Myeloid and plasmacytoid dendritic cells transfer HIV-1 preferentially to antigen-specific CD4+ T cells

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Dendritic cells (DCs) are essential antigen-presenting cells for the induction of T cell immunity against pathogens such as human immunodeficiency virus (HIV)-1. At the same time, HIV-1 replication is strongly enhanced in DC–T cell clusters, potentially undermining this process. We found that immature CD123+ plasmacytoid DCs (PDCs) and CD11c+ myeloid DCs (MDCs) were susceptible to both a CCR5- and a CXCR4-using HIV-1 isolate in vitro and were able to efficiently transfer that infection to autologous CD4+ T cells. Soon after HIV-1 exposure, both PDCs and MDCs were able to transfer the virus to T cells in the absence of a productive infection. However, once a productive infection was established in the DCs, newly synthesized virus was predominantly spread to T cells. HIV-1 exposure of the MDCs and PDCs did not inhibit their ability to present cytomegalovirus (CMV) antigens and activate CMV-specific memory T cells. As a result, both PDCs and MDCs preferentially transmitted HIV-1 to the responding CMV antigen–specific CD4+ T cells rather than to nonresponding T cells. This suggests that the induction of antigen–specific T cell responses by DCs, a process crucial to immune defense, can lead to preferential HIV-1 infection and the deletion of responding CD4+ T cells.

HIV-1 infection is associated with a gradual loss of CD4+ T cells and severe dysfunction of the immune system. Of particular note, HIV-1 preferentially infects CD4+ T cells specific for HIV-1 antigens, as compared with T cells with other antigen specificities within infected individuals (1). Resting CD4+ T cells are the major reservoir for HIV-1 infection in vivo (2–4). DCs are important for stimulating antigen-specific CD4+ T cell responses from the resting memory T cell pool, as well as priming naive T cells (5). Because DCs can be productively infected with HIV-1, and infected DCs can be found in mucosal tissues soon after viral transmission (6, 7), they could potentially serve as Trojan horses, delivering the signals necessary for inducing CD4+ T cell responses while simultaneously delivering a lethal dose of HIV-1 to those responding T cells. In fact, the DC–T cell microenvironment has been described as an “explosive” site for HIV-1 propagation, as activated T cells become highly susceptible to HIV-1 and thereby further enhance viral replication (8–12). Still, it is unknown whether DCs are as central to the pathogenesis of HIV-1 infection as they are for generating effective, adaptive anti–HIV-1 immunity.

Although studies of the role of DCs in HIV-1 infection have substantially intensified over the last decade, most knowledge has been revealed using in vitro monocyte-derived DCs to model this process, often using allogeneic T cell lines as a surrogate for antigen-specific T cells. To provide a more physiologic system, we modeled the spread of HIV-1 during specific CMV antigen presentation by DC subsets to autologous CMV-specific CD4+ T cells isolated directly from blood. We investigated CD123+ plasmacytoid DCs (PDCs) and CD11c+ myeloid DCs (MDCs), both of which have previously been found to be susceptible to HIV-1 infection (13–16).

Here, these subsets of DCs were infected with HIV-1 ex vivo and were subsequently co-cultured with autologous CD4+ T cells. Using flow cytometric analyses, we documented that MDCs and PDCs displayed differential susceptibility to the R5- and X4-HIV-1 strains.
BaL and IIIB, respectively, and both DC subsets were able to preferentially transmit either virus strain to the antigen-specific T cells that were responding to the antigen expressed by the DCs. Irrespective of the virus isolate, both PDCs and MDCs were able to transfer the virus to T cells in the absence of DC infection soon after HIV-1 exposure. However, once a productive infection was established in the DCs, they predominately spread the newly synthesized virus to the responding T cells. The process of virus transfer to antigen-specific T cells occurred predominantly within DC–T cell clusters.

**RESULTS**

**Productive HIV-1 infection of MDCs and PDCs**

Recently, we established a direct isolation procedure whereby we purify relatively high numbers of human CD123+ PDCs and CD11c+ MDCs from elutriated monocytes (17). The overnight culture of freshly isolated DCs leads to development of the characteristic DC morphology and immature phenotype defined by low or moderate expression of the costimulatory molecules CD40, CD80, CD86, and MHC class II (HLA-DR; reference 17). We found that both DC subsets expressed CD4, CCR5, and CXCR4, receptors used for HIV-1 infection (Fig. 1 A). Prior studies have found HIV-1 RNA or DNA in both of these DC subsets after HIV-1 exposure (13, 14, 16). We found that high infection frequencies in DCs could be obtained by exposing PDCs and MDCs to highly concentrated and purified virus isolates for 72 h (16). Viral replication was detected in MDCs and PDCs as early as 3 h after exposure by the presence of viral negative strand DNA in the DCs, and HIV-1 p24 antigen could be detected intracellularly after 24 h of exposure (16). No additional stimulation of the DCs was required to induce viral replication. PDCs and MDCs were shown to be susceptible to both CCR5- and CXCR4-using HIV-1 (BaL and IIIB, respectively, and both DC subsets were able to preferentially transmit either virus strain to the antigen-specific T cells that were responding to the antigen expressed by the DCs. Irrespective of the virus isolate, both PDCs and MDCs were able to transfer the virus to T cells in the absence of DC infection soon after HIV-1 exposure. However, once a productive infection was established in the DCs, they predominately spread the newly synthesized virus to the responding T cells. The process of virus transfer to antigen-specific T cells occurred predominantly within DC–T cell clusters.

Both DC subsets differentiated partially in response to HIV-1 exposure alone, as found by up-regulation of CD40, CD80, CD83, and CD86 (Fig. 1 E). However, Toll-like receptor (TLR)7/8 ligation with R-848 led to complete maturation exceeding the differentiation induced by HIV-1 exposure alone (Fig. 1 E). We did not find that R-848-induced activation of HIV-1–exposed MDCs or PDCs led to different cell surface phenotype than the activation of uninfected DCs (16).

**HIV-1–infected DCs transfer the virus to autologous CD4+ T cells**

Prior studies have shown that conjugates of DCs and T cells are predominant sites for HIV-1 replication both in vivo and in vitro (8–12). We examined the specific transfer of HIV-1
between HIV-1–exposed subsets of DCs and autologous CD4+ T cells during antigen presentation. To establish such an experimental system, we first evaluated the time required for the transmission of HIV-1 from infected DCs to uninfected autologous T cells. MDCs or PDCs, isolated from healthy CMV-seropositive donors, were exposed to HIV-1 for 72 h, washed thoroughly, and incubated briefly with inactivated CMV antigen lysate or Staphylococcal enterotoxin B (SEB), and then co-cultured with sorted CD4+ T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). The proliferation of CMV antigen–specific memory T cells responding to DCs presenting processed CMV peptides, or of T cells activated through TCR Vβ–restricted SEB cross-linking between DCs and T cells, was assessed at different time points after co-culture (1.5, 3.5, and 7.5 d) by flow cytometry. To analyze only T cells, gates were set on CFSE-labeled T cells excluding CD11c+ MDCs or CD123+ PDCs. HIV-1 p24 expression and CFSE dilution in the T cells were thereafter assessed and compared with T cells cocultured with uninfected DCs (Fig. 2). At 1.5 d after co-culture, there was no T cell division and no or very low intracellular HIV-1 p24 in the T cells (Fig. 2 A). At 3.5 d, T cells expressing HIV-1 p24 were detected and were more frequent in cultures where T cell activation and division had been stimulated (CMV or SEB) than in those where no stimulation had occurred (Fig. 2 B). The frequency of p24+ T cells increased over time. At 7.5 d after co-culture, a large percentage (ranging from 3 to 19%) of the T cells in the cultures that had received CMV or SEB stimulation expressed p24, whereas T cell infection in the nonstimulated cultures remained minimal (Fig. 2 C). HIV-1–infected MDCs and PDCs were able to activate CMV–specific T cells or SEB–stimulated T cells to proliferate to a similar extent as uninfected donor–matched DCs (Fig. 2, C and D). Both HIV-1BaL– and HIV-1H11001–exposed MDCs or PDCs were able to transfer the virus to CD4+ T cells as p24+ T cells were detected after 3.5 d of co-culture, irrespective of which virus isolate or DC subset was used. A time period of 3.5 d for cell co-culture was therefore chosen for further studies. To ensure that virus saturation had not been reached in our culture system, we exposed DCs to 10-fold dilutions of the virus stocks before co-culture with T cells. Proportionally fewer T cells became infected when co-cultured with DCs that had been exposed to lower amounts of either HIV-1BaL (Fig. 2 E) or HIV-1H11001 (unpublished data).

**Differential transfer of HIV-1BaL and HIV-1H11001 from MDCs and PDCs to CD4+ T cells**

As mentioned in Productive HIV-1...PDCs section, we found that MDCs were more susceptible to R5-using HIV-1BaL than to X4-using HIV-1H11001, whereas no preference was found for PDCs (Fig. 1, B and C). We therefore asked how efficiently the different virus strains were transferred from the DC subsets to T cells during co-culture. MDCs were shown to efficiently transfer both HIV-1BaL and HIV-1H11001 to T cells (Fig. 3, A and E). We found no significant difference in the efficiency of transfer and/or propagation of the different virus isolates by the MDCs (P = 0.42; Fig. 3 E). In contrast, HIV-1H11001 was more efficiently transferred to and propagated in T cells by PDCs, as compared with HIV-1BaL (P = 0.03; Fig. 3, B and E). Production of p24 in infected T cells represents a late stage of the virus replication cycle and may not reflect the absolute amount of virus that is transferred from DCs to T cells (20). In addition, the infection of T cells that appeared 3.5 d after co-culture may represent a direct
transfer of the virus from infected DCs, but may also represent T cell to T cell propagation of HIV-1 after an initial transfer of the virus from DCs to T cells. To distinguish between these possibilities, we added the protease inhibitor Indinavir to the co-cultures (see Materials and methods). Indinavir allows the formation of p24 in infected cells, but blocks the production of infectious viral particles and the subsequent spread of the virus between CD4+ T cells. The addition of Indinavir led to a reduction of p24 T cells after coculture with HIV-1–infected DCs, indicating that HIV-1 is primarily propagated by T cell to T cell spread after the initial transfer from DCs in this in vitro system (Fig. 3, A–D). With Indinavir present in the co-culture, significantly higher frequencies of p24+ T cells were found after co-culture with HIV-1BaL as compared with HIV-1IIIB–exposed MDCs (P = 0.02; Fig. 3, C and E). In contrast, HIV-1IIIB–exposed PDCs were again able to infect a higher number of T cells as compared with HIV-1BaL–exposed PDCs (P = 0.03; Fig. 3, D and E). HIV-1IIIB was transferred equally well by PDCs and MDCs (P = 0.81). In contrast, HIV-1BaL was more efficiently transferred to T cells by MDs than PDCs (P = 0.008; Fig. 3, C–E). Although neither of the virus isolates appeared to reach virus saturation at the inoculum concentration (1 μg p24/ml) used for this comparison, it is still possible that different virus strains may have different transfer efficiencies from DC subsets to T cells at different virus titers. Several factors may therefore influence the selection and propagation of virus strains taking place in vivo.

Different pathways for transfer of the virus can be used by MDCs and PDCs

There are at least two pathways for the binding of HIV-1 by DCs. In addition to the conventional CD4- and coreceptor-mediated infection, HIV-1 can also bind to C-type lectin receptors such as DC-SIGN (CD209), which may lead to internalization by DCs (21–27). To examine whether a productive infection by isolated PDCs and MDCs is required to transfer the virus to T cells, we supplemented the culture media with AZT during the 72 h of HIV-1 exposure of DCs. AZT was thereafter washed out together with a residual virus before the DCs were co-cultured with autologous CD4+ T cells for 3.5 d. Co-culture of T cells with AZT-treated DCs resulted in no p24 expression in the T cells (Fig. 4, A–D). This was true for both PDCs and MDCs exposed to either HIV-1BaL or HIV-1IIIB (Fig. 4, A–D). These data show that longer term HIV-1 transfer by PDCs and MDCs to T cells is dependent on the productive infection of DCs. It has been shown that the time of virus exposure influences the pathway for transfer of the virus from DCs to T cells (27). To examine whether MDCs and PDCs have the ability to transfer the early–captured virus in the absence of infection, we limited the virus exposure time to 6 h, washed, and then added T cells for 3.5 d. Both MDCs and PDCs were able to pass the virus to T cells using this shorter virus exposure period (Fig. 4, E and F). Thus, in line with previous reports using other types of DCs, the MDCs and PDCs isolated from blood also transfer HIV-1 to T cells in the absence of a productive infection of the DCs soon after virus exposure.

DCs are predominantly of an immature phenotype when they first encounter HIV-1 at peripheral sites, but are mainly mature when they are located in lymph nodes to induce T cell activation and, presumably, the transfer of HIV-1. Therefore, to mimic the in vivo situation, immature MDCs and PDCs were exposed to HIV-1 for 72 h and stimulated with R–848 for the final 24 h to induce differentiation of DCs. R–848 stimulation led to a phenotypic maturation of both

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**Figure 3.** MDCs and PDCs transfer CCR5–using BaL and CXCR4–using IIIB with different efficiencies. Sorted CD11c+ MDCs (A and C) and CD123+ PDCs (B and D) were exposed to HIV–1BaL or HIV–1IIIB for 72 h, washed, and co-cultured with autologous CFSE-labeled CD4+ T cells with SEB for 3.5 d in the absence (A and B) or presence (C and D) of the protease inhibitor Indinavir. Cultures were analyzed for T cell proliferation (CFSE dilution) and frequency of HIV–1 infection (intracellular p24 staining). The graphs show CD4+ T cells gated on size and lack of CD11c or CD123 expression to exclude DCs from the analysis. The numbers in each gate indicate the percentages of p24+ cells out of all cells. One representative experiment of at least five is shown. (E) The graphs show the frequency of p24+ T cells (mean ± SEM) after co-culture with MDCs (black bars) or PDCs (white bars) exposed to the respective virus strains. The co-cultures were performed in the absence or presence of Indinavir, as indicated. Significant differences between the groups were assessed by Mann-Whitney’s unpaired t test (*, P ≤ 0.05).
MDCs and PDCs (Fig. 1 E; reference 17). Maturation did not lead to increased viral production in either MDCs or PDCs, as measured by intracellular p24 expression (16). Despite this, mature MDCs more efficiently transferred and/or induced propagation of both HIV-1BaL and HIV-1HIV in T cells than in donor-matched immature MDCs (P = 0.007 and P = 0.03, respectively; Fig. 4, A and B). There was no increase in the viral transfer of either of the isolates after maturation of PDCs (P = 0.62 and P = 0.84, respectively; Fig. 4, C and D). As for immature DCs, viral transfer to T cells by mature DCs did not occur when each of the DC subsets had been exposed to AZT during the 72-h infection period (Fig. 4, A–D).

MDCs and PDCs preferentially transfer HIV-1 to antigen-specific CD4+ T cells

Viral transfer from DCs to T cells has been shown to occur within an “infectious synapse” formed between a DC and an adjacent CD4+ T cell (27–30). Still, it is not known if T cells become infected when DCs form a tight cluster with T cells and the immunological synapses between the cells also serve as infectious synapses. We have previously shown that HIV-1–specific CD4+ T cells are preferentially infected by HIV-1 in vivo (1). This would suggest that antigen-specific T cells responding to a given antigen presented by an HIV-1–infected DC would become infected more frequently than T cells not responding to specific signals transmitted by the DC. We investigated the relationship between a DC-mediated HIV-1 infection and the specific activation of CD4+ T cells in more detail. Again, the HIV-1–infected DCs were incubated with CMV antigens and then co-cultured with autologous CD4+ T cells. To ensure that HIV-1–infected T cells were infected exclusively by the DCs and not indirectly by T cell to T cell spread in the co-culture, the protease inhibitor Indinavir was added to the co-cultures. We initially examined the frequency of p24+ T cells or CD4+ T cells that did or did not produce the effector cytokines IFN-γ, TNF-α, and/or IL-2 in response to CMV antigen presented by the DCs (Fig. 5 A). In addition, as displayed by CFSE dilution, we gated on each cell division within the cytokine-producing and noncytokine-producing T cells. We found significantly higher frequencies of p24+ T cells among the cytokine-producing T cells as compared with the noncytokine-producing T cells (P < 0.0001). This indicates that among the T cells present in the co-cultures, the CMV-specific T cells that were responding to CMV antigens presented by DCs were most likely to become HIV-1 infected. The highest proportions of p24+ cells were found in undivided T cells or T cells that had divided only once. Because induction of cytokine production occurs rapidly after memory T cell activation and before proliferation (31), this indicates that a majority of the p24+ T cells were recently both activated and infected after their encounter with CMV antigen–presenting and/or HIV-1–infected DCs. Productive infection of T cells may lead to death before division and, therefore, it is not surprising that the frequency of infected T cells decreased in later divisions. In addition, HIV-1 Vpr causes cell cycle arrest and may also explain why most of the p24+ T cells had not divided. Next, we examined whether p24+ or p24− T cells most often colocalized with the production of effector cytokines (Fig. 5 B). Again, each cell division was analyzed. There was more than twice the frequency of cytokine-producing CD4+ T cells among the p24+ cells than p24− T cells. The greatest induction of preferential infection of antigen-specific cells occurred in the nondivided T cell population (Fig. 5, A and B). We also examined all the responding T cells in the co-cultures, i.e., both divided and cytokine-producing T cells (Fig. 5 C). Responding CMV-specific T cells were more frequently infected than nonresponding T cells (P < 0.0001; Fig. 5 C). The HIV-1–infected p24+ cells within the responding T cells were more likely to express cytokines as compared with the p24− T cells. The same pattern was observed when each
DC subset exposed to either HIV-1\textsubscript{Bal} or HIV-1\textsubscript{HIV} was co-cultured with T cells. This general trend—that HIV-1 infection was most frequent in responding T cells—was true both in the presence of CMV or SEB using either immature or mature DCs. This indicates that the majority of the infected T cells had interacted with an HIV-1–infected DC that simultaneously presented antigen to which the TCR of the T cell had specificity. Alternatively, the responding antigen-specific T cells received an HIV-1 infection from HIV-1–infected DCs that were in close proximity to the antigen-specific T cells. This observation also held for MDCs and PDCs. We gated on different cell populations within the co-cultures and examined them for p24 versus cytokine expression (Fig. 7, A and B). Single MDCs or PDCs, expressing the DC-specific markers CD11c or CD123, respectively, but not associated with CFSE-labeled T cells, were analyzed, as were undivided and divided T cells not colocalized with DC markers. A separate

**HIV-1 is transferred in conjugates of infected DCs and T cells**

We have previously shown that in co-cultures of HIV-1–infected skin DCs (Langerhans cells) and T cells, the infected p24\textsuperscript{T} T cells predominantly appear in tight conjugates of T cells and CD1a\textsuperscript{+} Langerhans cells (32). We analyzed whether this observation also held for MDCs and PDCs. We gated on different cell populations within the co-cultures and examined them for p24 versus cytokine expression (Fig. 7, A and B). Single MDCs or PDCs, expressing the DC-specific markers CD11c or CD123, respectively, but not associated with CFSE-labeled T cells, were analyzed, as were undivided and divided T cells not colocalized with DC markers. A separate
population of cells that coexpressed DC markers and CFSE that represented cell conjugates was also analyzed. The presence or absence of DCs and T cells within these different cell populations was confirmed by polychromatic flow cytometry examining the expression of CD11c/CD123, CD3, CD4, CD8, CD14, and CD20 (32, 33). Conjugate formation between DCs and T cells was observed among both undivided and dividing T cells. Higher proportions of MDCs, as opposed to PDCs, were found in clusters with T cells (Fig. 7, A–D). In addition, DC maturation led to a further increase in cluster formation (unpublished data). Cell conjugates were observed in all cultures whether unexposed or exposed to either HIV-1 BaL or HIV-1 IIIB. The DC–T cell conjugates in co-cultures with either PDCs or MDCs contained significantly higher frequencies of infected p24+ cells as compared with undivided T cells not associated with DCs (n = 10, P = 0.008). T cells that had undergone cell division, and thus been activated by DCs at a prior time point, displayed similar, but most often lower, frequencies of p24+ T cells compared with the T cells in the conjugates. The highest frequencies of cytokine-producing cells were found in the population consisting of cell conjugates as compared with the other three populations (n = 10, P = 0.008). In addition, the proportion of p24+ cells that also produced cytokines was the highest in the conjugates of DCs and T cells as compared with either undivided or divided T cells (Fig. 7, A and B).

DISCUSSION

It is well documented that DCs can efficiently transfer HIV-1 to T cells. However, the exact nature of the T cells that re-
receive the infection is still unknown. We describe a novel coculture and flow cytometry model system to examine the CD4+ T cells that become infected by HIV-1 from blood-derived subsets of DCs (MDCs and PDCs) during antigen presentation. Our results suggest that DC-mediated HIV-1 infection of CD4+ T cells does not occur randomly. In fact, HIV-1 usurps the antigen presentation and activation functions of DCs to specifically transfer HIV-1 to the T cells that are responding to the antigens that these DCs are presenting. This is possible because HIV-1 infection by itself does not notably diminish the antigen-processing and T cell-activating properties of DCs. HIV-1 produced by DCs is therefore provided an optimally activated CD4+ T cell population in which to establish a productive infection that can lead to the depletion of the specific T cells that are responding to cognate antigens. This would result in a specific loss of important T cell specificities.

It has been suggested that HIV-1–infected DCs will present infectious HIV-1 to HIV-1–specific CD4+ T cells in vivo (1). However, directly testing this in vitro is difficult because it would require the use of DCs and T cells from HIV-1–infected subjects, and few patients have adequate numbers of HIV-1–specific CD4+ T cells to carry out these experiments (34, 35). Therefore, we used healthy CMV+ subjects with preserved CMV-specific CD4+ T cells to model the transfer of HIV-1 from infected DCs to antigen-specific T cells. In our in vitro system, we showed that HIV-1–infected MDCs and PDCs presenting CMV antigens both activated preexisting autologous CMV-specific memory CD4+ T cells and preferentially transferred the virus to them. This pattern was also seen when using SEB that induced the cross-linking of TCRs as an alternative to using CMV antigens to activate T cells. This mechanism of preferential transfer to antigen-specific T cells may explain why HIV-1–specific T cells in infected individuals more frequently contain HIV-1 than T cells with other TCR specificities (1). It should be noted that the mechanism of infection identified here is not the only route of infection of CD4+ T cells. We have previously shown that naive T cells are infected in vivo (33), and this is unlikely to occur from DCs according to antigen specificities. In addition, we show here that once HIV-1 is transferred from a DC to an antigen-specific T cell, that infection can rapidly spread from T cell to T cell.

Immunological synapses are formed between DCs and T cells during antigen presentation. MHC class II peptide-bearing compartments within a DC have been shown to move to the contact point between the DC and the interacting TCR peptide–specific T cell (36, 37). HIV-1 may exploit this cellular machinery for transmission from infected DCs to CD4+ T cells. Viruses that spread from DCs to T cells have been shown to be directed to the connection site between the cells (28). DCs replicate HIV-1 poorly as compared with activated CD4+ T cells. Therefore, the efficient transfer of a virus from DCs to T cells may require cell–cell contact (38). We found that HIV-1 p24 was preferentially detected in conjugates of DCs and T cells, suggesting that T cells become activated and infected through cluster formation with infected DCs rather than from a free virus in the culture media secreted by HIV-1–infected DCs. This is also supported by the fact that we found early transcripts of HIV-1 primarily in CMV-specific cytokine-producing T cells, as opposed to nonresponding T cells, only 10 h after coculture with infected DCs. Still, we have not demonstrated that it is the HIV-1–infected DC that also presents CMV to, and activates, CMV-specific T cells. Therefore, we cannot exclude a scenario in which an uninfected DC presents CMV antigens to a responding CMV-specific T cell that will receive an HIV-1 infection from HIV-1–infected DCs that are in close proximity.

Preferential transfer of the virus to antigen-specific T cells occurred despite the differential susceptibility of DC subsets to R5 (BaL)- and X4 (HXB)-using virus isolates. We have previously found that TLR7/8 ligation using R-848 led to differentiation of both MDCs and PDCs, which in turn enabled them to activate higher frequencies of antigen-specific T cells (17). We found that R-848–matured MDCs transferred and propagated HIV-1 in T cells more efficiently than immature MDCs. More conjugates were formed between MDCs and T cells when the MDCs were mature, which would facilitate the milieu for viral transfer. Furthermore, the superior activation of T cells induced by mature MDCs can lead to enhanced replication in T cells. Therefore, although mature MDCs are better APCs and are essential for the induction of primary immune responses, they are also better at transferring the virus. This ability of HIV-1 to infect DCs and usurp their functions may accelerate the immune depletion in an HIV-1 infection by specifically depleting responding CD4+ T cells and allowing for an early lacunar defect in the HIV-1–specific repertoire. We did not see an increased transfer of the virus upon maturation of PDCs. Although we have previously found that R-848–matured PDCs are better APCs than immature PDCs (17), they may not be superior at transferring the virus to T cells. This may derive from the fact that exposure to either HIV-1 or R-848 will induce IFN-α production in PDCs (17–19), which can prevent viral replication in T cells (39). In addition, PDCs are highly sensitive to long-term culture and stimulation, and may die during the relatively long-term coculture used in our in vitro system.

Because of the low frequencies of DCs in vivo and the difficulties in isolating these cells, several different sources for DCs have been used to study the biology of DCs in the context of HIV-1 infection. Numerous in vitro studies have shown that there are at least two pathways by which HIV-1 interacts with DCs (21–27). These include the conventional CD4- and coreceptor-mediated infection by HIV-1, as well as the binding of the virus via DC-SIGN and other C-type lectins. The mechanism of viral transfer from DCs to T cells may first be dependent on the type of DC that is used.
Monocyte-derived DCs have been shown to capture HIV-1 without infection and transmit the virus to T cells (23, 24, 27, 29). Viral transmission to T cells from isolated Langerhans cells has been shown to occur in a CD4-dependent manner (22, 26, 40), but Langerhans cells can also bind HIV-1 in a CD4-independent manner (25). The mechanism of viral transfer may also be dependent on the time of viral exposure (27). It has been shown that the initial phase of HIV-1 transfer from monocyte-derived DCs to T cells is not dependent on a productive infection. The virus captured soon after DC exposure may thereafter be degraded and a productive infection of DCs is then required for viral transfer to T cells to occur at later time points (27). We found that transmission of the virus to T cells by PDCs and MDCs required productive infection by the DCs after 72 h of virus exposure, whereas soon after virus exposure (within 6 h), DCs could pass the virus to T cells without being productively infected. Thus, despite phenotypic and functional differences between MDCs, PDCs, and other DC types, they may all use similar mechanisms to transfer HIV-1 to T cells.

It may be possible to prevent the spread of HIV-1 by blocking conjugate formation between DCs and T cells, and thereby decrease T cell activation and proliferation. On the other hand, as the interaction between DCs and T cells is essential for elicitation of primary and secondary immune responses against pathogens, inhibiting this general interaction would not be without consequences. That said, a better understanding of the process by which HIV-1 targets antigen-specific T cells and that leads to their productive infection will certainly aid in our discovery of new and better approaches to vaccine and therapeutic agents.

MATERIALS AND METHODS

DC isolation. This study was approved by the National Institutes of Health Institutional Review Board. Our sorting procedures for the direct approaches to vaccine and therapeutic agents.

HIV-1 virus growth and preparation. The CCR5-using HIV-1NL4-3 isolate and the CXCR4-using HIV-1HXB isolate (NIH AIDS Research and Reference Reagent Program) were grown on PHA- (Sigma-Aldrich) and IL-2– (Chiron Corp.) stimulated PBMCs. The p24 Gag level in the cell culture supernatant was monitored by p24 ELISA and the viruses were harvested at a peak time point. To concentrate the virus and to minimize the presence of bystander activation factors in the supernatant that could influence DC growth, the isolates were concentrated with an ultracentrifugation machine at 30,000 rpm for 70 min at 4°C (Sorvall Surespin 630; Kendro). The virus pellet was resuspended in fresh RPMI with 10% FCS to obtain a 5–10-fold concentrated clean virus stock.

Characterization of HIV-1 stocks. The viral titers of the HIV-1 stocks were determined by p24 ELISA (PerkinElmer) according to the manufacturer’s instructions. The highly concentrated HIV-1NL4-3 stock had 10 μg/ml HIV-1 p24Gag and the HIV-1HXB stock had 5 μg/ml HIV-1 p24. Virus tissue culture ID50 was determined by a sensitive 14-d endpoint titration assay using PHA- and IL-2–stimulated PBMCs, as previously described (41). The HIV-1NL4-3 stock had 2.6 × 106 tissue culture ID50/ml and the HIV-1HXB stock 1.75 × 106 tissue culture ID50/ml.

HIV-1 infection and stimulation of DCs. The DC populations were cultured at 106 cells/ml (with no less than 0.25 × 106 cells/tube) for 12 h at 37°C using polystyrene round-bottom tubes (Becton Dickinson). HIV-1NL4-3 or HIV-1HXB (normalized by a final concentration of 1 μg p24/ml, which translated into a multiplicity of infection of 0.2–0.4) was thereafter added to the DCs, and the cells were cultured for 6 or 72 h similar to as described earlier (16, 42). For dose-response comparisons, the virus isolates were used at 0.01–1 μg p24/ml. In some experiments, 0.1 μM AZT, a nucleoside analogue that inhibits reverse transcription (Sigma-Aldrich), was added to the cultures before addition of the virus isolate. To induce maturation, the DCs were stimulated by the TLR7/8 ligand R-848 (1 μg/ml, 4-aminoo-2-ethylhexyl-aminoethyl-methyl, α-dimethyl-1H-imidazoguine-1-ethanol; GLSynthesis Inc.) as previously described (17). The DCs were exposed to HIV-1 for 72 h and stimulated with R-848 during the final 24 h.

Phenotypic characterization and quantification of HIV-1 protein in DCs. Cells were harvested in PBS supplemented with 0.5% BSA, and surface stained with different combinations of anti-CD11c, -CD123, -CD14, -CD20, and –HLA-DR antibodies (BD Biosciences). Cells were then washed, fixed, and permeabilized using a 2× fixation–permeabilization solution (Becton Dickinson). The frequency of HIV+–infected DCs was determined by intracellular staining for the HIV-1 gag protein p24 (anti-p24, clone KC57; Beckman Coulter; references 16, 42). The DCs were also phenotyped using anti–DC-SIGN (R&D Systems), anti-CXCR5, and anti-CXCR4 (BD Biosciences). Cell viability was evaluated by propidium iodide staining. The cells were analyzed on a flow cytometer (FACS Calibur; Becton Dickinson).

Measurement of IFN-α release. The supernatants from HIV-1–infected or uninfected DCs were harvested and analyzed by ELISA (Biosource International) according to the manufacturer’s instructions (17).

Isolation and CFSE labeling of CD4+ T cells. Enriched populations of autologous negatively selected CD4+ T cells, depleted of CD14+, CD15+, CD19+, CD56+, CD1c+, BDCA-4+, and CD8+ cells, were isolated using microbeads (Miltenyi Biotec) on elutriated lymphocytes and sequential separation on AutoMACs. The purity was >96%. The sorted CD4+ T cells were frozen in 10% DMSO (Fisher Scientific) in FCS and stored at −85°C until use. At the time of co-culture, the CD4+ T cells were thawed in the presence of 0.1 U/ml DNase I (RNase-free; Roche), washed thoroughly, and labeled with 0.25 μM CFSE (Molecular Probes) as described previously (31, 32). CFSE–labeled cells were washed and reuspended in medium. Cell viability was determined by Trypan blue exclusion before co-culture with DCs.

Co-culture of HIV-1–infected DCs with autologous CD4+ T cells. HIV-1–exposed or unexposed DCs were thoroughly washed with 3× repetitive centrifugation in 4 ml of prewarmed media to minimize the trans-
fer of residual no-cell-associated virus. The DCs were pulsed with whole CMV antigen lysate. The whole CMV antigen preparation (Microflex Biosystems) was derived from the CMV AD-169 virus strain grown in MRC-5 cells and inactivated by gamma irradiation. The CMV preparation was titrated to find a dose that was not toxic to the cells yet adequate to activate CMV specific memory T cells (10 μl/ml). In some experiments, 0.2 μg/ml of the TCR superantigen SEB (Sigma-Aldrich) was used as an antigen instead of CMV. The DCs were then co-cultured with sorted autologous CFSE-labeled CD4+ T cells in a DC/T cell ratio of 1:10 in complete media in polytetrfluoroethylene tubes. The DC–T cell co-cultures were carried for 1–8 d with the addition of 1 μg/ml Brefeldin A (Sigma-Aldrich) during the last 12 h of co-culture. In some experiments, 1 μM of the protease inhibitor Indinavir (Sigma-Aldrich) was added at initiation or 10 h of co-culture.

Analysis of a DC-mediated transfer of HIV-1 to antigen-specific T cells. Co-cultures of DCs and T cells were harvested at the indicated time points. Cells were surface stained with anti-CD11c PerCP-Cy5.5, anti-CD46 APC-Cy7, and anti–CD80 APC (BD Biosciences), and anti–IL-2 APCs (Caltag Laboratories). The cells were then fixed on a FACScalibur flow cytometer to prevent leakage of CFSE dye from the cells. To stentify define the cell types in the co-cultures, a phenotypic analysis was performed in detail with polychromatic flow cytometry using anti-CD11c Cy5-PE/CD123 Cy5-PE, CD3 Cy7-PE, CD4 Cy7-APC, CD14 Cy7-APC, and CD20 Cy7-APC as previously described (32, 33). Stained cells were analyzed on a modified LSR II (Becton Dickinson). All data were evaluated by FlowJo software (Treestar Inc.).

T cell sorting for the analysis of HIV-1 gag DNA content. DCs were exposed to HIV-1gag or HIV-1env for 72 h and co-cultured with autologous CD4+ T cells as described in Co-culture of...cells section. Co-cultures were harvested at indicated time points and four different fractions of T cells were then sorted on a FACSVantage SE/DiVa (Becton Dickinson). Cells were gated on CD11c−/CD123− CFSE-labeled T cells; subsequently, undivided cytokine-producing, dividing cytokine-producing, undivided noncytokine-producing, and dividing noncytokine-producing T cells were sorted. 15,000–50,000 T cells in each fraction were collected and the pellets were frozen.

Viral DNA assessment. The HIV-1 strong stop DNA (an early event of reverse transcription), full-length p17 gag DNA, and human albumin were quantified by real-time PCR on an ABI7900HT (Applied Biosystems) as previously described (1). In brief, the sorted cells were lysed in 0.1 mg/ml proteinase K buffer in Tris-Cl (pH 8.0) at 100,000 cells per 10 μl of buffer by incubation at 56°C for 1 h and then at 95°C for 10 min. The lysate was cleared by centrifugation at 14,000 g for 2 min. 5 μl of the lysate was used in a reaction volume of 25 μl with 500 nM of primers (AA55, CTGCTA-GCATTTCACACAGCTG; M676, TAACTAGGAACCCACTG-GCTTAA; gag FWD, GGTCG GGAC CGTCG CAGTATTTAAAG; gag REV, AGGGCT CCGTC GCTAC CTGCATTTA; human Fwd, ggtggaaagagcagaata; human Rev, atgtgcggctccgacaa; Albumin Fwd, tgcatgagaaaacgccagtaa; Albumin Rev, atgcggccgtgctccagc). BioSource International), 200 μM of probes (strong stop DNA probe, FAM-AGAGTCACACACAGGAGGACACACT-BHQ1; gag probe, FAM-AAAACTGCCTTTGACCTTGA-gtacctggcgctggc (Microsource International), 200 nM of dNTPs, 3.5 mM MgCl2, and 1.25 mM Blue 636 reference dye with 0.625 U Platinum Taq in the buffer supplied. The conditions were 95°C for 15 s and 60°C for 1 min for 50 cycles after a 2-min activation at 95°C. Quantitation was performed by running standard curves for p17 gag and albumin controls.

Statistical analyses. Statistical analyses were performed using Wilcoxon’s paired t test or Mann-Whitney’s unpaired t test with GraphPad Prism software.

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