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Virus replication begins in dendritic cells during the transmission of HIV-1 from mature dendritic cells to T cells

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Virus replication begins in dendritic cells during the transmission of HIV-1 from mature dendritic cells to T cells

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Background: To initiate immunity, dendritic cells (DCs) capture antigens or viruses at body surfaces, undergo maturation to express T-cell costimulatory molecules, and then migrate to lymphoid organs. DCs at body surfaces can capture human immunodeficiency virus 1 (HIV-1), but mature DCs do not support replication of the virus unless T cells are added. The initial site for HIV-1 replication remains unknown and it is unclear whether replication can take place in DCs or whether the virus must first be transmitted from DCs to T cells.

Results: We generated mature DCs from monocyte precursors. Upon infection with HIV-1, reverse transcription was completed only when T cells were added. When the reverse transcriptase inhibitor azidothymidine was added to the DCs during exposure to HIV-1, the DCs remained fully infectious, as long as the drug was removed just before culturing the DCs with T cells. HIV-1 variants that were engineered to undergo only one cycle of replication were able to infect DCs and replicate once in these cells. When T cells were added, newly produced HIV-1 Gag protein was exclusively localized to the DCs. With wild-type virus, subsequent rounds of replication took place in T cells. Soluble CD40 ligand (CD40L) and CD40L-transfected fibroblasts stimulated HIV-1 replication in purified mature DCs.

Conclusions: Mature DCs provide a drug-resistant reservoir for HIV-1. This reservoir is activated within DCs by CD40L and upon interaction with T cells, and the virus then spreads rapidly to other T cells.

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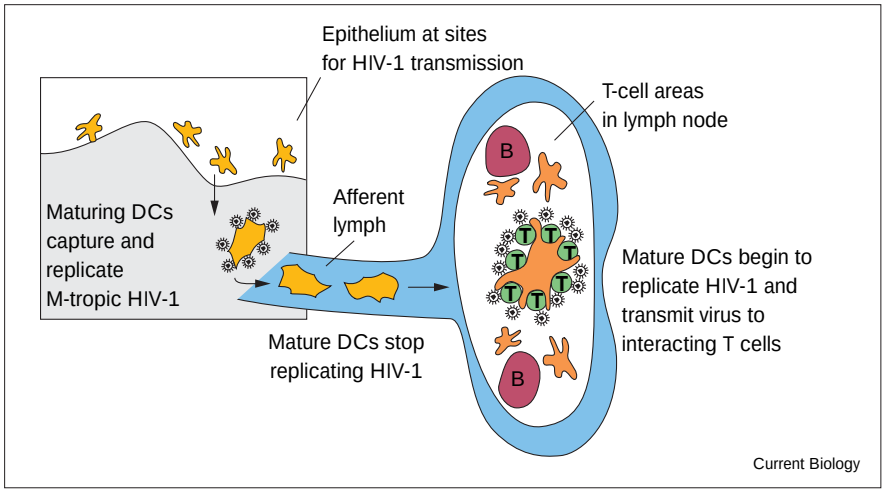
Background

During the early stages of infection, HIV-1 probably exploits the antigen-presentation ability of dendritic cells (DCs). At body surfaces, DCs are specialized to capture and process antigens but the cells also express the important coreceptors for HIV-1 entry — CD4 and the chemokine receptor CCR5 [1–3]. These DCs are termed 'immature' because, at this stage, they do not express the high levels of the cell surface molecules (such as, CD40, CD54, CD86) that are required for potent T-cell stimulation. Immature DCs selectively capture macrophage (M)-tropic strains of HIV-1 [2,4,5] (Figure 1), which predominate during transmission of the virus [6,7].

Upon mobilization from a body surface, the DCs enter the lymph to migrate to lymphoid organs [8–12] (Figure 1). The cells also mature to express T-cell stimulatory molecules [13,14]. The fully matured DCs activate antigen-specific T cells in lymphoid tissues [15]. Activated T cells are in turn permissive for HIV-1 replication and die by apoptosis [16]. Purified mature DCs are unable to support viral replication, however [5,17–22], as the life cycle of the virus appears to be blocked shortly after entry. Mature DCs therefore support the early but not the later stages of reverse transcription of the virus, and the formation of these early transcripts requires functional chemokine receptors on DCs [5,23].

Nevertheless, when mature DCs are incubated with virus and then mixed with T cells, as would occur when DCs stimulate immunity in lymph nodes, a strong productive infection occurs (Figure 1). This infection is noteworthy because of its rapidity and its magnitude, and because it does not require addition of mitogens, interleukin-2 (IL-2) or heterologous sera [5,17,19,20,22]. Here, we address the events that take place when mature DCs interact with T cells in culture. Large numbers of mature DCs were generated from blood monocytes using a standard tissue culture system [24–26] and these cells were further purified on the basis of high expression of CD83 [25,27]. We used pseudotyped variants of HIV-1 that are only able to undergo one cycle of replication to show that mature DCs are responsible for the first cycle of HIV-1 replication, whereas subsequent cycles occur primarily in T cells.

Figure 1



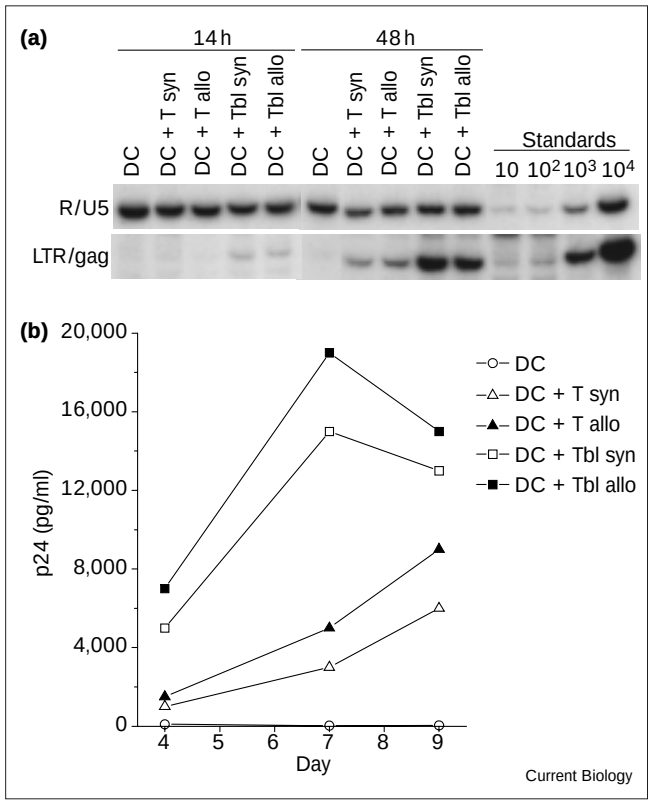
A diagram of the proposed function of immature and mature DCs at body surfaces and lymphoid organs, respectively. Tissue culture studies indicate that immature DCs directly capture and replicate M-tropic – R5 – viruses, whereas mature DCs support HIV-1 replication only when T cells are present. *In vivo*, immature DCs are found in the stratified, skin-like epithelia in the vagina, cervix and anus through which HIV-1 is probably transmitted. During migration into the lymph and upon reaching the T-cell areas of lymph nodes, the DCs mature and become potent stimulators of the immune response in lymphoid organs. In the lymph node, mature DCs would encounter T cells, setting up the kind of cellular environment that is permissive for HIV-1 replication. B, B-cell areas of lymph nodes.

Results

Virus-pulsed mature DCs initiate a productive infection upon co-culture with T cells

Mature DCs were purified from cultures of blood monocytes that had been treated with granulocyte macrophage colony-stimulating factor (GM-CSF), IL-4 and lipopolysaccharide (LPS) to drive maturation into DCs. When incubated or pulsed with HIV-1 for 2 hours, the mature DCs did not directly replicate virus, although a strong infection took place when T cells were added, with activated T cells having a greater effect than unstimulated T cells (Figure 2). In this and other experiments, we removed adsorbed virus by trypsinizing the DCs and then culturing for 24–48 hours prior to washing and addition of T cells. When the infection was monitored at the level of production of HIV-1 DNA, the purified DCs did not accumulate complete reverse transcripts, that is, sequences comprising the long terminal repeat/gag region (LTR/gag; Figure 2a). LTR/gag sequences quickly appeared upon the addition of T cells, either unstimulated T cells from blood or T-cell blasts activated in a mixed leukocyte reaction (Figure 2a) or with phytohemagglutinin (PHA; data not shown). By day 4 of the DC–T-cell co-culture, the p24 Gag protein began to accumulate in the medium (Figure 2b). The T cells were permissive whether syngeneic or allogeneic (that is, matched or mismatched human leukocyte antigen (HLA)-DR3, respectively) to the infected DCs, and stimulated T cells (T blasts) produced higher levels of virus (Figure 2). Purified, monocyte-derived, mature DCs therefore behave comparably to DCs from other sources [5,17,19,20,28,29], that is, the cells

Figure 2



Mature, HIV-1-pulsed DCs transmit virus to syngeneic (syn) and allogeneic (allo) T cells. Purified DCs were pulsed for 2 h with wild-type (BaL) HIV-1, washed and cultured for 24 h. The DCs were washed again and co-cultured with unstimulated T cells or activated T cells (Tbl) at a ratio of 1:3. **(a)** The cells were collected 14 h and 48 h after co-culture, and the lysates analyzed by PCR for early and complete reverse transcripts using R/U5-specific and LTR/gag-specific primers respectively. Reverse transcripts were compared with standards corresponding to graded doses of amplified DNA from ACH-2 cells that harbor one copy of viral DNA per cell. **(b)** Supernatants were collected 4, 7 and 9 days after DC–T-cell co-culture, and p24 antigen levels measured by enzyme-linked immunosorbent assay (ELISA).

do not replicate virus directly but do so upon co-culture with T cells.

HIV-1 in purified, mature DCs represents a drug-resistant reservoir of infectious virus

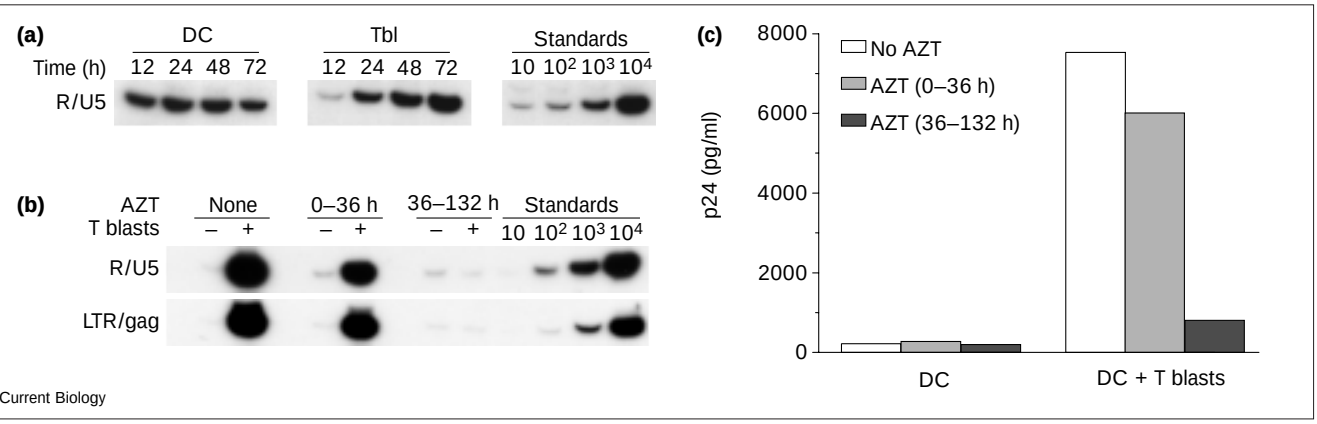
Previous work had shown that HIV-1 entered mature DCs as long as the appropriate chemokine receptors were expressed, but the life cycle of the virus was then blocked after the early stages of reverse transcription [2,23]. Virus replication resumed when the DCs were co-cultured with T cells. Following a 2 hour incubation of mature DCs with virus, reverse transcription began (as detected with R/U5 primers which detect the early stages of reverse transcription), but complete LTR/gag-containing sequences did not accumulate [23]. Before determining how infection was stimulated in co-cultures of mature DCs and T cells, we had to formally rule out that trace — but infectious — levels of reverse transcription were taking place in DCs. Therefore, we infected and cultured the DCs in the reverse transcriptase inhibitor azidothymidine (AZT) prior to mixing with T cells. AZT is more efficient in blocking late stages of reverse transcription than early stages, but the effect of AZT is reversible [30].

When DCs were pulsed with HIV-1, the early stages of reverse transcription were detected using primers for R/U5-containing sequences, and these sequences reproducibly persisted for 72 hours (Figure 3a). In contrast, HIV-1-infected T blasts initially had lower levels of R/U5 sequences than HIV-1-infected DCs, but the signal increased with time because the virus was replicating (Figure 3a). Next, DCs were pretreated with 1 μM AZT before and during the pulse with HIV-1, washed and then cultured for 36 hours with the drug. After 36 hours, AZT was washed out and uninfected T blasts were added to the infected DCs. Figure 3b shows that the DCs that had been treated for 36 hours with AZT (0–36 hours) were still able to transmit infection to T cells. This was documented both at the level of proviral DNA (Figure 3b) and by the release of p24 antigen into the medium (Figure 3c) in more than a dozen experiments. In contrast, if AZT was added to the DC–T-cell cultures at 36 hours and kept in the medium for the next 96 hours (36–132 hours), virus replication was blocked, as monitored by the appearance of LTR/gag-containing sequences (Figure 3b) and the accumulation of p24 (Figure 3c). We found that DCs can transmit infection to T cells even 3 days after exposure to virus (data not shown), although much less efficiently, presumably because the DCs were dying. These results indicate that DCs infected in the presence of AZT remained infectious once the AZT was removed. Therefore, we set up experiments to determine the site where virus replication begins when DCs are added to T cells, that is, whether HIV-1 replicates in the DCs or in the T cells.

Infection of DCs with pseudotyped viruses

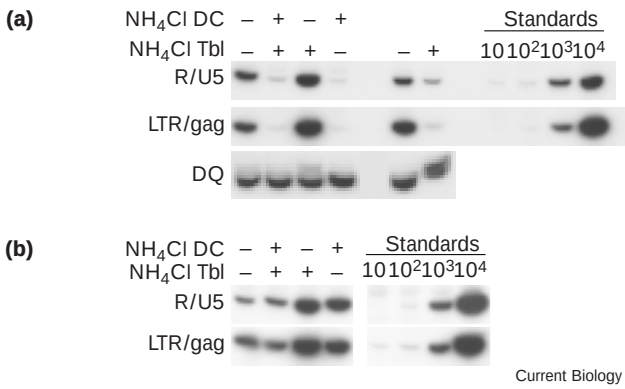
HIV-1 particles can be pseudotyped by envelope glycoproteins such as vesicular stomatitis virus glycoprotein (VSV-G) [31,32]. Pseudotyped viruses allow the study of early events in viral transmission, because the viruses are only capable of one cycle of replication due to their chimeric form. Using a VSV-G-pseudotyped virus, the

Figure 3



HIV-1 in virus-pulsed DCs is present in a drug-resistant form. (a) Longevity of R/U5 reverse transcripts in HIV-1-infected cells. Mature DCs and activated T cells were infected with HIV-1. At the indicated times, cells were lysed and DNA amplified by PCR for R/U5 sequences (5×10^4 cells were analyzed per lane). (b,c) AZT-pretreated mature DCs are competent to transfer HIV-1 to T cells. Mature DCs were incubated for 30 min with or without 1 μM AZT. Cells were infected with HIV-1 and maintained for 36 h in the presence or absence of AZT. Cultures were washed to remove AZT, and the DCs were cultured with or without non-infected T blasts. To block residual AZT, dNTPs (25 μM) were added to the cells. In one set of cultures, AZT was added at 36 h (36–132 h) during the addition of T cells. In (b), HIV-1 reverse transcripts were amplified after 72 h of co-culture, while in (c) productive infection was measured using ELISA for p24 antigen levels in the culture medium. Standards in (a,b) correspond to those used in Figure 2.

Figure 4



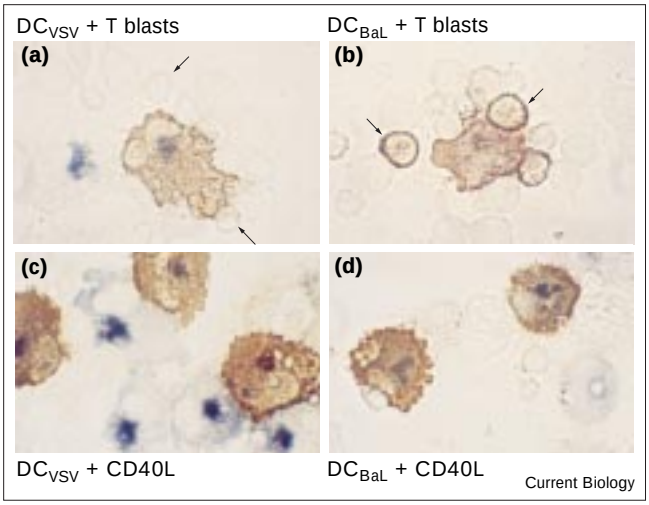
Infection of DCs with (a) VSV-pseudotyped HIV-1 and (b) wild-type (BaL) HIV-1. Mature DCs, sorted as $CD83^+$, were treated with 10 mM NH_4Cl for 1 h before and during a 2 h pulse with virus. The DCs were washed, cultured for 24 h with or without NH_4Cl, washed again, and T blasts that had been treated with or without NH_4Cl were added. After 72 h of co-culture, cell lysates were analyzed by PCR for detection of reverse transcripts. In (a), to determine that the samples we amplified for reverse transcripts contained the same amount of DNA, we also amplified HLA-DQ sequences as a control. Standards correspond to those used in Figure 2.

first cycle of HIV-1 replication could therefore be followed to determine whether it occurred in DCs or T cells.

VSV-G-pseudotyped virus enters cells through an endocytic, pH-sensitive pathway [33]. To test whether HIV-1–VSV required low pH levels for the infection of DCs, the cells were infected in 10 mM NH_4Cl, an inhibitor of endosomal acidification. $CD83^+$ DCs were pretreated for 1 hour with NH_4Cl and then pulsed with HIV-1–VSV for 2 hours in the continued presence of NH_4Cl. The DCs were washed and incubated for 24 hours before addition to T blasts, which had also been treated or untreated with NH_4Cl. After 72 hours, the cells were harvested and analyzed by PCR. NH_4Cl inhibited infection when it was applied with HIV-1–VSV to the DCs (Figure 4a, lane 4) but not when it was added to the DC–T-cell co-culture (Figure 4a, lane 3). This means that infection was taking place in the DCs, and not in the T cells. To rule out toxicity, we showed that NH_4Cl treatment did not significantly inhibit infection by an HIV-1 variant that was pseudotyped with the pH-insensitive JRFL M-tropic envelope (data not shown) or by wild-type (BaL) HIV-1 (Figure 4b).

To verify that the DCs were supporting viral protein synthesis upon co-culture with T cells, samples of the cultures were cytospun onto slides and immunolabeled for HIV-1 Gag with anti-p24 monoclonal antibody. The mature DCs, selected initially on the basis of expression of the CD83 marker, were double-labeled for a second marker, DC-LAMP, which is localized to the lysosomes of

Figure 5



Immunostaining of infected DC–T-cell co-cultures. Sorted $CD83^+$ cells were infected with either (a,c) HIV-1–VSV or (b,d) wild-type (BaL) HIV-1. After 24 h, T blasts were added and the DC–T-cell mixtures cultured for 4 days (a,b), or the DCs were cultured with CD40L-transfected fibroblasts for 4 days (c,d). Cytospins were stained for p24 expression (brown) and for the lysosomal marker that is expressed in mature DCs, DC-LAMP (blue). (a) With HIV-1–VSV, DCs became infected upon co-culture with T blasts but the T blasts (arrows) were not infected. (b) With wild-type HIV-1, DCs also become infected upon co-culture with T blasts, but the T cells are $p24^+$ (T blasts, arrows). (c,d) Likewise, CD40L-expressing cells activate HIV-1–VSV and wild-type HIV-1 infection of DCs.

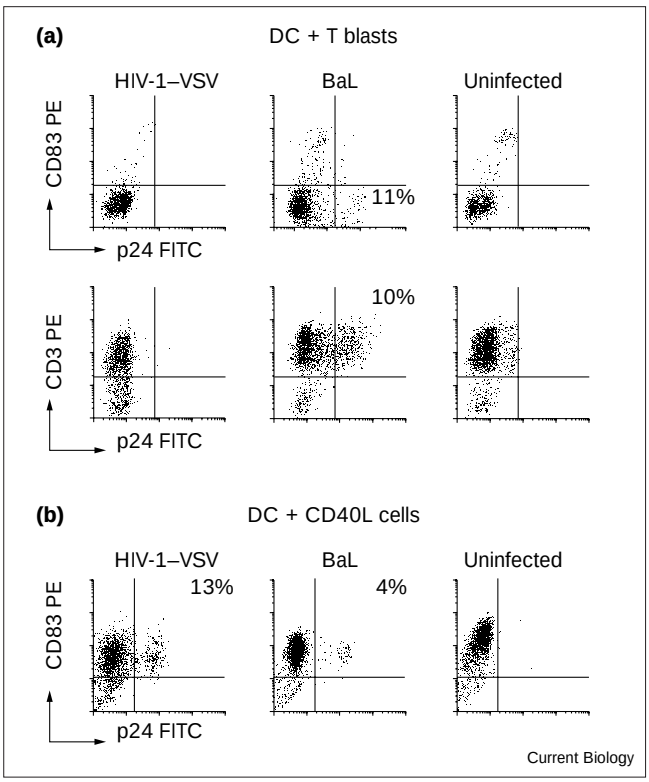
mature DCs [34]. All of the $p24^+$ cells were large, irregularly shaped and expressed DC-LAMP (Figure 5). In contrast, when DCs were infected with wild type HIV-1, so that cell–cell transmission of the infection could occur, round $p24^+$, DC-$LAMP^-$ T cells predominated (Figure 5b; note lack of blue DC-LAMP staining on the small, brown cells as marked by arrows).

These immunocytochemical observations were extended using fluorescence-activated cell sorting (FACS) approaches. When DCs were pulsed with wild-type virus and cultured with T cells for 4 days, many $p24^+$ cells were evident and the vast majority were $CD3^+CD83^-$, that is, T cells (Figure 6a). When VSV-G-pseudotyped virus was used to infect DCs, few infected cells were evident (Figure 6a). In these experiments, the T cells proliferated and vastly outnumbered the DCs. We conclude, therefore, that infection began in the DCs and, with wild-type virus, the infection continued primarily in T cells.

Ligation of CD40 on DCs triggers productive HIV-1 infection

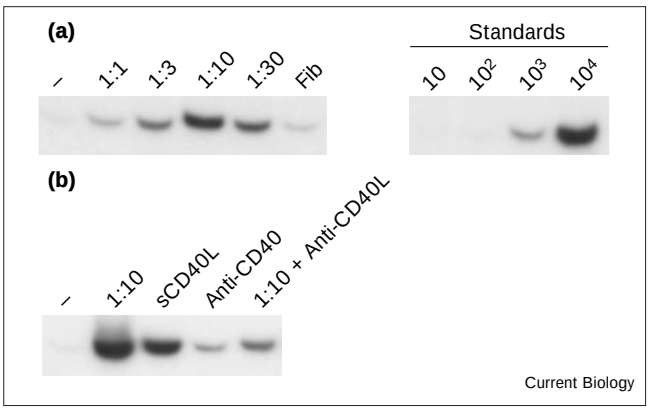
To identify a mechanism whereby T cells could be activating HIV-1 replication in DCs, we considered the role of CD40 ligand (CD40L), a tumor necrosis factor (TNF) family member that is expressed on activated T cells

Figure 6



FACS analysis of HIV-1-infected DCs cultured with T blasts or CD40L-expressing cells. Sorted CD83$^+$ cells were infected with wild-type (BaL) HIV-1 or HIV-1–VSV. (a) T blasts or (b) CD40L cells were added to the infected DCs after 24 h. After 4 days, the cells were collected, double-labeled with anti-p24 antibody conjugated to fluorescein isothiocyanate (FITC) and either anti-CD3 antibodies (for T cells) or anti-CD83 antibodies (for DCs) conjugated to phycoerythrin (PE).

Figure 7



Effect of CD40 ligation on infected DCs. (a) CD83$^+$ DCs were infected with wild-type (BaL) HIV-1 and then cultured at different ratios with irradiated (3000 rads) CD40L-transfected cells, as indicated (for example, 1:10 = 1 CD40L cell to 10 DCs) or with the control, irradiated non-transfected fibroblasts (fib; 1:10). Standards correspond to those used in Figure 2. (b) CD83$^+$ DCs were infected with pseudotyped virus and cultured as indicated: 1 irradiated CD40L cell to 10 DCs in the absence or presence of the anti-CD40L monoclonal antibody LL48 (10 μg/ml); soluble CD40L (sCD40L; 1:100 dilution of baculovirus supernatant); anti-CD40 monoclonal antibody 89 (anti-CD40; 10 μg/ml). After 72 h, cells were harvested and analyzed for the presence of LTR/gag sequences.

[35,36]. CD40L maintains the viability and function of mature DCs in culture [37,38]. We first tested whether fibroblasts transfected with CD40L could activate the first cycle of viral replication in HIV-1-pulsed, mature DCs. Again, we purified CD83$^+$ mature DCs by FACS prior to co-culture for 4 days with stably transfected, irradiated CD40L-expressing fibroblasts. Figure 5c,d shows that crosslinking with CD40L-expressing cells induced productive infection in mature, CD83$^+$ and DC-LAMP$^+$ DCs infected with pseudotyped and wild-type virus. Quantitation of the infected DCs after CD40 crosslinking was assessed by FACS (Figure 6b). The results of several experiments indicated that the percentage of DCs replicating HIV-1 was in the range of 1 to 15%. Figure 7 shows that complete reverse transcripts (detected using LTR/gag primers) were undetectable in the cultures containing DCs alone, but were increased in the DC–CD40L co-cultures or in DCs cultured with soluble CD40L (Figure 7b, lanes 2,3). A ratio of 1 CD40L-expressing fibroblast per 10 DCs was optimal (Figure 7a). Again, CD40L activated HIV-1 replication in purified DCs following infection with either wild-type (Figure 7a) or pseudotyped viruses (Figure 7b). The effect of CD40L was blocked by more than 50% by the addition of anti-CD40L antibody (Figure 7b, lane 5). Interestingly, a blocking anti-CD40 antibody could sustain the viability of the DCs but, unlike CD40L, this only modestly enhanced HIV-1 infection of DCs (Figure 7b, lane 4).

Discussion

Mechanism of imitation of HIV-1 replication in mature DCs following co-culture with T cells

It is important to study the interaction of HIV-1 with DCs, because these cells capture viruses and other antigens at body surfaces and initiate specific immunity in the draining lymphoid tissue (reviewed in [1]). Specific immunity includes responses in CD4$^+$ T cells, the major site for HIV-1 replication in patients. Initially, there were discordant findings on the capacity of DCs to support HIV-1 replication. Some reports concluded that DCs were explosive sites for infection, and others concluded that DCs were not a site for infection. Subsequently, it became apparent that HIV-1 does not replicate in mature DCs, which are unusually potent at stimulating T-cell-mediated immunity. DCs can support HIV-1 replication under two circumstances, however: either as highly enriched populations of immature DCs [2,4,5,39], that is, those DCs at the stage of development where they can capture and process antigens but are not yet endowed with full T-cell stimulatory activity; or as mature DCs

Figure 8

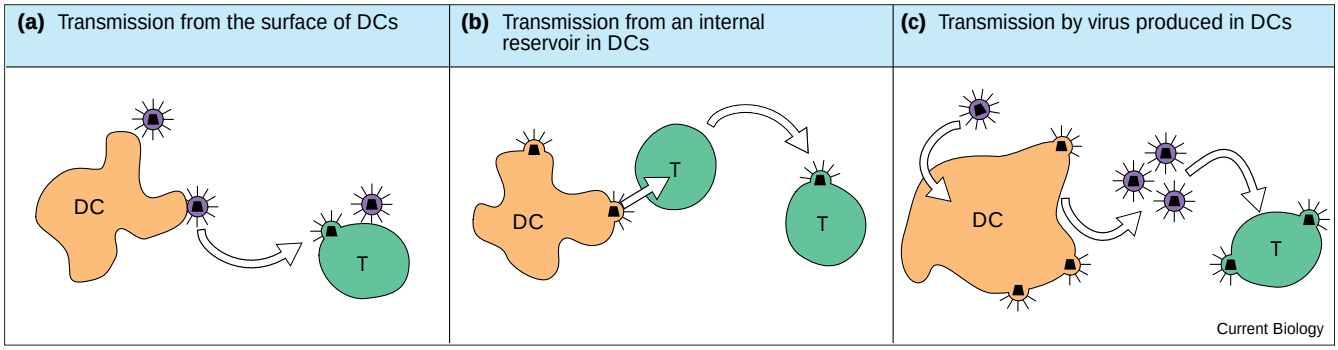


Diagram of possible DC mechanisms in transmitting HIV-1. (a) Passive transmission of viral particles trapped on the surface of DCs to T cells. (b) Fusion of viral particle at DC membrane via HIV-1 coreceptors and transmission to T cells upon DC–T-cell contact. (c) Replication of virus in DCs and transmission to T cells, which take over the infection in the culture because of higher levels of CD4 and CCR5 than mature DCs.

that are co-cultured with T cells [5,17–20,28,29,40,41]. In the latter instance, the bulk of the HIV-1 replication is taking place in $CD3^+$ T cells [17,18], or in DC–T-cell syncytia [19,20,42].

The roles played by DCs and T cells during infection of DC–T-cell mixtures has been unclear. It is important to decipher underlying mechanisms, because during pathogenesis (Figure 1 and see below), it is likely that HIV-1 is captured at body surfaces by immature DCs which then mature as they migrate to the lymphoid organs to engage and activate T cells. In other words, during acute infection with HIV-1, mature virus-exposed DCs should encounter T cells in lymphoid tissues. We have addressed the three main possibilities whereby DCs might initiate infection during T cell co-culture: the virus could simply be adsorbed to the DC surface and enter the T cell directly (Figure 8a); the virus could fuse with the DC but remain at the surface and be transmitted to the T cell upon DC–T-cell contact (Figure 8b); or the virus might undergo a single cycle of replication in DCs and then infect T cells (Figure 8c). Our findings reveal the latter to be the pathway that occurs in our tissue culture model, and we show that the block to viral replication in mature DCs can be overcome by co-culture with T cells and by signals delivered by CD40L. After the first cycle of infection in the DC, the T cell becomes the major site for replication.

Mature DCs were generated from blood monocytes and further purified by FACS on the basis of CD83 expression [27]. The $CD83^+$ cells had all the expected characteristics of mature DCs [25,26], expressing high levels of the human leukocyte antigen HLA-DR and of CD86, and having several features not found in immature DCs such as DC-LAMP expression and very potent stimulating activity for the mixed leukocyte reaction. With these monocyte-derived DCs, we confirmed the results of prior studies using mature DCs isolated directly from blood [5,17,18] and from skin [19,20], that is, the cells do not replicate HIV-1 directly but do initiate a strong infection upon co-culture with T cells. Prior to encounter with T cells, the infectious virus in the DCs is in an AZT-resistant state (Figure 3), confirming that the viral life cycle is blocked in DCs at a very early stage in reverse transcription [5].

To identify the cell type that initially replicated HIV-1 in DC–T-cell co-culture, we used pseudotyped virus. The pseudotyped virus was derived by cotransfecting 293T cells with separate plasmids encoding viral envelope proteins (from either JRFL or VSV in our studies) and the envelope-deficient HIV-1 NL4.3. These pseudotyped viruses could therefore undergo only a single cycle of virus replication. We found that the first cycle began in the DCs (Figure 8c) and not in the T cells (Figure 8a,b). NH_4Cl, which blocks the fusion of VSV-G-pseudotyped virus, only blocked infection of the DC and not the T cell (Figure 4). When the cells were examined directly, p24 was only being produced in DCs at the single-cell level (Figure 5). The DCs were mature on the basis of several markers, notably preselection for CD83 staining and expression of DC-LAMP.

A major pathway for T-cell-mediated activation of HIV-1 replication in DCs may involve a CD40L–CD40 interaction. CD40 is functionally active in DCs [37,43], whereas CD40L is expressed by activated T cells. When we tested the effects of CD40L-transfected fibroblasts or soluble CD40L, both could trigger the replication of wild-type and pseudotyped HIV-1 in mature DCs directly. Interestingly, anti-CD40 monoclonal antibodies had, at best, modest effects in triggering the first round of HIV-1 replication in DCs (Figure 7). We considered that CD40L could be the active molecule for the induction of HIV-1 replication in DCs by T cells. Addition of anti-CD40L monoclonal antibodies to DC–T-cell co-cultures, however, did not inhibit replication significantly. The explanation for this may be

that it is difficult to block the function of CD40L in cultures of growing T cells, or that additional molecules other than CD40L could be operating on T cells.

Possible *in vivo* relevance

The distribution of DCs *in vivo*, coupled with these findings and other studies [4,5,19,28,29,39] provide a model whereby DCs are critical for the transmission and early replication of HIV-1 in infected individuals. At body surfaces, such as the vagina, cervix and anus, immature DCs are found and can begin to replicate virus [5,29,44]. The virus that is produced is highly infectious for T cells [5] and macrophages [45].

In addition to picking up viruses and other antigens at body surfaces, DCs can undergo two other sets of changes: one is to leave the body surface and migrate to the lymph node via the lymphatic system; the other is to mature to become potent stimulators of immunity. These two sets of changes are coordinated, because the levels of CD80, CD86 and major histocompatibility (MHC) II molecules are increased when cells are stimulated to migrate from the skin or are examined in lymph. When immature DCs are infected and then a maturation stimulus added, HIV-1 replication ceases [5]. Likewise, when mature DCs are exposed to HIV-1, the virus enters but is blocked at an early stage of reverse transcription [23,46]. HIV-1 only replicates when DCs encounter T cells. We now show that the first cycle of replication occurs in the DCs, but that the bulk of the subsequent replication occurs in T cells, which also are a major site for viral replication during the acute stages of simian immunodeficiency virus (SIV) infection in macaque lymph nodes [47,48]. We would argue that the virus preferentially accesses activated T cells, because T cells have much higher levels of the HIV-1 coreceptors CD4 and CCR5 than mature DCs (at least a log higher fluorescence signal by FACS analysis [3]). Mature DCs are able to capture virus when they are the main cell type being exposed to the virus, as would be expected to occur at a body surface where there are relatively few T cells. The ominous feature is that once the mature DC encounters a T cell, the interaction between the two cell types — which is so efficient in generating T-cell-mediated immunity — sets up a permissive environment for the virus without further addition of experimental stimuli such as IL-2 and mitogens. The T cell drives the first cycle of replication in the mature DC, and the DC sustains the activation of T cells for subsequent rounds of replication.

Although we have discussed the exploitation of DCs during pathogenesis, our findings have another consequence. If single-cycle virus could be delivered to DCs, virus would not be transmitted to T cells but viral peptides should still be presented efficiently to $CD4^+$ and $CD8^+$ T cells. For example, influenza-infected DCs elicit very strong $CD4^+$ and $CD8^+$ T-cell responses [49]. Our data show that HIV-1 protein is being synthesized in DCs (Figure 5,6), so we would expect that these DCs would act as potent antigen-presenting cells for HIV-1-specific $CD4^+$ and $CD8^+$ cells. Both types of T cells have been implicated in resistance to HIV-1.

Conclusions

The DC system of antigen presentation constitutes a physiological mechanism for activating $CD4^+$ T-cell-mediated immunity. When mature immunostimulatory DCs are pulsed with HIV-1 in culture, virus replication only proceeds if T cells are added. Using pseudotyped viruses, we show that the first round of HIV-1 replication occurs within the DC. The T cells then serve as permissive hosts for the virus that is produced by the DCs. We propose that these events take place *in vivo* when HIV-1 is captured by DCs in blood or at body surfaces, and the virus is then transmitted by DCs to T cells in the T-cell-dependent regions of lymph nodes.

Materials and methods

Cells

DCs were generated from the blood of normal donors, usually from buffy coats obtained from the New York Blood Center [5,25,26]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by sedimentation in Ficoll-Hypaque, and 5×10^7 PBMC plated in 100 mm tissue culture plastic dishes in RPMI supplemented with 5% human serum. After 1 h, the floating cells were removed, and the adherent cells incubated for 6 days in GM-CSF (100 IU/ml, Leukine; Immunex) and IL-4 (1000 U/ml; Genzyme). The cells were fed on day 2 and day 4 with the same dose of cytokines. At day 6, most of the non-attached cells were immature DCs, being $CD14^-$, $CD4^+$, HLA-DR^+ and $CD86^+$, but lacked expression or only had weak expression of the CD83, p55, DC-LAMP and CD25 markers of mature, more stimulatory DCs. To promote maturation of the DCs, we replated the cells for 3 days in 6-well plates at 10^6 cells/well in 3 ml medium supplemented with cytokines as above and either lipopolysaccharide (LPS; 20 ng/ml) or monocyte-conditioned medium (MCM) [25,26]. Following maturation, the DCs expressed higher levels of surface HLA-DR and CD86 and now expressed CD83 [27] and the DC-LAMP antigen [34], as well as having strong antigen-presenting capacity in the mixed leukocyte reaction even at ratios of 1 DC to 1000 responder T cells. In many experiments, the purity of the mature DCs was enhanced by sorting $CD83^+$ cells on a FACStar^{PLUS}. T blasts were generated by stimulation of PBMC with PHA (1 μg/ml; Burroughs Wellcome) for 3 days, or in an allogeneic mixed leukocyte reaction for 5 days. CD40L-transfected fibroblasts, anti-CD40 (monoclonal antibody 89), and anti-CD40L (LL48) antibodies were kindly provided by J. Banchereau.

Viruses and infection of cells

We were kindly provided with a VSV-G expression construct pHCMV-G from J. Burns (University of California, San Diego), and the plasmid expressing JRFL env from N. Landau (Aaron Diamond AIDS Research Institute, New York). For production of wild-type HIV-1 and pseudotyped viruses, 293 T cells were cotransfected in 100 mm dishes with 20 μg each of envelope-minus proviral DNA (pNL4.3 env minus) and an envelope expression plasmid or with pNL4.3/BaL DNA using a calcium phosphate method. The cells were washed and incubated with 10 ml DMEM supplemented with 10% FCS 4 h after transfection. Virus stocks were prepared by collecting the culture supernatants after 48 h and filtering them through 0.45 μm Millipore filters. Virus stocks were stored in aliquots at –80°C. Viral titers (infectious units) were quantitated in HeLa MAGI cells as described by Vodicka *et al.* [50] and by

p24 ELISA. DCs were infected with pseudotyped viruses or BaL grown in mitogen-stimulated PBMC at an multiplicity of infection of 2–20 ng p24 antigen or 10^3–10^4 infectious units per 10^5 cells. To reduce HIV-1 DNA in the virus-containing supernatants, the latter were treated with RNAse-free DNA (50 U/ml at room temperature for 30 min). After 2 h at 37°C, non-absorbed virus was washed out and the cells incubated for the desired time in RPMI 5% human serum.

Detection of HIV-1 in infected cells

To analyze viral DNA, infected cells were collected at the indicated times, washed twice with PBS, and lysed in lysis buffer (10 mM Tris HCl pH 8, 1 mm EDTA, 0.001% Triton X100-sodium dodecyl sulphate, and proteinase K at 1 mg/ml). Samples were incubated at 60°C for 1 h and at 98°C for 10 min. Reverse transcripts were amplified by PCR, early transcripts with R/U5 primers (sense, 5′-GGTAACTAGGGAAC-CCACGT-3′; antisense; 5′-CTGCTAGAGTTTTCCCACTGAC-3′); and full-length transcripts with LTR/gag primers (sense 5′-GGCTAAC-TAGGGAACCCACGT-3′; antisense, 5′-CCTGCGTCGAGAGAGC-TCCTCTGG-3′). Amplified products were resolved on non-denaturing 8% polyacrylamide gels and visualized by autoradiography of the dried gels. At different times after infection, aliquots of culture supernatant were harvested and stored at –20°C. Samples were assayed for p24 antigen by ELISA (Coulter).

Immunohistochemistry was performed on 5×10^3 cells cytospun onto slides (Shandon). Slides were dried and fixed for 10 min in acetone at room temperature. For double labeling, the DC-LAMP antigen first was visualized with monoclonal antibody, kindly provided by S. Lebecque [34], followed by anti-mouse antibody conjugated with alkaline phosphatase (DAKO) and nitroblue tetrazolium (NBT) as substrate. Then p24 antigen was identified with rat anti-p24 monoclonal antibody (Biosource) and anti-rat Ig conjugated with horseradish peroxidase (Amersham) and diaminobenzamide as substrate. DC-LAMP expression is restricted to mature DCs, where it is confined to perinuclear lysosomes [34].

Infected cells were fixed with 4% paraformaldehyde in PBS for 30 min on ice. Cells were permeabilized for 30 min in 1% saponin on ice and stained with anti-p24 followed by a FITC-labeled secondary antibody (TAGO), and double-stained with anti-CD3 PE (Becton-Dickinson) or anti-CD83 PE (Coulter).

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