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Productive infection of human immunodeficiency virus type 1 in dendritic cells requires fusion-mediated viral entry

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Abstract

Human immunodeficiency virus type 1 (HIV-1) enters dendritic cells (DCs) through endocytosis and viral receptor-mediated fusion. Although endocytosis-mediated HIV-1 entry can generate productive infection in certain cell types, including human monocyte-derived macrophages, productive HIV-1 infection in DCs appears to be dependent on fusion-mediated viral entry. It remains to be defined whether endocytosed HIV-1 in DCs can initiate productive infection. Using HIV-1 infection and cellular fractionation assays to measure productive viral infection and entry, here we show that HIV-1 enters monocyte-derived DCs predominately through endocytosis; however, endocytosed HIV-1 cannot initiate productive HIV-1 infection in DCs. In contrast, productive HIV-1 infection in DCs requires fusion-mediated viral entry. Together, these results provide functional evidence in understanding HIV-1 *cis*-infection of DCs, suggesting that different pathways of HIV-1 entry into DCs determine the outcome of viral infection.

Keywords: HIV-1; Dendritic cells; Infection; Entry pathway; Fusion; Endocytosis

Introduction

Dendritic cells (DCs) are among the first target cells that encounter human immunodeficiency virus type 1 (HIV-1) at the mucosa. DC-mediated HIV-1 transmission to $CD4^+$ T cells contributes to the initial stages of viral infection (Piguet and Steinman, 2007; Wu and KewalRamani, 2006). DCs express relatively small amounts of HIV-1 receptor and coreceptors and support low levels of viral replication (Dong et al., 2007; Garcia et al., 2008; Granelli-Piperno et al., 1998; Pion et al., 2007, 2006). HIV-1 infection of DCs can lead to virus production and long-term viral transmission (Burleigh et al., 2006; Dong et al., 2007; Ganesh et al., 2004; Granelli-Piperno et al., 1998; Lore et al., 2005; Nobile et al., 2005; Smed-Sorensen et al., 2005; Turville et al., 2004), suggesting that HIV-1-infected DCs may be a viral reservoir *in vivo*.

To better understand HIV-1 *cis*-infection of DCs, it is important to define viral entry pathway that leads to productive infection in DCs. Our recent study suggests that HIV-1 enters DCs primarily through endocytosis rather than fusion between HIV-1 envelope (Env) and viral receptors at the plasma membrane (Dong et al., 2007). Although endocytosis-mediated HIV-1 entry can generate productive infection in certain cell types, including human monocyte-derived macrophages (Daecke et al., 2005; Fackler and Peterlin, 2000; Maréchal et al., 2001), productive HIV-1 infection in DCs appears to be dependent on fusion-mediated viral entry (Burleigh et al., 2006; Cavrois et al., 2006; Dong et al., 2007; Nobile et al., 2003; Nobile et al., 2005; Pion et al., 2007). DCs can efficiently capture and take up HIV-1 through multiple attachment factors (Gummuluru et al., 2003; Turville et al., 2001, 2002); however, it is unclear whether endocytosed HIV-1 can initiate productive infection in DCs.

Immunofluorescence and electron microscopy studies indicated that internalized HIV-1 particles are sorted and concentrated into a low-pH, non-lysosomal compartment in DCs

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(Garcia et al., 2008, 2005; Izquierdo-Useros et al., 2007; Kwon et al., 2002; Trumpfheller et al., 2003; Turville et al., 2004; Wang et al., 2007b; Wiley and Gummuluru, 2006). The intracellular vesicle that confines internalized HIV-1 in DCs has been characterized as a non-conventional endocytic compartment enriched in tetraspanins (Garcia, et al., 2008, 2005) or a multivesicular endosomal body-like compartment (Wiley and Gummuluru, 2006). Rapid HIV-1 degradation in DCs (Moris et al., 2004; Nobile et al., 2005; Turville et al., 2004; Wang et al., 2007b) suggests that the majority of HIV-1 trapped in the intracellular compartments could be eventually degraded. However, it remains to be confirmed whether endocytosed HIV-1 in DCs represents a dead end for viral infection.

Previous studies using a cellular fractionation assay indicated that the level of HIV-1 Gag p24 in the cytosolic fraction is correlated with productive viral infection in HIV-1 permissive cell lines and monocyte-derived macrophages (Maréchal et al., 1998, 2001). Therefore, the detection of cytosolic p24 shortly after exposure to HIV-1 is used as an index for productive viral infection (Maréchal et al., 1998, 2001), which appears to be a useful assay to study the correlation between the HIV-1 entry pathway and productive infection.

In this study, by using HIV-1 infection and cellular fractionation assays to measure productive viral infection and entry, we demonstrate that HIV-1 enters monocyte-derived DCs predominately through endocytosis; however, endocytosed HIV-1 cannot initiate productive HIV-1 infection in DCs. In contrast, productive HIV-1 infection in DCs requires the fusion-mediated viral entry. These results provide functional evidence in understanding HIV-1 *cis*-infection of DCs, suggesting that different pathways of HIV-1 entry into DCs determine the outcome of viral infection.

Results

HIV-1 infection in DCs is blocked by a reverse transcriptase inhibitor

To confirm productive HIV-1 infection in DCs, a reverse transcriptase inhibitor, 3′-azido-3′-dideoxythymidine (AZT), was used to block HIV-1 infection in DCs. R5-tropic HIV-1 was used in this study given that the infection of X4-tropic HIV-1 in DCs is significantly lower than that of R5-tropic HIV-1 (Ganesh et al., 2004; Granelli-Piperno et al., 1998; Nobile et al., 2005; Pion et al., 2007; Wang et al., 2007a). To examine the expression of the HIV-1 receptor CD4 and coreceptor CCR5, immature monocyte-derived DCs were stained with the specific monoclonal antibodies (MAbs) and analyzed by flow cytometry. Modest CD4 (86% positive populations) and low levels of CCR5 (12% positive populations) were detected on DC surfaces (Fig. 1A). DCs were infected with single-cycle, luciferase-reporter HIV-1 pseudotyped with R5-tropic Env of HIV-1_{JRFL} (HIV-JRFL) (Wang et al., 2007a). The HIV-1 proviral genome has *env* deleted and *nef* inactivated with a luciferase-reporter insertion, but contains all other viral genes (Yamashita and Emerman, 2004). Viral infection was determined 3, 5 and 7 day post-infection (dpi) by measuring the luciferase activity of DC lysates. HIV-JRFL infection in DCs was abolished in the presence of AZT (Fig. 1B). Similarly, AZT inhibited the viral replication of R5-tropic replication-competent HIV-1_{NLAD8} in DCs as viral replication was measured by supernatant p24 of infected DCs (Fig. 1C). No significant cytotoxicity of AZT was observed at the tested concentration. These data confirm that the measurement of the luciferase activity in cell lysates and p24 levels in the supernatants of the infected DCs represents *de novo* viral protein synthesis resulting from the infections.

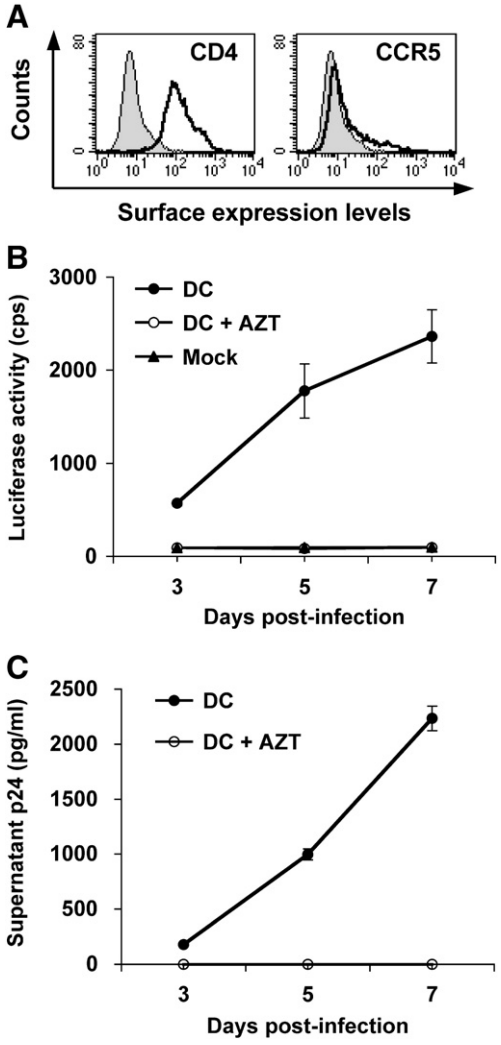

Fig. 1. HIV-1 infection in immature DCs is blocked by AZT. (A) Surface expression of CD4 and CCR5 on DCs. Monocyte-derived immature DCs were stained with specific MAbs and analyzed by flow cytometry. DCs were stained with either a murine IgG isotypic control antibody (filled peaks) or CD4 MAb (thick line, left panel) or CCR5 MAb (thick line, right panel). (B) DCs were infected with R5-tropic HIV-1 Env (JRFL)-pseudotyped, single-cycle luciferase-reporter HIV-1 (MOI, 0.8) in the presence or absence of AZT. DCs were lysed 3, 5, and 7 dpi to determine viral infection by measuring the luciferase activity of cell lysates. Mock infection was used for a background control for the detection. cps, counts per second. (C) DCs were infected with HIV-1_{NLAD8} in the presence or absence of AZT. HIV-1 infection was determined at 3, 5 and 7 dpi by measuring the p24 levels in supernatants. All data show the means±standard deviations (S.D.) of triplicate samples. Data for one representative experiment out of three are shown.

Comparison of HIV-1 infection with different HIV-1 pseudotypes in DCs and CD4$^+$ T cells

To compare the relative efficiency of viral infection between different HIV-1 psuedotypes, single-cycle, luciferase HIV-1 pseudotyped with vesicular stomatitis virus G protein (HIV-VSV-G) and HIV-JRFL were used to infect DCs and CD4$^+$ Hut/CCR5 T cells. Hut/CCR5 cells express high levels of the HIV-1 receptor CD4 and coreceptors CCR5 (Wang et al., 2007a). HIV-VSV-G enters cells through an endocytic pathway and mediates viral fusion in a low-pH endosome after endocytosis (Aiken, 1997). HIV-1 particles devoid of Env (HIVΔEnv) were used as a negative control in the infection assay.

When the same p24 amounts of HIV-1 were used in the infection assay, HIV-VSV-G infection of DCs was 7-fold more efficient relative to HIV-JRFL infection at 4 dpi, while HIV-JRFL infection of DCs was only 5% higher when compared with the negative control HIVΔEnv (Fig. 2A). In contrast, HIV-VSV-G infection of Hut/CCR5 cells was 51-fold more efficient relative to HIV-JRFL infection, whereas HIV-JRFL infection of Hut/CCR5 cells was 10-fold higher relative to the negative control HIVΔEnv (Fig. 2A). Moreover, HIV-JRFL and HIV-VSV-G infection of Hut/CCR5 cells was 8-fold and 56-fold higher than that of DCs, respectively (Fig. 2A).

To confirm the results of HIV-1 infection using luciferase-reporter HIV-1 pseudotypes, single-cell-based HIV-1 infection was performed using a green fluorescent protein-expressing HIV-1 vector (HIV-GFP). The HIV-1-derived vector was generated from a pNL4-3-based HIV-1 provirus that has deletions in the *env*, *vif*, *vpr*, *vpu*, and *nef* genes; the *GFP* gene was inserted in place of the *nef* open reading frame (Unutmaz et al., 1999). DCs and Hut/CCR5 cells were separately infected with HIV-1$_{JRFL}$ (HIV-GFP/JRFL) or VSV-G (HIV-GFP/VSVG).

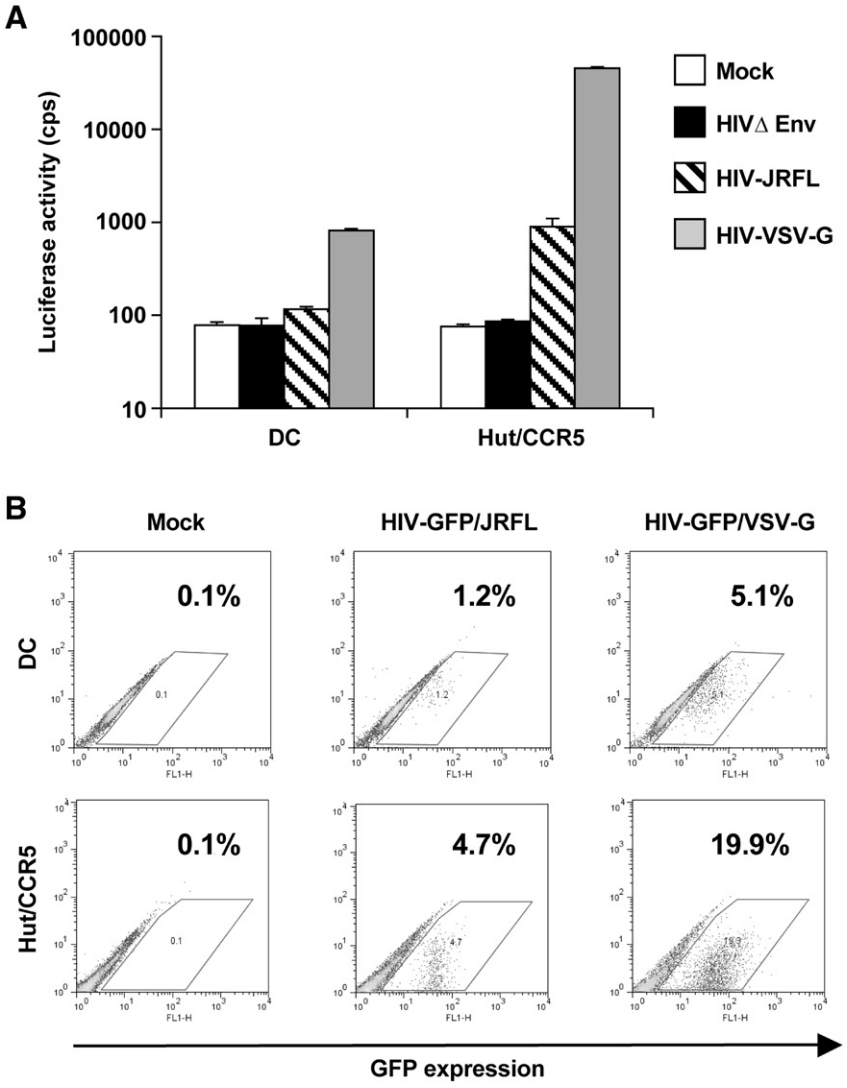

Fig. 2. Comparison of HIV-1 infection in DCs and CD4$^+$ T cells with different HIV-1 pseudotypes. (A) Direct infection of DCs or Hut/CCR5 cells with HIV-JRFL and HIV-VSV-G; the same amount of HIVΔEnv was used as a negative control. DCs were pulsed with different HIV-1 pseudotypes separately for 2 h at 37 °C, washed and cultured 4 dpi before measuring the luciferase activity of cell lysates. Mock infection was used for a background control for the detection. cps, counts per second. All data show the means ± S.D. of triplicate samples. (B) Direct infection of DCs or Hut/CCR5 cells with HIV-GFP/JRFL or HIV-GFP/VSV-G. Infected cells were analyzed by flow cytometry for GFP expression 4 dpi. Percentages of GFP-positive cells are shown in the plots. Data for one representative experiment out of three are shown.

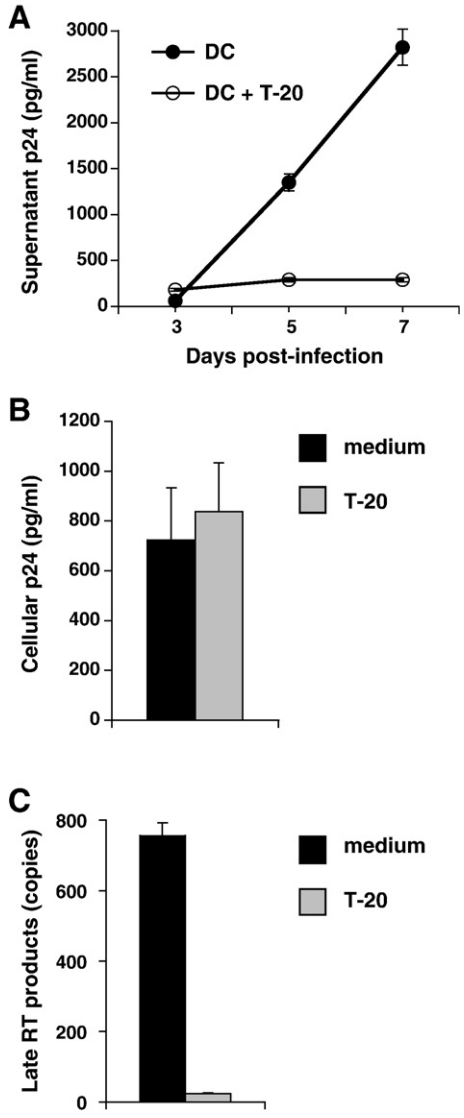

Fig. 3. Productive HIV-1 infection in DCs requires fusion-mediated viral entry. (A) Blockade of fusion-mediated HIV-1 entry into DCs diminishes viral replication. DCs were infected with HIV-1_{NLAD8} for 2 h at 37 °C in the presence or absence of T-20. Supernatants of infected DCs were measured for p24 levels at 3, 5 and 7 dpi. (B) T-20 does not impair HIV-1_{NLAD8} endocytosis into DCs. DC-associated p24 was measured after incubation with HIV-1_{NLAD8} for 2 h at 37 °C in the presence or absence of T-20. HIV-1-pulsed DCs were extensively washed, trypsinized and lysed for p24 detection. (C) Endocytosed HIV-1 in DCs does not generate significant amounts of late reverse transcription (RT) products. DCs were infected with HIV-1_{NLAD8}, washed, trypsinized, and cultured for 12 h before the cells were lysed for real-time PCR detection (40 ng of cellular DNA per sample was used). T-20 was present during the viral incubation and the 12-h culture. All data show the means±S.D. of triplicate samples. Data for one representative experiment out of four are shown.

The infected cells were analyzed for GFP expression by flow cytometry at 4 dpi. Infection of DCs and Hut/CCR5 cells with HIV-GFP/VSV-G was approximately 4-fold higher than that with HIV-GFP/JRFL (Fig. 2B). Infection of Hut/CCR5 cells with HIV-GFP/JRFL or HIV-GFP/VSV-G was also 4-fold higher than that of DCs (Fig. 2B). The results of HIV-GFP vector infection in DCs are in accordance with those of luciferase-reporter HIV-1, and those in a previous study using the same HIV-GFP vector (Pion et al., 2006).

Productive HIV-1 infection in DCs requires fusion-mediated viral entry

To investigate whether productive HIV-1 infection in DCs requires fusion-mediated viral entry, DCs were infected with HIV-1_{NLAD8} in the presence of HIV-1 fusion inhibitor T-20, a small synthetic peptide inhibiting fusion-mediated HIV-1 entry (Wild et al., 1993). The T-20 treatment efficiently blocked HIV-1_{NLAD8} infection in DCs to background levels (Fig. 3A), suggesting that fusion-mediated viral entry is required for productive HIV-1 replication in DCs. No significant cytotoxicity of T-20 was observed at the tested concentration.

To examine whether T-20 blocked HIV-1 entry into DCs, DCs were pulsed with HIV-1_{NLAD8} in the presence or absence of T-20, and trypsinized after extensive washes to strip cell-surface bound HIV-1. The p24 levels in the lysates of DCs were then quantified. Blockade of viral fusion with T-20 did not result in a reduction of total viral entry into DCs (Fig. 3B). Furthermore, to determine whether endocytosed HIV-1_{NLAD8} in DCs can complete the reverse transcription process, DCs were pulsed with HIV-1_{NLAD8} and cultured for 12 h before detection of late reverse transcription products by real-time PCR. In the presence of T-20, HIV-1 late reverse transcription products were

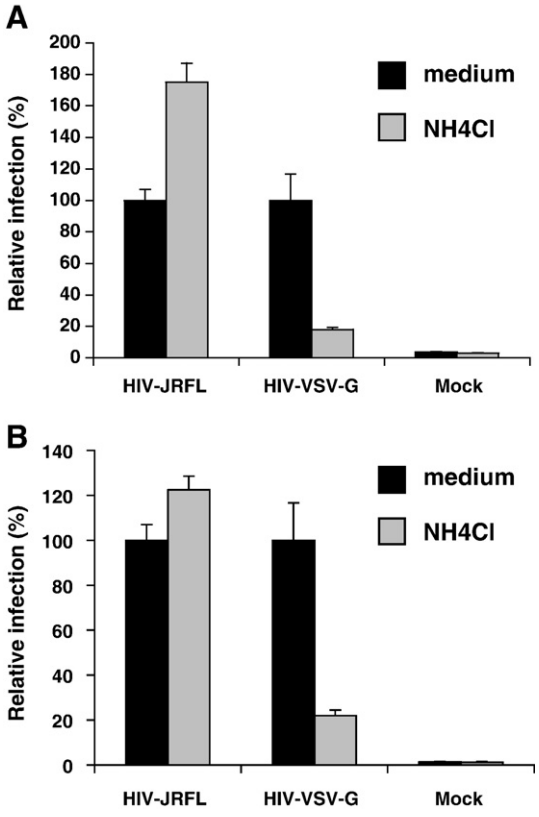

Fig. 4. HIV-1 infection of DCs is pH-independent. HIV-JRFL and HIV-VSV-G infection of (A) DCs and (B) $CD4^+$ Hut/CCR5 T cell line. DCs or Hut/CCR5 cells were incubated with NH_4Cl at 37 °C for 30 min and then pulsed separately with HIV-VSV-G and HIV-JRFL (MOI, 0.5) at 37 °C for 2 h in the presence of NH_4Cl. NH_4Cl was washed away after the 2.5-h incubation with DCs. HIV-1 infection was detected 3 dpi by measuring the luciferase activity of cell lysates and relative infection is shown. Values for medium controls were set at 100%. Data for one representative experiment out of three are shown.

efficiently blocked to basal level (24 copies in 40 ng cellular DNA, the detection limit was 10 copies) (Fig. 3C), indicating that fusion-mediated viral entry is necessary for productive synthesis of viral DNA. These results are in agreement with those in a previous report (Nobile et al., 2005). Together, these results suggest that HIV-1 enters DCs primarily through endocytosis; however, fusion-mediated viral entry is required for productive infection. These results also suggest that endocytosis-mediated HIV-1 entry into DCs cannot lead to productive viral infection.

HIV-1 infection of DCs is pH-independent

To block HIV-VSV-G infection, DCs were infected with HIV-VSV-G in the presence of the weak base ammonium chloride (NH_4Cl), which blocks acidification of endosomal compartments. NH_4Cl was washed away after the 2.5 h incubation with DCs; no significant cytotoxic effects on DCs were observed after 3 days in culture, as we recently reported (Wang et al., 2007b). Compared with the medium control, the NH_4Cl treatment significantly decreased HIV-VSV-G infection in DCs by 82% (Fig. 4A, $P<0.01$, Wilcoxon's paired t test). In contrast, HIV-JRFL infection in DCs was increased by 1.7-fold with the NH_4Cl treatment (Fig. 4A). Similar results were obtained using Hut/CCR5 T cells in the infection (Fig. 4B). These data confirm that HIV-VSV-G infection of DCs is low pH-dependent, while R5-tropic HIV-JRFL infection of DCs is pH-independent.

Analysis of HIV-1 entry into DCs by cellular fractionation

To better understand HIV-1 entry into DCs, HIV-pulsed DCs were analyzed by cellular fractionation as previously described (Maréchal et al., 2001). DCs were separately exposed to the same p24 amounts of HIV-JRFL and HIV-VSV-G. HIVΔEnv was used as a background control to assess non-specific viral uptake. After extensive washes, DCs were treated with pronase to remove viral particles adsorbed on the cell surfaces, and then the plasma membranes of DCs were permeabilized with digitonin, a mild nonionic detergent. DCs were then fractionated by centrifugation, and intracellular p24 levels in the cytosolic and vesicular fractions were quantified.

The average results of four independent experiments of the cellular fractionation indicate that the total intracellular p24 in HIV-VSV-G-exposed DCs was 2.4-fold ($P<0.05$) and 1.4-fold

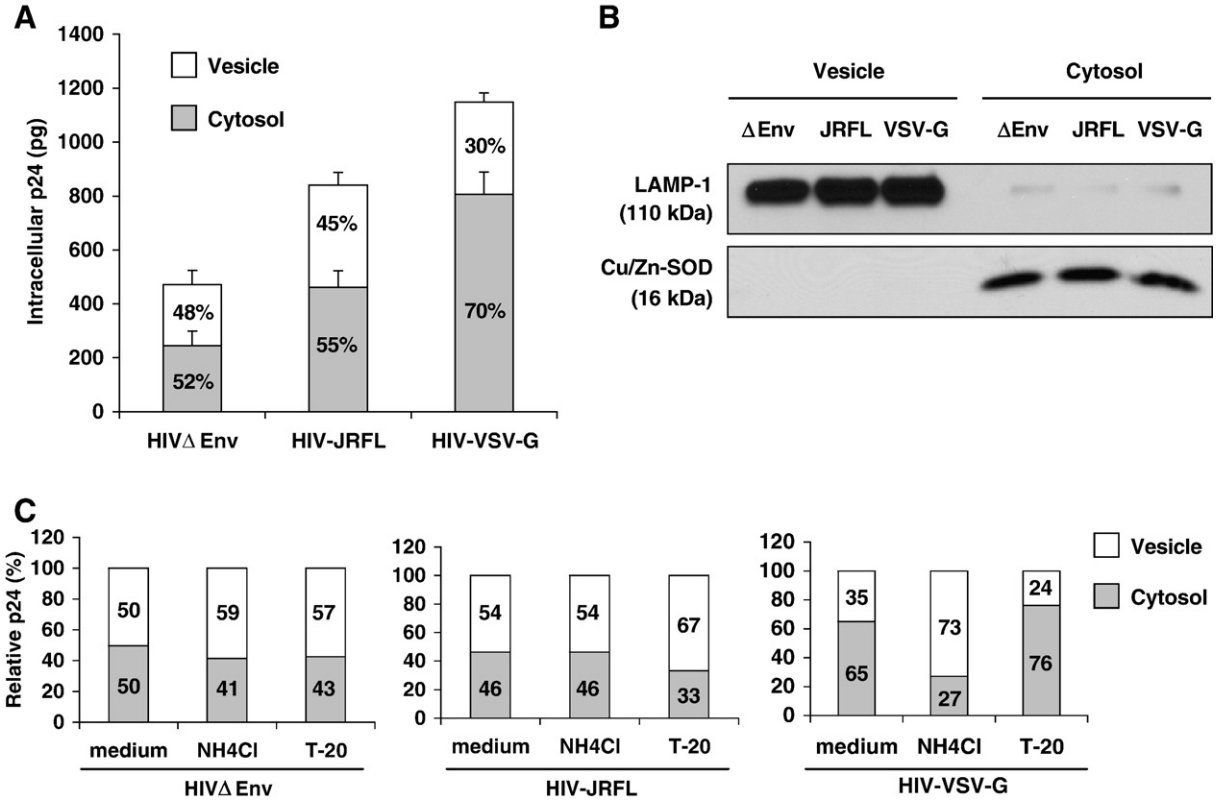

Fig. 5. Analysis of HIV-1 entry into DCs by cellular fractionation. (A) Cellular fractionation of HIV-pulsed DCs. DCs were separately exposed to HIVΔEnv, HIV-JRFL, and HIV-VSV at 37 °C for 2 h. After extensive washes, DCs were treated with pronase to remove HIV-1 bound on cell surface. DCs were then washed, permeabilized, and fractionated. Intracellular p24 levels were measured in the cytosolic and vesicular fractions. The percentages of cytosolic and vesicular p24 are shown inside the bars. The average results of four independent experiments using DCs from different donors are shown. (B) Western blot analysis of the cytosolic and vesicular fractionations of HIV-infected DCs. HIV-pulsed DCs were fractionated as described above in (A) and analyzed by Western blotting with antibodies specific for Cu/Zn-SOD (cytosol marker) and LAMP-1 (vesicle marker). Data for one representative experiment out of three are shown. (C) Relative p24 of the cytosolic and vesicular fractionations of DCs treated with fusion inhibitors. DCs were pretreated separately with $NHCl_4$ and T-20, pulsed with different HIV-1 pseudotypes in the presence of the appropriate inhibitors. DCs were then washed and fractionated, and the p24 levels were measured as described above in (A). The relative percentages of cytosolic and vesicular p24 are shown inside the bars. Data for one representative experiment out of three are shown.

higher than that in HIVΔEnv- and HIV-JRFL-exposed DCs, respectively (Fig. 5A). These data indicate that DCs take up HIV-VSV-G more efficiently than HIVΔEnv and HIV-JRFL. The total p24 of HIV-JRFL-exposed DCs was 1.8-fold higher than that of HIVΔEnv-exposed DCs. Interestingly, 70% of the total p24 was detected in the cytosolic fraction of HIV-VSV-G-exposed DCs, which is significantly higher ($P<0.001$, Dunnett's multiple comparison test) than 52% and 55% of p24 cytosolic fractions in HIVΔEnv- and HIV-JRFL-exposed DCs, respectively (Fig. 5A). These results suggest that HIV-VSV-G has a more productive entry than HIV-JRFL due to efficient viral fusion within endosomal compartments.

Notably, the cytosolic p24 fraction detected in HIV-JRFL-exposed DCs was only 3% higher than that in HIVΔEnv-exposed DCs (Fig. 5A), indicating that HIV-JRFL enters DCs mainly through endocytosis despite appropriate interactions between viral receptors and Env. The cytosolic p24 fraction in HIV-JRFL-exposed DCs appears to correlate well with the HIV-1 infection results, in which HIV-JRFL infection of DCs was only 5% higher than that of control HIVΔEnv (Fig. 2A). Together, these data suggest that the cytosolic p24 fraction can be used as an indicator for productive infection. These results also imply that, unlike HIV-VSV-G, endocytosed HIV-JRFL in DCs likely failed to escape from intracellular compartments and to initiate productive infection.

To verify the specificity of the fractionation assay, the cellular fractions were analyzed in parallel by Western blotting with the antibodies specific for the markers of cytosolic and vesicular fractions. Copper/zinc superoxide dismutase (Cu/Zn-SOD) was used as a specific marker for the cytosolic fractions (Crapo et al., 1992), while lysosomal-associated membrane protein 1 (LAMP-1) was used as a maker for the vesicular fractions (Chen et al., 1988; Maréchal et al., 1998). As expected, Cu/Zn-SOD was only detected in the cytosolic fractions, but not in the vesicular fractions, whereas LAMP-1 was highly enriched in the vesicular fractions (Fig. 5B). The trace amounts of LAMP-1 observed in the cytosolic fractions (Fig. 5B) might result from minimal contamination. Thus, these Western blotting results validate the cellular specificity of the fractionation assay.

To investigate whether the presence of fusion inhibitors can alter the cytosolic p24 fractions of HIV-1 pseudotyped with different envelope proteins, T-20 and NH_4Cl were separately used to inhibit the fusion of HIV-JRFL and HIV-VSV-G to DCs in the fractionation assays. DCs were pretreated separately with $NHCl_4$ and T-20, pulsed with different HIV-1 pseudotypes in the presence of the appropriate inhibitors. DCs were then fractionated, and the p24 levels in the cytosolic and vesicular fractions were measured. For HIV-JRFL-infected DCs, the T-20 treatment reduced the cytosolic p24 fraction from 46% to 33% (relatively, 28% reduction), whereas the NH_4Cl treatment did not affect the p24 fractions (Fig. 5C, central panel). For HIV-VSV-G-infected DCs, the NH_4Cl treatment efficiently reduced the cytosolic p24 fraction from 65% to 27% (relatively, 58% reduction) and reversed the relative p24 in the vesicle and cytosol fractions compared with the medium control, while the T-20 treatment did not strongly alter the cytosolic p24 fraction (Fig. 5C, right panel). For the HIVΔEnv controls, the NH_4Cl and T-20 treatments slightly decreased the cytosolic p24 fractions by 9% and 7%, respectively, which were likely due to experimental variations (Fig. 5C, left panel). Together, these results suggest that specific fusion inhibitors can alter the cytosolic p24 fractions in DCs infected by different HIV-1 pseudotypes.

Discussion

DCs can efficiently take up HIV-1 and release infectious viruses to cocultured $CD4^+$ T cells through viral trafficking (Dong et al., 2007; Ganesh et al., 2004; Garcia et al., 2005; Gummuluru et al., 2003; Kwon et al., 2002; McDonald et al., 2003; Turville et al., 2004; Wang et al., 2007b; Wiley and Gummuluru, 2006). In addition to DC-mediated HIV-1 *trans*-infection, HIV-1-infected DCs can act as infectious viral reservoirs and mediate long-term viral dissemination (Piguet and Steinman, 2007; Wu and KewalRamani, 2006). Defining HIV-1 entry pathways that lead to productive viral infection in DCs can enhance our understanding of HIV-DC interactions. In this study, using viral infection and cellular fractionation assays, we characterized HIV-1 entry and infection of DCs with replication-competent and single-cycle HIV-1. Our results suggest that HIV-1 enters monocyte-derived DCs predominately through endocytosis; however, endocytosis-mediated HIV-1 entry into DCs is not able to generate productive viral infection. In contrast, productive HIV-1 infection in DCs depends on the fusion-mediated viral entry pathway (depicted as a proposed model in Fig. 6).

Low levels of CD4 and CCR5 expression in DCs (Fig. 1A) could contribute to inefficient fusion between JRFL-pseudotyped HIV-1 and DCs at the plasma membrane, resulting in less productive viral infection in DCs (Fig. 2). Postentry restriction of HIV-1 infection in DCs also plays a role in a less productive viral infection (Peng et al., 2007; Pion et al., 2006). Our results of DC infection with VSV-G-pseudotyped HIV-1 vectors also suggest potential postentry restriction of HIV-1 infection in DCs. For instance, HIV-VSV-G infection of DCs was only 7-fold more efficient compared with HIV-JRFL infection (Fig. 2A). In contrast, the relative infectivity of the same stock of HIV-VSV-G (2193 infectious units/ng p24) was 26-fold higher than that of HIV-JRFL (85 infectious units/ng p24) in a flow cytometry-based titration assay. The viral titration was performed using GHOST/R5 cells, which are human osteosarcoma cells expressing high levels of CD4 and CCR5. These cells contain a GFP gene under the control of the HIV-2 LTR promoter, which is expressed during HIV-1 infection via Tat transactivation, acting as a sensitive indicator of HIV-1 infection (Cecilia et al., 1998).

Although T-20 efficiently blocked HIV-1 infection in DCs (Fig. 3A), HIV-1 endocytosis in DCs could not be impaired by T-20 (Fig. 3B). A previous study reported that T-20 efficiently inhibits HIV-1 *cis*- and *trans*-infection of monocyte-derived DCs (Ketas et al., 2003). However, T-20 was present in the DC-T-cell cocultures in that study, which could block HIV-1 infection in cocultured T cells rather than the viral transmission process. HIV-1 endocytosed in DCs can be rapidly and efficiently transmitted to cocultured $CD4^+$ T cells (Dong et al.,

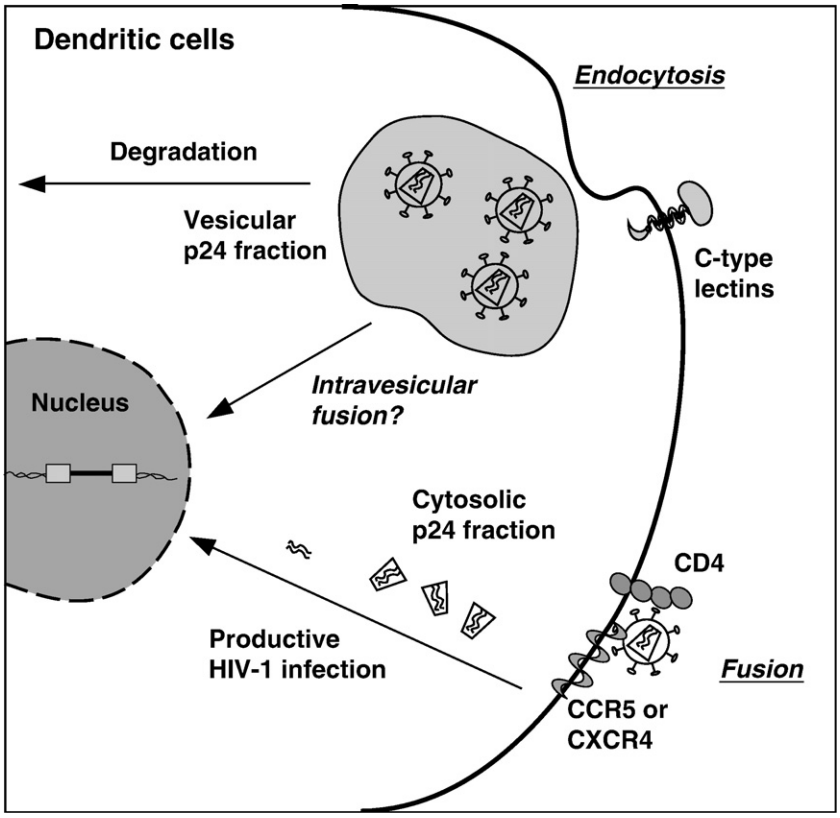

Fig. 6. A proposed model for HIV-1 entry and productive infection in DCs. HIV-1 enters DCs through the endocytosis and plasma membrane fusion pathways. The endocytic pathway appears to be the predominate route of HIV-1 entry into DCs; however, only fusion-mediated HIV-1 entry can lead to productive HIV-1 infection. In the cellular fractionation assay, the vesicular p24 fraction represents the HIV-1 confined in the endocytic compartments; whereas the cytosolic p24 fraction represents the HIV-1 entered through the fusion pathway. Endocytosed HIV-1 could be eventually degraded. However, it is currently uncertain whether a small proportion of internalized authentic HIV-1 in DCs can escape the vesicular compartment via potential intravesicular fusion.

2007; Garcia et al., 2005; McDonald et al., 2003; Turville et al., 2004; Wang et al., 2007a). The inefficient block of HIV-1 uptake in DCs with T-20 implies that endocytosed HIV-1 in DCs may recycle to the cell surface and mediate *trans*-infection at the infectious/virological synapse (Arrighi et al., 2004; Garcia et al., 2008, 2005; McDonald et al., 2003; Turville et al., 2004).

The block of HIV-1 infection in DCs by T-20 indicated the requirement for Env-mediated viral fusion; however, this result could not address the question where the fusion occurs. T-20 presented during the HIV-1 incubation with DCs could be internalized together with HIV-1 into the endocytic compartment of DCs. Although the acidic environment of the vesicular compartment might inactivate T-20, it is possible that T-20 could block HIV-1 fusion within the low-pH vesicles. However, it is currently uncertain whether a small proportion of internalized authentic HIV-1 in DCs can escape the vesicular compartment via intracellular fusion (Fig. 6), which is the fusion mechanism of VSV-G-pseudotyped HIV-1 (Aiken, 1997). In rare cases, intravesicular fusion between HIV-1 and the vesicular membrane was observed in monocyte-derived macrophages by electron microscopy (Maréchal et al., 2001), suggesting that a small part of intravesicular HIV-1 may escape the endocytic pathway and enter the cytoplasm. However, we were not able to observe such intravesicular viral fusion in our electron microscopy studies of HIV-1 interactions with DCs [(Wang et al., 2007b) and Wang and Wu, unpublished results].

A virion-based HIV-1 fusion assay has been used to measure the fusion of HIV-1 virions to DCs (Cavrois et al., 2006; Pion et al., 2007). Low levels of viral fusion (2–6% of DC populations) were detected by flow cytometry with the fusion assay using a high viral input (300 to 500 ng of p24 per 2×10^5 DCs) (Cavrois et al., 2006), suggesting inefficient HIV-1 fusion to DCs. The assay is based on the delivery of beta-lactamase (BlaM)-Vpr-incorporated HIV-1 into the cytoplasm of target cells upon virion fusion (Cavrois et al., 2002). BlaM can cleave a fluorescent substrate CCF2 dye loaded in the target cells and change its fluorescence emission, thereby allowing viral fusion to be detected. Although the virion-based fusion assay detects entry of HIV-1 by fusion, but not by endocytosis, given that intravesicular fusion of HIV-1 unlikely occurs efficiently (Cavrois et al., 2002), this assay might not be able to distinguish HIV-1 plasma membrane fusion from potential intravesicular fusion.

Inhibition of endosome acidification can increase HIV-1 infection by decreasing viral degradation (Fredericksen et al., 2002) and enhancing viral fusion (Schaeffer et al., 2004). In our experiments, the NH_4Cl treatment of DCs and $CD4^+$ T cells slightly increased HIV-JRFL infection (less than 2-fold), but blocked HIV-VSV-G infection (Fig. 4). These observations are consistent with previous results obtained from various cell types

(Aiken, 1997; Daecke et al., 2005; Fackler and Peterlin, 2000; Fredericksen et al., 2002; Nobile et al., 2005; Schaeffer et al., 2004).

In a previous study using the cellular fractionation assays, after exposure of HIVΔEnv to macrophages, the detection of 27% of total p24 in the cytosolic fraction of macrophages likely represents background contamination (Maréchal et al., 2001). In contrast, an average of 52% (ranged from 27% to 71% in four independent experiments) of total p24 was detected in the cytosol fraction of HIVΔEnv-exposed DCs (Fig. 5A), which might be due to the incomplete removal of viral particles bound on DC surfaces. It is also possible that during the permeabilization of the plasma membrane with digitonin, DC surface bound viral particles were released into the cytosolic fraction, resulting in an increased p24 background in the cytosolic fraction. Our recent study indicates that a portion of DC-associated HIV-1 is protected from pronase treatment, and is able to mediate *trans*-infection of cocultured $CD4^+$ T cells (Wang et al., 2007b). Based on our titration of the pronase concentration used for DCs (Wang et al., 2007b), to ensure the integrity of DC plasma membranes before the permeabilization, the pronase concentration used in our study was 4-fold lower than that used in macrophage fractionations (Maréchal et al., 2001). Our Western blot analysis confirmed the specificity of the subcellular fractions, indicating no strong cross contamination between the vesicular and the cytosolic fractions (Fig. 5B). The Western blot analysis has not been shown in the previous studies using the cellular fractionation assay (Maréchal et al., 1998, 2001).

Notably, the total p24 of HIV-JRFL-exposed DCs was about 2-fold higher than that of HIVΔEnv-exposed DCs, which might result from multiple receptor-mediated binding and internalization of HIV-JRFL, in addition to non-specific endocytosis-mediated viral uptake. HIV-1 Env interactions with CD4, the C-type lectin DC-SIGN, and other attachment factors on DCs can facilitate viral binding and endocytosis (Gummuluru et al., 2003; Turville et al., 2001, 2002; Wang et al., 2007a,b). Interestingly, the treatment with specific fusion inhibitors in HIV-1-infected DCs had altered cytosolic p24 distributions. The T-20 and NH_4Cl treatment of DCs efficiently reduced the cytosolic p24 fraction in HIV-JRFL-infected DCs and HIV-VSV-G-infected DCs, respectively. These results further validate that the cytosolic p24 fraction could be used as an indirect indicator to reflect productive HIV-1 infection in DCs.

The majority of endocytosed HIV-1 in intracellular compartments in DCs will eventually be degraded. Indeed, rapid intracellular HIV-1 degradation has been reported in DCs (Moris et al., 2004; Nobile et al., 2005; Turville et al., 2004; Wang et al., 2007b). A recent study suggests that Langerin-mediated HIV-1 internalization results in viral degradation, thereby inhibiting HIV-1 replication in Langerhans cells (de Witte et al., 2007). HIV-1 enters intraepithelial vaginal Langerhans cells primarily by multiple receptor-mediated endocytosis, and virions remained intact within the cytoplasm for several days without productive replication (Hladik et al., 2007). However, when HIV-1-bearing DCs encounter $CD4^+$ T cells prior to viral degradation, efficient HIV-1 *trans*-infection of $CD4^+$ T cell can occur *in vitro* (Dong et al., 2007; Hladik et al., 2007; McDonald et al., 2003; Izquierdo-Useros et al., 2007; Turville et al., 2004), suggesting that DCs may act as a potential viral reservoir to disseminate HIV-1 infection *in vivo*.

Materials and methods

Cell culture

Immature DCs were generated from purified $CD14^+$ monocytes treated with 50 ng/ml of granulocyte-macrophage colony-stimulating factor and interleukin 4 for 5 days as described previously (Dong et al., 2007). Immature DCs were more than 98.5% pure by DC-SIGN, HLA-DR, CD11b, and CD11c staining, but negative for CD3 and CD14 (Wang et al., 2007b). The human embryonic kidney cell line HEK293T, CD4 T-cell line Hut/CCR5, and HIV-1 indicator cell line GHOST/R5 have been described (Wang et al., 2007a).

Flow cytometry

DCs were stained with specific MAbs or isotype-matched immunoglobulin G (IgG) controls as previously described (Wang et al., 2007a). Phycoerythrin-conjugated mouse anti-human CD4 (clone S3.5) and goat anti-mouse IgG were purchased from Caltag Laboratories. Purified mouse anti-human CCR5 (clone 45531) was purchased from R&D Systems. Stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest program (Becton Dickinson) or FlowJo software (Tree Star).

HIV-1 stocks

Single-cycle, luciferase-reporter HIV-1 stocks were generated by cotransfections of HEK293T cells with pLai3ΔenvLuc2 (Yamashita and Emerman, 2004), an *env*-deleted and *nef*-inactivated HIV-1 proviral construct (a kind gift from Michael Emerman, Fred Hutchinson Cancer Research Center, Seattle, WA) and expression plasmids for VSV-G or R5 HIV-1 envelope JRFL as previously described (Dong et al., 2007; Wang et al., 2007a). HIV-GFP pseudotyped viral stocks were generated by cotransfections of HEK293T cells with pHIV-eGFP (a kind gift from Vineet KewalRamani, National Cancer Institute-Frederick, MD) and pJRFL or pVSV-G as previously described (Unutmaz et al., 1999). The infectivities of the virus stocks were evaluated by limiting dilution on GHOST/R5 cells (Wu et al., 2002).

Replication-competent HIV-1_{NLAD8} was generated by transfections of HEK293T cells with the proviral construct pNLAD8 (Freed et al., 1995) (a kind gift from Eric Freed, National Cancer Institute-Frederick, MD). To minimize the contamination of HIV-1_{NLAD8} stocks with plasmid DNA, Hut/CCR5 cells were infected with HEK293T-derived HIV-1_{NLAD8}, and supernatants were harvested 5 dpi as previously described (Dong et al., 2007). Gag p24 concentrations of HIV-1_{NLAD8} stocks were measured using an enzyme-linked immunosorbent assay (ELISA, anti-p24-coated plates were purchased from the AIDS Vaccine Program, SAIC, Frederick, MD).

HIV-1 entry, infection and transmission assays

To measure HIV-1 entry, DCs were pulsed with HIV-1_{NLAD8} for 2 h at 37 °C, cells were trypsinized after extensive washes, and then lysed with 200 μl of 1% Triton X-100 buffer for Gag p24 quantification by ELISA as previously described (Wang et al., 2007a). HIV-1 direct infection assays using luciferase-reporter HIV-1 were performed as previously described (Dong et al., 2007). Cell lysates were analyzed for luciferase activity with a commercially available kit (Promega). For infection of HIV-1_{NLAD8}, DCs (2×10^5) were pulsed with HIV-1_{NLAD8} (20 ng p24) at 37 °C for 2 h. When indicated, DCs were pretreated with 1 μM of HIV-1 fusion inhibitor T-20 (the NIH AIDS Reagents Program) at 37 °C for 30 min, and T-20 was maintained at the same concentration during the viral incubation (Dong et al., 2007). For infection using HIV-GFP pseudotypes, DCs (2×10^5) or Hut/CCR5 cells (1×10^5) were infected with either HIV-GFP/JRFL or HIV-GFP/VSV-G (multiplicity of infection [MOI], 0.5 ~ 1). Infected cells were fixed and analyzed for GFP expression by flow cytometry as previously described (Unutmaz et al., 1999). A total of 1×10^4 cells was analyzed for each sample.

Real-time PCR quantification of HIV-1 DNA in infected DCs

DCs (2×10^5) were infected with HIV-1_{NLAD8} (20 ng p24) that have been pretreated with 60 U/ml of DNase I (Fermenta) at 37 °C for 1 h as previously described (Dong et al., 2007). Total cellular DNA was extracted 12 h post-infection from the infected DCs with a Blood DNA kit (Qiagen). Cellular DNA was quantified and normalized with real-time PCR quantification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as described (Dong et al., 2007). Primers, probes, and real-time PCR conditions for the detection of HIV-1 late reverse transcription products were previously described (Dong et al., 2007).

Analysis of HIV-1 entry into DCs by cellular fractionation

DCs were separately exposed to HIVΔEnv, HIV-JRFL and HIV-VSV (225 ng of p24 per 1×10^6 DCs) for 2 h at 37 °C. After extensive washes, DCs were treated with 0.25 mg/ml of pronase E (Sigma-Aldrich) to remove HIV-1 bound on cell surface. DCs were then washed, permeabilized with digitonin (Sigma-Aldrich), and fractionated by centrifugation as previously described (Maréchal et al., 2001). Intracellular p24 levels in the cytosolic and vesicular fractions were measured by ELISA as previously described (Wang et al., 2007a). For T-20 and NH_4Cl inhibitor treatments, DCs were preincubated with 1 μM of T-20 or 10 mM of NH_4Cl at 37 °C for 30 min before the HIV-1 incubation. Inhibitor concentrations were maintained at the same concentration during the virus incubation.

Western blotting

DC lysates were quantified and normalized by bicinchoninic acid protein assay (Pierce) according to the product instruction. Samples were separated on a 13% sodium dodecyl sulfate-polyacrylamide gel, and transferred to Immobilon-P membranes (Millipore) for Western blotting. The membrane was probed sequentially with anti-Cu/Zn-SOD (1:1000; Calbiochem) and anti-LAMP-1 (1:2000; clone H4A3, BD Pharmingen) as the primary antibodies. Horseradish peroxidase (HRP)-conjugated anti-sheep IgG (1:5000, Jackson ImmunoResearch Laboratories) or anti-mouse IgG (1:5000; Promega) was used as secondary antibodies. Restore Western Blot Stripping buffer (Pierce) was used to strip antibodies from probed membranes. SuperSignal West Pico chemiluminescence reagents (Pierce) were used to detect HRP-conjugated secondary antibody.

Statistical analyses

Statistical analyses were performed with the Wilcoxon paired *t* test or Dunnett's multiple comparison test with Prism software.

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