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A Clathrin, Caveolae, and Dynamin-independent Endocytic Pathway Requiring Free Membrane Cholesterol Drives HIV-1 Internalization and Infection in Polarized Trophoblastic Cells

Gaël Vidricaire and Michel J. Tremblay*

Research Center in Infectious Diseases, CHUL Research Center, and Department of Medical Biology, Faculty of Medicine, Laval University Quebec, Canada G1V 4G2

In human trophoblastic cells, a correlation between early endosomal trafficking of HIV-1 and virus infection was previously documented. However, if HIV-1 is massively internalized in these cells, the endocytic pathway(s) responsible for viral uptake is still undefined. Here we address this vital question. Amongst all the putative endocytic pathways present in polarized trophoblastic cells, we demonstrate that HIV-1 infection of these cells is independent of clathrin-mediated endocytosis and macropinocytosis. Importantly, treatment with the cholesterol-sequestering drug filipin severely impairs virus internalization, whereas the cholesterol-depleting compound methyl-β-cyclodextrin has no impact on this pathway. Moreover, viral internalization is unaffected by overexpression of a mutant dynamin 2 or treatment with a kinase or tyrosine phosphatase inhibitor. Thus, HIV-1 infection in polarized trophoblastic cells occurs primarily *via* a clathrin, caveolae, and dynamin-independent pathway requiring free cholesterol. Notably, even though HIV-1 did not initially co-localize with transferrin, some virions migrate at later time points to transferrin-enriched endosomes, suggesting an unusual transit from the non-classical pathway to early endosomes. Finally, virus internalization in these cells does not involve the participation of microtubules but relies partly on actin filaments. Collectively these findings provide unprecedented information on the route of HIV-1 internalization in polarized human trophoblasts.

*Corresponding author

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Introduction

An estimated 2.1 million children are currently living with HIV-1 worldwide and mother-to-child transmission (MTCT) is the primary cause of infection by this retrovirus in infants.[1] Importantly, recent statistics published by UNAIDS indicate that women are now becoming infected at a higher frequency than men. Hence women of child-bearing age are at greater risks to become infected by HIV-1 and in this context, MTCT is becoming a serious public health concern.[1] MTCT may occur *in utero*, at birth during delivery or through breastfeeding.[2,3] When considering the timing of vertical transmission, it is estimated that approximately 30% of infants become infected during pregnancy.[4] *In utero* transmission of HIV-1 has been documented by several groups.[5–8] HIV-1 has been found in aborted foetuses as early as after eight weeks of gestation[9] and in first and second trimester foetuses.[10–13] However, the mechanism underlying *in utero* HIV-1 transmission remains poorly understood.

In order for *in utero* transmission to occur, HIV-1 must first cross this highly protective placental barrier to ultimately reach the fetal circulation. The placenta is composed of a double layer of polarized epithelial-type cells (i.e. cytotrophoblasts and syncytiotrophoblasts). These cells separate the maternal and foetal blood circulations and control fluxes

Abbreviations used: HIV-1, human immunodeficiency virus type 1; MTCT, mother-to-child transmission; Tfn, transferrin; GPI, glycosylphosphatidylinositol; PMA, phorbol myristate acetate; LTR, long terminal repeat; Ctx, cholera toxin subunit B.

E-mail address of the corresponding author: michel.j.tremblay@crchul.ulaval.ca

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between them throughout pregnancy. Current models propose that following infection of trophoblasts and/or transcytosis across these cells, HIV-1 is released from the basolateral pole of the trophoblasts (facing the fetal circulation) leading to productive infection of the underlying fetal cells.[14–17] The molecular mechanism associated with HIV-1 transcytosis is currently poorly understood. It was shown that efficient cell-free virus transcytosis is inducible upon RGD-dependent integrin cross-linking. Moreover, this process is inhibited by antibodies to galactosyl ceramide.[14,18]

In regards to transmission *via* infection, we and others have shown that trophoblasts can be productively infected by HIV-1 both *in vitro* and *in vivo*,[14–17] but like other epithelial cells, their permissiveness is lower than that of $CD4^+$ T cells.[19] For instance, pro-inflammatory cytokines (e.g. TNF-α or IL-1) and a rescue with cells highly permissive to HIV-1 infection are necessary to reveal virus production in such cells.[16] These data are meaningful because they show that while trophoblastic cells constitute a natural barrier to HIV-1, this obstacle is not impenetrable.

Recent data indicate that the mechanism through which HIV-1 can infect trophoblasts appears fundamentally different from what is known for $CD4^+$ T cells. First, expression of CD4 receptor and coreceptors (i.e. CXCR4 and CCR5) is low or absent in trophoblasts and virus infection seems to be independent of these cell surface components.[20–24] Second, normally HIV-1 entry into susceptible cells is pH-independent and results from fusion at the cell membrane.[25,26] Contrary to this, endosomal trafficking of incoming virions is an obligatory early step for virus infection to ensue in trophoblasts. Two major discoveries support this claim. First, scientists have relied extensively on the endosome inhibitors bafilomycin A1 and ammonium chloride (NH_4Cl) to differentiate between pH-dependent (e.g. influenza virus and Semliki Forest virus) and pH-independent viral entry. It was demonstrated that HIV-1 infection in trophoblasts is pH-dependent, since it is highly sensitive to both inhibitors.[19,27] Second, upon contact with trophoblasts, HIV-1 is trafficked through several intracellular organelles.[16] To test whether this pathway is mandatory to infection, we transfected cells with a dominant negative Rab5 and found that this greatly reduced HIV-1 infection, indicating that early endosomes are part of the trophoblast infection pathway.[19] Dominant negative Rab7 also inhibited infection, demonstrating that trafficking to late endosomes is also an obligatory step for infection to proceed in trophoblasts.[19] Hence, we have thus far established a tight association between endocytosis and viral infection in trophoblasts. However, if we know that HIV-1 is massively internalized by trophoblastic cells, we still do not know how this event occurs. In other words, the endocytic pathway(s) responsible for viral internalization, an event preceding endosomal trafficking and ultimately infection, is still undefined. Here we address this vital question.

Previous studies revealed the rather high complexity of the endocytic pathways occurring at the plasma membrane of mammalian cells. These include (i) phagocytosis, (ii) macropinocytosis, (iii) the classical clathrin-mediated endocytosis, (iv) dynamin-dependent endocytosis *via* caveolae or (v) glycolipid rafts, (vi) clathrin, caveolae, and dynamin-independent endocytosis that requires lipid rafts, and (vii) dynamin-independent endocytosis that does not involve clathrin-coated pits, caveolae or lipid rafts.[28,29] Macropinocytosis is a cell-type specific and receptor-independent endocytic pathway. Upon cell stimulation, large vacuoles called the macropinosomes are formed from the closure of plasma membrane ruffles.[30] During clathrin-dependent endocytosis, specific receptors are recognized by the adaptor protein 2 complex and directed into clathrin-coated pits. Vesicles fuse with early endosomes (positive for EEA-1 marker), where receptors are sorted for either recycling or transit to late endosomes and lysosomes.[31] Caveolae and caveolae-related endocytic processes relying on glycolipid rafts are related endocytic pathways characterized by their clathrin independence, dynamin dependence and sensitivity to cholesterol depletion, and the morphology and lipid composition of the vesicular intermediates.[30] Caveolae-mediated endocytosis involves (i) clustering of lipid raft components on the plasma membrane into caveolae and (ii) signal transduction pathways leading to invagination and internalization. The caveolar vesicles are delivered to pre-existing caveolin-1-enriched organelles, the caveosomes, which are cholesterol-rich structures that are devoid of markers of the classical endocytic pathway.[28] It is also emerging that aside from these pathways, two other less characterized pathways also exist. First, clathrin, caveolae and dynamin-independent endocytosis that requires lipid rafts. For instance, internalization of non-clustered glycosylphosphatidylinositol (GPI)-anchored proteins (e.g. folate receptor, decay accelerating factor and CD59), which reside in lipid rafts at the plasma membrane, is independent of clathrin and dynamin. Moreover, these proteins do not co-localize with caveolin-1.[32,33] Secondly, evidences suggest that dynamin-independent endocytosis that does not involve clathrin-coated pits, caveolae or lipid rafts, is also possible. Indeed, major histocompatibility class-I (MHC-I) molecules and Tac (alpha subunit of the IL-2 receptor) are endocytosed through this rather unusual pathway.[34] Several putative modes of internalization are possible in trophoblastic cells. The fact that trophoblasts are epithelial-like cells that have been shown to resemble macrophages[35] supports the possibility of macropinocytosis. Caveolae-dependent endocytosis is excluded because caveolin-1 and -2 are not expressed by trophoblasts.[36] However, endocytosis *via* glycolipid rafts requiring dynamin or dynamin and lipid raft-independent internalization may operate also in these cells.

All in all, the internalization route discovered here is a striking example of a clathrin, caveoale,