Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection

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HIV-1 infection triggers lateral membrane diffusion following interaction of the viral envelope with cell surface receptors. We show that these membrane changes are necessary for infection, as initial gp120–CD4 engagement leads to redistribution and clustering of membrane microdomains, enabling subsequent interaction of this complex with HIV-1 co-receptors. Disruption of cell membrane rafts by cholesterol depletion before viral exposure inhibits entry by both X4 and R5 strains of HIV-1, although viral replication in infected cells is unaffected by this treatment. This inhibitory effect is fully reversed by cholesterol replenishment of the cell membrane. These results indicate a general mechanism for HIV-1 envelope glycoprotein-mediated fusion by reorganization of membrane microdomains in the target cell, and offer new strategies for preventing HIV-1 infection.

INTRODUCTION

HIV-1 infects permissive cells by binding to the CD4 cell receptor (Maddon *et al.*, 1986), followed by gp120–gp41-mediated fusion of viral and target cell membranes (Dimitrov, 1997). In addition to CD4, several members of the chemokine receptor family act as necessary co-receptors for HIV-1 fusion and infection. The initial interaction with CD4 promotes a conformational change in gp120, exposing cryptic regions of the viral glycoprotein for further interaction with CXCR4 or CCR5 (Dimitrov, 1997). Chemokine receptor usage varies depending on the viral strain, and is the primary determinant of viral tropism (Moore *et al.*, 1997; Berger *et al.*, 1998; Littman, 1998). The discovery of

chemokine receptors as essential receptors for HIV and simian immunodeficiency virus entry into the cell has challenged our understanding of HIV infection, although several crucial pieces of the puzzle are still missing.

In addition to CD4 and chemokine receptors, other cell surface molecules modulate HIV-1 infection, either by direct interference with viral attachment or through signaling pathways. HIV-1 infection appears to depend on multiple intermolecular interactions at the cell surface. Indeed, CD26, CD28 and CD44 increase cell permissivity to HIV-1, whereas CD38 decreases susceptibility to infection (Callebaut *et al.*, 1993; Dukes *et al.*, 1995; Savarino *et al.*, 1999). Physicochemical studies of the gp120 interaction with defined glycosphingolipids (GSL) (Harouse *et al.*, 1991; Hammache *et al.*, 1999) and inhibition of HIV-1 *in vitro* infection after treatment of cells with anti-GSL antibodies (Harouse *et al.*, 1991) suggest that GSL may also be important in HIV-1 infection. This is of the utmost relevance since HIV-1 infection activates cell surface lateral associations whose mechanisms nonetheless remain elusive.

Accumulated evidence indicates that GSL–sphingomyelin–cholesterol-enriched membranes, so-called rafts, are fundamental in the lateral organization of the plasma membrane (Harder *et al.*, 1998). Raft microdomains are the result of the preferential packaging of GSL, sphingomyelin and cholesterol in the membrane external leaflet. Owing to the high melting point of these lipids, membrane rafts are found in a rigid liquid crystalline state (L_o), distinct from the surrounding fluid liquid phase membranes (L_d) (Brown and London, 1998). These lipid rafts retain substantial lateral and rotational mobility, such that rafts

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are viewed as moving platforms of highly ordered membranes that carry specific proteins. CD4 associates with membrane rafts in T cells (Xavier *et al.*, 1998), and we recently showed that CCR5 is also found in these rafts (Mañes *et al.*, 1999). Moreover, permissive molecules that favor HIV-1 infection, such as CD44 and CD28, are found in and mediate reorganization of lipid rafts in living cells (Viola *et al.*, 1999). It is thus plausible that raft microdomains have an essential role in the HIV-1-induced lateral associations required for viral infection.

RESULTS AND DISCUSSION

At temperatures below 12°C, antibody-induced cross-linking of membrane-associated proteins triggers their lateral clustering on the membrane plane without internalization, and stabilizes protein–lipid interactions. Only membrane proteins that prefer identical or compatible lipid environments will be clustered into the same patch (Harder *et al.*, 1998). To test for HIV-1 gp120 in lipid domains, we analyzed the gp120 interaction with rafts by co-patching gp120 and the cholera toxin β -subunit (CTx), a specific marker for the raft-associated GSL GM1. Mock or CD4-expressing HEK-293 (293-mock or 293-CD4) cells were incubated with recombinant gp120, followed by cross-linking with an anti-gp120 antibody to induce glycoprotein multimerization. Under these conditions, gp120 does not bind to 293-mock cells (Figure 1A, a1–a3), indicating the requirement for a primary interaction with CD4 for subsequent binding to other cell receptors, including CXCR4. In contrast, in 293-CD4 cells, we observed extensive co-localization of gp120 with CTx, indicating that gp120 associates with proteins distributed in rafts (Figure 1A, a4–a6). Co-patching of gp120 and CTx leads to coalescence of rafts in discrete membrane regions, giving a polarized pattern not observed in 293-mock or in 293-CD4 cells in the absence of gp120 (Figure 1B). Activation of raft-associated proteins mediates the acquisition of polarized cell morphology (Mañes *et al.*, 1999), and the polarized pattern detected after gp120 co-patching in 293-CD4 cells is consistent with this observation. The specificity of gp120-induced raft aggregation was further demonstrated by monoclonal antibody (mAb) cross-linking of the transferrin receptor (TfR), a protein excluded from raft membranes (Harder *et al.*, 1998), showing that anti-TfR-induced patches do not co-localize with those observed using CTx (Figure 1A, a7–a9).

We next analyzed the role of membrane rafts in the formation of higher order molecular complexes between gp120, CD4 and CXCR4 in 293-CD4 and peripheral blood mononuclear cells (PBMC). In the absence of gp120, both CD4 and CXCR4 show extensive co-localization with CTx in 293-CD4 cells (Figure 1B, b1 and b2) and PBMC (Figure 1B, b9 and b10). Nonetheless, CD4 and CXCR4 show weak co-localization in the absence of gp120 (Figure 1B, b3 and b11). These results concur with the reported poor association between CD4 and CXCR4 (Xiao *et al.*, 1999), suggesting that CD4 and CXCR4 are not physically associated in the absence of gp120. Since CD4 and CXCR4 are found in the membrane raft fraction, gp120-induced patching must trigger the lateral aggregation and coalescence of rafts containing CD4–gp120–CXCR4 complexes. To test this, we used anti-gp120 antibody to study co-patching of gp120-incubated 293-CD4 or PBMC cells. Anti-gp120 triggers the formation of large clusters of CD4 or CXCR4 with gp120 and

CTx (Figure 1B, b5–b7, b13–b15). Conversely, the non-raft TfR is excluded from these clusters (Figure 1B, b8 and b16). These results suggest that gp120 induces the lateral reorganization of rafts, bringing the CD4–gp120 complexes together with rafts containing the chemokine receptor.

This hypothesis is further supported by membrane cholesterol depletion in 293-CD4 cells using methyl- β -cyclodextrin (CD), which prevents the formation of lateral lipid assemblies. This treatment neither inhibits gp120 binding to cells (red and yellow staining, Figure 1C, c1–c3) nor prevents gp120–CD4 co-localization (Figure 1C, c1), although GM1 shows <10% co-localization with gp120 (Figure 1C, c2). Co-localization of gp120 with CXCR4 is not observed in CD-treated cells (Figure 1C, c3), indicating that raft integrity is required for lateral assembly of CD4–gp120 complexes with the CXCR4. Replenishment of membrane cholesterol in CD-treated cells restores formation of CD4–gp120–CXCR4 trimolecular complexes, confirming that the CD effect is limited to cholesterol removal.

Rafts are resistant to solubilization by Triton X-100 (TX-100) at 4°C, leading to the isolation of a light membrane fraction, the detergent-insoluble GSL-enriched membranes (DIG). The DIG fraction is enriched in specific membrane proteins, considered to be raft proteins, whereas others are excluded from this fraction, including the insoluble complexes formed by cytoskeleton association. Untreated and gp120-treated cells were fractionated following TX-100 extraction. Approximately 60% of the gp120 bound to 293-CD4 cells is detected in DIG, whereas gp120 is undetectable in 293-mock cells (Figure 2). Co-purification of the raft protein caveolin-1 and full solubilization of the non-raft protein TfR confirm the quality of the preparation. We also observed that although CXCR4 and CD4 are present in the DIG fraction, gp120 binding to 293-CD4 cells recruits a larger number of both of these HIV-1 receptors to the lightest fraction, due to the enhanced raft stability after gp120-induced clustering.

These results suggest that gp120 regulates the lateral clustering of rafts containing CD4 and CXCR4; this prompted us to evaluate the possible engagement of lipid domains in HIV-1 infection. Lateral raft aggregation is abolished either by cholesterol depletion (Harder *et al.*, 1998) or by reducing GSL membrane levels (Ledesma *et al.*, 1999). Inhibitors of GSL biosynthesis prevent HIV-1 infection (Mizrahi *et al.*, 1996). One interpretation may be that reduction in GSL synthesis affects raft formation in the plasma membrane, and hence prevents the lateral clustering of HIV-1 receptors required for viral infection. If this is the case, cholesterol depletion of the plasma membrane should inhibit HIV-1 infection as efficiently as GSL downregulation.

To evaluate the structural raft integrity requirement for viral entry, MT-2-CCR5 cells, which express both CXCR4 and CCR5, were CD treated before infection with NL4-3 (X4) or BaL (R5) HIV-1 strains. After viral infection, p24 levels were recorded daily as an indirect measurement of HIV-1 production. The cytopathic effect of the X4 virus was also monitored as giant cell (syncytia) formation and balloon degeneration. Cholesterol depletion reduced both X4 infection, demonstrated by the p24 production delay in CD-treated compared with untreated cells (Figure 3A), as well as the cytopathic effect of this virus strain (Figure 3C). Similar inhibition was observed for CD-treated R5-infected cells (Figure 3B), confirming CCR5 association with rafts. CD treatment also reduced X4 and R5 infection of activated PBMC by 82 ± 3.5 and $72 \pm 8.5\%$, respectively.

Fig. 1. Membrane raft integrity is required for gp120-induced lateral association of CD4 and CXCR4. **(A)** Confocal images of co-patching between biotinylated CTx (a2, a5, a8) and either an anti-gp120 polyclonal antibody (a1, a4) or anti-TfR mAb (a7) in mock-transduced and CD4⁺ cells. Primary reagents were visualized by addition of streptavidin-Cy5 and anti-mouse or anti-rabbit Cy3 secondary antibodies. The overlay is shown (a3, a6, a9). **(B)** 293-CD4 (b1–b8) or PBMC (b9–b16) cells, alone (–) or incubated with gp120 (+), were co-patched with anti-gp120, biotinylated CTx, anti-TfR mAb, FITC-labeled anti-CD4 mAb, or biotinylated or FITC-anti-CXCR4 mAb as indicated, and analyzed by confocal microscopy. Only the three-color overlay is shown. **(C)** Co-patching with anti-gp120 (red) and anti-CD4, anti-CXCR4 or CTx (green) in CD-treated and cholesterol-replenished CD-treated 293-CD4 cells. Only the overlay image of a representative confocal section is shown. Bar: 5 μ m for 293 cells and 10 μ m for PBMC.

Fig. 2. gp120 is found in the DIG fraction of the cell membrane. TX-100-insoluble membranes were isolated from gp120-treated or untreated 293-mock or 293-CD4 cells, and fractions analyzed by western blotting with antibodies to gp120, CD4 (Leu3A), CXCR4, TfR (Zymed Labs, South San Francisco, CA) and caveolin-1 (Cav; Santa Cruz Biotech., Santa Cruz, CA).

Membrane cholesterol replenishment by addition of free cholesterol before HIV-1 infection restores both p24 production and syncytia formation in CD-treated cells, indicating that the CD inhibitory effect is limited to membrane cholesterol removal.

We next studied the blockage level induced by CD treatment. Membrane rafts have been implicated in HIV-1 budding (Nguyen and Hildreth, 2000), and it would be of interest to determine whether CD pre-treatment of target cells interferes with events in the HIV-1 replicative cycle, such as the infectivity of the virions released. The equivalent slopes of the p24 production kinetics curve in untreated and CD-treated cells (Figure 3D), besides the unchanged p24 production after CD treatment of NL4-3-infected cells (Figure 3E), support the view that CD pre-treatment does not affect post-infection viral replication events. Furthermore, cholesterol depletion drastically inhibits infection of a replication-defective NL4-3 variant, whereas it does not affect the entry of either vesicular stomatitis virus (VSV) G- or murine leukemia virus (MLV) envelope-pseudotyped replication-defective NL4-3 variants (Figure 3F). These results show that CD-induced inhibition of HIV-1 infection is specific for viruses using receptors that partition in rafts, and support the view that the CD effect is probably exerted at initial phases of HIV-1 infection.

We next analyzed the CD effect on virus binding to cells. Recombinant gp120 binding to untreated or CD-treated MT-2 cells was quantitated by FACS (Figure 4A), showing that cholesterol removal does not affect the initial gp120–CD4 interaction. Furthermore, CD treatment does not affect NL4-3 binding to MT-2 cells as analyzed by western blotting (Figure 4B), suggesting that CD does not affect primary virus–CD4 interaction. The inhibitory effect of CD was also studied by monitoring newly reverse-transcribed HIV-1 proviral DNA in a semi-quantitative PCR. CD treatment drastically reduced the amount of HIV-1 DNA in MT-2-infected cells compared with untreated

cells. Collectively, these results suggest that cholesterol depletion inhibits HIV-1 infection at the entry stage.

Finally, we analyzed whether raft integrity is necessary for HIV-1 envelope fusion with the target cell in a cell–cell fusion assay. HeLa-CD4 cells transfected with a luciferase reporter gene under the control of the p7.5 promoter were mixed with BSC40 cells transduced with a recombinant vaccinia virus encoding the HIV-1_{IIIb} env gene. HeLa–BSC40 fusion results in transactivation of the p7.5 promoter and, hence, an increase in luciferase reporter gene transcription (Figure 4C). Cholesterol depletion of HeLa-CD4 cells reduces luciferase activity in these cells; this inhibition is reversed by cholesterol replenishment after CD treatment (Figure 4C). These results show that raft integrity is required for HIV-1 envelope fusion with the target cell membrane. When fusion was performed with cholesterol-depleted BSC40-env cells and untreated HeLa-CD4 cells, luciferase activity in the latter was unaffected (not shown). This suggests that cell–envelope fusion requires raft integrity only in the target cell, in which CD4 and CXCR4 must come together to enable viral infection.

Several reports have pointed out the role of GSL as cofactors in HIV-1 cell fusion. Indeed, CD4⁺ cell lines become susceptible to HIV-1 env-mediated fusion following complementation with Gb3 isolated from human erythrocytes (Puri *et al.*, 1998). It has further been suggested that gp120 from various HIV-1 strains has differential binding affinity for distinct GSL species (Hammache *et al.*, 1999); this indicates that the role of the GSL as alternative HIV-1 entry cofactors is mediated by their direct association with the viral envelope in a CD4-dependent manner. We show here that membrane cholesterol is essential for fusion of the HIV-1 envelope and the cell membrane, probably by enabling gp120-induced lateral association of CD4 and CXCR4. A decrease in cellular GSL or cholesterol levels disrupts raft structure and abolishes HIV-1 infection. Since GSL and cholesterol are both basic components of lipid rafts, we propose that the

Fig. 3. Cholesterol depletion specifically inhibits HIV-1 infection. p24 antigen levels at day 2 post-infection in NL4-3-infected cells (A) and at day 4 post-infection in BaL-infected cells (B) in untreated, CD- and cholesterol-replenished (CD+Cho) cells. Data shown are the mean $\pm$ SD of triplicate points ($n = 4$). *Statistically significant difference, $p < 0.001$ (two-tailed t -test). (C) NL4-3-induced syncytia in a representative field of control, CD- and CD+Cho-infected MT-2-CCR5 cells (100 $\times$). (D) Representative p24 production kinetics in untreated (control), CD-treated and cholesterol-replenished (CD+Cho) NL4-3-infected cells. (E) NL4-3-infected MT-2-CCR5 cells (day 2 post-infection) were CD and cholesterol treated. After extensive washing, 10^6 cells were plated in complete RPMI and p24 production measured in ELISA after 24 h (black bars) and 48 h (white bars). (F) Untreated or CD-treated MT-2 cells were infected with a replication-defective NL4-3 variant, or with a VSV-G- or MLV-*env*-pseudotyped NL4-3 virus. Two days later, infected cells were determined. The percentage of CD-induced inhibition for each viral type is shown.

effect of these lipids is mediated by lateral diffusion on the cell surface of the HIV-1 receptors, CD4 and CXCR4 or CCR5, a multimolecular organization critical for viral entry. The data presented here, added to the recently described budding of HIV-1 in rafts (Nguyen and Hildreth, 2000), indicate that membrane rafts may be a target for new strategies to prevent and/or block HIV-1 infection. This potential therapeutic approach would be suitable for both X4 and R5 viral strains, and would obviate the problem of resistance mutants generated using current treatments.

METHODS

Immunofluorescence and gp120-induced patching. For gp120 co-patching experiments, 293-mock cells, 293-CD4 cells or

Fig. 4. Cholesterol depletion inhibits *env*-mediated fusion. (A) Untreated (control) or CD-treated MT-2 cells were incubated alone (gray area) or with gp120 (dotted line) and binding monitored in flow cytometry. (B) Untreated or CD-treated MT-2 or HEK-293, as a CD4⁺ cell, were incubated with NL4-3 and, after removal of unbound virus, cell lysates prepared. Total lysates were resolved by SDS-PAGE and sequentially blotted with anti-gp120 and anti-tubulin antibodies, as indicated. (C) Untreated or CD-treated MT-2 cells were exposed to NL4-3 as in Figure 3, and after 6 h culture, total DNA was amplified by PCR with primers specific for HIV *gag*. Either 100 or 10 ng of total DNA from the PCR products were hybridized in solution with an internal ³²P-labeled *gag* probe, resolved by SDS-PAGE and autoradiographed. Serial dilutions of proviral 8E5 DNA were run in parallel for standardization. HLA-DQ α amplification is shown as a loading control. (D) Untreated, CD-treated and cholesterol-replenished (CD+Cho) HeLa-CD4 cells were mixed with BSC40-*env* cells and cell fusion events measured. *Statistically significant difference, $p < 0.001$ (two-tailed t -test).

PBMC were incubated with recombinant gp120 (T-cell line-adapted X4 virus, isolate IIIB) produced in CHO cells (Intracel, Seattle, WA) for 30 min at 12°C, then washed at 4°C in phosphate-buffered saline (PBS) + 0.2% bovine serum albumin. Cells were incubated (30 min, 12°C) with a rabbit polyclonal anti-gp120 raised in our laboratory, in combination with biotinylated CTx (Sigma, St Louis, MO), fluorescein isothiocyanate (FITC)-anti-CD4 (OKT4; Ortho Diagnostics, Raritan, NJ), biotinylated or FITC-anti-CXCR4 (FAB172; R&D Systems, Minneapolis, MN) or anti-TfR (Oncogene Research, Cambridge, MA) mAbs. Finally, anti-rabbit or anti-mouse Cy3 antibodies and streptavidin-Cy5 (Jackson ImmunoResearch, West Grove, PA) were added (30 min, 12°C). Cells were fixed for 5 min with 3.7% paraformaldehyde in PBS on ice, followed by 10 min with methanol at -20°C before mounting in Vectashield medium (Vector Laboratories, Burlingame, CA), and visualized by confocal laser scanning microscopy (Leica, Cambridge, UK). When two or three colors were used, the signal intensities detected for each fluorophore were covered by a linear scale of pixel intensities. Digital images were processed using Photoshop software (Adobe Systems, Mountain View, CA).

Cholesterol depletion and co-patching experiments. 293-CD4 cells were treated (30 min, 37°C) with 10 mM CD (Sigma), washed, then left untreated (CD) or replenished with 2 μ g/ml free cholesterol for 30 min (CD+Cho); cholesterol was

subsequently removed, the cells incubated with gp120 and co-patching performed with anti-gp120 (red) and anti-CD4, anti-CXCR4 or FITC-CTx (green). Further clustering was achieved by incubation with appropriate Cy2 and Cy3 secondary antibodies (except for FITC-CTx).

TX-100 flotation experiments. 293-mock and 293-CD4 cells were incubated with gp120 at 4°C and, after washing, scraped in 300 µl of TXNE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2% TX-100) containing a protease cocktail. DIG were isolated by ultracentrifugation (170 000 g for 4 h at 4°C) in a 30–35% gradient of Optiprep (Nycomed Pharma, Oslo, Norway) as described (Mañes *et al.*, 1999). Normalized protein amounts were analyzed for each fraction.

HIV-1 infection of MT-2-CCR5 cells and PBMC. Prior to infection with HIV-1 strains, MT-2-CCR5 cells and phytohemagglutinin-activated PBMC were untreated (control) or CD treated as above. Some CD-treated cells were then replenished by adding serum-free medium containing cholesterol. Just after treatment, control, CD- and cholesterol-replenished (CD+Cho) cells were incubated with NL4-3 or BaL viral stocks (2 ng of p24 antigen/10⁶ cells; 2 h, 37°C). Cells (0.5 × 10⁶/ml) were plated and cultured in complete RPMI medium. Cell-free supernatants were collected daily and tested for p24 antigen using a commercial ELISA.

Generation of recombinant replication-defective HIV pseudotypes. For single-round assays, a green fluorescent protein (GFP) version of pNL4-3.Luc.R-E-, an HIV-1 expression vector lacking *env* and *vpr* (Dr N. Landau, AIDS Research and Reference Reagent Program, NIAID, NIH) was constructed by subcloning EGFP (Clontech) at the *NotI*–*XhoI* site to generate pNL4-3-EGFP. pNGVL-VSV-G was generated by subcloning the VSV-G *env* into pNGVL3 (a gift of Dr G. Nabel, University of Michigan). Recombinant lentiviruses were produced by pNL4-3-EGFP co-transfection with pNGVL-4070Aenv, coding for the MLV amphotropic *env* (Dr G. Nabel), pNGVL-VSV-G or pCMV-HIV-1_{IIIIB}gp160 in 293-T cells as described (Yang *et al.*, 1999). Supernatants containing pseudotyped or *trans*-complemented viral particles were obtained 48 h later and filtered (0.45 µm pore). Infectious titers were determined using D17 cells. Control and CD-treated MT-2 cells were transduced with the supernatants (1 and 0.1 m.o.i., 2 h) and infected cells determined after 48 h.

Virus binding assays. Untreated and CD-treated MT-2 or 293 cells were incubated with NL4-3, washed four times to remove unbound virus, and lysed with RIPA buffer. Total cell lysates (25 µg/lane) were assayed sequentially with anti-gp120 and anti-tubulin (Sigma) antibodies. In flow cytometry analysis, control and CD-treated MT-2 cells were incubated with recombinant gp120 (10 µg/ml, 30 min, 4°C) and, after washing, incubated with anti-gp120 antibody in ice-cold PBS. gp120 binding was visualized using phycoerythrin-conjugated goat anti-rabbit IgG (Caltag, San Francisco, CA). Control samples were processed similarly, omitting gp120.

PCR amplification of proviral DNA. Control or CD-treated MT-2 cells (2 × 10⁶) were infected with NL4-3 (200 ng of HIV-1 p24) and cultured for 6 h in complete RPMI medium. Total DNA was amplified with primers specific for the HIV-1 *gag* gene (SK38 and SK39; Kellog and Kwok, 1990) and the reaction products hybridized in solution with ³²P-labeled *gag* inner probe SK19,

resolved by 8% PAGE and developed by autoradiography. Serial dilutions of proviral 8E5 DNA, used as positive control, were run in parallel. HLA-DQα was amplified under the same conditions for standardization.

gp160-induced cell–cell fusion. pScluc plasmid harboring the luciferase gene under the control of the vaccinia virus 7.5 promoter (a gift of Dr D. Rodríguez) was introduced in stably CD4-expressing HeLa cells. After 24 h, HeLa-CD4-transfected cells (2 × 10⁴) were left untreated, treated with CD, or with CD+Cho. HIV-1_{envIIIIB} was introduced into effector BSC40 cells by infection (1 h, 37°C) with recombinant vaccinia virus; 12 h post-infection, 10⁵ effector cells cultured in rifampicin (100 µg/ml) were mixed with HeLa-CD4 cells. After incubation (6 h, 37°C), luciferase activity was measured in cell lysates (25 mM Tris-phosphate pH 7.8, 1% TX-100, 1 mM EDTA, 1 mM dithiothreitol, 8 mM MgCl₂, 15% glycerol). Target cells were CD or CD+Cho treated as above immediately before co-cultivation with effector cells.

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NOTE ADDED IN PROOF

Following acceptance of this manuscript, we became aware of a report by Hug *et al.* (2000) indicating that membrane rafts are crucial elements in organizing gp120–gp41, CD4 and an appropriate chemokine receptor into a membrane fusion complex. These results are consistent with our findings and strengthen the importance of membrane raft integrity in HIV-1 infection.

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