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The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1

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A PUTATIVE chemokine receptor that we previously cloned and termed LESTR[1] has recently been shown to function as a co-receptor (termed fusin) for lymphocyte-tropic HIV-1 strains[2]. Cells expressing CD4 became permissive to infection with T-cell-line-adapted HIV-1 strains of the syncytium-inducing phenotype after transfection with LESTR/fusin complementary DNA. We report here the identification of a human chemokine of the CXC type, stromal cell-derived factor 1 (SDF-1), as the natural ligand for LESTR/fusin, and we propose the term CXCR-4 for this receptor, in keeping with the new chemokine-receptor nomenclature. SDF-1 activates Chinese hamster ovary (CHO) cells transfected with CXCR-4 cDNA as well as blood leukocytes and lymphocytes. In cell lines expressing CXCR-4 and CD4, and in blood lymphocytes, SDF-1 is a powerful inhibitor of infection by lymphocyte-tropic HIV-1 strains, whereas the CC chemokines RANTES, MIP-1α and MIP-1β, which were shown previously to prevent infection with primary, monocyte-tropic viruses[3], are inactive. In combination with CC chemokines, which block the infection with monocyte/macrophage-tropic viruses, SDF-1 could help to decrease virus load and prevent the emergence of the syncytium-inducing viruses which are characteristic of the late stages of AIDS[4].

LESTR (leukocyte-expressed seven-transmembrane-domain receptor) is an orphan receptor with structural similarity to chemokine receptors. Despite extensive testing of a large number of chemokines, the ligand for LESTR remained elusive[1]. Murine SDF-1 was described as a factor that is produced by bone-marrow stromal cells and shown to induce proliferation of B-cell progenitors[5,6] as well as recruitment of T cells[7]. The human homologue, which was cloned subsequently, is virtually identical to murine SDF-1 (see Methods). SDF-1 is a CXC chemokine with the typical four-cysteine motif and the first two cysteines separated by one amino acid[8].

When human SDF-1 was tested on the CHO-1C2 clone which stably expresses LESTR, a transient rise of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was observed (Fig. 1*a*). This response, which is characteristic of the action of chemokines on blood leukocytes, was not observed with parental CHO cells. Other chemokines, including RANTES (for regulation-upon-activation, normal T expressed and secreted) macrophage inflammatory protein (MIP), MIP-1α and MIP-1β, were not active. Monocytes, neutrophils and phytohaemagglutinin (PHA)-activated peripheral-blood lymphocytes (PBLs) were also stimulated by SDF-1, as shown by $[Ca^{2+}]_i$ changes and chemotaxis (Fig. 1*b*, *d*). Real-time recordings of Ca^{2+} mobilization after sequential stimulation are a reliable way to assess receptor usage by chemokines[8]. Stimulation with a chemokine (at saturating concentrations) causes receptor desensitization, and no response is observed when the cells are restimulated within a short time by a chemokine acting on the same receptor. As shown in Fig. 1*c*, monocytes stimulated with SDF-1 remained fully responsive to subsequent stimulation with MCP-1, RANTES or MIP-1α, and when applied first, none of these chemokines affected the $[Ca^{2+}]_i$ changes induced by SDF-1 as the second stimulus (not shown), indicating that SDF-1 does not share receptors with the three CC chemokines. The functional responses and the desensitization results show that LESTR is selective for SDF-1, the only ligand identified so far. The structure of receptor and ligand being known, we propose to rename LESTR/fusin as CXCR-4, in keeping with the receptor nomenclature established at the 1996 Gordon Research Conference on chemotactic cytokines.

The effects of SDF-1 on HIV-1 infection were tested first on HeLa cells transfected with CD4 and carrying an integrated HIV-1 long terminal repeat (LTR)-driven reporter gene, *lacZ* from *Escherichia coli*, which is induced by the HIV-1-encoded trans-activating protein Tat. The induction of β-galactosidase, the product of *lacZ*, reflects transactivation by Tat and therefore HIV-1 infection. Like leukocytes and several tissue cells, the human epithelial carcinoma cell line HeLa constitutively expresses CXCR-4 (refs 2, 9). The cells were infected with two

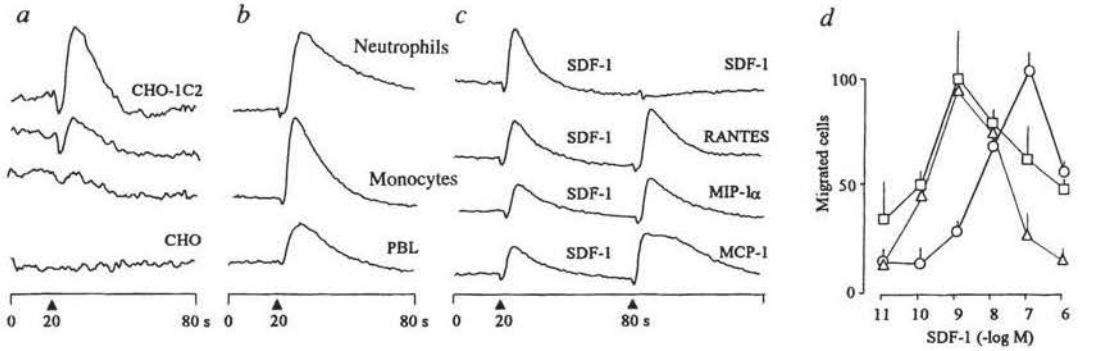

FIG 1. SDF-1 is the ligand for CXCR-4 and is active on blood monocytes, neutrophils and on PHA-activated PBLs. *a*, SDF-1 induced $[Ca^{2+}]_i$ changes in the CHO-1C2 clone expressing CXCR-4 (100, 10, 1 nM, from top to bottom) and in parental CHO cells (100 nM). *b*, SDF-1-mediated $[Ca^{2+}]_i$ changes in human neutrophils, monocytes and PHA-activated PBL. SDF-1, RANTES, MIP-1α and MCP-1 were used at 100 nM. *c*, Receptor desensitization in monocytes as assessed by $[Ca^{2+}]_i$ changes. *d*, *In vitro* chemotaxis of blood monocytes (squares), neutrophils (circles) and PHA-activated PBLs (triangles) in response to SDF-1.

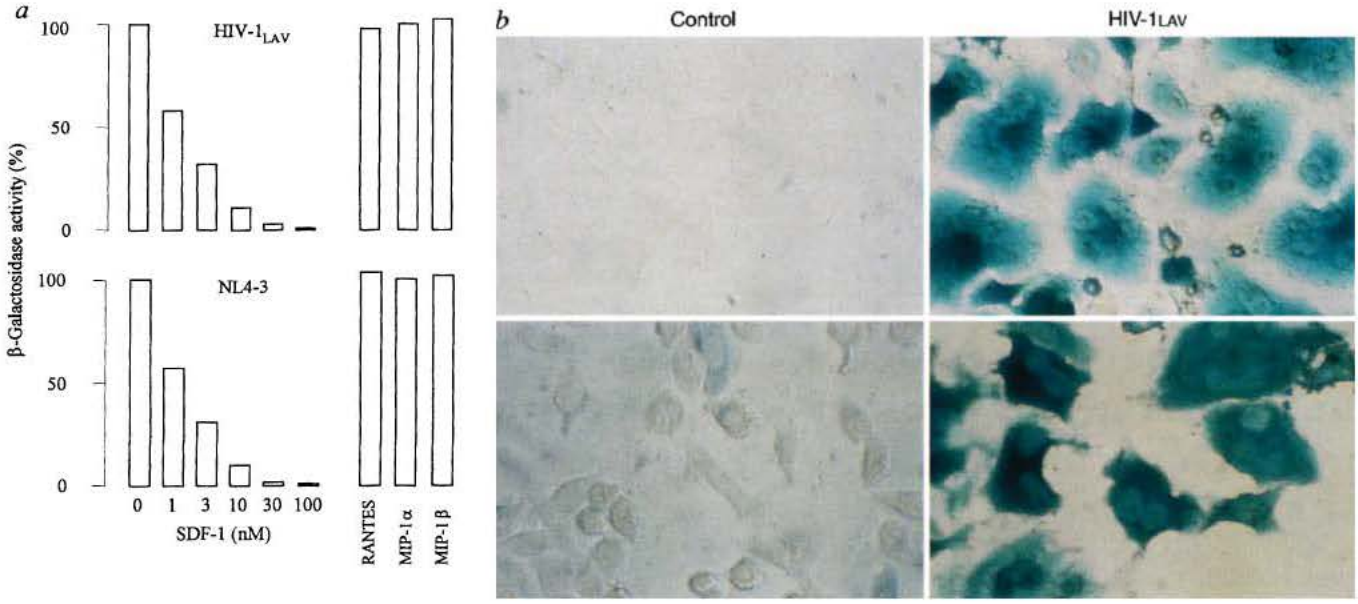

FIG. 2 SDF-1 inhibits infection of CD4$^+$ HeLa cells by HIV-1 SI strains. a, SDF-1 (1–100 nM) but not RANTES, MIP-1α or MIB-1β (all 100 nM) inhibited infection by HIV-1_{LAV} and NL4-3. Mean per cent activity of triplicate values are shown; the data are representative of four similar experiment. *b*, Formation of syncytia is inhibited by SDF-1 but not by RANTES. Cells infected with HIV-1_{LAV} and cultured for 24 h were fixed with 0.5% glutaraldehyde, and syncytia formation was visualized by staining for β-galactosidase.

viruses, HIV-1 Lai, LAV isolate (HIV-1_{LAV}) or the HIV-1 clone NL4-3, which harbours the LAV envelope (Env) glycoprotein[10]. SDF-1 was a very potent inhibitor of infection by both viruses, as shown by the decrease in β-galactosidase expression, which amounted to about 50, 70 and >95% at 1, 3 and 30 nM SDF-1, respectively (Fig. 2*a*). By contrast, no inhibition was observed when SDF-1 was replaced by 100 nM RANTES, MIP-1α or MIP-1β. These chemokines recognize the receptors CCR-5 and in part, CCR-1 and CCR-3 (refs 11–15), but they do not recognize CXCR-4 (Fig. 1). The dramatic inhibition of infection and syncytia formation by SDF-1 and the lack of activity by RANTES are shown in Fig. 2*b*. No expression of β-galactosidase was seen in infected cells cultured in the presence of the reverse-transcriptase inhibitor azidothymidine (AZT), indicating that the reporter gene was only induced upon infection (data now shown). To verify that the action of SDF-1 depends on the presence of CXCR-4, the receptor-expressing CHO-1C2 cells were transfected with CD4 and infected with NL4-3 carrying a luciferase reporter gene (kindly provided by V. Planelles). As in HeLa cells, SDF-1 inhibited the expression of the reporter gene, indicating inhibition of infection by NL4-3.

The inhibitory effect of SDF-1 was also studied by monitoring newly reverse-transcribed HIV-1 proviral DNA generated after viral entry, using the polymerase chain reaction (PCR) technique. Extracts of CD4$^+$ HeLa cells collected 6 hours after addition of HIV-1_{LAV} contained specific proviral DNA. In the presence of SDF-1, the appearance of proviral DNA was drastically reduced, whereas no effect was observed in the presence of RANTES. No transcripts were obtained when the reverse transcriptase inhibitor AZT was added to the cultures (Fig. 3*a*).

It was important to assess whether SDF-1 inhibits infection by competing with the virus for binding to CXCR-4, or by interfering with a post-entry event. For this purpose, we used an HIV-1 NL4-3 pseudotype expressing the G glycoprotein of vesicular stomatitis virus (VSV) instead of the LAV Env protein[16] (kindly provided by A. Miyanohara). NL4-3 (ΔEnv)G readily infects cells through the interaction of the G glycoprotein with membrane phospholipids, and does not depend on HIV-specific receptors. As shown by the expression of β-galactosidase, SDF-1 had no effect on the replication of NL4-3 (ΔEnv)G, in contrast to the marked concentration-dependent inhibition obtained for NL4-3 (Fig. 3*b*). These results rule out an effect of SDF-1 on the expression of HIV-1 proviral DNA, and suggest that SDF-1 inhibits HIV infection at stages that precede reverse transcription and presumably interferes with the interaction of the virus with CD4 and CXCR-4.

Upon activation, human peripheral blood mononuclear cells

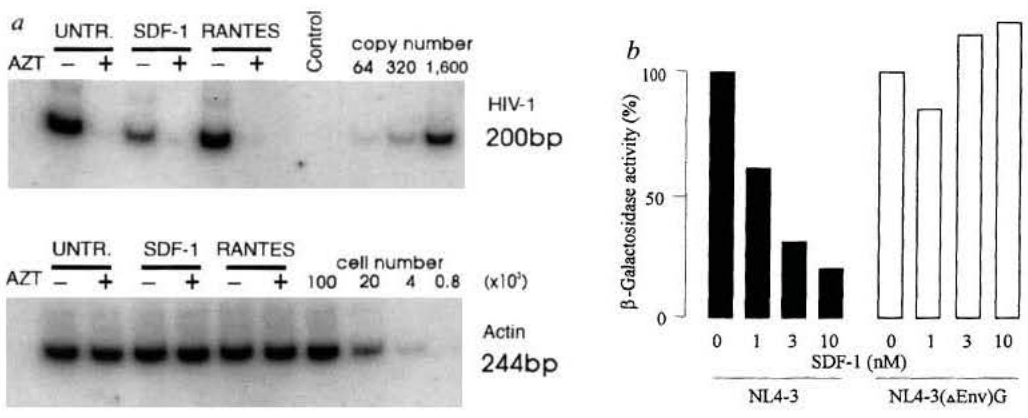

FIG. 3 SDF-1 blocks entry of HIV-1_{LAV} in CD4$^+$ HeLa(*lacZ*) cells. a, PCR amplification of retrotranscribed proviral DNA. The effect of SDF-1 or RANTES (both 100 nM) or buffer alone (UNTR.) on the generation of NL4-3 proviral DNA in CD4$^+$ HeLa(*lacZ*) was examined. AZT (50 μM) was added (+) or excluded (–) during infection to verify the retroviral origin of the PCR template. PCR products were analysed by agarose-gel electrophoresis and autoradiography. Three experiments were performed with similar outcome. *b*, Lack of inhibition of post-entry events by SDF-1 after infection by NL4-3 (ΔEnv)G. Data are representative of four different experiments.

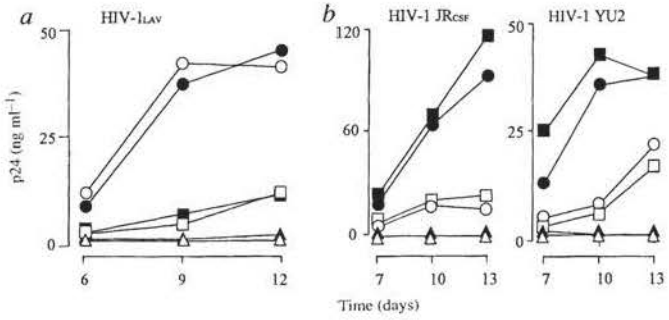

FIG. 4 SDF-1 is selective for lymphocyte-tropic HIV-1. *a*, Inhibition of HIV-1_{LAV} infection in PBMCs by SDF-1. 100 nM SDF-1 was added during and after infection (△, ▲), after infection (□, ■), or omitted (○, ●). Cultures with PBMCs from two different donors (open and filled symbols) were continued for 6 to 12 days, and p24 production assayed. *b*, No inhibition of HIV-1 JR_{JSF} of HIV-1 YU2 infection in PBMCs by SDF-1. PBMCs from two different donors (open and filled symbols) were cultured for 7 to 13 d in the presence of 100 nM SDF-1 (○, ●), 100 nM RANTES (△, ▲) or in the absence of chemokine (□, ■), and p24 production assayed.

(PBMCs) support replication of HIV-1 *in vitro*. To test the inhibitory activity of SDF-1, PBMCs were activated by PHA before infection with HIV-1_{LAV} and the accumulation of soluble HIV-1 p24 protein was measured. When SDF-1 was present from the time of infection until the end of the experiment (day 12), protection was virtually complete (>98%), and a high degree of protection was observed even when SDF-1 was added after the period of infection (Fig. 4*a*). Infection with monocyte/macrophage-tropic HIV-1 (JR_{CSF} or YU2), by contrast, was inhibited by RANTES but was not affected by SDF-1 (Fig. 4*b*).

The clear-cut discrimination of the mechanisms of entry by viruses with different tropism is highlighted by the inhibition of lymphocyte-tropic infection by SDF-1 and monocyte-macrophage-tropic infection by RANTES, MIP-1α and MIP-1β. This may be important when considering the progression of AIDS. Through its remarkable variability, the HIV-1 genome is likely to adapt the Env protein to different chemokine receptors. After years of asymptomatic infection with monocytotropic quasispecies viruses, strains may emerge that accelerate disease progression by rapid replication in leukocytes expressing CD4 with CXCR-4 as co-receptor. HIV adaptation to entry mediated by CXCR-4 into cells must represent a major biological advantage for the virus, which would find a much broader repertoire of infectable cells. In fact, CXCR-4 is constitutively expressed at high levels in PBMC[1], as well as in some tissue cells[1,9,17] where expression of CC chemokine receptors is low. It is therefore of major importance to find means to block infection by CXCR-4-adapted syncytium-inducing viruses. SDF-1 administration or induction may inhibit dissemination of such viruses and possibly prevent their emergence in patients with chronic HIV infection. □

Methods

Responses to SDF-1 in CXCR4 transfected CHO cells and blood leukocytes. The 67-amino-acid form of human SDF-1 (accession number U16752), which differs from murine SDF-1α (refs 5–7) by a single substitution (Val instead of Ile at position 18) and deletion of the C-terminal Lys, was prepared by chemical synthesis[18]. Neutrophils[19], monocytes[20] and PBLs[21] were prepared according to established methods. PBLs were stimulated for 72 h with 1 μg ml^{-1} PHA. Real-time recordings of $[Ca^{2+}]_i$ changes in CXCR-4-transfected CHO cells[1] and blood leukocytes loaded with Fura2/AM were performed as described[22]. Receptor desensitization was tested in monocytes by monitoring $[Ca^{2+}]_i$ changes upon repeated chemokine stimulation (100 nM) at 60-s intervals as described[20]. Chemotaxis was performed in 48-well chambers with 10^5 cells per well using bare (neutrophils and monocytes) or collagen-coated (PBLs) polyvinylpyrrolidone-free polycarbonate membranes with 5-μm pores.[20] Migrated cells were counted in five randomly selected fields at 1,000-fold magnification after migration for 1 h (neutrophils and monocytes) or 3 h (PBLs).

Generation of HIV-1 particles. HIV-1_{LAV} and NL4-3 infectious supernatants were obtained from infected CEMX174 and MT4 lymphoblastoid cell lines, respectively. NL4-3 particles[10] were generated by transient transfection of COS7 cells with cloned pNL4-3 DNA. NL4-3(ΔEnv)G, a pseudotyped NL4-3 variant with a replacement of the Env protein with the G glycoprotein of VSV, was produced by co-transfection of COS7 cells with a plasmid encoding NL4-3 with a *Bgl*II–*Bgl*II detection in the Env gene (provided by V. Planelles) plus pCMV vector carrying the DNA for VSV G glycoprotein. HIV-1 JR_{CSF}[23] and YU[24] particles were obtained by transfection of COS7 cells with plasmids carrying full-length proviral DNA.

HIV-1-entry and cell-fusion assays. A CD4$^+$ HeLa(*lacZ*) cell clone was generated in HeLa cells carrying a stably integrated *lacZ* gene under HIV-1 LTR control by stable transfection with a retroviral vector containing CD4 cDNA[25]. 1.5×10^4 CD4$^+$ HeLe(*lacZ*) cells per well in 96-well plates were cultured for 18 h before infection with 250 μl infectious supernatants (HIV-1_{LAV}, NL4-3 or NL4-3(ΔEnv)G at 500, 50 and 1 ng ml^{-1} HIV-1 p24, respectively) in the presence or absence of chemokines. After 24 h, β-galactosidase activity was determined in cell lysates from triplicate wells. After removing supernatants, cells were lysed in 100 μl 60mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 10 mM $MgSO_4$, 2.5 mM EDTA, 50 mM β-mercaptoethanol and 0.125% NP-40, and mixed with 100 μl 80 mM sodium phosphate, pH 7.4, 10 mM $MgCl_2$, 50 mM β-mercaptoethanol and 6 mM chlorophenol red-β-galactopyranoside monosodium salt and incubated for 20 min at 37 °C before measuring absorbance at 540 nm.

PCR amplification of proviral DNA. 2×10^6 cells in 4 ml were infected for 2 h with 2 ml NL4-3 infectious supernatant (100 ng ml^{-1} HIV-1 p24). The cultures were washed 3 times, supplemented with 100 nM SDF-1, 100 nM RANTES or the vehicle, and cultured for an additional 6 h in the presence (+) or absence (−) of 50 μM AZT. After extraction of total DNA[26], PCR was performed with primers for proviral DNA (3′-region primer, CCTGCGTCGAGAGAGCTCCTCTGG-3′; and 5′-region primer, GGCTAACTAGGGAACCCACTG-3′; end-labelled with ^{32}P) in 50 μl 0.25 mM dNTPs, 50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mg ml^{-1} BSA and 1.5 units of Taq DNA polymerase (Amersham) for 25 cycles. The primers correspond to nucleotide positions 496–516 (first nucleotide is at position 42 downstream of the transcription initiation site) and 695–672 (complementary sequence in *gag*) in the HIV-1 JR_{CSF}. Primers to β-actin DNA (5′-region primer, GTGGGGCGCCCCAGGCACCA-3′, and 3′-region primer, CGGTTGGCCTTGGGGTTCAGGGGGG-3′) yielding a 244-bp amplified product were used for standardization.

Infection of PBMCs. PBMCs from healthy blood donors were stimulated for 48 h with 1 μg ml^{-1} PHA (Wellcome) in RPMI 1640, 10% fetal bovine serum (FCS), and then infected for 2 h with 10–20 ng of HIV-1 p24 (10^7 cells per infection in 4 ml) in the presence or absence of chemokines. Infected PBMCs were washed four times with PBS and cultured in RPMI 1640, 10% FCS, plus 20 ng ml^{-1} IL-2 with or without chemokines. Cultures were refed every 3 d with IL-2 and chemokines, and soluble HIV-1 p24 was determined by ELISA (DuPont de Numours, France).

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