Macropinocytosis-Like HIV-1 Internalization in Macrophages Is CCR5 Dependent and Leads to Efficient but Delayed Degradation in Endosomal Compartments

Lise-Andrée Gobeil,a Robert Lodge,a,b Michel J. Tremblaya,b
Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec-CHUL,a and Département de Microbiologie-Infectiologie et Immunologie, Faculté de médecine, Université Laval,b Québec, Canada

HIV-1 endocytosis by a macropinocytosis-like mechanism has been shown to lead to productive infection in macrophages. However, little is known of this pathway. In this study, we examined HIV-1 endocytosis using biochemical approaches and imaging techniques in order to better understand the mechanisms that allow for productive infection of these cells via the endosomal pathway. We show here that this macropinocytosis-like mechanism is not the sole pathway involved in HIV-1 endocytosis in macrophages. However, this pathway specifically requires CCR5 engagement at the cell surface, which in turn suggests that the virus and its coreceptor are present in the endosomal environment simultaneously. Furthermore, although we observed efficient viral degradation following endocytosis, analyses of HIV-1 transport through the endolysosomal pathway revealed that viral degradation is delayed following endosomal internalization, possibly allowing the virus to complete its fusion.

Productive entry of enveloped virus into susceptible target cells requires envelope mixing with the cellular membrane (1) and can occur either directly at the plasma membrane (plasma membrane fusion [PMF]) or from inside endosomes after cellular engulfment of the viral particle (fusion after endocytosis [FAE]). These two distinct entry mechanisms are often defined as pH independent or pH dependent. In PMF, receptor binding is necessary and sufficient to trigger conformational changes associated with fusion events, whereas in FAE, the low-pH environment inside the endolysosomal machinery is also required for these changes to occur. FAE, however, can also be triggered by proteolysis, a mechanism involving low-pH-activated proteases but that is nonetheless considered to be pH independent (2, 3).

Viruses can exploit a variety of different endosomal pathways for their cellular entry. The most studied and the best described are clathrin-mediated endocytosis (CME), caveola-dependent endocytosis, and macropinocytosis (4). In CME, viruses and their transmembrane receptors are packaged into clathrin-coated vesicles. Receptor nucleation at the site of endocytosis is coordinated by adaptor proteins such as AP-2 (5). Caveolae are lipid raft-enriched flask-shaped plasma membrane invaginations that contain caveolin, which is a cholesterol binding protein (6). Involvement of caveola structures in viral internalization was described for simian virus 40 (SV40) (7) and echovirus 1 (8). In macropinocytosis, large vesicles called macropinosomes are used by the cell to internalize a large amount of solute and membranes. Their formation usually occurs in highly ruffled regions of the plasma membrane and results from its fusion with lamellipodia that fold back onto themselves (9). Following internalization via one of the above-mentioned mechanisms, viruses are usually transported to lysosomes through endosomal vesicles of increasing acidity (10), although they can also be transported, specifically for caveola-mediated endocytosis, through neutral pH routes and organelles such as caveosomes (11). Endosomal trafficking offers many advantages for viruses that are able to circumvent degradation in the unfriendly environment of the endolysosomal pathway. It allows, for example, viruses to escape immune surveillance and to bypass restriction factors or physical obstacles such as the actin cortex. It also provides viral particles with rapid transport to the cell nucleus (4).

Although PMF and FAE were previously thought to be mutually exclusive, several studies suggest that some viruses are able to exploit both pathways. Indeed, Newcastle disease, vaccinia, and herpes simplex viruses are capable of pH-independent PMF. However, they may also infect target cells by pH-dependent FAE (12–14). Another example is Epstein-Barr virus, for which fusion occurs by both PMF and FAE, depending on the cell type, although in that case, FAE is not triggered by pH acidification (15).

Human immunodeficiency virus type 1 (HIV-1) is another example of a virus that can use both PMF and FAE for cell entry. It has been suggested that FAE may be the result of a rapid virus engulfment after its specific primary receptor (i.e., CD4) and coreceptors (the most commonly used being CXCR4 and CCR5) are engaged at the cell surface (and are thus pH independent). The occurrence of this event would therefore depend on the balance between cellular internalization/degradation rates and the virus fusion kinetics (16). In agreement with this model, the process of HIV-1 entry seems to be highly cell type dependent. We and others showed that endocytosis has a major role in HIV-1 infection of transformed cell lines such as trophoblasts (17) and epithelial cells of intestinal (18) or cervical (19) origin. However, studies using primary human CD4+ T cells, the principal cellular targets of HIV-1 in peripheral blood, showed that both PMF and FAE occur in these cells (20), whereas only PMF seems to be important for infection of dendritic cells (21).
Macrophages are important targets of HIV-1 and contribute to disease progression by persisting after infection, allowing a constant and stable dissemination of viruses to other tissues. Their functions as phagocytes and antigen-presenting cells (APCs) are reflected by a constitutive high rate of fluid-phase pinocytosis and vesicle fusion with lysosomes (22). Furthermore, they have evolved to express a high level of lysosomal proteases and are therefore able to degrade antigens more efficiently than other APCs such as dendritic cells (23). Although these characteristics might impact the ability of HIV-1 to use the endosomal pathway to productively infect these cells, two previous reports point to a role for macrophinocytosis or a macropinocytosis-like mechanism (the pathway of HIV endocytic entry in macrophages [PHEEM]) in HIV-1 infection of macrophages (24, 25). However, the mechanisms allowing for FAE of HIV-1 in these cells are not completely understood. In the present study, we combined biochemical approaches and imaging techniques to analyze HIV-1 endocytosis and trafficking in macrophages in order to better understand these two processes. Our results suggest that PHEEM is not the sole approach and imaging techniques to analyze HIV-1 endocytosis and trafficking in macrophages in order to better understand these two processes. Our results suggest that PHEEM is not the sole pathway involved in HIV-1 endocytosis in macrophages. However, CCR5 engagement is required to induce HIV-1 internalization by PHEEM, suggesting that both HIV-1 and CCR5 are present in endosomal compartments after this specific type of endocytosis. Furthermore, although HIV-1 endocytosis led to efficient viral degradation in endolysosomal compartments, virus transport analyses showed that its degradation was delayed, possibly allowing a relatively small number of virions to complete fusion.

**MATERIALS AND METHODS**

**Reagents and antibodies.** Chlorpromazine (CPZ) was obtained from Calbiochem (EMD Millipore, Toronto, ON, Canada) and kept as a stock solution of 10 mg/ml in ethanol (EtOH). Dimethyl amiloride (DMA), 5-((N-ethyl-N-isopropyl)amiloride (EIPA), and bafilomycin A1 were all obtained from Sigma-Aldrich (St. Louis, MO) and kept in dimethyl sulfoxide (DMSO) at concentrations of 100 mM, 25 mM, and 100 μM, respectively. Maraviroc (MVC) was obtained from the NIH AIDS Research and Reference Reagent Program (Germantown, MD) and was kept in DMSO at the concentration of 10 mM. The anti-p24 hybridomas 31-90-25 and 183-H12-5C were respectively obtained from the American Type Culture Collection (Manassas, VA) and the NIH AIDS Research and Reference Reagent Program and were used in our in-house sandwich enzyme-linked immunosorbent assay (ELISA) (26). Both antibodies were purified using MabTrap protein affinity columns (Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions. For immunofluorescence microscopy, mouse anti-EA1 (BD Biosciences, Franklin Lakes, NJ), mouse anti-CD63 (H5C6; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and mouse anti-Lamp1 (H4A3, DSHB, University of Iowa) were used in combination with Alexa 555-coupled goat anti-mouse IgG (Molecular Probes, Invitrogen, Life Technologies, Burlington, ON, Canada). DRAQ5 was obtained from Biostatus (Leicestershire, United Kingdom) and Lyso-tracker Red from Lonza (Allendale, NJ).

**Preparation of macrophages.** Peripheral blood mononuclear cells from healthy donors were isolated by ficoll-hypaque gradient centrifugation. Monocytes were separated from other cells, mostly lymphocytes, by adherence for 2 h at 37°C in 150- by 20-mm tissue culture dishes followed by washing with endotoxin-free phosphate-buffered saline (PBS) (Sigma-Aldrich). Monocytes were then allowed to differentiate for 4 days in RPMI 1640 medium supplemented with 5% autologous plasma, 25 ng/ml macrophage colony-stimulating factor (M-CSF), and antibiotics and then washed and allowed to differentiate for 3 more days. Monocyte-derived macrophages (here called macrophages) were recovered by scraping following incubation with Accutase (eBioscience, San Diego, CA) and further cultured in RPMI 1640 medium supplemented with 5% autologous plasma and antibiotics. Experiments were performed in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics.

**Molecular constructs.** The X4-tropic pNL4-3 (27) and the R5-utilizing pYU-2 (28, 29) full-length molecular HIV-1 clones were obtained from the NIH AIDS Research and Reference Reagent Program. The pNL4-3Balenv R5-tropic molecular clone was a kind gift of R. Pomerantz (Thomas Jefferson University, Philadelphia, PA) (30). The pNL4-3env molecular clone was obtained by introducing a 1-frame shift in the envelope (Env) precursor and was a gift from D. E. Ott (National Cancer Institute, Frederick, MD). pRFLenv and pHCMV-G were from N. R. Landau (NYU School of Medicine, New York, NY) and J. C. Burns (UCSD, San Diego, CA), respectively. The pNL4-3gag-iCFPBalenv construct was obtained by replacing the X4-tropic NL4-3 env gene with that of the Bal virus in the pNL4-3gag-iCFP construct, which was kindly provided by B. K. Chen (Mount Sinai School of Medicine, New York, NY) (31), using the Sall and BamHI restriction sites. Both pNL4.3Balenv and pNL4.3gag-GFPBalenv were obtained by replacing the X4-tropic NL4-3 env gene with that of the Bal virus in pNL4.3gag-eGFP and pNL4.3 constructs, which were kindly provided by H. G. Krausslich (Heidelberg University, Heidelberg, Germany) (32), using the same restriction sites. The integrity of all constructs was verified by restriction analysis and by sequencing.

**Virus production.** Viruses were produced by calcium phosphate transfection of 293T cells as described previously (26). JRFLenv (R5-tropic) and vesicular stomatitis virus (VSV)-G-pseudotyped viruses were obtained by cotransfection of pNL4-3env with pRFLenv or pHCMV-G, respectively. To obtain NL4-3gag-eGFPBalenv particles, 293T cells were cotransfected using equimolar amounts of pNL4.3Balenv and pNL4.3gag-eGFPBalenv. For live-cell experiments, viruses were inactivated with 2-aldrithiol (AT-2). This mild oxidizing reagent induces covalent modification of the free sulfhydryl groups of the cysteines of internal virion proteins, in particular, the nucleocapsid proteins. This inactivation method preserves the structural and functional integrity of Env glycoproteins on the virus surface and therefore has no impact on its interaction with cell surface receptors (33). Briefly, viral suspensions were incubated overnight at 4°C in the presence of 1 mM AT-2 and then purified by ultracentrifugation. A sandwich ELISA previously developed in our laboratory (26) was used to quantify the amount of p24 in each of these viral preparations. Virus infectivity was determined using the TZM-bl reporter cell line (34, 35).

**Viral intracellular p24 assay.** Macrophages (10^5 cells/well in 24-well plates) were either left untreated or preincubated with CPZ, DMA, EIPA, MVC, MCV+C+I, or their respective drug vehicle for 30 min and then put in contact with different virus stocks (20 ng of p24/10^5 cells) for 2.5 h at 37°C to allow for virus internalization (i.e., fusion and endocytosis). Final drug concentrations were 10 μg/ml, 100 μM, 25 μM, and 200 nM for CPZ, DMA, EIPA, and MVC, respectively. Cells were then washed and treated with trypsin for 5 min to remove uninternalized viruses from their surface. This treatment efficiently removed 75 to 80% of the viruses on the cell surface (20 ng of p24/10^5 cells) after a 2-h binding experiment at 4°C (data not shown). After additional washes, cells were disrupted in lysis buffer (PBS, 0.05% Tween 20, 2.5% Triton X-100, and 0.02% thimerosal), and intracellular p24 was evaluated by ELISA (26), with a limit of detection of 31 pg/ml. The experiment was performed in triplicate, and the amount of intracellular p24 was normalized on cellular viability measured on drug-treated cells. In data illustrated below (see Fig. 3), ultracentrifuged viruses were used to ensure that no free p24 was present in viral preparations. Typical p24 concentrations in virus preparations used in this work ranged from 0.2 to 3.6 ng/ml, depending on the blood donors, type of viruses, and drug treatments.

**Viral degradation assay.** Macrophages (10^5 cells/well in 24-well plates) were put in contact with different virus stocks (20 ng of p24/10^5 cells) at 37°C to allow for virus internalization (i.e., fusion and endocytosis). Following 2 h of incubation, cells were washed and treated with tryp-
sin for 5 min to remove uninternalized viruses from their surface (time zero) and then incubated at 37°C to allow for virus degradation. Cell lysis was performed at time zero and at 1.5 and 3 h, and the intracellular p24 content was measured at every time point using our in-house ELISA (26). As controls, macrophages were preincubated with bafilomycin A1 (100 nM) for 30 min before addition of viruses and were left in contact with the drug for the whole duration of the experiment. The experiment was performed in triplicate.

**Cellular viability assay.** Cellular metabolism was monitored by incubating control drug vehicle- or drug-treated cells (10^5 cells in 24-well plates) with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI) for 1 h at 37°C according to the manufacturer’s instructions. The supernatant absorbance at 490 nm was read using an ELx808 Absorbance Microplate Reader (BioTek, Winooski, VT). The assay was performed in triplicate.

**Live cell imaging.** Macrophages (10^5 cells/well) were cultured in 8-well Lab-Tek II chambered coverglasses (Nalge Nunc International, Rochester, NY) and loaded with Lysotracker Red (100 nM) for 1.5 h at 37°C. AT-2-inactivated NL4-3 labeled viruses (20 ng of p24/105 cells) were then allowed to bind to cells at 4°C for 2 h (in order to obtain synchronized viral entry). Cells were then transferred to warm media containing Lysotracker Red to allow for virus internalization (time zero). Cells were imaged (12 fields) on a WaveFx Spinning Disk confocal microscope (Quorum Technologies, Guelph, ON, Canada) equipped with the appropriate lasers and filters.

**Immunofluorescence microscopy.** Macrophages (10^5 cells/well) were seeded on coverslips in 24-well plates and then allowed to interact with NL4-3-gag-eGFPBalenv (20 ng of p24/10^5 cells) at 4°C for 2 h for synchronization of virus entry. Cells were then removed to unbound viruses and transferred to 37°C to allow for virus internalization (i.e., fusion and endocytosis). After the different incubation times indicated, cells were fixed with 4% paraformaldehyde (in PBS) for 30 min and then processed for imaging as follows: fixed cells were first permeabilized and blocked with a 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich), 1% (wt/vol) bovine serum albumin (Sigma-Aldrich), 10% (vol/vol) human plasma, and 20% (vol/vol) normal goat serum (Jackson Immunoresearch, West Grove, PA) solution. Macrophages were stained for EEA1 (1/100), CD63 (1/300), or Lamp1 (1/300) for 1 h, washed in PBS, and further stained with secondary antibody (1/500) and the DNA probe DRAQ5 (1/1,000) for 30 min. Microscope slides were then mounted using Fluoromount G (Southern Biotech, Birmingham, AL). Cells were imaged (4 fields per marker per time point) by confocal laser scanning microscopy, using a Fluoview FV3000 microscope (Olympus, Center Valley, PA) equipped with the appropriate lasers and filters.

**Image processing and deconvolution and colocalization analyses.** Image processing was made using either the Velocity 4.2.1 (PerkinElmer, Waltham, MA) or NIH ImageJ 1.42 software. Image deconvolution, co-localization analyses, and the number of intracellular viral particles at a given time point were obtained using Volocity 4.2.1 (PerkinElmer, Waltham, MA). Cells were imaged with appropriate lasers and filters.

**RESULTS**

Macropinocytosis is involved in HIV-1 endocytosis in macrophages. Two separate groups have shown that HIV-1 replication is reduced in macrophages infected in the presence of amiloride derivatives such as DMA and EIPA (24, 25). These drugs specifically inhibit macropinocytosis by acting on Na+/H+ exchange and submembranous pH, thereby affecting F-actin remodeling (36). The authors concluded that macropinocytosis is involved in HIV-1 infection in such cells. However, only EIPA was efficient in reducing HIV-1 integration, as opposed to DMA, which had no significant effect (24). Therefore, we set out to determine if this discrepancy could reflect an unspecific effect of amiloride derivatives on the late steps of the viral cycle. We designed an intracellular HIV-1 p24 assay that allows for quantification of virus internalization and measures the effects of these drugs on the early events of the virus cycle. In this case, intracellular p24 represents both endosomal and cytosolic viral particles at a given time point and does not discriminate between fusion (PMF and FAE) and unproductive endocytosis. However, the assay will detect any significant reduction in HIV-1 endocytosis, as the vesicular fraction was shown to represent at least 45% of total intracellular p24 in macrophages in previous fractionation studies (25).

Cells were first pretreated with EIPA or DMA for 30 min before allowing for the internalization of viral particles for 2.5 h at 37°C. After a 5-min trypsin treatment, cell lysis was performed and intracellular p24 was quantified by ELISA. Data were normalized according to cellular metabolic activity as measured by an in vitro cellular proliferation assay (as described in Materials and Methods). We first tested the reliability of our assay using a VSV-G-pseudotyped virus, as its entry occurs exclusively by endocytosis and as it has been shown to exploit macropinocytosis in some cells (37–39). As expected, both EIPA and DMA reduced VSV-G internalization in macrophages, as mean intracellular p24 concentrations were, respectively, 1.27 ng/ml, 0.79 ng/ml, and 0.92 ng/ml for DMA-, EIPA-, and DMA-treated cells (Fig. 1A). However, this effect was greater for EIPA, with a reduction of 38% compared to controls.
to 24% with DMA. These results confirm that our assay is sensitive enough to detect variations in HIV-1 endocytosis. Therefore, we tested a fully replicative R5-tropic virus, NL4-3Balenv, in the same assay. EIPA successfully reduced NL4-3Balenv internalization in macrophages (mean measured intracellular p24 concentrations of 0.64 ng/ml for DMSO and 0.53 ng/ml for EIPA, for a reduction of 17% after normalizing for cell viability), although with a less pronounced effect than with the VSV-G-pseudotyped virus (Fig. 1B). However, DMA failed to reduce HIV-1 endocytosis (mean intracellular p24 concentration of 0.74 ng/ml), confirming the previously reported lower efficiency of this drug (24). Considering our observations using EIPA, we conclude that a macropinocytosis-like mechanism is involved in HIV-1 endocytosis in macrophages.

Binding of gp120 to CCR5 is required for HIV-1 macropinocytosis in macrophages. In order to investigate the requirements for HIV-1 macropinocytosis in macrophages, we repeated the experiment illustrated in Fig. 1 without DMA, using viruses that bind different receptors, i.e., NL4-3env−/JRFLenv and YU-2 (R5-tropic), which can bind both CD4 and CCR5, and NL4-3 (X4-tropic), which can bind both CD4 and CXCR4. Their internalization in the presence of EIPA was compared with that of an Env-deficient virus (i.e., NL4-3env−) that can bind none of the above and was used as a control. As shown in Fig. 2A, the presence of EIPA reduced the mean intracellular p24 concentration following a 2.5-h virus internalization of either NL4-3env−/JRFLenv (1.18 ng/ml for DMSO and 0.92 ng/ml for EIPA) or YU-2 (0.79 ng/ml for DMSO and 0.68 ng/ml for EIPA), confirming our results obtained with the NL4-3Balenv virus. As for NL4-3 and NL4-3env− viruses, we could not significantly reduce their endocytosis using EIPA (mean measured intracellular p24 concentrations of 0.43 ng/ml and 0.51 ng/ml and of 0.38 ng/ml and 0.53 ng/ml for DMSO- and EIPA-treated cells for NL4-3 and NL4-3env−, respectively). These results suggest that gp120 binding to CD4 is not sufficient and that CCR5 interaction is required to promote an HIV-1 macropinocytosis-like mechanism in macrophages.

In order to confirm the requirement of CCR5 in such a mechanism, we tested HIV-1 endocytosis in the presence of MVC, a CCR5 antagonist. As shown in Fig. 2B, NL4-3Balenv internalization was only slightly reduced (0.98 ng/ml, compared to 1.03 ng/ml for DMSO) in the presence of MVC, whereas that of NL4-3env−/VSV-G was increased (1.09 ng/ml, compared to 0.94 ng/ml for DMSO). However, the effect of EIPA on NL4-3Balenv internalization was significantly reduced by MVC (Fig. 2C), while that on NL4-3env−/VSV-G was not. Indeed, the mean intracellular p24 concentrations measured for NL4-3Balenv in cells treated with DMSO or EIPA were 1.03 ng/ml or 0.77 ng/ml in the absence of MVC, respectively, and 0.98 ng/ml or 0.82 ng/ml, with MVC. These results correspond to reductions of viral internalization in the presence of EIPA of 25% and 16%, respectively, following normalization for cell viability. As for NL4-3env−/VSV-G, we obtained 0.94 ng/ml and 0.53 ng/ml of intracellular p24 in DMSO- and EIPA-treated cells, as opposed to 1.09 ng/ml and 0.57 ng/ml in DMSO+MVC- and EIPA+MVC-treated macrophages, respectively, corresponding to reductions in intracellular p24 of 56% and 53%. These data confirm the role of CCR5 in the macropinocytosis-like HIV-1 endocytosis.

CCR5 binding is involved but not required for efficient HIV-1 endocytosis in monocyte-derived macrophages (MDMs). As CCR5 binding by gp120 is required to induce macropinocytosis of HIV-1 in macrophages, we investigated if this interaction was required for its efficient endocytosis. We therefore compared the amounts of intracellular p24 of the different viruses following a 2.5-h internalization at 37°C. In these experiments, we used ultra-centrifuged viruses, eliminating the possibility that free HIV-1 p24 present in our viral preparations could be internalized and affect results. As expected, R5-tropic viruses were all internalized with the same efficiency (Fig. 3A), as similar mechanisms are involved in their internalization. However, endocytosis efficiency was...
highly variable for macrophages derived from one blood donor to another (0.5 to 2.4 ng of p24/ml). The amount of intracellular p24 measured following endocytosis of the VSV-G-pseudotyped virus was greater than that of other viruses (1.0 to 3.2 ng of p24/ml). This was also expected, as the endocytosis of this virus is known to be very efficient, and its degradation rate is expected to be lower than that of R5-tropic viruses. Intracellular p24 detected following endocytosis of NL4-3env− virus was only slightly lower than that of its R5-tropic counterparts (0.4 to 1.7 ng of p24/ml), and similar amounts of intracellular p24 were obtained for NL4-3 (0.5 to 2.4 ng of p24/ml) and R5-tropic viruses, even though NL4-3 and NL4-3env− do not bind CCR5. As these experiments were all performed using the same set of viral productions, and to ensure that results were reproducible, we repeated the same experiment with 3 different viral productions in macrophages derived from the same blood donor and obtained similar results (Fig. 3B), although we observed different endocytosis efficiency levels from one viral production to another. Taken together, these results indicate that efficient HIV-1 endocytosis can occur in the absence of CCR5 binding.

CME is not significantly involved in HIV-1 endocytosis in macrophages. As CCR5 binding is not required for efficient HIV-1 engulfment in macrophages, macropinocytosis is thus not the sole mechanism involved in virus endocytosis. We therefore investigated which other endocytosis mechanism(s) could be involved in HIV-1 internalization in macrophages. We first discarded caveolin-mediated endocytosis, given that Carter and colleagues previously showed that both caveolin-1 RNA transcripts and protein were not detectable in macrophages (24). Although we did detect caveolin-1 RNA transcripts in our macrophage preparations, we were unable to detect the protein by either Western blotting or immunofluorescence microscopy using antibodies from different sources (data not shown). We then investigated whether CME could be involved in HIV-1 endocytosis. CME is unlikely to lead to productive HIV-1 infection in macrophages, given that previous reports have shown that inhibition of this pathway by CPZ has no effect on HIV-1 integration (24). However, electron microscopy studies of macrophages that had internalized HIV-1 particles determined that the virus can be found in clathrin-coated vesicles (25), suggesting that CME may be involved in CCR5-independent HIV-1 internalization. Thus, we took advantage of our HIV-1 internalization assay, which detects intracellular p24 following either productive or nonproductive infection, in order to investigate the role of CME in endocytosis of HIV-1 in macrophages. Cells were pretreated with CPZ or its vehicle for 30 min prior to the internalization of different virus particles for 2.5 h at 37°C. Macrophages were then treated with trypsin for 5 min and extensively washed prior to lysis and quantification of intracellular p24 by ELISA, and data were normalized according to cellular metabolic activity. We found that CPZ had either no effect or slightly increased HIV-1 internalization in macrophages derived from most of the blood donors tested (Fig. 4), therefore suggesting that CME is not a major internalization pathway in macrophages.

HIV-1 particles are targeted for degradation following their endocytosis in macrophages. In an attempt to investigate the fate of virions following endocytosis and vesicular transport in macrophages, we adapted our in vitro intracellular p24 assay to follow intracellular p24 in a time course study. In this experiment, treatment with bafilomycin A1, an inhibitor of vesicular H+−ATPase (40), blocks vesicular acidification and hence should inhibit viral degradation. Briefly, macrophages were treated with bafilomycin A1 for 30 min or left untreated prior to their contact with NL4-3Balenv, NL4-3env−, or NL4-3env−/VSV-G viruses and then further incubated for 2 h at 37°C to allow for virus internalization. Cells were then washed and treated with trypsin for 5 min to remove bound viruses (time zero). Macrophages were then returned to 37°C, in the presence or absence of the drug, and intracellular p24 amounts were measured over time using our homemade p24 ELISA, following cell lysis at different time points. We observed a loss of intracellular p24 over time, in untreated macrophages, for all the viruses tested (Fig. 5). However, the reduction in intracellular p24 was much slower, over time, for the VSV-G-pseudotyped virus, which is consistent with efficient viral escape from endosomes following their acidification. Surprisingly, we did not detect a significant difference between the rates of loss in intracellular p24 for the NL4-3Balenv and NL4-3env− viruses, suggesting that the great majority of HIV-1 particles are degraded in macrophages following virus internalization. Furthermore, bafilomycin A1 almost completely inhibited intracellular p24 decrease over time for all the viruses, confirming that the decrease of

FIG 3 CCR5 binding is involved but not required for efficient HIV-1 endocytosis in macrophages. Cells were allowed to internalize equal amounts (standardized in term of p24 content) of the listed virus stocks (i.e., ultracentrifuged viral productions) for 2.5 h at 37°C. After a 5-min trypsin treatment to remove membrane-bound viruses, cell lysis was performed. Total intracellular p24 was quantified by ELISA. Results are expressed as percentages of NL4-3Balenv intracellular p24 and represent mean values from 5 independent experiments performed with the same viral production on macrophages derived from 5 different blood donors (A) or mean values from a single experiment performed with 3 different viral productions on macrophages derived from a single blood donor (B). Asterisks denote statistically significant differences (*, P < 0.05; **, P < 0.01) compared with macrophages exposed to NL4-3Balenv viruses (considered 100%).
intracellular p24 over time was due to endosomal degradation. It can be concluded that the majority of internalized HIV-1 particles are degraded after their internalization in macrophages.

**HIV-1 is transported into compartments of increasing acidity following endocytosis in macrophages.** We next studied HIV-1 transport into vesicular compartments in live cell experiments. Macrophages were first labeled using Lysotracker Red, a cell-permeant dye that gradually becomes fluorescent when exposed to the low pH of acidified vesicles. Next, NL4-3gag-iCFP-Balenv fluorescent HIV-1 particles were allowed to bind cells at 4°C for 2 h. The temperature was then shifted to 37°C (time zero) to permit viral internalization. This technique allowed us to synchronize viral endocytosis and to increase viral detection, therefore facilitating colocalization studies. Cells were thus imaged every 20 to 30 min. As depicted in Fig. 6, the average virus colocalization with Lysotracker Red-labeled vesicles increased over time, reaching a peak level at 60 min following the temperature shift, and then decreased slowly after 90 min. This suggests that HIV-1 particles are transported to acidified vesicles following their endocytosis in macrophages. Peak colocalization coefficients varied from 0.50 to 0.75 in cell samples derived from the three blood donors tested. However, overall HIV-1 colocalization with highly acidified (highly positive for Lysotracker Red) endosomes was not significant (data not shown), thus suggesting that viruses were efficiently degraded before reaching these intracellular compartments. These results indicate that HIV-1 is transported and degraded in acidified vesicles following its endocytosis in macrophages.

The endolysosomal pathway is involved in HIV-1 transport and degradation following endocytosis in macrophages. The endolysosomal route is the main vesicular pathway involved in cargo transport to lysosomes for degradation, and it has also been shown to be involved in transport of macropinocytosed cargos (41). We therefore sought to determine its involvement in HIV-1 transport.
in macrophages. We first allowed NL4-3 gag-eGFPBal env viruses to bind to macrophages grown on coverslips for 2 h at 4°C and then shifted the temperature to 37°C to synchronize viral internalization. Macrophages were then fixed at different time points, stained for EEA1, CD63, and Lamp1, which are specific markers for early endosomes, multivesicular bodies/late endosomes, and lysosomes, respectively, and mounted for microscopy. As expected, we observed colocalization between HIV-1 and all the endolysosomal markers tested (Fig. 7). In all cases, colocalization was first low or absent and then increased to reach a peak level as viruses entered specific intracellular compartments and finally decreased as viruses progressed in the pathway or were being degraded.

Peak colocalization for EEA1, CD63, and Lamp1 was sequential in time, suggesting viral transfer from early to late endosomes and then to lysosomes. However, colocalization remained high for EEA1 throughout the experiment, indicating that some viruses remained in this compartment for a longer time period before being transferred. The loss of green fluorescent protein (GFP)-labeled particles occurred 30 min after the temperature shift, corresponding to peak Lamp1 colocalization. This suggests that viral degradation begins when viruses reach Lamp1-positive compartments, corresponding to lysosomes, and that transport to these compartments is important for HIV-1 degradation. Overall, these results indicate that the endolysosomal pathway is involved in HIV-1 transport and degradation following endocytosis in macrophages.

**DISCUSSION**

Previous studies have shown that HIV-1 internalization by macropinocytosis, specifically PHEEM, could lead to productive infection in macrophages (24, 25), even though these cells were shown to degrade antigens more efficiently than other APCs such as dendritic cells (23), which are restrictive to FAE and only allow PMF (21). In the current study, we confirmed the contribution of a macropinocytosis-like pathway in the process of HIV-1 infection in macrophages and identified factors that may contribute to the occurrence of FAE.

Our intracellular p24 assay confirmed the role of macropinocytosis in HIV-1 internalization in macrophages, although the effect of EIPA was only moderate compared to previously re-
ported observations (24). However, this is attributable to the nature of our assay, which measures all viruses in endosomal compartments, whether these pathways are productive or not. Furthermore, this assay does not discriminate between endosomal and cytosolic p24. We did not specifically fully characterize this macropinocytosis as PHEEM. Nevertheless, we obtained results similar to those of Carter and colleagues concerning the effects of DMA and EIPA on the early stages of the HIV-1 viral cycle. In-
deed, we observed a significant effect of EIPA on HIV-1 endocytosis, whereas DMA had no significant effect. Considering that DMA reduced VSV-G-pseudotyped virus internalization by only 24% compared to 38% for EIPA, it is clear that DMA is less efficient than EIPA in reducing macropinocytosis, as suggested by Carter et al. This is much more likely to explain the discrepancy between the effects of DMA on HIV-1 infection and on viral integration presented in their paper, rather than a nonspecific effect of amiloride derivatives on the late steps of the viral cycle.

In addition to confirming previously reported macropinocytosis (24, 25), our assay further allowed us to show that macropinocytosis is not the only pathway involved in HIV-1 endocytosis in macrophages. Indeed, treating the cells with EIPA did not significantly impact the internalization of X4-tropic and Env-deficient viruses, although virus internalization efficiency was the same for R5- and X4-tropic viruses. Furthermore, using the same p24 intracellular assay, we were able to identify CCR5 binding as a prerequisite for macropinocytosis of HIV-1. When comparing different viruses, we observed that macropinocytosis was involved only in R5-virus internalization. Furthermore, using MVC, we significantly inhibited macropinocytosis of NL4-3Balenv. Although it may be surprising that MVC does not significantly reduce NL4-3Balenv internalization, this can be easily explained by the drug’s action. Indeed, while EIPA allows for the binding of HIV-1 to CCR5 without internalization, MVC blocks this binding. In such an event, the virus will use an alternative endocytosis receptor.

The involvement of CCR5 in macropinocytosis is significant, as the FAE route can occur only when both HIV-1 and its coreceptor are present simultaneously in the same endosomal compartment, as a result of coreceptor engagement and engulfment at the plasma membrane (16). This unique characteristic of macropinocytosis explains its specific disposition for HIV-1 infection. Indeed, this pathway is the only one that has been associated with productive HIV-1 infection in macrophages (24, 25).

We were unable to specifically identify another major route of HIV-1 endocytosis in this specific cell type. It is possible that a variety of receptors are involved in nonproductive internalization of HIV-1 in these cells. It is known that gp120 can bind to a variety of cell surface receptors present at the cell surface, specifically via interactions of its glycosylated residues with sugar-binding proteins or via electrostatic interactions between its positive charges and the negative charges of surface molecules (42). Furthermore, different cellular constituents can be incorporated onto the viral envelope upon viral budding and may mediate HIV-1 internalization via interaction with their natural coreceptor at the target cell surface (43). These molecules are likely to be responsible for endocytosis of Env-deficient HIV-1 particles used in our experiments. It should be further taken into consideration that these yet-to-be-characterized pathways may vary in macrophages derived from different blood donors and that the membrane expression of different receptors is dependent on the cellular activation status of macrophages (44). In this regard, we previously demonstrated that HIV-1 is efficiently internalized by CME in M1- and M2a-activated MDMs in a CCR5-independent manner and that macropinocytosis is not a major internalization pathway in these cells (45).

Following endocytosis, virus degradation was monitored by measuring intracellular p24 in a time course experiment. Surprisingly, we did not detect any difference in degradation between NL4-3Balenv and NL4-3env− viruses, although NL4-3Balenv is fusion competent and some of these viruses should undergo PMF or efficiently escape degradation in endosomal compartments by FAE. This unexpected result is not due to a loss in NL4-3Balenv cytosolic p24 following fusion (PMF + FAE). Indeed, intracellular p24 of VSV-G-pseudotyped HIV-1, which is mostly cytosolic given this virus’s efficient pH-dependent FAE, did not decrease significantly over time in untreated cells. Furthermore, any potential loss of intracellular p24 following fusion (PMF + FAE), in the case of NL4-3Balenv virus, should translate into a time-dependent decrease in capsid protein even in bafilomycin A1-treated cells; we did not observe such a decrease. Another possible explanation for the observed NL4-3Balenv degradation rate would be that the majority of internalized viruses are present in endosomal compartments and therefore that cytosolic p24 contribution to total intracellular p24 is negligible. If such is the case, HIV-1 fusion, either at the plasma or at the endosomal membranes, is a relatively rare and inefficient event in macrophages. This hypothesis is in contradiction with fractionation studies that showed that cytosolic p24 represents about 55% of total intracellular p24 in macrophages after a 2-h viral internalization of fusion-competent R5-tropic viruses (25). However, cytosolic p24 is likely to be overestimated in this study, as the author showed that 30% of an Env-deficient virus was also detected in the cytosolic fraction.

The gradual gain and following loss in colocalization of HIV-1 with Lysotracker Red in live cell confocal microscopy suggest that viral particles are efficiently transported to compartments of gradually lowering pH after their endocytosis-mediated internalization in macrophages. Although colocalization with Lysotracker Red was highly significant, colocalization with endosomes that were highly positive for Lysotracker Red remained low throughout the experiment. This suggests that HIV-1 does not reach highly acidic compartments and thereby confirms the efficient degradation of viral particles during their transport. Our further observations showed that viral particles are efficiently transferred from early to late endosomes and then to lysosomal compartments, following their internalization. However, a decrease in GFP-positive particle detection, which corresponds to viral degradation, was observed only after 30 min of internalization, correlating with HIV-1 entry into Lamp1-positive compartments. Therefore, during macropinocytosis, as the HIV-1 coreceptor is engaged before internalization, few viral particles may undergo fusion before their subsequent degradation. Indeed, blocking HIV-1 fusion at a temperature-sensitive step that allows for virus binding to its coreceptor (temperature-arrested stage) and shifting to 37°C result in rapid fusion, occurring in as fast as 5 min (46).

Furthermore, although we observed that the majority of HIV-1 particles progressed efficiently into the endolysosomal pathway following internalization, some particles remained in EEA1-positive compartments. Therefore, during macropinocytosis, as the HIV-1 coreceptor is engaged before internalization, few viral particles may undergo fusion before their subsequent degradation. Indeed, blocking HIV-1 fusion at a temperature-sensitive step that allows for virus binding to its coreceptor (temperature-arrested stage) and shifting to 37°C result in rapid fusion, occurring in as fast as 5 min (46).

Furthermore, although we observed that the majority of HIV-1 particles progressed efficiently into the endolysosomal pathway following internalization, some particles remained in EEA1-positive compartments. Therefore, during macropinocytosis, as the HIV-1 coreceptor is engaged before internalization, few viral particles may undergo fusion before their subsequent degradation. Indeed, blocking HIV-1 fusion at a temperature-sensitive step that allows for virus binding to its coreceptor (temperature-arrested stage) and shifting to 37°C result in rapid fusion, occurring in as fast as 5 min (46).
in HIV-1 transport, as well as their possible implication in FAE, particularly given that HIV-1 was previously observed in transferrin-positive sorting compartments in macrophages (24).

Taken together, our data suggest that although endocytosis represents a dead end for the majority of HIV-1 particles in macrophages, macropinocytosis specifically offers favorable conditions for FAE, as this pathway depends on the previous interaction of HIV-1 gp120 with CCR5. Furthermore, a small number of virus particles may benefit from a slow progression into the endolysosomal pathway, resulting in their delayed degradation.

ACKNOWLEDGMENTS

We thank Julie-Christine Lévesque, Ann Rancourt, and Sachiko Sato (IDRC BioImagery Platform) for helpful discussions and assistance. We also thank Odette Simard, Caroline Côté, and Marc-André Roy for technical help.

This study was supported by an Emerging Team Grant in HIV Pathogenesis to M.J.T. from the Canadian Institutes of Health Research (CIHR) (grant number HET-85519). L.-A.G. is the recipient of CIHR and Fonds de la Recherche en Santé du Québec Doctoral Awards. M.J.T. holds the Canada Research Chair in Human Immunovirology (Tier 1 level).

REFERENCES


