Human immunodeficiency virus type 1 (HIV-1) protease inhibitors block cell-to-cell HIV-1 endocytosis in dendritic cells

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Sexual transmission is now the most frequent means of diffusion of human immunodeficiency virus type 1 (HIV-1). Even if the underlying mechanism is still largely unknown, there is a consensus regarding the key role played by mucosal dendritic cells (DCs) in capturing HIV through contact with infected subepithelial lymphocytes, and their capacity to spread HIV by *trans*-infection. We found that HIV protease inhibitors (PIs) reduced virion endocytosis strongly in monocyte-derived immature (i) DCs contacting HIV-1-infected cells, and that this phenomenon led to dramatically impaired *trans*-infection activity. This inhibitory effect was not mediated by the block of viral protease activity, as it was also operative when donor cells were infected with a PI-resistant HIV-1 strain. The block of virus maturation imposed by PIs did not correlate with significant variations in the levels of virus expression in donor cells or of Gag/Env virion incorporation. Also, PIs did not affect the endocytosis activity of DCs. In contrast, we noticed that PI treatment inhibited the formation of cell–cell conjugates whilst reducing the expression of ICAM-1 in target iDCs. Our results contribute to a better delineation of the mechanisms underlying HIV-1 *trans*-infection activity in DCs, whilst having implications for the development of new anti-HIV microbicide strategies.

INTRODUCTION

The sexual transmission of human immunodeficiency virus type 1 (HIV-1) implies its penetration through mucosal tissues (Haase, 2005; Hladik & McElrath, 2008; Wu, 2008). The mechanism of HIV transmission across the mucosa still remains an unresolved enigma, despite outstanding studies that have already shed light on relevant issues (Bomsel, 1997; Bouschbacher *et al.*, 2008; Collins *et al.*, 2000; Gupta *et al.*, 2002; Hladik *et al.*, 2007; Hu *et al.*, 2000; Kawamura *et al.*, 2000; Spira *et al.*, 1996; Tan & Phillips, 1996; Zhang *et al.*, 1999). In particular, two cell types are considered targets of HIV infection at the port of entry, i.e. CD4/CCR5 subepithelial lymphocytes and dendritic cells (DCs). These latter cells are supposed to capture cell-free HIV virions either from the intralumenal space upon the passage of virus across the epithelial barrier by transcytosis or, most efficiently, by contacting infected subepithelial lymphocytes. Thereby, DCs deliver virions to peripheral lymph nodes, where close contact with densely packed cells facilitates HIV cell-to-cell transmission and spread.

The consequences of HIV–DC interaction are still a matter of debate. DCs can be infected productively, even if with low efficiency (Piguet & Steinman, 2007). Alternatively, DCs can *trans*-infect bystander cells by releasing virions that were internalized and stored in tetraspanin-rich endosomal compartments (Garcia *et al.*, 2005; Geijtenbeek *et al.*, 2000; Turville *et al.*, 2004; Wiley & Gummuluru, 2006). On this subject, it has been also proposed that *trans*-infection occurs through the transmission of virions adhering to the DC membrane (Cavrois *et al.*, 2007, 2008; Yu *et al.*, 2008).

Whatever the mechanism, cell-to-cell virus transmission is a key phenomenon in HIV primary infection. In this regard, we recently reported that the contact between HIV-1-infected and uninfected CD4$^+$ cells leads to high levels of HIV-1 endocytosis in target cells. This was observed reproducibly using CD4$^+$ primary lymphocytes, macrophages (Ruggiero *et al.*, 2008) and monocyte-derived DCs (Muratori *et al.*, 2009) as target cells. Whilst this massive endocytosis does not generate detectable HIV-1 replication, it is associated with high levels of HIV-1 antigen-presenting activity in target DCs (Muratori *et al.*, 2009). Conversely, the extent to which the cell-to-cell HIV-1 endocytosis in DCs contributes to *trans*-infection has not yet been established.

HIV protease inhibitors (PIs) are small molecules designed specifically for blocking the activity of the viral aspartyl protease (Flexner, 1998). The ultimate consequence of the

PI treatment of HIV-infected cells is the release of immature virions that, although still able to enter target cells, then abort their life cycle at a post-entry step (Kaplan *et al.*, 1993; Schatzl *et al.*, 1991). PIs are now part of highly active antiretroviral therapy, which allows millions of infected people to co-exist with the virus and experience a good quality of life.

Here, we originally describe the strong inhibition induced by PIs on HIV-1 endocytosis in immature (i) DCs contacting HIV-1-infected cells, and its correlation with impaired *trans*-infection activity.

METHODS

Cell cultures and co-cultures. U937$_{HIV-1}$ is a cell line infected chronically with the human T-lymphotropic virus$_{IIIB}$ HIV-1 isolate. U937, U937$_{HIV-1}$ and CEM$_{GFP}$ cells (Gervaix *et al.*, 1997) were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % heat-inactivated fetal calf serum (FCS). Both 293T cells and 293/GPR inducible HIV-1-packaging cells (Sparacio *et al.*, 2001) were grown in Dulbecco's modified Eagle's medium plus 10 % FCS. CD4$^+$ lymphocytes were isolated from peripheral blood mononuclear cells and cultivated as described previously (Ruggiero *et al.*, 2008). iDCs were differentiated from purified human primary monocytes as described previously (Muratori *et al.*, 2009).

Co-cultivations were typically set up by seeding 2×10^5 donor and target cells in 48-well plates in 0.5 ml RPMI + 10 % FCS supplemented with both 500 U GM-CSF (granulocyte–macrophage colony-stimulating factor; Serotec) ml^{-1} and 30 ng interleukin-4 (IL-4; R&D Systems) ml^{-1}. T-20, PIs (i.e. ritonavir, saquinavir and indinavir) and recombinant Env gp120 MN strain were obtained from the NIH AIDS Research and Reference Reagent Program. Cytochalasin D and chlorpromazine were from Sigma-Aldrich.

HIV-1 infection and detection. Preparations of different HIV-1 strains pseudotyped with the G protein of vesicular stomatitis virus (VSV-G) were obtained from the supernatants of 293T cells co-transfected with the respective expressing vectors by the Lipofectamine 2000 (Invitrogen)-based method. In particular, the (VSV-G) X4 HIV-1 strain was produced by transfecting the pNL4-3 HIV-1 molecular clone, whereas the (VSV-G) R5 HIV-1 strain was recovered by using the (AD-8 Env) pNL4-3 strain. Virus preparations were titrated by measuring both Gag CAp24 contents by quantitative ELISA (Innogenetics) and reverse transcriptase activity. Cell infections were carried out by spinoculation at 400 *g* for 30 min at room temperature (r.t.) using 100 ng CAp24 equivalent of (VSV-G) HIV-1 per 10^5 cells. Then, virus adsorption was prolonged for an additional 2 h at 37 °C and, finally, cells were washed and re-fed with complete medium.

Cell and virus fluorescence-activated cell sorting (FACS) analysis. The iDC phenotype was scored routinely by FACS analysis as described previously (Muratori *et al.*, 2009). To score HIV-1 endocytosis in target iDCs, T-20-treated co-cultures were incubated with trypsin for 15 min at 37 °C, labelled with fluorescein isothiocyanate (FITC)-conjugated anti-CD1a mAb (Dako) and then permeabilized with Cytofix–Cytoperm (BD Biosciences) for 30 min at r.t. Afterwards, cells were labelled for 1 h at r.t. with a 1 : 100 dilution of phycoerythrin (PE)-conjugated anti-HIV-1 Gag CAp24 KC-57 mAb (Coulter Corp.). For the detection of HIV-1 Env gp120, cells were labelled with 1 : 100-diluted anti-gp120 4G10 mAb for 1 h at 4 °C, and then with either FITC- or PE-conjugated anti-mouse IgGs.

The same procedure was adopted for the detection of CD4, DC-SIGN and ICAM-1, which was carried out using mAbs from e-Bioscience.

FACS-based determination of the composition of HIV-1 virions was carried out by using surfactant-free white aldehyde/sulfate latex beads (Invitrogen Molecular Probes) as described previously (Muratori *et al.*, 2006).

Macropinocytosis activity in iDCs was evaluated by FACS analysis of iDCs pre-treated with PIs or left untreated, and then incubated with different amounts (i.e. from 500 ng to 2 µg per 10^5 cells) of FITC–dextran (Sigma-Aldrich) at either 4 or 37 °C.

Preparations of fluorescent virus-like particles (VLPs) were obtained from the supernatants of 293/GPR cells co-transfected with vectors expressing green fluorescent protein (GFP) fused at its N terminus with the $^{G}3^{C}$ HIV-1 Nef mutant and VSV-G as described previously (Muratori *et al.*, 2006). For the iDC challenge, 500 ng CAp24 equivalent of fluorescent (VSV-G) HIV-1 VLPs per 10^5 cells was adsorbed for 1 h at 37 °C in a volume of 20 µl per 10^5 cells. Then, 0.1 ml complete medium was added and, finally, the iDCs were analysed by FACS after incubation with trypsin for 15 min at 37 °C.

***Trans*-infection assay.** iDCs were co-cultivated with HIV-1-infected U937 cells at a 1 : 1 cell ratio for 16 h in the presence of either 5 µM PIs or, as a control, 100 ng recombinant HIV-1 Env gp120 ml^{-1} and 1 µg T-20 ml^{-1}. Then, iDCs were separated from donor cells with a two-step immunomagnetic-based selection procedure. In detail, co-cultures were first incubated with anti-CD14-conjugated microbeads (Militenyi Biotec) and CD14$^+$ cells were discarded. The remaining cell population was then incubated with anti-DC-SIGN mAbs, and then with anti-mouse IgG-conjugated microbeads. DC-SIGN$^+$ iDCs were then put into co-culture with indicator cells, i.e. either CEM$_{GFP}$ or autologous CD4$^+$ lymphocytes, at a 3 : 1 cell ratio, and the number of HIV-1-infected indicator cells was scored by FACS 72 h later. In particular, CEM$_{GFP}$-based mixed cultures were analysed for GFP expression, whereas iDC/CD4$^+$ lymphocyte mixed cultures were first labelled with PE-conjugated anti-CD3 mAb, then permeabilized and finally incubated with FITC-conjugated anti-CAp24 KC-57 mAb.

Analysis of cell–cell conjugates. Enumeration of cell–cell conjugates was performed through an already described FACS-based assay (Sourisseau *et al.*, 2007). Briefly, donor cells and target iDCs were labelled with CM-Dil C7000 and carboxyfluorescein succinimidyl ester (CFSE) (both from Molecular Probes), respectively, following the manufacturer's recommendations. Then, co-cultures were set up as described above in the presence or absence of PIs, harvested at different times and analysed by FACS. Doublets were identified as the events simultaneously displaying intensity of both fluorescences as high as those displayed by each cell type.

Statistical analysis. When appropriate, data are presented as means + SD. Statistical analysis was performed according to a paired Student's *t*-test and confirmed by using the non-parametric Wilcoxon rank sum test. *P*-values of <0.05 were considered significant.

RESULTS

PIs inhibit the massive virion endocytosis occurring in iDCs contacting HIV-1-infected cells

High numbers of HIV-1 virions are captured by CD4$^+$ target cells shortly after contact with HIV-1-infected cells (Ruggiero *et al.*, 2008). This phenomenon was revealed through the development of an assay designed to evaluate

the levels of virion endocytosis in target cells co-cultured with HIV-1-infected cells. This is based on FACS analysis of co-cultures treated with T-20 and labelled with anti-HIV-1 CAp24 mAb after 15 min incubation with trypsin. The treatment of co-cultures with T-20, i.e. a fusion inhibitor blocking virus–cell and cell–cell fusion (Kilby *et al.*, 1998), was mandatory to exclude the detection in target cells of both *de novo*-synthesized viral proteins following infection events, and Gag products transmitted by donor cells upon cell–cell fusion.

Very recently, we also described high levels of HIV-1 endocytosis in monocyte-derived DCs contacting HIV-1-infected cells (Muratori *et al.*, 2009). Further investigation led to the observation that when HIV-1-infected cells, for instance $U937_{HIV-1}$ cells, were co-cultivated with human primary iDCs in the presence of both 5 µM PIs (i.e. ritonavir, saquinavir or indinavir) and 1 µg T-20 ml^{-1}, a strong reduction of HIV-1 Gag accumulation occurred in target cells (Fig. 1a). Since, as we demonstrated previously (Ruggiero *et al.*, 2008), under these conditions the Gag-specific labelling in target cells measures the extent of HIV-1 endocytosis, these results indicated that PIs potently reduced the levels of HIV-1 uptake in target cells. This inhibition appeared similar when using the three different PIs, could be detected starting 4 h after co-culture set-up (not shown) and was fully operative within the 2–10 µM concentration range (data not shown). Of note, no obvious effects on the fluorescence excitation/emission by the fluorochromes (i.e. PE and FITC) of the anti-CAp24 mAb that we used throughout were noticed in PI-treated cells (Fig. 1b and data not shown).

We also found that the inhibition of cell-to-cell HIV-1 endocytosis in iDCs did not depend on the tropism of the virus expressed by donor cells, as similar inhibitory effects were observed when donor cells were infected with (VSV-G) X4 or (VSV-G) R5 HIV-1 strains (Fig. 1c). Finally, the same effect was observed in the case when HIV-1-infected primary CD4$^+$ lymphocytes were used as donor cells (Fig. 1d), suggesting strongly that the PI-induced inhibition of virion endocytosis does not depend on the donor-cell type used.

We concluded that PI treatment strongly impairs the HIV-1 endocytosis in iDCs that occurs upon contact with infected cells.

The block of viral protease activity is not involved in the PI-induced inhibition of HIV-1 endocytosis

To evaluate the involvement of the viral protease in the inhibition of HIV-1 endocytosis induced by PIs, we reproduced co-culture experiments using donor cells infected with an HIV-1 strain resistant to PIs (here referred to as PM4) as a consequence of four amino acid substitutions in the protease gene, i.e. $^M46^I$, $^L63^P$, $^V82^T$ and $^I84^V$ (Condra *et al.*, 1995). We first tested the actual PI resistance of PM4 by infecting PI-treated or untreated CEM$_{GFP}$ cells, i.e. a CD4$^+$ lymphoblastoid cell line

expressing GFP in the presence of Tat, with either wild-type (wt) or PM4 HIV-1 (Fig. 2a). Then, the levels of cell-to-cell virion endocytosis of wt HIV-1 in iDCs were compared with those of the PM4 mutant. As shown in Fig. 2(b), in the presence of tightly similar percentages of infected donor cells, no significant differences between the levels of endocytosis of the two HIV-1 strains were detected. When the co-cultures of PM4-infected U937 cells and iDCs were carried out in the presence of PIs, we observed inhibition of endocytosis of PM4 virions in iDC target cells (Fig. 2c) similar to that detected with wt HIV-1 (Fig. 1a).

Thus, the inhibition of HIV-1 endocytosis in target iDCs induced by PIs was not a consequence of inhibition of the viral protease activity.

PI-induced inhibition of HIV-1 endocytosis in iDCs leads to impaired *trans*-infection

It was reported previously that HIV-1 endocytosis in DCs could be part of the mechanism of *trans*-infection (Geijtenbeek *et al.*, 2000). We were interested in establishing whether cell-to-cell HIV-1 endocytosis could contribute to the *trans*-infection process and, more interestingly, whether PI treatment interferes with this phenomenon. *Trans*-infection experiments were carried out as summarized in Fig. 3 using donor cells expressing either PI-resistant or, as a control, wt HIV-1. For the sake of clarity, the cultures of HIV-1-infected cells with iDCs are referred to as co-cultures, whereas the subsequent co-cultures of purified iDCs with indicator cells (i.e. CEM$_{GFP}$ or autologous CD4$^+$ lymphocytes) are referred to as mixed cultures.

HIV-1-infected donor cells were co-cultivated with iDCs for 16 h in the presence of 5 µM PI and 1 µg T-20 ml^{-1}. As a control, co-cultures were also carried out in the presence of 100 ng recombinant HIV-1 gp120 ml^{-1}, which strongly reduces HIV-1 endocytosis in iDCs, probably by inhibiting the binding of Env gp120 expressed by donor cells with iDC Env receptors (Muratori *et al.*, 2009). Then, target iDCs were isolated through two immunomagnetic-based separation steps, i.e. an anti-CD14 negative selection followed by an anti-DC-SIGN positive one. The separation efficiency was tested routinely by FACS analysis (Fig. 4a). Afterwards, purified iDCs were incubated with trypsin for 15 min at 37 °C to remove residual virions still attached to the cell membrane. Finally, mixed cultures with HIV-1-susceptible indicator cells were set up at a 1:1 cell ratio. After 3 days, HIV-1 expression in indicator cells was evaluated by scoring the percentages of GFP-positive or, in the case of CD4$^+$ primary lymphocytes, HIV-1 Gag-positive cells. We found that iDCs isolated from co-cultures with wt or PM4 HIV-1-infected cells *trans*-infected indicator cells with similar efficiency (Fig. 4b, c). When these co-cultures were carried out in the presence of PIs, similar reductions of HIV-1-infected CEM$_{GFP}$ (Fig. 4b) and CD4$^+$ lymphocyte (Fig. 4c) indicator cells were detected. The treatment of co-cultures with recombinant Env gp120
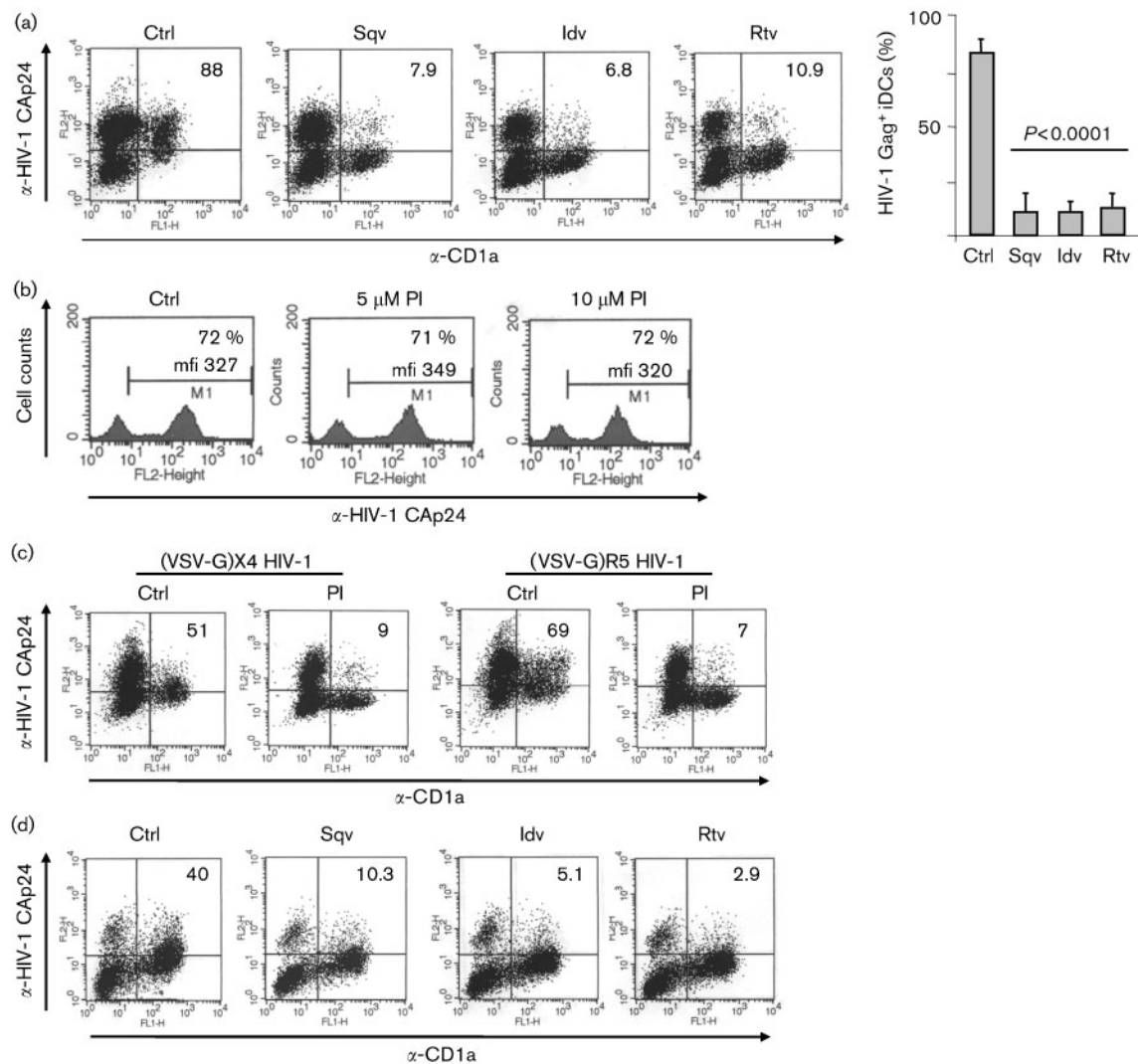
Fig. 1. PIs inhibit cell-to-cell HIV-1 endocytosis in human iDCs. (a) FACS analysis for the presence of both HIV-1 Gag products and cell-membrane CD1a in iDCs after 16 h co-cultivation with U937$_{HIV-1}$ cells in the absence (Ctrl) and presence of 5 μM saquinavir (Sqv), indinavir (Idv) or ritonavir (Rtv), and of 1 μg T-20 ml^{-1}. On the left, the results from a representative experiment are shown. Percentages of HIV-1 Gag-positive iDCs are indicated. On the right, the mean + SD percentages of Gag-positive iDCs as calculated from the data for six independent experiments are shown. (b) Anti-Gag CAp24 analysis of U937$_{HIV-1}$ cells treated and untreated (Ctrl) with the indicated concentrations of PIs. Both percentages of Gag CAp24-positive cells and mean fluorescence intensities (mfi) are reported. M1 identifies the range of fluorescence positivity. The results are representative of two independent experiments. (c) FACS analysis for the presence of HIV-1 Gag and CD1a in co-cultures of iDCs with U937 cells infected 2 days before with (VSV-G) X4 or (VSV-G) R5 HIV-1 strains. The co-cultures were carried out for 16 h in both the absence (Ctrl) and the presence of 5 μM PI and of 1 μg T-20 ml^{-1}. Percentages of HIV-1 Gag-positive iDCs are indicated. The results are representative of two independent experiments. (d) FACS analysis for the presence of both HIV-1 Gag and CD1a in co-cultures of iDCs with autologous CD4^{+} lymphocytes infected 2 days before with (VSV-G) HIV-1. The co-cultures were run for 16 h in both the absence (Ctrl) and the presence of 5 μM saquinavir, indinavir or ritonavir and of 1 μg T-20 ml^{-1}. Percentages of HIV-1 Gag-positive iDCs are indicated. The percentages of HIV-1 Gag-expressing lymphocytes ranged from 21 to 26 %. The results are representative of two independent experiments.

blocked *trans*-infection (Fig. 4b, c). Notably, the result from this control condition excluded the possibility that contaminating HIV-1-infected donor cells and/or residual virions still adhering to the iDC membrane interfered with the outcome of the *trans*-infection assays.

These data indicate that the inhibition of HIV-1 endocytosis in iDCs induced by PIs in the cell–cell context is associated with a reduced *trans*-infection efficiency, and that this phenomenon did not depend on the inhibition of viral protease activity. The functional correlation between
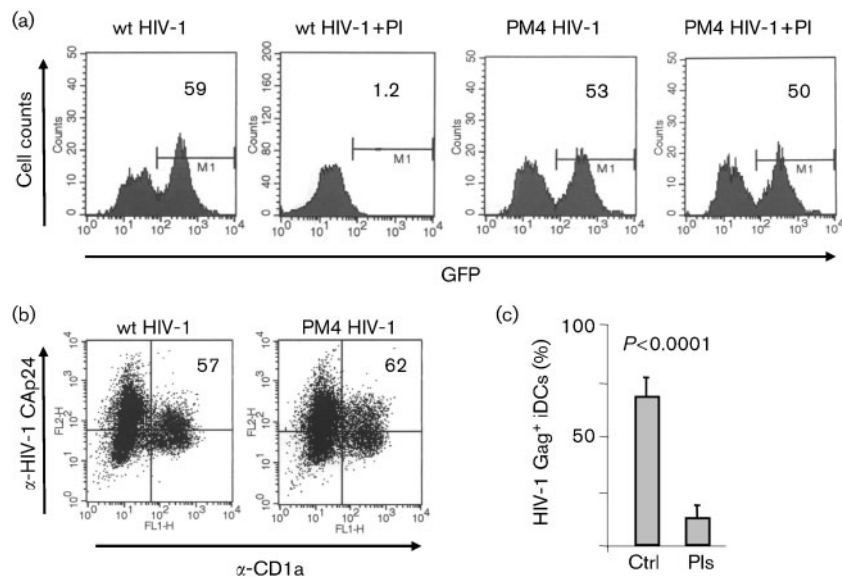
Fig. 2. PIs inhibit the endocytosis of both PI-susceptible and -resistant HIV-1 strains. (a) Analysis of the PI resistance of the PM4 HIV-1 strain. FACS analysis of GFP expression in CEM_{GFP} cells infected 5 days before with 500 ng CAp24 equivalent per 10^5 cells of either wt or PM4 HIV-1 strains in both the presence and the absence of 5 µM PIs. Percentages of GFP-expressing cells are indicated. M1 identifies the range of fluorescence positivity. (b) Endocytosis of wt HIV-1 compared with PM4 HIV-1 in co-cultures of infected U937 cells with iDCs. U937 cells were infected with either (VSV-G) wt or (VSV-G) PM4 HIV-1 strain and, after 2 days, put in co-culture with iDCs in the presence of 1 µg T-20 ml^{-1}. Sixteen hours later, the co-cultures were analysed by FACS for the detection of CD1a and HIV-1 Gag products. Percentages of HIV-1 Gag-positive cells are indicated. The results are representative of two independent experiments. (c) Effects of PIs on the endocytosis of the PM4 HIV-1 strain. The mean + SD percentages of Gag-positive iDCs after co-cultivation with U937 cells infected 2 days before with (VSV-G) PM4 HIV-1 are shown. The co-cultures were carried out in the presence of 1 µg T-20 ml^{-1} and 5 µM of the indicated PIs. The results were calculated from the data for three independent experiments.

HIV-1 capture in DCs and *trans*-infection suggests strongly that cell-to-cell HIV-1 endocytosis could be part of the *trans*-infection mechanism.

PIs do not affect either the expression/production of HIV-1 products or the endocytosis activity of iDCs

We demonstrated previously that HIV-1 endocytosis following cell–cell contact depends strictly on the Env expression on producer cells, and is mostly a consequence of the rapid internalization of virions released by producer cells (Ruggiero *et al.*, 2008). Hence, it is conceivable that alterations in the Env expression in producer cells and/or in virion composition/production would be at the basis of the PI inhibitory effect. To test this possibility, we analysed Env expression on the membrane of producer cells, virus production from PI-treated compared with control infected cells, as well as the Gag/Env composition of virions released from untreated and PI-treated cells. FACS analysis for Env cell-membrane expression failed to reveal effects of PIs on Env expression in infected cells (Fig. 5a). Also, no PI-dependent inhibition of the release of HIV-1 particles was detectable by quantitative ELISA (Fig. 5b). Finally, FACS analysis of virions bound on aldehyde/sulfate

latex beads did not reveal significant effects of PIs on the Gag/Env composition of the virions (Fig. 5c). We concluded that the PI-induced drop in HIV-1 uptake was not a consequence of negative effects on expression/production of the virus and/or of its structural components.

Alternatively, PIs could influence the endocytosis activity of iDCs. This hypothesis has been evaluated by measuring the effects of PIs on macropinocytosis as well as on the endocytosis of cell-free virions in iDCs. For the macropinocytosis assay, 10^5 iDCs untreated or treated with PI or, as a control, with cytochalasin D were incubated with different doses (i.e. 0.5–2.0 µg) of FITC–dextran. Cells were then harvested after 2 and 4 h incubation at 37 °C, washed extensively and analysed by FACS for FITC-associated fluorescence. As an additional control, FITC–dextran-treated iDCs were incubated at 4 °C. No significant differences were detectable between the fluorescence profiles of control and PI-treated iDCs treated with 1 µg FITC–dextran (Fig. 5d). Similar data were obtained with 0.5 or 2.0 µg FITC–dextran (not shown), as well as in the context of co-cultures with HIV-1-infected cells (data not shown). Hence, we concluded that PIs do not affect macropinocytosis activity in iDCs.
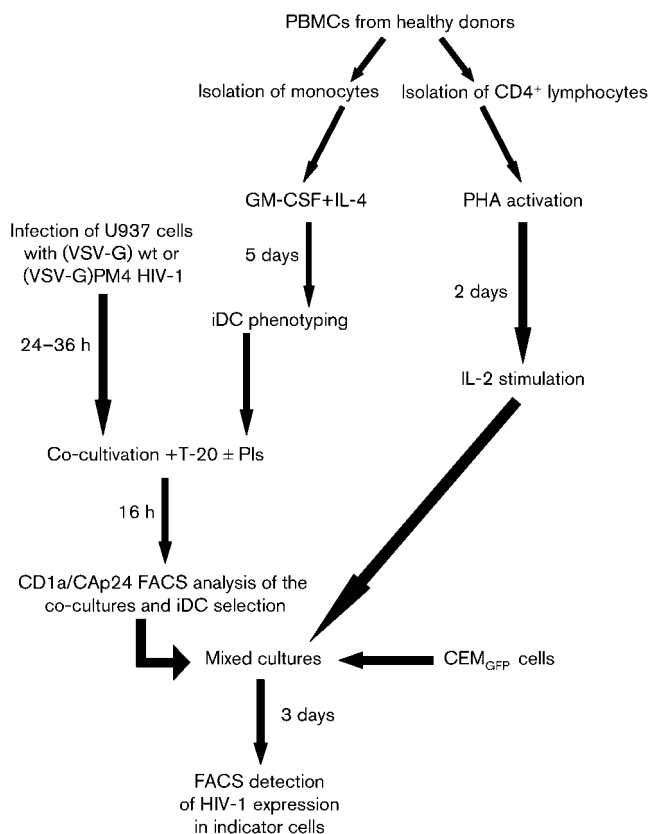
Fig. 3. Summary of the experimental procedures for the *trans*-infection assay.

To study the PI effects on the endocytosis of cell-free HIV-1 particles, iDCs were challenged with fluorescent HIV-1 particles pseudotyped with VSV-G, which drives virus entry by endocytosis. Indeed, this was expected to be the mode of HIV-1 entry in iDC target cells, where the presence of T-20 hinders Env/CD4-dependent fusion. In detail, 10^5 iDCs untreated or treated with PIs or, as a control, with the endocytosis inhibitor chlorpromazine were challenged with 0.5 μg CAp24 equivalent of GFP-labelled (VSV-G) HIV-1-based VLPs. After 2 and 4 h incubation at 37 °C, the cells were treated with trypsin for 15 min and finally analysed by FACS. As a control, the iDC challenge was also run at 4 °C. As shown in Fig. 5(e), VLP internalization in iDCs was not affected significantly by PI treatment, thus indicating that the inhibition of HIV-1 endocytosis in PI-treated iDCs in the context of co-cultures was not the consequence of detrimental effects on the endocytosis of cell-free virus particles.

PIs impair ICAM-1 expression on iDCs and reduce the number of cell–cell conjugates

Our previous results supported the idea that cell–cell adhesion is a key event for the efficiency of HIV-1 endocytosis in target cells, as suggested by the critical role

of Env expression in donor cells, of CD4 in lymphocyte target cells (Ruggiero *et al.*, 2008) and of DC-SIGN in iDC target cells (Muratori *et al.*, 2009). On the basis of these results and in an effort to clarify the mechanism underlying the PI inhibitory effect on HIV-1 endocytosis, we investigated possible effects of PIs on the expression of CD4 and DC-SIGN in iDC target cells and of the cell-adhesion molecules ICAM-1 and LFA-1 in both donor and target cells. Interestingly, we found that PI treatment downmodulated the cell-membrane expression of ICAM-1 in iDCs in the absence of apparent effects on CD4, DC-SIGN (Fig. 6a) and the major ICAM-1 counter-receptor, i.e. LFA-1, in both donor and target cells (not shown). On the basis of the mean fluorescence values, we estimated approximately 50 % reduction of ICAM-1 expression on the iDC membrane (not shown).

The impaired expression of the key adhesion molecule ICAM-1 was suggestive of some effects of PIs on cell–cell adhesion. To evaluate the influence of PIs on cell–cell adhesion directly, we enumerated the cell–cell conjugates in co-cultures by a FACS-based assay. To this end, before co-culture, U937$_{HIV-1}$ or uninfected U937 cells were labelled with the red-fluorescent cell tracker Dil, whereas iDCs were labelled with CFSE (green fluorescence). Then, co-cultures were set up in both the presence and the absence of PIs and the cells were harvested at different times. FACS analysis revealed an increase of about twofold in the number of cell–cell conjugates in co-cultures with HIV-1-infected cells compared with those with control U937 cells (Fig. 6b). PI treatment reduced the number of cell–cell conjugates in HIV-1-infected co-cultures significantly (Fig. 6b) in the presence of the usual inhibition of HIV-1 endocytosis in iDCs (not shown). Conversely, no apparent effects of PIs were detectable in uninfected co-cultures (Fig. 6b). Of importance, the PI treatment reproducibly led to slightly higher Dil-associated fluorescence levels in donor cells with respect to control conditions. The identification of cell doublets took account of these subtle variations and was confirmed by side/forward scatter dot-plot analysis (not shown).

These data suggested strongly that PIs affect the formation of cell–cell conjugates, but only in co-cultures comprising HIV-1-infected donor cells. In this context, the PI-induced downmodulation of ICAM-1 could contribute to the reduced cell–cell adhesion activity.

DISCUSSION

Cell-to-cell HIV transfer can occur through the formation of virological synapses (Chen *et al.*, 2007; Gousset *et al.*, 2008; Groot *et al.*, 2008; Jolly *et al.*, 2004; McDonald *et al.*, 2003; Rudnicka *et al.*, 2009) and upon the establishment of filopodial bridges (Sherer *et al.*, 2007) and nanotubes (Eugenin *et al.*, 2009; Sowinski *et al.*, 2008). On this subject, we recently found that virus–cell and cell–cell fusion, as well as high levels of HIV-1 endocytosis, are the
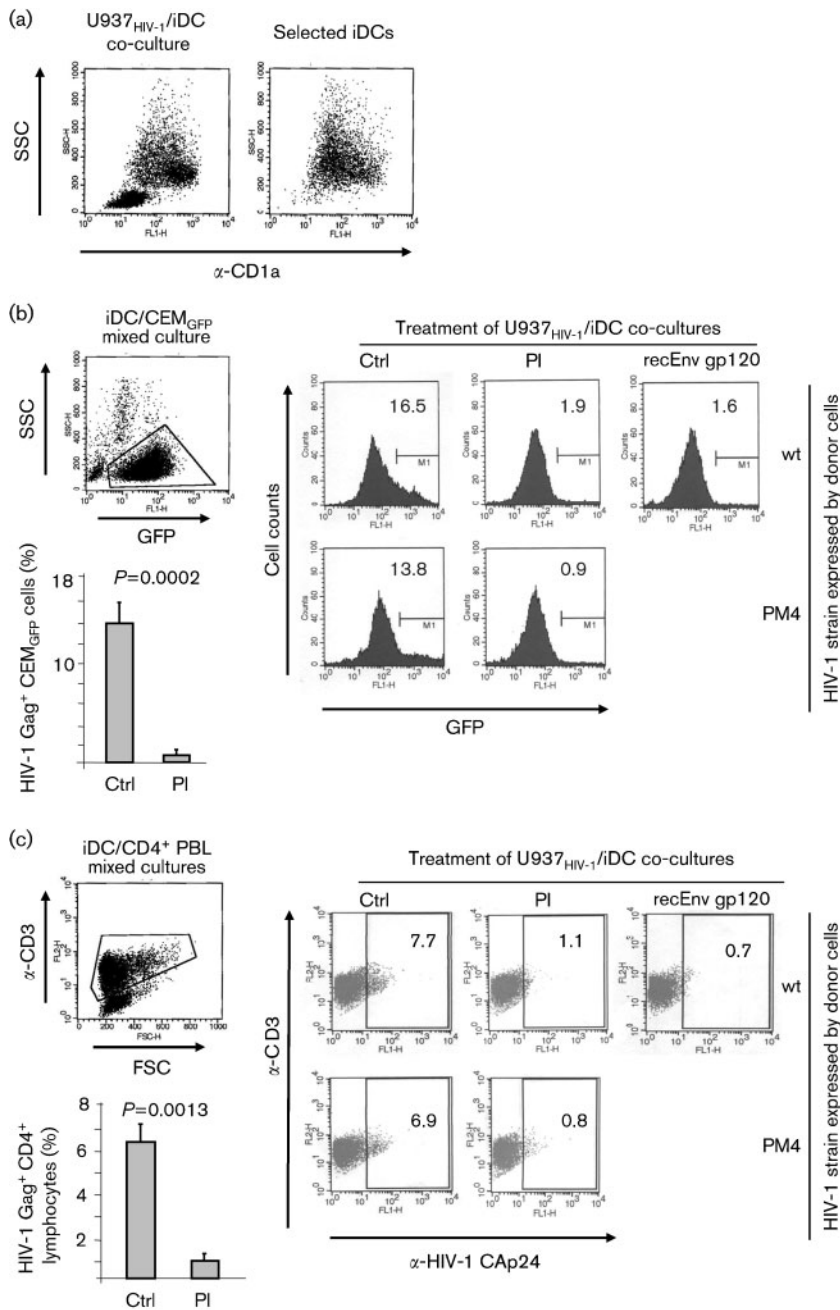
Fig. 4. Pls inhibit the *trans*-infection of both wt and PM4 HIV-1. (a) Representative CD1a FACS analysis for evaluating the purity of iDCs selected from the co-cultures with U937$_{HIV-1}$ cells. (b) At the top left, GFP/side scatter (SSC) FACS analysis of mixed cultures of CEM$_{GFP}$ cells with iDCs is shown. The gate selected the indicator cells scored for GFP expression. On the right, FACS analyses for GFP expression in CEM$_{GFP}$ cells 3 days after the set-up of mixed cultures with iDCs isolated from co-cultivation with HIV-1-infected cells under the indicated conditions are shown. Percentages of GFP-positive cells are indicated in each histogram. M1 identifies the range of fluorescence positivity. At the bottom left, mean + SD percentages of GFP-positive CEM$_{GFP}$ indicator cells as calculated from the data for three independent experiments are shown. (c) At the top left, anti-CD3/forward scatter (FSC) FACS analysis of mixed co-cultures of CD4$^+$ lymphocytes with naïve iDCs is shown. The gate selected the indicator cells scored for HIV-1 expression. On the right are shown the FACS analyses for Gag CAp24 expression in indicator CD4$^+$ lymphocytes 3 days after the set-up of mixed cultures with iDCs isolated from co-cultivation with HIV-1-infected cells under the indicated conditions. Percentages of Gag CAp24-positive cells are indicated. At the bottom left, mean + SD percentages of Gag CAp24-positive indicator CD4$^+$ lymphocytes as calculated from the data for three independent experiments are shown.

most relevant early events following the contact between HIV-1-infected and CD4$^+$ target cells (Ruggiero *et al.*, 2008). Although no detectable HIV-replication activity couples with endocytic virus internalization in target cells (Ruggiero *et al.*, 2008), this phenomenon shows alternative aspects of pathogenetic relevance, especially when DCs are considered as target cells.

Here, we report that treatment with ritonavir, indinavir or saquinavir inhibits cell-to-cell HIV-1 endocytosis strongly in iDC target cells and, worthy of note, also when primary CD4$^+$ lymphocytes were used as donor cells. Of importance, these results were not generated by a reduced binding

of the anti-CAp24 mAb to the Gag p55 precursor, as (i) the overall Gag-related fluorescence levels in PI-treated HIV-1-infected donor cells never differed from those detectable in untreated ones, also considering single cell cultures (Fig. 5a), and (ii) overlapping Gag-fluorescence profiles were also observed by analysing the same numbers of HIV-1 particles from control and PI-treated infected cells (Fig. 5c).

Interestingly, we observed that the PI-dictated inhibition of HIV-1 endocytosis in iDCs correlated with a strongly reduced *trans*-infection activity. The results that we obtained using the PM4 HIV-1 mutant imply that at least
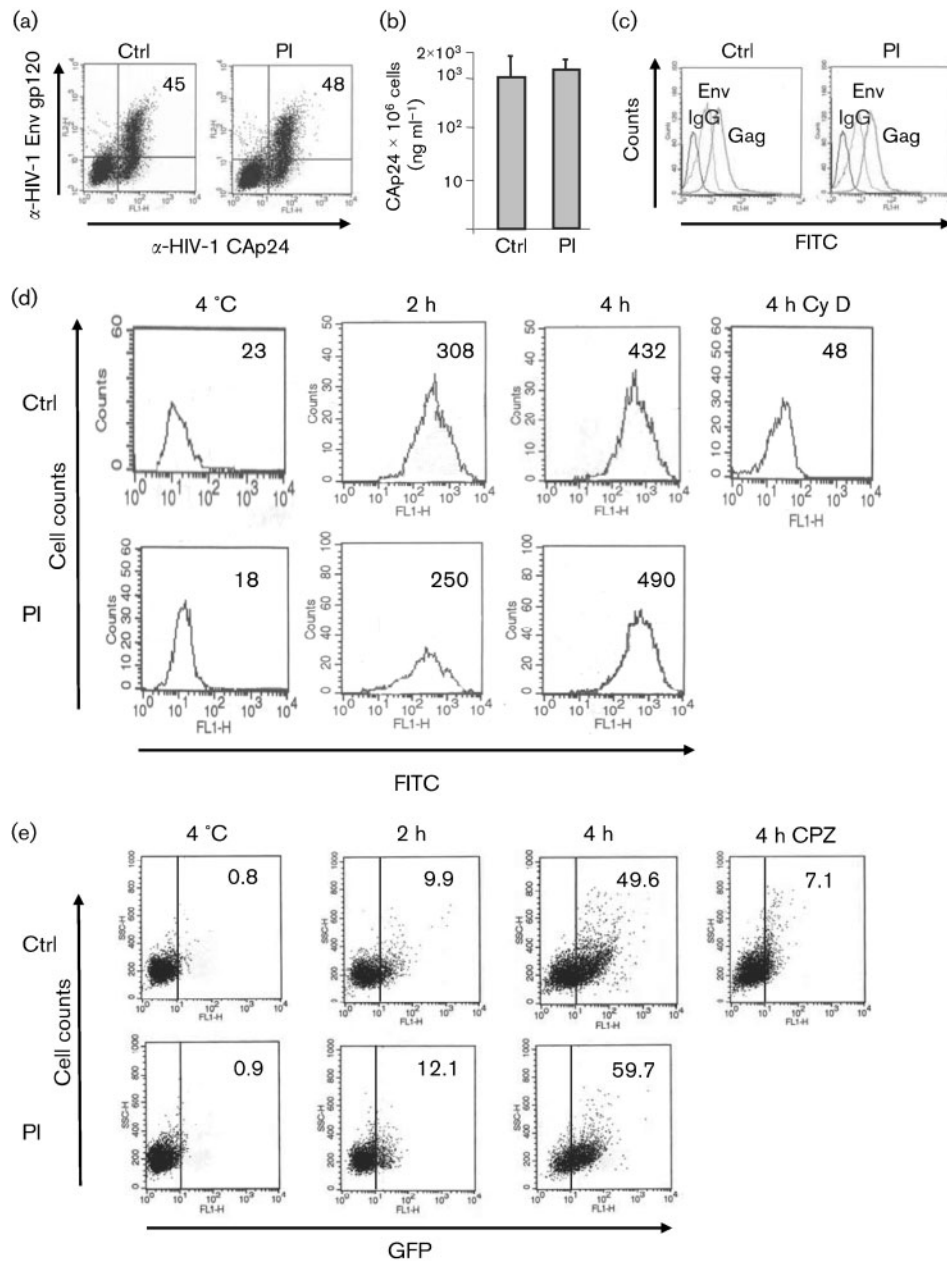
Fig. 5. PIs do not affect either the expression of HIV-1 products or macropinocytosis/virus endocytosis in iDCs. (a) FACS analysis for the detection of both Gag and cell-membrane Env in U937$_{HIV-1}$ cells treated and untreated with PIs. Percentages of Env/Gag double-positive cells are indicated. (b) HIV-1 CAp24 amounts in the supernatants of U937$_{HIV-1}$ cells treated and untreated (Ctrl) with PIs for 16 h. Viability of the cell cultures at the time of supernatant collection was >90 %. The results are given as means + SD for 10^6 cells calculated from three independent experiments. (c) Comparative analysis of the Gag and Env contents in HIV-1 particles from U937$_{HIV-1}$ cells treated or not with PIs for 16 h. HIV-1 particles from the supernatants of PI-treated or untreated (Ctrl) U937$_{HIV-1}$ cells were complexed with surfactant-free white aldehyde/sulfate latex beads and incubated with either anti-HIV-1 Env mAbs or the IgG isotype, and then with FITC-conjugated anti-mouse IgG. Alternatively, the virus–bead complexes underwent permeabilization and labelling with either FITC-conjugated anti-Gag CAp24 mAbs or FITC-conjugated IgG isotypes. The negative-labelling controls for Env and Gag generated overlapping slopes represented by a unique histogram (IgG). The results are representative of two independent experiments. (d) PIs do not affect iDC macropinocytosis. iDCs treated or not (Ctrl) with 5 µM PIs for 16 h were incubated for the indicated times with 1 µg FITC–dextran ml^{-1} at 37 °C and analysed by FACS. As a control, both iDCs treated with the same amounts of FITC–dextran for 4 h at 4 °C and iDCs pre-treated with 5 µM cytochalasin D (Cy D) were analysed. Mean fluorescence intensities are indicated. The results are representative of two independent experiments. (e) PIs do not affect virus endocytosis in iDCs. FACS analysis of iDCs treated and untreated (Ctrl) for 16 h with 5 µM PIs challenged with GFP-labelled (VSV-G) HIV-1 VLPs for the indicated times in the continuous presence of PIs. As a control, both iDCs challenged at 4 °C and iDCs pre-treated with 10 µM chlorpromazine (CPZ) were analysed. Percentages of FITC-positive cells are indicated. The results are representative of two independent experiments.
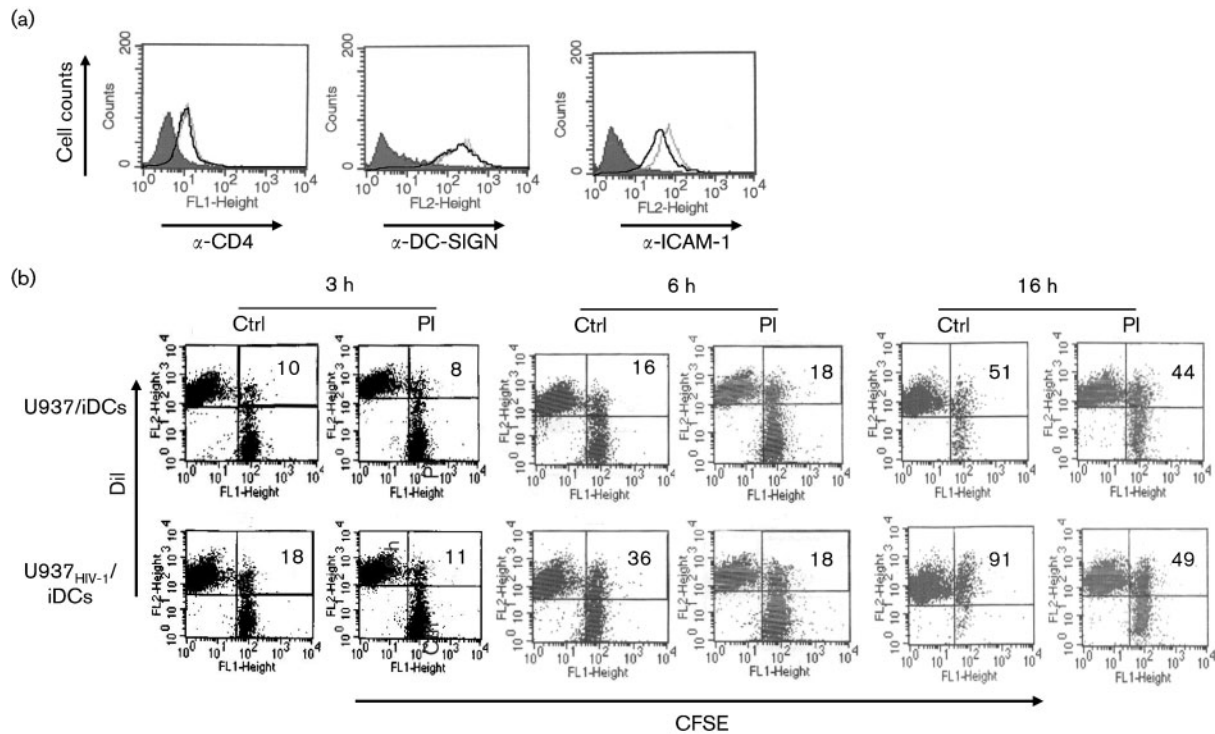
Fig. 6. PIs inhibit ICAM-1 expression in iDCs and the formation of cell−cell conjugates in HIV-1-infected co-cultures. (a) FACS analysis of CD4, DC-SIGN and ICAM-1 on the iDC membrane. iDCs were treated for 16 h with 5 μM PIs (black lines) or left untreated (Ctrl, grey lines) and then labelled with the indicated mAbs. Shaded histograms refer to fluorescence generated by respective IgG isotypes. The results are representative of three independent experiments. (b) Analysis of cell−cell conjugates in co-cultures. CFSE-labelled iDCs were co-cultivated with DiI-labelled U937 or U937$_{HIV-1}$ cells in the presence or absence of 5 μM PIs. After 3, 6 and 16 h, cells were harvested and analysed by FACS. Percentages of bright double-fluorescent events over the total number of iDCs are indicated. The results are representative of two independent experiments.

part of HIV-1 contacting iDCs is stored intracellularly, ready to be released upon contact with susceptible cells. As an alternative interpretation, PIs would inhibit the 'stickiness' of the virion on the iDC membrane. However, we excluded this hypothesis, as no differences in the attachment of fluorescent HIV-1 to the cell membrane of PI-treated iDCs compared with control cells were observed (not shown).

Our observations contribute to the still-unresolved debate concerning the relevance of HIV-1 internalization in DCs for the *trans*-infection process. In this regard, our data are consistent with the idea that iDCs can *trans*-infect indicator cells by releasing endocytosed virions. In fact, as the *trans*-infection of PI-resistant PM4 HIV-1 was also inhibited by PIs, we deduced that the possible presence of residual cell-surface virions resisting the trypsin treatment and that could be transmitted in an endocytosis-independent way did not alter the outcome of our *trans*-infection assays. This supports the hypothesis that HIV endocytosis is part of the DC-directed *trans*-infection process; however, this does not exclude additional mechanisms.

The observation that iDCs form cell–cell conjugates more efficiently with HIV-1-infected cells than with uninfected cells was consistent with the results obtained previously with human T CD4$^+$ cell lines (Chen *et al.*, 2007; Sourisseau *et al.*, 2007). We found that PIs reduced ICAM-1 expression in iDCs cultivated alone or co-cultivated with uninfected or HIV-1-infected cells. Thus, the ICAM-1 downmodulation cannot per se explain the PI-dependent reduction of cell–cell conjugates in HIV-1-infected co-cultures. On the other hand, the number of cell–cell conjugates in PI-treated HIV-1-infected co-cultures resembled that of PI-treated or control uninfected co-cultures. Thus, we may hypothesize that some ICAM-1-related cell-membrane activity induced by HIV-1 and inhibited by PIs would be involved in the cell-to-cell HIV-1 endocytosis mechanism. This does not seem to be the case for LFA-1, whose expression was not changed by HIV-1 expression in donor cells (not shown), but the influence of HIV-1 on the expression of alternative ICAM-1 counter-receptors (e.g. Mac-1) deserves additional investigation. Their identification would shed light on the mechanisms of *trans*-infection and, thus, of HIV spread at the port of

entry. In any case, considering that the percentages of cell–cell conjugates in the presence of PIs reproducibly overcame the levels of HIV-1 endocytosis in iDCs, functions not strictly correlated with cell adhesion would be also involved in the PI inhibitory effect.

The PI-induced inhibition of HIV endocytosis could also be regarded as a phenomenon of relevance in terms of anti-HIV prophylaxis, considering that DCs play a key role in HIV mucosal transmission. In this respect, inhibiting the DC-mediated delivery of HIV from the port of entry to bystander lymphocytes and peripheral lymph nodes would mean practising an effective anti-HIV microbicide strategy. In view of the disappointing outcomes from most recent vaccine trials (Sekaly, 2008), as well as from past generations of microbicides (Van Damme et al., 2008), finding new ways to control HIV epidemics remains a goal of a major relevance. Our data would be a great encouragement for a preclinical evaluation of PIs as anti-HIV microbicides.

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