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A clathrin–dynamin-dependent endocytic pathway for the uptake of HIV-1 by direct T cell–T cell transmission

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ABSTRACT

Cellular contacts between HIV-1-infected cells and target primary T CD4+ lymphocytes trigger the formation of a structure known as the virological synapse. As a consequence, viral production in HIV-1-infected cells is polarized towards the virological synapse and nascent viral particles are directly transferred to target T CD4+ lymphocytes. In this study, we performed short time cocultures of target primary T CD4+ lymphocytes with effector T cells infected by either HIV-1 NL4-3 or BaL. Using flow cytometry and immuno-confocal analyses, we investigated the transfer of HIV-1 virion antigens. We found that after 3 h of coculture, unstimulated T CD4+ lymphocytes captured complete HIV-1 virions from infected T cells during cell–cell contacts. Virus transfer occurred through a dynamin-dependent pathway and could be inhibited by chlorpromazine, an inhibitor of clathrin-dependent endocytosis. Transferred HIV-1 virions were located in compartments close to the surface of the target cell in a polarized manner. These compartments were positive for clathrin and the early endosomal marker EEA1 but were negative for caveolin-1. Furthermore, the great majority of internalized HIV-1 particles did not colocalize with Lamp1, a well-known marker for the lysosomal-degradative pathway. Similar results were observed when stimulated primary T CD4+ lymphocytes were the target cells. Our results suggest a mechanism of cell to cell HIV-1 transfer through a clathrin- and dynamin-dependent early endocytic pathway where internalized HIV-1 particles would not reach Lamp1 positive compartments, suggesting that during HIV-1 transfer by cell–cell contacts, virions can be taken up by endocytosis but not be degraded in lysosomes.

1. Introduction

HIV entry is a complex process which first relies on a number of cellular factors (Lama and Planelles, 2007). HIV-1 utilizes CD4 as its primary receptor followed by engagement with a coreceptor (CCR5 or CXCR4), and leading to TMgp41-dependent membrane fusion. Agents targeting virus entry potently block HIV replication (Esté and Telenti, 2007). Contacts between infected and non-infected target cells increase the efficiency with which the virus is transmitted to non-infected T CD4+ cells. This process of cell to cell HIV-1 transmission through the virological synapse (VS) implies polarization of both the viral production in the infected cell and the viral receptor and coreceptor in the target cell. In the presence of the appropriate coreceptor, VS formation triggers a rapid and efficient infection of target cells (Jolly et al., 2004). On the contrary, in the absence of the appropriate coreceptor, these cellular contacts may induce the transfer of high amounts of HIV-1 particles from infected cells to T CD4+ cells without immediate virus–membrane fusion (Blanco et al., 2004b). HIV-1 transmission may only depend on the interaction between the viral envelope SUgp120 and the cellular CD4 receptor (Bosch et al., 2005; Chen et al., 2007). HIV-1 particles taken up by T CD4+ cells in a coreceptor independent manner may reside in large intracellular vesicles, suggesting an endocytic process (Blanco et al., 2004b). The classical model of HIV-1 infection of T CD4+ lymphocytes establishes that envelope-mediated fusion at the plasma membrane is required for of HIV-1 core entry into the cytoplasm, often leading to a productive infection (Gallo et al., 2003). In addition, free HIV-1 can also enter target cells using the endocytic route (Daecke et al., 2005; Fackler and Peterlin, 2000; Schaeffer et al., 2004). However, this process may promote the inactivation and degradation of internalized viral particles, as a consequence of the gradual acidification of the endocytic compartments and the lysosomal degradation (Fredericksen et al., 2002; Wei et al., 2005). Nevertheless, we have shown that cells containing virus in

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endosomes after cell to cell transfer, may discharge viral particles that remain infectious to the extracellular medium (Blanco et al., 2004b; Bosch et al., 2005).

Characteristic membrane structures can be identified in each endocytic pathway (Conner and Schmid, 2003). Caveolae, required for the caveola-mediated endocytosis, are microdomains at the plasma membrane which contain caveolin-1 (Cav-1). On the other hand, the assembly at the plasma membrane of cytosolic coat proteins, such as clathrin, into coated pits triggers the formation of clathrin-coated vesicles. Dynamin is a GTPase that promotes the pinching off of endocytic vesicles from the inner leaflet of the plasma membrane, and it is involved in most of these portals of entry into the mammalian cells (Conner and Schmid, 2003). Once a clathrin-coated vesicle is formed, its cargo is delivered into early endosomes (Gruenberg, 2001) which contain the early endosome antigen 1 (EEA1) (Mu et al., 1995; Rubino et al., 2000). The degradation pathway continues towards late endosomes and lysosomes where the lysosome-associated membrane protein 1 (Lamp1) is highly enriched.

Here, we show that during cell to cell contacts, transferred HIV-1 particles were colocalized with dynamin, clathrin and EEA1 markers but not with Lamp1. Our results suggest that infected T cells may transfer a pool of HIV-1 virions through a clathrin-dependent endocytic pathway to T CD4+ lymphocytes which, in turn, may act as transitory recipients of HIV-1 viral particles in the absence of virus replication.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMC) from healthy donors were purified by Ficoll-Hypaque sedimentation. Unstimulated T CD4+ lymphocytes were immediately purified (>90%) from PBMC by negative selection using the T CD4+ cell enrichment kit (StemCell Technologies, Vancouver, Canada). This method renders purified T CD4+ cells without any interaction with the CD4 protein, in contrast to the CD4 positive selection, which uses CD4 antibodies that may alter CD4 function. When activated T CD4+ lymphocytes were required, PBMC were stimulated with phytohemagglutinin (PHA, Sigma, Madrid, Spain) at 4 μg/ml and 6 U/ml interleukin (IL)-2 (Roche) for 72 h. Then, activated PBMC were subjected to a negative selection to obtain the activated T CD4+ lymphocytes. Media were supplemented with 10% or 20% heat inactivated fetal calf serum (Invitrogen, Madrid, Spain), 100 U/ml penicillin, 100 μg/ml streptomycin. Chronically HIV-1-infected MOLT cells were generated in the laboratory after infection of MOLT-4/CCR5, a CD4+, CXCR4+ and CCR5+ lymphocytic cell line, with the following recombinant viruses carrying the HIV-1 envelope sequences corresponding to the X4 HIV-1 strain NL4-3 or the R5 HIV-1 strain BaL constructed in a HIV_{HXB2} backbone (Blanco et al., 2001; Moncunill et al., 2008). Persistently infected cultures of $HIV\text{-}1_{NL4\text{-}3}$ (MOLT-NL4-3) and $HIV\text{-}1_{BaL}$ (MOLT-BaL) were grown and characterized for Env expression and virus production (Blanco et al., 2004a). Uninfected MOLT-4/CCR5 cells (MOLT-uninfected) were used as negative controls in all experiments.

2.2. Cocultures of infected and uninfected cells

Primary cells (purified T CD4+ cells, 2×10^5 cells for flow cytometry experiments or 2.5×10^6 cells for immuno-confocal microscopy experiments) were cultured with effector (uninfected or infected) MOLT cells at a 1:1 ratio in the absence or the presence of the following HIV-1 inhibitors: 5 μg/ml of the TMgp41 inhibitor C-34 (Service of Peptide Synthesis, University of Barcelona) (Armand-Ugón et al., 2003, 2005); 1 μg/ml of the reverse transcriptase (RT) inhibitor AZT (Sigma); 0.25 μg/ml of the anti-CD4 mAb Leu3a (BD, Madrid, Spain); 10 μg/ml of the CXCR4 antagonist AMD3100; 5 μg/ml of the CCR5 antagonist TAK779. Cocultures were incubated at 37 °C for 3, 4 or 6 h to evaluate HIV-1 transfer as described below. In some cocultures, we used chlorpromazine (Sigma), an inhibitor of clathrin-dependent endocytosis, at 8 or 2 μg/ml. In this case, T CD4+ lymphocytes were treated with chlorpromazine for 30 min at 37 °C prior the addition of infected MOLT cells for 4 h. Cocultures with stimulated T CD4+ lymphocytes were incubated in the presence of 10 U/ml of IL-2.

2.3. Evaluation of chlorpromazine activity

Primary T CD4+ lymphocytes were resuspended in RPMI supplemented with 0.2% of bovine serum albumin (BSA, Sigma) and pretreated with or without 8 or 2 μg/ml of chlorpromazine for 30 min at 37 °C. Then, pretreated T CD4+ lymphocytes were cultured in the presence or the absence of phorbol 12-myristate 13-acetate (PMA, Sigma) at 0.1 or 0.01 μg/ml for 30 min at 37 °C. Cells were fixed with 2% formaldehyde for 15 min at room temperature, and washed with phosphate-buffered saline (PBS) and stained for CD4 expression with the Leu3a anti-CD4 mAb conjugated with the fluorochrome FITC (BD, Madrid, Spain). Cells were analyzed in a FACScalibur flow cytometer (BD) and identified by morphology.

2.4. Evaluation of HIV-1 transfer

In the cocultures, HIV-1 p24 antigen (CAp24) transfer was evaluated. Cells were trypsinized to eliminate HIV-1 particles bound to the cell surface. For trypsin treatment, cells were washed with PBS and treated (10 min, room temperature) with 0.25% trypsin solution (Invitrogen, Madrid, Spain). The action of trypsin was controlled by the disappearance of the Leu3a epitope of T CD4+-uninfected lymphocytes (Blanco et al., 2004b). Trypsin was stopped by addition of fetal calf serum. Cells were then washed with PBS and stained with Leu3a anti-CD4 mAb. Then, for intracellular staining cells were fixed, permeabilized (Fix & Perm, Caltag, Burlingame, CA) and stained with KC57 anti-HIV-CAp24 antigen mAb (Coulter, Barcelona, Spain). Cells were analyzed in a FACScalibur flow cytometer (BD, Madrid, Spain) and identified by morphology. Quantification of HIV-1 transfer was assessed by the percentage of CAp24 positive cells (using cocultures between primary T CD4+ lymphocytes and MOLT-uninfected cells as negative controls).

2.5. Immunofluorescent staining

Cocultures were fixed in 3% paraformaldehyde for 20 min. Then, cells were washed with PBS, stuck over a coverslip and free aldehydes were quenched with 50 mM NH_4Cl. Cells were permeabilized using 0.2% Triton X-100 for 5 min and blocked in 1% BSA (diluted in PBS) for 15 min. Then, cells were incubated for 1 h at room temperature with the following primary monoclonal antibodies: rabbit anti-HIV-MAp17 (NIH AIDS Reagent Program), mouse anti-HIV-CAp24 (#P3D10G9B8), goat anti-human-EEA1 (Santa Cruz Biotechnology Inc.), rabbit anti-human-caveolin-1, human anti-HIV-gp120 (b12, NIH AIDS Reagent Program), mouse anti-human-Lamp1 (Santa Cruz Biotechnology Inc.) and goat anti-human-Dynamin (Santa Cruz Biotechnology Inc.). Clathrin staining was performed at 37 °C for 1 h using the monoclonal antibody mouse anti-human-clathrin (BD, Madrid, Spain). For CD4, staining the mouse L120.3 anti-human-CD4-FITC (BD, Madrid, Spain) mAb was used. Cells were washed three times in 1% BSA and incubated for 1 h at room temperature with the corresponding fluorescent

Alexa 488-, 546- and 633-conjugated secondary antibodies (Molecular Probes, Orlando, FL). Coverslips were washed three times with PBS and mounted with Mowiol (Sigma). Samples were observed on Axioplan 2 Zeiss CLSM 510 confocal microscope with Argon 488/458 and HeNe 543 and HeNe 633 lasers and a plan Apochromat 63x1.4 oil objective, supplied with LSM 510 3.4 software. Percentage of Gag (MAp17 or CAp24) colocalization with endosomal markers was evaluated by the Metamorph software.

3. Results

3.1. Location of HIV-1 in a dynamin-dependent endocytic pathway

To study cell to cell HIV-1 transfer we incubated cocultures of unstimulated primary T CD4+ lymphocytes with uninfected or persistently infected with HIV-1_{NL4-3} or HIV-1_{BaL} MOLT cells. Both cell types, primary lymphocytes and MOLT cells, could be clearly distinguished by morphological parameters, as seen by flow cytometry or confocal laser scanning microscopy (CLSM) (Fig. 1A). As previously shown (Blanco et al., 2004b; Bosch et al., 2005), short time cocultures between effector MOLT-NL4-3 or MOLT-BaL cells with unstimulated primary T CD4+ lymphocytes triggered the transfer of high amounts of CAp24 antigens in trypsin-resistant compartments in the target cell (Fig. 1B). This process was only inhibited by the Leu3a anti-CD4 mAb, which specifically blocks the interaction between CD4 and SUgp120. Neither the fusion inhibitor C-34 or the coreceptors antagonists AMD3100 and TAK779 were able to inhibit the cell to cell HIV-1 transmission. CLSM showed that T CD4+ lymphocytes cocultured for 3 h with the uninfected control cells exhibited the CD4 receptor distributed all along the plasma membrane (Fig. 1C, upper panels). However, when target cells were cocultured with MOLT-BaL cells, they exhibited the CD4 receptor polarized where HIV-1 antigens had been transferred (Fig. 1C, lower panels). Taken together, these results confirmed that cellular contacts between T CD4+ lymphocytes and HIV-1-infected MOLT cells triggered the transfer of high amounts of HIV-1 particles at the same time that CD4 receptor was recruited at the contact site. This

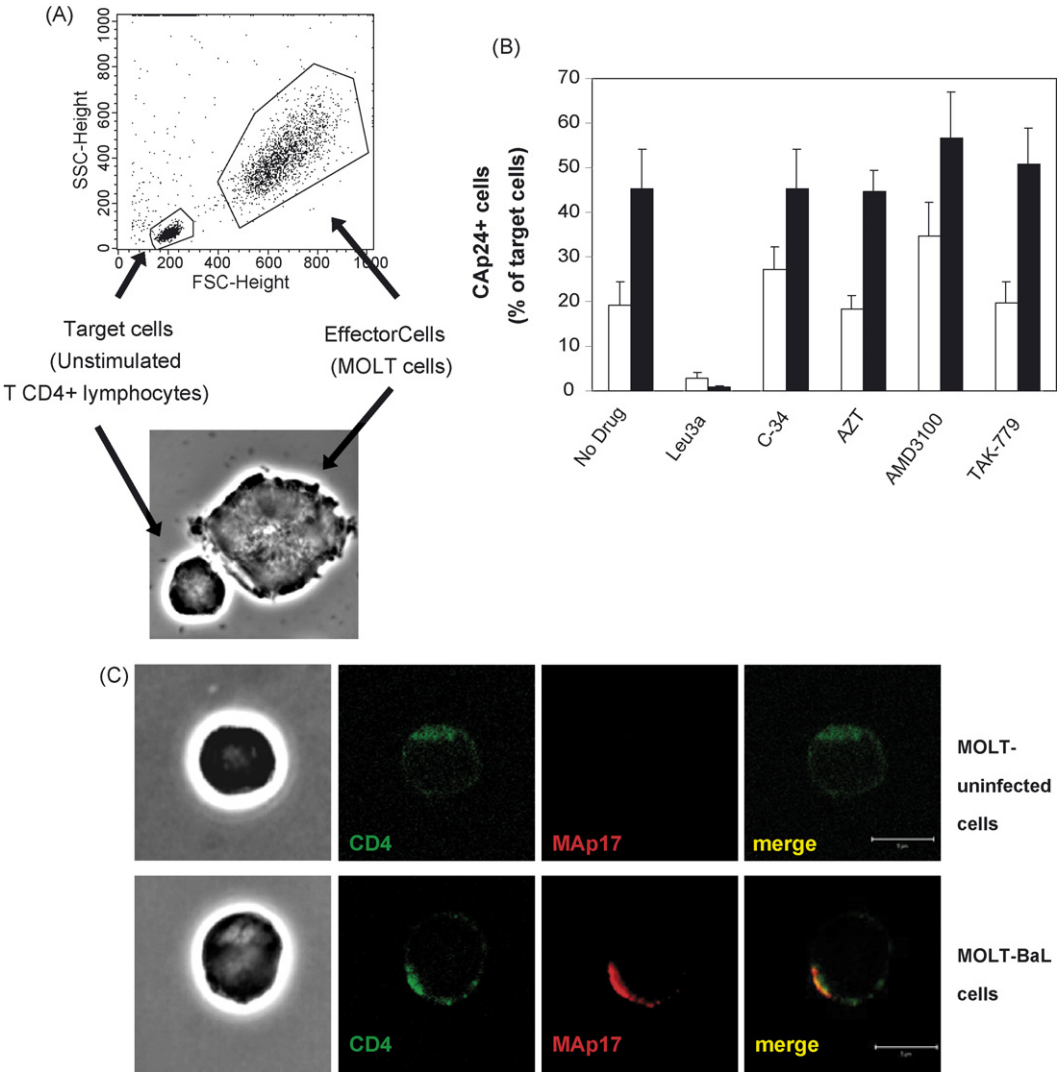

Fig. 1. HIV-1 is internalized into trypsin-resistant compartments of T CD4+ lymphocytes by a CD4 receptor-dependent process. (A) Cocultures between MOLT-uninfected cells and T CD4+ lymphocytes observed by flow cytometry (upper panel) or CLSM (lower panel). (B) T CD4+ lymphocytes were cocultured for 4 h with MOLT-NL4-3 (white bars) and MOLT-BaL (black bars) cells in the absence or the presence of mAb Leu3a at 0.25 μg/ml, C-34 at 5 μg/ml, AZT at 1 μg/ml, AMD3100 at 10 μg/ml and TAK-779 at 5 μg/ml. CAp24 antigen was stained after trypsin treatment. Cells were analyzed by flow cytometry and identified by morphology. Data are the mean ± S.D. of three independent experiments. (C) Three-hour cocultures between T CD4+ lymphocytes and MOLT-uninfected (upper panel) or MOLT-BaL cells (lower panel) were treated with trypsin and stained for CD4 (green) and MAp17 (red). Colocalization is shown in yellow. One T CD4+ lymphocyte representative of each coculture is shown and are representative of two independent experiments.

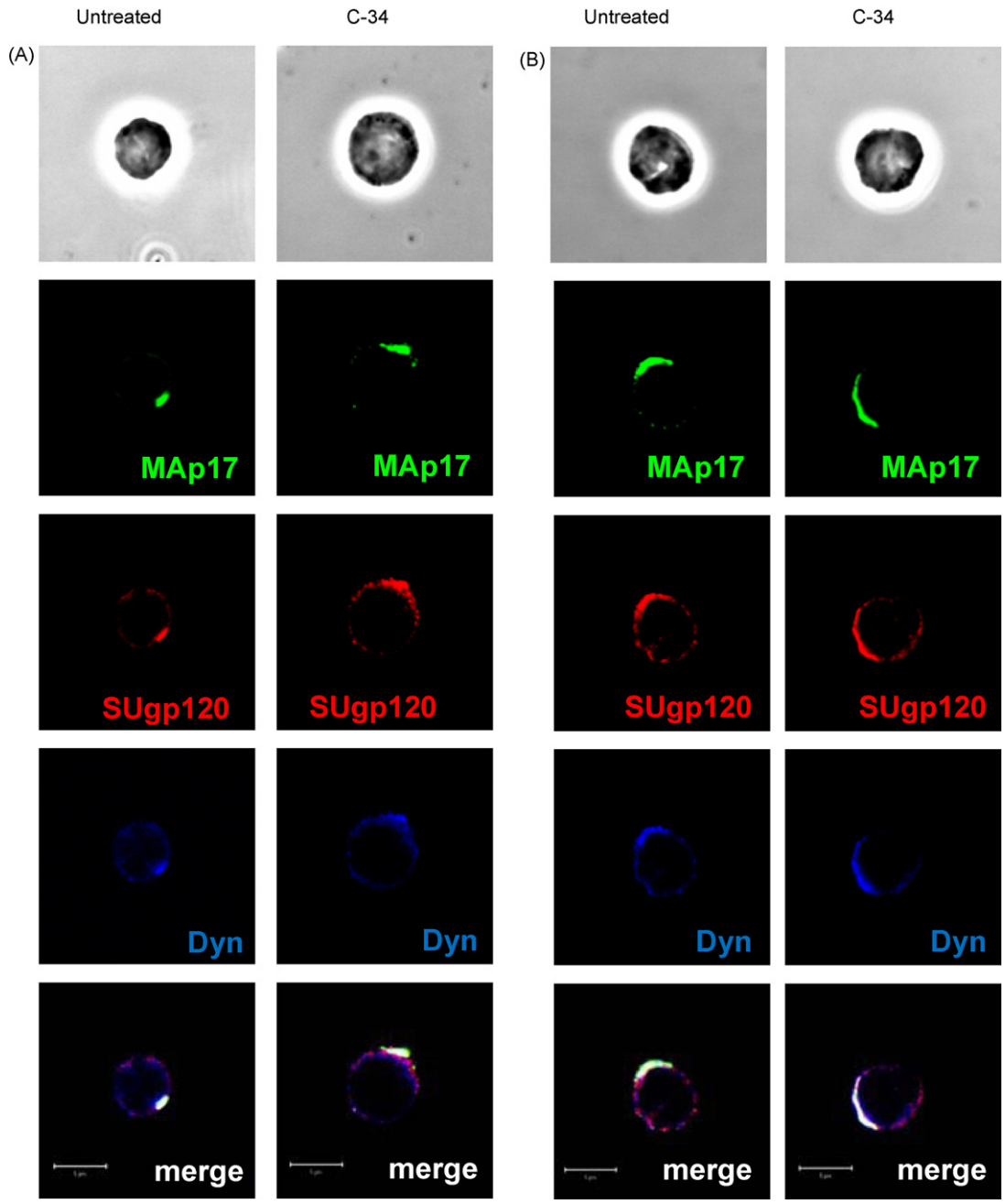

Fig. 2. Transfer of HIV-1 particles into T CD4+ lymphocytes through a dynamin-dependent pathway. Primary T CD4+ lymphocytes were cocultured with MOLT-NL4-3 (A) or MOLT-BaL (B) cells for 3 h in the presence or the absence of 5 μg/ml of C-34. After coculturing, cells were stained for MAp17 (green), SUgp120 (red) and dynamin (Dyn, blue). Colocalization is shown in white. Cells were identified by morphology. One T CD4+ lymphocyte representative of each coculture is shown.

process was dependent on the interaction between SUgp120 and CD4.

Transferred HIV-1 particles via cell–cell contacts located in intracellular vesicles (Blanco et al., 2004b). To identify the pathway by which these viral particles were internalized in target T CD4+ lymphocytes, we studied the location of different endosomal markers after 3 h cocultures between unstimulated T CD4+ lymphocytes and MOLT-NL4-3 (Fig. 2A) or MOLT-BaL cells (Fig. 2B). Cocultures were untreated or treated with C-34 or AZT to inhibit productive infection of target cells by blocking fusion events mediated by the TMgp41 glycoprotein and the retrotranscriptase activity, respectively. After coculturing, cells were trypsinized and flow cytometry analysis showed that 24% and 55% of T CD4+ lymphocytes cocultured with MOLT-NL4-3 and MOLT-BaL effector cells, respectively, were positive for intracellular CAp24. Immunofluorescence staining revealed that MAp17 and SUgp120 antigens colocalized close to the plasma membrane with a polarized phenotype, and at the same time they colocalized with dynamin. Quantification of Gag colocalization with dynamin determined that at least 67% of transferred Gag antigens colocalized with this GTPase, independently of the viral strain used and the presence or the absence of C-34 and AZT (Table 1). These results suggest that complete HIV-1 particles were transferred from the effector cells to target primary T CD4+ lymphocytes and that the endocytic pathway involved in this transfer would require the presence of dynamin.

3.2. Internalization of HIV-1 particles through a clathrin-mediated endocytic pathway

Dynamin plays a role in caveola-dependent, clathrin-dependent and in some clathrin- and caveola-independent endocytic pathways. The mechanisms involved in the clathrin- and caveola-independent endocytosis are poorly understood, whereas clathrin and caveolin-1 are well established markers for the clathrin-mediated endocytosis and caveola-mediated endocytosis, respectively. The lack of colocalization between CAp24 and caveolin-1 (Fig. 3A; Table 1) suggested that caveola-mediated endocytic pathway was not involved in T cell to T cell HIV-1 transfer. The simultaneous labeling of MAp17, SUgp120 and clathrin showed a triple colocalization of these three markers independently of the viral strain used and the presence or the absence of C-34 or AZT (Fig. 3B; Table 1). To confirm the role of clathrin-dependent endocytosis in T cell to T cell HIV-1 transfer, we cocultured unstimulated primary T CD4+ lymphocytes with MOLT-infected cells in the presence of chlorpromazine for 4 h. Chlorpromazine is an inhibitor of the clathrin-dependent endocytosis preventing the assembly of clathrin coated pits at the cellular membranes. At 8 μg/ml, a non-cytotoxic concentration where the production of HIV-1 particles from MOLT cells and the fusion capability of the envelope expressed by MOLT cells were unaltered (data not shown), chlorpromazine significantly inhibited HIV-1 transfer from MOLT-NL4-3 ($53 \pm 6\%$ inhibition, $p = 0.0049$, paired t-test) and MOLT-BaL cells ($44 \pm 13\%$ inhibition, $p = 0.0293$, paired t-test) to T CD4+ lymphocytes (Fig. 3C). Inhibition by chlorpromazine appears to be specific since the formation of clathrin-coated pits, and the downregulation of CD4 receptor in primary PMA-stimulated T CD4+ lymphocytes were prevented (Fig. 3D). Indeed, phorbol ester-induced downregulation of CD4 is a process involving a clathrin-dependent endocytic pathway (Pelchen-Matthews et al., 1993). Taken together, these data suggested that HIV-1 particles from infected MOLT cells may enter unstimulated primary T CD4+ lymphocytes through a clathrin-dependent endocytic pathway.

3.3. Internalized HIV-1 particles are not targeted to Lamp1 positive compartments

Receptors and other cell-surface components internalized through clathrin-mediated endocytosis are delivered to early endosomes (Gruenberg, 2001). Indeed, transferred viral particles were found to colocalize with EEA1, independently of the viral strain used and the presence of C-34 or AZT (Fig. 4; Table 1), suggesting that internalized HIV-1 particles were routed to the lysosomal endocytic pathway (Gruenberg, 2001). HIV-1 can enter the target cell by fusion or endocytosis. Fusion promotes the productive infection of the target cell (Gallo et al., 2003), and it is widely accepted that endocytosis generally causes HIV-1 degradation in lysosomes (Fredericksen et al., 2002; Wei et al., 2005). To clarify the fate of internalized HIV-1, in late endosomes or lysosomes, we analyzed the staining of MAp17, SUgp120 and Lamp1 in trypsin-treated cocultures of unstimulated primary T CD4+ lymphocytes with MOLT-NL4-3 or MOLT-BaL cells. Lamp1 is a marker of the lysosomal-degradative pathway that has been previously used to detect the presence of viral particles in the lysosomal compartments (Chu and Ng, 2004). MAp17, SUgp120 and Lamp1 staining revealed that, independently of the viral strain used, only a negligible fraction of MAp17 was localized in Lamp1 positive compartments (Fig. 5A; Table 1). As expected, MAp17 colocalized with SUgp120 (Fig. 5A, yellow arrows). Therefore, most of the incoming HIV-1 particles appeared to be excluded from the lysosomal-degradative pathway. This observation is in accordance with our previous findings where HIV-1 particles internalized into primary T CD4+ lymphocytes were released in the extracellular medium and remained infectious (Blanco et al., 2004b).

Finally, we investigated if the endocytic internalization of HIV-1 antigens in unstimulated primary T CD4+ lymphocytes could also be observed in target cells where HIV-1 can establish a productive infection. Thus, we tested cocultures using MOLT-NL4-3 as effector cells and PHA/IL-2 stimulated primary T CD4+ lymphocytes as target cells. Cocultures were treated with trypsin and tested for MAp17–EEA1–clathrin and MAp17–EEA1–Lamp1. As observed with unstimulated target cells, HIV-1 antigens in PHA/IL-2 stimulated T CD4+ lymphocytes colocalized with clathrin and EEA1, but not with Lamp1 (Fig. 5B). Thus, cell-to-cell HIV-1 transfer through the clathrin- and dynamin-dependent endocytic pathway could also occur in cells were virus fusion and productive infection occurred. HIV-1 particles internalized in the stimulated T CD4+ lymphocytes were not targeted to the lysosomes as Lamp1 and MAp17 colocalization was not observed.

4. Discussion

HIV viral transfer, through cell-to-cell contacts and viral synapses, has been shown to play an important role in HIV spread and transmission, and different transfer mechanisms have been described (Jolly and Sattentau, 2007; Lehmann et al., 2005; Sowinski et al., 2008).

Here, we examined the endocytic pathway by which HIV-1 was internalized into T CD4+ lymphocytes. After cellular contacts, MAp17 and SUgp120 were located in the same subcellular compartment of the target cell, suggesting that complete viral particles had been transferred and found that this process may be mediated by dynamin and clathrin. Dynamin is a GTPase required for clathrin-mediated endocytosis, caveola-mediated endocytosis and some clathrin- and caveola-independent endocytic pathways (Conner

Table 1
Quantification of Gag colocalization with endosomal markers[a]

Effector cell	Treatment	Endosomal marker				
		Dynamin	Caveolin-1	Clathrin	EEA1	Lamp1
MOLT-NL4-3	ND	81 ± 3.6	9 ± 7.8	48 ± 16.6	59 ± 0.2	0
	C-34[b]	74 ± 5.3	3 ± 5.1	75 ± 16.2	71 ± 16.5	0
	AZT[c]	73 ± 18.2	3 ± 1.3	68 ± 10.5	80 ± 22.4	0 ± 0.6
MOLT-BaL	ND	67 ± 5.6	6 ± 2.8	72 ± 9.3	74 ± 12	5 ± 6.3
	C-34[b]	71 ± 20.1	6 ± 2.8	84 ± 6.4	70 ± 11.2	1 ± 0.8
	AZT[c]	67 ± 12.1	2 ± 1.9	84 ± 4	77 ± 12.2	2 ± 0.5

[a] The percentage of Gag colocalization with the corresponding endosomal markers was evaluated using the Metamorph software. Values are the mean ± S.D. of three representative quantifications.
[b] C-34 was used at a final concentration of 5 μg/ml.
[c] AZT was used at a final concentration of 1 μg/ml.

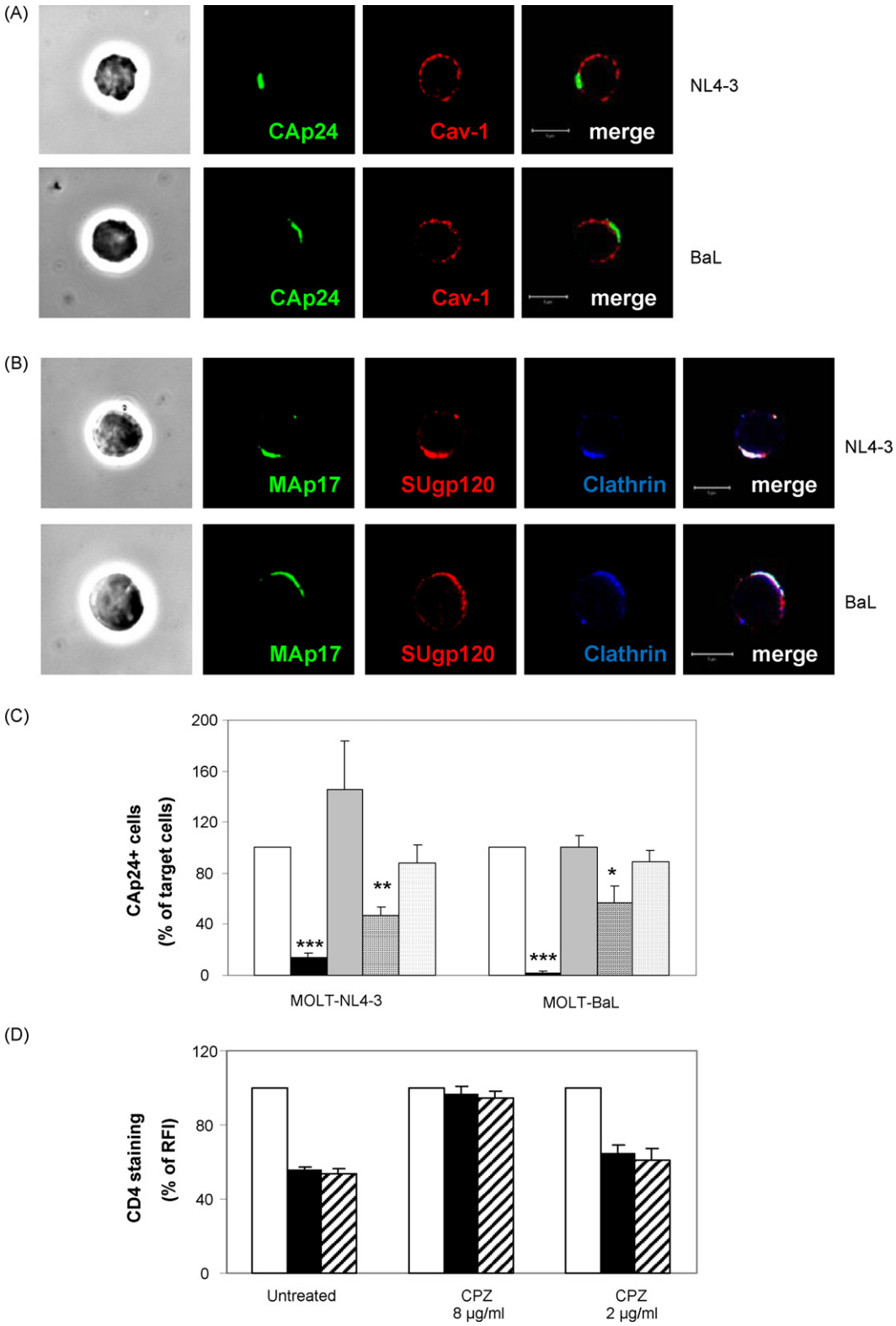

Fig. 3. Transfer of HIV-1 particles via a clathrin-dependent endocytic pathway. Primary T CD4+ lymphocytes were cocultured with MOLT-NL4-3 or MOLT-BaL cells for 3 h with C-34 at 5 μg/ml. Then, cocultures were treated with trypsin and stained for (A) Cap24 (green) and caveolin-1 (Cav-1, red) or (B) MAp17 (green), SUgp120 (red) and clathrin (blue). Triple colocalizations are shown in white. One T CD4+ lymphocyte representative of each coculture is shown. (C) T CD4+ lymphocytes were cocultured for 4 h with MOLT-NL4-3 or MOLT-BaL cells in the absence (white bars) or the presence of Leu3a (black bars), C-34 (grey bars), CPZ at 8 μg/ml (striped bars) or CPZ at 2 μg/ml (dotted bars). After 4 h of coculturing, cells were treated with trypsin, stained for CAp24 antigen and analyzed by flow cytometry. Cells were identified by morphological parameters. Data are the mean ± S.D. of three independent experiments (***$p < 0.001$, **$p < 0.005$, *$p < 0.05$, paired t-test). (D) T CD4+ lymphocytes were pretreated for 30 min with or without 8 and 2 μg/ml of chlorpromazine (CPZ) prior being cultured in the absence (white bars) or the presence of PMA at 0.01 μg/ml (black bars) or 0.1 μg/ml (striped bars) for 30 min. Then, cells were fixed with 2% of formaldehyde and surface CD4 expression was evaluated with the anti-CD4 mAb Leu3a-FITC. Cells were analyzed by flow cytometry and identified by morphology. Data are the mean ± S.D. of two independent experiments.

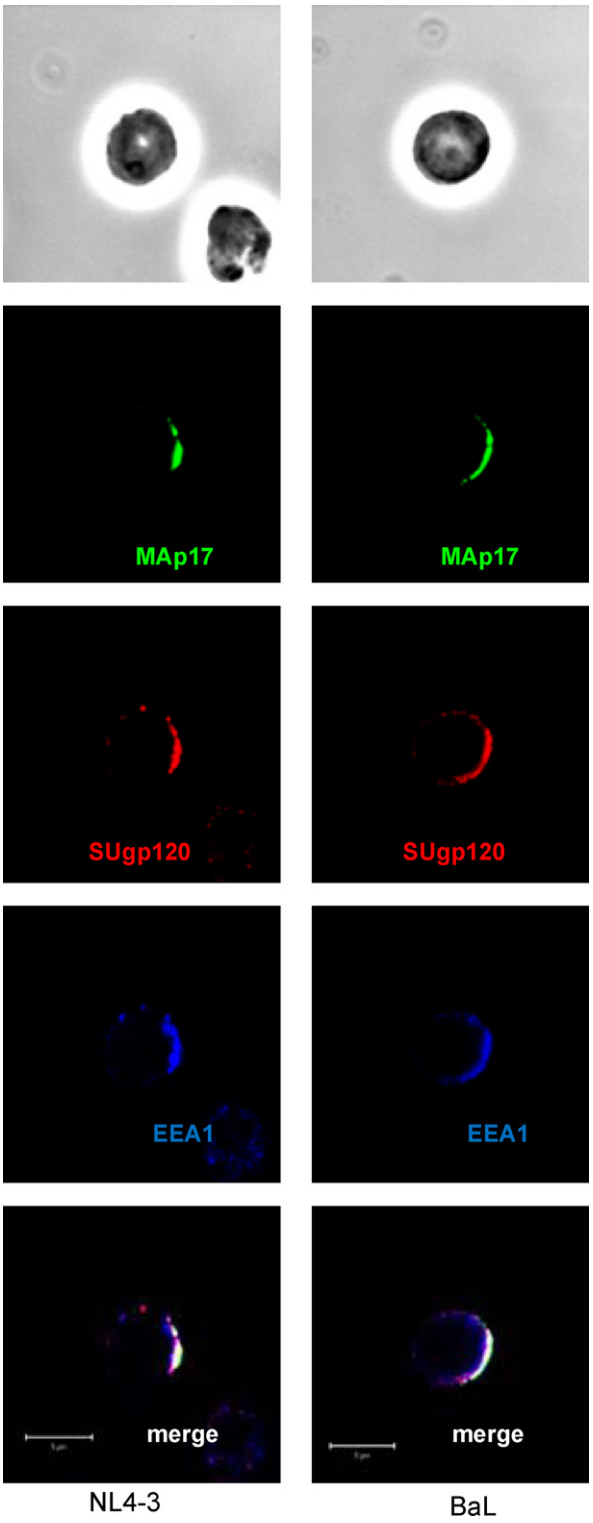

Fig. 4. Colocalization of HIV-1 particles with EEA1. Primary T CD4+ lymphocytes were cocultured with MOLT-NL4-3 (left panels) or MOLT-BaL cells (right panels) for 3 h in the presence of C-34 at 5 μg/ml. Then, cocultures were treated with trypsin and stained for MAp17 (green), SUgp120 (red) and EEA1 (blue). Triple colocalization is shown in white. One T CD4+ lymphocyte representative of each coculture is shown.

and Schmid, 2003). Clathrin-mediated endocytosis involves the concentration and assembly of transmembrane receptors and their bound ligands into coated pits on the plasma membrane, clathrin being the main assembly unit (Conner and Schmid, 2003; Hinshaw, 2000). On the other hand, caveolin-1 structurally organizes caveolae necessary for caveola-mediated endocytosis (Conner and Schmid, 2003). Thus, localization of HIV-1 antigens in compartments positive for dynamin and clathrin but not for caveolin-1 suggested that the endocytic pathway involved in T cell to T cell HIV-1 transfer was coated pit-mediated. Moreover, chlorpromazine, an inhibitor of clathrin assembly that prevents the formation of clathrin-coated pits (Blanchard et al., 2006; Stuart and Brown, 2006), was able to significantly inhibit the transfer of HIV-1 antigens into the target cells. Higher inhibition values could not be achieved due to the cytotoxicity of chlorpromazine at higher concentrations (Blanchard et al., 2006; Stuart and Brown, 2006). Thus, we cannot exclude that other cellular mechanisms might be participating in HIV-1 internalization in target T CD4+ lymphocytes.

The role of clathrin-dependent endocytosis in the entry of cell-free HIV-1 into HeLa cells has been recently described (Daecke et al., 2005) but a different pathway of entry may be at work (Pelchen-Matthews et al., 1991). However, our results clearly point to a clathrin–dynamin-dependent HIV-1 entry mechanism but not to a Lamp1+ compartment during transfer of HIV-1 to primary T CD4+ lymphocytes via cell-to-cell contacts.

Detection of viral antigen in primary T CD4+ lymphocytes was not dependent on virus replication in these target cells, as virus was transferred to target cells at short times post-exposure and could not be prevented by the HIV-1 inhibitors AZT or C-34. Activated lymphocytes may become infected and appear in flow cytometry measurements as CAp24+ and morphologically similar to MOLT cells. Thus, MOLT cells may contaminate the activated PBMC gate. Nevertheless, virus transfer to PBMCs in the presence of relatively high concentrations of TAK-779, AMD3100 and AZT suggest that the possibility of lymphocytes becoming infected is very low.

Coreceptor inhibitors, that block virus fusion and replication, did not prevent cells from capturing HIV particles that are later internalized. Commonly, this process lead to virus particle degradation (dead-end pathway); however, we showed that, alternatively, virus may be “regurgitated” and remain infectious. Moreover, even though only a small percentage of unstimulated primary T CD4+ lymphocytes express the chemokine receptor CCR5, an endocytic internalization of viral particles was observed using an R5 strain, suggesting that the presence of the appropriate coreceptor was not required for T cell to T cell viral transfer (Blanco et al., 2004b). However, we cannot discard that the presence of the appropriate coreceptor on the HIV-1 containing vesicle could trigger the productive infection of the target cell. In this regard, it has been recently described that some HIV-1 strains may be endocytosed and may fuse with the endosomal limiting-membrane in a CXCR4-dependent manner, establishing a productive infection (Maurin et al., 2007). In our model, the same endocytic internalization was observed in stimulated primary T CD4+ lymphocytes where HIV-1 could actively replicate, showing that the transfer mechanism also occurred in cells susceptible to classical fusion-dependent HIV entry (Blanco et al., 2004b).

It has been widely reported that endocytosis of HIV-1 cannot result in a productive infection, since the endocytic pathway may lead viral particles into acidic compartments where they can be inactivated and finally degraded in the lysosomes or the proteosome (Fredericksen et al., 2002; Schaeffer et al., 2004; Wei et al., 2005). However, in our model, internalized viral particles into T CD4+ lymphocytes apparently did not reach Lamp1 positive compartments. HIV-1 antigens did not colocalize with the Lamp1 marker, suggesting that HIV-1 particles escape from the

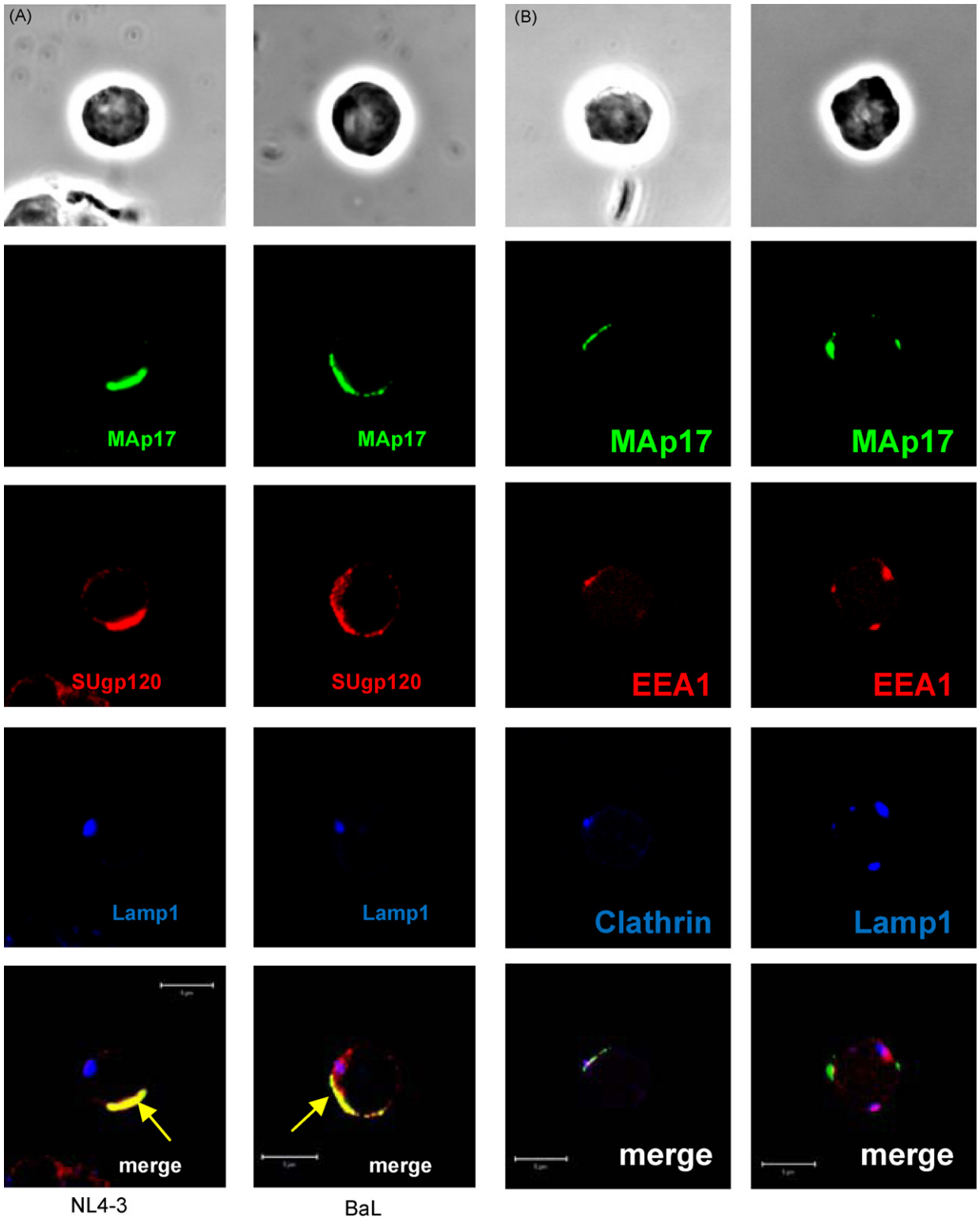

Fig. 5. No-colocalization between incoming HIV-1 particles and Lamp1. (A) Primary T CD4+ lymphocytes were cocultured with MOLT-NL4-3 cells (left panels) or MOLT-BaL cells (right panels) for 3 h in the presence of C-34 at 5 μg/ml. Then, cocultures were treated with trypsin and stained for MAp17 (green), SUgp120 (red) and Lamp1 (blue). Merge images show colocalization of MAp17 with SUgp120 (yellow arrows). One T CD4+ lymphocyte representative of each coculture is shown. (B) Stimulated T CD4+ lymphocytes were cocultured with MOLT-NL4-3 cells 6 h in the presence of C-34 at 5 μg/ml. Then, cocultures were treated with trypsin and stained for MAp17 (green), EEA1 (red) and clathrin (blue) or MAp17 (green), EEA1 (red) and Lamp1 (blue) Triple colocalizations are shown in white. One T CD4+ lymphocyte representative of each coculture is shown.

lysosomal-degradative pathway. This result would be in agreement with our previous findings when we observed that HIV-1 particles, once internalized into primary T CD4+ lymphocytes, could be released back into the extracellular medium and remained infectious (Blanco et al., 2004b; Bosch et al., 2005). Furthermore, since HIV-1 antigens were found in EEA1 positive but not in Lamp1 positive compartments, viral particles would escape from the endocytic pathway after reaching early endosomes before acidification and degradation of viral particles.

The mechanism proposed here suggests a possible viral mechanism to escape from the immune system or antiviral treatments. Infectious full virus particles may reside in vesicles of T cells, hence being inaccessible to the action of drugs or the immune system. Moreover, T cells harbouring these viruses may migrate through the organism, allowing the virus to infect cells in other body tissues or compartments.

5. Conclusions

Our work provides new insights into the mechanism of HIV-1 cell to cell transfer that can occur through the clathrin–dynamin-mediated endocytosis and suggests that both stimulated and unstimulated T CD4+ lymphocytes may act as carriers of HIV-1 particles in a manner that is reminiscent of dendritic cells (Wiley and Gummuluru, 2006). Furthermore, HIV-1 may enter cells via an alternative entry route that relies on endosome trafficking (Maurin et al., 2007), suggesting that cell-to-cell endocytic transfer of virus may promote productive infection. As transitory recipients of infectious HIV-1, endocytic virus transfer may serve as a mechanism of evasion of free viral particles to the immune system (Chen et al., 2007), antiviral drugs and virus clearance until the appropriate *milieu* and conditions may be reached for virus fusion, uncoating and productive infection.

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