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Human Plasmacytoid Dendritic Cells Efficiently Capture HIV-1 Envelope Glycoproteins via CD4 for Antigen Presentation


Advances in HIV-1 vaccine clinical trials and preclinical research indicate that the virus envelope glycoproteins (Env) are likely to be an essential component of a prophylactic vaccine. Efficient Ag uptake and presentation by dendritic cells (DCs) is important for strong CD4+ T cell responses and the development of effective humoral immune responses. In this study, we examined the capacity of distinct primary human DC subsets to internalize and present recombinant Env to CD4+ T cells. Consistent with their specific receptor expression, skin DCs bound and internalized Env via C-type lectin receptors, whereas blood DC subsets, including CD1c+ myeloid DCs, CD123+ plasmacytoid DCs (PDCs), and CD141+ DCs exhibited a restricted repertoire of C-type lectin receptors and relied on CD4 for uptake of Env. Despite a generally poor capacity for Ag uptake compared with myeloid DCs, the high expression of CD4 on PDCs allowed them to bind and internalize Env very efficiently. CD4-mediated uptake delivered Env to EEA1+ endosomes that progressed to Lamp1+ and MHC class II+ lysosomes where internalized Env was degraded rapidly. Finally, all three blood DC subsets were able to internalize an Env-CMV pp65 fusion protein via CD4 and stimulate pp65-specific CD4+ T cells. Thus, in the in vitro systems described in this paper, CD4-mediated uptake of Env is a functional pathway leading to Ag presentation, and this may therefore be a mechanism used by blood DCs, including PDCs, for generating immune responses to Env-based vaccines.


Dendritic cells (DCs) are the most potent APCs and essential for establishing effective adaptive immune responses. Their capacity for uptake and presentation of vaccine Ags is likely to dictate the quality and efficiency of the ensuing vaccine-induced response. A primary goal of a prophylactic HIV-1 vaccine is to elicit potent, broadly neutralizing Abs that provide the first line of defense against virus exposure. The HIV-1 envelope glycoproteins (Env) are the only surface-exposed, virally encoded proteins of the HIV-1 virion and as the sole target for neutralizing Abs are likely to be included in a vaccine (1, 2).

One of the critical factors in the establishment of an effective humoral response is the provision of appropriate CD4+ T cell-mediated help. Thus, the efficiency with which DCs stimulate CD4+ T cells following vaccination is likely to influence the quality of a developing B cell response.

The diversity of DC subsets suggests they play distinct roles in stimulating innate and adaptive immune responses. In the event of an i.m. vaccination, DCs are likely to infiltrate the muscle from the blood and potentially also the skin and interact with vaccine Ags early in the immune response. Myeloid DCs (MDCs) found in the skin and blood have potent Ag uptake capacity (3) and express a variety of receptors including C-type lectin receptors (CLRs), which they use for endocytic or phagocytic uptake of exogenous Ag. In contrast, plasmacytoid DCs (PDCs) have a more restricted capacity for uptake of soluble Ag (4–7). Different receptor expression and uptake pathways can lead to different intracellular routing and consequently a different outcome in the mode or efficiency of Ag presentation (5, 8–10). DC subsets also have intrinsic differences in their Ag-processing capacity because of variations in the expression level of proteins involved in MHC processing (3, 11, 12). MDCs are generally considered more potent APCs for T cell priming. The distinctly different PDCs play an important role in immediate antiviral responses through their capacity to produce large amounts of type I IFNs, which among a plethora of functions support B cell differentiation and Ab production (13–16). However, the contribution of human PDCs in presentation of exogenous Ag to naive CD4+ T cells to prime adaptive immune responses is less well defined (5, 7, 17, 18).

In this study, we examined key interactions between soluble HIV-1 Env gp120 of the YU2 strain with human skin and blood DC subsets to understand the fate of these interactions. We found that skin DC subsets used CLRs to bind and internalize Env very ef-
CD4 uptake and blocking assays

DCs at 1 × 10^6/ml were pulsed with 10 μg/ml AF488-conjugated HIV-1 Env Ags or Influenza HA for 90 min at 37°C. For blocking studies, the cells were preincubated with 5 mg/ml mannan (Sigma-Aldrich) or 100 μg/ml CD4 Ab (RPA-T4) for 15 min at 4°C or 37°C prior to addition of Ag. Cells were then washed and analyzed by flow cytometry.

CD4 internalization

Whole PBMCs (for T cells) or monocyte-enriched PBMCs (for DCs) were incubated with anti-CD4 mAb (clone SK-3; BD Biosciences) at 4°C for 20 min or 10 μg/ml Env-AF488 at 4°C for 45 min. Cells were then washed in cold PBS and cultured in cold R10 on ice or at 37°C for 0, 60, or 120 min. At each time point, cells were washed twice in either cold PBS or acidic buffer (0.2 M acetic acid and 0.15 M NaCl [pH 3]) to remove residual surface CD4 Ab or Env and resuspended in PBS/10 mM HEPES to neutralize the pH. Cells were then surface stained with HLA-DR, lineage markers, and either CD1c, CD123, or CD141 to identify DC populations by FACS.

Confocal microscopy

Purified DC subsets were exposed to Env Ags for between 10 and 120 min at 37°C, then washed, applied to glass adhesion slides (Marianfeld, Lauda-Königsfeld, Germany), fixed in 4% paraformaldehyde for 20 min at 4°C, and permeabilized with 0.05% saponin for 20 min. The slides were blocked with 10% normal donkey serum (Sigma-Aldrich) for 30 min at 37°C, then stained for 45 min at 37°C with EEA1 (clone 14; BD Biosciences) or Lamp-1 (clone HSG11; Santa Cruz Biotechnology, Santa Cruz, CA), diluted in 10% normal donkey serum, followed by Alexa Fluor 555–conjugated donkey anti-mouse IgG for 30 min at 37°C. The cell surface was then stained with Alexa Fluor 647–conjugated HLA-DR (BioLegend, San Diego, CA) and coverslips mounted using Pro-Long Gold containing DAPI (Molecular Probes). Analysis was done with a Leica DMIRE2 microscope and Leica TCSSP2 confocal system (Leica Microsystems, Heidelberg, Mannheim, Germany). Colocalization data were quantified using JaCOP (ImageJ (26)) and Manders’ M1 coefficient for the percentage of Env overlapping with an endolysosomal marker is shown.

Ag presentation assays

Blood DC subsets, sorted to high purity by flow cytometry from donors with detectable CMV pp65–specific CD4+ T cell recall responses, were pulsed for 4°C for 1 h with Env-pp65, Env-D368R-pp65, or pp65 (Miltenyi Biotec) using equivalent amounts of pp65 (5–10 μg/ml). Alternatively, overlapping 15-mer CMV pp65 peptides were used (Proimmune). The cells were then washed and cocultured overnight with autologous CD4+ T cells (isolated using Rosettesep CD4+ T cell enrichment kit; StemCell Technologies) at a DC:T cell ratio of 1:10 in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich). Cells were then washed and stained for DC and T cell surface markers and intracellular IFN-γ, TNF, and IL-2 (BD Biosciences).

Statistical analyses

One-way ANOVA was used to test for differences in the degree of uptake of wt and D368R Env in different DC subsets, and Tukey’s posthoc comparisons were then used to compare the groups. Paired two-tailed Student t tests were performed for comparisons of colocalization and Ag presentation (Prism 6; GraphPad Software, La Jolla, CA) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001).

Results

Primary human DC subsets express distinct repertoires of receptors capable of binding HIV-1 Env

To define the uptake pathways of soluble HIV-1 Env by different DC subsets, immature primary DCs isolated from skin or blood were compared for their expression of a range of known and putative Env–binding receptors. During HIV-1 infection, Env binds the primary receptor CD4, which triggers a conformational change.
that allows the virus to engage CCR5 or CXCR4, leading to viral fusion. CLRs can also bind HIV-1 through their interaction with high mannose oligosaccharides on Env (27, 28), but these interactions do not mediate viral fusion. Consistent with earlier reports, we found that the most well recognized Env-binding CLRs, including Langerin, MR, and DC-SIGN, were all expressed on skin DCs. Freshly isolated HLA-DR+CD1a+ LCs from epidermis showed a characteristically high expression of Langerin (Fig. 1). Conversely, HLA-DR+ DDCs predominantly expressed MR and low levels of MR in the presence of GM-CSF (Supplemental Fig. 1). All three blood DC subsets expressed DEC-205 and DC-SIGN, whereas CD14+ DDCs expressed DC-SIGN and low amounts of MR. DEC-205 DDCs expressed DC-SIGN and low amounts of MR. DEC-205 and DCIR, more recently proposed to bind HIV-1 (29, 30), were also expressed on both LCs and DDCs, particularly the CD1a+ DDCs. Finally, we detected low levels of CD4 expression on skin DCs as reported previously (31–33).

The blood DCs were divided into three distinct populations: CD11c+CD1c+ MDCs (referred to as MDCs), the rare CD11cint CD141 (BDCA-3)+ MDCs (referred to as BDCA-3+ DCs), and CD123+ PDCs. In contrast to the skin, blood DCs displayed high levels of CD4, in particular PDCs, but were largely devoid of DC-SIGN, MR, and Langerin (Fig. 1). MDCs could be induced to express low levels of MR in the presence of GM-CSF (31–33).

We next investigated the impact of this differential receptor expression on the capacity of the respective DC subsets to internalize a model Env immunogen, the YU2-based gp120 monomer. We used fluorophore-conjugated Ags (22) to compare the degree of Env binding (at 4˚C) and uptake (at 37˚C) in the different DCs alongside another glycosylated viral surface Ag, influenza virus HA. The latter comprises the main component of the seasonal influenza vaccine and was used in this study as a benchmark for Ag uptake of a clinically effective vaccine. Among the skin DCs, we found that DDCs were superior to LCs at both HA and Env uptake (Fig. 2A, 2B, upper panels). Of the blood DCs, MDCs are generally known as having superior Ag uptake capacity over PDCs. Consistent with this, MDCs displayed a higher capacity for HA uptake than PDCs, but strikingly, donor-matched PDCs were as efficient, if not more efficient, than MDCs for Env binding and uptake (Fig. 2A, 2B, lower panels). BDCA-3+ DCs showed the lowest capacity for uptake of these two Ags (Fig. 2A, 2B, lower panels). Consistent with these data, the rates of internalization of Env in MDCs and PDCs at 37˚C were very similar, plateauing around 90 min (Fig. 2C). BDCA-3+ DCs tended to lag behind in their accumulation of Env, with significantly lower uptake at 60 and 90 min compared with PDCs (p < 0.05).

Because Env and HA are both glycosylated, the uptake by DCs of both proteins may be mediated by CLRs. However, the discrepancy in uptake by PDCs indicates that Env can be internalized using alternative pathways. To dissect the contribution of CLRs versus CD4 for Env uptake by the different DC subsets we used mannan, a polymer of mannose that efficiently binds and blocks CLRs, as well as a gp120 variant containing a mutation in the CD4 binding site, which abolishes its ability to bind CD4 (Env-D368R) (Fig. 2D) (22). In both LCs and DDCs, Env uptake was largely inhibited by the presence of mannan, whereas uptake of Env-D368R was only slightly reduced compared with wt Env. This indicates that these cells rely heavily on CLR-mediated uptake of Env. Conversely, blood DC subsets showed very poor uptake of Env-D368R compared with wt Env, and exposure to mannan had, at most, a modest effect, indicating that Env uptake in these cells is predominantly dependent on CD4. This was confirmed in PDCs by blocking Env uptake with a CD4 neutralizing Ab (Fig. 2E). However, because of the high expression of CD4 and internalization of CD4 Ab that occurs in PDCs over time, and possibly also because of a difference in affinity, 10-fold excess of Ab was required for efficient blocking of Env. Thus, the mutated version of Env, abolishing the CD4 binding site, was a more convenient and definitive control for our studies. Although the reliance on CD4 was virtually complete for PDCs and BDCA-3+ DCs, the mannan block and uptake of Env-D368R were more pronounced in MDCs, which may indicate low level usage of CLRs or nonspecific Ag uptake mechanisms. This pattern of differential receptor usage between skin and blood DCs is consistent with previous findings (36), and despite expression of some proposed Env-binding CLRs in blood DCs, we found little evidence of their usage. This pattern of receptor usage...
was also confirmed using fibrin-stabilized, soluble gp140 (gp140-F) trimers (Supplemental Fig. 2) (37).

**CD4 mediates internalization of Env in blood DCs**

The primary function of CD4 is to act as a coreceptor during TCR communication with an APC. CD4 helps stabilize the immune synapse by binding directly to MHC class II and also amplifies the signal generated by the TCR. Because blood DCs were largely dependent on CD4 binding for uptake of Env, we next determined whether CD4 itself also acts as an endocytic receptor in these cells. Earlier studies showed that CD4+ T cells are unable to internalize CD4 because of their expression of protein tyrosine kinase p56 \(\text{ lyn}\), which stabilizes CD4 on the cell surface, whereas monocytes, which lack this molecule, can internalize CD4 (38). Although endocytosis of HIV-1 virions can occur in DCs (39, 40), it is not known whether CD4 can internalize Env and deposit it in intracellular Ag presentation compartments in these cells. To test their ability to internalize CD4, blood DC subsets were surface-labeled with an anti-CD4 Ab at 4°C, followed by a period at 37°C to allow internalization of the receptor. Residual CD4 Ab remaining on the cell surface was then removed by an acid buffer (pH 3), prior to analysis by flow cytometry. At 4°C, CD4 Ab is only bound to the cell surface, and the total signal is thus susceptible to acid stripping. At 37°C, the proportion of CD4 Ab that is internalized becomes protected from acid stripping and the signal is retained (Fig. 3A). We found that MDCs, BDCA-3+ DCs, and PDCs all internalized CD4 within 60 min, whereas CD4+ T cells maintained their CD4 expression on the surface (Fig. 3B). Furthermore, PDCs internalized Env with similar kinetics to the CD4 Ab, whereas Env remained surface-bound on CD4+ T cells (Fig. 3C). These results demonstrate that DCs, which do not use CLRs for Env-binding, in particular blood DC subsets including PDCs, are instead able to internalize Env via CD4.

Uptake of Env via either CLRs or CD4 leads to Ag compartmentalisation in MHC class II+ lysosomes

The cell type and the receptor used for Ag uptake determine the intracellular destination of an Ag and have implications for Ag

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**FIGURE 2.** DC subsets use different receptors for Env uptake. Skin and blood DCs were exposed to 10 μg/ml AF488-labeled Influenza HA or HIV-1 Env for 90 min at 4°C to allow Ag binding or 37°C to allow Ag uptake and analyzed by flow cytometry. (A) Data from one representative skin donor and one representative blood donor are shown. (B) The degree of Ag uptake (mean fluorescence intensity [MFI] 37°C – MFI 4°C) in each DC subset is shown for matched donors. (C) The kinetics of Env uptake over the 90-min period are shown in blood DC subsets for three matched donors. (D) DC subsets were incubated with Env or the CD4-binding defective Env-D368R in the absence or presence of mannan for 90 min at 4 or 37°C or alternatively. (E) PDCs were incubated with CD4 neutralizing Ab (RPA-T4) prior to incubation with Env. The degree of uptake was calculated as for (B) and normalized to Env alone. Data are the mean+SD for three to five donors. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
matched PDCs or CD4+ T cells were used in the experiment described.

mean fluorescence intensity (MFI) for donor-matched subsets. (A) The presence of acetic acid indicates the proportion of CD4 that has been internalized into EEA1+ early endosomes within 10 min but rarely colocalized with Lamp1 (Fig. 4A, bottom panel) and likely represents a compartment for MHC class II loading. From here, MHC class II-peptide complexes could be transported to the cell surface and presented to CD4+ T cells. To dissect the intracellular trafficking of Env taken up via CLRs versus CD4, we took advantage of the fact that MR expression could be induced in MDCs by culturing them in GM-CSF. Such cells displayed an equal usage of CLRs and CD4 for Env uptake (Supplemental Fig. 1). To study the CLR pathway separately, we pulsed MDCs with the CD4-binding defective Env-D368R. Conversely, to isolate the CD4 pathway, we preincubated MDCs with mannan to block CLR-mediated uptake of wt Env. A similar pattern of decreasing colocalization with EEA1 and increasing colocalization with Lamp1 and HLA-DR, as described above, was observed for both routes of uptake (Fig. 4B). When Env colocalization with EEA1 or Lamp1 was quantified, comparing MDCs and PDCs (Fig. 4C) or CLR-dependent versus CD4-dependent Env uptake (Fig. 4D), the same trends were revealed, and no statistically significant differences were detected. Although Env rarely appeared to colocalize with Lamp1 visually at early time points, during quantification, the diffuse nature of the Lamp1 stain resulted in a higher level of background colocalization than could be discerned by eye. Importantly, there was increasing colocalization with Lamp1 over time, and for MDCs, this was most pronounced in the comparison between CD4 and CLR-mediated uptake (Fig. 4D). Thus, despite distinctly different DC types or routes of uptake, Env was targeted to MHC class II+ lysosomes.

CLR-mediated and CD4-mediated endocytosis of Env result in similar kinetics of Ag processing

As we found that Env accesses a compartment for potential MHC class II loading of peptides in DCs, we evaluated Ag processing in the different DC subsets. For these studies, we used FITC-conjugated Env because this fluorophore is sensitive to the low pH in acidic lysosomal compartments and would therefore be extinguished along with degradation of the Ag. DCs were loaded with Ag (Env or HA for comparison), and the fluorescent signal was tracked over time (Fig. 5A). In both skin and blood DC subsets, there was a rapid decay of the Env-FITC signal within 2–4 h, followed by relative preservation of a fraction of the Ag (~10%) for 24 h (Fig. 5B). This rate of degradation was previously described for other Ags in primary DCs (42–44) and is similar to the degradation kinetics observed for HIV-1 virions (45). The rate of Env degradation also closely mirrored that of HA (Fig. 5B). Furthermore, when the CLR and CD4-mediated pathways were studied separately in MDCs by using the conditions described above, there were no significant differences in the rate of degradation of Env internalized via either route (Fig. 5C). To confirm that this decay represented real proteolytic digestion of Env and not simply quenching of the FITC signal, the experiment was performed in MDCs in the presence of leupeptin, a competitive lysosomal protease inhibitor, at 4˚C where Ag is not internalized. Under these conditions, the majority of the Env-FITC signal was preserved (Fig. 5A, 5C), indicating that the observed decay is due to proteolytic digestion of the protein. Also, Western blot analysis for the presence of Env in cell lysates, collected at the indicated time points, confirmed that most of the protein was degraded within 2–4 h and that this was inhibited by leupeptin (data not shown). Thus, Env internalized via CD4 or CLRs and by all DC subsets analyzed in this study is efficiently degraded in MHC class II compartments, suggesting the potential for Ag presentation.

CD4-mediated endocytosis of Env results in MHC class II presentation to CD4+ T cells

CLR-mediated Ag uptake is known to result in Ag presentation on MHC class II by DCs (46–49), but it is not known whether...

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**FIGURE 3.** CD4 mediates Env internalization in blood DCs, in contrast to T cells. Enriched MDCs, BDCA-3+ DCs, PDCs, and CD4+ T cells were incubated with CD4-PE Ab (SK3 clone) for 20 min at 4˚C to allow surface binding and then washed thoroughly. The cells were then incubated at 4 or 37˚C to allow internalization of the Ab for 0, 60, or 120 min. The cells were then either washed in PBS to preserve the total CD4 signal or acetic acid buffer to strip surface-exposed CD4 Ab. Any remaining signal in the absence of acetic acid indicates the proportion of CD4 that has been internalized into EEA1+ early endosomes within 10 min but rarely colocalized with Lamp1 (Fig. 4A, top panel). By 90 min, the degree of colocalization with EEA1 had decreased, and Env had progressed to colocalize with Lamp1+ lysosomes in both DC subsets (Fig. 4A, bottom panel). This staining overlapped with HLA-DR (Fig. 4A, bottom panel) and likely represents a compartment for MHC class II loading. From here, MHC class II-peptide complexes could be transported to the cell surface and presented to CD4+ T cells. To dissect the intracellular trafficking of Env taken up via CLRs versus CD4, we took advantage of the fact that MR expression could be induced in MDCs by culturing them in GM-CSF. Such cells displayed an equal usage of CLRs and CD4 for Env uptake (Supplemental Fig. 1). To study the CLR pathway separately, we pulsed MDCs with the CD4-binding defective Env-D368R. Conversely, to isolate the CD4 pathway, we preincubated MDCs with mannan to block CLR-mediated uptake of wt Env. A similar pattern of decreasing colocalization with EEA1 and increasing colocalization with Lamp1 and HLA-DR, as described above, was observed for both routes of uptake (Fig. 4B). When Env colocalization with EEA1 or Lamp1 was quantified, comparing MDCs and PDCs (Fig. 4C) or CLR-dependent versus CD4-dependent Env uptake (Fig. 4D), the same trends were revealed, and no statistically significant differences were detected. Although Env rarely appeared to colocalize with Lamp1 visually at early time points, during quantification, the diffuse nature of the Lamp1 stain resulted in a higher level of background colocalization than could be discerned by eye. Importantly, there was increasing colocalization with Lamp1 over time, and for MDCs, this was most pronounced in the comparison between CD4 and CLR-mediated uptake (Fig. 4D). Thus, despite distinctly different DC types or routes of uptake, Env was targeted to MHC class II+ lysosomes.

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CD4-mediated uptake has the same result. We investigated this in the context of Env in the blood DC subsets. To enable these studies, we designed a fusion protein between Env and the immunodominant CMV protein pp65 (65 kDa lower matrix phosphoprotein) as an epitope tag. The latter is a tegument protein, not exposed on the surface of CMV, and is not involved in virus binding to cells. We reasoned that if the Env-pp65 fusion protein was targeted to, and endocytosed via, the Env receptors, namely CD4 in blood DCs, it would follow a similar processing route to Env alone. As detectable pre-existing memory T cell responses to pp65 are common in the human population, CD4+ T cell memory responses to the pp65 epitope tag could then be measured as a surrogate readout for Env presentation. The wt (Env-pp65) and the CD4-binding defective (Env-D368R-pp65) fusion proteins were characterized biochemically prior to the Ag presentation assays (Supplemental Fig. 3). The fusion proteins were 180–190 kDa, glycosylated, and preserved both pp65 epitopes and key Env epitopes. Critically, only wt Env-pp65 was recognized by the CD4 binding site–directed mAb b12. When binding of fluorescently conjugated versions of the fusion proteins was assessed in MDCs and PDCs, the results were similar to those observed for Env and Env-D368R (Fig. 6A). Both MDCs and PDCs were able to bind wt Env-pp65 at 4°C, with PDCs demonstrating slightly higher binding, as was observed for Env alone. Binding of the Env-D368R-pp65 fusion protein was markedly reduced although still detectable in MDCs, whereas in PDCs, binding was abolished, suggesting that CD4 mediates the majority of the uptake of the fusion proteins in these cell types. Recombinant pp65 protein alone was also bound by a small proportion of MDCs, but there was no detectable binding in PDCs. Neither the fusion proteins nor pp65 induced maturation of MDCs or PDCs in terms of upregulation of costimulatory molecules.

FIGURE 4. Env is delivered to MHC class II+ lysosomes in DCs via both CLRs and CD4. (A) MDCs and PDCs were exposed to 10 μg/ml Env-AF488 (green) at 37°C for 10–90 min. At various time points, the cells were washed, fixed, and permeabilized and then stained for EEA-1 (red, early endosomes) or Lamp1 (red, lysosomes) and HLA-DR (blue, MHC class II). For visual clarity, only green and red images are merged, and colocalization appears yellow, although the distribution of HLA-DR can be seen to overlap with these areas. Images shown are from 10 and 90 min and were taken at ×63 magnification. (B) MDCs were exposed to 10 μg/ml Env-D368R-AF488 as above to follow CLR-mediated uptake or preincubated with mannan for 15 min at 37°C, followed by exposure to Env-AF488 to follow CD4-mediated uptake. Cells were stained and imaged as above. Colocalization between Env and EEA1 or Lamp1 in (C) MDCs versus PDCs and (D) CLR-dependent versus CD4-dependent uptake was quantified. Manders’ coefficients (M1 only) describing the percentage of Env that overlaps with the endolysosomal marker are shown. Between 6 and 20 cells were analyzed for each time point.

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A representative MDC donor showing the percentage of positive cells. The mean fluorescence intensity (MFI) for each time point was normalized to the 0 min 37°C control for each Ag, and the data shown in this figure are the mean ± SD of three to five donors for Env and 1 donor for HA. (C) Alternatively, MDCs were incubated with Env in the presence of mannan or Env-D368R to isolate degradation via CD4 and CLR-mediated uptake pathways, respectively. Leupeptin was also included in these experiments as a control. Data shown in this figure are the mean ± SD of two donors.

**FIGURE 5.** Both CLR-mediated and CD4-mediated endocytosis of Env result in similar kinetics of Ag processing. Cells were incubated with 10 μg/ml FITC-labeled Env or HA for 90 min at 4°C or 37°C and then washed twice and cultured at 4 or 37°C for up to 24 h. In some cases, MDCs were preincubated, and the experiment was carried out in the presence of 1 mM leupeptin (Leu) to inhibit proteolytic degradation. Samples were then stained with DC markers for analysis by flow cytometry and gated on HLA-DR+CD11c+ (MDCs), CD123+ (PDCs), CD1a+ (LCs), or HLA-DR+ (DDCs). (A) Representative MDC donor showing the percentage of positive cells. (B) The mean fluorescence intensity (MFI) for each time point was normalized to the 0 min 37°C control for each Ag, and the data shown in this figure are the mean ± SD for three to five donors for Env and 1 donor for HA. (C) Alternatively, MDCs were incubated with Env in the presence of mannan or incubated with Env-D368R to isolate degradation via CD4 and CLR-mediated uptake pathways, respectively. Leupeptin was also included in these experiments as a control. Data shown in this figure are the mean ± SD of two donors.

**Discussion**

Due to their anatomical distribution in peripheral tissues, DCs are likely to be one of the first immune cells to come into contact with vaccine Ags after delivery. Through their unique capacity to transport Ag to the lymph nodes and prime naive CD4+ T cells, they provide the critical link between the administration of a vaccine and the development of a specific response. Thus, the efficiency with which DCs take up the vaccine Ag and present peptides to CD4+ T cells is considered central to the induction of potent vaccine responses. In this study, we analyzed the interactions between multiple primary human DC subsets and soluble HIV-1 Env as a HIV-1 immunogen. We show that skin and blood DC subsets take up Env to varying degrees via different CLR ligands and CD4, respectively, dictated by their respective receptor expression repertoire, supporting previous work by Turville et al. (36). Strikingly, PDCs, which are generally poor at uptake of soluble Ag and are thought to only present Ag to CD4+ T cells under certain conditions, showed a particularly high capacity for internalization of Env via CD4. We further demonstrated that this route is a functional Ag uptake pathway in multiple DC subsets to deliver Env to the endolysosomal system where it is degraded and subsequently presented to CD4+ T cells.
Whereas CLRs are known pattern recognition receptors used by DCs for the capture of a broad range of Ags, facilitating their role in immune surveillance, CD4 does not have an established role in Ag capture and processing. Specific CLRs such as the MR, DC-SIGN, and Langerin have a higher functional affinity for Env than CD4 does, which may include avidity effects (27), and this has been suggested as a mechanism by which DCs expressing CLRs can redirect the virus from conventional infection toward endocytosis and degradation (52). Alternatively, CLR-mediated uptake can lead to trans infection where internalized virions are neither degraded nor establish a productive infection but are transferred to other susceptible cells (28, 53). Although DCs are unlikely to be important in clearance of the virus, their capacity to endocytose, degrade, and subsequently present HIV-1 Ags to T cells is critical for the development of HIV-specific adaptive immune responses.

DC subsets with low expression of high-mannose binding CLRs such as blood DCs may therefore be more vulnerable to infection and/or use CD4 more readily as a receptor to endocytose the virus. The latter is supported by the finding that blocking BDCA-2, a CLR expressed on PDCs with binding affinity for Env, was shown not to reduce endocytosis of HIV-1 virions (40).

Extensive efforts have been directed toward targeting vaccine Ags to DCs via specific CLRs to improve Ag uptake and presentation (54–57). This strategy can result in enhancement of T and B cell responses by orders of magnitude and reduce the dose of Ag required in a vaccine setting. However, depending on the choice of targeting receptor and the expression pattern of that receptor on DC subsets, the immunological outcome will likely differ. MDCs are generally superior to PDCs for MHC class II Ag presentation (58–60), whereas BDCA-3+ DCs excel at cross-
presentation on MHC class I molecules (3, 61). Restricted receptor expression and lower endocytotic activity are thought to account for the less efficient uptake and presentation of exogenous Ag by PDCs than MDCs (62). In the case of Env, its high glycosylation naturally targets it to DCs via their CLRs. In addition, the data presented in this study suggest that the high-affinity interaction between Env and CD4, which is highly expressed on PDCs, also enables efficient uptake of Env in these cells.

Apart from Ag uptake, the weaker capacity of PDCs for exogenous Ag presentation on MHC class II has been related to their failure to reduce the turnover rate of MHC class II molecules, in contrast to other DC subsets. As a consequence, PDCs are unable to generate stable MHC class II:peptide complexes that facilitate extended engagement of CD4+ T cells and optimal T cell activation (63, 64). This is consistent with what we observed in peptide dose-response experiments. However, under the right conditions, such as when provided with temporal TLR stimulation (5), Ag complexed to immune serum (17), or specific receptor usage (18, 65), PDCs have been shown to function well as APCs and even comparable to MDCs. In the current study, we found that donor-matched PDCs, MDCs, and BDCA-3+ DCs presented Env pp65 on MHC class II and stimulated CD4+ T cells with similar efficiencies when identical experimental conditions were used. The enhanced presentation capacity of PDCs in this instance compared with earlier studies is likely a result of their efficient uptake and therefore potentially the ability of PDCs to contribute to Env presentation in vivo does not compromise peak CD4+ T cell helper response. However, more subtle effects of the Env-CD4 interaction on vaccine-induced immune responses in the absence of a strong adjuvant were not investigated in this study. Furthermore, others have shown that Env binding to CLRs on DCs stimulates an immunosuppressive response (66) and DC apoptosis (67), and blocking or removing the mannose moieties on Env, and thus precluding endocytosis via CLRs, enhanced Env-specific humoral responses in vivo (68, 69). Thus, uptake and presentation pathways not relying on CLRs may be relevant to Env vaccination.

In summary, we have identified a pathway by which DCs expressing limited or no Env-binding CLRs may be involved in binding and uptake of Env-based vaccines and the subsequent priming of a naive CD4+ T cell response. This CD4-mediated pathway may be especially relevant in the context of i.m. vaccinations where blood DCs are likely to constitute the main infiltrating DC population at the site of inoculation.

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Disclosures
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