Identification of a major co-receptor for primary isolates of HIV-1

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Entry of HIV-1 into target cells requires cell-surface CD4 and additional host cell cofactors. A cofactor required for infection with virus adapted for growth in transformed T-cell lines was recently identified and named fusin. However, fusin does not promote entry of macrophage-tropic viruses, which are believed to be the key pathogenic strains *in vivo*. The principal cofactor for entry mediated by the envelope glycoproteins of primary macrophage-tropic strains of HIV-1 is CC-CKR-5, a receptor for the β-chemokines RANTES, MIP-1α and MIP-1β.

THE human immunodeficiency viruses infect $CD4^+$ macrophages and T helper cells[1]. Although HIV-1 entry requires the cell-surface expression of CD4, to which the viral envelope glycoprotein (Env) binds, several studies have suggested that it is not sufficient for fusion of the viral envelope to the cellular plasma membrane. Early studies showed that, although human cells expressing a transfected CD4 gene were permissive for virus entry, murine cells expressing human CD4 were not[2–4]. These findings led to the suggestion that a species-specific cell-surface cofactor is required, in addition to CD4, for HIV-1 entry. Subsequent studies showed that strains of HIV-1 that had been adapted for growth in transformed T-cell lines (T-tropic strains) could infect primary T cells, but not primary monocytes or macrophages. In contrast, many primary viral strains were found to infect monocytes, macrophages and primary T cells, but not transformed T-cell lines[5–8]. This difference in tropism was found to be a consequence of specific sequence differences in the gp120 subunit of Env[9,10], suggesting that multiple cell-type-specific cofactors may be required for entry, in addition to CD4.

The nature of the cofactors required for HIV entry proved elusive until the recent identification of fusin[11], a member of the seven-transmembrane G-protein-coupled receptor family. Fusin was shown to act as a co-receptor for T-tropic strains, but it did not support syncytium formation of $CD4^+$ cells with cells expressing Env of macrophage-tropic viruses. These viruses more closely resemble those that predominate in infected individuals throughout the course of the disease, particularly in the asymptomatic phase. In addition, these strains appear to be responsible for HIV-1 transmission, both sexually and by the transfer of infected blood[12,13]. Rare individuals who are resistant to sexual transmission of HIV-1 have T cells that are readily infected by T-tropic virus, but cannot be infected by macrophage-tropic virus, further supporting a role for macrophage-tropic virus in the transmission of HIV-1 (ref. 14).

The inhibitors of HIV-1 replication present in supernatants of $CD8^+$ T cells have recently been characterized[15] as the β-chemokines RANTES (regulated-upon-activation, normal T expressed and secreted), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β. Chemokines are chemotactic cytokines that are released by a wide variety of cells to attract macrophages, T cells, eosinophils, basophils and neutrophils to sites of inflammation (reviewed in refs 16,17). There are two classes of chemokines, C-X-C (α) and C-C (β), depending on whether the first two cysteines are separated by a single amino acid (C-X-C) or are adjacent (C-C). The α-chemokines, such as interleukin-8 (IL-8), neutrophil-activating protein-2 (NAP-2) and melanoma growth stimulatory activity protein (MGSA) are chemotactic primarily for neutrophils, whereas β-chemokines, such as RANTES, MIP-1α, MIP-1β, monocyte chemotactic protein-1 (MCP-1), MCP-2, MCP-3 and eotaxin are chemotactic for macrophages, T-cells, eosinophils and basophils. The chemokines bind specific cell-surface receptors belonging to the family of G-protein-coupled seven-transmembrane-domain proteins (reviewed in ref. 18). On binding their cognate ligands, chemokine receptors transduce an intracellular signal though the associated trimeric G protein, resulting in a rapid increase in intracellular calcium concentration. There are at least seven human chemokine receptors that bind or respond to β-chemokines with the following characteristic pattern: CC-CKR-1 (refs 19, 20) (MIP-1α, MIP-1β, MCP-3, RANTES), CC-CKR-2A and CC-CKR-2B (MCP-1, MCP-3), CC-CKR-3 (ref. 21) (eotaxin, RANTES, MCP-3), CC-CKR-4 (ref. 22) (MIP-1α, RANTES, MCP-1), CC-CKR-5 (ref. 23) (MIP-1α, RANTES, MIP-1β), and the Duffy blood-group antigen[24] (RANTES, MCP-1).

Several lines of evidence implicate chemokine receptors as possible accessory factors in infection by primary strains of HIV-1. First, fusin is a member of the seven-transmembrane-domain family of chemokine receptors. It is most closely related to the IL-8 receptor, having a homology of 39% in the transmembrane domains[25]. Presumably, fusin is a receptor for some currently unknown chemokine or neuropeptide. Second, the finding that the β-chemokines RANTES, MIP-1α and MIP-1β inhibit infection by primary or macrophage-tropic HIV-1, but not by T-tropic virus[15], suggests that chemokine receptors are involved in HIV-1 replication and implicates the macrophage-tropic Env in this process. Third, $CD4^+$ cells of individuals that have been multiply

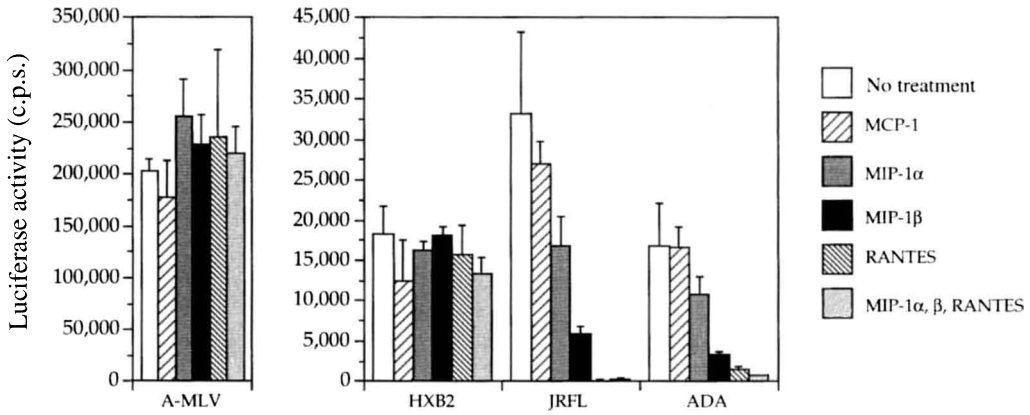

FIG. 1 Chemokines block infection at the level of viral entry. PM1 cells were infected with luciferase reporter viruses pseudotyped by Envs of HIV-1 macrophage-tropic (ADA and JRFL) or T-cell-line adapted virus (HXB2) or with A-MLV Env in the presence or absence of individual β-chemokines or a mixture of MIP-1α, MIP-1β and RANTES. Luciferase activity was measured 4 days later, as described below. This experiment was repeated 4 times with similar results.

METHODS. NL4-3-Luc-R$^-$E$^-$[27] virus stocks pseudotyped by various Envs were generated by transfecting 293T cells with 10 μg each of pNL4-3-Luc-R$^-$E$^-$ and pcDNA-I/Amp-based expression vectors (InVitrogen) encoding JRFL. ADA[28], BaL[29], HXB2 or amphotropic MLV Env[30]. Virus-containing supernatants were collected 48 h post-transfection and frozen at −80 °C. Viruses were quantified by p24 ELISA assay. Cells (5×10^4) were seeded in 48-well dishes in DMEM containing 10% fetal bovine serum and infected with luciferase reporter virus (50 ng p24) in a total volume of 400 μl, with or without 30 min pretreatment with each of the chemokines listed (500 ng ml^{-1}, Peprotech). After 16 h, 0.5 ml medium was added to the wells. After 4 days of additional culture, 100-μl lysates were prepared and luciferase activity in 20 μl was assayed using commercially available reagents (Promega).

exposed to HIV-1 are highly resistant to infection *in vitro* by primary and macrophage-tropic strains of HIV-1 (ref. 14). Resistance to infection was correlated with an overproduction of chemokines. Taken together, these findings suggest a role for chemokines or chemokine receptors in the replication of primary but not T-cell-line adapted virus. These studies did not determine the step in the viral life cycle that was blocked by chemokines.

Here we report that β-chemokines inhibit HIV-1 replication by blocking entry of the virus into CD4$^+$ cells. In light of this finding and those described above, we surmised that one or more of the β-chemokine receptors may serve as a required accessory factor for entry by macrophage-tropic HIV-1. We therefore tested the major members of the CC-CKR family for their ability to facilitate infection with macrophage-tropic HIV-1 strains and fusion with cells expressing Env proteins from these strains. Our results show that the product of the recently identified gene encoding CC-CKR-5 acts together with CD4 to allow entry of primary macrophage-tropic strains of HIV-1.

Chemokines block entry of primary HIV-1

To test whether β-chemokines block entry of macrophage-tropic HIV-1, we infected the T cell line PM1, which is highly susceptible to infection with both macrophage-tropic and T-tropic virus[26], with HIV-1-based luciferase reporter viruses[27]. The luciferase reporter viruses infect cells in a single round but are not competent for further replication because of a frameshift mutation inserted into the *env* gene. Thus measurement of luciferase activity in cells infected with pseudotypes of this virus allows comparison of the relative efficiency of entry mediated by different Envs. In these studies we used HXB2 as a representative T-tropic Env, and three commonly studied macrophage-tropic Envs, JRFL[6], ADA[28] and BaL[29]. To control for possible post-entry or nonspecific effects of β-chemokines, we also prepared virus pseudotyped with amphotropic murine leukaemia virus (A-MLV) Env[30].

The β-chemokines inhibited infection of PM1 cells with virus pseudotyped by macrophage-tropic Env (JRFL and ADA). In contrast, the β-chemokines had no effect on infection with virus bearing T-tropic (HXB2) or A-MLV Envs (Fig. 1). Strongest blocking was observed with RANTES, followed in order of effectiveness by MIP-1β and MIP-1α. We found that MCP-1, MCP-3 and eotaxin had no inhibitory effect (Fig. 1, and data not shown). This same order was observed in inhibition of primary HIV-1 replication by β-chemokines[15]. Taken together, these findings strongly suggest that β-chemokine inhibition of viral replication is due to a block to entry of macrophage-tropic, but not T-tropic, HIV-1.

CC-CKR-5 is a potent co-receptor

Given that β-chemokines block HIV-1 entry, and CC-CKRs belong to the same gene family as fusin[11], the recently described accessory factor for the entry of T-tropic virus, we surmised that macrophage-tropic viruses use a β-chemokine receptor for fusion and entry into target cells. To test this hypothesis, we expressed the known β-chemokine receptors or fusin in several human and murine cell lines, and then tested their relative infectivity using HIV-luciferase pseudotyped with the different Envs.

Human embryonic kidney 293T cells transiently co-transfected with CD4 and the different chemokine receptor expression vectors were readily infected with virus pseudotyped by amphotropic or T-tropic Env, but not by virus lacking Env (Fig. 2*a*). Cells transiently transfected with expression vectors for CD4 plus CC-CKR-1, CC-CKR-2B, CC-CKR-3 or CC-CKR-4 were resistant to infection with virus pseudotyped with macrophage-tropic envelopes when compared with vector-transfected control cells (Fig. 2*a*). However, cells coexpressing CD4 and CC-CKR-5 displayed increases of three to four orders of magnitude in sensitivity to infection with viruses pseudotyped by ADA, BaL or JRFL Env (Fig. 2*a*). Almost identical findings were made with CC-CKR-5 cDNAs amplified from three different individuals (data not shown).

Infection of the 293T cells expressing both CD4 and CC-CKR-5 was completely blocked by the anti-CD4 monoclonal antibody Leu-3a (Fig. 2*b*). In addition, when pcCD4 was omitted from the transfection, CC-CKR-5 failed to support virus entry (Fig. 2*c*). Taken together, these findings indicate that CC-CKR-5 and CD4 must function cooperatively to mediate entry of macrophage-tropic virus.

Murine cells transfected with human CD4 are resistant to infection with all tested strains of HIV. To determine whether chemokine receptors could confer susceptibility to infection, the different receptor genes were stably introduced into murine 3T3.CD4 cells. Cells expressing CC-CKR-1, CC-CKR-2B, CC-CKR-3, CC-CKR-4, Duffy or fusin were all resistant to infection with HIV-luciferase pseudotyped with macrophage-tropic Envs, but were infected with virus bearing amphotropic Env (Fig. 3*a*, and data not shown). Expression of CC-CKR-5 allowed infection with the macrophage-tropic pseudotypes, but these cells were resistant to infection mediated by HXB2 Env (Fig. 3*a*). Only fusin-expressing 3T3.CD4 cells were permissive for infection with this

T-tropic virus (Fig. 3*a*). The β-chemokine receptors were expressed on the surface of the 3T3.CD4 cells, as assessed by mobilization of intracellular free Ca^{2+} in response to the appropriate chemokines (Fig. 3*b*, and data not shown). Cells expressing CC-CKR-5 responded to RANTES, MIP-1α and MIP-1β, which is consistent with known β-chemokine reactivities[23]. Infection of the 3T3.CD4 cells expressing CC-CKR-5 with macrophage-tropic virus was blocked by a mixture of the three chemokines that efficiently activate this receptor, as well as by anti-CD4 antibody (Fig. 3*a*). Infection of the fusin-expressing cells with T-tropic virus was also blocked by anti-CD4, but was completely refractory to treatment with chemokines. Thus these results suggest that only CC-CKR-5 mediates entry of macrophage-tropic Envs, that T-tropic envelope glycoproteins do not use this co-receptor for entry, and that β-chemokines block entry of the macrophage-tropic virus by specifically binding to this receptor.

Stable expression of CC-CKR-5, but not of the other β-chemokine receptors, in human HOS.CD4, HeLa.CD4 and U87MG.CD4 (ref. 31) cells also conferred susceptibility to infection with macrophage-tropic HIV-1 (Fig. 3*c*, *d*, and data not shown). As observed in the transient transfections, stable coexpression of both CC-CKR-5 and CD4 was required for viral entry into the HeLa cells (Fig. 3*d*). Infection of these cells with macrophage-tropic virus was reduced by 70–80% when they were treated with a mixture of chemokines (Fig. 3*d*). Unexpectedly, high levels of β-chemokines failed to inhibit infection of HOS.CD4 cells (data not shown). In general, inhibition with β-chemokines was consistently less efficient in the non-lymphoid cells expressing CD4 and CC-CKR-5 than in the PM1 cells.

CC-CKR-5 promotes Env-mediated fusion

Fusion of the HIV-1 envelope with the cellular plasma membrane can be simulated by co-cultivating cells that express Env with human cells that express CD4, thus resulting in formation of syncytia[3]. Murine cells expressing human CD4 fail to support this fusion. Expression of fusin renders murine cells fusogenic for cells expressing T-tropic, but not macrophage-tropic, Env[11]. To test whether CC-CKR-5 would support fusion with cells expressing macrophage-tropic Env, we transfected 293T cells with different Env expression vectors and co-cultivated them overnight with cell lines stably expressing transfected CD4 and CC-CKR-5 genes. As shown in Fig. 4, 293 T cells expressing JRFL Env formed large syncytia with murine 3T3.CD4 cells expressing CC-CKR-5, but not with cells expressing fusin. Conversely, 293T cells expressing HXB2 Env were found to fuse to cells expressing fusin, but not to cells expressing CC-CKR-5. Similar results were obtained with U87MG.CD4 cells transfected with either fusin or CC-CKR-5 (not shown). Thus macrophage-tropic Env-mediated fusion occurs in a manner that is highly specific for the entry cofactor.

Replication of macrophage-tropic virus

To test whether CC-CKR-5 expression allows for full replication and spread of macrophage-tropic virus, we infected HOS.CD4 cells expressing CC-CKR-5 and control cells (HOS.CD4-BABE, transduced with the puromycin-resistance vector alone) with the replication-competent reporter viruses HIV–HSA (HSA, heat-stable antigen)[32] and HIV(BaL)–HSA. These viruses contain the gene for HSA (CD24) in place of *nef*, allowing for quantification of the infected cells by fluorescence-activated cell sorting (FACS) after staining with anti-HSA monoclonal antibody[32]. Both viruses are based on the T-cell-line adapted virus NL4-3, but the latter contains the BaL macrophage-tropic Env. Both replicated in PM1 cells (Fig. 5*a*), but HIV(BaL)–HSA fails to replicate in T-cell lines, such as CEMX174, and in HOS.CD4 (data not shown).

The HOS.CD4-BABE cells remained uninfected with both viruses six days after infection, but nearly all of the HOS.CD4-CKR5 cells were infected with HIV(BaL)–HSA (Fig. 5*a*, bottom panel). Sampling of the HIV(BaL)–HSA-infected cultures over a period of several days indicated that an increasing percentage of the cells became infected over time, confirming the ability of the virus to spread in the culture (Fig. 5*b*). HIV–HSA failed to replicate in the HOS.CD4-CKR5 cultures, consistent with the restriction of this T-tropic virus to using fusin, which is expressed at very low levels in these cells (data not shown). Expression of CC-CKR-5 in 3T3.CD4 cells also led to HIV(BaL)–HSA virus replication, but this was rather limited, presumably owing to inefficient viral gene expression in murine cells (data not shown).

Expression of CC-CKR-5

The initial description of the CC-CKR-5 gene suggested that its expression is limited to granulocyte precursors, and absent in peripheral blood mononuclear cells (PBMCs)[23]. To be a major co-receptor *in vivo*, however, this molecule should be expressed in T cells and monocyte/macrophages, the predominant cell-types targeted by the virus. Northern blot analysis with CC-CKR-5 cDNA as probe does not readily distinguish between CC-CKR-5

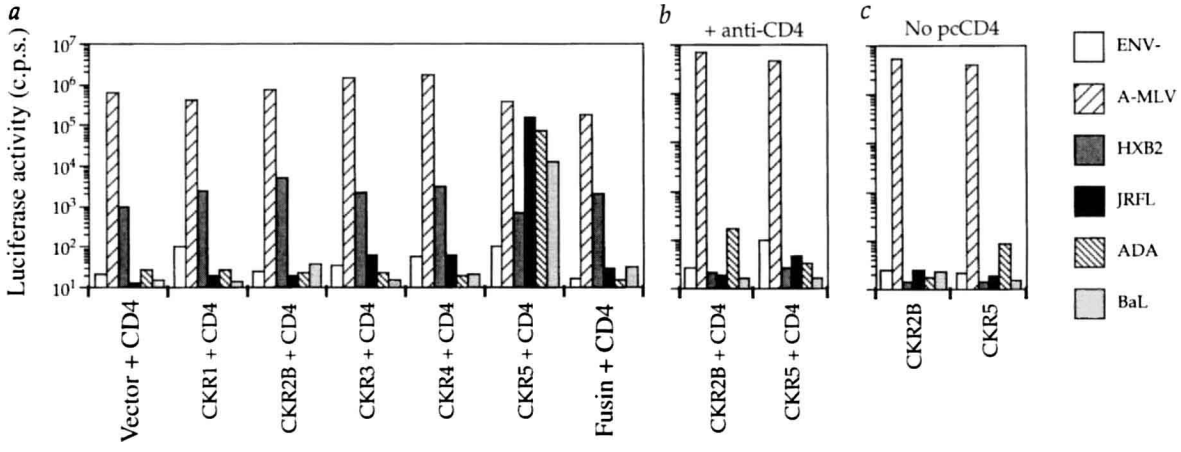

FIG. 2 CC-CKR-5 mediates entry of macrophage-tropic HIV-1. cDNAs encoding CC-CKR-1, CC-CKR-2B, CC-CKR-3, CC-CKR-4 and CC-CKR-5 were amplified from activated PBMC RNA using primers hybridizing to the respective 5′ and 3′ untranslated regions. Amplified products were cloned into pcDNA-I/Amp and pBABE-puro[40] expression vectors. Each of the cDNAs was sequenced and determined to correspond to that previously reported. *a*, Transfection of 293T cells with 5 μg CD4 expression vector pcCD4[41] and 15 μg pcDNA-I expression vectors for each of the CC-CKR genes. The next day the cells were plated in 24-well dishes (2×10^4 per well), and one day later were infected with luciferase reporter viruses (20 ng p24) in a volume of 300 μl. Luciferase activity was measured 4 days later, as described above. *b*, As *a*, with addition of anti-CD4 monoclonal antibody Leu3a (20 μg ml^{-1}) 30 min before adding virus. *c*, As *a*, except that pcCD4 was omitted from the transfection and replaced by pcDNA-I control vector DNA.

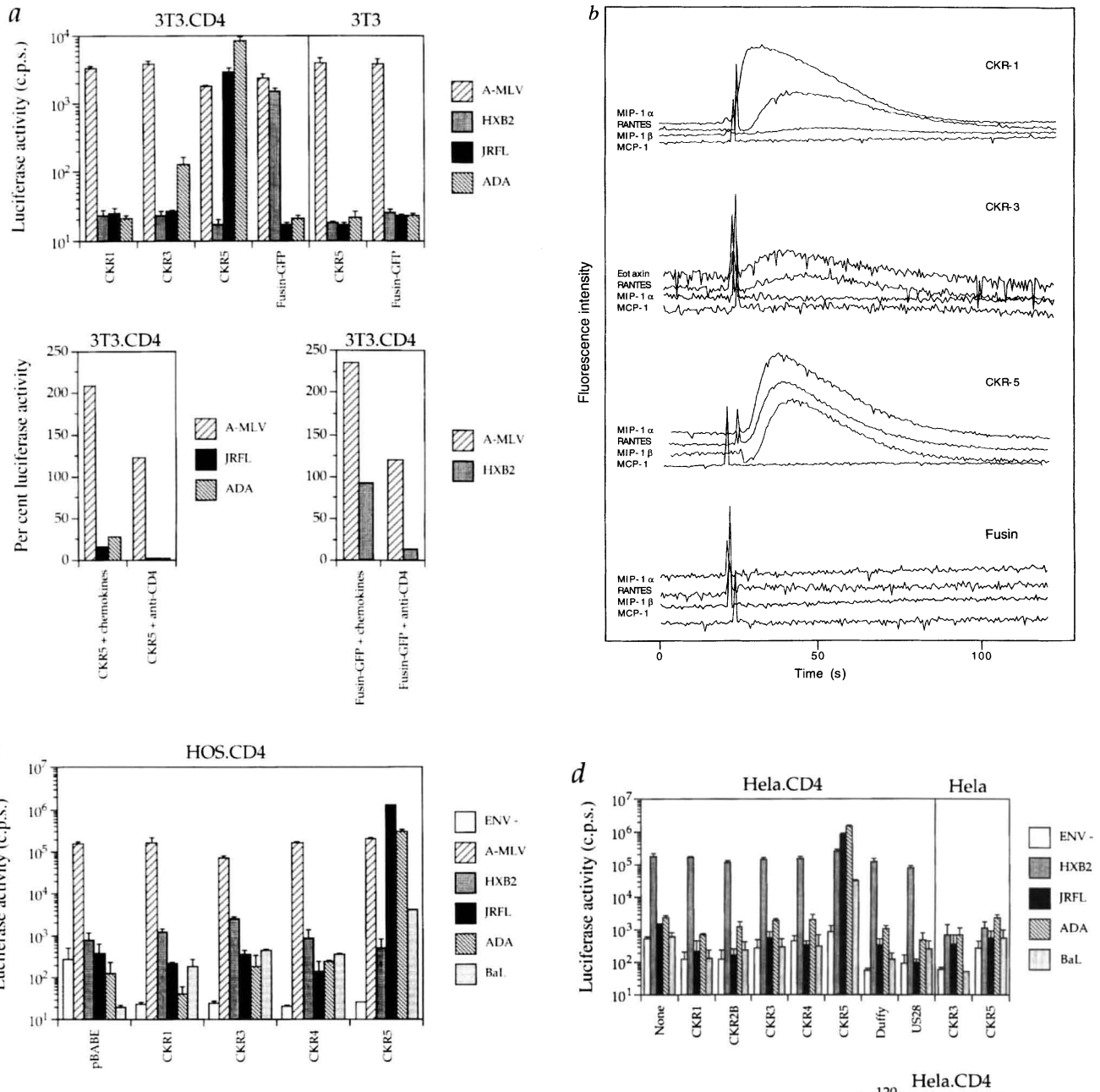

FIG. 3 Stable expression of CC-CKR-5 confers susceptibility to HIV-1 entry that can be inhibited by anti-CD4 monoclonal antibody or chemokines. Cell lines stably expressing β-chemokine receptors were infected with pseudotyped luciferase reporter viruses. *a*, Infection of NIH3T3.CD4 cells (murine fibroblast) expressing different β-chemokine receptors or fusin–GFP, a fusion protein in which green fluorescent protein (GFP) has been attached to the C terminus of fusin (D.U. *et al.*, unpublished). In the lower panels, β-chemokine and anti-CD4 blocking are expressed as a percentage of luciferase activity in the presence of blocking agent as compared to untreated controls. *b*, Comparison of cytoplasmic Ca^{2+} levels in 3T3.CD4-CKR stable transfectants expressing CC-CKR-1, CC-CKR-3, CC-CKR-5 or fusin, upon challenge with the listed β-chemokines, which were added through an injection port after ~20 s (the sharp spike in each record) to a final concentration of 100 nM. The rise in $[Ca^{2+}]_i$ is represented by the rapid increase in relative fluorescence. *c*, Infection of HOS.CD4 cells (human osteosarcoma). *d*, Infection of HeLa.CD4 (human carcinoma); US28 (ref. 42) is a β-chemokine receptor encoded by human cytomegalovirus. Duffy antigen is a promiscuous chemokine receptor expressed primarily on erythroid cells[24].

METHODS. Cell lines stably expressing chemokine receptors or fusin–GFP were established as described[43,44]. Briefly, cDNAs encoding the indicated receptors were subcloned into pBABE-puro[40]. Amphotropic virus stocks were prepared by transfecting BING packaging cells[44] with the resulting plasmids or by a previous method[43,44], with the substitution of 293T cells for COS cells. Supernatants were collected 48 h later and used to infect NIH3T3 (3T3), 3T3.CD4, HOS, HOS.CD4, HeLa, and HeLa.CD4 cells. After another 48 h, cells were selected in medium containing 1 μg ml^{-1} puromycin. One week later, puromycin-resistant populations were tested for infectability by pseudotyped luciferase reporter virus (100 ng p24 per infection). For antibody blocking experiments, cells were preincubated with anti-CD4 monoclonal antibody Leu3a (5 μg ml^{-1}, Becton Dickinson) at 10 μg ml^{-1} for 1 h before infection. For chemokine blocking experiments, cells were preincubated with a mixture of MIP-1α, MIP-1β and RANTES (1 μg ml^{-1}). After 30 min, an equal volume of reporter virus was added without additional chemokines, and luciferase activity was measured 2 days later. For calcium mobilization assays, cells were loaded with the calcium indicator indo-1/AM (2 mM) in complete growth medium at 20 °C for 45 min. Cells were washed, resuspended in Na-HBSS (in mM: 2 $CaCl_2$, 145 NaCl, 5 KCl, 1 $MgCl_2$, 5 D-glucose, 20 HEPES, pH 7.3) containing 1% BSA, at 20 °C for up to 2 h. Fluorescence measurements to determine $[Ca^{2+}]_i$ used a spectrofluorimeter (Photon Technologies) with ~ 5 × 10^5 cells suspended in 2 ml Na-HBSS, at 37 °C in a constantly stirred acrylic cuvette. The excitation wavelength was 350 nm (4 nm bandwidth) and dual simultaneous monitoring of emission was at 405 and 485 nm (10 nm bandwidth). The ratio of emission at 405/485 nm was measured at a rate of 2 Hz.

and the closely related CC-CKR-2 transcripts, so we performed reverse-transcriptase polymerase chain reaction (RT–PCR) on isolated subsets from PBMCs. CC-CKR-5 transcripts were detected in both the monocyte/macrophage and macrophage-depleted T-cell fractions (Fig. 6*a*). In addition, we found that PM1 and HUT78 cells both expressed the gene. Significantly more CC-CKR-5 transcript was detected in PM1 cells, consistent with the higher infectivity of these cells by macrophage-tropic and primary HIV-1 isolates[26].

Discussion

Here we have shown that CC-CKR-5 acts as a potent coreceptor that, together with CD4, allows entry of macrophage-tropic HIV-1 into cells. Both CD4 and CC-CKR-5 are required, just as CD4 and fusin are required for the entry of T-cell-line adapted virus[11]. Co-receptor usage appears to be highly sequence specific. Other β-chemokine receptor family members tested showed no detectable co-receptor activity, including the closely related CC-CKR-2, which is 76% identical to CC-CKR-5 (ref. 23).

Several lines of evidence suggest that CC-CKR-5 serves as a major co-receptor for primary macrophage-tropic strains of HIV-1 *in vivo*. The three independently derived macrophage-tropic Envs used in our study were specific for CC-CKR-5. These Envs were derived from virus after limited growth in PBMCs and are therefore likely to maintain co-receptor usage similar to that of primary virus. We also found that a variety of human and murine cells transfected with human CD4 and CC-CKR-5 expression vectors became highly prone to infection by macrophage-tropic virus. Thus these proteins are likely to function as co-receptors in cells in which they are coexpressed *in vivo*. Finally, the importance of macrophage-tropic virus in HIV-1 transmission[33] and the demonstration of high levels of β-chemokines in T-cell cultures of individuals resistant to infection[14] further imply a key role for CC-CKR-5 *in vivo*.

Although our data support a central role for CC-CKR-5 *in vivo*, we cannot rule out the possible significance of other potential co-receptors. CC-CKR-5 seemed to be expressed in T lymphocytes and macrophages, but it remains possible that these cells express a currently unidentified co-receptor. Furthermore, although the macrophage-tropic Envs that we tested used only CC-CKR-5, infected individuals could harbour viruses specific for other members of the β-chemokine receptor family. Resolution of these issues will await definitive demonstration that virus entry into macrophages and T cells can be prevented by treatment with CC-CKR-5-specific antibodies.

The identification of CC-CKR-5 as a macrophage-tropic virus co-receptor, together with the recent identification of fusin as a co-receptor for T-tropic viruses[11], suggests a resolution for the long-standing puzzle of Env-related differences in HIV-1 tropism (as shown in Fig. 6*b*). Thus adaptation of primary HIV-1 isolates for growth in transformed T-cell lines may be the result of selection for Env sequences that use fusin rather than CC-CKR-5, presumably owing to loss of expression of the latter in transformed cells. *In vivo*, the phenotypic switch from macrophage-tropic, non-syncytium-inducing (NSI) to T-tropic, syncytium-inducing (SI) viruses that occurs in many infected individuals before an increase in the severity of the disease[34,35] would reflect a change in predominant co-receptor usage form CC-CKR-5 to fusin. Although the driving force for this change in receptor usage has yet to be determined, it could be the result of selection for viruses that can replicate in the presence of high levels of β-chemokine, or that can infect a wider variety of cell types. With the availability of cell lines expressing the co-receptors it will now be possible to evaluate the receptor usage of viruses sampled at different stages of HIV disease progression.

The V3 loop of gp120 clearly plays a central role in determining viral tropism. Alteration of only a few amino acids of the loop alters the ability of the virus to infect macrophages or transformed T-cell lines[29,36,37]. Presumably, the V3 loop plays a critical role in determining which co-receptor is used. These findings lead us to

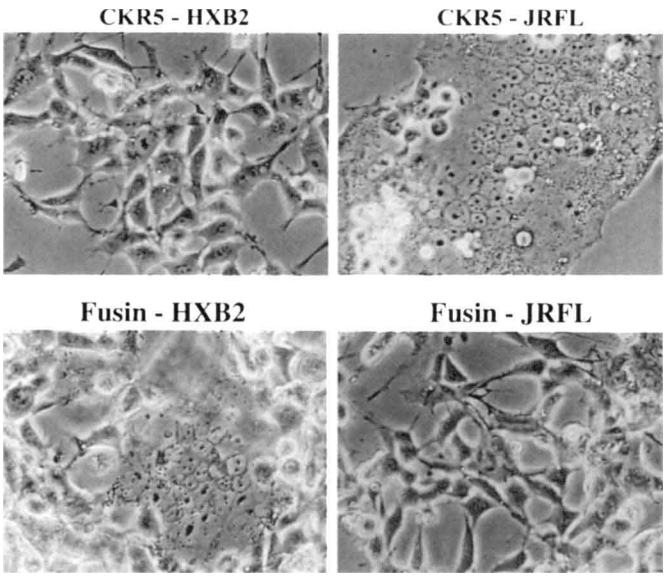

FIG. 4 CC-CKR-5 mediates Env-dependent fusion. We transfected 293T cells with equal amounts of pcDNA-I-based Env and Rev expression vectors. After 2 days the transfected cells (1.5×10^5) were seeded with 3T3.CD4-CKR5 or 3T3.CD4-fusin (3.0×10^5) cells. The next day the cells were stained with Giemsa stain and photographed.

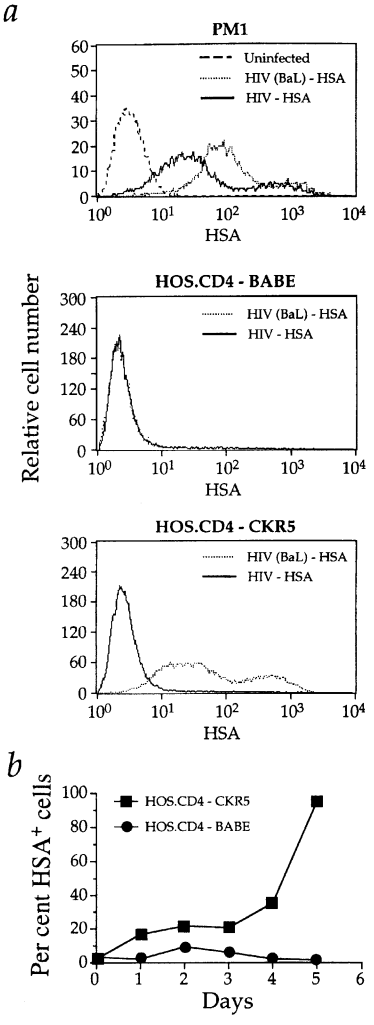

FIG. 5 CC-CKR-5 supports macrophage-tropic, but not T-cell-line adapted, virus replication in human cells. *a*, PM1, HOS-CD4-BABE and HOS-CD4-CKR-5 cells (5×10^5) were plated in 6-well dishes, and the next day infected with replication competent T-cell-line adapted HIV–HSA[32] or macrophage-tropic HIV(BaL)–HSA reporter viruses (50 ng p24). HIV–HSA is based on the T-cell-line adapted virus pNL4-3, but contains, in place of *nef*, the gene encoding the small cell-surface protein, HSA (or CD24). HIV(BaL)-HSA virus is similar except that its *env* gene has been replaced by the *SalI–BamHI* restriction fragment containing the macrophage-tropic Env of BaL. HIV(BaL)–HSA replicates in PM1 cells but not in CEM cells, while HIV–HSA replicates in both cell types (data not shown). Both viruses show a characteristic bimodal distribution of HSA-staining cells. This is likely to reflect whether the cells are in the early or late phase of the replication cycle. After 6 days the cells were stained with fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-conjugated anti-HSA monoclonal antibody (Pharmingen) and analysed in a Becton-Dickinson FACS-caliber. Both viruses failed to replicate in HOS.CD4-BABE cells, resulting in superimposed curves. *b*, Time course of HIV(BaL)–HSA virus replicating in HOS.CD4-CKR5 cells. Cells were infected with HIV(BaL)–HSA and analysed by FACS on the indicated days.

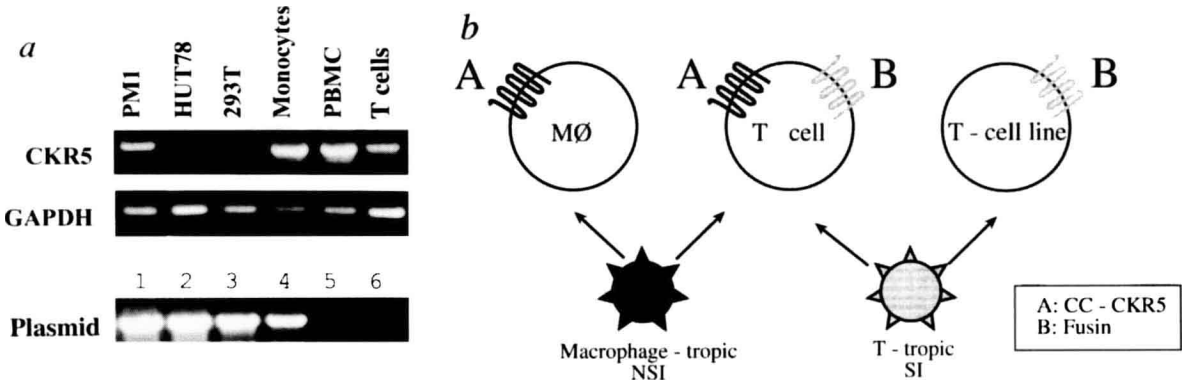

FIG. 6 CC-CKR-5 is expressed in T cells and monocyte/macrophages. *a*, Total RNA was prepared from the indicated cell-types using Triazol reagent (Gibco/BRL), treated with RNase-free DNase (Bochringer-Mannheim), and used in RT–PCR reactions. First-strand cDNA was primed with oligo-dT using Superscript reverse transcriptase (Gibco/BRL), and products were amplified with primers hybridizing to the 5′ and 3′ untranslated regions of CC-CKR-5 (upstream CTCGGATCCGGTGGAACAAGATGGATTAT; downstream CTCGTCGACATGTGCACAACTCTGACTG) or to glyceraldehyde-3-phosphate dehydrogenase using a *Taq/Pwo* polymerase mixture (Bochringer Mannheim). To control for the presence of genomic DNA, control cDNA reactions in which reverse transcriptase was omitted were prepared in parallel. These were uniformly negative (data not shown). To test the linearity of amplification, a 10-fold dilution series (lanes 1–5), starting at 1 pg of pcCKR5 plasmid DNA, was amplified under conditions identical to those above. In lane 6, no DNA was added. Monocytes were prepared by overnight adherence to plastic. T cells were prepared from the monocyte-depleted preparation by adherence to anti-CD2-coated beads (Dynal). *b*, Model for HIV-1 co-receptor usage. Macrophage-tropic viruses (dark shading), analogous to the NSI viruses that predominate early in infection, are proposed to use CC-CKR-5, which is expressed in macrophages and primary T cells. T-tropic viruses (light shading), analogous to SI viruses, use fusin, which is presumed to be expressed on primary T cells and transformed T-cell lines.

speculate that CD4 binding induces a conformational change in Env that exposes a co-receptor binding domain. This interaction site could either contain the V3 loop, or be conformationally influenced by it. In support of this model, increased exposure of the V3 loop upon CD4 binding to Env has been reported[38]. A successful interaction with the co-receptor would trigger a conformational change in gp41, releasing its N-terminal hydrophobic peptide to initiate membrane fusion. Such a mechanism has a precedent in the low-pH-mediated activation of influenza haemagglutinin[39].

The identification of co-receptors required for HIV-1 entry raises several questions. The mechanism of chemokine blocking remains unclear: it may involve binding-site competition or desensitization of the receptor through downregulation or conformational changes. The inefficient chemokine blocking that we observed with several cell lines suggests that competition for a binding site on the receptor may not be sufficient. In addition, it will be of interest to determine whether polymorphic differences in human *CC-CKR-5* alleles influence the susceptibility of individuals to HIV-1 transmission. It will also be important to determine whether signalling through the co-receptor is important for HIV-1 entry, for subsequent events in viral replication, or for elimination of T-helper cells. Transgenic mice that express human CD4 and the co-receptors may provide a means of addressing these questions. □

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