD3100 has been shown to increase T-cell trafficking in the central nervous system, leading to significant improvement in the survival of West Nile virus encephalitis (29). Given its highly potent and selective inhibition of SDF-1–CXCR4 interaction and its bioavailability when administered orally, it is important to address whether KRH-3955 can also be used for such clinical applications.

One important issue to be addressed is whether HIV-1 strains resistant to other CXCR4 antagonists show cross-resistance to KRH-3955. In our preliminary studies, AMD3100-resistant HIV-1 (kindly provided by M. Baba, Kagoshima University) (4) showed ~19-fold resistance to KRH-3955 compared with parental NL4-3, whereas the resistant virus showed ~40-fold resistance to both AMD3100 and AMD070 in MT-4 cells (data not shown). Interestingly, the AMD3100-resistant HIV-1 strain was relatively sensitive to T22, another prototype CXCR4 antagonist. Thus, KRH-3955 target sites on CXCR4 seem to partially overlap those of AMD3100, although experiments with CXCR4 mutants do not support this idea. It is important to establish KRH-3955-resistant mutants and investigate whether they also show cross-resistance to other CXCR4 antagonists. Long-term culture experiments with PM1/CCR5 cells that express both CXCR4 and CCR5 infected with X4 HIV-1 in the presence of KRH-3955 are in progress.

In conclusion, KRH-3955 is a small-molecule antagonist of the CXCR4 receptor that is bioavailable when administered orally. The compound potently and selectively inhibits X4 HIV-1 infection both in vitro and in vivo. Thus, KRH-3955 is a promising antiviral agent for HIV-1 infection and should be evaluated for its clinical efficacy and safety in humans.

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Follicular Dendritic Cells Activate HIV-1 Replication in Monocytes/Macrophages through a Juxtacrine Mechanism Mediated by P-Selectin Glycoprotein Ligand 1[1]

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Follicular dendritic cells (FDCs) are located in the lymphoid follicles of secondary lymphoid tissues and play a pivotal role in the selection of memory B lymphocytes within the germinal center, a major site for HIV-1 infection. Germinal centers are composed of highly activated B cells, macrophages, CD4$^+$T cells, and FDCs. However, the physiological role of FDCs in HIV-1 replication remains largely unknown. We demonstrate in our current study that FDCs can efficiently activate HIV-1 replication in latently infected monocytic cells via an intercellular communication network mediated by the P-selectin/P-selectin glycoprotein ligand 1 (PSGL-1) interaction. Upon coculture with FDCs, HIV-1 replication was significantly induced in infected monocytic cell lines, primary monocytes, or macrophages. These cocultures were found to synergistically induce the expression of P-selectin in FDCs via NF-κB activation and its cognate receptor PSGL-1 in HIV-1-infected cells. Consistent with this observation, we find that this response is significantly blocked by antagonistic Abs against PSGL-1 and almost completely inhibited by PSGL-1 small interfering RNA. Moreover, a selective inhibitor for Syk, which is a downstream effector of PSGL-1, blocked HIV-1 replication in our cultures. We have thus elucidated a novel regulatory mechanism in which FDCs are a potent positive bystander that facilitates HIV-1 replication in adjacent infected monocytic cells via a juxtacrine signaling mechanism. *The Journal of Immunology,* **2009, 183: 524–532.**

The natural progression of HIV-1 infection consists of acute and chronic stages (1, 2). In the acute phase of viral infection, an initial peak level of plasma viremia appears within a couple of weeks of transmission. At this early time point in the course of infection, HIV-1 has disseminated to the lymphoid organs and viral reservoirs and latency have been established. The HIV-1 viral load stabilizes at a relatively low level after a period of acute viral infection, defined as the "set point," during which an immunological activation against HIV-1 is initiated. However, in tandem with seroconversion, HIV-1 production in reservoir or latently infected cells will eventually resume upon specific immunological responses such as host cytokine secretion or cell-mediated immune reactions (3–6).

Lymphoid organs have been proposed to function as a major reservoir for HIV-1 (7). During the course of HIV infection, T cells and macrophages in secondary lymphoid organs also become major reservoir cells for HIV-1 (8). Several in vitro studies have now identified potentially stable reservoirs of inducible latently infected CD4$^+$ cells carrying an integrated form of the viral genome (7–9). In addition to CD4$^+$ T cells, monocytes are thought to be major reservoirs for HIV-1 in vivo, since a number of blood monocytes are maintained in HIV-1-infected patients even during the late disease stages when T cells can be practically undetectable (10, 11). These observations suggest that infected CD4$^+$ T cells and macrophages provide sites as a stable reservoir and producer of HIV-1, causing the persistent production of progeny virus in lymphoid organs. However, it has not been well investigated how these reservoir cells can maintain sufficient levels of viral replication that will retain a sufficient viral load during the long course of this disease.

It is generally believed that the central point in the immune system is the lymphoid organs and germinal centers (GCs)[3] where several immune cell types are localized, although these circulate throughout the whole body (12–14). The GCs of secondary lymphoid tissues are composed of B cells, CD4$^+$ T cells, macrophages, and follicular dendritic cells (FDCs) (15–17). FDCs are characterized by the expression of CD21, CD35, CD40, and specific cell surface adhesion molecules including ICAM-1, VCAM-1, and the surface dendritic cell (DC) markers DC-SIGN and DRC-1 (16, 18–21). The FDCs play an important role in the

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[3] Abbreviations used in this paper: GC, germinal center; FDC, follicular dendritic cell; DC, dendritic cell; PSGL-1, P-selectin glycoprotein ligand 1; Syk, spleen tyrosine kinase; LTR, long terminal repeat; MOI, multiplicity of infection; siRNA, small interfering RNA.

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immune response by interacting with $CD4^+$ T or B cells and in organization of the follicular structure.

In HIV infection, human FDCs can capture and retain infectious HIV particles in a stable manner on their cell surfaces for several months or even years via Fc receptors or other molecules (22–25). Unlike conventional DCs, FDCs are not themselves infected with HIV despite expression of chemokine receptors and DC-SIGN (24). Furthermore, active HIV infection is largely confined to sites surrounding the FDCs (24), suggesting that this microenvironment is highly conducive to infection with this virus. FDCs have also been shown to transmit signals to the GC microenvironment which also appears to increase HIV infection and replication (24, 25). Our previous study showed that FDCs stimulated virus production in MOLT-4 T cells preexposed to HIV-1(23). Very recently, Thacker et al. (26) also reported that FDCs contributed to virus replication in $CD4^+$ T cells infected with HIV-1 obtained from peripheral blood and GCs by increasing viral transcription mediated by TNF-α upon coculture. However, the role of FDCs in HIV-infected monocytes/macrophages is largely unknown.

We here report that FDCs can activate HIV-1 production in surrounding infected monocytes or macrophages via a cell-cell interaction with a clear mechanistic distinction from $CD4^+$ T cells reported by Thacker et al. (26). This enhancement in monocytic cells was found to be mediated mainly by an association between P-selectin on FDCs, acting as a ligand, and P-selectin glycoprotein ligand 1 (PSGL-1), the cognate receptor, on HIV-1-infected cells. Furthermore, we delineate the biological significance of the PSGL-1/spleen tyrosine kinase (Syk) pathway in the FDCs-mediated switch to induce HIV-1 replication. Our current findings thus shed new light on mechanisms involved in the HIV replication pathway that are mediated through intercellular communication and provide clues for the design of future novel therapeutic interventions against AIDS and related disorders.

Materials and Methods

Cell culture and reagents

Several FDC lines were established from fresh human palatine tonsils and maintained as described previously (23). Briefly, FDCs were isolated from fresh palatine tonsils surgically removed. Tonsils were cut into pieces in the thickness of 2–3 mm and then digested for 15 min at 37°C with collagenase (type I; Wako). Following rinsing with RPMI 1640 by centrifugation at 400 × *g* for 7 min, cells were filtered through at 70-μm nylon mesh and overlaid on a 1.25, 2.5, and 5% continuous BSA gradient at 1 × *g* for 2 h. The lowest fraction with a higher density fraction was resuspended and cultured in RPMI 1640 medium with 10% FCS. Cell clusters in the lowest fraction included cells positive for DRC-1, a specific maker of FDCs. One week after the culture, adherent spindle-shaped FDCs appeared from the cell clusters after having released lymphoid cells and spontaneously proliferated without additional cytokines or growth factors. The character of FDCs was checked with expression of FDCs makers such as CAN-42, S-100α, CD54, DC-SIGN, and CXCR4 on its surface. After culturing along more than 2 wk, FDCs were stocked in −80°C before use. PBMCs were separated from three healthy donors in accordance with the guidelines of the ethics committee of Tokyo Medical and Dental University. PBMCs were cultured in RPMI 1640 containing 10% FBS at 37°C in 5% CO_2. Primary monocytes were obtained from three healthy donors with Rosette Sep (StemCell Technologies) according to the manufacturer's instructions. Primary macrophages were differentiated from monocytes by culturing in RPMI 1640 containing 10% AB serum (Sigma-Aldrich) and 20 ng/ml M-CSF (R&D Systems) for 7 days. HIV-1 chronically infected monocytic cell line U1 cells (27) were cultured in RPMI 1640 supplemented with 10% FBS (Invitrogen Life Technologies). Coculturing and Transwell assays were performed using 1×10^5 HIV-infected cell lines or 2×10^5 primary cells with 1×10^4 FDCs. For the FDC supernatant assay, filtered (0.2 μm) supernatants from FDC cultures were collected and added to HIV-1-infected cells at a 1:4 volume supernatant:total volume of fluid ratio. In the cell fixation assay, FDCs incubated with 3% paraformaldehyde in PBS for 2 h were washed three times with PBS and then twice with RPMI 1640 before coculturing.

Virus preparation and infection

$HIV\text{-}1_{JR\text{-}FL}$ or $HIV\text{-}1_{NL4\text{-}3}$ viruses were generated by transfection of the pJR-FL or pNL4-3 construct in 293T cells, respectively. Virus preparations were passed through a 0.4-μm filter and titrated using a conventional method as described previously (28). For the HIV-1 infection of primary cells, PBMCs were infected with $HIV\text{-}1_{JR\text{-}FL}$ for 24 h following stimulation with PHA-P (3 μg/ml) for 3 days. To adjust the culture condition for monocytes/macrophages with that for PBMCs, monocytes or macrophages were also infected with $HIV\text{-}1_{JR\text{-}FL}$ for 24 h following stimulation with PHA-P (3 μg/ml) for 3 days. All primary cell cultures were maintained in the absence of IL-2. Jurkat or FDCs were infected with $HIV\text{-}1_{NL4\text{-}3}$ (multiplicity of infection (MOI) = 0.05) for 1, 3, or 5 days.

Antibodies

Polyclonal Abs raised against phospho-p65 (Ser^{536}), phospho-Syk (Tyr^{352}), phospho-IκBα (Ser^{32}), and unmodified Syk were purchased from Cell Signaling Technology. Anti-p65 polyclonal, actin, and PSGL-1 (KPL1) mAb were purchased from Santa Cruz Biotechnology. Anti-α-tubulin mAb was purchased from Sigma-Aldrich. Neutralizing Abs targeting PSGL-1 or ICAM-1 were purchased from R&D Systems. Anti-HIV-1 p24 mAb (2C2; mouse IgG1) was produced by Y. Tanaka (University of Ryukyus, Okinawa, Japan).

Isolation of total RNA from cells and quantitative RT-PCR

U1 cells and FDCs were harvested after coculturing and washed three times with PBS. Total RNA was then extracted using Isogen (Nippongene) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed using Superscript III (Invitrogen) before semiquantitative RT-PCR, and quantitative RT-PCR was performed using a SYBER Green One-step Real-time PCR kit (Invitrogen) with mRNA-specific primer pairs. Analyzed genes and corresponding primers are listed in supplemental Table I.[4]

Neutralization assay

HIV-1-infected cells were pretreated with neutralizing Abs (anti-PSGL-1, anti-ICAM-1, or control mouse IgG) for 2 h before and during coculturing. Optimal concentrations were determined by the IC_{50} values in accordance with the manufacturer's instructions. Culture supernatants were collected after 3 days and subjected to measurement of HIV-1 p24.

Chemicals and inhibitory assays

BAY11-7082 and JNK inhibitor II were purchased from Merck. The Syk-specific inhibitor ER-27319 (29, 30) was purchased from Sigma-Aldrich. Cells were pretreated with 30 μM ER-27319, 1 μM JNK inhibitor II, 1–2 μM BAY11-7082, or DMSO (Sigma-Aldrich) for 2 h. The inhibitor treated/untreated cells were then cocultured with FDCs in the presence of Syk or NF-κB inhibitor. In small interfering RNA (siRNA) experiments, U1 cells were transfected with control or PSGL-1 siRNA (Santa Cruz Biotechnology) by Nucleofector (Amaxa) and then cocultured with FDCs. Lysates and supernatants were collected from these cultures after 3 days for measurement of p24 and Western blotting analysis.

Flow cytometry

Cells were washed twice with staining buffer (3% FBS and 0.09% NaN_3/PBS) and then stained with PSGL-1-RP-E (BD Biosciences) for 30 min on ice. Cells were then washed twice and processed for flow cytometry.

Measurement of HIV-1 p24

Cell culture supernatants were collected after centrifugation at 4000 rpm for 5 min at 4°C and then processed for measurement of HIV-1 p24 by using Lumipulse (Fujirebio) according to the manufacturer's instructions. Assays were performed in triplicate.

Results

FDCs activate HIV-1 production in adjacent HIV-1-infected monocytic cells

To address whether FDCs can also activate HIV-1 replication in the surrounding infected monocytes/macrophages as an effective bystander or stimulator, several primary FDCs were established from fresh palatine tonsils of three healthy human donors (23).

[4] The online version of this article contains supplemental material.

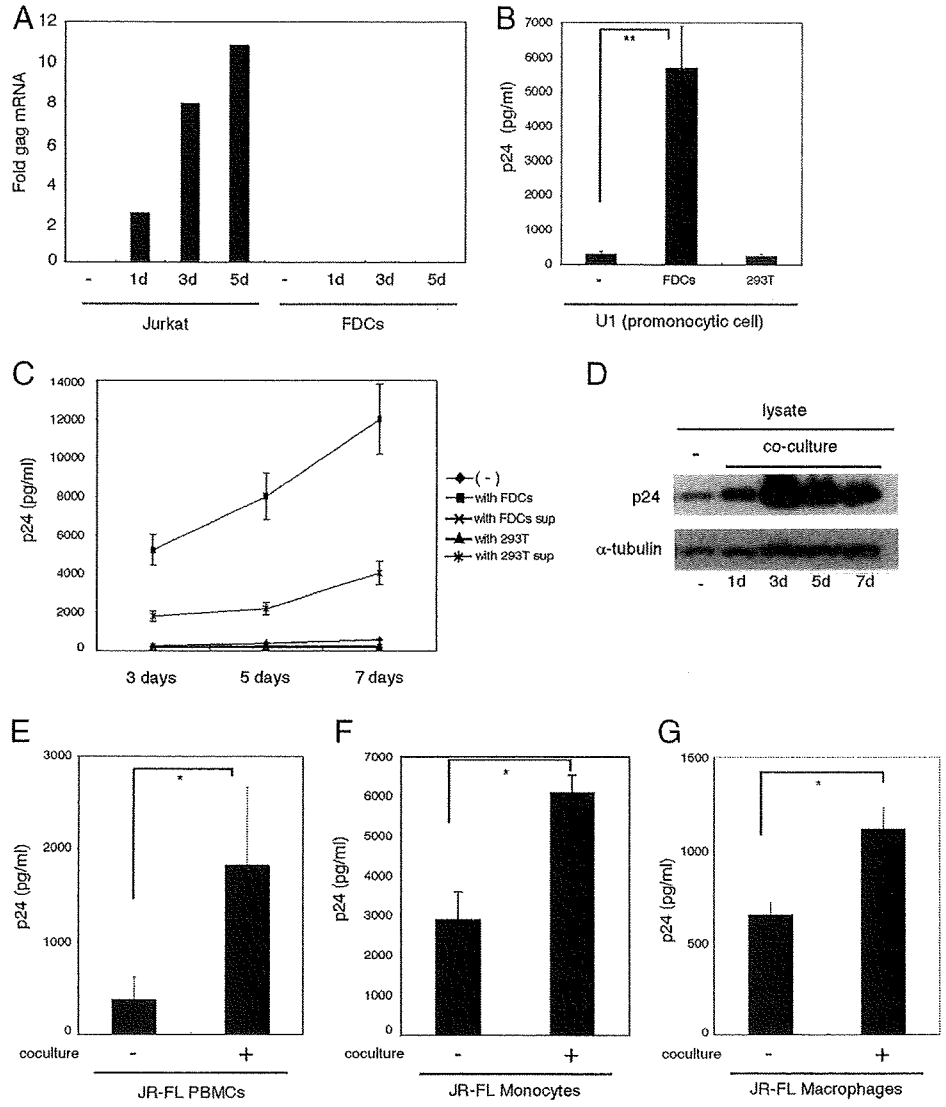

FIGURE 1. FDCs activate HIV-1 production in adjacent HIV-1-infected cells. *A*, Jurkat or FDCs were infected with HIV-1_{NL-4-3} (MOI = 0.05) for 1, 3, or 5 days. Cells were collected and then lysed for the separation of total RNA. Total RNA were treated with DNase I followed by quantitative RT-PCR with specific primer sets for either HIV-1 Gag or G3PDH. The data shown are the fold inductions normalized by G3PDH. *B*, U1 cells (1×10^5 cells/well) were cultured alone or in coculture with either FDCs or 293T cells (1×10^4 cells/well) for 3 days. Cell supernatants were then collected and assayed for measurement of p24. *C*, p24 levels in culture supernatants were monitored at 3, 5, and 7 days. *D*, p24 levels in lysates were monitored at 1, 3, 5, and 7 days by Western blot. *E*, PBMCs separated from a healthy donor were cultured with 3 μg/ml PHA for 3 days followed by infection with HIV-1_{JR-FL} (MOI = 0.05) for 24 h. The PBMCs (2×10^5 cells/well) were then cocultured with FDCs (1×10^4 cells/well) in the absence of IL-2 for 14 days. Culture supernatants were then assayed for measurement of p24. *F*, Monocytes separated from a healthy donor were cultured with 3 μg/ml PHA for 3 days followed by infection with HIV-1_{JR-FL} (MOI = 0.05) for 24 h. The monocytes (1×10^5 cells/well) were then cocultured with FDCs (1×10^4 cells/well) for 14 days. *G*, Primary differentiated macrophages were cultured with 3 μg/ml PHA for 3 days followed by infection with HIV-1_{JR-FL} (MOI = 0.05) for 24 h. The macrophages (1×10^5 cells/well) were then cocultured with FDCs (1×10^4 cells/well) for 7 days. Culture supernatants were then assayed for measurement of p24. The data shown in *B* are the average ± SD of at least three independent experiments. The data presented in *E–G* were obtained using samples of three donors (*, $p \leq 0.05$ and **, $p \leq 0.01$ by the Student *t* test).

Since each of these established cell lines was very similar in nature, exhibiting typical properties of FDCs (positive for CAN-42, S-100α, CD54, DC-SIGN, and CXCR4; morphological character such as spine-like spiculae and intercellular gap junction), the FDC 1 line was mainly used in subsequent experiments. FDCs themselves were not productively infected with HIV-1 (Fig. 1*A*), consistent with previous reports (22–25).

Initially, the FDCs were cocultured with chronically HIV-1-infected monocytic cell line U1 to examine whether they had the ability to activate HIV-1 replication. After 3 days of growth, HIV-1 production was analyzed for HIV-1 p24. The results showed that coculturing with FDCs significantly induced HIV-1 replication in the two infected cell types tested, whereas no such induction was observed when the U1 cells were cultured with 293T cells (Fig. 1*B*). A parallel kinetic study further demonstrated that the p24 levels in supernatants and lysates were increased in a time-dependent manner in U1 cells grown under these coculture conditions (Fig. 1, *C* and *D*). To address whether this trend occurred also in primary cells, FDCs were cocultured with PBMCs from healthy donors after infection with R5 (HIV-1_{JR-FL}) virus. As shown in Fig. 1*E*, the virus production was considerably augmented in coculture with FDCs. Furthermore, parallel experiments revealed that the virus production in monocytes or macrophages purified from PBMCs was also increased by coculturing with

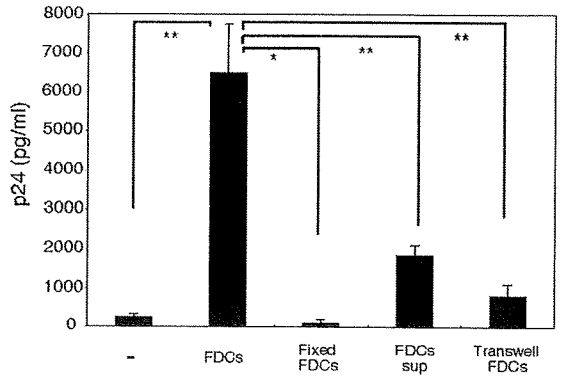

FIGURE 2. The enhancement of HIV-1 production by FDCs requires direct cell-cell interactions. U1 cells (1×10^5 cells/well) were cocultured with regular or paraformaldehyde-fixed FDCs (1×10^4 cells/well), cultured separately with FDCs on Transwell plates, or grown in medium supplemented with FDC culture supernatant at a 1:4 ratio of volume supernatant:total volume of fluid. Cell supernatants were collected after 3 days and assayed for measurement of p24. The data shown are the average ± SD of two independent experiments (*, $p \leq 0.05$ and **, $p \leq 0.01$ by the Student t test).

FDCs (Fig. 1, *F* and *G*). These data thus strongly indicate that FDCs can indeed activate viral replication monocytes/macrophages infected with HIV-1.

The enhancement of HIV-1 production by FDCs requires direct cell-cell interactions

To investigate whether this stimulation by FDCs was achieved by direct cell-cell interaction or soluble factors, we used two different cell culture methods for FDCs and U1 cells as follows: 1) FDCs were separately cultured with U1 cells using Transwells and 2) U1 cells were grown in culture medium supplemented with FDC supernatant. Although both culture systems could partially induce HIV-1 replication in U1 cells, these effects were ~20–30% of the full induction of those observed following coculture with FDCs (Fig. 2). This suggested that direct cell-cell interactions might be required for the full induction of HIV-1 replication in monocytic cells, although certain soluble factors may also activate HIV-1 replication to a lesser degree. Furthermore, the fixation of FDCs with paraformaldehyde before coculture completely abrogated the induction of HIV-1 replication in U1 cells, suggesting a requirement for bioactive cell surface molecules in this response.

Taken together, these data indicate that direct interactions via cell surface bioactive molecules are important to fully stimulate HIV-1 replication in monocytic U1 cells by FDCs.

Activation of NF-κB in both FDCs and HIV-1-infected cells following coculture

Our initial analysis demonstrated that FDCs can enhance HIV-1 replication in infected cells via cell-cell interaction. We thus examined whether this induction is initiated by the activation of the HIV-1 long-terminal repeat sequence (LTR). Quantitative and semiquantitative RT-PCR analyses revealed that the levels of HIV-1 mRNA were increased in U1 cells in tandem with increased supernatant p24 levels under coculture conditions with FDCs (Fig. 3, *A* and *B*).

HIV-1 replication has been shown to be regulated by host transcription factors such as NF-κB, NF-AT, Sp1, and AP-1 that are

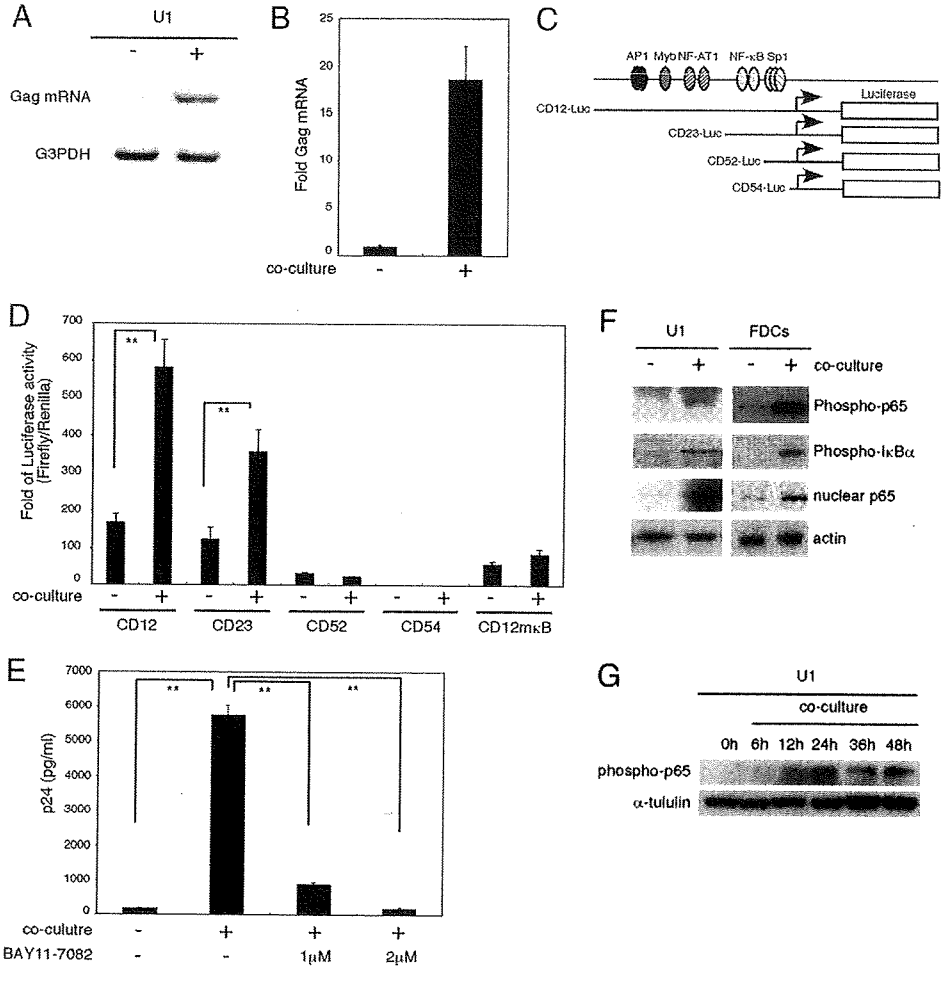

FIGURE 3. Activation of NF-κB in both FDCs and HIV-1-infected cells. *A* and *B*, U1 cells (1×10^5 cells/well) were cocultured with FDCs (1×10^4 cells/well) for 5 days and the mRNA levels for the indicated genes were measured by RT-PCR (*A*) or quantitative RT-PCR (*B*). *C*, Schematic representation of HIV-1 LTR-derived luciferase reporter constructs. *D*, U1 cells (1×10^5 cells/well) were initially transfected with the indicated reporter constructs and then cocultured with FDCs (1×10^4 cells/well) for 48 h, which was followed by a gene reporter assay. *E*, U1 cells (1×10^5 cells/well) were pretreated with the indicated concentrations of BAY 11-7082 for 2 h and then cocultured with FDCs (1×10^4 cells/well) for 3 days in the presence of the same concentration of BAY 11-7082. Cell supernatants were then collected and assayed for measurement of p24. *F*, U1 cells (1×10^5 cells/well) were cocultured with FDCs (1×10^4 cells/well) for 3 days and both cell types were collected and subjected to immunoblotting analysis for phospho-p65 (Ser^{536}), phospho-IκBα (Ser^{32}), p65, or actin. *G*, U1 cells (1×10^5 cells/well) were cocultured with FDCs (1×10^4 cells/well) and collected at the indicated time points. Cell lysates were subjected to immunoblot analysis using either phospho-p65 (Ser^{536}) or α-tubulin Abs. The data shown are the average ± SD of three independent experiments (*, $p \leq 0.05$ and **, $p \leq 0.01$ by the Student t test).

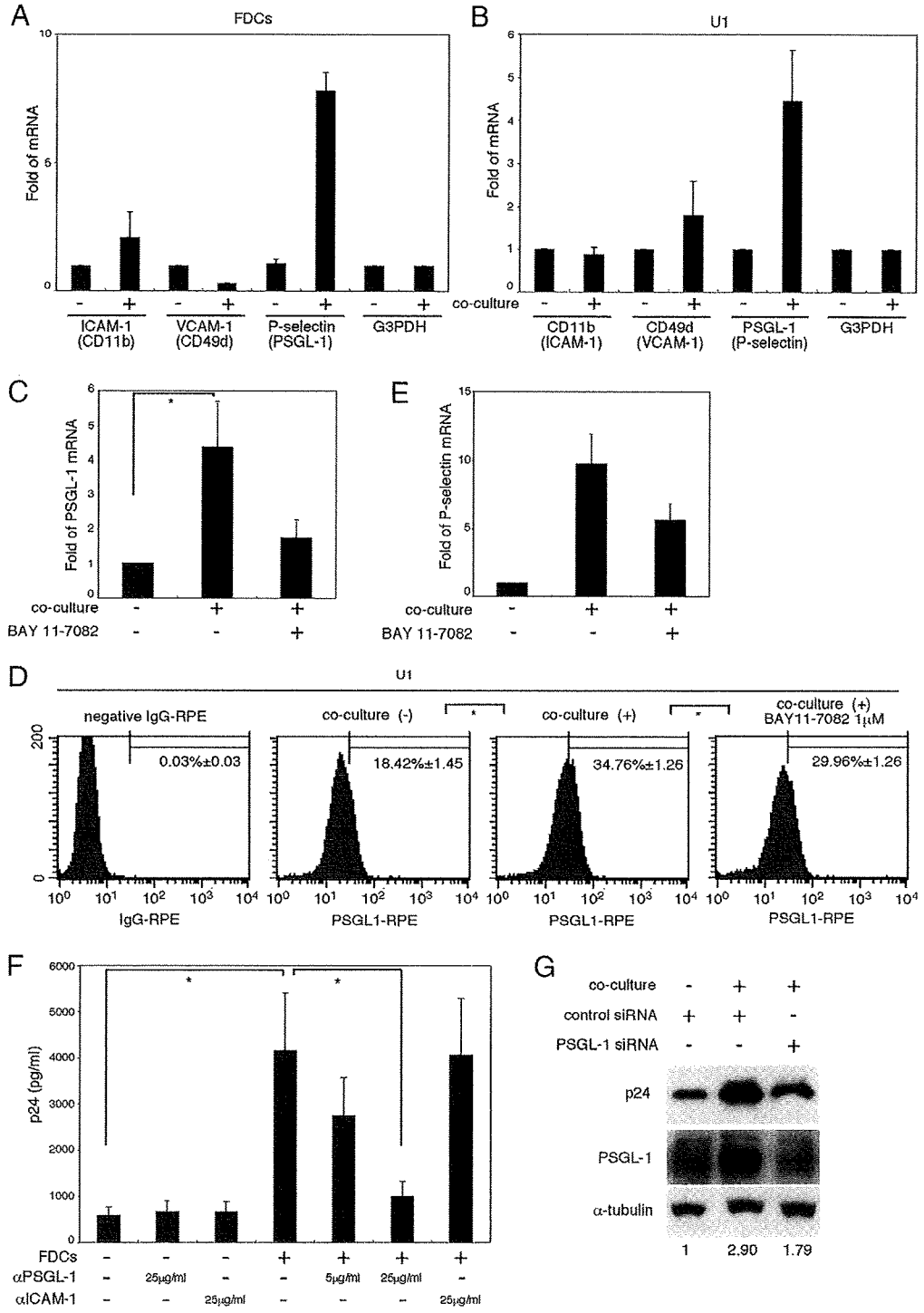

FIGURE 4. Involvement of P-selectin/PSGL-1 in reactivation of HIV-1 replication by FDC. *A* and *B*, U1 cells (1×10^5 cells/well) were cocultured with FDCs (1×10^4 cells/well) for 3 days. The mRNA levels of the indicated genes were then measured by quantitative RT-PCR. Labels inside parentheses indicate counterpart ligand or receptor molecules. *C–E*, U1 cells (1×10^5 cells/well) were cocultured with FDCs (1×10^4 cells/well) in the presence of BAY 11-7082 (1 μM) for 3 days and the levels of PSGL-1 in these cells were then analyzed by quantitative RT-PCR (*C*). Cell surface PSGL-1 was analyzed by flow cytometry using an anti-PSGL-1 Ab (*D*). M1 denotes the range of positive cell populations. *E*, P-selectin expression in FDCs analyzed by quantitative RT-PCR. *F*, U1 cells (1×10^5 cells/well) were untreated or pretreated with either PSGL-1 or ICAM-1 Ab for 1 h. Cells were then cocultured with FDCs (1×10^4 cells/well) for 3 days followed by measurement of p24. *G*, U1 cells (1×10^5 cells/well) were transduced with either control or PSGL-1 siRNA (final 6 nM) by Nucleofector according to the manufacturer's instructions. Cells were then cocultured with FDCs (1×10^4 cells/well) for 3 days followed by Western blot analysis with the indicated Abs. Numerical values below the blots indicate p24 signal intensities normalized by α-tubulin intensity derived by densitometry. The data shown are the average ± SD of three independent experiments (*, $p \leq 0.05$ and **, $p \leq 0.01$ by the Student t test).

recruited and bind directly to the HIV-1 LTR (31–33). To determine the identity of the *cis*-regulatory element(s) within the HIV-1 LTR that are the targets of FDC-mediated transcriptional activation, we examined various 5′-deletion mutants of these region as described in Fig. 3*C* (34). Coculturing of U1 cells with FDCs resulted in the activation of CD12 and CD23 reporter constructs

that harbor a NF-κB-binding sequence. However, the CD52 and CD54 constructs lacking this NF-κB consensus site were not activated, suggesting the involvement of NF-κB in the HIV-1 replication response (Fig. 3*D*). Consistent with this notion, the reporter construct CD12 that contains a site-directed mutation within the NF-κB binding site, CD12mκB, was not responsive to FDC stimulation. These results together indicate that the stimulation of HIV-1 in infected cells by FDCs is mediated via the activation of the HIV-1 LTR via NF-κB.

To further address this in terms of biological function, cells were treated with the NF-κB inhibitor BAY 11-7082 to further delineate the role of NF-κB in FDC-mediated HIV-1 replication. Treatment with BAY 11-7082 significantly suppressed HIV-1 production from U1 cells, even when growing in coculture with FDCs (Fig. 3*E*), although the viability of both cell types was not significantly affected by this exposure (data not shown). Taken together, our data thus indicate that intercellular communication pathways triggered by FDCs can promote and augment HIV-1 production in infected cells via NF-κB activation.

We next investigated whether NF-κB is in fact activated in FDCs as well as in U1 cells under coculture conditions. Consistent with our above gene reporter data, NF-κB activation was confirmed in U1 cells as revealed by the phosphorylation status of NF-κB p65 and IκBα (Fig. 3*F*). Interestingly, parallel experiments showed NF-κB activation in FDCs also in our coculture system, as revealed by immunoblotting with phospho-specific Abs (Fig. 3*F*). Furthermore, fractionation analysis demonstrated that the nuclear p65 (RelA) levels were significantly enhanced in both U1 and FDCs, indicating the nuclear accumulation of activated NF-κB (Fig. 3*F*). Parallel kinetic analysis revealed that NF-κB activation in U1 cells was initiated at 12 h and persisted for at least 48 h (Fig. 3*G*). These findings thus support our contention that cell-cell interactions between FDCs and U1 cells results in the constitutive activation of NF-κB in both cell types and that this is likely to be involved in the amplification of HIV-1 replication signals.

FDCs activate HIV-1 production via a P-selectin-PSGL-1 interaction

We were prompted to examine whether NF-κB up-regulates a specific cell surface ligand and its cognate receptor in FDCs and HIV-1-infected monocytic cells, eventually contributing to the amplification of HIV-1 replication signals via NF-κB activation. To this end, we examined the expression of different cell surface ligands and their cognate receptors which are known to be regulated by NF-κB. We chose three ligand/receptor combinations based upon a database search, ICAM-1/CD11b, VCAM-1/CD49d, and P-selectin/PSGL-1, and the expression of these molecules was analyzed by quantitative RT-PCR. Although the mRNA levels of ICAM-1 and VCAM-1 were not significantly altered upon stimulation, transcripts for P-selectin (CD62P/SLBP) were dramatically increased in FDCs (Fig. 4*A*). Interestingly, transcripts for the cognate receptor for P-selectin, PSGL-1, were found to be significantly up-regulated in U1 cells grown in coculture with the FDCs (Fig. 4*B*), but this was not the case for the CD11b and CD49d receptors. Quantitative RT-PCR and FACS analysis revealed that treatment with the NF-κB inhibitor BAY11-7082 significantly inhibited the increase of PSGL-1 mRNA expression and, consequently the cell surface expression of PSGL-1, in U1 cells cocultured with FDCs (Fig. 4, *C* and *D*). This suggested a crucial role for NF-κB signaling in the induction of PSGL-1 during this coculture in HIV-1-infected cells. Likewise, we found that BAY11-7082 treatment also decreased the induction of P-selectin mRNA in FDCs, indicating that the NF-κB activation in FDCs could play

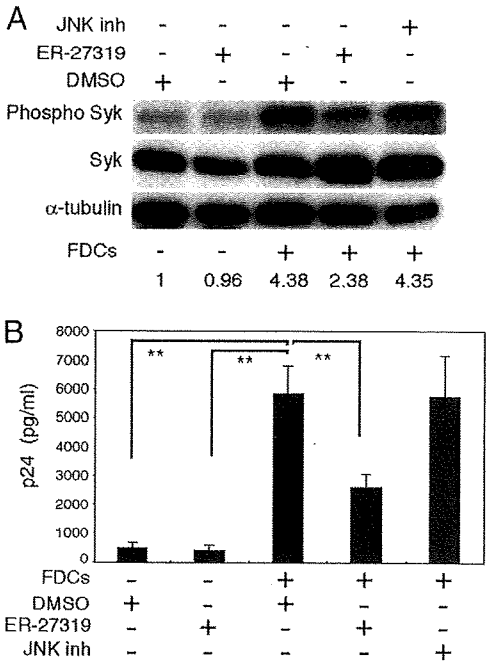

FIGURE 5. Syk is a mediator of P-selectin/PSGL-1 signaling for HIV-1 replication in U1 cells. *A* and *B*, U1 cells (1×10^5 cells/well) were untreated or pretreated with either ER-27319 (30 μM) or JNK inhibitor II (1 μM) for 1 h. Cells were then cocultured with FDCs (1×10^4 cells/well) for 3 days in the presence or absence of inhibitor. Cells were collected and subjected to immunoblotting analysis for phosphorylated Syk (Tyr^{352}), unmodified Syk, and α-tubulin (*A*). The numbers below the blot indicate the band intensity ratios. Cell supernatants were assayed for measurement of p24 (*B*). The data shown are the average ± SD of two independent experiments (*, $p \leq 0.05$ and **, $p \leq 0.01$ by the Student *t* test).

a crucial role in the induction of P-selectin during the coculture with HIV-1-infected monocytic cells (Fig. 4*E*).

Next, to test the biological significance of a P-selectin-PSGL-1 interaction in terms of HIV-1 induction in our FDC coculture system, U1 cells were pretreated with blocking Ab against PSGL-1 before setting up these cultures. Treatment with PSGL-1 Ab, but not an ICAM-1 Ab, specifically suppressed HIV-1 production in a dose-dependent manner (Fig. 4*F*). Consistent with this result, targeted disruption of PSGL-1 by specific siRNA significantly decreased HIV-1 production in U1 cells coculturing with FDCs (Fig. 4*G*). These results together indicate that a juxtacrine signaling mechanism mediated by PSGL-1/P-selectin underlies the activation of HIV-1 replication in infected monocytic cells stimulated by FDCs.

Syk acts as a downstream effector of PSGL-1 during HIV-1 replication

Several previous reports have demonstrated that the cytoplasmic domain of PSGL-1 can directly interact with a Src family kinase, the Syk (35). Syk consists of two N-terminal Src homology 2 domains, which bind phosphorylated ITAM sequences, and a C-terminal tyrosine kinase domain (35–37). The phosphorylation of Syk at Tyr^{352} has been shown to be a hallmark of its activation. Indeed, phosphorylated Syk was found in our present analyses to be significantly increased in U1 cells during their cocultivation with FDCs (Fig. 5*A*).

To next examine the possible biological functions of Syk during HIV-1 replication, we used a specific inhibitor of the molecule ER-27319 (29, 30) in our FDC cocultures. Treatment with ER-27319 significantly decreased HIV-1 production and this was accompanied by a reduction in the phosphorylated Syk levels in U1

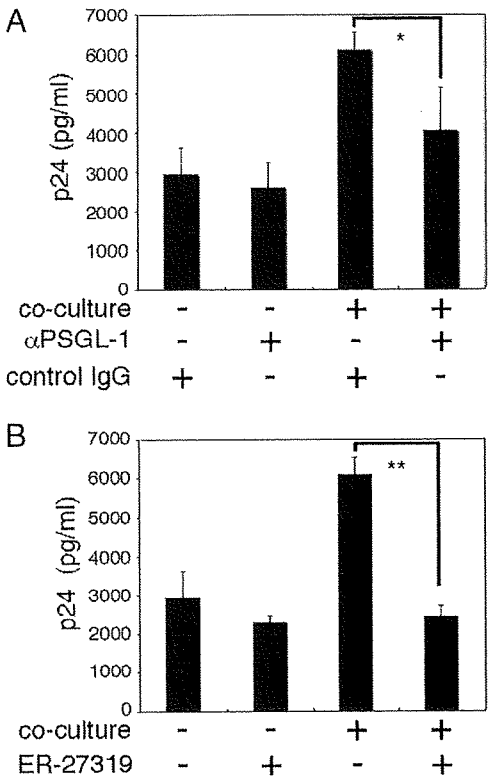

FIGURE 6. Inhibition of the PSGL-1/Syk pathway abrogates FDC-induced HIV-1 replication in primary monocytes. *A* and *B*, Primary human monocytes were separated from three healthy donors as indicated in *Materials and Methods* and these cells were then treated with 3 μg/ml PHA for 3 days. After stimulation, the cells (1×10^5 cells/well) were infected with HIV-$1_{JR\text{-}FL}$ (MOI = 0.05) for 24 h and subsequently cocultured with FDCs (1×10^4 cells/well) in the presence of PSGL-1 Ab (25 μg/ml; *A*) or ER-27319 (30 μM; *B*) at 14 days, followed by measurement of p24 (*, $p \leq 0.05$ and **, $p \leq 0.01$ by the Student *t* test).

cells (Fig. 5), whereas JNK inhibitor II had no such effects. These results indicate that the juxtacrine signaling between FDCs and HIV-1-infected monocytic cells mediated by P-selectin/PSGL-1 results in the activation of Syk, which serves as a mediator of the function of NF-κB activation in the HIV-1 replication pathway.

PSGL-1 and Syk inhibition blocks FDC-induced HIV-1 replication in primary monocytes

Finally, we addressed whether FDCs can also activate HIV-1 production in infected primary cells via P-selectin/PSGL-1 pathway, in this case human primary monocytes from healthy donors that had subsequently been exposed to HIV-$1_{JR\text{-}FL}$. At 24 h after viral infection, the primary monocytes were cocultured with FDCs in the presence or absence of either PSGL-1 Ab or the Syk inhibitor ER-27319. Both of these treatments significantly inhibited HIV-1 production in the primary monocytes in a manner similar to U1 cells (Fig. 6). These results indicate that similar to U1 cells, the PSGL-1/Syk signaling is likely to be a major pathway mediating FDC-induced HIV-1 replication in primary monocytes.

Discussion

Previous studies have indicated that HIV-1 infection is largely confined to the GCs of secondary lymph nodes where FDCs commonly reside (15–17). This microenvironment could thus provide the site for highly productive HIV-1 infection whereby FDCs might execute "on-switch" signaling to increase HIV replication. Furthermore, cell-cell infection appears to be far more efficient for viral spread than cell-free virus infection (38, 39). We here report that FDCs can facilitate HIV-1 replication in adjacent infected monocytes/macrophages via a cell-cell interaction mechanism.

FDCs have been shown to interact with B or $CD4^+$ T cells in the GCs of normal lymph nodes (16, 17, 20). It is also reported that in tonsils, CD150 (SLAM)$^+$ monocytes were localized not only in T cell areas, but also within GCs, suggesting they play a role in B cell activation (40). Moreover, substantial numbers of HIV-infected macrophages were observed in GCs during the course of HIV infection (41). Thus, FDCs can interact with HIV-infected monocytes or macrophages under these conditions during HIV-1 infection. Furthermore, the dysfunctional FDC network is observed in secondary lymph nodes of lymphadenopathy, where the degeneration of the FDC network is usually seen following highly active antiretroviral therapy or administration of therapeutic vaccine in HIV or SIV infection (42–45). One of the most common histological features of HIV-1-associated lymphadenopathy is hyperplastic lymphoid follicles that subsequently undergoes follicululolysis, in which FDCs can be scattered to the extra-GC within the lymph nodes such as cortical sinuses and mantle bodies (46, 47). Our results with immunohistochemical analysis indicate that FDCs reside with various types of HIV-1-infected cells including monocytes or macrophages in lymphoid organs of HIV-1-associated lymphadenopathy (supplemental Fig. 1). Therefore, our current proposed model for cell-cell interaction between FDCs and HIV-1-infected monocytic cells may reflect the biological or pathological aspects of the natural HIV infection in vivo. However, we could not determine the specific cell surface molecules for activating HIV-1 replication via the cell-cell interaction in vivo. Moreover, it is not well confirmed whether a multitude of other cells, cytokines, and other factors in vivo could influence the cell-cell interaction observed in our in vitro coculture system. Further careful analysis should be performed using human tissues as well as a humanized mouse model inoculated with HIV-1-infected human cells.

We clearly demonstrated here that FDCs, derived from human tonsils, can enhance HIV-1 production in infected monocytic cells in a coculture system. This enhancement requires direct cell-cell interactions via a juxtacrine signaling pathway that is mediated by P-selectin/PSGL-1. Our results are summarized as follows: 1) FDCs can activate HIV-1 replication in infected cells through cell-cell interactions; 2) HIV-1 replication is activated at the transcriptional level and is accompanied by the activation of the HIV-1 LTR through NF-κB; 3) P-selectin expression in FDCs and the up-regulation of its cognate receptor PSGL-1 in HIV-1-infected monocytes cells are facilitated via NF-κB activation; 4) the pathways leading to HIV-1 induction in cell lines also function in human primary monocytes and macrophages infected with HIV-1; and 5) selective inhibitors of PSGL-1 or Syk can efficiently block HIV-1 production in U1 and primary monocytes. These data together indicate for the first time that FDCs are a potent inducer of HIV-1 replication in surrounding infected monocytes and macrophages and that PSGL-1/Syk signaling plays a crucial role in this induction of HIV-1.

Very recently, Thacker et al. (26) reported a similar but distinct role of FDCs in the induction of HIV-1 replication in $CD4^+$ T cells obtained from PBMCs and GCs. We also confirmed that FDCs could stimulate HIV-1 replication in MOLT-4 T cells (23) as well as in primary $CD4^+$ T cells (data not shown). However, FDCs-induced HIV-1 replication in $CD4^+$ T cells might be mediated by a distinct mechanism from HIV-1-infected monocytic cells since the involvement of the PSGL-1/Syk pathway in $CD4^+$ T cells was found to be not prominent (K. Ohba, A. Ryo, and N. Yamamoto, unpublished observation). Therefore, the molecular mechanism for

FDCs to stimulate HIV-1 replication in surrounding infected cells could be attributable to cell type specific.

Intercellular interactions via a ligand/receptor juxtacrine signaling system has been implicated in several virus infections. Tsukamoto et al. (48) reported that the juxtacrine function of the IL-15/IL-15 receptor system in human B cell lines might play a role in the infectivity of EBV (48). Pilotti et al. (49) have demonstrated a crucial protective role for CCL3L1/CCL3 (MIP-1αP/LD78α) signals in both HIV infection and subsequent disease progression. These intercellular communication processes may play an important role in the sustained infection of viruses in different microenvironments within lymphoid organs. Further careful analyses will be required in the future to elucidate the variety of intercellular communication systems that may operate during HIV-1 infection.

There is now some evidence for a role of PSGL-1 as a signal-transmitting receptor in neutrophils (50), monocytes (51), and T lymphocytes (52). This molecule has been reported to associate with Syk through its interaction with moesin and promotes the tyrosine phosphorylation and thus the activation of Syk (35). In addition, signals elicited through PSGL-1/Syk can induce the activation of downstream effectors such as ERK, c-Fos, and NF-κB (53). The activation of NF-κB via PSGL-1 has also been demonstrated in platelet-stimulated monocytes, although the details of the molecular pathways leading to NF-κB activation in this manner have not yet been elucidated (51). Consistent with this result also, we found from our current analyses that PSGL-1/Syk signaling can activate NF-κB. This observation suggests a linkage between PSGL-1 signaling and HIV-1 replication through the activation of NF-κB.

Recently, Gilbert et al. (54) have reported that Src and Syk tyrosine kinases play important roles in the spread of HIV-1 from immature monocyte-derived DCs to CD4$^+$ T cells. They found that these kinases play a suppressive role in virus transfer in vitro probably by inhibiting the formation of the virological synapse. However, it has not been well characterized whether these signaling molecules contribute to the cell-cell interaction between HIV-1-infected cells and adjacent noninfected cells for virus replication. We showed in this current study that the activation of Syk through the PSGL-1 positively regulates HIV-1 replication in infected monocytic cells. Thus, Syk could be involved at multiple points in HIV-1 infection and its role could be dependent on each step of HIV-1 life cycle.

In summary, we demonstrate in our current study that FDCs are a potent activator of HIV-1 replication in surrounding infected monocytic cells. Furthermore, the PSGL-1/Syk pathway is important for this activation of HIV-1 replication. These results shed valuable new light on our understanding of the natural progression of HIV-1 infection over the long term and could provide a means for designing novel therapeutic interventions against AIDS and related disorders.

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Disclosures

The authors have no financial conflict of interest.

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