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HIV-1 Capture and Antigen Presentation by Dendritic Cells: Enhanced Viral Capture Does Not Correlate with Better T Cell Activation

Maria T. Rodriguez-Plata,* Alejandra Urrutia,†,1 Sylvain Cardinaud,†,1 Maria J. Buzón,* Nuria Izquierdo-Users,* Julia G. Prado,* Maria C. Puertas,* Itziar Erkizia,* Pierre-Grégoire Coulon,† Samandhy Cedeño,* Bonaventura Clotet,* Arnaud Moris,† and Javier Martinez-Picado*‡

During HIV-1 infection, dendritic cells (DC) facilitate dissemination of HIV-1 while trying to trigger adaptive antiviral immune responses. We examined whether increased HIV-1 capture in DC matured with LPS results in more efficient Ag presentation to HIV-1-specific CD4+ and CD8+ T cells. To block the DC-mediated trans-infection of HIV-1 and maximize Ag loading, we also evaluated a noninfectious integrase-deficient HIV-1 isolate, HIV_{NL4-3ΔIN}. We showed that higher viral capture of DC did not guarantee better Ag presentation or T cell activation. Greater HIV_{NL4-3} uptake by fully LPS-matured DC resulted in higher viral trans-infection to target cells but poorer stimulation of HIV-1-specific CD4+ and CD8+ T cells. Conversely, maturation of DC with LPS during, but not before, viral loading enhanced both HLA-I and HLA-II HIV-1–derived Ag presentation. In contrast, DC maturation with the clinical-grade mixture consisting of IL-1β, TNF-α, IL-6, and PGE2 during viral uptake only stimulated HIV-1–specific CD8+ T cells. Hence, DC maturation state, activation stimulus, and time lag between DC maturation and Ag loading impact HIV-1 capture and virus Ag presentation. Our results demonstrate a dissociation between the capacity to capture HIV-1 and to present viral Ags. Integrase-deficient HIV_{NL4-3ΔIN} was also efficiently captured and presented by DC through the HLA-I and HLA-II pathways but in the absence of viral dissemination. HIV_{NL4-3ΔIN} seems to be an attractive candidate to be explored. These results provide new insights into DC biology and have implications in the optimization of DC-based immunotherapy against HIV-1 infection. The Journal of Immunology, 2012, 188: 6036–6045.

Dendritic cells (DC) are the most potent APCs in the immune system and act as a link between innate and adaptive immunity (1, 2). DC reside as immature cells in peripheral tissues, where they monitor their environment for danger signals, capture pathogens, and migrate to draining lymph nodes. During migration, DC process captured Ags while acquiring a mature phenotype by upregulating costimulatory and HLA molecules at the cell membrane. Once they reach the T cell areas of the lymph nodes, mature DC (mDC) can present pathogen-derived peptides to naive T cells in association with HLA molecules. This process initiates a cellular immune response that involves CD4+ Th cells and CD8+ CTL, as well as a humoral immune response that requires activation of B cells (3). Nevertheless, during HIV-1 exposure in vivo, DC can also contribute to the spread of HIV-1 (4). At portals of viral entry, such as the mucosa, DC are among the first cells to encounter HIV-1; subsequently, the DC move to lymphoid tissues, where they interact with CD4+ T cells, the main targets of HIV-1 (4).

Several studies demonstrated that, upon maturation, DC improve their efficiency in transmitting HIV-1 through trans-infection (5–9), a process in which DC can retain and transfer infectious virions without becoming infected themselves. It was proposed that HIV-1 takes advantage of a pre-existing exosome Ag-dissemination pathway intrinsic to mDC to enable final trans-infection of CD4+ T cells (10–12), whereas others investigators adduce that only DC surface-bound HIV-1 are able to trans-infect target cells (13). However, both models have been reconciled, demonstrating that LPS-matured DC (mDC LPS) concentrate HIV-1 in a tetra-

*Institut de Recerca de la SIDA IrsiCaixa, Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Spain; INSERM, Unité Mixte de Recherche Scientifique 945, Infection et Immunité, Université Pierre et Marie Curie, 75013 Paris, France; and †Institut Català de Recerca i Estudis Avançats, Barcelona, Spain

1A.U. and S. Cardinaud contributed equally to this study.

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M.T.-P. designed and performed research, analyzed data, and wrote the manuscript; A.U. and S. Cardinaud designed and performed research, contributed vital analytical tools, and analyzed data; M.J.B., N.I.-U., J.G.P., M.C.P., and I.E. contributed vital analytical tools; designed research, provided technical assistance, and analyzed data; P.G.C., S. Cedeño, and B.C. contributed vital analytical tools; A.M. contributed vital analytical tools, designed research, analyzed data, and wrote the manuscript; and J.M.-P. designed research, analyzed data, and wrote the manuscript.

Address correspondence and reprint requests to Dr. Javier Martinez-Picado, Institut de Recerca de la SIDA IrsiCaixa, Hospital Germans Trias i Pujol, Carretera de Canyet s/n, 08916 Badalona, Spain. E-mail address: jmpicado@irsicaixa.es

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Abbreviations used in this article: AZT, azidothymidine; BlaM, β-lactamase; DC, dendritic cell; iDC, immature dendritic cell; iDC+ITIP, IL-1β, TNF-α, IL-6, and PGE2-matured dendritic cell; mDC LPS, LPS-matured dendritic cell; MPL, monophosphoryl lipid A; NIH, National Institutes of Health; NVP, nevirapine.

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spanin-rich compartment that remains physically connected to the extracellular milieu (9). Generally, all maturation signals upregulate expression of HLA and costimulatory molecules, although the functional ability of the resulting mDC varies (14). Consequently, depending on their qualitative maturation state, DC are able to polarize various T cell responses (15). The ability of mDC to transfer HIV-1 is also largely influenced by the maturation stimuli and the resulting DC subsets (8). For instance, DC matured in the presence of PGE2 are inefficient in HIV-1 transmission to T lymphocytes (8), whereas other maturation factors, such as TNF and LPS, are very potent in transmission of HIV-1 (16). We previously showed that mDC LPS (i.e., both monocyte-derived and blood-derived myeloid DC) capture higher amounts of HIV-1 than do immature DC (iDC) (6) and that this finding correlated with a greater ability to transfer HIV-1 to susceptible target cells (6, 9, 17). In addition, plasma LPS levels, which are significantly augmented in chronically HIV-1–infected individuals as the result of increased microbial translocation, might also support the contribution of mDC to the spread of HIV-1 in vivo (18).

Considering that DC are crucial in the generation and regulation of immune responses, they may play a dual role in HIV-1 infection by increasing the spread of HIV-1 while trying to trigger an adaptive response against viral infection. Although it is well documented that the higher viral capture of mDC LPS results in increased trans-infection to target cells (4, 6, 8), little is known about the Ag-presentation ability of this DC subset. It was recently suggested that *Mycobacterium tuberculosis* promotes HIV-1 trans-infection similarly to LPS, while suppressing class II Ag processing by DC (19). In this study, we address the efficiency of mDC LPS for presenting HIV-1–derived Ags to both CD4+ and CD8+ T cells. Using a more clinical approach, we extended the amount of HIV-1 particles, this resulted in higher viral transmission, whereas maturation of DC with ITIP during viral loading enhanced both HLA-I and HLA-II HIV-1–derived Ag presentation with LPS during, but not before, viral loading (iDC+LPS) significantly limiting viral Ag degradation. In addition, DC maturation with LPS during, but not before, viral loading (iDC+LPS) enhanced both HLA-I and HLA-II HIV-1–derived Ag presentation, whereas maturation of DC with ITIP during viral loading (iDC+ITIP) only stimulated the HLA-I pathway. Finally, compared with replication-competent HIVNL4-3, integrase-deficient HIVNL4-3ΔIN was equally captured and presented to HIV-1–specific T cells by DC. Therefore, HIVNL4-3ΔIN appears to be a promising immunogen for anti–HIV-1 vaccine development.

**Materials and Methods**

**Cells**

PBMC from HIV-1–seronegative donors were purchased from the Banc de Sang i de Teixits (Barcelona, Spain) or the Etablissement Français du Sang of the Pitié-Salpêtrière Hospital (Paris, France). Purified monocyte populations were isolated with CD14+ magnetic beads (Miltenyi Biotec) and cultured with RPMI 1640 containing 10% FBS (Invitrogen), 1000 U/ml GM-CSF, and IL-4 (R&D Systems) for 5 days to obtain iDC. On day 5, mDC were obtained by adding 100 ng/ml LPS (Sigma-Aldrich) or 300 U/ml IL-1β, 1000 U/ml IL-6, 1000 U/ml TNF-α (all from CellGenix), and 1 μg/ml PGE2 (Sigma-Aldrich) for an additional 2 days. Monocytes, iDC, and mDC were immunophenotyped using flow cytometry at day 7 (FACSCalibur Flow Cytometer; BD Biosciences). The mAb used for cell immunophenotyping were as follows: CD14-FITC and -PE (clone M5E2; BD Pharmingen), CD209-PE and -allophycocyanin (clone DCN46; BD Pharmingen), CD4-PerCP (clone SK3; BD Biosciences), HLA-DR–PE and -PerCP (clone L243; BD Biosciences), HLA class I-FTTC (clone W6/32; Sigma-Aldrich), CD86-FITC (clone 2331; BD Pharmingen), CD83-PE and -allophycocyanin (clone HB15e; BD Pharmingen), and CD80-PE-Cy5 (clone L307.4; BD Pharmingen). The institutional review boards of University Hospital Germans Trias i Pujol and Pitié-Salpêtrière Hospital approved this study.

**Virus stocks and plasmids**

Replication-competent full-length CXCR4-tropic HIV-1 (HIVNL4-3) and integrase-deficient CXCR4-tropic HIV-1 (HIVNL4-3ΔIN) stocks were generated by transfecting the proviral construct pNL4-3 (NIH AIDS Research and Reference Reagent Program: from M. Martin) and pNL4-3ΔIN (23) with calcium phosphate (Clontech) in HEK-293T cells. Both mNL4-3 and pNL4-3ΔIN were modified to express the optional epitope (SLYNTVATL) (24) and escape epitope (SLFNTIVAT) (25) for p17Gag 77–85 (SL9 peptide) and restricted by HLA-A*02 (22) were used to evaluate HLA-II Ag presentation. The T cell clones were restimulated and expanded, as previously described (21, 22), using irradiated feeders and autologous lymphoblastoid cell lines loaded with cognate peptides in T cell-clone-lining medium (RPMI 1640 containing 5% serum AB [Institut Jacques Boy], 100 U/ml recombinant human IL-2, and 1 μg/ml PHA [PAA] supplemented with nonessential amino acid and sodium pyruvate [Life Technologies]). At least 4 h before coculture with DC, T cell clones were thawed and allowed to rest in cloning medium without PHA.

**Viral stocks and trans-infection assays**

The F12 and N2 CD4+ T cell clones specific for HIV p24Gag (aa 271–290), restricted by HLA-DR*01/HLA-DRB*04 and HLA-DRB*04 (21), respectively, were used to monitor HLA-II Ag presentation. The SL9-2 and EM40-F21 CD8+ T cell clones specific for HIV p17Gag (aa 77–85, SL9 peptide) and restricted by HLA-A*02 (22) were used to evaluate HLA-I Ag presentation. The T cell clones were restimulated and expanded, as previously described (21, 22), using irradiated feeders and autologous lymphoblastoid cell lines loaded with cognate peptides in T cell-clone-lining medium (RPMI 1640 containing 5% serum AB [Institut Jacques Boy], 100 U/ml recombinant human IL-2, and 1 μg/ml PHA [PAA] supplemented with nonessential amino acid and sodium pyruvate [Life Technologies]). At least 4 h before coculture with DC, T cell clones were thawed and allowed to rest in cloning medium without PHA.

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HIVNL4-3Vpr-GFP was obtained by cotransfecting the plasmids mNL4-3 and pEGFP-Vpr with calcium phosphate (Clontech) in HEK-293T cells, as previously described (26). Supernatants containing virus were collected 48 h after transfection, filtered (Millex HV, 0.45 μm; Millipore), and frozen at −80°C until use.

The p24Gag content of all viral stocks was measured using an ELISA (PerkinElmer). Titers of all viruses were determined using the TZM-bl reporter cell line. Cells were assayed for luciferase activity 48 h postinfection (Bright-Glo Luciferase Assay System; Promega) in a Fluoroskan Ascent FL Luminometer.

**DC viral capture and trans-infection assays**

A total of 2.5 × 106 DC and mDC matured for 48 h with ITIP (mDC ITIP) or LPS (mDC LPS) from at least seven donors was incubated at 37°C for 6 h with 50 ng p24Gag of HIVNL4-3 or HIVNL4-3ΔIN at a final concentration of 1 × 106 cells/ml. During viral pulse, some iDC were matured with ITIP (iDC+ITIP), others were matured with LPS (iDC+LPS), and the rest were immature (iDC). After incubation, cells were extensively washed with PBS to remove uncaptured viral particles and lysed with 0.5% Triton X-100 at a final concentration of 5 × 105 cells/ml. Lysates were clarified of cell debris by centrifugation to measure intracellular p24Gag Ag content using an ELISA (PerkinElmer), as described elsewhere (6). Before lysis, 106 HIV-1–pulsed DC were cocultured with the TZM-bl reporter cell line at a ratio of 1:1/well in a 96-well plate at a final volume of 100 μl. Cells were assayed for luciferase activity after 48 h of coculture (Bright-Glo Luciferase Assay System; Promega) using a Fluoroskan Ascent FL Luminometer.

**Ag-presentation assays**

To evaluate the impact of the time lag between DC maturation and Ag loading on the stimulation of HIV-1–specific T cell clones, Ag presentation assays were designed as follows. For HIV-1 Ag-presentation experiments, monocytes from HLA-A*02 donors were used to generate DC. iDC and mDC matured for 48 h with

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ITP (mDC ITP) or LPS (mDC LPS) were exposed for 48 h to the viruses (500 ng p24Gag/ml per 1 × 10^5 cells) at 37°C and 5% CO₂ in culture medium with IL-4, GM-CSF, 5 μM azidothymidine (AZT), and 1.2 μM nevirapine (NVP) (AZT and NVP were both from Sigma-Aldrich). During viral incubation, iDC were simultaneously matured with ITIP (mDC+ITIP) or LPS (mDC+LPS) or remained unstimulated (iDC). Cells were then extensively washed with PBS to remove unbound viruses and cocultured for 16–18 h with SL9-2 or EM40-F21 CTL clones. T cell activation was monitored using the IFN-γ ELISPOT assay, as previously described (22). Negative controls consisting of non-HIV-1–exposed DC-CD8⁺ T cell clone cocultures were included to exclude nonspecific activation of the T cell clone. As a positive control, DC were loaded with 0.1 μg/ml cognate peptide.

For HLA-II Ag-presentation experiments, monocytes from HLA-DR*04 or HLA-DRB1*04/HLA-DRB1*01 donors were used to generate DC. iDC and mDC matured for 48 h with ITP (mDC ITP) or LPS (mDC LPS) were exposed for 6 h to the viruses (285 ng p24Gag/ml per 1 × 10^5 cells) at 37°C and 5% CO₂ in culture medium with 5 μM AZT and 1.2 μM NVP. Cells were then washed extensively with PBS to remove unbound viruses and cultured overnight in medium with IL-4, GM-CSF, AZT, and NVP. During viral incubation, iDC were simultaneously matured with ITIP (mDC+ITIP) or LPS (mDC+LPS) or remained unstimulated (iDC). Cells were then washed with PBS and cocultured for 16–18 h with F12 or N2 CD4⁺ T cell clones. T cell activation was monitored using an IFN-γ ELISPOT assay. Negative controls consisting of non–HIV-1–exposed DC-CD4⁺ T cell clone cocultures were included to exclude non-specific activation of the T cell clone. As a positive control, DC were loaded with 0.1 μg/ml cognate peptide.

Although differing in the time lag between maturation and viral loading, as well as in the exposure time to maturation stimuli, all DC conditions were exposed to virus for the same amount of time in the HLA-I or HLA-II Ag-presentation assays.

Virion fusion assay

HIV-1 virions containing the β-lactamase (BlaM)-Vpr chimera were produced, as previously described (27). In brief, HEK-293T cells were cotransfected with 60 μg pNL4-3 or pNL4-3ΔIN proviral DNA, 20 μg pCMV–BlaM-Vpr (Addgene), and 10 μg pXdVantage vectors (Promega). Supernatants containing virus were collected 48 h later, filtered (Millipore, HV, 0.45 μm; Millipore), ultracentrifuged at 72,000 × g for 90 min at 4°C, and frozen at −80°C until use. The p24Gag content of viral stocks was determined using ELISA (PerkinElmer).

The viral fusion assay was performed, as previously described (27). Briefly, 5 × 10⁵ Jurkat T cells were infected with 400 ng p24Gag of HIVNL4-3 or HIVNL4-3ΔIN containing BlaM-Vpr, or 600 pg p24Gag for 90 min at 4°C and incubation for 2.5 h at 37°C 5% CO₂ in the presence or absence of 5 μg/ml C34 fusion inhibitor (NHL AIDS Research and Reference Reagent Program). Cells were then washed in CO₂-independent medium (Life Technologies, Invitrogen) to remove free virions and loaded with 1 mM CCF2-AM dye (Invitrogen) for 1 h at room temperature, as described by the manufacturer. After two washes with phosphate-buffered saline (PBS)-buffered medium supplemented with 10% FBS, the BlaM cleavage of CCF2 reaction was allowed to proceed in the dark for 16 h at room temperature in 200 μl development medium. Finally, cells were washed in PBS and fixed in a 1:2 paraformaldehyde solution. The degradation of CCF2-AM by BlaM cleavage and its change in emission fluorescence were measured by flow cytometry using a BD LSR II (Becton Dickinson). Data were collected with FACSDiva software (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

Confocal microscopy

A total of 2 × 10⁶ iDC and mDC matured for 48 h with ITP (mDC ITP) or LPS (mDC LPS) was incubated at 37°C for 5 h with 120 ng p24Gag of HIVNL4-3-Vpr-eGFP. Cells were stained with DAPI and, after extensive washing, were fixed with 2% formaldehyde and cytospun onto glass slides. Cells were then mounted with Dako fluorescent media for analysis under a confocal microscope with hybrid detector (Leica TCS SPS AOBBS; Leica Microsystems). To obtain three-dimensional reconstructions, confocal z stacks were collected every 0.13 μm and processed with Imaris software v.7.2.3 (Bitplane), using the maximum fluorescent intensity projection for HIVNL4-3-Vpr-eGFP and surface modeling for DAPI-stained nuclei.

Electron microscopy

For electron microscopy analysis of viral capture by DC, cells were processed as described elsewhere (6). In brief, 3 × 10⁶ mDC LPS were pulsed at 37°C overnight with 1600 ng p24Gag of HIVNL4-3 or HIVNL4-3ΔIN. Cells were then extensively washed with PBS and fixed in 2.5% glutaraldehyde for 1 h. Finally, cells were processed for analysis of ultrathin sections using a Jeol JEM 1010 electron microscope.

Statistical analysis

All statistical analyses were performed using GraphPad software Prism v.5.

Results

Maturation of DC with LPS, but not with ITP, enhances HIV-1 capture and trans-infection

We previously showed that mDC LPS capture greater amounts of HIV-1 than do iDC, thus facilitating viral transmission to T lymphocytes (6). Before evaluating the Ag-presentation abilities of iDC and mDC, we studied the viral capture and trans-infection capabilities of DC matured for 48 h with LPS or with ITP, the gold standard for DC maturation in immunotherapy (14, 20).

Therefore, we assessed the replication-competent HIVNL4-3 and an integrase-deficient HIV-1 isolate, HIVNL4-3ΔIN, which allowed us to exploit viral uptake of DC in the absence of trans-infection.

iDC and mDC (i.e., matured with ITP [mDC ITP] or LPS [mDC LPS]) were incubated with HIVNL4-3 or HIVNL4-3ΔIN for 6 h (Fig. 1, left panel). After extensive washing, we lysed some cells to determine the amounts of DC-associated HIV-1. The remaining washed cells were cocultured with TZM-bl to assay DC-mediated trans-infection. As expected, maturation with LPS (mDC LPS) enhanced HIV-1 capture compared with iDC (>5-fold, p = 0.0039, Fig. 1, middle panel), resulting in higher trans-infection of HIVNL4-3 to target cells (>7-fold, p = 0.0156, Fig. 1, right panel). Unexpectedly, maturation with ITP (mDC ITP) increased neither uptake nor trans-infection of HIVNL4-3ΔIN, and it remained at similar levels to iDC (Fig. 1), although phenotypic markers of maturation and differentiation did not diverge between mDC LPS and mDC ITP (Fig. 2A). To further address these differences in viral capture, HIV-1-pNL4-3-Vpr-eGFP–pulsed iDC, mDC ITP, and mDC LPS were monitored by confocal microscopy (Fig. 2B). Confirming our results by p24Gag ELISA (Fig. 1, middle panel), mDC LPS captured higher amounts of viral particles, concentrating them in a large sac-like compartment, as previously described (10, 28). In contrast, mDC ITP showed a random distribution of captured HIV-1, similar to iDC than to mDC LPS.

Because vaccine adjuvants boost immune responses, mainly because they are DC activators (29, 30), we wondered whether timing of maturation during Ag loading affected viral capture and trans-infection to HIV-1–susceptible cells. Thus, we compared the viral uptake and trans-infection abilities of DC matured with LPS (mDC+LPS) or with ITP (mDC+ITIP) during viral capture with those DC fully matured before HIV-1 incubation. Surprisingly, both iDC+ITIP and iDC+LPS exhibited a lower capacity to capture and transfer HIV-1, which was similar to iDC than to mDC LPS.

Fig. 1 (middle panel) shows that HIVNL4-3ΔIN was captured by DC as efficiently as was replicative-competent HIVNL4-3. As expected, functional trans-infection of HIVNL4-3ΔIN was completely abrogated, even for mDC LPS (Fig. 1, right panel). Because infection of target cells was not detected, we checked whether the HIVNL4-3ΔIN preserved its envelope integrity and functionality, despite lacking the whole integrase-coding region. Using viral fusion assays (27), we confirmed that HIVNL4-3ΔIN was as fusog-
genic as the wild-type HIVNL4-3 and equally susceptible to the C34 fusion inhibitor (Supplemental Fig. 1A). Additionally, to evaluate whether HIVNL4-3DIN followed the same intracellular trafficking as did the wild-type HIVNL4-3 in DC, both viral particles were monitored in parallel in mDC LPS using electron microscopy (Supplemental Fig. 1B). The HIVNL4-3 and HIVNL4-3DIN virions had an identical structure, with a characteristic electron-dense core and similar accumulation in intracellular compartments in DC. Altogether, these findings indicated that the lack of integrase in HIVNL4-3DIN did not alter viral fusogenicity or morphology and that HIVNL4-3DIN behaved as a wild-type virus, despite not being infectious. Thus, HIVNL4-3DIN seems to be an attractive vaccine candidate to be explored.

Enhanced viral capture does not correlate with better T cell activation
To elucidate whether viral capture correlates with Ag presentation and T cell activation, we evaluated the abilities of iDC, mDC ITIP, mDC LPS, iDC+ITIP, and iDC+LPS, to present HIV-1-derived Ags to CD4+ and CD8+ T cells. To this end, we used various previously generated HIV-1-specific T cell clones (21, 22). To monitor HLA-I HIV-1 Ag presentation, we used two CD8+ T cell clones, SL9-2 and EM40-F21, which are specific for HIV-1 p17Gag (aa 77–85), restricted by HLA-A*02, and derived from two HIV-1–infected patients (22). Because the wild-type HIVNL4-3 and its derived HIVNL4-3DIN did not present the consensus SL9 epitope restricted by HLA-A*02, we engineered HIVNL4-3 and HIVNL4-3DIN to express the optimal SL9 sequence (SLYNTVATL) (24) or the escape variant (SLFNTIAVL) (25) of the SL9 epitope. Two CD4+ T cell clones, F12 and N2, which are specific for HIV-1 p24Gag (aa 271–290) and restricted by HLA-DRb*04/HLA-DRb*01 and HLA-DRb*04, respectively, were used to evaluate HLA-II Ag presentation (21). HLA-matched DC for each HIV-1–specific T cell clone were exposed for 24 h (HLA-I assays) or 6 h (HLA-II assays) to HIVNL4-3 or HIVNL4-3DIN (Fig. 3). T cell activation was monitored using IFN-γ ELISPOT after overnight coculture of HIV-1–pulsed DC with HIV-1–specific T cell clones. Background values based on non–HIV-1–exposed DC–TZM-bl cocultures were subtracted for each cell condition. In all cell subsets, viral capture correlates with viral trans-infection of HIVNL4-3 to target cells, with higher ability of mDC LPS (p = 0.0156, Wilcoxon matched-pairs test). DC-mediated trans-infection of HIVNL4-3DIN was completely abrogated under all cell conditions (p = 0.0156, Wilcoxon matched-pairs test). Data are expressed as mean and SEM and are from three independent experiments including cells from at least seven different donors.

FIGURE 1. Complete maturation of DC with LPS, but not with ITIP, enhances HIV-1 capture and trans-infection. Left panel, Protocol for HIV-1 capture assay of fully mature DC matured with LPS (mDC LPS) or ITIP (mDC ITIP) for 48 h before viral incubation, iDC, and iDC that were matured with LPS (iDC+LPS) or with ITIP (iDC+ITIP) during viral exposure. Middle panel, Comparative capture of HIVNL4-3 and HIVNL4-3DIN under each cell condition described in the left panel. The amount of DC-associated HIV-1 was determined using p24Gag ELISA after viral incubation at 37˚C for 6 h. Complete maturation of DC with LPS (mDC LPS), but not with ITIP (mDC ITIP), enhanced HIV-1 capture (p = 0.0390, Wilcoxon matched-pairs test). HIVNL4-3DIN was captured with the same efficiency as was the wild-type HIVNL4-3 by DC (p = NS, Wilcoxon matched-pairs test). Conversely, maturation of DC with LPS during viral pulse (iDC+LPS) did not enhance HIV-1 capture to levels observed in mDC LPS. Right panel, Transmission of HIVNL4-3 and HIVNL4-3DIN captured under each cell condition described in the left panel to the TZM-bl reporter cell line. Luciferase activity was assayed after 48 h of coculture. Background values based on non–HIV-1–exposed DC–TZM-bl cocultures were subtracted for each cell condition. In all cell subsets, viral capture correlates with viral trans-infection of HIVNL4-3 to target cells, with higher ability of mDC LPS (p = 0.0156, Wilcoxon matched-pairs test). DC-mediated trans-infection of HIVNL4-3DIN was completely abrogated under all cell conditions (p = 0.0156, Wilcoxon matched-pairs test). Data are expressed as mean and SEM and are from three independent experiments including cells from at least seven different donors.

To elucidate whether viral capture correlates with Ag presentation and T cell activation, we evaluated the abilities of iDC, mDC ITIP, mDC LPS, iDC+ITIP, and iDC+LPS, to present HIV-1–derived Ags to CD4+ and CD8+ T cells.  

To this end, we used various previously generated HIV-1–specific T cell clones (21, 22). To monitor HLA-I HIV-1 Ag presentation, we used two CD8+ T cell clones, SL9-2 and EM40-F21, which are specific for HIV-1 p17Gag (aa 77–85), restricted by HLA-A*02, and derived from two HIV-1–infected patients (22). Because the wild-type HIVNL4-3 and its derived HIVNL4-3DIN did not present the consensus SL9 epitope restricted by HLA-A*02, we engineered HIVNL4-3 and HIVNL4-3DIN to express the optimal SL9 sequence (SLYNTVATL) (24) or the escape variant (SLFNTIAVL) (25) of the SL9 epitope. Two CD4+ T cell clones, F12 and N2, which are specific for HIV-1 p24Gag (aa 271–290) and restricted by HLA-DRb*04/HLA-DRb*01 and HLA-DRb*04, respectively, were used to evaluate HLA-II Ag presentation (21). HLA-matched DC for each HIV-1–specific T cell clone were exposed for 24 h (HLA-I assays) or 6 h (HLA-II assays) to HIVNL4-3 or HIVNL4-3DIN (Fig. 3). T cell activation was monitored using IFN-γ ELISPOT after overnight coculture of HIV-1–pulsed DC with HIV-1–specific T cell clones. Because it is known that, after activation with LPS, DC transiently produce IL-12 subsequently becoming refractory to further stimulation (31), and that DC activation with PGE2 suppresses IL-12 production (32), we included several negative controls consisting of non–HIV-1–exposed DC–T cell clone cocultures or SL9 escape HIV-1 variants in the ELISPOT assays, to exclude the nonspecific activation of the T cell clones and eliminate potential background due to IL-12 secretions. Fig. 3 shows a representative experiment using SL9-2 and F12 clones for HLA-I and HLA-II presentation, respectively. All assays were performed in the presence of NVP and.
AZT to prevent viral replication and guarantee that activation of HIV-1–specific CD8+ and CD4+ T cell clones was not due to the presentation of de novo viral proteins synthesized in DC.

Remarkably, compared with iDC, mDC LPS loaded with HIVNL4-3 or HIVNL4-3ΔIN did not enhance HIV-1–specific CD8+ T cell activation (Fig. 3). This contrasts with the extremely high viral capture and HLA molecule expression observed in mDC LPS (Figs. 1, 4A, 4C). Furthermore, inducing full DC maturation with ITIP (mDC ITIP) had a moderate effect, if any, on HIV-1–specific CTL activation (Fig. 3, middle panel). It was reported that HLA-I–restricted exogenous HIV-1 Ag presentation requires fusion of viral and cellular membranes in a CD4/coreceptor–dependent manner and release of HIV-1 Gag capsid into the cytosol of DC for proteasomal processing and HLA-I loading (33, 34). However, maturation of DC is associated with a decrease in HIV-1 fusion (35), which, in turn, has a direct impact on the ability of mDC to support viral replication (16, 35). These observations most likely explain why DC matured with either LPS or ITIP (mDC LPS and mDC ITIP, respectively), exhibiting very high levels of HLA-I and costimulatory molecules, induced very low stimulations of HIV-1–specific CD8+ T cell clones.

Interestingly, compared with mDC ITIP and mDC LPS, iDC induced a 3-fold increase in HIV-1–specific CD4+ T cell clone activation (Fig. 3, right panel), in contrast to their reduced expression of HLA-II and costimulatory molecules and their poor capacity to capture HIV-1 virions (Figs. 1, 4B, 4D). These results strongly suggest that HIV-1 capture by mDC LPS does not route HIV-1 virions toward degradation compartments and HLA loading. Our results are reminiscent of the observations that localization of captured virus differs between iDC and mDC (36), because mDC, but not iDC, accumulate whole virions in a nonconventional endocytic compartment rich in tetraspanins with a mildly acidic pH (6, 10, 37). In addition, HLA-II Ag presentation depends on viral degradation in acidified endosomes (34); however, inhibition of endosomal acidification preserves HIV-1 infectivity (38).

FIGURE 2. mDC LPS and mDC ITIP display similar phenotypic profile but different HIV-1 localization. (A) Immunophenotyping of iDC and mDC matured for 48 h with ITIP (mDC ITIP) or with LPS (mDC LPS). Both maturation stimuli conferred a mature phenotype to DC, by upregulating costimulatory molecules (CD80, CD83, CD86) and HLA class-I and class-II molecules (HLA-A, -B, -C, and -DR) at the cell surface. (B) Confocal microscopy images of iDC and mDC matured for 48 h with ITIP (mDC ITIP) or with LPS (mDC LPS) and exposed to HIVNL4-3-Vpr-eGFP for 5 h. For three-dimensional reconstructions, confocal z stacks were collected every 0.13 μm and processed with Imaris software, using the maximum fluorescent intensity projection for HIVNL4-3-Vpr-eGFP and the surface modeling for DAPI-stained nucleus. mDC LPS captured higher amounts of viral particles, concentrating them into a large sac-like compartment, whereas mDC ITIP showed a random distribution of captured HIV-1, similar to that observed in iDC. Scale bar, 3 μm.
DC maturation with LPS during viral capture enhances both HLA-I and HLA-II Ag presentation

We next examined the effect of maturation of iDC during HIV-1 loading on Ag presentation by HLA-I and HLA-II molecules. iDC were simultaneously matured with ITIP or LPS (iDC+ITIP and iDC+LPS, respectively) and pulsed with HIVNL4-3 or HIVNL4-3IN for 24 h in HLA-I Ag-presentation assays or 6 h in HLA-II experiments. After overnight coculture of HIV-1–pulsed DC with HIV-1–specific T cell clones, Ag presentation was quantified by ELISPOT (Fig. 3). All assays were performed in the presence of NVP and AZT to ensure that Ags did not derive from neosynthesized HIV-1 proteins.

In contrast to fully matured DC (mDC LPS and mDC ITIP), DC stimulated with LPS or ITIP simultaneously with virus loading (iDC+LPS and iDC+ITIP) induced greater activation of HIV-1–specific CD8+ T cells than did iDC (Fig. 3, middle panel). Minor differences were observed in HLA-I Ag presentation between DC matured with LPS and ITIP, although iDC+LPS were associated with a 3-fold increase in IFN-γ secretion and iDC+ITIP were associated with a 1.5-fold change compared with their fully matured counterparts. It is worth noting that HIV-1–specific CD8+ T cell activation levels induced by peptide-loaded mDC LPS and mDC ITIP were higher than levels induced by peptide-loaded iDC, iDC+LPS, and iDC+ITIP (Fig. 3, middle panel), most likely reflecting higher HLA-I molecule expression (Fig. 4C). In contrast, upon loading of the DC subsets with HIVNL4-3 or HIVNL4-3IN, neither viral capture nor HLA-I molecule expression correlated with better CD8+ T cell activation (Fig. 4A, 4C). As expected, neither HIVNL4-3 nor HIVNL4-3IN expressing the escape variant (SL_{LNTIAY}L) of the SL9 epitope induced responses in the SL9-2 CD8+ T cell clone under any DC condition (Supplemental Materials and Methods).
As stated elsewhere (35), DC maturation restricts viral fusion, which is crucial for cytosolic proteasomal processing of Gag proteins and proper HLA-I Ag presentation (33, 34). Thereafter, although iDC capture fewer virions, HIV-1 is more prone to fuse, potentially enhancing viral uptake and HLA-I Ag presentation of HIV-1–derived peptides.

Compared with mDC LPS and mDC ITIP, LPS maturation of iDC simultaneously with viral pulse (iDC+LPS) increased activation of HIV-1–specific CD4+ T cells, reaching levels similar to those induced by iDC (Fig. 3, right panel). Once again, the ability to activate HIV-1–specific CD4+ T cell clones did not correlate with the ability to capture HIV-1 or HLA-II expression levels (Fig. 1, 4B, 4D). In contrast, maturation of iDC with ITIP during Ag uptake (iDC+ITIP), rather than before Ag uptake (mDC ITIP), did not improve HLA-II Ag presentation (Fig. 3). These results show that maturation of DC with ITIP simultaneously with Ag loading (iDC+ITIP) does not guarantee efficient CD4+ T cell activation. The type of activation (ITIP induced or LPS induced) also determines the ability to process and present Ags.

Interestingly, DC loaded with integrase-deficient HIV$_{NL4-3\text{MIN}}$ induced comparable activation of both HIV-1–specific CD8+ and CD4+ T cell clones than DC loaded with HIV$_{NL4-3}$, further demonstrating that no viral replication was needed for HIV-1–derived Ag presentation. HIV$_{NL4-3\text{MIN}}$ is a promising immunogen for the development of an HIV-1 vaccine, because it was processed and presented by DC as wild-type HIV-1 without being infectious.

**Discussion**

DC play a pivotal role in the generation and regulation of immune responses, and they might also facilitate in vivo viral dissemination during HIV-1 infection. The classic DC paradigm proclaims that iDC have abundant endocytic ability and Ag-processing activity but a reduced ability to activate T cells, whereas mDC show low endocytic activity with enhanced Ag presentation and immunostimulatory function (1). In contrast, mDC LPS have a greater ability to uptake and transmit viral particles than do iDC (4, 6, 8). In fact, it was recently shown that *M. tuberculosis* is able to mature DC, promoting similar phenotype of HIV-1 trans-infection and viral sequestration to those seen in LPS-stimulated DC, while suppressing class II Ag processing (19). Although both agonists would mature DC primarily by MyD88-dependent TLR-mediated mechanisms, little is known about the fate of captured particles in mDC LPS and whether they represent a source of viral Ags for HLA loading and T cell activation. Therefore, we assessed the ability of mDC LPS to present HIV-1–derived Ags to HIV-1–specific CD4+ and CD8+ T cells. To apply a more clinically oriented approach, we also analyzed DC matured with the proinflammatory mixture ITIP, the standard stimulus for DC maturation in immunotherapy. Furthermore, we evaluated a noninfectious integrase-deficient HIV-1 isolate, HIV$_{NL4-3\text{MIN}}$, to maximize Ag loading of DC while blocking DC-mediated trans-infection of HIV-1.

We first confirmed that maturation of DC with LPS (mDC LPS) increased subsequent HIV-1 capture and trans-infection compared
with iDC. Surprisingly, although mDC LPS and mDC ITIP displayed a similar phenotypic profile (with regard to DC-SIGN, CD80, CD83, and CD86, as well as HLA-A, -B, -C, and -DR), maturation of DC with ITIP did not improve the uptake or transmission of HIV-1. Moreover, both cell types exhibited distinct intracellular localization of viral particles by confocal microscopy. Consistent with our p24\textsuperscript{Gag} ELISA results, mDC LPS displayed a large sac-like compartment in which viral particles were concentrated, whereas mDC ITIP showed a random distribution of HIV-1, similar to that observed in iDC. Several activation signals have been used for DC maturation, including polyinosinic:polycytidylic acid, R848, LPS, IFN-γ, CD40L, and TNF-α; however, although all maturation stimuli confer a mature phenotype, the functional ability of the resulting mDC for polarization, secretion, or migration of Th cell function varies (4, 14).

In addition, comparable results to those of iDC were obtained when DC were matured with LPS (mDC iDC+LPS) or ITIP (iDC+ITIP) during viral uptake, suggesting that complete LPS activation can provide DC with an exceptional ability to capture HIV-1 particles. In fact, we recently showed that DC matured with LPS for 48 h (our mDC LPS) exhibit maximal viral capture ability, whereas DC matured for 6 h (our iDC+LPS) show a viral uptake similar to that of iDC (28). Altogether, our results indicate that DC-meditated HIV-1 capture and transmission are not only dependent on the DC maturation state (6), they are also dependent on the activation stimulus used for maturation (8), as well as the time lag between DC maturation and Ag loading.

DC are able to process exogenous Ags and present them through HLA-I and HLA-II for stimulation of CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells, respectively. However, we have observed that, despite greater viral uptake and increased HLA molecule expression levels in mDC LPS, this DC condition induced poor stimulation of HIV-1–specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell clones. On the contrary, when DC are matured with LPS during viral capture (iDC+LPS), Ag presentation of HIV-1–derived Ags was efficiently triggered through the HLA-I and HLA-II pathways. Although iDC+LPS trapped five times fewer virions than did mDC LPS, they were more efficient in eliciting HIV-1–specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell responses. Conversely, DC maturation with ITIP during viral capture (iDC+ITIP) was able to efficiently activate HIV-1–specific CD8\textsuperscript{+} T cells but not CD4\textsuperscript{+} T cells. Like other investigators (39), we have observed that DC maturation differentially regulated exogenous HLA-I and HLA-II presentation pathways, although in our case, LPS activation of DC during viral uptake facilitated presentation of HIV-1–derived Ags to CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Our experiments showed that HIV-1–derived Ag presentation was not directly associated with viral capture but was mainly affected by DC status and the activation stimulus used for DC maturation.

mDC efficiently trapped more intact viral particles than did iDC, with a completely different localization of internalized HIV-1–derived proteins, indicating different intracellular fates for captured virions (36). Trapped infectious HIV-1 in mDC LPS are concentrated in unconventional compartments rich in the tetraspanins CD81, CD82, CD9, and CD63 but not in the lysosomal marker LAMP-1 (6, 9, 10, 37). This large vesicle, not present in iDC, is suggestive of a multivesicular body; in addition, its mildly acidic pH preserves HIV-1 infectivity (6, 37). However, HLA-II Ag presentation depends on endosomal/lysosomal maturation and acidification (34), and inhibition of endosomal acidification was reported to increase HIV-1 infectivity (38). Studies even showed that intracellular HIV-1 degradation occurs faster in iDC than in mDC LPS (6, 37, 40). Therefore, the low ability to activate HIV-1–specific CD4\textsuperscript{+} T cells observed in mDC is probably the result of the accumulation of virions in a nondegrading neutralized endosome, thus protecting HIV-1 infectivity and hampering proper lysosomal degradation. Consequently, intact virions confined in this slow-degrading vesicle in mDC would be routed to a trans-infection pathway rather than to Ag presentation, thus enabling dissemination of HIV-1 infection. In contrast, Ag presentation to CD4\textsuperscript{+} T cells is initiated upon LPS activation of DC (41, 42) and is dependent on the presence of TLR4 ligands, such as LPS, with the cargo within the individual phagosome (41). Furthermore, maturation of DC with LPS activates the vacuolar proton pump that acidifies the lysosomes and facilitates Ag proteolysis and efficient formation of peptide–HLA-II complexes (43). Our results are consistent with these observations, because DC maturation with LPS, but not with ITIP, during Ag loading notably increases HIV-1–derived Ag presentation to CD4\textsuperscript{+} T cells.

Envelope integrity and virion functionality are crucial for exogenous presentation of HIV-1 Ags through the HLA-I pathway, because adequate fusion of viral and cellular membranes via CD4 and coreceptor enables cytosolic cleavage of Gag protein by the proteasome (33, 34). Nevertheless, maturation of DC is associated with a decline in HIV-1 fusion (35), which, in turn, has a direct impact on the ability of mDC to support viral replication (16, 35). In addition, other restriction factors, such as SAMHD1 (44) or APOBEC3G (45), in mDC were reported to limit HIV-1 replication upon viral fusion. Consistent with these findings, our results showed that mDC matured with either LPS (mDC LPS) or ITIP (mDC ITIP) had a limited capacity for cross-presenting HIV-1–derived Ags, probably owing to the reduced viral fusion in these cell subsets. Interestingly, although iDC capture smaller amounts of virions, HIV-1 would be more able to fuse in iDC rather than in mDC, facilitating cytosolic degradation of viral Ags. Therefore, these findings support our observations, given that HLA-I Ag presentation of HIV-1–derived peptides was triggered more efficiently when iDC matured during Ag uptake.

Because of the exceptional ability of DC to generate cellular and humoral immune responses, they have been used as tools for immunotherapy of HIV-1 infection (46). An anti–HIV-1 therapeutic vaccine should induce a specific and efficient immune response against the virus while regulating chronic activation of the immune system. CD4\textsuperscript{+} T cells are required for the development of cytotoxic CD8\textsuperscript{+} T cells, which lyse infected cells by HLA-I–dependent mechanisms. Thus, Ag presentation by HLA-A1 or HLA-II molecules is mandatory for the development of a cognate T cell response (47). However, the qualitative response of a DC-based vaccine will be determined primarily by the adjuvants used, because these are DC activators (29, 30). The most widely used DC-maturation stimulus in immunotherapy is the proinflammatory mixture ITIP (14, 20); however, our results show that it only boosts HLA-I Ag presentation when DC mature during viral capture. In contrast, maturation of DC with LPS during Ag loading was the best approach when eliciting both HLA-I and HLA-II Ag-specific responses. LPS is a potent adjuvant in vivo, although it is not used in clinical practice because of its high toxicity. Furthermore, it was reported that levels of circulating LPS increase significantly in chronically HIV-1–infected individuals as a result of microbial translocation, contributing to chronic immune activation (18). However, our results indicate that other TLR-4 ligands are worthy of evaluation. Monophosphoryl lipid A (MPL), a derivative form of lipid A, retains the immunostimulatory activity of LPS but with lower toxicity (48). MPL was recently successful in mice as an adjuvant in immunization with liposomes containing HIV-1 p24\textsuperscript{Gag}, because it induced HIV-1 p24\textsuperscript{Gag}–specific CD8\textsuperscript{+} T cells, effector CD4\textsuperscript{+} T cells, and cytokines with a Th1-type profile (49). Moreover, stimulation of human DC with MPL induces maturation, migration, survival...
signals, and secretion of cytokines (50, 51). Although MPL-matured DC secrete lower levels of IL-12 than do mDC LPS (51), IL-12 secretion can be rescued by maturing DC with MPL and IFN-γ to induce more potent Th1 polarization, which is essential for the induction and maintenance of the CD8+ T cell response (52). Although further investigation is required, our iDC +LPS approach seems feasible in an immunotherapeutic context when MPL is used as a maturation stimulus.

Several protocols have been used in DC-based immunotherapy to stimulate the presentation of peptide–HLA complexes with high efficiency. Chemical inactivation with AT-2, which preserves the native morphology of viral particles (53), provided encouraging immune results in eliciting HLA-I responses (22, 54, 55), but its use in human clinical trials is not approved by European regulatory authorities. Thus, novel delivery tools for cell therapy vaccination and new methods to enhance the immunogenicity and antiviral efficacy of DC-based vaccines for HIV-1 infection are needed. We evaluated HIVNL4-3, an isolate-deficient HIVNL4-3 isolate that lacks the whole integrase-coding region. We showed that HIVNL4-3 preserves intact virion morphology and envelope functionality, despite not being infectious. Interestingly, in the context of Ag-presentation assays, HIVNL4-3 behaves as a wild-type virus, because it is efficiently captured and presented by DC in the absence of viral replication. Consequently, HIVNL4-3 could be an attractive immunogen for future vaccine candidates.

In summary, our results clarify the different intracellular trafficking routes of HIV-1 in DC, diverting the processing and presentation pathway from trans-infection, depending on DC maturation status and the activation signal used for DC maturation. Moreover, we observed that greater viral capture in DC does not guarantee better Ag presentation or T cell activation. These results provide new insights into DC biology and have implications in the optimization of DC-based immunotherapy against HIV-1 infection.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. (A) Viral fusion assay of HIV\textsubscript{NL4-3} and HIV\textsubscript{NL4-3ΔIN} in the Jurkat T-cell line using HIV-1 virions containing a β-lactamase-Vpr chimeric protein. Experiments were performed in the presence or absence of C34 fusion inhibitor. HIV\textsubscript{NL4-3ΔIN} was as fusogenic as wild-type HIV\textsubscript{NL4-3} and equally susceptible to the C34 fusion inhibitor.

(B) Comparative electron microscopy images of mDC LPS exposed to HIV\textsubscript{NL4-3} (left panel) or HIV\textsubscript{NL4-3ΔIN} (right panel), showing similar large vesicle location. Red arrows indicate captured particles, which have the characteristic electron-dense structure of the HIV-1 core. Processing and analysis using a Jeol JEM 1010 electron microscope.

Supplementary Figure 2. Comparative analysis of IFN-γ production by the SL9-2 CD8\textsuperscript{+} T-cell clone when stimulated with DC pulsed with HIV\textsubscript{NL4-3} (A) or HIV\textsubscript{NL4-3ΔIN} (B) expressing the optimal (SLYNTVATL) or the escape (SLFNTIAVL) variant of SL9 epitope. Both HIV\textsubscript{NL4-3} and HIV\textsubscript{NL4-3ΔIN} expressing the escape variant for the SL9 epitope did not induce activation of the HIV-1–specific CD8\textsuperscript{+} T-cell clone response.
Supplementary Figure 1.

A.

![Images showing flow cytometry plots for different conditions: Mock, HIV\textsubscript{NL4-3}, HIV\textsubscript{NL4-3} + C34, HIV\textsubscript{NL4-3,32IN}, and HIV\textsubscript{NL4-3,32IN} + C34. The plots indicate the percentage of cells in the Uncleaved CCF2 (450nm) and Cleaved CCF2 (447nm) channels.]

B.

![Images showing electron micrographs for HIV\textsubscript{NL4-3} and HIV\textsubscript{NL4-3,32IN}. The micrographs highlight the differences in the structures of the cells under the two conditions, with red arrows indicating specific features.]
Supplementary Figure 2.

A.

B.