HIV-1 ENTRY INTO T-CELLS IS NOT DEPENDENT ON CD4 AND CCR5 LOCALIZATION TO SPHINGOLIPID-ENRICHED, DETERGENT-RESISTANT, RAFT MEMBRANE DOMAINS.

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SUMMARY

The contribution of raft domains to HIV-1 entry was assessed. In particular, we asked whether the CD4 and CCR5 HIV-1 receptors need to associate with sphingolipid-enriched, detergent-resistant membrane domains (rafts) to allow viral entry into primary and T-cell lines. Based on Triton X-100 solubilization and confocal microscopy, CD4 was shown to distribute partially to rafts. In contrast, CCR5 did not associate with rafts and localized in non-raft plasma membrane domains. HIV-1-receptor partitioning remained unchanged upon viral adsorption, suggesting that viral entry probably takes place outside rafts. To directly investigate this possibility, we targeted CD4 to non-raft domains of the membrane by preventing CD4 palmitoylation and interaction with p56Lck. Directed mutagenesis of both targeting signals significantly prevented association of CD4 with rafts, but did not suppress the HIV-1 receptor function of CD4. Collectively, these results strongly suggest that the presence of HIV-1 receptors in rafts is not required for viral infection. We show, however, that depleting plasma membrane cholesterol inhibits HIV-1 entry. We therefore propose that cholesterol modulates the HIV-1 entry process independently of its ability to promote raft formation.
INTRODUCTION

Numerous studies dealing with biological membrane organization and composition have emphasized the non-random distribution of lipids and proteins into distinct membrane domains (1). Domains composed of cholesterol and saturated lipids, e.g. sphingolipids, or rafts have recently been shown to support a wide range of cellular events, including signal transduction, sorting and cellular trafficking of proteins and lipids, as well as pathogen entry into cells (2). Non-ionic detergent insolubility of these domains at 4°C was found to result from tight packing of cholesterol and sphingolipids in a liquid-ordered state (3). This property allows recovery of rafts as low-density, floating membranes by gradient centrifugation and makes it possible to characterize raft lipids and proteins (4).

Entry of human immunodeficiency virus type 1 (HIV-1) into host cells relies primarily upon interaction of the viral glycoprotein envelope (Env) gp120 subunit with cell surface CD4 (5). Conformational changes of gp120 upon CD4 binding trigger interactions of the Env with HIV-1 coreceptors CCR5 or CXCR4 (6). Subsequently, this binding to coreceptors exposes the Env gp41 trans-membrane subunit and promotes fusion of viral and cellular membranes (7). It has been proposed that oligomeric assembly of Env proteins (8,9) facilitates the recruitment of viral receptor (CD4 and coreceptors) molecules and ultimately HIV-1 entry (10). Since a correlation between the cell surface density of HIV-1 receptors and efficiency of infection has been emphasized (10), the clustering of CD4 and the coreceptor in delimited plasma membrane domains would be expected to favor HIV-1 entry.

The CD4 antigen is among the few membrane-spanning proteins found to partition into raft domains enriched in sphingolipids (e.g. the GM1 ganglioside, a prototypic marker of these domains) (11-13). To establish the contribution of rafts to HIV-1 entry, inhibition of
glycosphingolipid synthesis (14,15) and depletion of cell plasma membrane cholesterol have been investigated (16-18). The inhibition of HIV-1 infection was shown in both instances and believed to result from disruption of raft integrity. However, considering that inhibition of glycosphingolipids synthesis is not detrimental to raft domain formation (19), and that cholesterol is distributed throughout plasma membranes (20-22), inhibition of HIV-1 entry following these two means of lipid perturbation (14-17) cannot be attributed solely to disruption of rafts.

In the present work, a requirement for an association of CCR5 and CD4 with rafts to support HIV-1 entry was specifically addressed. A large body of evidence points to fatty acylation (specially, post-translational palmitoylation of cysteine residues) as a critical signal for targeting several inner leaflet signaling proteins to rafts (23) and for a few raft-seeking transmembrane proteins (24-26). It is conceivable that C-terminal palmitoylation of CD4 (27) might account for localization of this protein to rafts. This prompted us to investigate whether interference with CD4 palmitoylation would alter the distribution of the CD4 receptor to rafts. Such a non-invasive approach led us to further investigate if CD4 maintains its HIV-1 receptor function when localized outside rafts.

Here we show that palmitoylation of CD4 and its interaction with the tyrosine kinase p56\textsuperscript{Lck} are important for the distribution of CD4 to rafts. In contrast to CD4, we present evidence that the CCR5 coreceptor preferentially partitions to non-raft domains, despite its palmitoylation on three C-terminal cysteine residues (28,29), both in T-cell lines and primary T cells. Importantly, when predominantly redistributed to non-raft domains, CD4 still displays full receptor function for monocytotropic (R5)-HIV-1 strains. Together, our results indicate that CCR5-dependent HIV-1 infection does not depend upon the presence of CD4 and CCR5 receptors in rafts. Furthermore, we show that depleting plasma membrane cholesterol in target
cells inhibits viral entry, suggesting that cholesterol-dependent membrane properties other
than rafts formation come into play to promote efficient HIV-1 infection.
EXPERIMENTAL PROCEDURES

Cell cultures and primary T cell blast preparation. T-cell lines were grown in RPMI medium (Life Technologies, Inc), supplemented with 10% Fetal Calf Serum (FCS) and 100U/ml penicillin, 100 µg/ml streptomycin (growth medium). Human embryonic kidney cells (HEK-293T), Baby Hamster Kidney cells (BHK) and human HeLa cells were cultured in DMEM growth medium with the same additives. A3.01, a CD4 HAT-sensitive variant of the CEM T-cell line (30) and its derivatives, CD4⁻ (A2.01) and CCR5⁺ (A2.01R5, A3.01R5) cells, were obtained from H-T He (Centre d'Immunologie INSERM/CNRS de Marseille-Luminy, France). Stable ectopic CCR5 expression was maintained by culturing CCR5⁺ T-cell lines in RPMI growth medium supplemented with 1 mg/ml of geneticin (G418) (Roche, Molecular Biochemicals). Both A3.01 and A2.01 T-cell lines have a defect in cholesterol biosynthesis and accumulate lanosterol, a cholesterol precursor, in FCS-depleted growth medium (31). However, we confirmed others' data (31) that in 10% FCS growth medium, the sterol contained in plasma membranes of these cells is mainly cholesterol, as assessed by thin layer chromatography (TLC) (our data not shown). Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors using Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. For lymphocyte experiments, freshly prepared PBMC at 2-3 X 10⁶ cells per ml were cultured in growth medium containing phytohemagglutinin (PHA (Sigma)) at 1 mg/ml for three days and afterwards growth medium was supplemented with recombinant interleukin 2 (IL-2) at 150 units/ml (Chiron) for two weeks. Cells were mostly CD14 negative and more than 70% CD4 positive, as assessed by FACscan analysis (results not shown).

Treatment of cells with methyl-β-cyclodextrin. In cholesterol extraction experiments, 30 X 10⁶ cells were rinsed once with PBS and incubated for 30 min with (cholesterol-depleted cells) or without (untreated cells) 5 mM methyl-β-cyclodextrin (MBCD, Sigma) in 25 ml RPMI
medium supplemented with 1% FCS. Experiments were carried out at 37°C under continuous, gentle stirring. Cells were washed twice with a Hepes-based saline buffer (Hepes 20mM, NaCl 150mM). To restore the cholesterol content of cholesterol-depleted cells, cholesterol-methyl-β-cyclodextrin (chol-MBCD) inclusion complexes were first prepared by adding small aliquots of a 180 mM propanol-2 solution of cholesterol to an aqueous solution of MBCD (1g/10 ml). This was performed at 50-80°C under continuous shaking up to a 1/10 MBCD to cholesterol final molar ratio. This chol-MBCD stock solution was further diluted in RMPI medium supplemented with 1% FCS to yield a 2.6 mM MBCD solution, stirred for 40 min at 40°C, then filtered through a Millipore filter (0.22 µm) prior to use. Loading of cholesterol was achieved following incubation of cholesterol-depleted cells for 30 min in a 25 ml final volume of the latter filtered solution. This procedure was performed at 37°C under continuous gentle stirring. Cells were then rinsed twice (Hepes 20mM, NaCl 150mM). The amount of cholesterol in treated cells was normalized to both their protein and phospholipid contents estimated in the whole cell extracts and in some case in plasma membranes extracted according to (31,32). Protein concentration was determined with the bicinchoninic acid protein assay reagent (Pierce) with bovine serum albumin as a standard. Total lipids were extracted according to Bligh and Dyer (33). The amount of phospholipid was estimated by a phosphate assay after total digestion in the presence of perchloric acid (34). Cholesterol was quantified using a colorimetric method based on the oxidation of the hydroxyl group at the carbon atom 3 in the β-position (Roche, Molecular Biochemicals).

**HIV-1 infection, Luciferase assays and real-time quantitative PCR (Taq-Man).** HIV-1 particles were produced and their concentration estimated by measuring both the HIV-1 Gagp24 antigen (ELISA detection kit (NEN™ Life Science Products)) and the β-galactosidase activity in infected Hela CD4LTRLacZ indicator cells, as previously described (29). Briefly, HEK-293T cells were transiently transfected either with the WT R5-HIV-1YU2
proviral DNA or the R5-HIV-1 JR-CSF-luc, which carries the firefly luciferase (luc) reporter gene instead of nef (a gift from Dr.V. Planelles, University of Utah School of Medicine, UT), or were co-transfected with an HIV-1_pNL4-3-luc envelope-deficient (env(-)), proviral DNA containing luc instead of nef and the R5-HIV-1-Ba-L Env-expressing vector. Luciferase activity was measured at the times indicated in the figure legends using a luminometer as described (29). Infection of T-cells with the indicated HIV-1 Gagp24 quantities is detailed in the figure legends. Genomic and HIV-1 reverse-transcribed DNAs were extracted 4 h post-infection after extensive cell washes, using a DNA isolation kit according to manufacturer's indications (Roche, Molecular Biochemicals). HIV-1 primers and probe were designed to detect reverse-transcribed HIV-1 DNA by real-time quantitative PCR using the TaqMan procedure. These were chosen according to Zack et al (35) to detect either all HIV-1 reverse-transcribed DNA (R/U5 region of the LTR) or the full-length DNA (LTR/gag) and modified according to the Primer Expressed™ program (Perkin-Elmer, Applied Biosystems, USA). The forward primer was the same for the both sets: [5′GCTAGCTAGGGAACCCACTGCT3′]. Reverse primers were: [5′CACTGCTAGAGATTTCACACTGAC3′] and [5′GTCTCTGCCTGAGAGATCTCCTCCT3′] mapping to the R/U5 and the R/gag regions, respectively. The probe [5′-FAM-CCTCAATAAAGCTTGCCTTGAGTGCTTCA-TAMRA-3′] carried the 6-carboxy-fluorescein (FAM) fluorescent dye and the 6-carboxy-tetramethylrhodamine (TAMRA) quencher at the 5’ and the 3’ ends, respectively. Quantification of HIV-1 DNA was normalized according to the amount of genomic DNA by measuring the alpha-L-iduronidase (IDUA) cellular gene with [5′ACTTGACCTTCTCAGGGAGAC3′] and [5′CACCTGCTTGCTCCAAAGTCA3′] as forward and reverse primers, respectively, and the probe [5′-FAM-CAGCGCTCAGGGCCACTTCA-TAMRA-3′] (a gift from Dr. J-M. Heard, Unité des Rétrovirus et Transfert Génétique, Institut Pasteur, Paris). Amplification was performed in a total volume of 50 µl containing 2 µl of DNA solution, 1 µM of probe, 50 nM
of each primer and 25 µl of TaqMan Universal PCR Mastermix Mix (Perkin-Elmer Applied Biosystems). Amplification and fluorescence detection were conducted in a 5700 Sequence Detector (Perkin-Elmer Applied Biosystems) with a program including first, 2 min at 50°C and 10 min at 95°C then 40 cycles, each one consisting of 15 sec at 95°C followed by 1 min at 60°C. Intensity was related to the initial number of DNA copies. Standards, consisting of HIV-1 YU2 and human IDUA cDNA-containing plasmids (seven dilutions from 4 to 4 X 10^5 and 38 to 6 X 10^5 copies, respectively), and an HIV-1 minus template control (four dilutions of non-infected cells DNA, in ng: 100, 20, 2 and 0.2) were included in each assay, which are performed in duplicate. Copy numbers of IDUA gene and HIV-1 genomes were determined for the four dilution of DNA samples and by reporting the Ct (the minimum cycle number at which fluorescence was detected) for each assay on the respective standard curves. Mean quantities and standard deviation over the four dilutions of DNA samples were then determined.

Detergent-resistant membranes (DRMs) isolation. DRMs were obtained by sucrose floatation after TX-100 cell lysis according to (36). Briefly, 30 or 60 X 10^6 cells were washed twice in ice-cold TKM buffer (50 mM Tris, pH 7.4, 25 mM KCL, 5 mM MgCl2, 1 mM EGTA) containing a cocktail of phosphatase (5 mM NAF, 10 mM P-Nitrophénylphosphate, 10 mM β-Glycerophosphate, 1 mM Orthovanadate, 200 mM Okadaic acid) and protease (Roche, Molecular Biochemical) inhibitors. Cells were then incubated for one hour on ice in TKM containing 1% TX-100 v/v. Cell lysates were loaded on a sucrose step gradient (in w/v : 5%-35%-40%, 5 or 11 ml gradients for 30 or 60 x 10^6 cell, respectively) and were centrifuged to equilibrium for 20h at 4°C and 200,000 g (Beckman L70-ultracentrifuge). Fractions were collected from the top of the gradient and protein contents estimated using the NanoOrange® quantification kit (Molecular Probes).
**Immunoblotting and Immuno-affinity purification.** Equal volumes of each gradient fraction were resolved by SDS-PAGE using a 4-12% NuPage (Invitrogen) in reducing (CD4, CCR5, p56Lck and LAT) or non-reducing (CD46 and CD55) conditions. For immunodetection, the following antibodies were used: CD4 (1f6, Novocastra), CCR5 (MC5, a gift from Dr M. Mack, Medical Policlinic, University of Munich, Germany), p56Lck (sc-13, Santa-Cruz), LAT (Transduction Laboratories), CD46 (J4-48 Immunotech) and CD55 (sc-9156 Santa-Cruz).

Immobilized antigen-antibody complexes were detected with secondary horseradish peroxidase conjugated anti-species-IgG- (Pierce), developed by enhanced chemiluminescence (ECL+, Amersham), and quantified using a LAS-1000 CCD camera (Image Gauge 3.4 software, Fuji Photo Film Co., Tokyo, Japan). GM1 Ganglioside was detected by slot-blot using peroxidase-coupled CTx (Sigma). For immunoprecipitation, DRM fractions (pooled fractions 3 to 5) were centrifuged at 150,000g for 1 hour at 4°C, pellets resuspended in 50 µl SDS (2 % w/v) for 15 min at room temperature, then diluted with 450 µl TKM containing 1% TX-100 before adding p56Lck or CD4 antibodies (OKT4, a gift from Dr. F. Lemonnier, Unité d’Immunité Cellulaire Antivirale, Institut Pasteur, Paris) and overnight incubation at 4°C. Protein G/A-Sepharose-coupled beads (Calbiochem) were added for 2 hours at 4°C and immunoprecipitates were resolved by SDS-PAGE. 

[^14]C]Palmitate and [^35]S]Met metabolic labeling were performed as previously described (29). Radiolabeled cells were lysed in TKM buffer containing 1% Brij 96 (w/v) and CD4 was immunoprecipitated using OKT4 antibody. Where indicated, cells were incubated in the presence of 2-bromopalmitate (2BP) (100 µM) for 16 hours throughout the labeling time.

**Immunofluorescence Microscopy.** A3.01 cells (1 X 10⁵) were treated to aggregate GM1 as described (26). Briefly, phycoerythrin (PE)-conjugated CTx (Sigma Chemical Co.) was applied for 30 min at 4°C, followed after washing by an anti-CTx monoclonal antibody (Sigma Chemical Co.) for 30 min at 4°C, then for 15 min at 37°C. After three washes in 0.5%
BSA-containing RPMI (blocking buffer), cells were fixed for 10 min in 4% paraformaldehyde (PAF) in PBS. Cells were then incubated in PBS containing 0.1 M glycine in blocking buffer for 30 min and fluorescein isothiocyanate (FITC)-conjugated anti-human-CD4 (SK3, BD Pharmingen) or -CCR5 (2D7, BD Pharmingen) for 30 min. Coverslips were mounted in Mowiol (Hoechst) and fluorescence observed with a confocal laser microscope (Leica TCS4D instrument) using a PL APO X63 oil immersion objective.

Plasmid constructs and stable expression in T-cell lines. CD4 cDNA carrying mutations in cysteines (Cys) interacting with p56Lck (Cys445-Ser/Cys447-Ser) (CD4 Lck-) was a gift from Dr. M. Marsh (MRC Laboratory for Molecular Cell Biology, University College, London, UK). Mutagenesis by PCR of the WT- or -Lck- CD4 cDNAs was performed to substitute Cys residues at position 419 and 422 with Ala residues (Palm- and Palm Lck-, respectively) using overlap extension with T7, Sp6 and two internal primers containing the mutation, as described previously (29). The forward and reverse primers were, respectively:  

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\begin{align*}
5' & \text{GCTGTCAGGGCCCGGCACCGAAGGCGCCAAGCAGAG} \\
3' & \text{GGCCCTGACAGCGAAGAAGATGCCTAGCCCAATGAAAAG}
\end{align*}
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The entire coding region of each construct (Palm-, Lck- or Palm Lck-) was confirmed by sequence analysis, before being cloned into the HIV-1-based lentiviral pTRIP vector (a gift from Dr. P. Charneau, Groupe de Virologie moléculaire et de Vectorologie, Institut Pasteur, Paris). The efficiency of the pTRIP vector relies on the presence of a triple-stranded DNA structure that acts as a cis-determinant of HIV-1 DNA import (37). Stable integration of the transgene into the host DNA permits efficient and long-term transgene expression without clone selection. Virus stock production and infection were as described in (37) in order to transduce and express CD4 variants (WT, Palm-, Lck- or Palm-Lck-) in the A2.01 CD4- T-cell line. Briefly, virus particles were produced after transient co-transfection of HEK-293T cells using a standard calcium phosphate method, with the p8.91 encapsidation plasmid, the pHCMV-G
vector encoding the VSV envelope and the pTRIP vector encoding CD4 variants (WT, Palm-, Lck- or Palm-Lck-). Viral titers were estimated as described above.

Fluorescence-based assay of syncitia induced by HIV-1 envelope. The fluorescence-based assay of syncytia formation induced by HIV-1 Env (FLASH method) is described elsewhere (38). Briefly, BHK cells were infected for eight hours using a Semliki forest virus-based strategy permitting surface expression of the R5-HIV-1-BX08 Env. Infected BHK cells were labeled with SNARF®-1 and A2.01R5 target cells expressing various CD4 variants (WT, Palm-, Lck- or Palm-Lck-) with CellTracker™-Green (CMFDA) for 30 min. Both cells were co-cultured in suspension at a 1:5 BHK to A2.01R5 cell ratio for 12h at 37°C. Fused cells were detected by FACscan analysis as doubly labeled syncitia, using CellQuest software. The dependence of this fusion assay upon the interaction between the HIV-1 Env and receptors was assessed using the following inhibitors, added at the start of the co-cultures: TAK779 (200nM) (NIH AIDS reagent program) and AMD3100 (200ng/ml) (AnorMed, Langley, Canada) that bind to CCR5 and CXCR4, respectively and the CD4 monoclonal antibody, Q4120, that inhibits CD4-dependent fusion (5 μg/ml) (MRC AIDS reagent program).
RESULTS

Role of plasma membrane cholesterol in HIV-1 entry

The effect of the modulation of plasma membrane cholesterol on HIV-1 entry was assessed using real-time quantitative polymerase chain reaction (PCR) in a CD4 lymphoid T-cell line (A3.01) that stably expressed a functional CCR5 transgene (A3.01R5) and in IL-2-expanded primary T-cells that are mostly CD4+. Measurement of newly reverse-transcribed HIV-1 proviral DNA reflects the extent of viral fusion and internalization into target cells.

Modifying the membrane cholesterol content was performed using methyl-β-cyclodextrins (MBCD) (39), known to remove plasma membrane sterol more specifically (40). We found that incubating cells with 5 mM MBCD resulted in a decrease in cholesterol to protein ratios: 10.1±2µg cholesterol per mg of proteins to 4.1±1.5µg cholesterol per mg of proteins in A3.01R5 cells and 19.5±3µg cholesterol per mg of proteins to 7.4±0.9µg cholesterol per mg of proteins in primary T-cells. Phospholipid to protein ratios were unaltered in cells treated with MBCD (124±18 nmoles phospholipid per mg of proteins) as compared to untreated cells (116±9). The same was observed in plasma membranes of MBCD-treated cells (1470 compared to 1365 nmoles phospholipid per mg of proteins in plasma membrane of untreated cells), thus demonstrating the specificity of this reagent for sterols. We controlled that the cholesterol deficit was maintained throughout the 4 hours post-infection experiment and was not detrimental to cell viability or plasma membrane expression of HIV-1 receptors CD4 and CCR5 (data not shown). Plasma membrane cholesterol extraction resulted in a 50 and 60% decrease in newly reverse-transcribed HIV-1-proviral DNA in A3.01R5 and in primary T-cells, respectively, compared to untreated, infected control cells (Figure 1, left and right panels, respectively). The results were the same using primers designed to detect either all HIV-1 reverse-transcribed DNA (R/U5 region of the
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LTR) (Figure 1) or only the full-length reverse-transcribed HIV-1 proviral DNA (R/gag region) (data not shown). The minute amounts of HIV-1 DNA detected in untreated cells infected in presence of a CD4 monoclonal antibody and the nucleotide analog azidothymidine (AZT) (αCD4 + AZT) indicate that this assay allows detection of newly synthesized viral DNA. We observed that re-loading cholesterol (cholesterol/protein ratios = 22.1±5µg/mg and 40.3µg/mg, respectively, in cholesterol-loaded A3.01R5 and primary T-cells) restored the capacity of cells to support HIV-1 entry. This is illustrated by the increase of proviral DNA to levels close to those detected in untreated infected cells (Figure 1, MBCD-Cholesterol). Such near-complete reversion again validates MBCD as a specific and non-deleterious tool. Collectively, these results confirm recent observations regarding the importance of target cell plasma membrane cholesterol in regulating viral entry (16-18) and extend them to primary CD4+ T cells, the natural and major target of HIV-1. The question remains whether the observed regulatory effect of plasma membrane cholesterol relates to perturbation of raft domain integrity.

CD4 but not CCR5 localizes in rafts of T lymphocytes

The insolubility of some lipids and proteins in cold non-ionic detergent Triton X-100 (TX-100) correlates well with their partitioning into ordered phases of biological membranes (3,41). This makes it possible to recover detergent-resistant membrane components as low-density material after centrifugation to equilibrium on a sucrose density gradient. Accordingly, the partitioning of CD4 and CCR5 was investigated in primary T (Figure 2A) and A3.01R5 cells (Figure 2B). Low-density fractions of primary T cells (Figure 2A, fractions 3-5) collected from the top of the gradient were enriched in gangliosides like GM1, as detected by specific binding of the choleratoxin subunit B (CTx). By loading equal volumes of each fraction, the relative quantities of the marker studied can be assessed, regardless of the total protein content of each fraction. Indeed, a minor amount of the total protein content was
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recovered in the low-density fractions and the bulk (90 to 95%) was present in the fractions of higher density (data not shown and (42)). We found that both the tyrosine kinase p56Lck, which is known to associate with the cytoplasmic leaflet (Figure 2A, Lck), and the exoplasmic CD55 leaflet-glycophosphatidylinositol-anchored protein float with insoluble material at the top of the gradient (Figure 2A, fractions 3-5), as reported by others (43,44). CD4 was observed to segregate partially within the insoluble low-density fractions (fractions 3-5) (Figure 2A), along with a marker of these fractions, the LAT transmembrane protein (Figure 2A) (24). In sharp contrast, CCR5 was fully solubilized in 1% TX-100 (Figure 2A, fractions 9-11). The same was observed for the membrane-spanning protein, CD46 (Figure 2A), a measles virus receptor also known to associate with TX-100-soluble membranes (45).

In A3.01R5 cells (Figure 2B, upper panel), CCR5 partitioning was similar to that of the endogenous CCR5 protein in primary T cells (Figure 2A), i.e. CCR5 remained fully solubilized in 1% TX-100 in both cell types. Moreover, in both A3.01R5 (Figure 2B) and A2.01R5 cells transduced with the wild-type (WT) CD4 molecule (Figures 4 and 5), the relative distribution of CD4 in both low- and high-density fractions was similar to that observed in primary T cells (Figure 2A). One may thus conclude that the behavior of CCR5 and CD4 after stable ectopic expression in T-cell lines is similar to that of their endogenous counterparts in primary T lymphocytes.

The above results agree with previously reported partitioning of CD4 to sphingolipid-enriched, detergent-resistant domains (11-13). This contrasts strongly with the distribution pattern of CCR5, which was recovered in 1% TX-100 soluble domains where GM1 remains only marginally localized. Sphingolipids (i.e., glycosphingolipids and sphingomyelin) differ from the other class of membrane lipids, glycerolipids, in that they contain long, highly saturated fatty acyl chains. In appropriate admixture with cholesterol, sphingolipids form
detergent-resistant domains in the plasma membrane (*i.e.* rafts) (46) that are sensitive to cholesterol depletion (22).

The nature of CD4- and GM1-enriched TX-100-resistant domains was further characterized following extraction of cholesterol-depleted A3.01R5 cells. As shown in Figure 2C, decreasing the cholesterol content of plasma membranes resulted in a sharp enrichment of CD4 in 1% TX-100-soluble membranes, where CCR5 resides. This correlated with a parallel redistribution of GM1 throughout the sucrose gradient (Figure 2C). These results illustrate the role that cholesterol plays in structuring the detergent-resistant membrane domains where GM1 and CD4 are found. Moreover, compared to published phase diagrams of cholesterol:phospholipid binary mixtures (47), the sterol content found in our isolated TX-100-resistant domains (24 mol %) is consistent with the lipid phase described in detergent-resistant membranes (48). These domains are henceforth referred to as *rafts*, in accordance with the previously proposed nomenclature for detergent-resistant membrane domains composed of cholesterol and sphingolipids (49).

Although the above results suggest that CCR5 and CD4 do not colocalize to 1% TX-100-resistant rafts in T cells, the hypothesis of a colocalization to raft domains of lesser detergent-resistance was envisaged. This possibility was based on the structural heterogeneity of membrane lipids (including cholesterol) that form a mosaic of domains in membranes (1,50). The fact that the solubility of membrane proteins depends on the concentration of the non-ionic detergent used is in keeping with this postulate (50,51). We thus investigated the possibility that CCR5 and CD4 colocalize to rafts of lesser detergent-resistance. A3.01R5 cells were lysed in decreasing TX-100 concentrations. At low TX-100 concentrations (0.1%), relatively less CD4 was found in the soluble pool (Figure 2B, fractions 8-10) and more was recovered from the low-density insoluble fractions (Figures 2B (fractions 3-5) and 2D). In contrast, the bulk of CCR5 always remained soluble and sedimented in the gradient.
independently of the TX-100 concentration (Figure 2B, middle and lower panels). CCR5 was not detected significantly in 0.3% TX-100-resistant membrane domains (Figure 2B, middle panel, fractions 3-5) and only a minor part was detected in 0.1% TX-100-resistant membrane domains (Figure 2B, lower panel, fractions 3-5 and Figure 2D). Moreover, quantification of CCR5 in low-density fractions expressed as a function of TX-100 concentration, revealed a distribution (Figure 2D) identical to that of CD46 (Figures 2B and D), which is representative of TX-100-soluble membrane proteins (45). These observations further support our previous conclusion that CCR5 is excluded from rafts and highlight that only a very small amount of the receptor is found in membrane domains of lesser detergent resistance.

To rule out that CCR5 might transiently distribute into CD4-containing rafts, we forced stabilization of these domains into visible patches by specific clustering of GM1 by cross-linking with CTx. The coalescence of Influenza Hemagglutinin (HA)- and GM1-containing domains following GM1-CTx cross-linking was taken to reflect the propensity of HA to localize to GM1-enriched rafts (26). Prior to CTx cross-linking, both CD4 and CCR5 receptors were found evenly distributed over the plasma membrane (Figures 3A and B, left panels). Cross-linking of GM1-CTx complexes with a rhodamine-labeled CTx antibody induced redistribution of CD4 into patches (Figure 3A, second-panel) that overlapped GM1 patches (Figure 3A, GM1 and overlay panels). In marked contrast, CCR5 distribution remained unchanged and did not significantly overlap with GM1-patches following CTx-dependent cross-linking (Figure 3B, GM1 and overlay panels). This clearly shows that CCR5 is mainly excluded from GM1-enriched domains and behaves differently from CD4 in this respect.

As concerns the requirement of HIV-1 entry on rafts, several possibilities remain. It was proposed that HIV-1 binding to CD4 might occur within rafts and that, subsequently, CD4-Env complexes redistribute to coreceptor-containing non-raft domains (52). Alternately,
supramolecular complexes of CD4, the viral Env and the coreceptor (53-58) could dock in raft domains (16-18). To test these possibilities, we investigated whether receptor cross-linking induced by R5-HIV-1 Env caused membrane redistribution of either CD4 or CCR5. A2.01R5 CD4 WT cells, exposed to R5-HIV-1-Ba-L virions for 45 or 90 min, were lysed in 1% TX-100, and fractioned on a sucrose gradient. As shown in Figure 4 adsorption of virions onto these cells did not modify CCR5 or CD4 distribution compared to uninfected control cells (Figure 4).

These results emphasize the distinct propensities of HIV-1 receptors to partition into raft domains where GM1 is enriched. Taking into account both the resting distribution of HIV-1 receptors at the cell membrane and the lack of redistribution upon adsorption of HIV-1 virions, we conclude that the necessary colocalization of CD4 and CCR5 required for viral entry (57,58) most likely occurs outside rafts. To directly address this issue we decided to force CD4 outside rafts and investigate the consequences of such CD4 redistribution on HIV-1 entry.

**Palmitoylation and interaction with p56Lck anchor CD4 in rafts of the membrane**

The possibility that palmitoylation of cysteine residues (Cys 419, Cys 422) at the boundary of the cytoplasmic and the membrane spanning regions of CD4 (27) could affect its distribution at the plasma membrane was explored. We stabilized expression of human CD4 WT in the CD4-negative A2.01R5 T cells using a lentivirus-mediated strategy. CD4 WT-expressing cells were then treated with 2-bromopalmitate (2BP), a palmitic acid analog that inhibits palmitoylation (29,59). The partition of CD4 in detergent-resistant and detergent-soluble fractions of untreated- and 2BP-treated cells was compared following extraction in 1% TX-100 and fractionation on sucrose gradients. Figure 5A shows that CD4 (CD4 WT, upper panel) distributed into low- (3-5) and high-density (9-11) fractions enriched in GM1
and CCR5, respectively, as previously observed (Figure 2). Importantly, blocking the metabolic incorporation of palmitate (Figure 5B, left panel, lane 3) resulted in an important redistribution of CD4 into high-density, non-raft fractions (9-11) (Figure 5A, CD4 WT + 2BP).

This non-raft distribution of CD4 in cells treated with 2BP indicated that palmitoylation is a key factor in regulating the distribution of this receptor within the cell membrane. Interestingly, 2BP also inhibited p56\textsuperscript{Lck}-palmitoylation and prevented its localization to rafts (59). CD4 interacts non-covalently with p56\textsuperscript{Lck} through its C-terminal cysteine residues (Cys 445 and Cys 447) (60) and it was proposed that this association occurs early in the secretory pathway, allowing both proteins to reach the plasma membrane together (61). Aside from blocking CD4 palmitoylation, 2BP treatment may indirectly affect CD4 distribution by preventing p56\textsuperscript{Lck} localization to rafts.

To explore these possibilities, CD4 cDNAs carrying mutations in cysteine residues involved in CD4-palmitoylation (CD4 Palm\textsuperscript{−}), in its interaction with p56\textsuperscript{Lck} (CD4 Lck\textsuperscript{−}) or both (CD4 Palm\textsuperscript{−}Lck\textsuperscript{−}), were expressed in CD4-negative A2.01R5 T-cells. Membrane distributions of these molecules were compared to that in CD4 WT cells following 1% TX-100 solubilization (Figure 6A). Similar to the effect of 2BP (Figure 5), direct prevention of CD4 palmitoylation by mutation (CD4 Palm\textsuperscript{−}, Figure 5B, left panel, lane 2) shifted CD4 to high-density fractions (Figure 6A). This strongly suggests that palmitoylation \textit{per se} directs membrane localization of CD4 without affecting its biosynthesis (Figure 5B, right panel \[^{35}\text{S}]\text{Met}\) labeling) and cell surface expression (data not shown). Similar results obtained for the CD4 Lck\textsuperscript{−} mutant show that, in addition, CD4/p56\textsuperscript{Lck} interaction also directs CD4 to rafts (Figure 6A, (CD4 Lck\textsuperscript{−})). In keeping with these observations, the double mutation (CD4 Palm\textsuperscript{−}Lck\textsuperscript{−}) almost abolished CD4 association with low-density fractions (Figure 6A and B, 6±2 % remaining in the low-density fractions). The distribution of the WT and mutant CD4 proteins
in 1% TX-100-soluble and resistant fractions are compared in Figure 6B. The lower recovery of the CD4 Palm’Lck’ mutant protein in TX-100-resistant low-density fractions (3-5) is consistent with an additive effect of palmitoylation and lack of interaction with p56Lck in the targeting of CD4 to rafts. In agreement with previous reports (61), the distribution of p56Lck at the plasma membrane in raft and non-raft domains (Figure 6C, D and S fractions, respectively) is independent of its interaction with CD4 (Figure 6D). The reduced cell surface expression of the CD4 protein in the Lck’ and Palm’-Lck’ cells, compared to its expression in CD4 WT or Palm’ cells (data not shown), is in keeping with CD4 down-regulation observed in cells lacking p56Lck (62).

**CD4 redistribution into non-raft domains does not impair CCR5-dependent HIV-1 entry**

The consequences of expression of the CD4 double mutant (Palm’Lck’) on R5-HIV-1 entry were explored. We first used an HIV-Env glycoprotein-mediated cell fusion assay that is independent of the post-entry steps of the viral cycle and allows accurate quantification of fusion events (38). This method relies on syncytia formation between cells expressing the HIV-1 R5 BX08 Env glycoprotein and CD4 -stabilized A2.01R5 target cells (Figure 7A). CD4-WT and CD4-Palm’Lck’ cells showed the same capacity to fuse with R5-Env expressing cells. Cell fusion was prevented by blocking with either monoclonal antibody Q4120 that recognizes the gp120-binding domain of CD4 (63), or TAK779 that binds to CCR5 (64).

Another alternative assay was used to verify the apparent independence of the HIV-1-R5 entry on the presence of CD4/CCR5 in rafts. CD4 WT and CD4 Palm’Lck’-expressing cells were inoculated with cell-free virions generated upon trans-complementation of a luciferase reporter HIV-1 provirus (Env’) with an R5-HIV-1 Ba-L Env (Figure 7B). This pseudotyped virus yields only a single-round of infection, thereby allowing evaluation of
HIV-1 entry by quantifying viral replication. Consistent with our findings in the cell-cell fusion assays (Figure 7A), R5-HIV-1 virus replicated to the same levels in both CD4 WT and Palm' Lck' cells (Figure 7B). Similar results were obtained with HIV-1 pseudotyped with an R5-strain Env (CSF) or the WT HIV-1 YU2 R5-strain (data not shown). These results demonstrate that CD4 delocalization outside rafts does impair neither HIV-1 Env-mediated cell-cell fusion, nor entry and replication of HIV-1 cell-free particles.

We conclude that adsorption of R5-viruses, which exposes fusogenic HIV-1 gp41 Env trans-membrane subunits allowing subsequent virus entry, does not require localization of viral receptors to detergent-resistant raft domains.
DISCUSSION

Given the critical structural role played by cholesterol in promoting raft domain formation, it was postulated that removal of cholesterol would interfere with HIV-1 infection by disrupting rafts. The aim of this work was to critically evaluate the contribution of membrane rafts to HIV-1 entry. In particular, we investigated whether the HIV-1 receptors, CD4 and CCR5, need to reside in rafts to promote viral entry into CD4 T lymphocytes.

CCR5 does not partition into rafts

Upon stable expression in T-cell lines, CCR5 did not associate with rafts. This result is in keeping with a recent observation by Nguyen et al. (65). Fluorescent microscope findings fully corroborated flotation studies, showing that the bulk of CCR5 localizes to non-raft domains of plasma membranes. Furthermore, in biologically relevant primary CD4 T-cells, the endogenous CCR5 receptor was found exclusively in detergent-soluble, non-raft domains.

It is likely that the recovery of CCR5 in 0.2% TX-100-resistant membranes previously reported in adenocarcinoma cells transiently over-expressing a CCR5 transgene (66) reflects spillover of over-expressed CCR5 in rafts. In the latter, the relatively high recovery of CCR5 (11-18%) in 0.2% TX-100-resistant membranes could have resulted from loading equal amounts of protein rather than equal volumes of each fraction of the gradient. Indeed, with most of the cellular proteins concentrated in soluble fractions ((42) and our data not shown), hence equalizing the amounts of protein may lead to an over-estimation of the amount of CCR5 in detergent-resistant fractions.

Although CCR5 appears to be present in detergent-soluble membrane domains, we envisaged that HIV-1 receptor engagement by viral Env might recruit CCR5 to rafts, to which CD4 and GM1 localize. This explanation was recently advanced to account for redistribution of CXCR4 by soluble X4-HIV-1 Env (16,18). However, the finding by Kozak et al. that
CXCR4 coreceptor distribution into detergent-soluble domains is not modified by virion adsorption (52) challenges previous observations (16,18). HIV-1 entry is expected to be a cooperative process requiring the assembly of HIV-1 virions with CD4 and coreceptors. We therefore investigated whether R5-HIV-1 virion binding, which facilitates initial CD4 cross-linking unlike soluble monomeric viral Env, would induce CCR5 redistribution and colocalization with CD4 in T-cell rafts. In flotation studies, bridging of viral receptors by R5-HIV-1 virions adsorbed on T-cells did not shift CCR5 to raft fractions. Taken together, our data strongly suggest that recruitment of CCR5, to rafts is not a prerequisite for virus entry.

One could speculate that CCR5-enriched detergent-soluble membrane domains may form a ring around a raft area where virus could interact with the "raft "fraction of CD4. This hypothetical situation would be similar to the membrane organization described in T lymphocytes interacting with antigen-presenting cells (67), where the T-cell receptor interacts with an MHC II-bound cognate peptide not included in rafts. The role played by CD4 residing in rafts on HIV-1 infection was explored expressly to test this hypothesis.

Redistribution of CD4 outside raft domains does not affect HIV-1 infection

In contrast to CCR5, CD4 was equally distributed in both TX-100-resistant and TX-100-soluble membranes. The non-raft redistribution of the non-palmitoylated CD4 protein strongly suggests that palmitoylation determines the targeting of this receptor to rafts. The role of fatty acylation in targeting membrane proteins to rafts (68,69) has been established for a number of integral palmitoylated proteins, such as LAT and CD8αβ (24,25). However, palmitoylation per se does not obligatorily direct the modified protein to rafts, as illustrated in our work showing exclusion of palmitoylated CCR5 (29) from rafts; this further illustrates that other molecular features of the protein determine its raft/non-raft distribution. For instance, both CD4 palmitoylation and its interaction with p56Lck contribute to the presence of CD4 in raft domains. The association of p56Lck with the non-palmitoylated CD4 outside rafts is actually...
decreased, as observed previously for p56\textsuperscript{Lck} and CD8 (25). In agreement with previous studies (61,70) we showed that p56\textsuperscript{Lck} partitioning into raft domains occurs independently of its association with CD4.

We used different mutants of CD4 to analyze the impact of CD4 delocalization from raft domains on infection of T-cells by R5-HIV-1 strains. Importantly, we observed that excluding most of the CD4 from rafts does not alter its capacity to support R5-Env–mediated cell-cell fusion and entry of R5-HIV-1 viruses. These data are fully consistent with previous observations suggesting that CD4-p56\textsuperscript{Lck} association is not necessary for HIV-1 infection (71,72). Our results reinforce the view that rafts are not sites where virus binding and entry actually take place. This conclusion is in keeping with biophysical considerations suggesting that membrane fusion is more favorable in non-raft membrane environments of lipids, such as glycerophospholipids (73).

**Membrane cholesterol is required for efficient HIV-1 entry in host cells**

MBCD-extraction of cholesterol inhibits entry of R5-HIV-1 into primary CD4 T-cells, its natural host cell. This confirms and extends recent observations in transformed cell lines stressing the requirement for cholesterol in HIV-1 infection (16-18,74). However, taking into account that cholesterol is present everywhere on the plasma membrane (21,22), inhibition of HIV-1 infection by cholesterol depletion does not necessarily implicate disturbances of rafts integrity. We showed that R5-HIV-1 fusion and entry are independent of CD4/CCR5 localization in membrane rafts. Consequently, it is unlikely that the inhibitory effect of cholesterol depletion on HIV-1 entry results from disorganization of rafts.

The role of cholesterol in HIV-1 entry can be interpreted differently. The membrane lipid environment, into which the HIV-1 gp41 fusion peptide partitions, plays an important role in the fusion of viral and cell membranes (75); plasma membrane cholesterol may be a critical component in this process. Membrane fusion is purported to set off local bending of
lipid bilayers converging to a common type of non-lamellar, highly curved, stalk intermediates (73,76). Indeed, fusion depends very much on lipids of spontaneous negative curvature (i.e. unsaturated phosphatidylethanolamine, cholesterol) to promote membrane bending (77,78). Likewise, synthetic peptides mimicking the HIV-1 gp41 fusion domain were found to promote negative curvature that facilitates formation of stalk intermediates (79). The fusogenic activity of these peptides was stimulated by insertion of cholesterol into a non-raft environment (80). Therefore, the observed interference of the lack of cholesterol with HIV-1 entry might reflect the altered capacity of plasma membranes to undergo viral-induced fusion.

Furthermore, the role played by cellular cholesterol in HIV-1 entry may be related to its capacity to modulate the activity of membrane proteins. This has been shown for several G-protein coupled receptors (81,82) and also applies to the HIV-1 coreceptors, CCR5 and CXCR4. Effectively, depletion of cell membrane cholesterol has been shown to modify binding of CCR5 and CXCR4 to their natural chemokine ligands, MIP1-β and SDF-1α, respectively (65,74). Nonetheless, whether diminished HIV-1 infection in cholesterol-depleted cells relates to modulation of CCR5 or CXCR4 still remains speculative.

In conclusion, our findings exclude a significant participation of membrane rafts in HIV-1 binding to host cells and point to an important role for cholesterol in the mechanisms of viral entry into primary CD4 T lymphocytes. Further study, using real time and quantitative approaches, are needed to address the particularly intriguing modulator effect of plasma membrane cholesterol on the HIV-1 entry process.
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FIGURE LEGENDS

FIG.1. **Effect of cholesterol on R5-HIV-1 entry.** Untreated or Methyl-β-Cyclodextrin (MBCD) treated A3.01R5 and primary T cells were tested for R5-HIV-1 entry. Depleted cells were cholesterol back-loaded (MBCD-Cholesterol). Cells ($5 \times 10^6$) were infected for 4 hours with HIV-1 YU$_2$ (75 or 300 ng of HIV-1 Gagp24 for A3.01 and primary T-cells, respectively). Untreated control cells were also infected in the presence of CD4 monoclonal antibody Q4120 (5 µg/ml) and the nucleoside analog AZT (50 µM) [αCD4 + AZT] throughout infection. Following infection, genomic- and reverse-transcribed HIV-1- DNAs were quantified by fluorescence-monitored, real-time PCR analysis. Values calculated as number of HIV-1 genomes/ cell genome are expressed relative to values obtained for untreated cells (taken as 100%).

FIG.2. **CCR5 does not localize into rafts in primary T cells, in contrast to CD4.** A, primary T-cell lysates (1% TX-100) were loaded onto a step sucrose gradient (11 ml). Equal volume fractions were analyzed for the presence of CD4, CCR5, Lck, LAT, CD46, CD55 and GM1. B, A3.01R5 cell were lysed in 1, 0.3 or 0.1% TX-100 and loaded onto a small gradient (5 ml). Equal volume fractions were collected and analyzed for the presence of CD4, CCR5, CD46 and GM1. C, A3.01R5 cells were subjected (MBCD-treated cells) or not (untreated cells) to cholesterol depletion. Following an additional incubation of cells for 1 hour at room temperature in 1% FCS supplemented RPMI medium, cells were extracted with 1%TX-100 and sucrose gradient fractions were analyzed for the presence of CD4, CCR5 and GM1. D, CD4, CCR5 and CD46 content of low-density fractions 3 to 5 (i.e.: detergent-resistant membranes, DRMs) collected from the gradient shown in (B) were quantified. Protein enrichment was expressed as % of the quantities present in DRM fractions over the total protein recovered. Standard deviations from four determinations are shown.
FIG. 3. **Patching of GM1 with Cholera toxin does not induce clustering of CCR5, in contrast to CD4.** Aggregation of GM1 clusters was initiated by incubation of the CTx-PE-labeled, A3.01R5 cells with CTx antibody (GM1). CD4 (A) and CCR5 (B) staining before (left hand panels) and after GM1 clustering (GM1-CTx-cross-linking) are shown. The merge of GM1 images with those of CD4 (A) or CCR5 (B) are also presented (overlay).

FIG. 4. **R5-HIV-1 binding does not redistribute CD4 and CCR5.** Binding of R5-HIV-1-Ba-L to A2.01-CD4R5 cells (24 µg of Gagp24 /60 X 10⁶ cells) was performed at 4°C for 45 min, then at 37°C for an additional 45 or 90 min accordingly to (54). Uninfected control cells were treated in parallel. DRMs were isolated from sucrose gradients of cell lysates and CD4 and CCR5 were detected for each condition. CD46 and GM1 representative profiles are shown. Luciferase activity (50 x 10³ U/µg of protein), detected 48 hours post-infection, controlled for the efficiency of HIV-1 entry and replication (data not shown).

FIG. 5. **CD4 palmitoylation and distribution into rafts are affected by 2-bromopalmitate.** A, A2.01R5 cells expressing CD4 WT were either left untreated or were treated overnight with 2BP (CD4 WT + 2BP). DRMs were isolated on sucrose gradients and CD4, CCR5, CD46 and GM1 were detected. B, CD4 palmitoylation was monitored in A2.01R5 cells expressing either the WT (CD4 WT) or the palmitoylation-deficient (CD4 Palm⁻) CD4 proteins after incorporation of labeled [¹⁴C]-palmitate (left panel). Cells were also metabolically labeled with [³⁵S]-Met (right panel).

FIG. 6. **Disruption of palmitoylation and p56Lck interaction sites determines exclusion of CD4 from rafts.** A, distribution pattern in sucrose gradients of CD4 WT protein and its
variants (Palm⁺, Lck⁻ or Palm⁻Lck⁻) stably expressed into A2.01R5 cells. B, CD4 was quantified in DRMs (3-5) or soluble (9-11) fractions and is expressed as % of the total CD4 in the whole gradient. Data represent the mean values of six independent experiments. C, p56Lck detection in pooled DRMs (3-5) and soluble fractions (9-11) of gradients shown in (A). D, interaction of p56Lck with CD4 WT and its mutants was monitored in DRMs (fractions 3-5) by co-immunoprecipitation and Western Blot.

FIG.7. Extensive CD4 delocalisation from rafts neither impairs fusion nor replication of R5-HIV-1. A, parental A2.01 cells (CD4 negative) or A2.01R5 cells stably expressing either CD4 WT or CD4 Palm⁻Lck⁻ mutants were co-cultured with BHK cells expressing the BX08 R5-Env. Syncitia formation was monitored by FACscan analysis. When indicated, inhibitors of HIV-1 entry were added 30 min before the starting of the fusion assay. B, These cell lines were infected with R5-HIV-1-Ba-L (75 ng of p24 for 10⁶ cells). Luciferase activity was analyzed 65 hours after infection. Standard deviations of two independent determinations done in triplicate are shown.
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Fig. 1
Fig. 2
Fig. 4
Fig. 6
Fig. 7
HIV-1 entry into T-cells is not dependent on CD4 and CCR5 localization to sphingolipid-enriched, detergent-resistant, raft membrane domains

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