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Human Immunodeficiency Virus Type 1 Virological Synapse Formation in T Cells Requires Lipid Raft Integrity

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Human immunodeficiency virus type 1 (HIV-1) can spread directly between T cells by forming a supramolecular structure termed a virological synapse (VS). HIV-1 envelope glycoproteins (Env) are required for VS assembly, but their mode of recruitment is unclear. We investigated the distribution of GM1-rich lipid rafts in HIV-1-infected (effector) T cells and observed Env colocalization with polarized raft markers GM1 and CD59 but not with the transferrin receptor that is excluded from lipid rafts. In conjugates of effector T cells and target CD4$^+$ T cells, GM1, Env, and Gag relocated to the cell-cell interface. The depletion of cholesterol in the infected cell dispersed Env and GM1 within the plasma membrane, eliminated Gag clustering at the site of cell-cell contact, and abolished assembly of the VS. Raft integrity is therefore critical for Env and Gag coclustering and VS assembly in T-cell conjugates.

Efficient spread between T cells by the retroviruses human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1 can take place in vitro via the formation of a virological synapse (VS) (3, 17). Previous studies have demonstrated that this mode of viral dissemination is productive and more efficient than that achieved by infection with cell-free virions (25, 31). HIV-1 dissemination via VS may contribute to the rapid spread of HIV-1 within secondary lymphoid tissues in vivo, particularly during early infection (5). Similarly, HIV-1-infected or uninfected but pulsed dendritic cells can efficiently transfer virus to T cells via a synapse (2, 21, 26), and this may represent an important pathway of viral dissemination after mucosal inoculation (28). Furthermore, the movement of HIV-1 across an adhesive junction may help to protect the virus from elements of the humoral immune response, such as antibodies and complement.

The HIV-1 VS assembles at the site of contact between an infected (effector) and receptor-expressing (target) cell and is exemplified by the recruitment of the viral receptors (CD4 and a chemokine receptor, CXCR4 or CCR5) and adhesion molecules on the target cell and of viral Env and Gag and cognate adhesion molecules on the effector cell (16, 17, 26). The assembly of these supramolecular complexes relies on engagement of the viral receptors by Env and on a functional actin cytoskeleton in the target cell (16). The recruitment of Env and Gag to the intercellular junction is followed by viral antigen transfer across the synapse into the target cell (16), which has been shown by others to lead to rapid de novo reverse transcription (8, 18, 31).

HIV-1 particles assemble under and bud through specific plasma membrane domains termed lipid rafts or related raft-like structures (6, 9, 15, 20, 22, 24). Rafts are biochemically and biophysically distinct regions of plasma membrane that are rich in cholesterol and sphingomyelin, maintain an ordered structure, and contain proteins important in immune cell signaling via the immunological synapse (IS) (13). Virion envelopes similarly contain a large proportion of cholesterol and sphingomyelin (1), which is important for the maintenance of virion structure and infectivity (7, 10). Virion assembly is targeted to rafts or raft-like regions of the plasma membrane via lipid interactions with acylated residues in viral Gag (6, 9, 15, 20, 22, 24) and Env (4, 29). What has not been investigated is the role of lipid rafts in the targeting of viral Env and Gag to the plasma membrane in infected T cells and in the subsequent assembly and function of the HIV-1 VS.

To localize HIV-1 Env and lipid rafts in the plasma membrane, we labeled T cells with the biotinylated cholera toxin β subunit (B-Ctx) to detect the raft marker ganglioside GM1 and Env with the gp41-specific monoclonal antibody (MAb) 50–69 (16). B-Ctx and 50–69 binding was detected using streptavidin-fluorescein isothiocyanate (FITC) and anti-human–tetramethyl rhodamine isocyanate (TRITC), respectively, and immunofluorescence was analyzed by laser scanning confocal microscopy (LSCM). Unless marked, images are single *x-y* optical sections through the middle of the T cell. Jurkat CE6.1 cells show strong GM1 membrane staining distributed around the entire plasma membrane, resting primary CD4$^+$ T cells show no detectable fluorescence, and 3-day phytohemagglutinin (PHA)-activated CD4$^+$ T-cell blasts show patchy diffuse membrane staining (Fig. 1A). This pattern of labeling agrees with previous studies showing a redistribution of GM1 from intracellular stores to the plasma membrane upon the activation of resting T cells (32). The infection of Jurkat cells with CXCR4-tropic HIV-1_{LAI} (Jurkat$_{LAI}$) led to the plasma membrane expression of Env, as detected by MAb 50–69. Env was generally cocapped with GM1 at one pole of the T cell, and both markers showed strong colocalization (Fig. 1B). This pattern of staining was observed whether labeling was carried out prior to or after fixation, excluding the possibility of ligand-induced capping. Similar results were obtained with PHA-activated primary CD4$^+$ T cells infected for 5 days with HIV-1_{LAI} (Fig. 1C). To confirm the lipid raft nature of the GM1-Env membrane compartment, we labeled the phosphati-

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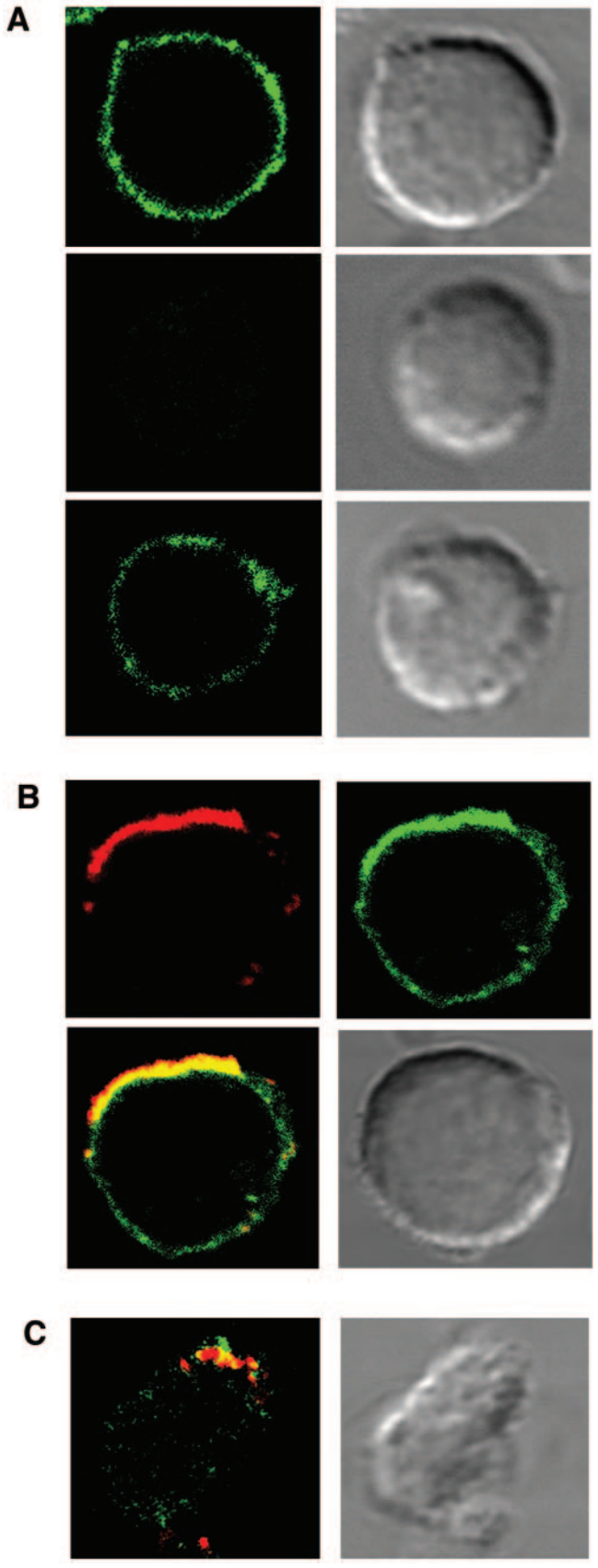

dylinositol-linked CD59 molecule that segregates into rafts. Jurkat$_{LAI}$ cells showed tight polarized clustering of GM1, CD59, and Env (Fig. 2A), whereas minimal copolarization of Env and GM1 was observed with the transferrin receptor (TfR), which is excluded from rafts (Fig. 2B) (14). Although colocalization by immunofluorescence labeling and LSCM suggests a cosegregation of molecules, the resolution of this technique is not sufficient to unequivocally demonstrate this. We therefore analyzed Jurkat$_{LAI}$ cells immunolabeled for Env and GM1 by cryo-electron microscopy. Figure 2C shows GM1 in the plasma membrane and colabeling of GM1 and Env in a budding virion, in accord with previous studies showing that HIV incorporates raft markers into the viral envelope (22, 30). In contrast, budding virions did not incorporate control markers such as LAMP-2 (data not shown).

The capping of GM1 in HIV-1-infected but not uninfected T cells implied that viral proteins were directing the aggregation of rafts in the plasma membrane. The most likely candidate for this is Gag, which was demonstrated by others using biochemical techniques to cluster rafts into large zones of Gag-raft aggregates, termed "barges" (20), with a higher density than that of noncoalesced rafts (9, 15, 24). We therefore analyzed the distribution of Gag in relation to GM1 staining in Jurkat$_{LAI}$ cells. Gag colocalized strongly with Env and GM1 at one pole of the T cell (Fig. 3A).

In the IS, many proteins required for T-cell activation are recruited to the cell-cell contact surface in the context of lipid rafts (12). To investigate whether HIV-1 Gag and Env are similarly enriched in rafts at the VS, we formed conjugates and probed for GM1, Gag, and Env in effector cells and CD4 in target cells. Within 1 h, 47% of conjugates had formed a VS, as defined by copolarization to the site of cell-cell contact, of HIV antigens in the effector cell and CD4 in the target cell (Table 1 and Fig. 3B). Of these VS, the majority (63%) showed Env and Gag polarization to the VS in the context of GM1 (Table

FIG. 1. GM1 and Env expression in uninfected and infected T cells. (A) Jurkat CE6.1 T cells (top panels), naïve CD4$^+$ T cells negatively enriched from peripheral blood mononuclear cells (PBMC) (middle panels), or 3-day PHA (5 μg/ml)- and interleukin-2 (10 IU)-activated primary CD4$^+$ T cells (bottom panels) were washed and resuspended in RPMI-1% fetal calf serum (WB), and 5×10^5 cells were allowed to settle onto poly-L-lysine-coated coverslips, fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline–1% bovine serum albumin, and stained for GM1-containing lipid rafts (green) with 10 μg/ml biotinylated B-Ctx (Sigma, United Kingdom) at 4°C followed by streptavidin-FITC (Jackson Immunoresearch). All staining with B-Ctx was performed on fixed cells to prevent Ctx-induced cross-linking and GM1 capping. (B) HIV-1$_{LAI}$-infected (Jurkat$_{LAI}$) effector cells were washed, allowed to settle onto poly-L-lysine-coated coverslips, and stained for surface Env (red) and GM1 (green) with 10 μg/ml of the human Env-specific antibody 50–69 (CFAR, United Kingdom) and 10 μg/ml B-Ctx followed by anti-human-TRITC and streptavidin-FITC, respectively. (C) HIV-1$_{LAI}$-infected 3-day PHA- and interleukin-2-activated primary CD4$^+$ T-cells negatively enriched from PBMC by magnetic selection (Miltenyi Biotech, United Kingdom) were stained for Env (red) and GM1 (green) as described above. All images represent single *x-y* sections through the center of a cell and were acquired using a Bio-Rad MicroRadiance laser scanning confocal microscope and subsequently processed using Metamorph v6 (Universal Imaging Corporation) and Photoshop 7.0 (Adobe Inc.). Fluorescence images are shown next to the corresponding Nomarski images, and the colocalization of red and green gives yellow staining.

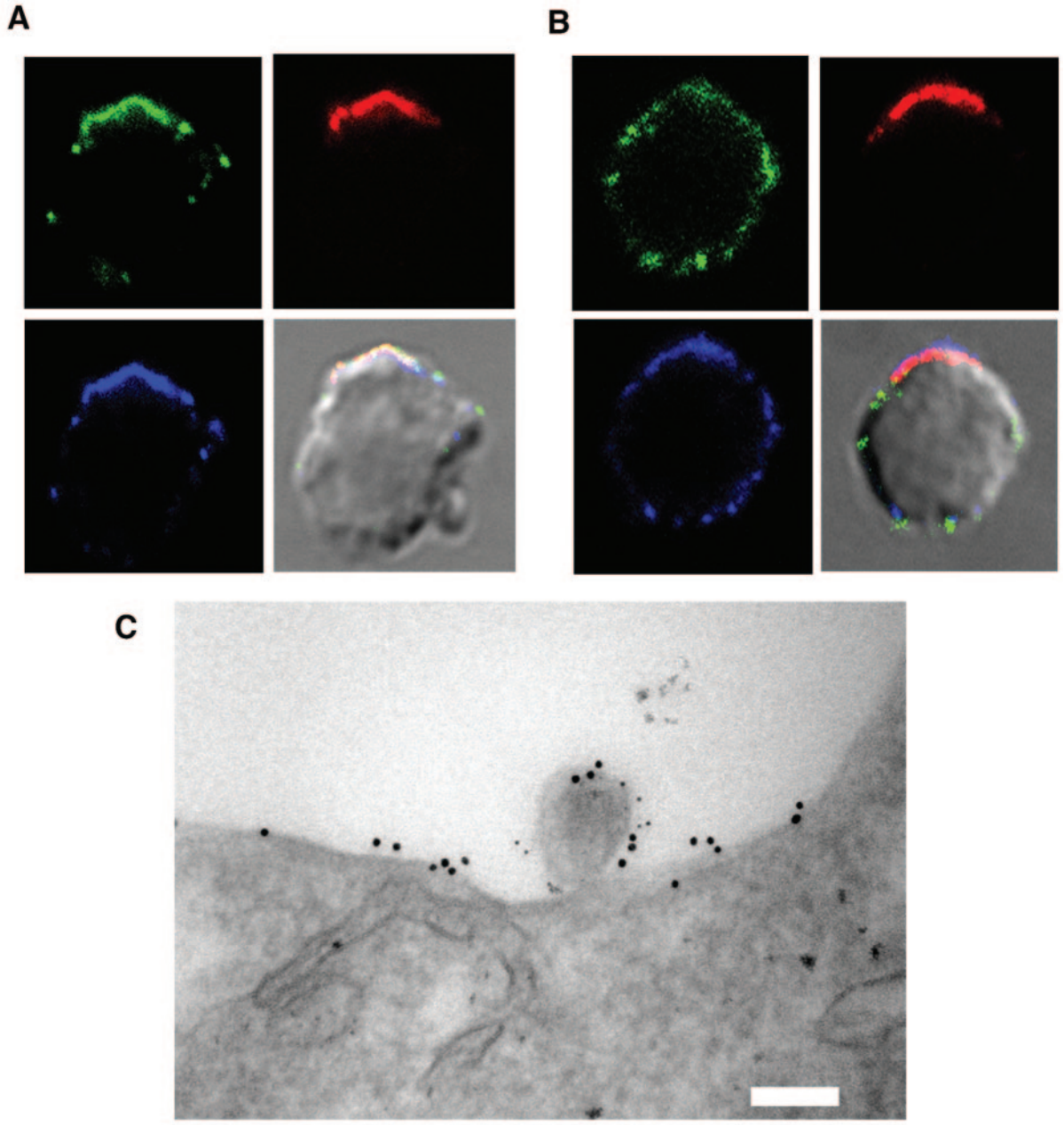

FIG. 2. HIV-1 Env colocalizes with the raft markers GM1 and CD59 but not with the nonraft marker TfR. Jurkat$_{LAI}$ cells were incubated for 1 h at 12°C with B-Ctx, the human Env-specific antibody 50–69, and either a mouse CD59-specific monoclonal antibody (Serotec) or a mouse anti-human TfR-specific antibody (from T. Harder, University of Oxford). Cells were washed at 4°C and incubated for a further 1 h at 12°C with anti-human-TRITC, streptavidin-Cy5, and anti-mouse-FITC to promote antibody-induced patching of CD59 and TfR as described previously (14). Cells were then washed, fixed in paraformaldehyde, and analyzed by confocal microscopy. (A) CD59 (green) colocalizes with HIV-1 Env (red) and GM1 (blue). (B) TfR (green) does not colocalize with HIV-1 Env (red) and GM1 (blue). (C) Incorporation of GM1 into a budding HIV-1 virion. Jurkat$_{LAI}$ cells were fixed sequentially in 4% and 8% paraformaldehyde, stained for Env with the human MAb 2G12 (20 μg/ml) and for GM1 with B-Ctx (20 μg/ml), washed, and labeled with anti-human immunoglobulin G and anti-biotin conjugated with 5-nm and 10-nm gold colloids, respectively (Agar, United Kingdom). Cells were then fixed in a mixture of glutaraldehyde and paraformaldehyde, washed, and postfixed in 1% osmium tetroxide in cacodylate buffer. After extensive washing, the cells were incubated in 0.5% magnesium uranyl acetate, dehydrated in ethanol and propylene oxide, and embedded in Epon resin. Ultrathin sections were examined using a Phillips FEI Technai 12 transmission electron microscope, and digital images were captured using Soft imaging software and processed using Photoshop. Bar, 100 nm.

1 and Fig. 3B and C). Furthermore, patches of Env and GM1 were observed in the target cell membrane (Fig. 3B), suggesting that fusion at the VS of virions with the receptor-expressing target cell had occurred, as we described previously (16).

To investigate whether lipid raft integrity is important in the assembly of the VS, we treated effector cells with 10 mM methyl-β-cyclodextrin (β-CD), an agent that depletes cholesterol from lipid bilayers, prior to washing and conjugate for-

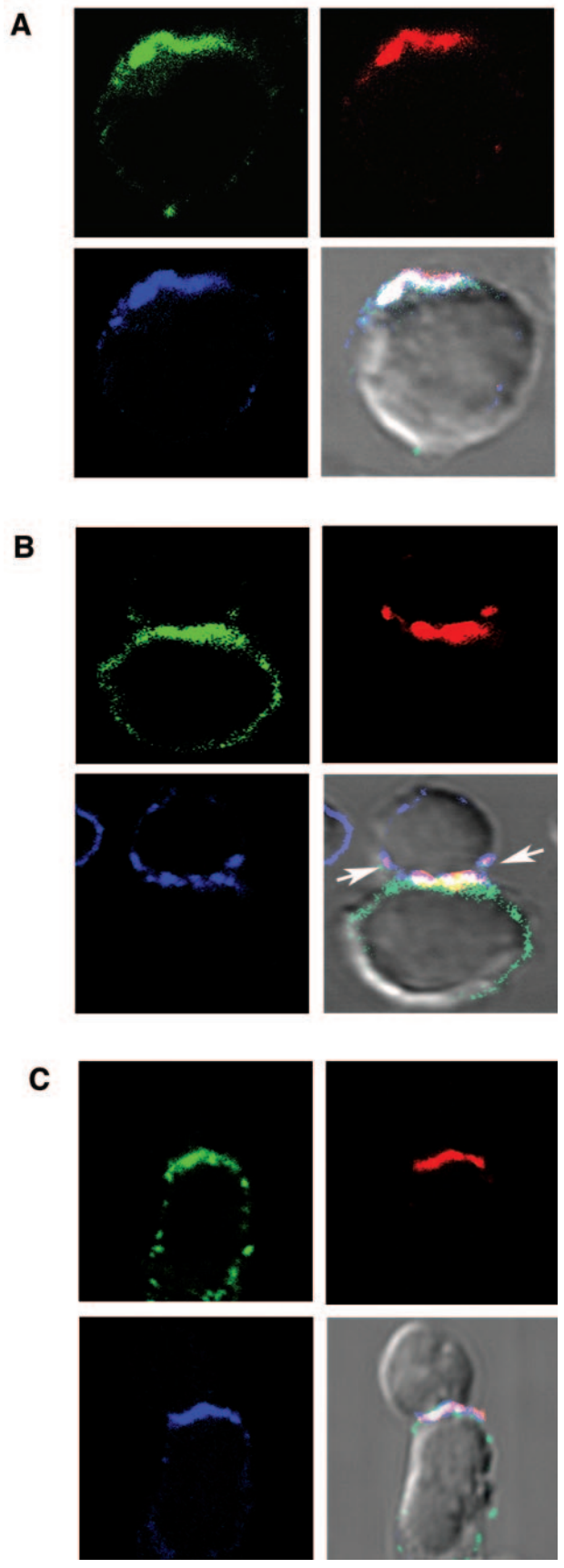

FIG. 3. GM1 colocalizes with Gag and Env and is polarized to the VS. (A) Jurkat$_{LAI}$ cells were stained with MAb 50–69 for surface-expressed Env (red) and with B-Ctx for GM1 (green), fixed, permeabilized with 0.1% Triton X-100, and incubated with rabbit antisera against HIV-1 Gag$^{p17/p24}$ (blue) obtained from CFAR, United Kingdom, followed by anti-human-TRITC, streptavidin-FITC, and anti-rabbit-Cy5. Merging of the three colors illustrates colocalization, which is superimposed in white on the corresponding Nomarski image. (B) Conjugates between equal numbers of Jurkat$_{LAI}$ effector cells (lower cell in conjugate) and freshly isolated resting primary CD4$^+$ T target cells negatively enriched from normal PBMC (upper cell) were made on poly-L-lysine-treated coverslips for 1 h at 37°C, during which cells were stained for CD4 with L120 (blue; CFAR, United Kingdom) and for Env with 50–69 (red). Conjugate evolution was arresting by fixing with paraformaldehyde prior to staining for GM1 with B-Ctx (green), followed by anti-mouse-Cy5, anti-human-TRITC, and streptavidin-FITC. Merging of the three colors illustrates colocalization, which is superimposed in white on the corresponding Nomarski image. Arrows point to regions of GM1 and Env in the target cell membrane. (C) Conjugates between Jurkat$_{LAI}$ effector and primary CD4$^+$ T target cells were made as described above, during which cells were stained for Env with 50–69 (red). Conjugate evolution was arrested by fixing with paraformaldehyde prior to staining with B-Ctx (green). Conjugates were then permeabilized in 0.1% Triton X-100 and labeled with rabbit antisera against Gag$^{p17/p24}$ (blue), followed by anti-human-TRITC, streptavidin-FITC, and anti-rabbit-Cy5. Merging of the three colors illustrates colocalization, which is superimposed in white on the corresponding Nomarski image.

TABLE 1. Quantification of GM1 recruitment to the VS[a]

Jurkat$_{LAI}$ effector cell treatment	% Conjugates[b]	% VS[c]	% GM1 polarization	n[d]
Untreated	42	47	63	129
β-CD treatment	35[e]	6	7	118

[a] Jurkat$_{LAI}$ effector T cells were either untreated or pretreated with 10 mM β-CD for 30 min at 37°C to deplete membrane cholesterol. Washed effector cells were mixed with primary CD4$^+$ target T cells for 1 h at 37°C in the presence of L120 to stain CD4. Conjugates were fixed and stained at 4°C with B-Ctx to label GM1 and then permeabilized and stained for HIV Gag. Conjugates were subsequently analyzed by LSCM for VS formation and GM1 polarization.

[b] The percentage of effector Jurkat$_{LAI}$ cells in conjugates with primary CD4$^+$ T cells was calculated by analyzing the number (n) of effector cells shown.

[c] VS formation was defined as copolarization to the conjugate interface of CD4 on the target cell and of HIV antigens in the effector cell as described previously (16, 17).

[d] n indicates the number of effector cells examined from randomly chosen low-power fields.

[e] Value does not differ significantly ($P < 0.05$) from the control values as calculated using an unpaired two-tailed student t test.

mation with target cells. Treatment of the effector cell with 10 mM β-CD dispersed GM1 staining in the effector cell membrane, prevented CD4 polarization in the target cell, and unexpectedly, completely eliminated the detection of Env expression (Fig. 4A). This effect was dose dependent, with lower concentrations of β-CD resulting in intermediate Env staining levels and the breaking up of Env clusters (Fig. 4B). This was the case whether Env was labeled with the gp41-specific 50–69 MAb, the gp120-specific 2G12 MAb, or polyclonal HIV immunoglobulin G (results not shown), implying that the loss of detectable staining was unlikely to be a result of conformational changes induced in Env. The loss of Env from the membrane by β-CD-induced shedding was ruled out by measuring extracellular Env using a sensitive enzyme-linked immunosorbent assay (ELISA) (27): insignificant differences were ob-

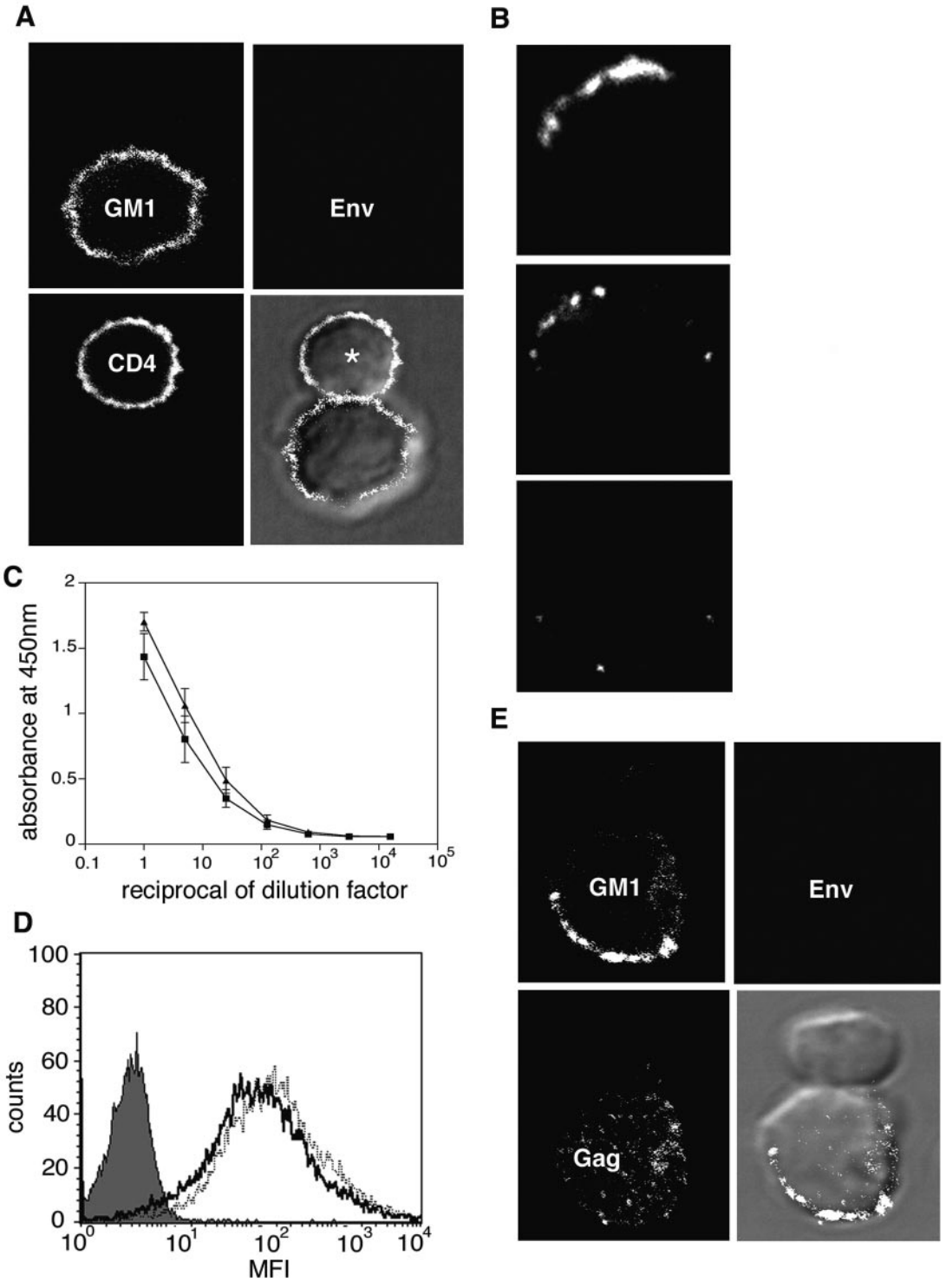

FIG. 4. Depletion of cholesterol eliminates Env detection and prevents VS assembly. Jurkat$_{LAI}$ effector cells were treated with 10 mM β-CD in RPMI for 30 min at 37°C to extract membrane cholesterol. Red, green, and blue staining was converted to white in all images by using Photoshop to increase contrast. (A) β-CD-treated Jurkat$_{LAI}$ cells (lower cell in conjugate) were washed, mixed with an equal number of primary CD4$^+$ target T cells (labeled with an asterisk), and incubated for 1 h at 37°C with the Env-specific MAb 50–69 (top right panel) and the CD4-specific MAb L120 (lower left panel). Conjugate evolution was arrested by fixing with paraformaldehyde prior to staining with B-Ctx to detect GM1 on the effector cell (top left panel). (B) Jurkat$_{LAI}$ effector cells were either left untreated (top panel) or treated with 5 mM (middle panel) or 10 mM (bottom panel) β-CD and stained with the HIV-1 Env-specific MAb 50–69 and an appropriate conjugate. (C) ELISA for soluble gp120. Jurkat$_{LAI}$ cells (1×10^7) were treated with 10 mM β-CD or left untreated, and supernatants were collected and assayed for their Env content by ELISA. Supernatants were serially diluted and captured on plates coated with the sheep anti-gp120 antibody D7320, and bound gp120 was detected using MAb 2G12 followed by anti-human-horseradish peroxidase and developed with Ultra-TMB ELISA substrate (Pierce). The data show the means

served (Fig. 4C) that could not explain the reduction in the detection of staining by confocal microscopy. Moreover, a flow cytometric analysis of surface Env expression showed only subtle differences between β-CD-treated and untreated cells (Fig. 4D). The labeling of Env with various antibodies after cell fixation and permeabilization and analysis by either LSCM or flow cytometry did not reveal a difference in the level of intracellular Env, making increased internalization an unlikely explanation (data not shown). The loss of Env detection is most likely explained, therefore, by Env dispersion in the plane of the plasma membrane coordinate with the disruption of lipid rafts, with the resultant molecular dilution reducing Env labeling to below the levels that are readily detectable by LSCM. These results are in accord with studies demonstrating that HIV-1 virions treated with β-CD retain Env despite losing their infectivity and some intravirion structures such as p24 and RNA (7, 10, 11).

In cultures of $Jurkat_{LAI}$ cells, 80 to 100% of the cells are infected. Nevertheless, to confirm HIV infection of the effector cells in the absence of Env staining and to investigate the localization of Gag with respect to GM1 staining after β-CD treatment, we costained conjugates for Gag, Env, and GM1. As before, Env staining was undetectable, whereas Gag was dispersed within the cytoplasm and completely uncoupled from GM1 staining, and GM1 was not polarized to the target-effector contact site (Fig. 4E). Neither capping of Env or Gag to the cell-cell contact site nor a transfer of Gag to the target cell was observed in target-effector conjugates, even after 3 h of incubation (results not shown). A statistical analysis of conjugates formed between target and effector cells showed an 88% reduction in VS formation ($P = 0.0001$) and an 89% reduction in GM1 polarization ($P = 0.0003$) after β-CD treatment of the effector cells (Table 1). Based on these data, we suggest that the depletion of cholesterol in the effector cell membrane disrupts both the Gag association with rafts at the inner leaflet of the plasma membrane and gp41-raft localization, preventing Gag-Env interaction and abrogating the polarization of these molecules to the site of effector-target cell contact. This would impede virion assembly and budding and would prevent sufficient Env engagement of receptors on the target cell to drive VS assembly. We were unable to restore capped Env staining by the reconstitution of β-CD-treated cells with exogenous cholesterol over a period of 24 h (data not shown). The disruption of Gag-Env clusters by raft dispersal may therefore be irreversible, or alternatively, reconstruction of Gag-Env assemblies in the plasma membrane is very slow following raft reconstitution. Moreover, it is possible that the disruption of HIV antigen localization is not solely a consequence of the dispersal of lipid rafts. β-CD disruption of Gag targeting to the plasma membrane may take place via sequestration of the lipid phosphatidylinositol 4,5-bisphosphate (PIP_2), which is normally concentrated in cholesterol-rich membrane domains and is involved in the organization of the actin cytoskeleton (19, 23). The perturbation of PIP_2 levels by cholesterol depletion using, among other agents, β-CD, causes Gag to be redirected away from the plasma membrane towards PIP_2-enriched endosomal membranes (23). Since it is documented that cholesterol depletion can also disrupt the underlying actin cytoskeleton and influence the lateral mobility of membrane proteins (19), we cannot rule out the possibility that some effects of β-CD treatment we observed were also the result of altered actin-mediated processes involved in HIV Env and Gag targeting and VS formation. However, we do not believe that this alters the principal interpretation of our data that correct raft structure and function, perhaps additionally influenced by components of the underlying cytoskeleton, are important in HIV Env and Gag localization and VS formation.

β-CD is toxic at high concentrations and could not reasonably be considered an antiviral strategy against HIV-1 cell-cell (and potentially cell-free) dissemination. However, the interruption of the HIV-1 Env and/or Gag association with lipid rafts may be effected by other, less toxic approaches that disrupt lipid rafts and/or alter PIP_2 distribution and should be explored as an avenue to novel prophylaxis in the form of microbicides or as a therapeutic intervention in HIV-1 infection.

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± 1 standard deviation for untreated (■) and β-CD-treated (▲) samples from two independent experiments performed in triplicate. (D) Flow cytometric analysis of surface-expressed Env. $Jurkat_{LAI}$ cells were left untreated (solid line) or treated with 10 mM β-CD (dotted line) and then were stained for surface-expressed Env using the MAb 2G12 followed by anti-human-phycoerythrin. Mean fluorescence intensities relative to the unstained control (shaded) are shown. (E) β-CD-treated $Jurkat_{LAI}$ effector cells were mixed with an equal number of primary $CD4^+$ target T cells, incubated for 1 h at 37°C, fixed, and stained for GM1 (top left panel) and Env (top right panel). Conjugates were permeabilized and labeled with rabbit antisera against HIV $Gag^{p17/p24}$ (lower left panel).

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