Glycosphingolipid Composition of Human Immunodeficiency Virus Type 1 (HIV-1) Particles Is a Crucial Determinant for Dendritic Cell-Mediated HIV-1 *trans*-Infection[▽]

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Interactions of human immunodeficiency virus type 1 (HIV-1) with dendritic cells (DCs) are multifactorial and presumably require nonredundant interactions between the HIV-1 envelope glycoprotein gp120 and molecules expressed on the DC surface that define the cellular fate of the virus particle. Surprisingly, neutralization of HIV-1 gp120-dependent binding interactions with DCs was insufficient to prevent HIV-1 attachment. Besides gp120, HIV-1 particles also incorporate host cell-derived proteins and lipids in their particle membrane. In this study, we demonstrate a crucial role for host cell-derived glycosphingolipids (GSLs) for the initial interactions of HIV-1 particles with both immature and mature DCs. Production of HIV-1 particles from virus producer cells treated with ceramide synthase inhibitor fumonisins B1 or glucosylceramide synthase inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) resulted in the production of virus particles that, although capable of binding previously defined HIV-1 gp120-specific attachment factors CD4, DC-SIGN, and syndecans, were attenuated in their ability to be captured by both immature and mature DCs. Furthermore, GSL-deficient HIV-1 particles were inhibited in their ability to establish productive infections in DC-T-cell cocultures. These studies provide initial evidence for the role of HIV-1 particle membrane-associated GSLs in virus invasion of DCs and also provide additional novel cellular targets, GSL biosynthetic pathways and GSL-dependent HIV-1 interactions with DCs, for development of antiviral therapy.

Dendritic cells (DCs) play a major role in human immunodeficiency virus type 1 (HIV-1) pathogenesis. Although DCs have been shown to process captured HIV-1 particles for antigen presentation pathways (50), the virus is able to exploit DC biology to promote its transmission to CD4⁺ T cells and macrophages. Two different mechanisms of DC-mediated HIV-1 transmission have been proposed: the *cis*- and *trans*-infection pathways. Productive or *cis*-infection of DCs, though feasible (10, 66), is ineffective for a myriad of reasons, including low CD4/coreceptor levels on the DC surface (40), as well as the presence of potent interferon-dependent (23, 48, 49, 52, 53, 68) and interferon-independent (23, 25) antiviral mechanisms, that restrict HIV-1 at different steps in the viral life cycle. In contrast to productive infection, DCs express a number of HIV-1 attachment factors that may serve to concentrate virus particles and retain them in an infectious state for an extended period of time (11, 19, 24, 55, 56). These virus-bearing DCs may then also facilitate a more efficient spread of virus to surrounding permissive CD4⁺ T cells by recruiting captured virus particles to the site of T-cell contact during the formation of “infectious synapses,” as defined by the *trans*-infection pathway (6, 11, 18, 27, 51, 55, 56, 81).

Members of the mannose binding C-type lectin receptor (MCLR) family, DC-SIGN and DCIR, expressed on DCs, have been implicated as HIV-1 gp120 binding factors (21, 39)

that can mediate transmission of captured HIV-1 particles to CD4⁺ T cells (2–4, 21, 39, 54). In addition to MCLR-dependent interactions, gp120-dependent binding to heparan sulfate proteoglycans (HSPG) or syndecans and gp41 ectodomain-dependent interactions with galactosylceramide have also been suggested to mediate HIV-1 attachment (7, 14, 42, 44, 60, 67). Recent studies in the literature have questioned these findings and have documented numerous instances of DC-SIGN- and HSPG-independent capture of virus particles by DCs (4, 26, 28, 74, 76) and transfer to CD4⁺ T cells (28). Furthermore, maturation of DCs either via infections with bacterial or viral pathogens or via exposure to Toll-like receptor agonists, such as bacterial lipopolysaccharide (LPS), results in enhanced capture of HIV-1 particles by mature DCs in an MCLR- and CD4-independent manner and robust transmission of captured HIV-1 particles to CD4⁺ T cells (15, 36). Thus, the molecular basis of the HIV-1 interactions with immature and mature DCs remains unclear.

In this report, we define a novel mechanism of virus capture by DCs that is independent of the HIV-1 envelope glycoprotein gp120. Capture of HIV-1 by both immature and mature DCs was dependent on the presence of glycosphingolipids (GSLs) in the virus particle membrane. Reduction of GSL content in the HIV-1 particle membrane by pharmacological inhibition of GSL biosynthesis pathways in virus producer cells resulted in the production of virus particles that were deficient for capture by DCs and for subsequent transfer to CD4⁺ T cells. These findings argue for an essential role for HIV-1 particle membrane-associated GSLs for virus interaction with DCs and for the establishment of DC-mediated HIV-1 *trans*-infection.

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MATERIALS AND METHODS

Cells. Primary monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors, using CD14-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA), as described previously (28). CD14⁺ monocytes (1×10^6 cells/ml) were cultured in RPMI-10% fetal bovine serum (complete RPMI) containing recombinant human granulocyte-macrophage colony-stimulating factor (0.5 μ g/ml) (Leukine; Berlex) and recombinant human interleukin-4 (IL-4), 1,000 U/ml (BD Biosciences) for 6 days, after which cells acquired a DC phenotype (CD14⁺, CD209⁺, HLA-DR⁺, CD86⁺), as described previously (28, 73). Mature DCs were obtained by culturing immature DCs at day 6 of culture for two additional days in the presence of 100 ng/ml of ultrapure *Escherichia coli* LPS (Sigma) and were found to be HLA-DR^{hi}, DC-SIGN^{lo}, and CD86⁺. Primary human CD4⁺ T cells were positively isolated from CD14-depleted PBMCs, using CD4-conjugated magnetic beads (Mitenyi Biotec) according to manufacturer's instructions. CD4⁺ T cells were activated using 2% phytohemagglutinin (Invitrogen) for 2 days and were washed and cultured in complete RPMI supplemented with 50 U/ml recombinant human IL-2 (Roche). HEK293T, Jurkat-CCR5, MAGI-CCR5, and GHOST/CD4/CXCR4/CCR5 cell lines have been described previously (46, 73).

Virus stocks and plasmids. Replication-competent HIV-1 molecular clones HIV/Lai (CXCR4-tropic) and HIV/Lai-YU2 *env* (expresses a CCR5-tropic *env*), single-cycle replication-competent luciferase-expressing provirus clone HIV-luc, HIV-1 *env*-deficient molecular clone Lai Δ *env*, and CXCR4-tropic HIV-1 gp160 (Lai *env*) expression constructs have been described previously (69, 73). Vesicular stomatitis virus G (VSV-G)-pseudotyped Lai or Lai Δ *env* virus particles were produced by cotransfection of HIV-1 proviral molecular clones with a VSV-G expression plasmid (H-CMVG; a gracious gift of Wei Chun Goh, NEMC). Wild-type, VSV-G-pseudotyped or *env*-deficient virus particles were generated by calcium phosphate-mediated transfections of HEK293T cells, as described previously (73). Jurkat and primary CD4⁺ T-cell-derived infectious or *env*-deficient virus particles were produced by infecting Jurkat-CCR5 or primary activated CD4⁺ T cells with VSV-G-pseudotyped Lai or Lai Δ *env* virus particles. Virus-exposed Jurkat and CD4⁺ T cells were washed extensively and cultured for 4 to 6 days. Cell-free virus supernatants were passed through 0.45- μ m filters and frozen at -80°C . To alter the lipid composition of infectious virus particles or virus-like particles (VLPs), HEK293T cells were treated with either 50 μ M fumonisin B1 (FB1; Cayman Biologicals) or 10 μ M 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP; Calbiochem) 24 h prior to transfection. On the day of transfection, fresh media containing FB1 or PDMP were added, and cells were transfected with gp120-expressing or -deficient proviral expression plasmids by Lipofectamine 2000 (Invitrogen). Virus-containing cell supernatants were harvested 2 days posttransfection, passed through 0.45- μ m filters, and frozen at -80°C until further use. Infectious virus (Lai or Lai/YU2) stocks were assayed for infectivity by GHOST/CD4/CXCR4/CCR5 or MAGI-CCR5 infections. The p24^{gag} content of all the virus stocks was quantitated by a previously described enzyme-linked immunosorbent assay (ELISA) (72), with slight modifications. Briefly, p24^{gag} was bound to HIV immunoglobulin (from NABI and National Heart Lung and Blood Institute)-coated wells and detected with an anti-p24^{gag} monoclonal antibody (clone 183-H12-5C from the NIH AIDS Research and Reference Reagent Program, contributed by Bruce Chesebro) and horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Sigma). Incorporation of gp120 into virus particles produced from FB1- and PDMP-treated HEK293T cells was confirmed by Western blot analysis. Briefly, equal amounts of virus containing cell-free supernatants (3,500 ng p24^{gag}) were layered on 20% sucrose cushions (20 mM HEPES [pH 7.4], 100 mM NaCl) and spun at 200,000 $\times$ g for 2 h in a SW55 Ti rotor (Beckman). Virus pellets were lysed in a sodium dodecyl sulfate-containing sample buffer and loaded on 10% sodium dodecyl sulfate-polyacrylamide gels and probed for gp120 expression, using a combination of two anti-HIV-1 gp120 monoclonal antibodies (catalog number 1121 [Immunodiagnosics]; catalog number 522 from the NIH AIDS Research and Reference Reagent Program, contributed by Bruce Chesebro) and Gag expression using anti-p24^{gag} monoclonal antibody (clone number 24-2 from the NIH AIDS Research and Reference Reagent Program, contributed by Michael Malim). Additionally virus pellets were probed for the presence of cellular proteins CD9 and CD63, using anti-CD9 (catalog number 555370; BD Biosciences) and anti-CD63 antibodies (clone H-193; Santa Cruz Biotechnology).

Soluble gp140 production. HEK293T cells were cotransfected with a plasmid encoding His-tagged soluble trimeric HIV-1 JRFL gp140 envelope glycoprotein (gift from Joseph Sodroski, DFCI) and an HIV-1 Tat expression plasmid (pCMV-tat) by calcium phosphate. Transfected cells were washed 16 h later, and cells were cultured for two additional days. Supernatants were harvested and concentrated $\sim$ 10-fold in Centricon Plus-70 Ultracel PL-100 (Millipore), and

aliquots were frozen at -80°C . Expression of soluble gp140 (sgp140) in cell supernatants was confirmed by Western blot analysis with biotin conjugated rabbit anti-His polyclonal antibody and streptavidin-HRP (Pierce), and the amount of sgp140 in supernatants was determined by the Bradford protein assay kit (Bio-Rad). To confirm functional expression of sgp140 in HEK293T cell supernatants, Jurkat-CCR5 cells were incubated with sgp140-containing or mock HEK293T cell supernatants for 1 h at 37°C and then challenged with Lai-*env* pseudotyped HIV/luc virus particles (multiplicity of infection [MOI] = 0.001). Cells were washed and placed in culture. Cells were harvested 2 days postinfection and lysed, and the luciferase activity of the cell lysates was determined according to the manufacturer's instructions (Promega), as a measure of productive virus infection.

sgp140 binding to DCs. Immature DCs (5×10^5) were incubated with 1.3 μ g of sgp140 or mock HEK293T cell supernatant (100 μ l volume) for 1 h at 4°C . Binding of sgp140 to DC surface was visualized by sequential staining with rabbit anti-His polyclonal antibody and streptavidin-conjugated fluorescein isothiocyanate (FITC) (BD Pharmingen). Cells were fixed with 1% paraformaldehyde, and the number of positively stained cells was determined by a fluorescence-activated cell sorter (FACS), using BD/FACScan and CellQuest software.

Dot blot analysis. Cell lysates from virus producer cells (HEK293T cells) that had been treated with PDMP as previously described or virus lysates derived via pelleting equal amounts of virus particle-containing supernatants (100,000 $\times$ g, 2 h, 4°C), were dotted onto 0.2- μ m nitrocellulose filters, using a dot blot apparatus (Bio-Rad). Nitrocellulose blots were blocked in Tris-buffered saline-0.1% Tween 20-5% nonfat milk powder, and the levels of GM1 were detected by incubating the blots with HRP-conjugated cholera toxin subunit B (CtxB; Sigma) in Tris-buffered saline-0.1% Tween 20-1% nonfat milk powder (35).

DC capture and transfer assays. Immature and mature DCs (3×10^5) were incubated with 10 ng of virus particles for 2 h at 37°C , washed three times with RPMI-10% fetal bovine serum, lysed, and cleared of cell debris by centrifugation, and the amount of cell-associated virus was determined by p24^{gag} ELISA. In experiments with single-cycle replication-competent virus, immature and mature DCs were incubated with 10 ng or 1 ng of virus particles, respectively. To potentially inhibit HIV-1 gp120-dependent virus particle capture by DCs, immature and mature DCs were incubated initially with 100 μ l (1.3 μ g) of sgp140 or mock HEK293T supernatant for 1 h at 37°C , followed by the addition of HIV/Lai or HIV/Lai-YU2 virus particles (1 ng p24^{gag}) for 2 h at 37°C . The percentage of virus capture by DCs was determined by the following formula: [(ng of p24^{gag} associated with DC)/(ng of input p24^{gag})] $\times$ 100. Alternatively, virus-exposed DCs were cocultured with 3×10^5 autologous primary CD4⁺ or Jurkat/CCR5 T cells. DC-T-cell cocultures were harvested 2 days postinfection, the DC-primary CD4⁺ T-cell supernatants were analyzed for p24^{gag} production via ELISA, or luciferase activity of the DC-Jurkat T-cell coculture lysates was determined as a measure of productive infection. Additionally, DC-primary CD4⁺ T-cell cocultures were stained for cell surface CD3 (BD Biosciences), fixed with 4% paraformaldehyde, permeabilized with Cytoperm solution (Becton Dickinson), stained for intracellular p24^{gag} expression (FITC-conjugated anti-p24^{gag} antibody; clone KC57 [Beckman Coulter]) and analyzed by FACS. The percentage of p24^{gag}⁺ CD3⁺ T cells in the infected cocultures was analyzed using CellQuest software (Becton Dickinson).

Virus infections. GHOST/CD4/CXCR4/CCR5 (4×10^4) cells, primary CD4⁺ T cells (3×10^5), or Jurkat-CCR5 cells (3×10^5) were infected with either HIV/Lai or Lai-luc virus particles (10 ng of p24^{gag}) in triplicate for 2 h at 37°C , washed, and cultured for 2 days. Infectivity of different virus preparations was determined by measurement of either green fluorescent protein (GFP) expression in GHOST/CD4/CXCR4/CCR5 cells by FACS, p24^{gag} content in infected CD4⁺ T-cell supernatants by ELISA, or luciferase activity in infected Jurkat-CCR5 cell lysates.

RESULTS

HIV-1 particles can be captured by DCs in an envelope glycoprotein-independent manner. We have previously reported that monomeric gp120 binding to DCs does not faithfully recapitulate all of the virus particle binding (28) and that studies that utilized gp120 to define HIV-1 particle binding to DCs in an MCLR-dependent manner (21, 64, 65) fail to account for binding epitopes expressed on viral membrane-anchored trimers of gp120 and gp41 ectodomains. Furthermore, attachment of HIV-1 to HSPG and galactosylceramide, at-

tachment factors that are also expressed on DCs, cannot be mimicked by monomeric gp120 (14, 42, 67), suggesting that conformational epitopes present on the trimeric envelope glycoprotein, the functional form of HIV-1 *env* found on virus particles, are functionally different from those expressed on the monomeric gp120 (17, 45, 61). Hence, we performed virus capture assays with infectious virus particles in the presence of the soluble trimeric HIV-1 gp140 envelope glycoprotein (77, 78). The soluble His-tagged gp140 glycoprotein containing T4 bacteriophage fibrin sequence stabilized trimers of gp120 and gp41 ectodomains (78) was derived from supernatants of HEK293T cells that had been transiently transfected with a His-gp140 expression plasmid. Expression of soluble His-tagged HIV-1 gp140 in HEK293T cell supernatants was confirmed by Western blot analysis, using a polyclonal anti-His antibody (Fig. 1A). Functional expression of the protein was confirmed by preincubating Jurkat T cells with increasing amounts of either gp140-containing HEK293T supernatants or mock supernatants for 1 h at 37°C prior to infection with single-cycle replication-competent HIV/Lai-luc virus particles. Preincubation with soluble gp140-containing supernatants, but not mock HEK293T supernatants, completely blocked Lai-*env* pseudotyped HIV/luc infection of Jurkat T cells (Fig. 1B). Furthermore, we could detect robust binding of soluble gp140 to cells expressing known HIV-1 gp120-dependent attachment factors, including immature and mature DCs and HeLa/DC-SIGN (DC-SIGN⁺ and HSPG⁺) cells (Fig. 1C).

We then tested the ability of soluble gp140 to block virus particle capture by immature DCs, mature DCs, and HeLa/DC-SIGN cells. Preincubation with soluble HIV-1 gp140 glycoprotein reduced HIV/Lai (CXCR4-tropic) and HIV/Lai-YU2 (CCR5-tropic) particle capture by HeLa/DC-SIGN cells by 90% (Fig. 1D and E). In contrast, preincubation with soluble HIV-1 gp140 glycoprotein resulted in only a 50% reduction in Lai-YU2 and a 30% reduction in Lai virus particle capture by immature DCs (Fig. 1D and E) and no impact on capture of either virus by mature DCs (Fig. 1D and E). These results suggest that inhibition of HIV-1 *env*-dependent interactions with previously defined DC-associated HIV-1 *env* attachment factors (CD4, DC-SIGN, galactosylceramide, and/or HSPGs) is insufficient to prevent virus particle capture by immature and mature DCs.

Since it has been previously suggested that interactions of HIV-1 particles with DCs can vary depending on the source of the virus producer cell (14, 67), we wanted to determine if virus particles derived from T cells could also be captured by DCs in a gp120-independent manner. HIV-1 gp120-deficient (Lai Δenv) or gp120-containing (Lai) particles were derived from either transient transfection of HEK293T cells or via infections of Jurkat T cells and primary CD4⁺ T cells with VSV-G pseudotyped Lai or Lai Δenv particles. Immature and mature DCs were challenged with equal amounts of Lai and Lai Δenv particles for 2 h at 37°C, washed, and lysed, and the amount of cell-associated virus particles was determined by a p24^{gag} ELISA. In contrast to recently published findings (14), the cellular origin of the virus particles had limited impact on *env*-deficient virus particle capture by DCs. Immature DCs could capture HEK293T, Jurkat, and primary CD4⁺ T-cell-derived Lai Δenv particles, though with variable efficiency (Fig. 2A to C). While capture of HEK293T and Jurkat-derived Lai

Δenv particles was reduced by only 50%, capture of primary CD4⁺ T-cell-derived Lai Δenv particles was reduced by 68%. Furthermore, HEK293T and Jurkat cell-derived Lai and Lai Δenv particles were captured with equal efficiency by LPS-matured DCs (Fig. 2A and B), while capture of primary CD4⁺ T-cell-derived Lai Δenv particles by mature DCs was reduced only by 50% (Fig. 2C). These results suggest that capture of HIV-1 particles by immature and mature DCs can occur independently of the HIV-1 envelope glycoprotein.

Changes in the sphingolipid composition of HIV-1 particles negatively impacts virus capture by DCs. Our recent findings have failed to find a role for host cellular proteins incorporated into the budding HIV-1 particle membrane in capture of HIV-1 Gag-derived VLPs by mature DCs but, rather, have suggested the involvement of sphingolipids present in the virus particle membrane for mediating gp120-deficient Gag VLP capture by mature DCs (37). To determine if alterations in the sphingolipid composition of HIV-1 particles can affect capture of infectious HIV-1 particles by immature DCs, HIV-1/Lai particles were produced from HEK293T cells in the presence of FB1, a mycotoxin that inhibits ceramide synthase enzyme, and hence inhibits synthesis of the simple sphingolipid, ceramide, and other downstream complex sphingolipids (70). Previous studies have demonstrated that FB1 treatment of HIV-1 producer cells results in a decrease in sphingolipid content in the plasma membrane of the virus producer cell and, hence, a decrease in sphingolipid content within the virus particle membrane (9), as it acquires the host lipids during the virus budding process. Treatment of HEK293T cells transfected with HIV-1 proviral plasmids with FB1 resulted in no significant difference in virus particle release upon FB1 treatment (data not shown), and virus particles had similar levels of gp120 incorporation (ratios of gp120 to p24^{gag} are similar between the two virus preparations [compare lanes 1 and 2 of Fig. 3A]). To determine if FB1 treatment during HIV-1 production from HEK293T cells has an effect on virus infectivity, GHOST/CD4/CXCR4/CCR5 cells were challenged with equal amounts of virus particles, and GFP expression was determined 2 days postinfection. In contrast to previous studies that had demonstrated a fivefold decrease in infectivity of HIV-1 particles derived from FB1-treated MT4 T-cell line (9), there was only a modest decrease in infectivity of HIV-1 particles produced from FB1-treated HEK293T cells (Fig. 3B), suggesting that FB1 affects HIV-1 infectivity in a producer cell-type-dependent manner.

Immature and mature DCs were challenged with HIV/Lai virus particles (10 ng of p24^{gag} per 3×10^5 cells), and the relative amounts of virus capture were determined by measuring the amount of cell-associated p24^{gag} by ELISA. In comparison to HIV/Lai particles produced from untreated HEK293T cells, there was a 60% and 68% reduction in virus capture by immature (Fig. 3C) and mature DCs (Fig. 3D), respectively, upon challenge with HIV-1 particles produced from FB1-treated HEK293T cells. In contrast, HIV/Lai capture by HeLa/DC-SIGN cells was unaffected by the production of virus particles from FB1-treated HEK293T cells (Fig. 3E), suggesting that FB1 treatment of virus-producing HEK293T cells had negligible effects on gp120-dependent virus particle capture by DC-SIGN- and HSPG-expressing cells. These capture experiments have been repeated with virus stocks

FIG. 1. Envelope glycoprotein-independent capture of HIV-1 particles to immature and mature DCs. (A) Western blot analysis of HIV-1 JRFL gp140 expression in HEK293T cell lysates and supernatants. Lane 1, mock HEK293T cell lysate; lane 2, sgp140-transfected HEK293T cell lysate; lane 3, mock HEK293T cell supernatant; lane 4, sgp140-transfected HEK293T cell supernatant. (B) Jurkat/CCR5 T cells were preincubated with mock or sgp140 (1.3 μ g)-containing supernatants prior to challenge with HIV/Lai-luc virus particles (MOI = 0.001). Luciferase activity in cell lysates was measured 2 days postinfection. The data shown are means of triplicate experiments $\pm$ standard errors of the mean. (C) Immature DCs, mature DCs, or HeLa/DC-SIGN cells (5×10^5) were incubated with 1.3 μ g of sgp140, washed, and sequentially stained with biotin-conjugated anti-His monoclonal antibody and streptavidin-conjugated FITC. Cells were fixed in 1% paraformaldehyde and analyzed by FACS. Filled histograms represent mock staining; open histograms with dashed lines indicate streptavidin-FITC alone; open histograms with solid lines represent gp140-specific staining. Immature DCs, mature DCs, or HeLa/DC-SIGN cells (3×10^5) were incubated with sgp140 (1.3 μ g) followed by challenge with either HIV/Lai (D) or HIV/Lai-YU2 (E) virus particles corresponding to 1 ng of p24^{gag}. The mean HIV/Lai or HIV/Lai-YU2 capture by DCs and HeLa/DC-SIGN cells in the presence of sgp140 was normalized to virus capture by DC in the presence of mock supernatants (set at 100%) and reported as relative p24^{gag} captures. The experiment was performed at least four independent times with DCs derived from four independent donors. The results shown here are the mean percentages of capture observed for all the experiments $\pm$ standard errors of the mean. P values were determined using an unpaired, two-tailed sample *t* test.

FIG. 2. Lack of virus producer cell effect on envelope glycoprotein-independent capture of HIV-1 to DCs. Immature and mature DCs (3×10^5) were challenged with HIV-1 gp120-containing (Lai) or gp120-deficient (Lai Δ env) virus particles corresponding to 10 ng p24^{gag} derived from HEK293T cells (A), Jurkat T cells (B), and primary CD4⁺ T cells (C). Cells were washed and lysed, and the cell-associated p24^{gag} content was determined via ELISA. The experiment was performed three (A), six (B), or four (C) independent times with DCs derived from at least three independent donors. The results represent mean percentages of p24^{gag} capture $\pm$ standard errors of the mean. *P* values were determined using an unpaired, two-tailed sample *t* test.

derived from at least three independent transfections of FB1-treated HEK293T cells with similar results, suggesting that sphingolipids present in the HIV-1 particle membrane play a crucial role in gp120-independent virus particle capture by immature and mature DCs.

GSLs in the HIV-1 particle membrane are crucial for virus particle capture by DCs. The next step in the GSL biosynthetic pathway following ceramide synthesis involves the glycosylation of ceramide to glucosylceramide, a precursor of all GSLs

in host cell membranes, and a reaction catalyzed by the enzyme glucosylceramide (GlcCer) synthase (34). Treatment of cells with a competitive inhibitor of GlcCer synthase, PDMP, a cationic lipid, has been shown to selectively deplete GSL content within the cells (63). To determine if GSLs that are incorporated in the membranes of budding HIV-1 particles play a role in mediating attachment of virus particles to immature DCs, HEK293T cells were treated with PDMP (10 μ M) for 24 h prior to transfection with proviral DNA. The drug con-

FIG. 3. Decrease in the sphingolipid content of HIV-1 particles negatively impacts virus particle capture by DCs. (A) Western blot analysis of HIV-1 particles (700 ng of p24^{gag}), purified via pelleting through 20% sucrose cushions, derived from untreated (lane 1) or FB1-treated (lane 2) HEK293T cells, and immunoblots probed for gp120 and viral capsid (p24^{gag}) expression. (B) Infectivity of HIV-1 particles derived from untreated or FB1-treated HEK293T cells was assayed by infections of GHOST/CD4/CXCR4/CCR5 cells. The data are represented as percentage of enhanced-GFP-positive cells obtained 2 days postinfection, following challenge with equal amounts of virus particles (10 ng p24^{gag}) and are the means of four independent experiments $\pm$ standard errors of the mean. Capture of HIV/Lai particles derived from untreated or FB1 (50 μ M)-treated HEK293T cells by immature DCs (C), mature DCs (D), or HeLa/DC-SIGN (E) was determined by incubating cells (3×10^5) with virus corresponding to 10 ng of p24^{gag}. The mean virus capture obtained with HIV/Lai particles derived from FB1-treated HEK293T cells was normalized to capture observed with virus particles derived from untreated HEK293T cells (set at 100%) and was reported as relative mean p24^{gag} capture $\pm$ standard errors of the mean. *P* values were determined using an unpaired, two-tailed sample *t* test. Virus capture studies with DCs were performed at least seven times with cells derived from at least five independent donors or performed three times with HeLa/DC-SIGN cells.

FIG. 4. GSL-depleted HIV-1 particles are attenuated for capture by DCs. (A) Dot blot analysis of cell lysates and HIV/Lai particles derived from untreated or PDMP-treated HEK293T cells. Lanes 1 through 3 on the dot blots represent 100 μ g, 50 μ g, and 25 μ g of cell lysate or 10 μ g, 5 μ g, and 2.5 μ g of virus particles, respectively. Blots were probed for GM1 expression using HRP-conjugated CtxB. (B) Western blot analysis of HIV-1 particles derived from untreated (lane 1) or PDMP (10 μ M)-treated (lane 2) HEK293T cells for gp120, capsid (p24^{gag}), CD63, and CD9 expression. (C) Infectivity of HIV-1 particles derived from untreated or PDMP-treated HEK293T cells was assayed by infections of GHOST/CD4/CXCR4/CCR5 cells. The data are represented as the percentages of enhanced-GFP-positive cells obtained 2 days postinfection, following challenge with equal amounts of virus particles (10 ng p24^{gag}) and are the means of three independent experiments $\pm$ standard errors of the mean. Capture of HIV/Lai particles derived from untreated or PDMP-treated HEK293T cells by immature DCs (D), mature DCs (E), or HeLa/DC-SIGN (F) was determined by incubating cells (3×10^5) with virus corresponding to 10 ng of p24^{gag} at 37°C for capture assays or at 4°C for binding assay in mature DCs. The mean virus capture obtained with HIV/Lai particles derived from PDMP-treated HEK293T cells was normalized to capture observed with virus particles derived from untreated HEK293T cells (set at 100%) and was reported as relative means of p24^{gag} capture $\pm$ standard errors of mean. *P* values were determined using an unpaired, two-tailed sample *t* test. Virus capture studies with DCs were performed at least five times with cells derived from at least five independent donors or performed three times with HeLa/DC-SIGN cells.

centration was maintained in the cell culture media during the duration of the virus particle production. To determine the effect of PDMP treatment on GSL content in HEK293T cells, equal amounts of nontreated or PDMP-treated HEK293T cell lysates or virus particles derived from nontreated or PDMP-treated HEK293T cells were run on dot blots, and the GM1 (a complex GSL) contents of cell lysates or virus particles were determined by incubating the blots with HRP-conjugated CtxB. Treatment of virus-producing HEK293T cells with 10 μ M PDMP led to a six- to eightfold decrease in the amount of GM1 in cell lysates and virus particles (Fig. 4A). PDMP treatment of HEK293T cells had no significant effect on virus particle release (data not shown), or incorporation of host cell-derived CD9 and CD63 tetraspanin proteins or virus-encoded gp120, into budding virus particles (Fig. 4B). Furthermore, production of virus particles from HEK293T cells treated with PDMP (10 μ M) had minimal effects on infectivity of virus preparations for GHOST/CD4/CXCR4/CCR5 cells (Fig. 4C), suggesting that GSL deficiency in HIV-1 particle membrane does not result in attenuation of virus infectivity in reporter cell lines in vitro.

To test the effect of GSL-depletion in virus producer cells on HIV-1 particle capture, immature and mature DCs were challenged with HIV/Lai particles produced in the presence of PDMP. There was a 55% inhibition of HIV/Lai particle capture by immature DCs (Fig. 4D) and a 53% inhibition of capture and binding at both 37°C and 4°C to mature DCs (Fig. 4E) upon PDMP-dependent depletion of virus particle-associ-

ated GSLs, suggesting that most of the gp120-independent capture of HIV-1 particles by immature and mature DCs can be accounted for by virus particle membrane-associated GSLs. In contrast, virus capture by HeLa/DC-SIGN cells was moderately affected by the production of virus particles from GSL-depleted (10 μ M PDMP-treated) HEK293T cells (Fig. 4F). Furthermore, capture of gp120-deficient Lai Δ env particles by immature and mature DCs was completely inhibited upon production of particles from PDMP-treated HEK293T or primary CD4⁺ T cells (data not shown). These results argue that GSLs incorporated into the HIV-1 particle membrane account for most of the gp120-independent capture of HIV-1 by immature and mature DCs.

GSLs incorporated into the HIV-1 particle membrane are crucial for DC-mediated HIV-1 *trans*-infection. Both immature and mature DCs can mediate transfer of captured HIV-1 particles to CD4⁺ T cells, a mechanism of HIV-1 *trans*-infection, and DC-T-cell cocultures have been shown to support explosive virus replication (27, 35, 50, 75). Since FB1 and PDMP treatments affected only the gp120-independent mechanism of virus capture, we wanted to determine if HIV-1 particles that were captured in a gp120-dependent manner by DCs could still account for most of the DC-mediated *trans*-infection, characterized in a number of previously published studies. We used three different experimental strategies to assay the ability of GSL-depleted HIV-1 particles to establish productive infection in DC-T-cell cocultures. Immature and mature DCs were challenged with HIV/Lai particles (MOI = 0.001) derived

FIG. 5. Virus particle-associated GSL-dependent interactions are crucial for DC-mediated HIV-1 *trans*-infection. Immature and mature DCs (3×10^5) or primary activated CD4⁺ T cells (3×10^5) were incubated with HIV/Lai virus particles corresponding to 10 ng of p24^{gag} produced from untreated, FB1-treated (panels A, C, and D) or PDMP-treated HEK293T cells (panels B, C, and D). DCs were washed and then cocultured with autologous primary CD4⁺ T cells (3×10^5) for 2 days, while directly infected CD4⁺ T cells were cultured alone. Supernatants from cocultures were harvested and analyzed for p24^{gag} content by ELISA (A and B). The mean relative virus transfer obtained with DCs incubated with HIV/Lai particles derived from FB1- or PDMP-treated HEK293T cells was normalized to virus transfer observed with HIV/Lai particles derived from untreated HEK293T cells (set at 100%) and was reported as relative virus transfer $\pm$ standard errors of the mean. Virus replication in CD4⁺ T cells obtained with HIV/Lai particles derived from FB1- or PDMP-treated HEK293T cells was normalized to the virus replication observed with HIV/Lai particles derived from untreated HEK293T cells (set at 100%) and was reported as relative virus infection $\pm$ standard errors of the mean. *P* values were determined using an unpaired, two-tailed sample *t* test. HIV-1 replication in DC-T-cell cocultures has been performed at least seven times with DCs and T cells derived from at least six independent donors. Cells from the cocultures were stained with anti-CD3-PE, fixed, permeabilized, and stained for intracellular p24^{gag} expression using FITC-conjugated anti-p24 monoclonal antibody. Stained cells were analyzed by FACS, and representative flow cytometric data for surface CD3 staining and intracellular p24^{gag} staining in immature DC-T-cell (C) and mature DC-T-cell cocultures (D) are shown.

from untreated or FB1- or PDMP-treated HEK293T cells and cultured alone or cocultured with autologous activated CD4⁺ T cells. Primary CD4⁺ T cells were also directly infected with untreated or FB1- or PDMP-treated HEK293T cell-derived virus particles (MOI = 0.001). The ability of the HIV-1 particles to establish productive infection in DC-T-cell cocultures was severely attenuated when virus particles were derived from HEK293T cells treated with GSL synthesis inhibitors. There was a 79% and a 73% inhibition of virus replication in immature and mature DC-T-cell cocultures, respectively, upon challenge of cocultures with virus derived from FB1-treated HEK293T cells, relative to infection with virus derived from untreated HEK293T cells (Fig. 5A). Similarly, there was

a 75% and a 63% inhibition in virus replication in immature and mature DC-T-cell cocultures, respectively, upon infection with HIV/Lai produced from PDMP-treated HEK293T cells (Fig. 5B). Interestingly, direct challenge of primary CD4⁺ T cells with HIV/Lai derived from either FB1- or PDMP-treated HEK293T cells had no significant effect on virus infectivity in comparison to infections with untreated HIV/Lai particles (Fig. 5A and B). As described before (73), replication of HIV/Lai virus particles in immature and mature DCs alone was near background levels at 2 days postinfection (data not shown), suggesting that majority of the p24^{gag} production in DC-T-cell cocultures is derived from productively infected T cells.

To confirm that attenuation of HIV-1 replication in DC-T

FIG. 6. DC-mediated *trans*-infection of single-cycle replication-competent HIV/luc particles is attenuated upon GSL depletion in the virus particle membrane. Immature or mature DCs (3×10^5) were incubated with HIV/luc virus particles (10 ng or 1 ng of p24^{gag}, respectively) produced from FB1-treated (A and B) or PDMP (D and E)-treated HEK293T cells. Cells were either lysed to analyze cell-associated p24^{gag} content via ELISA (A and D) or cocultured with Jurkat-CCR5 T cells for 2 days (B and E). Alternatively, Jurkat/CCR5 cells were infected directly with cell-free HIV/luc corresponding to 5 ng (C) or 10 ng (F) of p24^{gag}. Infected cultures were lysed 2 days postinfection and cell lysates analyzed for luciferase activity as a measure of virus replication. The mean virus capture and infection ($\pm$ standard errors of the mean) observed with FB1- or PDMP-treated HEK293T cell-derived virus particles were normalized to those observed with virus particles derived from untreated HEK293T cells (set at 100%). The experiment was performed at least three independent times with DCs derived from three independent donors. *P* values were determined using an unpaired, two-tailed sample *t* test.

cell cocultures upon challenge with virus particles derived from FB1- or PDMP-treated HEK293T cells is due to the inability of DCs to mediate *trans*-infection of GSL-deficient virus particles to T cells, virus-exposed DC-CD4⁺ T-cell cocultures were harvested 2 days postinfection and stained for cell surface CD3 and intracellular p24^{gag} antigen expression. The percentage of productively infected CD4⁺ T cells in the cocultures, positively identified by cell surface CD3 expression, was determined by intracellular p24^{gag} expression (Fig. 5C and D), and was found to correlate well with p24^{gag} production in cell-free supernatants (Fig. 5A and B). Immature and mature DC-T-cell cocultures infected with virus particles derived from FB1-treated HEK293T cells displayed a fivefold decrease in the number of productively infected CD4⁺ T cells, relative to those infected with viruses derived from untreated HEK293T cells (Fig. 5C and D), while infection with virus particles produced from PDMP-treated HEK293T cells resulted in a threefold and fourfold decrease in the number of p24^{gag}⁺ CD3⁺ T cells in immature and mature DC-T-cell cocultures, respectively (Fig. 5C and D).

To confirm that virus particles derived from FB1- or PDMP-treated HEK293T cells were attenuated in their ability to establish productive DC-mediated *trans*-infection of T cells, single-cycle replication-competent luciferase-expressing reporter virus HIV-luc (73), derived from FB1- or PDMP-treated HEK293T cells, was incubated with immature and mature DCs and washed extensively to remove unbound virus particles, and then DCs were cultured alone or cocultured with Jurkat T cells. Similar to the results described above with replication-competent virus particles, FB1 or PDMP treatment of virus producer cells also resulted in

HIV-luc particles that were deficient for capture by immature and mature DCs (65% and 69% inhibition upon FB1 treatment and 55% and 57% inhibition upon PDMP treatment [Fig. 6A and D, respectively]). While both immature and mature DCs infected and cultured alone had very low levels of luciferase activity (data not shown), immature and mature DC-T-cell cocultures displayed high levels of luciferase activity that were decreased 88% and 84%, respectively, upon challenge with FB1-treated virus particles (Fig. 6B). Furthermore, there were 75% and 67% decreases in luciferase activity in immature and mature DC-T-cell cocultures, respectively, upon challenge with Lai-luc particles derived from PDMP-treated HEK293T cells relative to that in virus particles derived from untreated virus producer cells (Fig. 6E). Note that there was no significant difference in infectivity when Jurkat cells were infected directly with Lai-luc particles derived from untreated or FB1 (Fig. 6C)- or PDMP (Fig. 6F)-treated HEK293T cells. These data imply a significant role for GSLs incorporated in the HIV-1 particle membrane in the initial interactions of virus particles with DCs and that GSL-dependent interactions of HIV-1 particles with DCs play a nonredundant role in targeting captured virus particles to the DC-mediated *trans*-infection pathway.

DISCUSSION

Although HIV-1 gp120-dependent interactions with CD4 and chemokine coreceptors are critical for the virus to establish productive infections in CD4⁺ T cells and macrophages, HIV-1 gp120-dependent interactions with DCs have long been proposed to be multifactorial. Depending on the attachment

factor mediating virus capture, HIV-1 interactions with DCs can lead to either virus particle degradation, establishment of productive virus infection, or capture and subsequent *trans*-infection of CD4⁺ T cells. In this report, we demonstrate that host cell-derived GSLs incorporated into the budding virus particle play a critical role in mediating HIV-1 capture by immature and mature DCs in a gp120-independent manner. Though inhibition of cholesterol or GSL biosynthesis in CD4⁺ target cells or cholesterol extraction from virus or cell membranes can cause a decrease in efficiency of HIV-1 infectivity (22, 29, 33, 38, 41, 43, 57), presumably by affecting gp120-dependent virus-cell fusion, these studies have not taken into account the direct effects of surface-exposed GSLs in HIV-1 attachment to DCs. Interestingly, accessing the GSL-dependent gp120-independent capture pathway in both immature and mature DCs was crucial for HIV-1 to be targeted to the *trans*-infection pathway, since DC capture of GSL-depleted HIV-1 particles was reduced by ~50%, while transfer of GSL-depleted virus particles to T cells was reduced by 70 to 80% (Fig. 5 and 6). These results suggest an active role for the putative DC receptor that interacts with virus particle membrane-associated GSLs in trafficking captured particles to DC–T-cell infectious synapses.

It is not surprising that HIV-1 gp120-independent DC binding mechanisms can exist, considering that there are only 7 to 14 trimeric gp120 spikes irregularly clustered on the surface of an HIV-1 particle (80), thus leaving open the possibility that host cell-derived determinants incorporated into the virus lipid bilayer can impact virus capture. Since HIV-1 budding occurs from cholesterol and GSL-enriched plasma membrane lipid rafts, thus ensuring a specific GSL composition in the viral membrane (9, 12), HIV-1 particles are imbued with a unique binding profile that is independent of gp120. In mammalian cells, sphingomyelin and GSLs comprise the two major classes of sphingolipids, both of which are synthesized from the hydrophobic molecule ceramide (34). The lipid portion of GSLs, ceramide, is embedded in the outer leaflet of the plasma membrane, while their oligosaccharide chain, having specific ligand binding activity, projects into the extracellular space at a defined angle to the axis of ceramide (34). Quantitative estimates on the number of lipid molecules per average HIV-1 particle place the number at ~296,000, including ~600 glucosylceramide molecules (9). Though estimates on various classes of GSLs in an HIV-1 particle membrane are unknown, the fact that at least 600 glucosylceramide-derived molecules per virus particle can in theory participate in HIV-1 attachment to DCs speaks to the robustness of GSL-dependent HIV-DC interactions. Hence, targeting of virus particles to bud from nonraft regions of the plasma membrane might result in the production of HIV-1 particles that are unable to efficiently access the DC-dependent *trans*-infection pathway.

It has been previously suggested that interactions of PBMC-derived HIV-1 particles with immature DCs can be completely accounted for by gp120-dependent interactions with DC-SIGN and syndecan-3 (14, 21). Though these studies have implied that the cellular source of virus plays a crucial role in defining the ability of virus particles to bind DCs (14), we observed gp120-independent capture of HEK293T, Jurkat T-cell-, or primary CD4⁺ T-cell-derived virus by immature DCs (Fig. 2A and B). Furthermore, this mechanism of gp120-independent

virus capture was significantly upregulated upon maturation of DCs (Fig. 2), an observation made previously by others as well (36, 71). We have recently reported that gp120-independent HIV-1 capture by mature DCs can be competitively inhibited by exosomes that bud from lipid raft-like plasma membrane domains (37). Interestingly, PBMCs and CD4⁺ T cells can produce large amounts of exosomes (5, 59, 62). The presence of exosomes in HIV-1 particle containing supernatants could competitively inhibit the gp120-independent capture mechanism of HIV-1 particles to DCs, thus possibly accounting for the previously described results that have failed to observe gp120-independent capture of PBMC-derived HIV-1 by immature DCs (7, 14, 39).

HIV-1 particles captured by immature and mature DCs can localize within tetraspanin-positive endosomal compartments (20, 36, 73) or deep membrane invaginations, suggested to be contiguous with the cell surface (79), which upon T-cell contact are rapidly translocated to the DC–T-cell contact zone via a trafficking pathway that is mimicked by exosomes (73). The data presented in this report and our previously published findings (37, 73) suggest a common entry mechanism for HIV-1 particles and exosomes within DCs that can lead virus particles and exosomes to common destinations within the cell and for cellular exit. Though HIV-1 gp120–DC-SIGN interaction has been implicated in targeting HIV-1 particles in immature DCs to sites of DC–T-cell infectious synapses (1, 2, 20), our data argue for DC-SIGN-independent, GSL-dependent interactions of virus particles with DCs as the critical determinant for capture and routing of particles within DCs for transfer of HIV-1 particles to T cells. It is possible that the DC-SIGN requirement for DC-mediated HIV-1 *trans*-infection involves signaling through DC-SIGN, initiated by gp120 binding that recruits the Rho guanine nucleotide exchange factor, LARG, a factor that might be necessary for the generation of optimal DC–T-cell infectious synapses (32). While our previous finding that cholesterol depletion from the DC membrane suppresses HIV-1 binding (28) argues for the presence of the GSL-recognizing attachment factor(s) within lipid raft-like membrane microdomains of DCs, the identity of this attachment factor is at this moment unknown. Interestingly, HIV-1 binding to activated Langerhans cells, derived from cord blood CD34⁺ stem cells (16), vaginal epithelial sheets (31), or human skin explants (13), was also enhanced upon maturation, and binding to these cells could not be completely explained by previously defined gp120-dependent attachment factors, suggesting that interactions of HIV-1 particles with DC subsets present in peripheral mucosa might also be dependent on GSLs.

Several GSLs present in the target cell surface have been proposed as gp120 ligands that are thought to facilitate virus entry upon CD4 engagement in T cells (30, 33, 47, 58). In contrast to these results, our studies implicate GSLs present in the virus particle membrane as potential host-derived cofactors for mediating interactions of virus particles with DCs. Pharmacological inhibition of GSL expression in the cell surface, hence, could inhibit not only HIV-1 transmission between T cells but also DC-mediated HIV-1 transmission. Interestingly, while the manuscript was in preparation, an exhaustive small-interfering-RNA screen to identify host factors necessary for HIV-1 replication identified cellular enzymes and transport proteins of GSL biosynthesis pathways as crucial host factors for the HIV-1 life cycle (8). These previously published studies

and our data argue for a unique role for GSLs in DC-mediated HIV-1 pathogenesis and provide a novel host target for the development of antiviral therapeutics.

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