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Innate Invariant NKT Cell Recognition of HIV-1–Infected Dendritic Cells Is an Early Detection Mechanism Targeted by Viral Immune Evasion

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Invariant NKT (iNKT) cells are innate-like T cells that respond rapidly with a broad range of effector functions upon recognition of glycolipid Ags presented by CD1d. HIV-1 carries Nef- and Vpu-dependent mechanisms to interfere with CD1d surface expression, indirectly suggesting a role for iNKT cells in control of HIV-1 infection. In this study, we investigated whether iNKT cells can participate in the innate cell–mediated immune response to HIV-1. Infection of dendritic cells (DCs) with Nef- and Vpu-deficient HIV-1 induced upregulation of CD1d in a TLR7-dependent manner. Infection of DCs caused modulation of enzymes in the sphingolipid pathway and enhanced expression of the endogenous glucosylceramide Ag. Importantly, iNKT cells responded specifically to rare DCs productively infected with Nef- and Vpu-defective HIV-1. Transmitted founder viral isolates differed in their CD1d downregulation capacity, suggesting that diverse strains may be differentially successful in inhibiting this pathway. Furthermore, both iNKT cells and DCs expressing CD1d and HIV receptors resided in the female genital mucosa, a site where HIV-1 transmission occurs. Taken together, these findings suggest that innate iNKT cell sensing of HIV-1 infection in DCs is an early immune detection mechanism, which is independent of priming and adaptive recognition of viral Ag, and is actively targeted by Nef- and Vpu-dependent viral immune evasion mechanisms. ***The Journal of Immunology*****, 2016, 197: 1843–1851.**

Invariant NKT (iNKT) cells express an invariant CD1d-restricted TCR and have innate-like characteristics (1, 2). iNKT cells respond rapidly in an innate manner with a broad range of effector and immunoregulatory functions upon recognition of glycolipid Ags presented by CD1d (3, 4). These Ags can be of exogenous microbial origin or be endogenous self-antigens presented at elevated levels and in an inflammatory milieu (5, 6). Glucosylceramide (GlcCer) with a 24:1 *N*-acyl chain paired with a d18:1 sphingosine base is one such endogenous CD1d-presented iNKT cell Ag (7, 8). Expression of this endogenous glycolipid Ag can be increased by innate pattern recognition of bacterial products such as LPS in dendritic cells (DCs), allowing triggering of an iNKT cell response (7, 8).

A role for iNKT cells in immune responses to viral infections is supported by studies in mouse models, where mice deficient in either CD1d or iNKT cells show increased susceptibility or impaired immune responses to several viruses (9). In humans the role of iNKT cells in viral immunity has been less studied and their involvement in immune protection and immunopathogenesis is less clear (10). In the context of HIV-1, early studies described the numerical decline of iNKT cells in chronic stages of infection (11–15). Furthermore, the residual iNKT cells in chronically HIV-1–infected patients have an exhausted phenotype with poor functionality (16–18), and in particular the $CD4^+$ subset of the residual iNKT cells displays viral load–driven activation (19). In SIV infection of nonhuman primates, a study comparing iNKT cells in AIDS-resistant mangabeys and susceptible macaques indicated that high iNKT cell levels and certain phenotypes of these cells were associated with slower disease progression (20). In humans a somewhat reminiscent pattern was observed where HIV-1 subtype A infection, which shows slower disease progression as compared with subtype D infection, was associated with preserved iNKT cell counts (21). iNKT cells in the gut may be particularly important in HIV immunopathogenesis, because loss of $CD4^+$ iNKT cells in this tissue was associated with systemic immune activation (22). In support this notion, maintenance of iNKT cell function in the gut mucosa in HIV-1 infection was

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Abbreviations used in this article: DC, dendritic cell; eGFP, enhanced GFP; GlcCer, glucosylceramide; iNKT, invariant NKT; IRS, immunoregulatory DNA sequence; LC-MS/MS, liquid chromatography–tandem mass spectrometry; mdDC, monocyte-derived dendritic cell; NB-DGJ, *N*-(*n*-butyl)deoxygalactonojirimycin; Δ*nef*Δ*vpu*, virus with defective *nef* and *vpu* genes; rh, recombinant human; UPLC, ultra-performance liquid chromatography; VSV, vesicular stomatitis virus; wt, wild-type.

recently reported to be associated with maintenance of a relatively healthy composition of the gut microbiome as well as low levels of microbial translocation (23).

In DCs productively infected by HIV-1, the accessory proteins Nef and Vpu act in concert to inhibit CD1d surface expression (24–27). In this context, previous findings indicate that Nef acts by inhibiting CD1d transport from the endoplasmic reticulum to the cell surface (25, 26), whereas Vpu interferes with CD1d recycling from endosomal compartments to the cell surface (24, 27). Although this suggests a role for iNKT cells in control of HIV-1, evidence that iNKT cells can directly recognize virus-infected cells in general, and HIV-infected cells in particular, is lacking.

HIV-1 is transmitted to a new host primarily across mucosal surfaces in the genital tracts where DCs are thought to be among the first immune cells in contact with the virus (28). DCs can be infected by HIV-1 and transfer the virus to $CD4^+$ T cells (29–31), and such events are probably important during exposure, transmission, and acute infection for the systemic spread and establishment of infection in a new host (32). The early stages of sexual HIV-1 transmission probably include local viral replication in cells of the immune system within the genital mucosa (33, 34). This phase represents a bottleneck for the virus and a window of opportunity for the innate immune system to control infection locally before systemic spread and establishment of the latent viral reservoir (35, 36).

In this study, we investigate lipid Ag presentation in HIV-1–infected DCs and iNKT cell responses to such infected cells. Our data indicate that infected DCs respond in a TLR7-dependent manner and upregulate presentation of the endogenous CD1d-presented iNKT cell Ag GlcCer. Interestingly, iNKT cells can recognize and respond specifically to productively infected DCs, and this response is at least partially inhibited by Nef- and Vpu-mediated immune evasion mechanisms. We propose a model where innate iNKT cell responses may play a role in the immune defense of the female genital mucosa during initial stages of HIV-1 transmission.

Materials and Methods

Blood and mucosal samples

Specimens of endometrial and cervical mucosa were obtained from women undergoing hysterectomy for nonmalignant and noninflammatory disorders. Blood samples were from healthy blood donors. Written informed consent was obtained from all study participants in accordance with study protocols conforming to the provisions of the Declaration of Helsinki and approved by the Regional Ethics Review Board in Stockholm (protocols 2007/772-32 and 2013/1377-31/2).

Construction of DHIV3 Δnef Δvpu *and DHIV3 enhanced GFP*

Proviral vectors DHIV3 wild-type (wt), DHIV3 Δ*nef*, and DHIV3 Δ*vpu* plasmids were provided by Dr. Edward Barker (Rush University, Chicago, IL) (37). To generate DHIV3 virus with defective *nef* and *vpu* genes (Δ*nef*Δ*vpu*), the defective *vpu* gene was cloned into the DHIV3 Δ*nef* construct. The enhanced GFP (eGFP) gene was cloned into the DHIV3 wt plasmid as previously described (38). DHIV3 is a replication-deficient HIV-1 construct based on the NL4-3 sequence carrying a deletion in the *env* gene and therefore requires vesicular stomatitis virus (VSV)–G pseudotyping of the viruses to ensure infectivity.

Cell culture and production of virus stocks

293T cells were cultured in RPMI 1640 (Life Technologies/Invitrogen, Carlsbad, CA), supplemented with 2 mM L-glutamine, 1% penicillin and streptomycin, and 10% heat-inactivated FCS. To obtain VSV-G pseudotyped virions, 293T cells were cotransfected with proviral DNA and pVPack VSV-G plasmid (Stratagene). Forty-eight hours after transfection, virus containing cell culture supernatants was harvested, cleared, and frozen. HIV-1 BaL virus and HIV-1 founder virus stocks were produced using the same protocol without VSV-G cotransfection. Founder virus plasmids encoding full-length transmitted/founder HIV-1 infectious molecular clones pCH077.t/2627, pRHPA.c/2635, and pTHRO.c/2626 were obtained through the National Institutes of Health AIDS Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health), originally from Dr. John Kappes and Dr. Christina Ochsenbauer (39).

HIV-1 infection of DCs

DCs were generated from human monocytes and infected as described (40). Briefly, buffy coats were obtained from healthy blood donors and monocytes were enriched from PBMCs using RosetteSep human monocyte enrichment mixture (Stemcell Technologies, Vancouver, BC, Canada) and cultured for 6 d in medium supplemented with 5% human serum (Sigma-Aldrich), 6.5 ng/ml recombinant human (rh)IL-4 (R&D Systems, Minneapolis, MN), and 250 ng/ml rhGM-CSF (PeproTech, Rocky Hill, NJ). DCs were infected with viral stocks in the presence of cytokines and serum.

Culture of iNKT cells

CD1d-restricted iNKT cell lines were established as described (24). Briefly, PBMCs of healthy donors were cultured in RPMI 1640 (Invitrogen, Paisley, U.K.) supplemented with 10% FCS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (Hyclone, Logan, UT), and 100 ng/ml αGalCer (Enzo Life Sciences, Plymouth Meeting, PA) to stimulate proliferation of iNKT cells. Twenty-four hours later, the medium was supplemented with 10 ng/ml rhIL-2 (PeproTech). After 10–14 d, iNKT cells were purified by immunomagnetic cell sorting using biotinylated anti-TCR Vα24 mAb (clone C15; Beckman Coulter, Marseille, France) and streptavidin-conjugated MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated iNKT cells was assessed by flow cytometry and routinely exceeded 95%. Purified cells were restimulated with gamma-irradiated (40 Gy) allogeneic monocytes loaded with αGalCer and maintained in culture medium supplemented with rhIL-2.

Flow cytometry and mAbs

The mAbs anti–HIV-1 p24-FITC (clone KC57), anti–Vα24-FITC (clone C15), and anti–Vβ11-PE (clone C21) were from Beckman Coulter; anti–CD1d-PE (clone CD1d42), anti-CD3 Alexa Fluor 700 (clone UCHT1), anti-CD4 Brilliant Violet 605 (clone RTA-T4), anti–CD11c-allophycocyanin (clone B-ly6), anti-CD11c PE-Cy5 (clone B-ly6), anti-CD45 PerCP (clone 2D1), anti-CD56 Alexa Fluor 700 (clone B159), anti-CCR5 allophycocyanin-Cy7 (clone 2D7/CCR5), anti–DC-SIGN v450 (clone DCN46), and anti–HLA-DR allophycocyanin (clone L243) were from BD Biosciences (San Jose, CA); anti-CD4 Brilliant Violet 711 (clone OKT4) and anti-CD8 Brilliant Violet 570 (clone RPA-T8) were from BioLegend (San Diego, CA); anti-CD14 PE–Texas Red was from Invitrogen; and anti-CD19 PE–Texas Red (clone SJ25-C1) was from Abcam (Cambridge, U.K.). Data were acquired on a BD LSRFortessa instrument (BD Biosciences) and analyzed using FlowJo version 9.7.5 software (Tree Star, Ashland, OR). In some experiments, DCs were treated with 5 μg/ml imiquimod (InvivoGen, Toulouse, France), 5 μg/ml polyinosinic-polycytidylic acid (InvivoGen), 1 μg/ml ssRNA40LyoVec (InvivoGen), 50 μM *N*-(*n*-butyl)deoxygalactonojirimycin (NB-DGJ) (Toronto Research Chemicals, Toronto, ON, Canada), 10 μM chloroquine (InvivoGen), or with 5.6 μM TLR7 oligonucleotide antagonist immunoregulatory DNA sequence (IRS)954 (5′-TGCTCCTGGAGGGGTTGT-3′) or the control oligonucleotide 5′-TCCTGCAGGTTAAGT-3 (Integrated DNA Technologies) (41).

iNKT cell activation assays

iNKT cell activation was assessed by microcopy as previously described (38). Briefly, iNKT cells were coincubated with infected DCs in the presence of brefeldin A for 4 h. After fixation with 4% paraformaldehyde, cell complexes were cytospun onto glass microscope slides and permeabilized with 0.1% saponin (Sigma-Aldrich). Cells were stained with anti–IFN-γ mAb (clone 25,718; 1:50; R&D Systems), blocked with 1% normal goat serum (Dako, Glostrup, Denmark), and incubated with goat anti-mouse Alexa Fluor 594 secondary Ab (1:500; Molecular Probes, Eugene, OR). To detect productively infected DCs, cells were stained with anti–HIV-1 p24 FITC (1:20), blocked with 1% rabbit serum, and then anti-FITC Alexa Fluor 488 (Invitrogen) and DAPI (Sigma-Aldrich) were added. All incubations with primary and secondary Abs and serum were done at room temperature for 30 min. Images were obtained on a Nikon A1R confocal system with a ×60/1.49 oil objective using NIS-Elements AR software (version 3.2). IFN-γ production by iNKT cells was analyzed by visual inspection of randomly captured images of DC–iNKT cell complexes, 20–50 pictures per condition, counting 100–1300 iNKT cells per condition. In some experiments, DCs were incubated with 10 μg of anti-CD1d (clone 51.1; eBioscience, San Diego, CA) for 20 min at 37°C before addition of iNKT cells. For the CD25 upregulation assay, the

iNKT cell clone HDD3 (provided by Steven Porcelli) (42), confirmed to be mycoplasma free by PCR, was cocultured with DCs with or without 5 μg/ml imiquimod for 24 h and then stained with anti-CD25 PE-Cy7 (clone M-A251; BD Biosciences). In some experiments, DCs were pretreated with 50 μM NB-DGJ for 24 h.

Quantitative PCR analysis

RNA was extracted with the RNeasy system (Qiagen, Valencia, CA), and cDNA was synthesized with the SuperScript VILO cDNA synthesis kit (Invitrogen). The QuantiTect SYBR Green quantitative PCR kit (Qiagen) and the Applied Biosystems 7500 real-time PCR system were used for quantitative PCR. Primers for *gapdh*, *ugcg*, and *b4galt6* were all obtained from Qiagen.

TLR phenotyping of monocyte-derived DCs by RT-PCR

RNA was isolated from monocyte-derived DCs (mdDCs) of three donors and cDNA was produced as described before. The quality of cDNA was confirmed by amplification of the *gapdh* housekeeping gene for each donor. For TLR expression phenotyping, the human TLR-RT primer set (rts-htlrs; InvivoGen) was used in combination with Pfu polymerase (MBI Fermentas).

Cell sorting

DCs were infected with the DHIV3 eGFP construct for 6 d and fixed for 30 min at room temperature with 4% paraformaldehyde (Sigma-Aldrich). Equal numbers of mock, $eGFP^-$, and $eGFP^+$ were then sorted using a FACSAria III instrument (Becton Dickinson). Sorting was performed using an 85-μm nozzle at 45 ψ and a yield mask of 32 to maximize rare event recovery. Mock-infected DCs were used as controls to define GFP^+ gates for sorting. Sorted cells were stored at −80°C.

Extraction and analysis of GlcCer 24:1

Sphingolipids were extracted using a modified Bligh and Dyer protocol and analyzed by electrospray ionization/liquid chromatography–tandem mass spectrometry (LC-MS/MS). Briefly, sorted DCs were thawed on ice, and 10 μl of the internal standard solution containing GlcCer 8:0 in MeOH (60 μM) (Avanti Polar Lipids, Alabaster, AL) was added. After that, 570 μl of MeOH/$CHCl_3$ (2:1, v/v) were added and samples were vortexed for 30 s followed by a 15-min sonication. Extraction was performed by sequential additions of H_2O, $CHCl_3$, and H_2O (150, 190, and 160 μl, respectively). Then, samples were centrifuged at 5000 × *g* for 10 min and the organic layer was transferred to another tube. After re-extraction of the aqueous phase with 300 μl of $CHCl_3$, both organic extracts were combined and reduced to dryness in a SpeedVac concentrator (Genevac, Ipswich, U.K.) and stored at −80°C until analysis. On the day of analysis, extracts were reconstituted in 200 μl of MeOH, sonicated for 2 min, vortexed, and filtered by centrifugation for 3.5 min at 3000 × *g* using 0.1-μm membrane spin filters (Merck Millipore, Billerica, MA). Extracts were then transferred into autosampler vials and 7.5 μl was injected in the chromatographic system. Electrospray ionization/LC-MS/MS analysis was performed on an ultra-performance liquid chromatography (UPLC) Acquity-Xevo TQS mass spectrometer (Waters, Milford, MA). Chromatographic separation was carried out on an Acquity UPLC BEH C8 column (130 Å, 1.7 μm, 2.1 × 150 mm) equipped with an Acquity UPLC BEH C8 VanGuard precolumn. GlcCer 24:1 was quantified by selected reaction monitoring using the following transition in the positive ionization mode: GlcCer 24:1 *m/z* 792.6→264.4.

Mucosal sample collection and tissue processing

Specimens of endometrial and cervical mucosa were obtained from women undergoing hysterectomy for nonmalignant and noninflammatory disorders. None of the women had any clinical signs or a history of sexually transmitted infections during the past 3 mo. Tissue samples were maintained in ice-cold RPMI 1640 and processed within 24 h of surgery. For each specimen, the mucosa was separated from underlying stroma and dissected into blocks as previously described (43). Tissue blocks were snap frozen immediately after dissection and stored at −80°C for in situ staining, or digested with 0.1 mg/ml DNase I (Roche, Basel, Switzerland) and 0.5 mg/ml collagenase II (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. Digested tissue blocks were mechanically disrupted with a pestle. The obtained cell suspension was filtered through a 70-μm cell strainer (BD Biosciences, San Jose, CA) and washed once in PBS before staining.

In situ staining

Immunofluorescence staining was performed on 8-μm-thick sections of cryopreserved endometrial and ectocervical biopsies as previously described (44). The tissue sections were preincubated with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) and stained sequentially. First, CD1d was detected by mouse anti-human CD1d mAb (clone 51.1) in combination with an Alexa Fluor 594–conjugated donkey anti-mouse IgG mAb (Molecular Probes). Second, HLA-DR was detected with biotinylated mouse anti-human HLA-DR mAb (clone L243) (BD Biosciences) and streptavidin-conjugated Alexa Fluor 488 (Molecular Probes). Finally, tissue sections were mounted with Vectashield containing DAPI (Vector Laboratories), and images were acquired on a DMR-X microscope (Leica, Wetzlar, Germany) with a Retiga 2000R camera (QImaging, Surrey, BC, Canada) using Image-Pro Premier 9.1 software (Media Cybernetics, Rockville, MD), which was also used for image processing.

Statistical analysis

All replicates are biological replicates. All statistical analysis was performed using GraphPad Prism version 5.0c for Mac OSX (GraphPad Software, La Jolla, CA). Groups where compared using the Mann–Whitney *U* test or the Wilcoxon signed rank test for paired samples. A p value <0.05 was considered statistically significant.

Results

Elevation of CD1d expression levels in productively HIV-infected DCs is inhibited by Nef and Vpu

We hypothesized that the CD1d-restricted iNKT cells may have an innate-like capacity to detect and respond to HIV-infected DCs. To address this possibility, human mdDCs were infected with wt DHIV3 HIV-1 or with DHIV3 Δ*nef*Δ*vpu*, and productive infection was detected by intracellular p24 staining. This Nef and Vpu double-defective virus lacks the two known mechanisms for HIV-1 interference with CD1d surface expression (24–27). mdDCs productively infected with the wt HIV-1 had decreased surface CD1d expression compared with uninfected mdDCs, consistent with our previous report (Fig. 1A, 1B) (24). Notably, however, mdDCs infected with HIV-1 Δ*nef*Δ*vpu* displayed CD1d surface expression levels that were significantly elevated compared with uninfected cells (Fig. 1C, 1D). Direct comparison of mdDCs infected with wt and Δ*nef*Δ*vpu* HIV-1 supported the conclusion that mdDCs upregulate CD1d in response to HIV-1 infection, and that the Nef and Vpu proteins expressed by wt HIV-1 together inhibit this upregulation (Supplemental Fig. 1A).

To investigate the extent to which the mechanisms of inhibition of the CD1d pathway are present in disease-relevant viral isolates, mdDCs were infected with HIV-1–transmitted founder strains. Three founder strains out of seven tested gave rise to detectable productive infection rates in the 0.1–0.5% range as measured by p24 staining on day 6: the pCH077.t/2627 virus, the pRHPA.c/2635 virus, and the pTHRO.c/2626 virus (Fig. 1E). The pTHRO.c/2626 virus downregulated CD1d at least as strongly as the BaL laboratory strain, whereas downregulation by the pCH077.t/2627 virus and the pRHPA.c/2635 virus was more modest and variable between mdDC donors. These findings indicate that early transmitted viral isolates from patients display some variability in their CD1d downregulation activity.

TLRs are involved in innate sensing of HIV infection (32, 45, 46). We therefore evaluated the ability of chloroquine to inhibit the increase in CD1d expression induced by HIV-1 Δ*nef*Δ*vpu*. Chloroquine did not alter the extent of CD1d downregulation by the wt virus. In contrast, CD1d up-regulation in response to HIV-1 Δ*nef*Δ*vpu* was modestly reduced (Fig. 1F). However, chloroquine has broad effects beyond inhibition of TLR signaling, such as inhibition of endocytosis. Thus, we next aimed to more specifically investigate the contribution of different TLRs. TLR3, 7, 8, and 9 have been implicated in DC sensing of HIV-1. The mdDCs expressed TLR3, 7, and 8, but not TLR9, as determined by RT-PCR (Supplemental Fig. 1B). Next, we tested whether agonists of TLR3, 7, and 8 could reproduce the increase

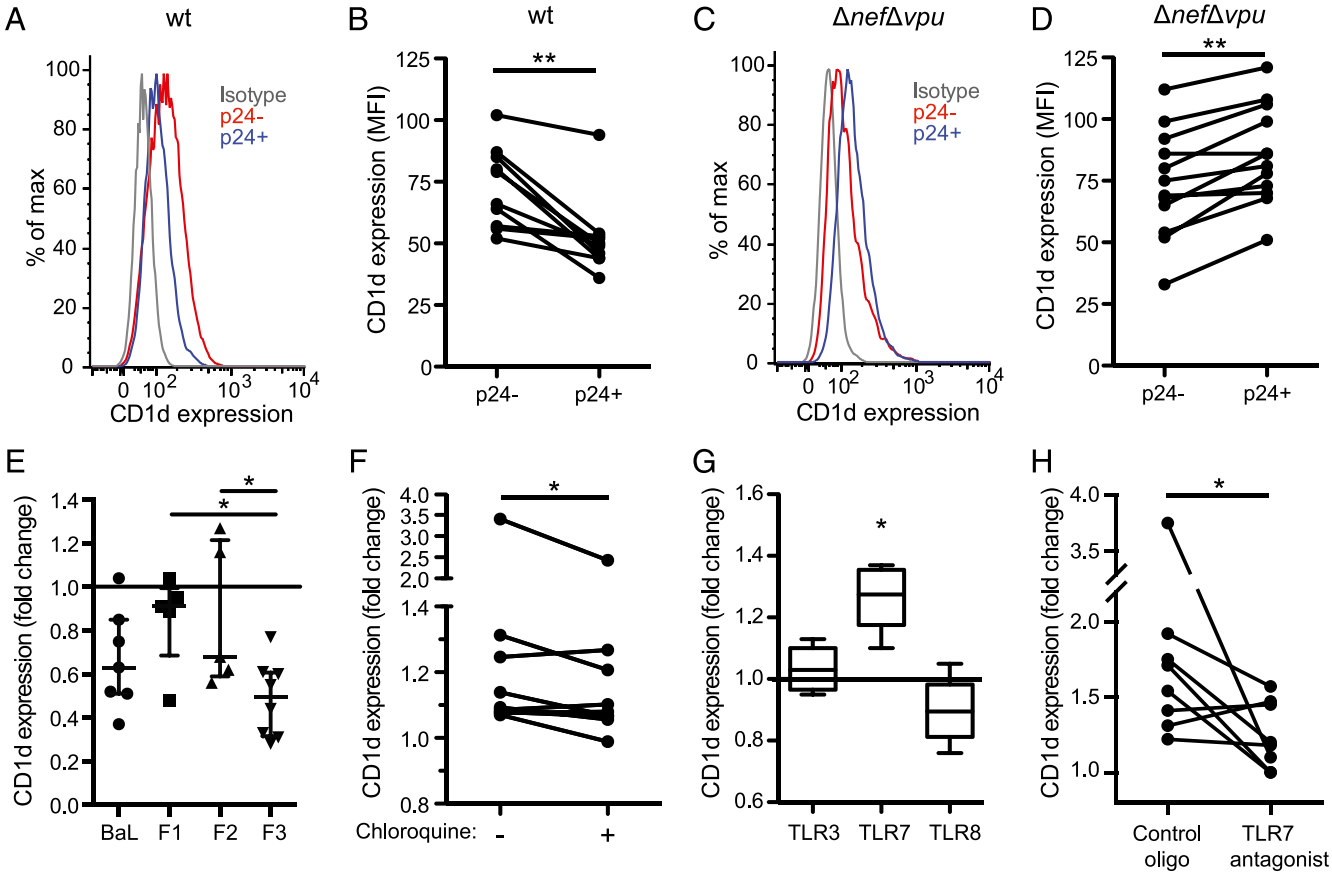

FIGURE 1. Elevated CD1d levels in response to TLR7 signals are antagonized by Nef and Vpu. mdDCs were infected with DHIV3 for 6–7 d and stained for surface CD11c, CD1d, and intracellular p24 and analyzed by flow cytometry. (**A**) Flow cytometry histogram of CD1d expression in mdDCs infected with wt DHIV3. (**B**) CD1d mean fluorescence intensity (MFI) on $p24^-$ and $p24^+$ mdDCs infected with wt ($n = 10$). (**C**) Flow cytometry histogram of CD1d expression in mdDCs infected with Δ*nef*Δ*vpu* DHIV3. (**D**) CD1d MFI on $p24^-$ and $p24^+$ mdDCs infected with Δ*nef*Δ*vpu* DHIV3 ($n = 12$). (**E**) Change in CD1d MFI was determined in $p24^+$ mdDCs in comparison with $p24^-$ mdDCs ($n = 8$) infected with BaL virus, or founder virus strains pCH077.t/2627 (F1), pRHPA.c/2635 (F2), and pTHRO.c/2626 (F3). mdDC donors where $p24^+$ infection rates in the 0.1–0.5% range were detected were included in the analysis. (**F**) mdDCs were infected with DHIV3 for 3 d ($n = 7$), 10 μM chloroquine was added for 24 h, and cells were stained for surface CD11c, CD1d, and intracellular p24 and analyzed by flow cytometry. (**G**) mdDCs were treated with TLR3 agonist polyinosinic-polycytidylic acid, TLR7 agonist imiquimod, or TLR8 agonist ssRNA40/LyoVec for 24 h and levels of surface CD1d were analyzed by flow cytometry ($n = 6$); box and whiskers, minimum to maximum. (**H**) mdDCs from eight independent donors were infected with DHIV3 Δ*nef*Δ*vpu*, treated with TLR7 oligonucleotide antagonist IRS954 or control oligonucleotide on day 4, and stained for surface CD11c, CD1d, and intracellular p24 and analyzed by flow cytometry on day 5. $*p < 0.05$, $**p < 0.01$ by Wilcoxon signed rank test. MFI, mean fluorescence intensity.

in CD1d observed after infection with HIV-1 Δ*nef*Δ*vpu*. Stimulation of mdDCs with the TLR7 agonist led to a significant increase in CD1d surface expression, whereas stimulation with agonists for TLR3 and TLR8 did not (Fig. 1G). Furthermore, CD1d upregulation in mdDCs in response to HIV-1 Δ*nef*Δ*vpu* was inhibited by addition of the TLR7 oligonucleotide antagonist IRS954 to infected cultures on day 4, whereas CD1d upregulation was not inhibited by addition of control oligonucleotide (Fig. 1H). Collectively, these results suggest that mdDCs upregulate CD1d after sensing of HIV-1 productive infection in a TLR7-dependent fashion, and that HIV-1 has evolved mechanisms to prevent induction of CD1d cell surface expression. The observation that CD1d is upregulated when mdDCs are productively infected is consistent with reports that HIV-1 sensing by DCs requires genome integration and expression of the viral capsid (47, 48).

TLR7 triggering modulates expression of enzymes in the sphingolipid pathway in DCs and supports iNKT cell activation

TLR4 stimulation of mouse DCs leads to accumulation of GlcCer with a 24:1 *N*-acyl chain paired with a d18:1 sphingosine base, reported to be an endogenous glycolipid Ag for iNKT cells, by increasing the expression of the Ugcg enzyme and decreasing the expression of the B4galt6 enzyme (7, 8). Furthermore, Kain et al. (49) recently reported that α-linked GlcCer is an endogenous ligand activating iNKT cells. To investigate whether stimulation via TLR7 leads to similar changes in *Ugcg* and *B4galt6* expression levels in human mdDCs, such cells were stimulated with imiquimod for 8–48 h and expression of *Ugcg* and *B4galt6* was assessed by quantitative PCR. Expression of *Ugcg* increased rapidly following imiquimod treatment and remained increased by 2-fold after 48 h (Fig. 2A). Reduction of *B4galt6* expression occurred with a maximum after 24 h of incubation with imiquimod, and levels remained slightly decreased after 48 h (Fig. 2B). A similar decrease in *B4galt6* expression was observed when mdDCs were infected with wt or Δ*nef*Δ*vpu* HIV-1 (Fig. 2C), whereas *Ugcg* expression was unchanged (Fig. 2D). Next, we investigated whether these changes in *Ugcg* and *B4galt6* expression, together with the increased surface CD1d expression induced by TLR7, could trigger iNKT cell activation as evaluated by CD25 upregulation. iNKT cells were activated after incubation with mdDCs that had been treated with imiquimod, and this response was inhibited by

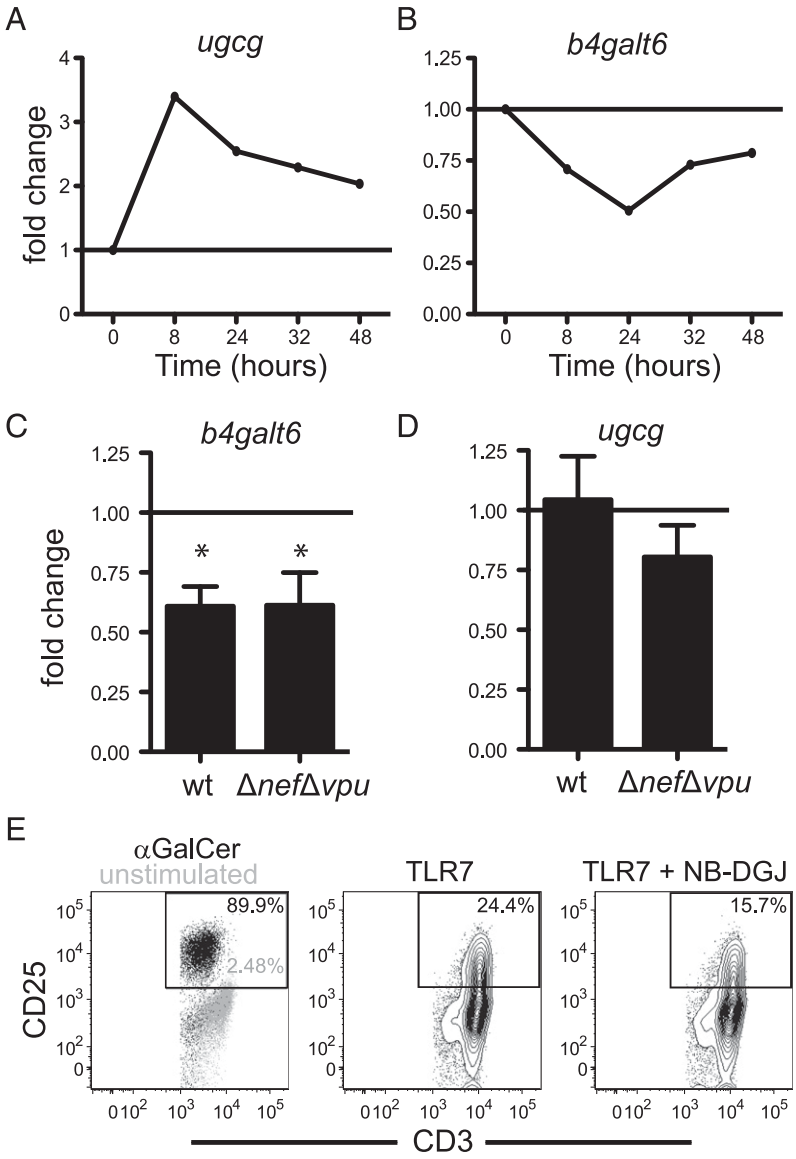

FIGURE 2. TLR7 stimulation induces changes in *ugcg* and *b4galt6* expression and iNKT cell activation. mdDCs were stimulated with imiquimod for 8–48 h and expression levels of (**A**) *ugcg* and (**B**) *b4galt6* were assessed by quantitative PCR; data are representative of three independent experiments. mdDCs were infected with DHIV3 wt or DHIV3 Δ*nef*Δ*vpu* for 6 d and expression of (**C**) *b4galt6* ($n = 7$) and (**D**) *ugcg* ($n = 8$) was assessed by quantitative PCR; mean and SD from independent experiments is shown. (**E**) The human iNKT cell clone HDD3 was cocultured with mdDCs in the presence of imiquimod for 24 h. As a positive control, αGalCer (100 ng/ml) was added to one coculture condition. mdDCs were left untreated or preincubated with 50 μM NB-DGJ for 24 h. FACS plots show one representative out of six independent experiments. $*p < 0.05$ by *t* test.

DC pretreatment with NB-DGJ inhibitor of glucosphingolipid synthesis (Fig. 2E). These data indicate that TLR7 signaling in mdDCs induces changes in the sphingolipid pathway and CD1d expression that support their ability to activate iNKT cells.

The endogenous CD1d-presented Ag GlcCer 24:1 accumulates in HIV-infected DCs

To investigate whether the abundance of GlcCer 24:1 changes in infected mdDCs, cells were infected with HIV-1 encoding eGFP. The eGFP$^-$ and eGFP$^+$ cells were purified by flow cytometric sorting followed by sphingolipid extraction and analysis by mass spectrometry (Supplemental Fig. 2A). The eGFP$^-$ fraction purity was typically 100%, and the eGFP$^+$ fraction had purity >80% (Supplemental Fig. 2B). Uninfected (mock) mdDCs and sorted eGFP$^-$ mdDCs had similar levels of GlcCer 24:1 (Supplemental Fig. 2C). Notably, sorted eGFP$^+$ mdDCs showed a significant increase in GlcCer 24:1 content compared with eGFP$^-$ cells (Fig. 3). These results indicate that sensing of productive HIV-1 infection leads to accumulation of an endogenous CD1d-presented iNKT cell-activating ligand in DCs.

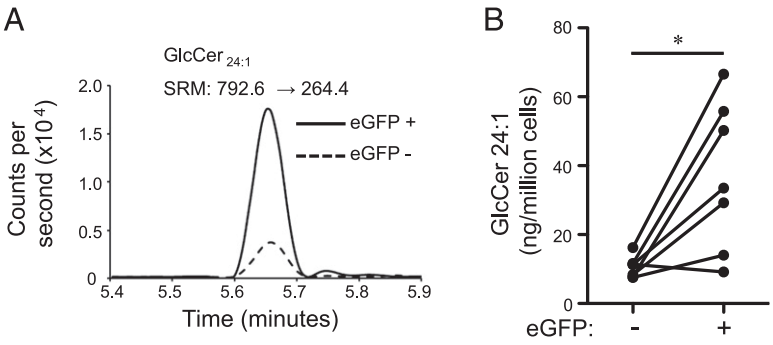

FIGURE 3. GlcCer 24:1 accumulates in HIV-1–infected DCs. mdDCs were infected with DHIV3 eGFP for 6 d and sorted based on eGFP expression. Pellets of sorted cells were extracted and then subjected to mass spectrometry analysis for glycolipid content. (**A**) Representative selected reaction monitoring (SRM) UPLC-MS/MS chromatograms of GlcCer 24:1. (**B**) Relative GlcCer 24:1 levels in eGFP$^-$ mdDCs compared with eGFP$^+$ mdDCs ($n = 7$). $*p < 0.05$ by Wilcoxon signed rank test.

To investigate whether this accumulation was involved in CD1d upregulation by DCs following TLR7 stimulation or HIV-1 infection, mdDCs were pretreated with NB-DGJ before addition of TLR7 agonist. Induction of CD1d surface expression was not affected by pretreatment with NB-DGJ (Supplemental Fig. 3A). Similarly, no change in CD1d surface expression induction was observed in NB-DGJ–treated HIV-1 Δ*nef*Δ*vpu*-infected mdDCs (Supplemental Fig. 3B), suggesting that the upregulation of CD1d in response to HIV-1 is independent of GlcCer 24:1 accumulation.

HIV-infected DCs trigger an iNKT cell response that is inhibited by Nef and Vpu

The combination of elevated CD1d surface expression and accumulation of GlcCer 24:1 in response to HIV-1 infection suggests that productively infected DCs may be able to trigger an iNKT cell response independently of viral Ag recognition. Because the frequency of productively HIV-infected mdDCs is low, we used a microscopy-based assay to investigate the iNKT cell response to infected DCs at the single cell level (Fig. 4A). We previously showed that this assay allows assessment of the iNKT cell response to the exogenous model Ag αGalCer (38). The iNKT cell IFN-γ production in response to mdDCs infected with wt HIV-1 was significantly reduced compared with uninfected mdDCs (Fig. 4B). In contrast, HIV-1 Δ*nef*Δ*vpu*–infected mdDCs triggered a significantly higher IFN-γ production in iNKT cells as compared with uninfected mdDCs (Fig. 4B). The rate of formation of DC–iNKT cell complexes was similar between wt-infected mdDCs and Δ*nef*Δ*vpu*-infected mdDCs as determined by flow cytometry (data not shown). The iNKT cell response to HIV-1 Δ*nef*Δ*vpu*–infected mdDCs was dependent on CD1d, as determined by preincubation of mdDCs with CD1d-blocking mAb (Fig. 4C). Pretreatment of the HIV-1 Δ*nef*Δ*vpu*–infected mdDCs with NB-DGJ inhibitor for 24 h also significantly reduced the iNKT cell IFN-γ response (Fig. 4C), supporting the notion that the endogenous GlcCer 24:1 Ag may be involved in the triggering of iNKT cell activation by HIV-infected mdDCs. This iNKT cell response was dependent on productive HIV-1 infection, as activation levels were similarly low when iNKT cells were in complex with $p24^-$ mdDCs in both wt or Δ*nef*Δ*vpu* HIV-1–infected DC cultures (data not shown). Furthermore, pretreatment of mdDCs with NB-DGJ did not alter iNKT cell activation by $p24^-$ mdDCs (data not shown). Taken together, these data suggest that iNKT cells specifically respond to productively HIV-1–infected DCs in the absence of functional Vpu and Nef expression, and this occurs in a CD1d-dependent and endogenous Ag-dependent manner.

iNKT cells and CD1d$^+$ DCs are present in the female genital mucosa

The main route of HIV-1 transmission is across genital mucosal surfaces, and therefore the presence of iNKT cells and $CD1d^+$ DCs in the female genital mucosa was investigated. Endometrial and cervical samples from women undergoing hysterectomy were enzymatically digested and analyzed for the presence of iNKT cells and mDCs by flow cytometry (Supplemental Fig. 4). iNKT cells were readily detected in the endometrium (0.001–0.58% of T cells, $n = 10$) as well as in the cervix (0.001–0.15% of T cells, $n = 10$) (Fig. 5A), at frequencies similar to what is usually observed in peripheral blood in the Swedish population (0.001–0.94% of T cells, $n = 74$). The three subsets of iNKT cells, that is, the $CD4^+$, $CD8^+$, and double-negative subsets, were detected in the tissues when sufficient events were recorded (Supplemental Fig. 4A). Next, DCs were identified as previously described (50) (Supplemental Fig. 4B) and found at similar frequencies in endometrial and cervical tissues. Interestingly, CD1d expression was more frequent in

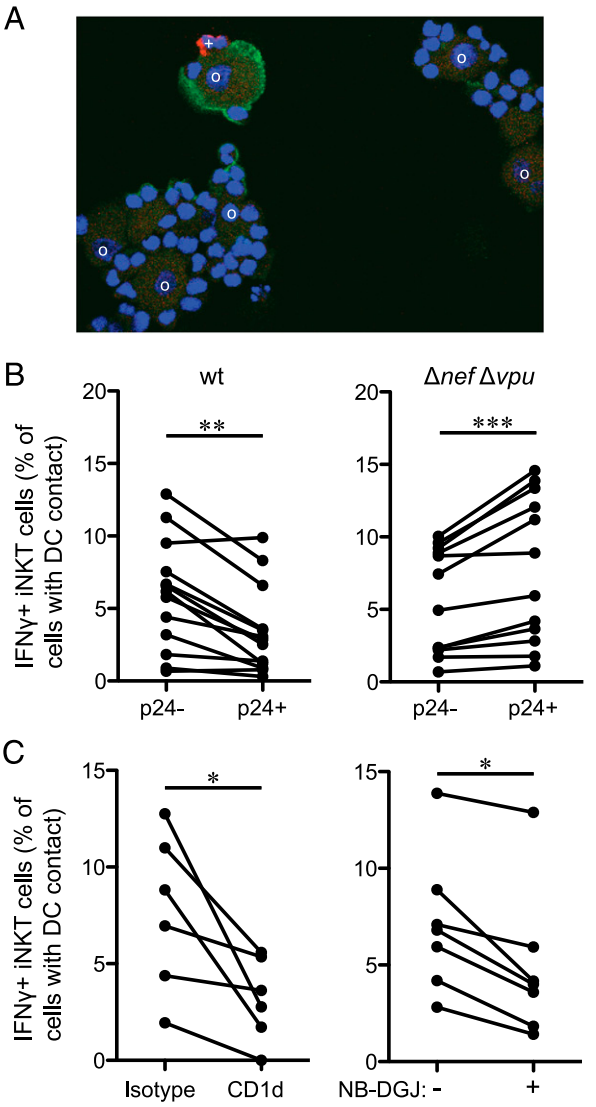

FIGURE 4. Innate targeting of HIV-1–infected cells by iNKT cells is inhibited by Nef and Vpu. (**A**) mdDCs were infected with Δ*nef*Δ*vpu* DHIV3 for 6 d before coculture with iNKT cells for 4 h in the presence of brefeldin A. Following cytospinning, cells were fixed, permeabilized, and stained with anti–IFN-γ mAb, anti-p24 mAb, and DAPI. IFN-γ, red; p24, green; DAPI, blue. +, IFN-γ^+ iNKT cells in contact with mdDCs; white circles, DCs. Image captured using a ×60 objective. (**B**) Quantification of iNKT cell IFN-γ production after coculture with DHIV3 wt or DHIV3 Δ*nef*Δ*vpu*–infected mdDCs ($n = 13$). (**C**) mdDCs infected with DHIV3 Δ*nef*Δ*vpu* were treated with anti-CD1d mAb ($n = 6$) or NB-DGJ inhibitor for 24 h ($n = 7$) before coculture with iNKT cells and quantification of iNKT cell IFN-γ production. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Wilcoxon signed rank test.

DCs in the endometrium than in the cervix (Fig. 5B). $CD1d^+$ DCs expressed higher levels of the main HIV-1 receptors CD4 and CCR5 compared with $CD1d^-$ DCs (Fig. 5C), suggesting that DCs more susceptible to HIV-1 infection express the CD1d lipid Ag presentation machinery. DC-SIGN was expressed at similar levels in $CD1d^+$ and $CD1d^-$ DCs (Fig. 5C).

To investigate the localization of $CD1d^+$ APCs in the mucosa by in situ staining, CD1d was costained together with HLA-DR, which was used as a surrogate marker for APCs in the tissues. The $CD1d^+HLA\text{-}DR^+$ cells displayed a scattered distribution in the lamina propria of the endometrium, mainly located adjacent to or within the glandular epithelium (Fig. 5D). In the ectocervix, the CD1d and HLA-DR coexpressing cells were located in close

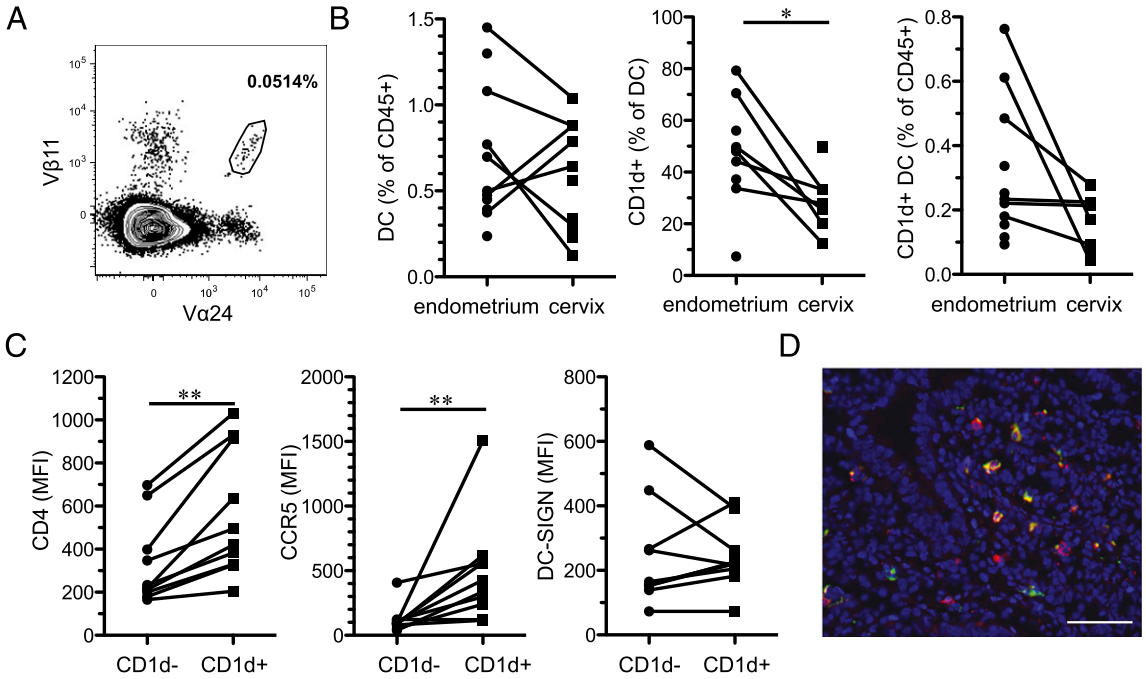

FIGURE 5. DCs that express CD1d, CD4, and CCR5 are present in the female genital mucosa. (**A**) Representative FACS plots for identification of iNKT cells in the female genital mucosa. (**B**) Frequency of DCs, CD1d expression on DCs, and frequency of $CD1d^+$ DCs in the endometrium and cervix ($n = 11$). Where paired samples were available, those data points are connected with a solid line. (**C**) Comparison of CD4, CCR5, and DC-SIGN expression mean fluorescence intensity (MFI) on $CD1d^-$ and $CD1d^+$ DCs in the endometrium ($n = 10$). (**D**) In situ staining for DAPI (blue), CD1d (red), and HLA-DR (green) in the endometrium, Scale bar, 120 μm. Images were collected with a ×20 objective. $^*p < 0.05$, $^{**}p < 0.01$ by Mann–Whitney *U* test in (B) and by Wilcoxon signed rank test in (C).

proximity to the basal membrane or in the parabasal layer of the epithelium. APCs expressing CD1d thus reside at the mucosal sites where HIV-1 transmission can occur.

Discussion

The results of the present study indicate that productive HIV-1 infection in DCs induces changes in expression of CD1d and endogenous glycolipid Ag. In the absence of functional Nef and Vpu expression, these two changes act as danger signals and allow an innate-like recognition and a rapid response by iNKT cells. The notion that this may represent a significant immune mechanism to defend the host during initial transmission events is supported by the observation that HIV-1 carries Nef- and Vpu-dependent mechanisms that inhibit this CD1d surface upregulation and Ag presentation (24–27). In this study, distinct transmitted founder viral isolates display differential CD1d downregulation capacity, and a similar pattern was evident in our recent analysis of a library of Vpu sequences showing a wide range of activity against CD1d (27). Different HIV-1 strains may thus be differentially successful in inhibiting the TLR7-mediated upregulation of the CD1d Ag presentation pathway (Fig. 6). This mode of immune evasion could be particularly relevant at the earliest stages of infection where iNKT cells may act at the site of transmission to prevent HIV-1–infected DCs from spreading the virus to peripheral secondary lymphoid organs.

Recognition of CD1-presented lipid and glycolipid Ags is important for immune responses against bacteria, whereas the importance of this mode of recognition is less obvious for viral infections, as viruses do not encode those types of Ags. Nevertheless, several viruses have the capacity to interfere with CD1d surface expression, including HSV-1 (51, 52), Kaposi sarcoma–associated herpes virus (53), human papillomavirus (54), and HIV-1 (24–26, 55). The results by Zeissig et al. (56) indicated that hepatitis B virus infection of hepatocytes induces alterations in endogenous lipids that can be recognized by both invariant and noninvariant NKT cells and contribute to hepatitis B virus–specific immunity in mice. Our finding that iNKT cells respond to HIV-1 infection by recognition of elevated levels of CD1d and endogenous lipid Ag indicates a novel immune defense strategy against HIV-1. It also supports the notion that the interference with CD1d expression observed in several viral infections may more generally represent viral immune evasion mechanisms from iNKT cell recognition of endogenous CD1d-presented Ag.

Activation of iNKT cells in tissues, including the mucosa, can be expected to have both local direct effects and systemic effects on both innate and adaptive immunity. In the interaction with the infected target cell, the iNKT cells may produce IFN-γ, with direct

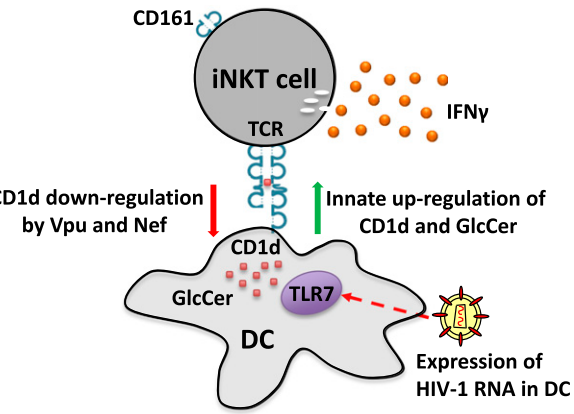

FIGURE 6. Proposed model of iNKT cell immune surveillance in the genital mucosa. Effectiveness of iNKT cell immune surveillance depends on competition between the immuno-evasive capacity of infecting HIV-1 strains as well as the TLR7-mediated upregulation of the CD1d Ag presentation pathway.

antiviral effects, and TNF as well as chemokines that will promote local inflammation. It is also possible that iNKT cells may mediate direct cytolysis of infected cells, although such targeting of cells may also be indirect via the recruitment of, and cross-talk with, NK cells. The recent observation that iNKT cells mature and accumulate in the small intestine already at the fetal developmental stage opens the possibility that this mode of immune recognition of viral infection may be operative at this site as well (57). At the systemic level, iNKT cell activation may promote both cellular and humoral adaptive immune responses, in line with the well-characterized adjuvant effect of these cells (58). The idea that this effect can be important in the genital mucosa is supported by the finding in mice that systemic and mucosal IgG responses against vaginal immunization with HSV-2 glycoprotein is strongly enhanced by inclusion of exogenous αGalCer Ag (59). It is possible that natural activation of iNKT cells by the mechanisms described in the present study will have similar effects, and approaches to harness this effect in vaccinology could be very fruitful. Exploitation of naturally presented endogenous ligands may avoid exhaustion of the iNKT cell compartment, which can be a problem with the artificial superagonist Ags (60). Interestingly, Kain et al. (49) recently reported that α-linked GlcCer is an endogenous CD1d-presented ligand for iNKT cells. Use of such endogenous Ags may open new possibilities for iNKT cell–based therapeutics and vaccine adjuvants.

The HIV-1 accessory proteins Vpu and Nef have multiple effects targeting the innate immune response (55). With regard to CD1d, the two proteins have complementary mechanisms to prevent CD1d-mediated Ag presentation, where Nef inhibits surface expression and triggers internalization from the surface, and Vpu inhibits CD1d recycling back to the surface from early endosomal compartments. That HIV-1 maintains these two mechanisms to prevent recognition of CD1d-presented Ag strongly supports the importance of such responses in immune defense against this virus. At the same time, the observation in this study that transmitted founder viral strains may differ in their ability to inhibit CD1d expression supports the notion that inhibition of iNKT cell targeting is incomplete and variable between viruses. This is further supported by our recent observation that the different Vpu proteins encoded by a broad set of HIV isolates are differentially effective in downregulating CD1d (27). Collectively, these observations suggest that there is a dynamic range of effectiveness of these viral immune evasion mechanisms, and we speculate that this allows iNKT cells to exert immune surveillance of variable efficiency during distinct transmission events. We can identify a population of DCs expressing CD1d in the female genital mucosa, along with detectable levels of iNKT cells. It is therefore possible that iNKT cell targeting of infected DCs is an important innate immune mechanism in the earliest stages of transmission to a new host. It will be important to study the detailed mechanistic aspects of HIV-1 Vpu and Nef inhibition of CD1d-mediated Ag presentation. The results of such studies may give clues as to how to therapeutically counteract these effects and possibly design novel microbicidal agents to help prevent HIV-1 infection.

In summary, we have investigated the ability of iNKT cells to recognize HIV-1–infected DCs. The findings indicate that productively infected DCs respond to HIV-1 replication in a TLR7-dependent manner and upregulate both CD1d and the endogenous GlcCer Ag. Importantly, this allows iNKT cells to recognize and respond specifically to rare HIV-1–infected DCs in a mixed culture, and this response is actively inhibited by viral immune evasion mechanisms. To our knowledge, these findings represent the first demonstration of human iNKT cell recognition of virus-infected DCs, and they support a model where innate iNKT cell responses may play a role in the immune defense of the female genital mucosa during the initial stages of HIV-1 transmission.

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Disclosures

The authors have no financial conflicts of interest.

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