Supporting Information

Auerbach et al. 10.1073/pnas.1207314109

SI Materials and Methods

Cells. The human cell lines MT-2, PM1, Sup-T1, and MAGI-CCR5 were obtained from the National Institutes of Health-AIDS Research and Reference Reagent Program (NIH-ARRRP). Primary human $CD4^+$ T cells were purified from the peripheral blood of healthy blood donors using immunomagnetic beads (Stem Cell Technologies) and stimulated with phytohemagglutinin (PHA; Sigma) and recombinant human interleukin-2 (IL-2; Roche Applied Science) in complete RPMI medium (Invitrogen) containing 10% (vol/vol) FBS (HyClone, Thermo Scientific), glutamine at 2 mM, streptomycin at 50 μg/mL, and penicillin at 100 units/mL for 72 h before infection. Primary human macrophages were differentiated in vitro from peripheral blood as follows: total peripheral blood mononuclear cells (PBMCs) obtained from healthy blood donors were resuspended in RPMI medium supplemented with 20% FBS and 10% human AB serum (Invitrogen) and cultured for 6 d at 37 °C at a concentration of 1×10^6 cells/cm^2 in 24-well tissue culture plates (Nunc). Nonadherent cells were then removed by extensive washing with prewarmed culture medium. Adherent macrophage cultures contained >90% $CD14^+$ cells, as assessed by flow cytometry.

Proteins and Antibodies. Recombinant human CXCL4 (PF-4), CCL3 (MIP-1α), CCL5 (RANTES), CXCL11 (I-TAC), and CXCL12 (SDF-1) were obtained from R&D Systems; native CXCL4 derived from activated human platelets was obtained from Calbiochem. Molar values were calculated on the basis of the molecular weight of the monomeric molecules. Recombinant 4-domain soluble CD4 was obtained from Progenics. Recombinant wild-type or mutated (D368R) gp120 produced in mammalian cells was a kind gift of John R. Mascola (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Peptide T20 was obtained from the NIH-ARRRP. Monoclonal antibodies (mAbs) to human CXCL4 (nos. 7951 and 7952), polyclonal goat IgG antibody to human CXCL4, and purified goat IgG were obtained from R&D Systems. Goat antisera PB1 and PB1 Sub7 raised against HIV-1 gp120 outer-domain fragments, as well as human mAbs to various regions of HIV-1 gp120 (2G12, IgG1b12, F105, 654-D, and A1G8) were obtained from the NIH-ARRRP; mAbs VRC01, VRC03, VRCPG04, and VRCCH31 were a gift from Dr. John R. Mascola; mAb 412D was a gift from James E. Robinson (Tulane University Medical Center, New Orleans, LA). Neutralizing mAb to human CD4 (clone RPA-T4) was obtained from eBioscience; fluorochrome-conjugated mAbs to human CD4, CXCR3, CXCR4, and CCR5 were obtained from BD Biosciences.

Coimmunoprecipitation Assays. To evaluate the direct interaction between CXCL4 and HIV-1 envelope glycoproteins, we performed coimmunoprecipitation experiments using native, trimeric viral envelope expressed on the surface of persistently infected PM1 cells. Five million PM1 cells persistently infected with HIV IIIB (X4) were washed with 20 mM of Hepes buffer, 0.9% NaCl, pH 7.3 and incubated with 5 μM recombinant human CXCL4 or CCL3 (both from R&D Systems) for 1 h at room temperature in Hepes buffer. The cells were then washed twice with Hepes buffer to remove the unbound chemokines and treated with 2 mM of the cross-linking agent DTSSP (Pierce) in Hepes buffer; after 30 min at room temperature, the cross-linking reaction was stopped by addition of 50 mM Tris/HCl buffer pH 7.4. The cells were then washed three times with PBS and incubated with polyclonal rabbit antihuman CXCL4 IgG antibody (Peprotech) or goat antihuman CCL3 IgG antibody (R&D Systems) at room temperature; after 30 min, the cells were washed twice with PBS, lysed with Triton X-100 0.5% for 10 min on ice, and then spun. The pelleted material was treated with RIPA lysis buffer (Sigma) for 20 min on ice. After spinning, the supernatant was incubated with magnetic beads coated with protein G (Invitrogen) under continuous rotation for 1 h at 4 °C. The beads were then washed three times with RIPA buffer and dissolved in SDS loading buffer containing 10% 2-mercaptoethanol. The samples were resolved by SDS electrophoresis on 8% Tris-glycine acrylamide gels, transferred to nitrocellulose membranes, and analyzed by Western blot using the anti-gp120 mAb B13, directed against a linear epitope in the protein N terminus (a kind gift of George K. Lewis, Institute of Human Virology, Baltimore, MD). Following incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Pierce), the samples were visualized with the supersignal West chemiluminescent kit (Thermo Scientific). To further evaluate the direct interaction between CXCL4 and HIV-1 gp120, coimmunoprecipitation was also performed using recombinant wild-type or mutated (D368R) monomeric gp120 derived from isolate YU2 (a kind gift of John R. Mascola) after cross-linking of CXCL4 or CCL3 using the zero-length two-step method (Pierce). Recombinant human CXCL4 or CCL3, each at 2.5 μM, were separately incubated with EDC at 6 mM and sulfo-NHS at 15 mM in 10 mM Mes, 50 mM NaCl, pH 6.0, for 30 min at room temperature. The first step of the reaction was stopped with 2-mercaptoethanol at 200 mM for 20 min at room temperature. Gp120 was added at 20 nM and incubated for 2 h at room temperature. The cross-linking reaction was quenched by addition of hydroxylamine at a final concentration of 10 mM and incubation for 15 min at 25 °C. Molecular complexes were immunoprecipitated by polyclonal rabbit anti-CXCL4 or goat anti-CCL3 antibodies prebound to magnetic beads and then washed three times with RIPA buffer. The samples were resolved by SDS electrophoresis on 12% Tris-glycine acrylamide gels, transferred to nitrocellulose membranes, and analyzed by Western blot using mAb B13 (a gift of G. K. Lewis).

Receptor Down-modulation Assay. Expression of CD4, CCR5, CXCL3, and CXCR4 was evaluated on primary $CD4^+$ T cells after treatment with CXCL4. The cells were incubated with recombinant human CXCL4 at the concentration of 640 nM for 30 min, 2 h, or 18 h at 37 °C. Positive control cultures were incubated in the presence of PMA (50 ng/mL), CCL5, CXCL11, or CXCL12 (each at 100 ng/mL). The expression of the four receptors was evaluated by flow cytometry using specific fluorochrome-labeled mAbs (all from BD Bioscience).

Enzyme Immunoassay for CD4 Binding. Binding of CD4 to CXCL4 was evaluated by enzyme immunoassay. Briefly, 96-well flat-bottom plates were coated with recombinant human CXCL4 at 2 μg/mL in 100 μL of PBS overnight at 4 °C and blocked with casein 0.5% (wt/vol); 4-domain soluble CD4 (sCD4) was added in the liquid phase at 1 μg/mL and then revealed with the anti-CD4 mAbs OKT4 (specific for domains D3 and D4) or RPA-T4 (specific for the gp120-binding site in domain D1). An anti-CXCL4 mAb (no. 7951) was used as a positive control. As a further control, binding of sCD4 to immobilized recombinant HIV-1 gp120 derived from isolate IIIB (X4) was tested. Background binding was measured by testing all of the reagents on uncoated plates treated only with the blocking agent (casein).

Calcium Mobilization Assay. Interference of CXCL4 with CCL5- or CXCL12-elicited intracellular signaling was tested by calcium mobilization on purified $CD4^+$ T cells using the Fluo-4 NW kit (Molecular Probes, Invitrogen) according to the manufacturer protocol for nonadherent cells. To evaluate the antagonistic activity of CXCL4, the cells were pretreated with CXCL4 at 320 nM for 1 min at room temperature and then exposed to CCL5 or CXCL12 at 25 nM; to evaluate the agonistic activity, the cells were exposed to the chemokines without pretreatment.

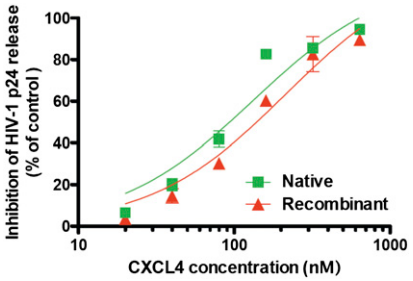

Fig. S1. Inhibition of HIV-1 replication by native and recombinant CXCL4. Dose-dependent inhibition of infection by a prototypic X4 HIV-1 strain (IIIB) in primary human $CD4^+$ T cells. Virus replication was assessed by measuring the amount of extracellular p24 Gag protein in culture by enzyme immunoassay. To reduce interexperimental variability, data were normalized with respect to the level of virus replication detected in control cultures (not treated with inhibitors). Data represent mean values (±SE) from two independent experiments, each performed in duplicate. Molar values were calculated on the basis of the molecular weight of the CXCL4 monomer (~7.8 K_d). Considering that CXCL4 is predominantly present as a tetramer in aqueous solution, with varying proportions of monomers, dimers, and higher-order aggregates, the values shown here represent the most conservative estimate of the antiviral potency of CXCL4.

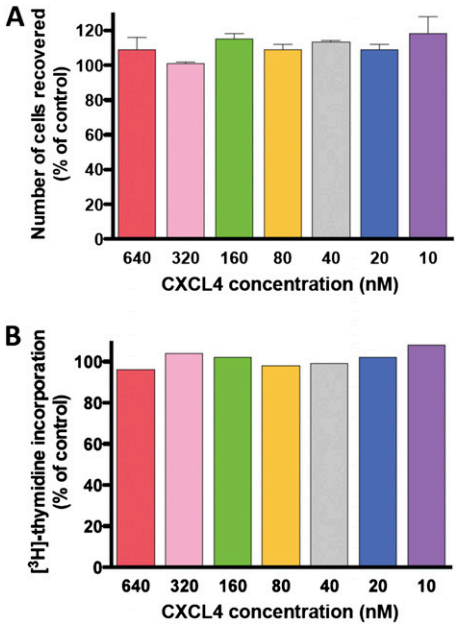

Fig. S2. Lack of inhibition of cell survival and proliferation by CXCL4. (*A*) Effect of CXCL4 on cell survival. Primary $CD4^+$ T cells, previously activated in vitro with PHA and IL-2 for 3 d, were treated with the indicated concentrations of recombinant human CXCL4 and then cultured for 7 d in the presence of recombinant human IL-2. Cell survival was assessed using timed flow cytometry by counting the absolute number of gated live cells in each culture. Data were normalized with respect to the number of cells present in control cultures (not treated with CXCL4). Data represent mean values (±SE) from two independent experiments, each performed in duplicate. (*B*) Effect of CXCL4 on cell proliferation. Freshly isolated primary $CD4^+$ T cells were treated with the indicated concentrations of recombinant human CXCL4 and then stimulated with an anti-CD3 monoclonal antibody cross-linked to plastic. The $[^3H]$-thymidine was added at 48 h of culture, and proliferation was measured by counting its uptake by the cells during the time window between 48 and 72 h. Data were normalized with respect to the proliferation detected in control cultures (not treated with CXCL4). Data are from a representative experiment of more than five performed with similar outcome.

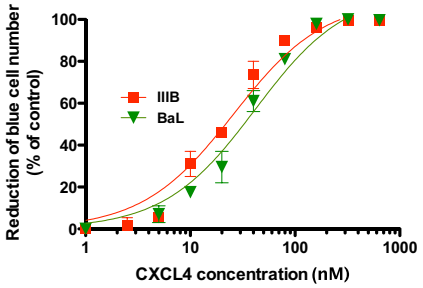

Fig. S3. Dose-dependent inhibition of single-cycle HIV-1 infection in MAGI-CCR5 cells by two biological variants of the virus (IIIB, X4; BaL, R5). MAGI-CCR5 cells express endogenous CXCR4 and recombinant human CD4 and CCR5, and contain a *LacZ* reporter gene under the control of the HIV-1 LTR. Enumeration of blue cells after 48 h denotes the completion of a single infectious cycle of HIV-1. To reduce interexperimental variability, data were normalized with respect to the number of blue cells detected in control cultures (not treated with inhibitors).

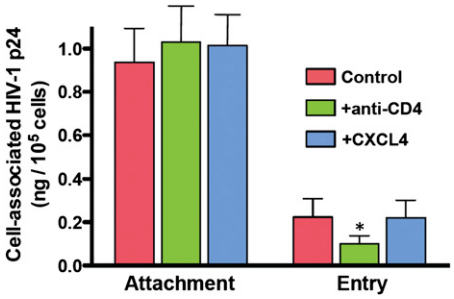

Fig. S4. Effects of CXCL4 on cell attachment and entry of a CXCL4-insensitive HIV-1 isolate (07USLR, X4). The test was performed as described in *SI Materials and Methods*. HIV-1 isolate 07USLR was also insensitive to the inhibitory effects of the anti-CD4 mAb on virion attachment (*Left*), but not entry (*Right*), suggesting the involvement of cell surface structures other than CD4 in the initial interaction of this virus with CD4$^+$ T cells. Data represent mean values (±SD) from three independent experiments. *$P < 0.05$ vs. untreated control by paired two-tailed Student *t* test.

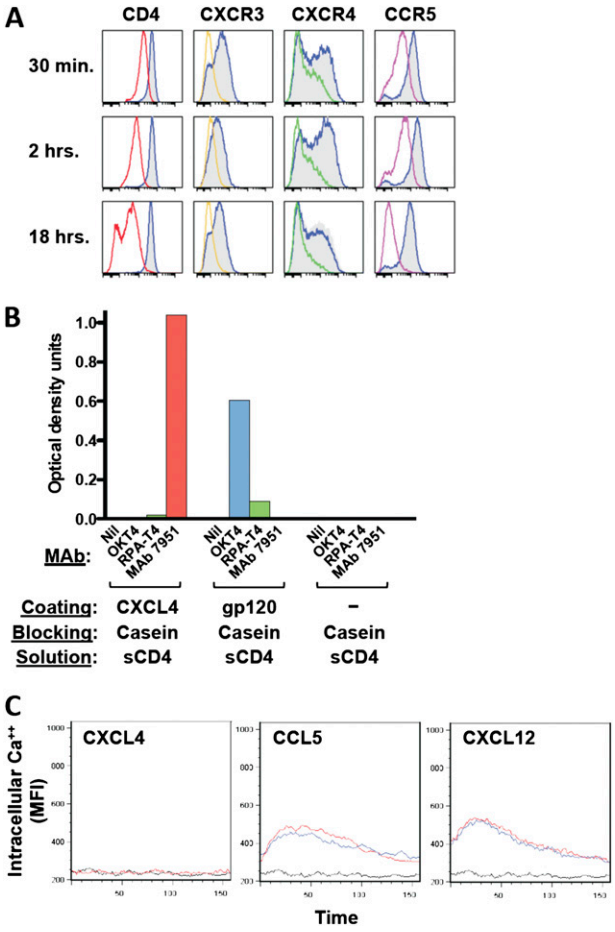

Fig. S5. Lack of CXCL4 interaction with CD4, CCR5, or CXCR4. (*A*) Lack of down-modulation of cell-surface CD4, CCR5, and CXCR4 expression upon treatment with CXCL4. Primary $CD4^+$ T cells were incubated with recombinant human CXCL4 at the concentration of 640 nM for 30 min, 2 h, or 18 h at 37 °C; control cultures were incubated in parallel with PMA (which down-modulates CD4) at 50 ng/mL, or with the specific receptor ligands CCL5 (CCR5), CXCL11 (CXCR3), or CXCL12 (CXCR4) each at 12.8 nM. The expression of the four receptors was evaluated by flow cytometry using specific fluorochrome-labeled mAbs. The solid gray histograms represent untreated cells; blue empty profiles represent cells treated with CXCL4; the other colored empty profiles represent cells treated with specific controls for each receptor: red, PMA (CD4); yellow, CXCL11 (CXCR3); green, CXCL12 (CXCR4); and purple, CCL5 (CCR5). (*B*) Lack of CD4 binding to CXCL4 in enzyme immunoassay. Plastic 96-well flat-bottom plates were coated with recombinant human CXCL4, and 4-domain soluble CD4 (sCD4) was added in the liquid phase and revealed with the anti-CD4 mAbs OKT4 (specific for CD4 domains D3 and D4) or RPA-T4 (specific for the gp120-binding site in CD4 domain D1). No binding of sCD4 to CXCL4 was detected, whereas an anti-CXCL4 mAb (no. 7951) used as a control showed a high level of binding. As a further control, we tested binding of sCD4 to immobilized recombinant HIV-1 gp120 derived from isolate IIIB (X4): as expected, binding of sCD4 to gp120 was detected using mAb OKT4, but not with mAb RPA-T4 directed against the gp120-binding site of CD4. Background signals measured with all of the reagents on uncoated plates treated only with the blocking agent were close to zero. The two coreceptors, CCR5 and CXCR4, could not be tested by enzyme immunoassay because these 7-transmembrane domain proteins cannot be stabilized in aqueous solution. (*C*) Lack of interference of CXCL4 with CCL5- or CXCL12-elicited intracellular calcium mobilization. Gray lines represent background signals recorded in the absence of stimulation; the red lines represent intracellular calcium signals elicited in the absence of competitors by the three chemokines (CXCL4 at 320 nM and CCL5 and CXCL12 at 25 nM); the blue lines represent the signals elicited by each chemokine in cells pretreated with CXCL4 (320 nM) for 1 min at room temperature.

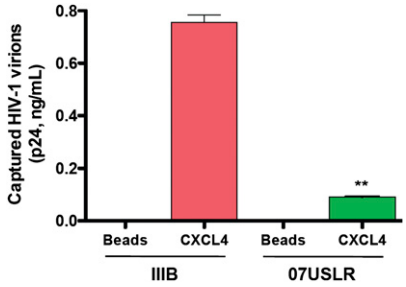

Fig. S6. Differential CXCL4-mediated virion capture of two HIV-1 isolates, one CXCL4 sensitive (IIIB) and one CXCL4 insensitive (97USLR). Data represent mean values (±SE) from two independent experiments. $^{**}P < 0.001$ for the difference between the amounts of p24 Gag protein captured for IIIB and 97USLR as analyzed by paired two-tailed Student *t* test. The virion-capture experiments were performed as described in *Materials and Methods*.

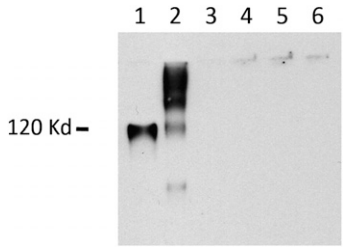

Fig. S7. Coimmunoprecipitation of monomeric HIV-1 gp120 by CXCL4. Lane 1, Recombinant gp120 (strain YU2) control (direct Western blot without immunoprecipitation); lane 2, gp120 plus CXCL4 precipitated with anti-CXCL4 rabbit antiserum; lane 3, gp120 precipitated with anti-CXCL4 rabbit antiserum alone (no CXCL4); lane 4, gp120 plus CXCL4 precipitated with irrelevant rabbit IgG; lane 5, gp120 plus CCL3 precipitated with anti-CCL3 rabbit antiserum; and lane 6, gp120 precipitated with anti-CCL3 rabbit antiserum alone (no CCL3). Presence of high molecular weight bands in lane 2 is presumably due to cross-linking of multiple gp120 molecules. Coimmunoprecipitation was performed as described in *SI Materials and Methods*.

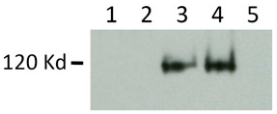

Fig. S8. Coimmunoprecipitation of wild-type (WT) and mutated (D368R) HIV-1 gp120 by CXCL4. Lane 1, Recombinant mutated (D368R) gp120 (strain YU2) precipitated with anti-CXCL4 rabbit antiserum alone (no CXCL4); lane 2, WT YU2 gp120 precipitated with anti-CXCL4 rabbit antiserum alone (no CXCL4); lane 3, mutated gp120 D368R plus CXCL4 precipitated with anti-CXCL4 rabbit antiserum; lane 4, WT gp120 plus CXCL4 precipitated with anti-CXCL4 rabbit antiserum; and lane 5, no gp120 control. Coimmunoprecipitation was performed as described in *SI Materials and Methods*.

Table S1. Effect of recombinant human CXCL4 against HIV-2, simian immunodeficiency virus (SIV), and human herpesvirus (HHV)-6A

Virus	Strain	CXCL4 IC_{50}, μM
HIV-2*	CBL-20	>1
HIV-2*	CDC310342	>1
HIV-2*	7924A	>1
SIV_{sm}†	smE660/307	>1
SIV_{mac}†	mac251	>1
SIV_{mac}†	mac251/745	>1
HHV-6A	GS	>1

*Primary HIV-2 isolates minimally passaged in vitro exclusively in primary human T cells.

†Primary SIV strains passaged in vivo in macaques (mac251 and mac251/745 in *M. mulatta;* smE660/307 in *M. nemestrina*).