Interaction of HIV-1 with dendritic cells: implications for pathogenesis
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ANTIBODY DISSOCIATION DURING HIV-1-IMMUNE COMPLEX PROCESSING BY DENDRITIC CELLS DEPENDS ON AVIDITY

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Antibody dissociation during HIV-1-immune complex processing by dendritic cells depends on avidity

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Summary

Neutralizing antibodies can block human immunodeficiency virus type 1 (HIV-1) infection of susceptible target cells. Ab-neutralized HIV-1 however, can be captured by dendritic cells (DCs) and transmitted in trans as infectious virus to CD4⁺ T lymphocytes. We previously demonstrated that this process is Ab-specific; HIV-1 neutralization with 2F5 was readily reversible, whereas neutralization with b12 was irreversible. Using confocal microscopy imaging we studied processing of 2F5- and b12-HIV-1 immune complexes (ICs) by DCs. Upon HIV-1-IC uptake 2F5 rapidly dissociated from the virus, whereas b12 did not. Although intact 2F5-HIV-1 ICs could be visualized in a CD81-enriched compartment in mature DCs, we could not identify a specific compartment responsible for dissociation of 2F5 from HIV-1. Envelope glycoprotein (Env) ELISA studies demonstrated that b12 was more resistant for Ab-dissociation at acidic pH and bound 3-log stronger to variant Envs compared to 2F5. Furthermore, viral infectivity data demonstrated that the neutralizing capacity of 2F5 was 6 to 20-fold lower compared to b12, suggesting that the weaker avidity of 2F5 for the Env cannot withstand processing of HIV-1 by DCs, whilst b12 can. Collectively, these data imply that only neutralizing Abs with high avidity for Env like b12 can block DC-mediated HIV-1 transmission.
Introduction

Dendritic cells (DCs) reside within the skin and mucosal epithelia in an immature state. There they capture and survey the environment for pathogens (Banchereau et al., 2000; Banchereau and Steinman, 1998). Non-self antigens are captured with an array of specific surface expression molecules designated as pattern recognition receptors (Akira et al., 2001; Cambi et al., 2005; Fvidor et al., 2002). DC-entrapped antigens are processed into small peptides to trigger innate and adaptive immune responses (Steinman, 2006; Steinman and Hemmi, 2006). Human immunodeficiency virus type 1 (HIV-1) has found a way to circumvent the processing and inactivation by DCs and hijacks this cell type for infection and viral dissemination (Garcia et al., 2005; Geijtenbeek and van Kooyk, 2003; Kwon et al., 2002; Trumpfheller et al., 2003). DCs have been implicated to transfer HIV-1 towards susceptible target CD4+ T lymphocytes in cis and in trans (Turville et al., 2004). HIV-1 transmission in cis is characterized by direct infection of DCs resulting in transfer of de novo produced virions. The production of these new viruses takes a few days (Cavrois et al., 2007; Holl et al., 2006b). Although HIV-1 can infect DCs like Langerhans cells, myeloid or plasmacytoid dendritic cells, production of new viral particles is limited and mainly CCR5-tropic (R5) viruses replicate in these cells (Canque et al., 1999; Ganesh et al., 2004; Smed-Sorensen et al., 2005). It is postulated that this mode of transmission is prevalent during acute infection (Hu et al., 2000; Sugaya et al., 2004). HIV-1 transmission in trans is mediated by capture of HIV-1 particles by DCs followed by transfer to CD4+ T lymphocytes, which occurs within a few hours (Cavrois et al., 2007; Geijtenbeek et al., 2000a). We recently showed that transmission in trans is more efficient for CXCR4 (X4) using HIV-1 variants than for R5 tropic variants (van Montfort et al., 2008). Transmission in trans will likely be more favourable over transmission in cis later in disease, when HIV-1 specific Abs are induced that efficiently block DC infection (Holl et al., 2006b; Holl et al., 2006a). Therefore our findings could help explain the emergence of X4 HIV-1 during disease progression (van Montfort et al., 2008).

Transmission of HIV-1 from DCs to CD4+ T lymphocytes occurs via formation of an immunological synapse upon cell-cell contact (Arrighi et al., 2004; Fackler et al., 2007; McDonald et al., 2003; Thoulouze et al., 2006). The mechanism of transmission of captured virus to its target cell remains unclear. It was recently suggested that only cell membrane bound virions are transmitted and that internalization of HIV-1 prevents trans-infection (Cavrois et al., 2007). Other studies have shown, however that internalized HIV-1 can be redirected to the immunological synapse where the virus is transmitted either as free virions or together with exosomes (Garcia et al., 2005; Geijtenbeek et al., 2000a).
Capture of HIV-1 by DCs occurs either via phagocytosis, pinocytosis, macropinocytosis or receptor-mediated endocytosis (Izquierdo-Useros et al., 2007; Marechal et al., 2001; Wang et al., 2008). C-type lectins like DC-SIGN (Geijtenbeek et al., 2000b; Geijtenbeek and van Kooyk, 2003), DEC205 (Guo et al., 2000), langerin (Braathen et al., 1987; de Witte et al., 2007b), DC-SIGN immunoreceptor (DCIR) (Lambert et al., 2008), and proteoglycans (Roderiquez et al., 1995) such as syndecan1-4 (Bobardt et al., 2003; de Witte et al., 2007a) have been shown to interact with the HIV-1 envelope glycoprotein (Env). Expression of these receptors vary on the different DC lineages which can result in altered capture and processing of HIV-1 virions by DCs as shown for Langerhans cells and conventional DCs (Turville et al., 2002; Turville et al., 2001).

Processing of gp120-coated nanoparticles by iDCs demonstrated internalization within 10 min after capture and degradation occurred within 90 minutes in lysosomes (Cambi et al., 2007). Experiments with intact virions however showed that LPS-matured DCs retained the virus in an internal compartment containing the CD81 tetraspanin (Garcia et al., 2005; Garcia et al., 2008). Similar results were also observed for immature DCs. Little colocalization was observed of HIV-1 with cell membrane markers or lysosomes for either cell type, suggesting that a considerable number of HIV-1 virions were able to escape lysosomal degradation. HIV-1 processing by DCs seem to be envelope glycoprotein (Env) dependent; R5-viruses are more vulnerable for degradation than X4-virions (Garcia et al., 2008).

We demonstrated previously that neutralized HIV-1 captured by DCs could regain infectivity upon viral transmission in trans to CD4+ T lymphocytes (van Montfort et al., 2007; van Montfort et al., 2008). Most broadly neutralizing Abs like 2F5 did not block DC-mediated viral transmission, with the b12 Ab as an exception, which efficiently blocked viral transfer. Here we followed processing of 2F5- and b12-neutralized HIV-1 in immature monocytes-derived DCs (iMDDCs) and mature monocytes-derived DCs (mMDDCs) to determine how viral neutralization is reversed during transfer to CD4+ T lymphocytes. During internalization, the 2F5 Ab readily dissociated from HIV-1, whereas the b12 Ab remained firmly bound. Intact 2F5-HIV-1-ICs were obtained in a tetraspanin CD81-rich compartment in mMDDCs. No specific compartment of the endocytic pathway could be identified to be responsible for dissociation of 2F5 from the virus as analyzed with confocal microscopy. With ELISA we could detect a 0.5 pH unit-difference in Ab-dissociation from the Env between 2F5 and b12, with b12-binding being more acid-resistant. The pH, however, in which Ab-dissociation occurs can only be reached in lysosomes, suggesting that acidification along the endocytic pathway does not cause Ab-dissociation in DCs. Moreo-
ver the binding of the b12 Ab to monomeric Env was 1000-fold stronger than the 2F5 Ab. Data with intact virions also demonstrated a strong decrease in HIV-1 neutralization with 2F5 compared to b12. Taken together our data suggest that binding of the 2F5 Ab to Env is too weak to withstand processing by DCs and dissociates from the virus, allowing trans-infection of permissive cells.

Results

Processing of neutralized HIV-1-Ab complexes by dendritic cells is antibody-dependent. We previously described the effect of various HIV-1-specific neutralizing antibodies on capture and transfer of HIV-1 from DCs to CD4+ T lymphocytes in trans. Pre-incubation of HIV-1 with the 2F5 Ab increased viral capture and transmission by DCs, whereas the b12 Ab blocked DC-mediated trans-infection, although viral capture was not inhibited (van Montfort et al., 2007). To dissect the differential effect of these neutralizing Abs on viral transfer we visualized the processing of 2F5- or b12-neutralized virus in DCs. GFP-HIV-1 and fluorescently labelled neutralizing Abs were visualized by fluorescence microscopy after 2 hour incubation with iMDDCs (Figure 1A) or polyI:C-activated mMDDCs (Figure 1B). Maturation of DCs was confirmed by FACS flow analyses (data not shown). Localization of Abs and GFP-HIV-1 virions was analyzed and plotted as percentage of total captured HIV-1 particles for each cell type (Figure 1C).

The 2F5 Ab co-localized with 11.9% of HIV-1 particles in iMDDCs and with 14.4% in mMDDCs. In contrast, 42.3% of the virus co-localized with the b12 Ab for iMDDCs and 42.6% for mMDDCs. Control IgG1-treated HIV-1 demonstrated an overlay of 5.2% for iMDDCs and 2.6% for mMDDCs (Figure 1C). Apparently, the b12 Ab is much stronger associated with HIV-1 in DCs compared to the 2F5 Ab. These results support our previous data that b12 is more potent at blocking trans-infection than 2F5 (van Montfort et al., 2007; van Montfort et al., 2008). Although the average number of intact HIV-1-IC for 2F5 and b12 are comparable for iMDDCs and mMDDCs, we observed that iMDDCs more often contained cells without intact HIV-1-IC compared to mMDDCs (Figure 1D). As seen for iMDDCs 57% (33/58) and 25% (14/57) of the cells did not contain 2F5-HIV-1-ICs, or b12-HIV-1-ICs respectively. For mMDDCs, on the contrary, 29% (15/51 for 2F5) and 7% (4/54 for b12) of the cells contained Ab-free HIV-1 particles. The two different cell-types therefore appear to process neutralized HIV-1 differently.

When comparing Ab-levels in iMDDCs, internalized by capturing HIV-1-ICs, we observed a 10-fold increase in Ab-uptake for b12 and only a 2.5-fold increase for 2F5, compared to the non-specific IgG1 control Ab (Figure 1E). These data suggest that the 2F5 Ab is removed from the virus before or after internalization, whilst the b12 Ab is not. Similar results were obtained for mMDDCs, with a 6.7-fold increase in Ab uptake of
Figure 1. Legend on subsequent page
b12, whilst a 2.5-fold for 2F5. Moreover, the average Ab-levels in mMDDCs was lower compared to iMDDCs (Figure 1E), which suggests that Ab-dissociation is slower in mMDDCs than in iMDDCs.

**HIV-1-Ab complexes are more efficiently captured by iMDDCs than by mMDDCs.** Capture of 2F5- and b12-neutralized HIV-1 by iMDDCs and mMDDCs was analyzed by measuring the number of captured HIV-1 particles. An increase in viral capture by iMDDCs was observed upon neutralization with either 2F5 (126%, $P \leq 0.05$) or b12 (127%, $P \leq 0.05$) in comparison to the IgG1 control Ab (Figure 2). The heightened capture of neutralized HIV-1 by iMDDCs is facilitated by Fc receptors as previously published (van Montfort et al., 2007). In contrast, HIV-1 neutralization with 2F5 (82%, $P \leq 0.05$) or b12 (81%, $P \leq 0.05$) mildly inhibited viral capture by mMDDCs. This could be caused by a reduced DC-SIGN expression on mMDDCs (Sanders et al.,

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**Figure 1. HIV-1-Ab complexes captured by DCs.** A) HIV-1-GFP particles were treated with 2F5, b12 or control IgG1 labeled Ab and captured by iMDDCs or B) mMDDCs for 2 hr. HIV-1 virions (green left panel), Abs (middle panel) and nuclear DNA (blue) were detected by confocal microscopy. HIV-1-Ab complexes were visualized by co-localization of HIV-1 and Ab signal and plotted as overlay in yellow (right panel). C) The number of HIV-1-Ab complexes for the different Abs as percentage of HIV-1 virions for each cell. D) Number of HIV-1 positive cells without viral-Ab complexes. E) IgG1, 2F5 or b12 Ab-signal derived from HIV-1-Ab complexes in DCs. Data from iMDDCs (light grey) and mMDDCs (dark grey) is depicted in 1A, D and E.
which is an important receptor for capturing HIV-1-ICs (van Montfort et al., 2007). Furthermore, we observed an 1.7-fold increase in capture of control-treated HIV-1 upon DC maturation with polyI:C. This was observed previously when LPS was used to induce DC maturation (Izquierdo-Useros et al., 2007; Sanders et al., 2002; Wang et al., 2007). Together these results show that maturation of the DC results in an altered or increased receptor expression pattern that increases capture of either control or neutralized virus.

**Dissociation of 2F5 from HIV-1 does not occur in a specific compartment of the endocytic pathway.** Since 2F5, but not b12 was easily dissociated from HIV-1 during DC-processing (Figure 1A), we examined the intracellular location of HIV-1 and 2F5-neutralized virus in DCs to determine where Ab-dissociation from the virus can occur. Plasma membrane (CD81, DC-SIGN), early endosomes (EEA1), multivesicular bodies (MVBs) (CD63, HLA-DM) and lysosomes (LAMP-1) were stained with Abs directed against the above markers and analyzed for co-localization with fluorescent GFP-HIV-1 neutralized with 2F5-labeled Ab. Localization of HIV-1 with the different markers is shown for iMDDCs (Figure 3A) and (Figure 3B). MHC class-II staining was used to visualize DC maturation since this molecule is mainly localized in MVBs in iMDDCs and is recruited to the plasma membrane upon maturation.

Most GFP-HIV-1 particles in iMDDCs were found to be clustered with CD81 (38%) and DC-SIGN (47%) (Figure 3C). Since DC-SIGN and CD81 localize on the cell membrane, although they are also involved in virus internalization, these data show that the remaining virus (53-62%) is intracellular, which

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**Figure 2. Capture of IgG1-control, 2F5 or b12-neutralized HIV-1 by iMDDCs and mMDDCs.** The number of HIV-1-GFP particles treated with either IgG1, 2F5 or b12 captured by iMDDCs (light grey) and mMDDCs (dark grey) were counted for each cell.
sis in line with previously published data (Garcia et al., 2005; Izquierdo-Useros et al., 2007; Wang et al., 2007). Strangely, only a relative small percentage of virus co-localized with the internal markers EEA1 (8.1%), MHC II (7.8%), CD63 (6.5%), HLA-DM (4.2%) and LAMP-1 (2.7%) in iMDDCs. The low viral presence in LAMP-1 positive lysosomes is likely caused by loss of the GFP-signal from intact virions due to viral degradation; such degradation is a likely scenario as we could find the capsid-p24 protein in lysosomes (data not shown). Since only ~25% of captured HIV-1 particles could be identified with markers from intracellular compartments may indicate that HIV-1 enters a DC-compartment different from the endocytic pathway. Nevertheless, the HIV-1 virions found in the endocytic pathway did not retain in a specific compartment, suggesting these particles are readily degraded.

As seen for mMDDCs a high amount of...
of HIV-1 co-localized with the CD81 marker (57%) (Figure 3D). DC-SIGN staining of mMDDCs was strongly reduced compared to iMDDCs (data not shown), nonetheless, 47% of the virus clustered with this marker in mMDDCs. HIV-1 co-localization with MHC-II molecules of mMDDCs (34%) increased compared to iMDDCs, which was caused by the recruitment of these molecules to the plasma membrane upon DC-maturation. HIV-1 co-localization with the internal endocytic pathway markers EEA1 (3.7%), CD63 (4.9%), HLA-DM (5.2%) and LAMP-1 (0.6%) was even lower than observed for iMDDCs, illustrating that mMDDCs transport virus less actively into the endocytic pathway.

The quantity of 2F5-clustered virions in each compartment was analyzed as a percentage of total neutralized viruses to determine in which compartment 2F5 dissociates from HIV-1 (Figure 3E and 3F). The localization of 2F5-HIV-1-ICs in the different compartments as measured with the mentioned markers was 28% for CD81, 35% for DC-SIGN and 10% or lower for the other markers of iMDDCs (Figure 3E). These co-localization numbers are similar to the HIV-1 distribution shown in Figure 3C, suggesting that 2F5-HIV-1-ICs are similarly processed as Ab-free HIV-1 virions.

The location of 2F5-HIV-1-ICs in endocytic compartments in mMDDCs (Figure 3F) was also highly similar as the distribution of Ab-free HIV-1 for these cells (Figure 3D). We did notice

Figure 4. The b12-Ab has higher avidity for the HIV-1 envelope than 2F5 at acidic pH. ELISA plates were coated with JRFLgp120, JRFLgp140 (R5), or HXB2gp140 (X4) envelopes and loaded with the 2F5 A) or b12 Ab B). Ab-dissociation was measured after washing with various acidic pH. Bound Ab was detected with alkaline phosphatase conjugated goat anti-human Fc and plotted as percentage of bound Ab at neutral pH.
however, that incidentally large clusters of intact HIV-1-2F5 complexes in mMDDCs were co-localized internally or in invaginations with CD81 or DC-SIGN and not with the other membrane marker MHC II (Figure 3B). These data illustrate that big HIV-1 clusters are internalized. A recent publication demonstrated that uptake of these large clusters of HIV-1 particles occurs via macropinocytosis (Wang et al., 2008; Wang et al., 2007). Moreover, these large HIV-1 clusters in the internal CD81-compartment are described to survive proteolytic degradation for a prolonged period (Garcia et al., 2005; Garcia et al., 2008). Together, these data illustrate that 2F5-HIV-1-ICs can enter different internal compartments, in which HIV-1 could be processed differently.

The 2F5 and b12 neutralizing Abs dissociate from HIV-1 Env at acidic pH. A decrease in pH along the endocytic pathway may cause Ab-dissociation. Therefore we examined binding of the 2F5 and b12 Ab to monomeric envelope glycoprotein (Env) of an R5 (JRFL) and X4 (HXB2) virus in ELISA at variable acidic pH. At pH 5.0 or above, 2F5 Ab bound to JRFLgp140 and HXB2gp140, whilst no binding was observed to the control JRFLgp120 lacking the 2F5-binding epitope (Figure 4A). At pH 4.5, binding of 2F5 was strongly decreased by ~75% for JRFLgp140 and ~90% for HXB2gp140; background binding remained at pH 4.0 for both Envs. The gp120-specific b12 Ab bound all three Envs (Figure 4B). At pH 4.5, ~30% of the binding of the b12 Ab was lost for JRFLgp120/140 and ~60% for HXB2gp140. At pH 4.0, only ~20% of the b12 Ab bound to JRFLgp120/140 and ~5% to HXB2gp140; at pH 3.5 only residual Ab-binding was observed. These results illustrate that the b12-Env complex is more resistant to acidic pH. Additionally, 2F5 and b12 Ab dissociated at acidic pH more easily from the CXCR4 HXB2gp140-molecule compared to the CCR5 JRFLgp140-molecule. Although the b12 Ab is more resistant to Ab-dissociation than the 2F5 Ab, it is not likely that pH causes HIV-1-Ab dissociation in DCs, since a pH of 4.5 can only be reached in lysosomes (Vyas et al., 2008). It is unlikely that HIV-1 can retain infectivity after dissociation of neutralizing Abs in lysosomes, as HIV-1 is degraded in this compartment.

The 2F5 neutralizing Ab binds weaker to the viral Env in comparison to the b12 neutralizing Ab. The more rapid dissociation of 2F5 from the Env at acidic pH, could indicate that 2F5 binds weaker to the Env, compared to b12. We therefore examined the binding of 2F5 and b12 to JRFLgp120/140 and HXB2gp140 at different Ab concentrations (Figure 5). The b12 Ab still bound to JRFLgp120 with mid-point titer of 0.01 μg/ml (Figure 5A), whereas 2F5 binding was negligible as expected. Binding of b12 to JRFLgp140 (Figure 5B) showed a high binding as seen for JRFLgp120 with a mid-point titer of 0.008 μg/ml. In contrast the 2F5 Ab showed only weak binding, with a mid-point titer of 4.7 μg/ml. Binding of both Abs to the CXCR4 HXB2gp140-molecule (Figure 5C) again
demonstrated weak binding of the 2F5 Ab (mid-point titer 7.6 μg/ml) and a stronger binding of b12 (mid-point titer 0.02 μg/ml). These results demonstrate that the avidity of the b12 Ab to Env is approximately 3-log higher than that of the 2F5 Ab. The binding capacity of both Abs 2F5 and b12 on intact R5 (JRFL) and X4 (LAI) HIV-1 was measured in an infectivity assay. Intact virus neutralized at different Ab concentrations were incubated on HIV-1 permissive CCR5/CXCR4 expressing TZM-bl cells and the 50% inhibitory concentration (IC$_{50}$) of both Abs was determined. The IC$_{50}$ for JRFL was 0.8 μg/ml with 2F5 Ab and 0.04 μg/ml with b12 Ab (Figure 5D). X4 (LAI) HIV-1 also demonstrated a higher IC$_{50}$ for 2F5 (2.4 μg/ml) than for b12 (0.4 μg/ml) (Figure 5E). Again these results show that the b12 Ab more potently binds to HIV-1 than the 2F5 Ab. This difference in binding helps to understand why b12 Ab does not dissociate from the Env during HIV-1 processing by DCs and therefore is able to prevent viral trans-infection. The 2F5 Ab on the contrary dissociates from the virus due to its weak binding and is unable to block DC-mediated viral transmission to CD4$^+$ T lymphocytes.

**Discussion**

In this study we visualized the differences in processing of 2F5- and...
b12-neutralized virus by iMDDCs and mMDDCs by confocal microscopy. The 2F5 Ab weakly clustered with HIV-1 in both types of DCs, whereas strong co-localization was observed for HIV-1 with the b12 Ab. No specific compartment of the endocytic pathway could be identified to be responsible for the dissociation of the 2F5 Ab from the virus. Ab-binding studies demonstrated however that the 2F5 Ab only weakly bound to the Env, whereas the b12 Ab bound 1000-fold more strongly. Viral infectivity data demonstrated that the neutralizing capacity of 2F5 was 6-20-fold lower compared to the b12 Ab, suggesting that the 2F5 Ab less strongly interacts with HIV-1. Taken together, the weak 2F5-virus interaction is responsible for the fast dissociation of the Ab from the virus and its escape from neutralization, allowing \textit{trans}-infection of permissive cells.

As previously reported by other groups we found that next to LPS-stimulated DCs polyI:C matured DCs were able to capture virus more efficiently than immature DCs (Izquierdo-Useros \textit{et al.}, 2007). DC-SIGN and syndecan-3 are the main receptors responsible for the capture of HIV-1 by iMDDCs (de Witte \textit{et al.}, 2007a). Capture of HIV-1 by mMDDCs on the other hand only weakly occurs via DC-SIGN (Wang \textit{et al.}, 2007) (data not shown). Furthermore, DC-SIGN expression on mMDDCs is reduced compared to iMDDCs (Sanders \textit{et al.}, 2002), which is in agreement with our observation of weak DC-SIGN expression in mMDDCs. Interestingly, the remaining DC-SIGN expression on mMDDCs strongly co-lo-calized with HIV-1 particles, suggesting that DC-SIGN is involved in capture of HIV-1 virions (Figure 3D). Co-localization of HIV-1 with DC-SIGN or CD81 was mainly found in large clusters of virions. Uptake of these HIV-1 clusters appear to occur via macropinocytosis (Frank \textit{et al.}, 2002; Marechal \textit{et al.}, 2001; Wang \textit{et al.}, 2008), which is a process of non-specific constitutive engulfment of large volumes of fluid by DCs. HIV-1 uptake via this process can than explain why DC-SIGN on mMDDCs is not involved in viral capture. Since DC-SIGN is not actively involved in capture of HIV-1 by mMDDCs we conclude that other receptors should facilitate HIV-1 capture by mMDDCs; these putative receptors remain to be identified.

Capture of neutralized HIV-1 by iMDDCs was more efficiently compared to untreated virus (Figure 2). DC-SIGN together with FcRs are responsible for the enhanced capture of HIV-1-ICs on iMDDCs as previously reported (van Montfort \textit{et al.}, 2007). In contrast, mMDDCs captured neutralized virus less efficiently. This could be caused by the reduced expression of DC-SIGN on mMDDCs and increased capture of HIV-1 by putative other cellular receptors. The reduced capture of HIV-1-ICs by mMDDCs may also explain the observed decrease in transmission of 2F5-neutralized HIV-1 by these cells, which was increased for iMDDCs (van Montfort \textit{et al.}, 2008). Capture of 2F5- and b12-neutralized virus by both DCs types also demonstrated a substantial quantity of Ab-free virus (Figure 1A-D). Our results
however with b12-neutralized virus demonstrated that this Ab efficiently blocked HIV-1 transmission. A possible explanation could be that, next to Ab-free virions and b12-HIV-1-ICs, also free b12 Ab is recruited to the immunological synapse and cross neutralization occurs during transmission. Since the 2F5 Ab less potently neutralizes HIV-1 and less 2F5 Ab is present in DCs, the possibility of cross neutralization is somewhat reduced.

By visualizing HIV-1 capture by DCs with confocal microscopy we observed that the majority of the HIV-1 particles were internalized in small spots in close proximity to the cell surface for iMDDCs and mMDDCs as well as in big clusters for the latter cell type. These small spots could represent invaginations of the plasma membrane. We determined that these particles are not part of the cell surface, but are internalized (Figure 3A and 3B). Based on colocalization with plasma membrane markers, CD81 for iMDDCs and MHC class-II for mMDDCs, we estimate that ∼60-70% of cell-bound virus is intracellular for iMDDCs as well as for mMDDCs. This finding is in line with results from other studies (Garcia et al., 2005; Izquierdo-Useros et al., 2007; Wang et al., 2008).

We observed discrepancies in location, size and number of HIV-1 spots in polyI:C-stimulated mMDDCs, compared to LPS-matured DCs (Garcia et al., 2005). PolyI:C mimics viral infection, and LPS a bacterial infection, both stimuli leading to DC-maturation. LPS-mMDDCs collect viruses in a few large clusters in internal CD81-positive compartments, whereas polyI:C treated DCs have multiple scattered viral spots close to the cell membrane. We note that exposure of HIV-1 for a longer period (24 hrs) with DCs decreased the total number of viral spots, with a simultaneous modest increase in spot-size (data not shown). These results illustrate that DC maturation or increased time exposure with HIV-1 particles have affect on the distribution of virus within the cell.

Internalization of gp120-coated beads by DCs occurred rapidly within 10 minutes and these dots reached the lysosome within 90 minutes (Cambi et al., 2007). Our results with intact virus could not identify a strong clustering of HIV-1 with lysosomes after a short incubation (2 hr) (Figure 3A-F). This was likely caused by the loss of signal due to degradation of intact HIV-1 particles. Marking intact and disassembled HIV-1 virions by staining CA-p24 we observed a large internal fluorescent compartment likely representing degraded HIV-1 in lysosomes (data not shown). This result than indicates that a substantial amount of HIV-1-GFP is already degraded within 2 hours. Trafficking and processing of HIV-1 particles was different for X4-enveloped virus compared to R5-tropic HIV-1 in LPS-stimulated mMDDCs. R5-HIV-1 was found to be stronger associated with the lysosomal marker LAMP-1 and CD63, whereas X4 HIV-1 was stronger clustered with CD81, illustrating that the viral Env can trigger an altered route or rate of processing of HIV-1 particles by DCs. All together, exposure time, the Env phenotype and DC-maturation
stimuli effect trafficking of HIV-1 in DCs, resulting in altered transmission in trans of HIV-1 to CD4+ T lymphocyte (Groot et al., 2006; Sanders et al., 2002).

Neutralization of HIV-1 with either 2F5 or b12 did not retain the virus in a particular compartment of the endocytic pathway nor did it dramatically alter trafficking in DCs (Figure 1). Our earlier model (van Montfort et al., 2007) that dissociation of the 2F5 Ab from HIV-1 occurs via a decrease in pH was supported by our ELISA data. Ab-dissociation from the Env, however occurred at pH of 4.0-4.5, which can only be reached in lysosomes. Confocal microscopy data demonstrated that no change in number in HIV-1-ICs was observed in the different endocytic compartments with various internal pH. These data demonstrate that Env-Ab-dissociation is pH sensitive, but a reduction in pH along the compartments of the endocytic pathway is probably not responsible for the observed Ab-dissociation.
cation from HIV-1 during processing by DCs. Interestingly the low pH of 4 can be obtained in vaginal fluids (Garcia-Closas et al., 1999; Thinkhamrop et al., 1999), suggesting that neutralized HIV-1 upon transmission may regain infectivity via sexual intercourse.

We illustrated that the b12 Ab has a high avidity for both R5 and X4 Env, in contrast to the 2F5 Ab. Based on all our data, we propose two following models specific for the Ab-dissociation of 2F5 (Figure 6). 2F5 Ab is removed from HIV-1 on the cell surface upon virus binding to a specific HIV-1-receptor, whereas the b12 Ab stays attached upon binding to the receptor and is internalized together with the HIV-1 particle. Alternatively, instable 2F5-HIV-1 ICs are internalized; Ab is pulled from the virus and Ab is recycled, for instance by the intracellular recycling neonatal Fc receptor termed FcRn (Ghetie and Ward, 2000; Popov et al., 1996; Raghavan et al., 1995); Ab-free infectious virus is further processed by DCs and can be transferred to susceptible CD4+ T lymphocytes. The FcRn receptor is strongly expressed in DCs (Zhu et al., 2001) and has a strong avidity at pH 6.5 for IgG1 Abs such as 2F5 and b12. The Ab bound to the FcRn is recycled back to the cell surface, where the Ab is released due to loss of receptor avidity at pH 7.0 (Datta-Mannan et al., 2007; Mezo et al., 2008; Vidarsson et al., 2006). The activity of the FcRn in DCs could therefore be responsible for the low amount of 2F5 Ab obtained in DCs.

We observed large spots of HIV-1 particles in mMDDCs strongly clustered with the 2F5 Ab, which could not be identified in iMDDCs. It could be that these 2F5-HIV-1-ICs clusters are captured by macropinocytoses by mMDDCS. Since HIV-1 captured by macropinocytosis does not enter the endolysosomal pathway (Wang et al., 2008), it could be that these big 2F5-HIV-1 clusters are not processed in classical internalization pathway. This could lead, therefore, to a reduced immune response as observed in patients, since less HIV-1 is loaded as processed peptides in MHC class molecules. It may therefore be interesting to target HIV-1 directly to the classical endolysosomal pathway to strengthen the neutralizing Ab response against HIV-1.

Monitoring HIV-1 transmission in trans we demonstrated that only the b12 Ab was able to block viral transfer to CD4+ T lymphocytes, whilst the other Abs only partially reduced viral trans-infection or had no effect (van Montfort et al., 2007). Since the main difference between 2F5 and b12 Ab-dissociation from HIV-1 was strongly correlated with avidity for the Env we postulate that strong avidity neutralizing Abs will likely be able to block DC-mediated viral transfer. It would be interesting to test the effect of other CD4bs Abs with lower avidity for the Env than the b12 Ab with regards to dissociation and blocking of viral transfer. Moreover Abs in sera from HIV-1 infected patients that bind the CD4 binding site (CD4bs) of gp120, like b12, have also the most potent broadly neutralizing activity compared to neutralizing Abs binding other parts of the Env (Li
et al., 2007). Taken together, to increase the efficacy of a vaccine against HIV-1 it to would be desirable to elicit CD4bs neutralizing Abs with b12-like avidity to minimize Ab-evasion induced by passing HIV-1 through DCs.

Materials and Methods

Antibodies and reagents. The human Abs 2F5 directed against the HIV-1 Env gp41 and b12 directed against gp120, were obtained from the National Institute for Biological Standards and Control (Potters Bar, U.K.) and used at 20 µg/ml or indicated in figures. Human sera obtained from a patient with an IgG1 B cell lymphoma was used as an HIV-1-nonspecific control Ab; the sera contained IgG1-λ M protein at 83 mg/ml and no detectable IgA or IgM (< 0.06 mg/ml). The Abs to stain DC-compartments, CD81, DC-SIGN, EEA1, HLA-DR, CD63, HLA-DM and LAMP-1 were obtained from BD Pharmingen. The secondary Abs AffiniPure donkey anti-human-Cy3, donkey anti-mouse-Cy5 was acquired from Jackson ImmunoResearch Laboratories and used at a 50-fold and 200-fold dilution respectively. Nuclear DNA was stained with Hoechst 33258.

Cells. The iMDDCs were prepared as previously described (van Montfort et al., 2007). In short, human blood monocytes were isolated from buffy coats by use of a Ficoll gradient and a subsequent CD14 selection step using a magnetic bead cell sorting system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purified monocytes were differentiated into iMDDCs by stimulating the cells twice with 45 ng/ml interleukin-4 (IL-4) (Biosource, Nivelles, Belgium) and 500 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Schering-Plough, Brussels, Belgium) on day 0 and day 3 and used on day 6. Mature monocyte-derived DCs (mMDDCs) were obtained on day 6 after stimulating iMDDCs on day 5 with 20 µg of polyI:C per ml (Sigma-Aldrich, St. Louis, MO). The phenotypes of both types of DCs were confirmed by flow cytometry as previously described (van Montfort et al., 2008). The HIV-1 permissive CD4, CCR5 and CXCR4 expressing TZM-bl reporter cell line (NIH AIDS Research and Reference Reagent Program) containing the luciferase and β-galactosidase genes under the control of the HIV-1 long-terminal-repeat promoter was cultivated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1x minimum essential medium nonessential amino acids, and penicillin-streptomycin (both at 100 units/ml) at 37°C with 5% CO₂.

Virus/Env production. Molecular cloned dual tropic GFP-fluorescent HIV-1 (299.10ΔgV3) (Pollakis et al., 2001) was generated by co-transfecting C33A cells with GFP-VPR and full infectious HIV-1 299.10ΔgV3 vectors in a 1:1 ratio. The supernatant, containing the produced virions was collected, three days after the transfection and was concentrated with Amicon Ultra filters (100,000 molecular weight cutoff). The concentration of viral capsid (CA-p24) was determined with ELISA. HIV-1-GFP was frozen in small aliquots.

The JRFLgp120, JRFLgp140 and HXB2-gp140 Envs were generated by transfecting C33A cells with Env encoding plasmids. Supernatant containing soluble Env was collected two days post-transfection and was frozen at -80°C in aliquots.

Virus neutralization and capture. HIV-1-GPF was incubated with either 20 µg/ml IgG1 control, 2F5 or b12 Abs for 45 min. Subsequently, a 50-fold dilution of secondary
anti-human Cy3 Ab was added for 15 min. iMDDCs or mMDDCs were inoculated for 2 hrs at 37°C with the viral Ab mixture. Unbound virus and antibody was removed by washing three times with PBS and cells were seeded on pretreated poly-L-lysine coated coverslips (1 mg/ml). Cells were immediately fixed in 3.7% paraformaldehyde (PFA) for 20 min. PFA was quenched with 50 mM NH₄Cl and cells were permeabilized with 0.1% saponin (Riedel de Haen, Germany), 10 mM NH₄Cl, and 1% BSA in PBS for 30 min. Subsequently, cells were stained with either primary mouse Ab CD81, DC-SIGN, EEA1, HLA-DR, CD63, HLA-DM or LAMP-1 and Cy5-coupled donkey anti-mouse secondary Ab; nuclear DNA was stained with Hoechst 33258. Excess Ab was removed by washing twice with permeabilization buffer, once with PBS, and twice with millipore filtered water. Cells were embedded in Vectashield and fluorescence was analyzed by confocal microscopy.

Confocal analyses. Fluorescent images were made with a Leica DM SP2 AOBS confocal microscope with a X63 HCX PL APO 1.32 oil objective. The whole cell was scanned in 20 images (512 x 512) with a pixel size of 232 nm and a step size of 340 nm that were acquired with the Leica confocal imaging processing software with a line average of two scans per image. For each cell the number of HIV-1-GFP spots was counted. Colocalization was analyzed by using a semi-automatic program based on DipImage (TU Delft, The Netherlands), custom-written in Matlab (Mathworks Inc., USA). Briefly, image channels containing relevant color information were thresholded using the Isodata algorithm. Next, colocalization was determined as percentages of the number of overlapping pixels for the different channels for each Z-stack per cell.

ELISA, Env coating. Wells of ELISA plates were coated overnight with 100μl sheep antibody D7324 (10 μg/ml; Aalto Bioreagents), directed to the gp120 C5 region, in 0.1 M NaHCO₃. After washing twice with Tris-buffered saline (TBS), wells were blocked with 1% BSA in TBS for 1 hr. Soluble Env protein 100 μl/well were bound to the sheep Abs for 2 hrs at RT. Unbound envelope was removed by washing three times with TSM (20 mM Tris, 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂) followed by 30 min incubation with block buffer (BB) (1% BSA in TSM) at RT.

neutralizing Ab dissociation at various pH. Env-coated ELISA plates were incubated with 20 μg/ml 2F5 or b12 Ab in BB pH 7.0 for 1 hour at RT. Unbound Ab was removed by washing three times in wash buffer (WB), BB supplemented with 0.05% Tween 20 at pH 7.0. Subsequently, wells were washed five times in WB at pH 6.0-3.0 over a 30 min period, followed by 2 wash steps in WB pH 7.0. Bound Ab was detected with alkaline phosphatase conjugated goat anti-human Fc (GaH-PO) (1:10,000, Jackson Immunoresearch) in 2% milk, 20% sheep serum, 0.5% Tween and absorbance at 450 nm was measured in an ELISA reader.

neutralizing Ab binding at various concentrations. Env-coated ELISA plates were incubated with a concentration range of 30-0.0005 μg/ml 2F5 or b12 Ab in BB pH 7.0 for 1 hr at RT. Unbound Abs was washed 5 times in wash buffer (WB) and bound Ab was detected with GaH-PO.

Single cycle infectivity assay. One day prior to infection, TZM-bl cells were plated on a 96-well plate JRFL and LAI ~3-5 ng CA-p24 was preincubated for 60 min at room temperature with serial diluted Ab concentrations (30-0.0015 μg/ml). Pre-neutralized virus was added to the TZM-bl cells in
the presence of 400 nM saquinavir (Roche, Mannheim, Germany) and 40 µg/ml DEAE in a total volume of 200 µl. Two days post-infection, the medium was removed and cells were washed once with phosphate-buffered saline (PBS) and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI) and a Glomax luminometer according to the manufacturer’s instructions (Turner BioSystems, Sunnyvale, CA). All infections were performed in duplicate. Uninfected cells were used to correct for background luciferase activity. The infectivity without Ab was normalized at 100%. Nonlinear regression curves were determined and 50% inhibitory concentration (IC$_{50}$) was calculated using Prism software version 4.0c.

**Statistical analysis.** Significance was determined with unpaired t test (two tailed) and indicated in figures with stars. *, $P \leq 0.05$; **, $P \leq 0.005$; *** $P \leq 0.0005$.

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