Fusion of monocytes and macrophages with HIV-1 correlates with biochemical properties of CXCR4 and CCR5

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Human macrophages can be infected more efficiently by M-tropic than by T-tropic HIV-1 strains, despite surface expression of both CXCR4 and CCR5 co-receptors. Western blot analyses of total cell extracts and surface proteins from multiple sets of monocytes and macrophages demonstrated substantial differences between CXCR4 molecules. CXCR4 was mainly a monomer in monocytes, but was mainly a species of higher molecular weight (90 kDa) on the surface of macrophages. CCR5 was monomeric in both cell types. A constitutive association between CD4 and the co-receptors was seen in monocytes and macrophages. However, CD4 co-precipitated with CCR5 and CXCR4 monomers, but not with the high-molecular-weight forms of CXCR4, indicating that the high-molecular-weight CXCR4 species in macrophages are not available for association with CD4, which may contribute to the inefficient entry of T-tropic strains into mature macrophages.

Macrophages are important in HIV transmission and propagation of viral infection. Macrophages can be found in the lamina propria adjacent to the mucosal epithelium, and in cases of genital ulceration or tears in the mucosal epithelium, macrophages may have direct contact with HIV. HIV-1 can be found within macrophages in the female reproductive tract[1–4]. In addition, macrophages may contribute to the development of neurologic and pulmonary complications in HIV-1 infection[5–7]. With the discovery that the chemokine receptors CXCR4 and CCR5 provide co-receptor functions for T-tropic and M-tropic HIV-1 strains, respectively[8–13], it was anticipated that monocytes (Mo) and monocyte-derived macrophages (MDM) would express CCR5 but not CXCR4, explaining the differential susceptibility of MDM to infection with M-tropic strains[14–15]. However, CXCR4 was initially cloned from a cDNA library derived from primary human macrophages[16], and SDF-1, the recently identified ligand for CXCR4, induces calcium (Ca^{2+}) mobilization in human monocytes[17,18] (Fig. 1). Freshly isolated monocytes fuse with several CXCR4 envelopes as well as CCR5 envelopes. In contrast, MDM retain their CCR5 envelope fusion but fuse poorly with CXCR4 envelopes[19]. Here we show that the biochemical properties of CXCR4 molecules differ in Mo and MDM. Furthermore, CCR5 molecules in both Mo and MDM and CXCR4 in Mo (but much less in MDM) are constitutively associated with CD4, the principal HIV-1 receptor.

CXCR4/CCR5 function in Mo and MDM

Studies of elutriated monocytes from more than 30 donors and the macrophages derived from them demonstrated that both Mo and MDM express CD4, CXCR4 and CCR5, although the levels of all three receptors varied considerably among individuals. Use of either polyclonal rabbit IgGs specific for co-receptors or murine monoclonal antibodies 12G5 and 2D7 demonstrated either increases, decreases or no changes in the densities of CXCR4 and CCR5 on differentiated MDM compared with their densities on the Mo from which they were derived. The average change in mean fluorescence channel (ΔMFC) in seven FACS experiments showed a modest decrease in CXCR4 densities and a modest increase in CCR5 densities on MDM compared with their densities on Mo (Table 1). In addition, surface CD4 density was usually reduced on MDM (Table 1). These findings are in general agreement with previous reports[19–21]. Both Mo and MDM mobilized Ca^{2+} in response to SDF-1α and MIP-β (CXCR4 and CCR5 ligands, respectively), but the magnitude of the Ca^{2+} flux was always lower in MDM (Fig. 1*a*). However, there were considerable differences between Mo and MDM in a fusion assay. MDM fused efficiently with cells expressing M-tropic envelopes and only minimally with T-tropic envelopes (in agreement with another study[22]), whereas Mo formed similar numbers of syncytia with either M-tropic or T-tropic envelopes (Table 1; ref 19). The fusion of Mo with the T-tropic envelopes was CXCR4-dependent and could be blocked by SDF-1α and by our polyclonal rabbit IgG against CXCR4 (data not shown). Entry of T-tropic, CXCR4-dependent viruses into MDM has been shown[23,24]. However, in earlier re-

Table 1 Elutriated monocytes, but not differentiated macrophages, fuse with cells expressing T-tropic envelope

Cells	Surface expression (mean ΔMFC)[a]			Number of syncytia[b]	
	CD4	CXCR4	CCR5	IIIB	JR-FL
Monocytes	140 ± 28	180 ± 65	135 ± 62	132 ± 6	173 ± 8
Macrophages	66 ± 24	113 ± 38	148 ± 44	5 ± 5	192 ± 45

[a]Delta mean fluorescence channels (ΔMFC) were calculated by subtracting control MFCs from experimental values. Data are represented as mean ± s.d. ΔMFC of seven separate experiments. [b]Data represent mean ± s.d. of three cultures. IIIB, recombinant vaccinia virus expressing T-tropic envelope; JR-FL, expressing M-tropic envelope.

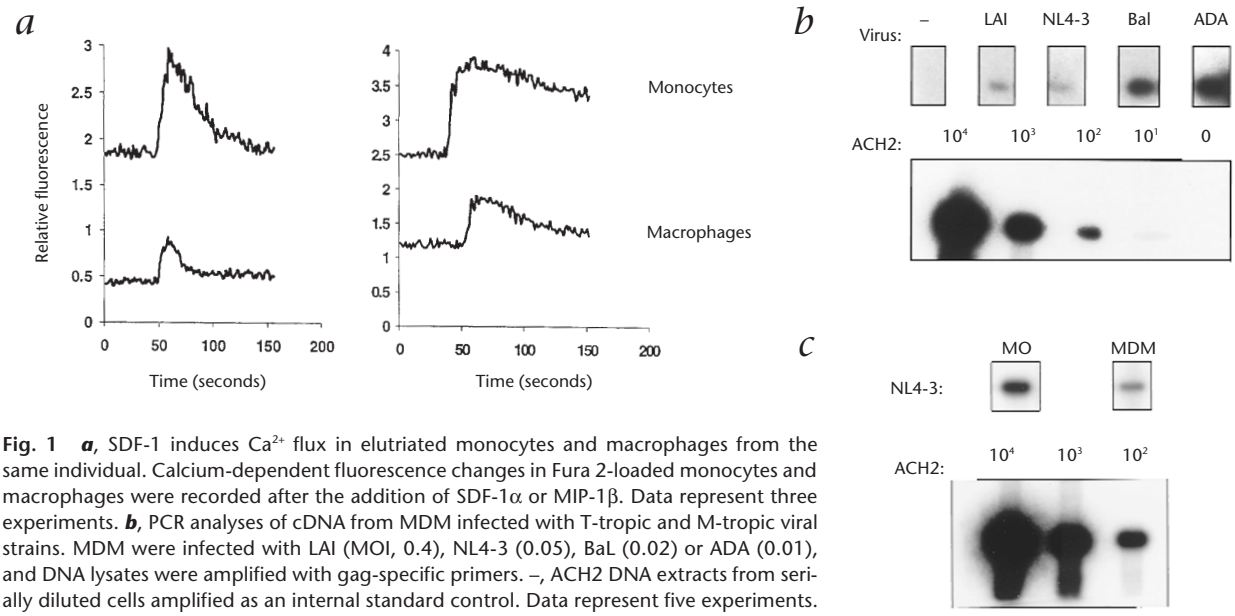


Fig. 1 ***a***, SDF-1 induces Ca^{2+} flux in elutriated monocytes and macrophages from the same individual. Calcium-dependent fluorescence changes in Fura 2-loaded monocytes and macrophages were recorded after the addition of SDF-1α or MIP-1β. Data represent three experiments. ***b***, PCR analyses of cDNA from MDM infected with T-tropic and M-tropic viral strains. MDM were infected with LAI (MOI, 0.4), NL4-3 (0.05), BaL (0.02) or ADA (0.01), and DNA lysates were amplified with gag-specific primers. –, ACH2 DNA extracts from serially diluted cells amplified as an internal standard control. Data represent five experiments. ***c***, PCR analyses of Mo and MDM (from the same individual) infected with the CXCR4 strain NL4-3 (MOI, 0.05); DNA lysates were amplified with gag-specific primers. ACH2 DNA extracts from serially diluted cells were amplified as an internal standard control.

ports, MDM were shown to be relatively resistant to infection with T-tropic strains[14,15,25]. The difference in findings may reflect differences in viral strains used, their MOIs and the assays used for detection. We also did multiple infectivity experiments with Mo and MDM and used PCR to detect viral cDNA 24–48 hours after cell entry. Even though the MOIs used for T-tropic strains (LAI and NL4-3) were 200–1,000% higher than those used for M-tropic strains (BaL and ADA), the signals detected after infection with the M-tropic strains were 500–1,000% higher (Fig. 1*b*). These data support the conclusion that entry of T-tropic strains into MDM does occur, but at an efficiency much lower than that of M-tropic strains. We also compared the entry of NL4-3 into Mo and MDM and found viral entry into MDM was much less than viral entry into Mo (Fig. 1*c*). Although on average the surface CXCR4 density was reduced by 25–30% on MDM compared with that on Mo, in several Mo/MDM pairs there was no reduction in CXCR4 expression, yet there was a substantial loss of CXCR4–virus fusion/cell entry. Therefore, post-translational modifications and/or other cellular factors may contribute to the functional differences between CXCR4 molecules in Mo and MDM, and possibly those between CXCR4 and CCR5 in MDM.

CXCR4 species of different sizes predominate in Mo and MDM

Total cell extracts from Mo and MDM were analyzed by western blot. CXCR4 molecules were detected with rabbit IgG against CXCR4 specific for either the N terminus (Fig. 2*a*) or the second extracellular loop (EC2) (Fig. 2*b*). Binding of these polyclonal IgGs was specific and could be blocked by the relevant peptides, but not with peptides from the corresponding regions in CCR5 (Fig. 2*a* and *b*). The CXCR4 molecules were considerably different in Mo and MDM. The size of the CXCR4 molecules in Mo was consistent, with a 62-kDa monomer (Fig. 2*a*, lanes 1 and 5) of higher molecular weight than the monomeric CXCR4 identified in HeLa cells and T-cell lines[8,26]. This difference in molecular weight is a property of Mo, as infection of this cell type with vaccinia-CXCR4 recombinant (vCBFY1; ref. 8) increased CXCR4 levels (200–300%), but only the 62-kDa monomer was seen in western blots (not shown). In addition, immunoprecipitation and immunoblots using commercially available rabbit antibodies against CXCR4 also resulted in precipitation of a 62-kDa CXCR4 species from Mo extracts (data not shown). Several differences were seen in CXCR4 western blots of MDM extracts compared with those of Mo extracts. CXCR4 monomers appeared as species of slightly lower molecular weight than the monomeric species in Mo (59 kDa and 62 kDa, respectively). Moreover, more than 50% of the CXCR4 molecules in MDM appeared as a species of higher molecular weight (90 kDa). The ratio of the high-molecular weight:low-molecular-weight species in MDM cell extracts ranged between 1:1 and 3:1 in different donors. Finally, our rabbit IgG against CXCR4/EC2 reacted in western blots only with the high-molecular-weight species in MDM, and did not react at all with CXCR4 molecules in Mo cell extracts (Fig. 2*b*), indicating that some EC2 epitopes may be either sequestered or conformationally different in the low- and high-molecular-weight species of CXCR4.

To further correlate the loss of CXCR4–envelope fusion in MDM with the appearance of the high-molecular-weight CXCR4 species, we did kinetics experiments with elutriated monocytes undergoing differentiation into MDM. The high-molecular-weight CXCR4 species appeared after 1–2 days of culture, and its density increased over the next 3 days. There was an inverse relationship between the appearance of the high-molecular-weight species and the number of syncytia formed with T-tropic envelopes (Fig. 2*c*). Surface CD4 expression did not correlate with fusion. It diminished on day 1 of culture, but then returned to its original surface density in this experiment.

Because CXCR4 may be found either on the cell surface or in intracellular stores[19], we determined which CXCR4 species are present on the surface of Mo and MDM. Extracts from surface-biotinylated Mo and MDM were precipitated with strepta-

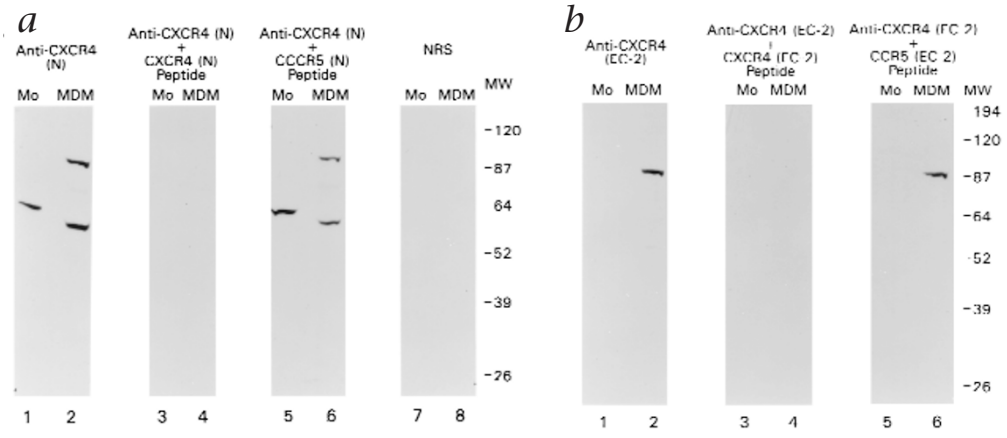
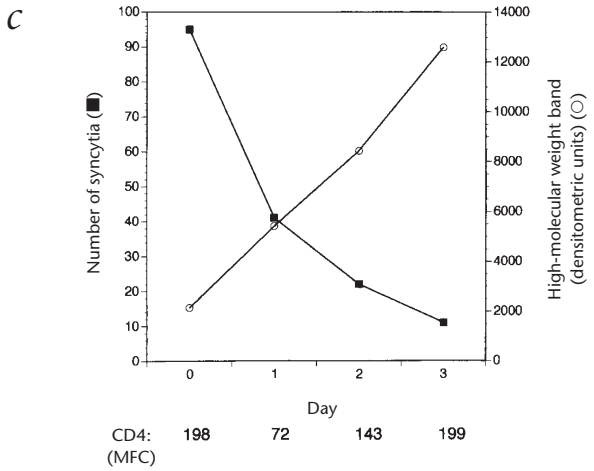


Fig. 2 Different forms of CXCR4 molecules are present in monocytes and macrophages. a and b, Total cell extracts of Mo and MDM were analyzed by western blot. MW (right margins), positions of molecular weight standards. ***a***, Rabbit IgG against CXCR 4 (specific for the N terminus) was incubated with no peptide (lanes 1 and 2) or with peptides corresponding to the N termini of CXCR4 (lanes 3 and 4) or CCR5 (lanes 5 and 6). Control blots (NRS) were reacted with pre-immune rabbit IgG (lanes 7 and 8). ***b***, Rabbit IgG against CXCR4/EC2 was preincubated with no peptide (lanes 1 and 2) or with EC2 peptides from CXCR4 (lane 3 and 4) or CCR5 (lanes 5 and 6). Data represent eight experiments. ***c***, Kinetics of cell fusion with IIIB envelope (left vertical axis) and the appearance of high-molecular-weight CXCR4 molecules (right vertical axis) and surface CD4 expression (horizontal axis) in elutriated Mo undergoing differentiation into MDM. Day-5 MDM gave results similar to those of day-3 MDM. Data represent three experiments. MFC, mean fluorescent channel.



vidin–Sepharose beads, and the precipitates were analyzed by SDS–PAGE and western blot. Use of rabbit IgG against CXCR4 (N terminus) demonstrated that the predominant forms of CXCR4 on the surface of MDM were a species of higher molecular weight (90 kDa), whereas mostly monomeric CXCR4 molecules were precipitated from Mo membranes (Fig. 3*a*). To further determine if CXCR4 exists in a form with higher molecular weight on the surface of MDM we mixed lysates from Mo and MDM (or intact cells before lysis) at a ratio of 1:1. There was no increase in the intensity of the high-molecular-weight species, demonstrating that the Mo monomer was not simply aggregating in the presence of cell lysates of MDM. In addition, as MDM are much larger than Mo, the resulting cell lysates have a higher protein concentration (data not shown). This MDM were lysed at several different cell concentrations. The band of higher molecular weight remained prevalent as long as we were able to detect CXCR4 in the immunoblots, indicating that the high-molecular-weight species are not simply the products of aggregation due to high protein concentration. We also stained cells with the rabbit IgG against EC2 that only recognizes the band of higher molecular weight in immunoblots. In FACS analyses, the rabbit IgG specific for the N terminus and the monoclonal antibody 12G5 stained Mo and MDM in a similar manner (Table 2). The rabbit IgG specific for EC2 was less efficient in surface staining, but in agreement with the western blots, it stained MDM but not Mo (Table 2). These data support the conclusion that the high-molecular-weight CXCR4 species are present on the surface of MDM and are not simply a post-cell-lysis phenomenon. The high-molecular-weight band of CXCR4 does not change in reducing conditions in the presence of urea. Thus, this form may represent covalent post-translational modifications or tight noncovalent protein–protein associations. This band does not react with polyclonal reagents against CD4 (data not shown). In the same experiments, membrane extracts were also reacted with rabbit IgGs against CCR5. CCR5 molecules appeared only as monomers in both Mo and MDM (Fig. 3*a*, lanes 3 and 4).

Table 2 Surface staining of monocytes and macrophages from the same donor

Cells	CXCR4 surface expression (ΔMFC)		
	12G5	RαNterminus	RαEC2
Monocytes	96	70	0
Macrophages	65	62	30

12G5, monoclonal antibody specific for murine CXCR4; RαN terminus, rabbit IgG specific for the N terminus of CXCR4; RαEC2, rabbit IgG specific for EC2 of CXCR4. ΔMFC was calculated by subtracting control MFCs from experimental values.

Co-precipitation of CXCR4 and CCR5 with CD4

A close association forms between CD4 and CXCR4 molecules on the surface of human CD4⁺ cell lines after treatment of the intact cells with soluble gp120 at 37 °C. In the promonocytic line U937, we consistently observed low levels of co-precipitation of CXCR4 and CD4 in the absence of gp120 treatment, which was substantially enhanced in cells treated with soluble gp120 (ref. 26). Because our data and those of others[27–29] indicated that the formation of trimolecular complexes is an essential step in the fusion process, we determined if a similar association between CD4 and CXCR4 occurs in Mo and MDM, and whether both low- and high-molecular-weight CXCR4 species can associate with CD4. CD4 molecules in Mo and MDM are constitutively associated with CXCR4, as demonstrated by their co-precipitation with a monoclonal antibody against CD4 (OKT4) from cells not treated with gp120 (Fig. 4*a*). In all experiments, the CD4 was immunoprecipitated to completion. In co-precipitation experiments done in parallel on

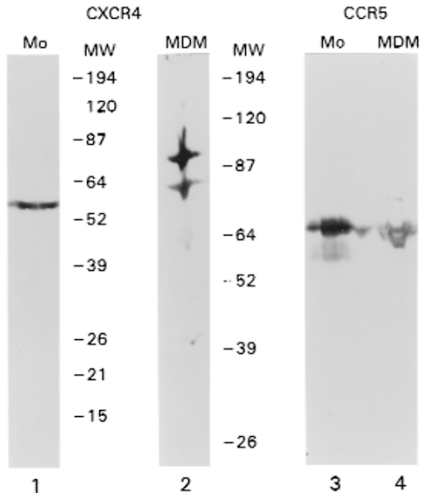


Fig. 3 CXCR4 and CCR5 on the surface of monocytes and macrophages. Mo and MDM were surface-biotinylated, lysed, precipitated with avidin-Sepharose beads, and analyzed by western blot with rabbit IgGs against CXCR4 (lanes 1 and 2) or CCR5 (lanes 3 and 4). No signal was detected in blots reacted with pre-immune rabbit IgG (not shown). MW (right margin), positions of molecular weight standards. Data represent four experiments.

Mo and MDM (from the same donors), over 300% more CXCR4 was co-precipitated with CD4 from Mo than from MDM (CXCR4:CD4 ratios determined by densitometry; Fig. 4*a*, lanes 1 and 2). Unexpectedly, treatment with gp120 induced either minimal or no increase in the amount of CXCR4 co-precipitated with CD4, whereas in the same experiment, gp120 did induce a substantial increase in the amount of CXCR4/CD4 co-precipitation in U937 cells (data not shown). In the same pairs of Mo/MDM, CCR5 also co-precipitated with CD4, and unlike CXCR4, similar ratios of CCR5/CD4 molecules were co-precipitated from Mo and MDM (Fig. 4*a*, lanes 3 and 4). Only the monomeric forms of CXCR4 and CCR5 were co-precipitated with CD4 (Fig. 4*a*). Thus, the failure of the high-molecular-weight CXCR4 in MDM to co-precipitate with CD4 indicates that they cannot associate efficiently with CD4. Based on these findings, one possible explanation for the reduced fusion of MDM with T-tropic envelopes and reduced infectability with T-tropic strains may be the lower numbers of pre-existing CD4/CXCR4 complexes in MDM.

CXCR4/CD4 co-precipitation in MDM infected with vaccinia-CD4

Because the density of CD4/CXCR4 complexes may be determined by the availability of both monomeric CXCR4 molecules and CD4, we infected MDM with a recombinant vaccinia virus (vCB3) expressing human CD4. The infected MDM expressed much higher levels of CD4 protein (Fig. 4*b*). There was no difference in the ratio of high-molecular-weight:low-molecular-weight CXCR4 species in the infected MDM (data not shown). However, a small increase in the amount of CXCR4 co-precipitating with CD4 was observed (Fig. 4*b*). This increase in CD4/CXCR4 co-precipitation correlated with a substantial increase in the ability of the MDM to fuse with cells expressing T-tropic envelope (Fig. 4*c*), in agreement with another study[22] . The syncytia formed were CXCR4-dependent, as they could be blocked with SDF-1 (but not with RANTES, which binds to CCR5), and with rabbit IgG against CXCR4 (but not against CCR5) (Fig. 4*c*). The MDM infected with vaccinia-CD4 also showed increased fusion with M-tropic (JR-FL and Ba-L) and dual-tropic (89.6) envelopes (data not shown).

Discussion

Our findings provide evidence for considerable differences in the biochemical properties of CXCR4 molecules in Mo and MDM that correlate with and may contribute to the reduced ability of CXCR4 in MDM to support entry of T-tropic HIV-1 strains. Additional cellular factors may limit the post-entry replication of CXCR4 strains in Mo/MDM, as suggested[23]. The monomeric form of CXCR4 in Mo/MDM is of a substantially higher molecular weight than that reported for cells transfected with co-receptor and continuous human cell lines. However, a commercially available antibody against CXCR4 that recognizes a 50-kDa pro-

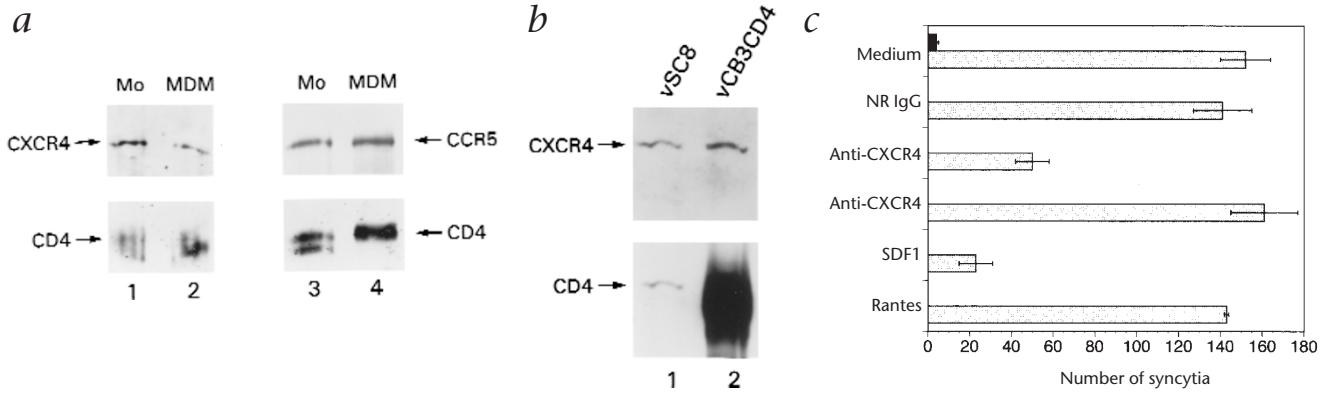


Fig. 4 Co-precipitation of CXCR4 and CCR5 with CD4 from monocytes and macrophages. ***a***, Mo or MDM were lysed, CD4 was immunoprecipitated, and samples were analyzed by western blot with rabbit polyclonal IgG raised against the N terminus of CXCR4 (lanes 1 and 2) or a 'cocktail' of rabbit polyclonal IgGs raised against peptides corresponding to the N-terminus and the three extracellular loops of CCR5 (lanes 3 and 4). The same membranes were 'stripped' and were then reacted with rabbit polyclonal antibody against CD4, then HRP-conjugated antibody against rabbit IgG, and bands were detected using chemiluminescence. Data represent five experiments. ***b***, Macrophages infected with recombinant vaccinia virus expressing human CD4 (vCB3) or control vSC8 vaccinia were lysed, CD4 was immunoprecipitated, and samples were analyzed by western blot. Data represent three experiments. ***c***, MDM infected with recombinant vaccinia virus expressing human CD4 can fuse with a T-tropic HIV envelope in a CXCR4-dependent manner. MDM infected with vCB3 (▨) or vSC8 (■) were mixed with effector cells expressing IIIB envelope. Some vCB3-infected MDM were treated with rabbit antibody against CXCR4 or CCR5, with preimmune rabbit IgGs or with SDF-1α or RANTES and then mixed with the effector cells. Data represent mean ± s.d. of triplicate wells.

tein from Jurkat cells by immunoblot (class I MHC, a 45-kDa protein, also appears as a 50-kDa protein in these gels) recognizes only a 62-kDa protein in an immunoblot of monocytes and can be used to precipitate a 62-kDa protein from biotinylated monocytes. In addition, we found enhanced expression of only the 62-kDa band in monocytes infected with a vaccinia that encodes CXCR4. These data indicate a lineage-specific processing of chemokine receptors. Study of other chemokine receptors known to be expressed in Mo/MDM is waranted. Experiments are underway to identify the post-translational modifications of CXCR4 in Mo/MDM and to determine whether other cellular components are associated with these receptors. The differential binding of the rabbit antibody against CXCR4 EC2 to the MDM high-molecular-weight species of CXCR4 in western blots further indicates that this region, which plays an important part in HIV fusion, is conformationally different in the low- and high-molecular-weight forms of CXCR4.

In addition to differences in the post-translational modifications in the co-receptors that may affect their ability to support fusion, diversity among CXCR4- or dual-tropic HIV-1 strains in their interactions with the CD4/CXCR4 on target cells may also have an effect. For example, MDM from CCR5 Δ32/Δ32 donor could support infection with the dual-tropic strain 89.6 but not with the T-tropic strain NL4-3 (ref. 25). The monoclonal antibody against CXCR4, 12G5, which binds to EC2, could block infection of PBL with 89.6, but not with T-tropic strains[30]. The 89.6 envelope (and some other dual-tropic or CXCR4 primary isolates[31]) may have an inherently higher avidity for CXCR4 and/or for CD4, allowing them to interact more efficiently with the limited numbers of CXCR4/CD4 complexes present on the surface of MDM (especially in MDM devoid of CCR5) compared with those on lab (T-cell line)-adapted CXCR4 strains.

Another explanation for the preferential fusion of MDM with M-tropic envelopes is that different chemokine receptors that are co-expressed in the same cell may be competing for association with CD4 molecules, especially in cells with low CD4 density (such as MDM). Specifically, CCR5 may preferentially associate with the limiting numbers of CD4 molecules in MDM. Thus, as the biochemical properties of surface chemokine receptors may vary, surface staining with limited number of antibodies may not provide an accurate estimate of the functionally fusion-active co-receptor molecules. Our findings support the theory that a successful fusion process requires a 'threshold' density of CD4–co-receptor complexes[32]. If a co-receptor is associated with CD4, then binding of Env to the co-receptor is likely to occur more quickly than if it has to wait for the co-receptor to move into close proximity. When CD4 density is limiting, there is a greater dependence on co-receptor density and, as shown here, the availability of monomeric co-receptors. Our results also indicate that stimuli that affect the processing, transport and/or recycling of surface CXCR4 molecules in MDM may alter the ratios of low-molecular-weight:high-molecular-weight CXCR4 molecules and change their susceptibility to infection with T-tropic strains.

Methods

Generation of rabbit IgGs against CXCR4 and CCR5. Synthetic peptides corresponding to the N termini or extracellular loops of CXCR4 and CCR5 were synthesized by B. Chandrasekhar and N.Y. Nguyen at the Facility for Biotechnology Resources (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland) using standard flourenyl methoxycarbonyl chemistry as described[19,26]. New Zealand White rabbits were immunized with the KLH-peptide conjugates, emulsified in complete Freund's adjuvant, at 1 mg per dose, followed by 'boosts' (3 weeks apart) in incomplete Freund's adjuvant. Immune and pre-immune IgG fractions were prepared by two rounds of precipitation with saturated ammonium sulfate. The following peptides were used: CXCR4 (N terminus), CMEGISITSDNYTEEMGSGDYDSMKEPCFRE ENANFNK; CXCR4 (EC2), CNVSEADDRYICDRFYPND; CCR5 (N terminus), CMDYQVSSPIYDINWT-SEPCQKINVKQIMR; CCR5 (EC1), CHYMMQWDFGNIM; CCR5 (EC2), CQKEGLHYTCSSHFPYSQYQFWKNFQTLKI; CCR5 (EC3), CQEFFGLNNC-SSSN RLDQ.

Generation of MDM. Elutriated Mo and differentiated MDM were 100% $CD3^-$, >85% $CD14^+$ and >95% $HLA\text{-}DR^+$, as determined by flow cytometry. MDM were derived from elutriated Mo in 5- to 7-day cultures in DMEM medium supplemented with rhGM-CSF (1,000 U/ml) and 10% 'pooled' human serum (heat inactivated).

Infection of MDM with CXCR4 and CCR5 HIV-1. Infections of MDM and PCR analyses were done as described. MDM (1×10^6) at day 6 of culture were infected with the following strains (MOI): LAI (0.4), NL4-3 (0.05), BaL (0.02) and ADA (0.01). After 24–48 h, cells were recovered and counted. DNA lysates (50 µl, the equivalent of 1×10^4 cells) were amplified by PCR with gag-specific primers (SK38 and SK 39), and the products were hybridized to a ^{32}P-ATP end-labeled SK19 probe as described[33]. Mo and MDM (from the same individual) infected with the CXCR4 strain NL4-3 were analyzed in a similar manner.

Co-precipitation of CD4/CXCR4 and CD4/CCR5. Mo or MDM were lysed at a concentration of 2×10^7cells/ml in buffer containing 1% BRIJ 97 (ref. 26). Cell lysates were incubated with protein G Sepharose beads conjugated to monoclonal antibody OKT4 at 4 °C for 3 h; preliminary data confirmed that in these conditions, CD4 was immunoprecipitated to completion. Beads were washed five times with lysis buffer and boiled with an equal volume of 2X Laemmli sample buffer containing 8 M urea. Samples (4×10^7 cells per lane) were separated by 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked[26] and were incubated either with a rabbit polyclonal antibody raised against a peptide corresponding to the N terminus of CXCR4 or a 'cocktail' of rabbit polyclonal antibodies against CCR5, raised against peptides corresponding to the N terminus and the three extracellular loops. The membranes were washed and incubated with horse radish peroxidase (HRP)-conjugated antibody against rabbit IgG diluted in blocking buffer at 4 °C (Amersham), followed by incubation with supersignal ultra chemiluminescent substrate (Pierce, Rockford, Illinois) for 5 min and then autoradiography. Membranes were 'stripped' by wetting in 100% methanol and washing with blotting buffer. They were incubated in 0.5 M glycine, pH 2.5 with 0.05% Tween for 30 min at 60 °C and washed in blotting buffer and blocked. The membranes were reacted with rabbit polyclonal antibody against CD4 (Intracel, Seattle, Washington), then HRP-conjugated antibody against rabbit IgG and chemiluminescent reagent, followed by autoradiography.

Western blots of CXCR4 and CCR5 in Mo/MDM. For total cell extracts, Mo and MDM were lysed at 1×10^7 cells per ml in buffer containing 1% NP40, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) and the following reagents: 940 mg/ml iodoacetamide, 0.2 mM EDTA, and 240 mg/ml 4-(2-aminoethyl)-benzene sulfonyl flouride, HCl (AEBSF; Sigma); 0.5 mg/ml leupeptin, 2 mg/ml aprotinin (Boehringer), 60.5 mg/ml chymostatin, 35 mg/ml Na-Tosyl-Phe Chloromethyl Ketone (TPCK), 40 mg/ml Bestatin trypsin inhibitor (Calbiochem, La Jolla, California) and 0.7 mg/ml pepstatin A (Boehringer). After a 20-min incubation on ice, nuclei were pelleted by centrifugation at 13,000*g* for 5 min, and samples were boiled with an equal volume of 2X Laemmli sample buffer containing 8 M urea. Samples corresponding to 2.5×10^5–5×10^5 cells were separated by 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. For peptide blocking, antibodies were preincubated overnight at 4 °C with a 50-fold molar excess of peptide. Membranes were reacted with rabbit polyclonal IgG specific for either the N terminus or the second extracellular domain of CXCR4.

Macrophages were infected with recombinant vaccinia virus expressing human CD4 (vCB3) or control vSC8 vaccinia at 10 PFU/cell. After 5 h, cells

were lysed in BRIJ lysis buffer, CD4 was immunoprecipitated, and samples were analyzed by western blot as described above.

For studies of surface CXCR4 and CCR5 molecules, Mo and MDM were surface biotinylated by the addition of 2 mM sulfo-NHS-LC-Biotin (Pierce, Rockford, Illinois) in 5 mM citrate, pH 5.0, to 2.5×10^7 cells/ml in PBS for 1 h on ice. The reaction was 'quenched' with 20 mM glycine for 15 min. Washed cells were lysed in NP40 lysis buffer and precipitated with avidin-Sepharose beads (Sigma). Samples were treated as described for co-precipitation, and blots were reacted with rabbit IgGs against CXCR4 or CCR5. No signal was detected in blots reacted with pre-immune rabbit IgG (not shown). Binding of IgGs against either CXCR4 or CCR5 could be completely blocked by pre-incubation with the relevant (but not irrelevant) peptides.

Chemokine-induced Ca^{2+} flux in Mo and MDM. Calcium-dependent fluorescence changes in Fura2-loaded Mo and MDM were recorded after the addition of SDF-1α or MIP-1β (Peprotech, Rocky Hill, New Jersey) at a final concentration of 50 nM as described[33].

FACS analysis of surface expression. Cells were stained with rabbit IgGs against CXCR4 or CCR5 or with pre-immune rabbit IgG as described[19], or with the murine monoclonal antibodies 12G5 (against CXCR4) and 2D7 (against CCR5). CD4 was stained with the monoclonal antibody OKT4 (Ortho Diagnostic, Raritan, New Jersey) or isotype-matched control antibody. Cells were analyzed using FL-1 (for FITC) channels on a FACScan (Becton Dickinson, San Jose, California) with Cell Quest software. Delta mean fluorescence channels (ΔMFC) were calculated by subtracting the control MFC from the experimental values.

Analysis of syncitium formation. Syncytium formation was measured using effector 12E1 cells infected with recombinant vaccinia virus expressing T-tropic envelope (vPE16, IIIB) or M-tropic envelope (vCB29, JR-FL). Syncytia were counted after 18 h of co-culture. Alternatively, MDM were infected with recombinant vaccinia virus expressing CD4 (vCB3) or control vSC8 virus at 10 PFU/cell for 5 h and then mixed with effector cells expressing IIIB envelope. The vCB3-infected MDM were sometimes treated with rabbit antibody against CXCR4 or CCR5 or preimmune rabbit IgGs (at 10 μg/ml), or with SDF-1α or RANTES (at 1 μg/ml) for 60 min at 37 °C and then mixed with effector cells expressing IIIB envelope. Syncytia were counted after 18 h.

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1. Pomerantz, R. *et al.* Human Immunodeficiency virus (HIV) infection of the uterine cervix. *Ann. Intern. Med.* **108**, 321–327 (1988).
2. Nuovo, G., Forde, G., MacConnel, P. & Fahrenwald, R. In situ detection of PCR amplified HIV-1 nucleic acid and tumor necrosis factor cDNA in cervical tissues. *Am. J. Pathol.* **143**, 40–48 (1993).
3. Spencer, L.T., Ogino, M.T., Dankner, W.M. & Spector, S.A. Clinical significance of human immunodeficiency type 1 phenotypes in infected children. *J. Infect. Dis.* **169**, 491–495 (1994).
4. Zhu T. *et al.* Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. *Science* **261**, 1179–1181 (1993).
5. Koenig S. *et al.* Detection of AIDS virus in macrophages in brain tissues from AIDS patients with encephalopathy. *Science* **233**, 1089–1093 (1986).
6. Chayt, K.J. *et al.* Detection of HTLV-III RNA in lungs of patients with AIDS pulmonary involvement. *J. Am. Med. Assoc.* **256**, 2356–2359 (1997).
7. Ghorpade A. *et al.* Human immunodeficiency virus neurotropism: An analysis of viral replication and cytopathicity for divergent strains in monocytes and microglia *J. Virol.* **72**, 3340–3350 (1998).
8. Feng, Y., Broder C.C., Kennedy, P.E. & Berger, E.A. HIV entry cofactor: functional cDNA cloning of a seven transmembrane G protein-coupled receptor. *Science* **272**, 872–877 (1996).
9. Alkhatib, G. *et al.* CC CKR5: a RANTES, MIP-1α, MIP-1(receptor as a fusion cofactor for macrophage-tropic HIV-1 *Science,* **72**, 1955–1958 (1996).
10. Choe, H. *et al.* The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135–1148 (1996).
11. Deng, H. *et al.* Identification of a major co-receptor for primary isolates of HIV-1 *Nature* **381**, 661–666 (1996).
12. Dragic, T. *et al.* HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5 *Nature* **381**, 667–673 (1996).
13. Doranz, B.J. *et al.* A dual-tropic primary HIV-1 isolate that uses fusin and β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149–1158 (1996).
14. Cheng-Mayer, C. Quiroga, M., Tung J.W., Dina D. & Levy, A.J. Viral determinants of human immunodeficiency type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. *J. Virol.* **64**, 4390–4398 (1990).
15. Cheng-Mayer, C., Liu, R., Landau N.R. & Stamatatos, L. Macrophage tropism of human immunodeficiency virus type 1 and utilization of the CC-CKR5 coreceptor. *J. Virol.* **71**, 1657–1661 (1997).
16. Loetscher, M. *et al.* Cloning of a human even-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes. *J. Biol. Chem.* **269**, 232–237 (1994).
17. Oberlin, E. *et al.* The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line adapted HIV-1. *Nature* **383**, 833–835 (1996).
18. Bleul, C.C. *et al.* A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1(SDF-1) *J. Exp. Med.* **184**, 1101–1109 (1997).
19. Zaitseva, M. *et al.* Expression and function CCR5 and CXCR4 on human Langerhans cells and macrophages: Implications for HIV primary infection. *Nature Med.* **3**, 1369–1375 (1997).
20. Ovarecz, T. *et al.* Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. *J. Exp. Med.* **184**, 1865–1872 (1997).
21. Di Marzio, P., Tse, J. & Landau, N.R. Chemokine receptor regulation and HIV type 1 tropism in monocyte-macrophages. *AIDS Res. Hum. Retrovir.* **14**, 129–138 (1998).
22. Broder, C.C. & Berger, E.A. Fusogenic selectivity of the envelope glycoprotein is a major determinant of human immunodeficiency virus type 1 tropism for $CD4^+$ T-cell lines vs. primary macrophages. *Proc. Natl. Acad. Sci. USA* **92**, 9004–9008 (1995).
23. Schmidtmayerova, H., Alfano, M., Nuovo, G. & Bukrinsky, M. Human Immunodeficiency virus type 1 T-lymphotropic strains enter macrophages via a CD4-and CXCR4-mediated pathway: Replication is restricted at a post entry level. *J. Virol.* **72**, 4633–4642 (1998).
24. Verani, A. *et al.* CXCR4 is a functional coreceptor for infection of human macrophages by CXCR4-dependent primary isolates. *J. Immunol.* **161**, 2084–2088 (1998).
25. Yi, Y., Rana, S., Turner, J.D., Gaddis, N. & Collman, R.G. CXCR-4 is expressed by primary macrophages and supports CCR5-independent infection by dual-tropic but not T-tropic isolates of human immunodeficiency virus type 1. *J. Virol.* **72**, 772–777 (1998).
26. Lapham, C.K. *et al.* Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. *Science* **274**, 602–605 (1996).
27. Wu, L. *et al.* CD4-induced interaction of primary HIV-1gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384**, 179–187 (1996).
28. Trokola, A. *et al.* CD4-dependent antibody-sensitive interaction between HIV-1 and its co-receptor CCR-5. *Nature* **384**, 184–187 (1996).
29. Hill, C.M. *et al.* Envelope glycoproteins from human immunodeficiency type 1 and 2 and simian immunodeficiency virus can use human CCR5 as a coreceptor for viral entry and make direct CD4-dependent interactions with this chemokine receptor. *J. Virol.* **71**, 6296–6304 (1997).
30. Strizki, J.M., Turner, J.D., Collman, R.G., Hoxie, J. & Gonzales-Scarano, F. A monoclonal antibody (12G5) directed against CXCR-4 inhibits infection with the dual-tropic human immunodeficiency virus type 1 isolate HIV-1 (89.6) but not the isolate HIV-1(HxB). *J. Virol.* **71**, 5678–5683 (1997).
31. Simmons, G. *et al.* CXCR4 as a functional coreceptor for human immunodeficiency virus type 1 infection of primary macrophages. *J. Virol.* **72**, 8453–8457 (1998).
32. Platt, E.J., Wehrly, K., Khumann, S.E., Chesebro, B. & Kabat, D. Effects of CCR5 and CD4 cell surface concentration on infections by macrophage-tropic isolates of human immunodeficiency virus type 1. *J. Virol.* **72**, 2855–2864 (1998).
33. Zaitseva, M.B. *et al.* CXCR4 and CCR5 in human thymocytes: Biological function and role in HIV-1 infection. *J. Immunol.* **161**, 3103–3113 (1998).